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THE ABSENCE OF ABCD2 REVEALS A NOVEL ROLE FOR PEROXISOMES IN THE PROTECTION FROM METABOLIC SYNDROME

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ABSTRACT OF DISSERTATION

Jingjing Liu

The Graduate School
University of Kentucky
2011
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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmaceutical Sciences at the University of Kentucky

By Jingjing Liu
Lexington, Kentucky

Director: Dr. Gregory Graf, Professor of Pharmaceutical Sciences
Lexington, Kentucky 2011

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ABSTRACT OF DISSERTATION

THE ABSENCE OF ABCD2 REVEALS A NOVEL ROLE FOR PEROXISOMES IN THE PROTECTION FROM METABOLIC SYNDROME

ABCD2 (D2) is a peroxisomal ATP binding cassette (ABC) transporter that is expressed in brain, adrenal and liver. D2 is transcriptionally regulated by key transcriptional factors that control lipid and glucose metabolism. Therefore, we examined its role in adipose tissue. These studies revealed that D2 is highly abundant in adipose tissue and upregulated during adipogenesis. However, D2 deficiency does not affect either adipogenesis or lipid accumulation. An examination of the lipid profile of adipose tissue revealed the accumulation of C20 and C22 fatty acids in D2 deficient (D2⁻⁻) mice. When challenged with a diet enriched in erucic acid (C22:1, 10% kcal), this lipid accumulated in both liver and adipose tissue. Following 8 weeks of diet, D2⁻⁻ mice showed increased adiposity, glucose intolerance, dyslipidemia and steatosis. Analysis of the hepatic lipid profile showed significant changes away from poly unsaturated fatty acids (PUFAs) and toward C18-22 mono-unsaturated fatty acids (MUFA). RT-PCR of the mRNA from the adipose tissue and liver revealed significant changes in lipogenic (ACC, SCD1 & 2) and PUFA synthesis (Δ5 & 6-desaturase) genes in D2⁻⁻ mice. The molecular mechanisms by which D2 regulates lipid metabolism in adipose tissue remains unclear. To explore potential mechanisms, the subcellular localization of D2 in adipose tissue was determined. Our results demonstrated that D2 resides in a distinct subclass of peroxisomes that does not containing classical peroxisomal markers such as pex19 or PMP70, but are positive for pex14. In conclusion, our studies reveal a novel role of D2 and peroxisomes in the protection from disruptions of lipid metabolism induced by dietary erucic acid and that D2 resides in a unique compartment within adipocytes that plays a yet to be elucidated role in the regulation of lipid metabolism.

KEYWORDS: Peroxisome, ABCD2, Nonalcoholic fatty liver disease, Obesity, Poly unsaturated fatty acids
Jingjing Liu, B.S.
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Oct 5th, 2011
Date
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Dissertation

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I dedicate this dissertation to my wonderful family. Particularly to my understanding and patient husband, Jian, who has brought happiness to my life. I must also thank my dear parents, who have always being supportive for my career.
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1. Chapter 1: Introduction

1.1. Obesity

Obesity is defined by World Health Organization as excess weight gain for a given height (BMI ≥ 30 kg/m^2). The over consumption of energy dense foods that contain high proportions of simple carbohydrates and saturated fats in Western societies directly contributes to the high incidence of obesity. The United States has the highest obesity rate in the world with 33.8% being obese (BMI ≥ 30) and 68.0% being overweight or obese (BMI ≥ 25) as of 2007(1). The hallmarks of obesity are expanded adipose tissue, impaired adipocyte function and chronic low degree inflammation. Obesity increases the risk of other diseases including type 2 diabetes mellitus (T2D), cardiovascular diseases (CVD), hypertension, and nonalcoholic fatty liver disease (NAFLD).

Adipose tissue serves as body's largest energy reservoir by storing triacylglycerols (TAGs) in lipid droplets during the fed state and releasing nonesterified free fatty acids (NEFA) during the fasted state for use in other tissues as fuel (Figure 1.1-1). Recently, studies have revealed an important role of adipose tissue as an active endocrine organ that secretes a variety of adipokines, adipose derived cytokines that regulate metabolic homeostasis, appetite, insulin sensitivity, inflammation and energy expenditure(2). Due to the disruption of the endocrine function of adipose tissue in obese patients, fat becomes a source of inflammation. TNF-α, IL-6, and free fatty acids create a proinflammatory environment and promote insulin resistance in liver, skeletal muscle and adipose tissue.
In the postprandial period, insulin is secreted from pancreatic β-cells in response to elevated blood glucose. As an important hormone that regulates energy and glucose homeostasis, insulin exerts three main functions: 1) lowering the postprandially elevated blood glucose level by increasing the disposal of glucose in liver and skeletal muscle and inhibiting gluconeogenesis, 2) promoting lipid synthesis in liver, and 3) lowering circulating NEFA by inhibit lipases that promote TAG hydrolysis in adipose tissue. In insulin resistant patients, glucose disposal by peripheral tissues is reduced and the antilipolytic action of insulin in adipose tissue is impaired. When NEFA are delivered to liver, they exacerbate hepatic insulin resistance, resulting in ectopic lipid accumulation and ER stress and contributing to NAFLD. In addition, hyperinsulinemia can further promote hepatic lipid accumulation by stimulating hepatic lipid synthesis(4, 5).
NAFLD is characterized by lipid accumulation, especially TAG, in the absence of significant alcohol consumption. The prevalence of NAFLD in the world-wide general population is estimated to be between 14% and 24% (6-8), and is around 30% in the United States adult population (9). Hepatic lipid accumulation results from the imbalance between lipid availability (fatty acid uptake from circulation and \textit{de novo} lipid synthesis) and lipid disposal (fatty acid β-oxidation or triglyceride rich lipoprotein secretion) (10). NAFLD is typically diagnosed in patients with insulin resistance and other metabolic syndromes (obesity, dyslipidemia and diabetes). Consequently, in obese individuals, the prevalence of NAFLD increases significantly to between 74% and 90% (4, 11).

To provide information for treatment, kinetic studies have been conducted to identify the origin of TAGs accumulated in the livers of NAFLD patients. The major sources are (Figure 1.1-2):

1) Lipid stored in adipose tissue in the form of TAG. Under energy demanding conditions, lipolysis of TAG generates and releases NEFA into circulation, which can be taken-up by liver and used for energy via β-oxidation (pathway 1),

2) Fatty acids made in liver through \textit{de novo} lipogenesis (DNL) from acetyl-CoA generated in Krebs cycle (pathway 2),

3) Dietary fatty acids, which can enter liver through spillover into plasma NEFA pool (pathway 3),

4) Internalization of chylomicron remnants (pathway 4).
Figure 1.1-2. Model of lipid flux through the liver (12).

Boxed numbers indicate the metabolic pathways traced using stable (nonradioactive) isotopes. DNL indicates new fat synthesis from 2-carbon precursors (e.g., dietary carbohydrate); chylomicrons are lipoproteins made in the intestine that carry dietary fat. FA, cellular fatty acids (fatty acids not esterified to glycerol but bound to a carrier protein).

The relative importance and contribution of each source to the content of liver TAGs accumulation has been studied(12). Using a multiple-stable-isotope labeling approach in patients of NAFLD, Donnelly et al. reported that this method labels up to 64% (mean ± SD, 38 ± 16%) of the hepatic lipids. Of the labeled TAGs in liver, 59% TAGs are from the plasma NEFA pool (pathway 1, 59.0% ± 9.9%), 26% are from DNL (pathway 2, 26.1% ± 6.7%) and 15% are from the diet (pathway 3 and 4, 14.9% ± 7.0%).
Also, the relative contribution of these sources to very-low-density lipoproteins (VLDL) TAGs paralleled the liver TAGs(10). In the fasting period, the NEFA, DNL and dietary NEFA pools contribute 81%, 7% and 10% of VLDL TAGs, respectively. In the fed period, the contribution of NEFA, DNL and dietary NEFA to VLDL TAGs are 61%, 9% and 26%. These data demonstrate that NEFA from adipose tissue contribute the most to both liver and VLDL in NAFLD patients, suggesting that lipid composition of adipose tissue will greatly affect the lipid composition of liver.

To better understand the development of NAFLD, important metabolic pathways of lipids will be discussed in detail below: 1) lipid absorption 2) hepatic de novo lipogenesis, 3) lipid storage and 4) lipid utilization.

1.2. Lipid metabolism

1.2.1. Lipid absorption

Dietary fat exists mostly in the form of triglycerides and represents about one third of the average daily caloric intake of humans. The fats in ingested foods are insoluble in water and aggregate into large lipid droplets in the upper portion of the stomach. The large lipid droplets are broken into smaller lipid droplets by contractile activity at the lower portion of the stomach and small intestine. Phospholipids and bile salts, which are secreted in the bile, serve as emulsifying agents and prevent the smaller droplets from reaggregating back into large droplets (Figure 1.2-1). Fat digestion occurs almost entirely in the small intestine. Fats are mostly digested by pancreatic lipase, which breaks the ester bond at the first and third carbon atoms of triglyceride, releasing two free fatty acids and a monoglyceride. Since pancreatic lipase is a water-soluble enzyme, it can only digest lipids at the surface of a lipid droplet. The emulsification process greatly increases lipid droplet surface area, providing access to the lipase and accelerating the rate of digestion.
The free fatty acids and monoglyceride then cluster together with bile salts and phospholipids to form micelles with the polar ends of each molecule oriented toward the surface and the nonpolar portions toward the core. When micelles break down, their contents diffuse across the luminal plasma membranes of the epithelial cells. During their passage through the epithelial cells, fatty acids and monoglycerides are resynthesized into triglycerides in the smooth endoplasmic reticulum and aggregates into small lipid droplets. The droplets are secreted in vesicles, which then fuse with the plasma membrane and releases chylomicrons into the interstitial fluid. The chylomicrons pass into lacteals, lymphatic capillaries in the intestinal villi, enter the lymph and eventually empty into systemic veins via the thoracic duct(13).
Figure 1.2-1 Fat absorption across the walls of the small intestine (from (13))
1.2.2. Lipid synthesis and regulation

Normally, the contribution of DNL to total hepatic TAG accumulation is small and accounts for only about 5% of FAs incorporated into secreted VLDL-TAG (14, 15) in healthy people; In patients with NAFLD, the contribution of DNL increases to 15% and 23% of FAs within hepatic triglycerides and VLDL-TAG (12, 15). Moreover, DNL pathway also serves as a metabolic regulator by affecting other pathways like FA oxidation. For example, Omega-3 PUFA (polyunsaturated fatty acids) synthesized by DNL and their metabolites are endogenous ligands for PPARα, which stimulates fatty acid uptake and oxidation. Also, malonyl-CoA, the precursor for DNL, inhibits CPT-1 (carnitine palmitoyltransferase 1 activity) and reduces FA β-oxidation rate (16).

1.2.2.1. Lipid synthesis

The general nomenclature of fatty acids is showed in Figure 1.2-2. Fatty acids that are referenced in this thesis are listed in Table 1.2-1.

![Fatty acid nomenclature](image)

n= chain length = 18 carbons
x= 2 double bonds
n-y= n-6 position of the first double bound from the methyl end

**Figure 1.2-2. Fatty acid nomenclature.**

As an example, the structure of linoleic acid (C18:2, n-6) is given.
Table 1.2-1. Common names of various fatty acids referred in thesis

<table>
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<tr>
<th>Class</th>
<th>Symbol</th>
<th>Common Name</th>
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<tr>
<td>Saturated fatty acids</td>
<td>C16:0</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td></td>
<td>C18:0</td>
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<td>C22:0</td>
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<td></td>
<td>C24:0</td>
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<td>Monounsaturated fatty acids</td>
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<td>Palmitoleic acid</td>
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<tr>
<td></td>
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<td>Sapienic acid</td>
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<tr>
<td></td>
<td>C18:1 (n-9)</td>
<td>Oleic acid</td>
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<tr>
<td></td>
<td>C20:1 (n-9)</td>
<td>Gadoleic acid</td>
</tr>
<tr>
<td></td>
<td>C22:1 (n-9)</td>
<td>Erucic acid</td>
</tr>
<tr>
<td>n-6 and n-3 polyunsaturated fatty acids</td>
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<td>Linoleic acid</td>
</tr>
<tr>
<td></td>
<td>C20:4 (n-6)</td>
<td>Arachidonic acid</td>
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<tr>
<td></td>
<td>C22:5 (n-6)</td>
<td>Docosapentaenoic acid</td>
</tr>
<tr>
<td></td>
<td>C18:3 (n-3)</td>
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</tr>
<tr>
<td></td>
<td>C22:6 (n-3)</td>
<td>Docosahexaenoic acid</td>
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Citrate formed in the Krebs cycle is shuttled to the cytosol and converted to acetyl-CoA by ATP citrate lyase (ACL). ACC1 (acetyl-CoA carboxylase 1) then converts acetyl-CoA to malonyl-CoA, which can by used by FAS (fatty acid synthase) for the sequential 2-carbon elongation reactions that produce palmitic acid (C16:0) in the cytosol (Figure 1.2-3).
Figure 1.2-3. Pathways for fatty acid biosynthesis.

(bold, italic letters) Enzymes: ACC, Acetyl-coenzyme A carboxylase; ACL, ATP citrate lyase; CoA, coenzyme A; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; ME, malic enzyme.

Palmitic acid can be elongated by the long chain fatty acyl elongase (ELOVL) to form stearic acid (C18:0). Both palmitic acid and stearic acid can be desaturated by SCD1 (stearoyl-CoA desaturase 1) to form palmitoleic acid (C16:1, n-9) and oleic acid (C18:1, n-9) (Figure 1.2-4). Oleic acid (37~49.7% mol%), palmitic acid (18.1~23.5 mol%) and linoleic acid (C18:2, n-6), (8.6~24.9 mol%) are the most abundant fatty acids of TAG in human adipose tissue(17).
1.2.2.1.1. Acetyl-CoA Carboxylase (ACC)

ACC catalyzes the first committed step in fatty acid synthesis. There are two isoforms of ACC in rodents and humans. ACC1 is highly enriched in the cytosol of hepatocytes and adipocytes, whereas ACC2 is located in mitochondria membrane in heart, skeletal muscle and liver (19). ACC1 generated malonyl-CoA is used by FAS as a 2-carbon donor for fatty acid synthesis in the cytosol. In contrast, ACC2 generates malonyl-CoA in the vicinity of CPT1 in mitochondria to allosterically inhibit CPT1, a protein responsible for transport of long chain FAs into mitochondria for β-oxidation (16). Thus, the intermediate of fatty acid synthesis inhibit the generation of acetyl CoA from oxidation to prevent fatal cycling.

ACC is regulated by three different mechanisms. 1) At the transcriptional level, ACC is regulated by SREBP-1c, ChREBP and LXR (20-22). Insulin induces LXR, which transcriptionally stimulates SREBP-1c (23). SREBP-1c then promotes the
transcription of ACC. Glucose activates ChREBP independent of insulin. ChREBP then induces lipogenic genes including ACC(24). 2) The short-term control of ACC activity is achieved via reversible phosphorylation and allosteric regulation. Phosphorylation of ACC by AMPK inhibits enzyme activity(25), whereas dephosphorylation of ACC by insulin signaling in the fed state increases enzyme activity(26). 3) Citrate generated in Krebs cycle is a feedforward activator of ACC with different potency for ACC1 and ACC2 (Ka is around 2mM)(27, 28). Citrate is converted by ACL (ATP citrate lyase) to acetyl-CoA, which is the substrate of ACC. High concentration of citrate induces the polymerization of ACC and thereby increases ACC activity. However, the concentrations of citrate required to increase ACC activity (> 5 mM) are much higher than its physiological concentration in cells (0.1-1mM)(29, 30). A novel regulator of ACC named MIG12 has recently been identified (31). MIG12 expression is regulated by SREBP-1c in a manner similar to other genes involved in fatty acid synthesis. MIG12 binds to ACC and significantly lowers the threshold for citrate activation into the physiological range (< 1 mM). Overexpression of MIG12 in liver induces ACC polymerization, increases FA synthesis, and results in TG accumulation and fatty liver.

ACC1 deletion is embryonically lethal(32). Liver specific ACC1 deletion of (LACC1KO) provided direct evidence that ACC1 is the principal pathway for conversion of acetyl–CoA to malonyl–CoA. LACC1KO mice displayed a 70-75% reduction in ACC activity and malonyl-CoA levels. When challenged with a fat-free diet, these mice showed significant decrease in de novo fatty acid synthesis and TAG accumulation in the liver, despite upregulation of PPARγ and lipogenic genes(33).

In contrast, ACC2−/− mice have a normal life span, but eat more food and gain less weight than their WT controls due to increased fatty acid oxidation rates(16). ACC2−/− mice are resistant to high fat/high carbohydrate diet induced obesity and insulin resistance(34).

In rats with high-fat diet-induced hepatic steatosis and insulin resistance, liver-specific ASO inhibition of both ACC1 and ACC2 expression reduces hepatic TAG, long
chain acyl-CoA and DAG (diacylglycerol) content and improves hepatic insulin sensitivity(35). This study suggested a promising therapeutic effect of ACC inhibition for treating multiple metabolic disorders.

1.2.2.1.2. Fatty acid synthase (FAS)

FAS catalyzes the biosynthesis of saturated fatty acids myristate (C14:0), stearate (C18:0) and mainly palmitate (C16:0) by using malonyl-CoA as substrate(36). FAS functions as a homodimer. Each monomer is about 260 kDa in size and contains eight functional domains. Six harbor the enzyme activities required for the initiation of synthesis and the elongation of fatty acid chains by two carbon increments: acyl transferase, β-ketoacyl synthase, β-ketoacyl reductase, β-hydroxylacyl dehydrase, enoyl reductase, and β-ketoacyl synthase. One domain, thioesterase, represents the enzyme activity required for releasing the mature fatty acid from the enzyme. The eighth domain represents the acyl carrier protein, the site to which the growing fatty acid is tethered during synthesis.

Deletion of FAS results in embryonic lethality(37). Liver specific FAS knockout mice, when challenged with low fat diet, develop hypoglycemia and hepatic steatosis due to a reduction in β-oxidation. These phenotypes were corrected with a PPARα agonist, which enhances fatty acid β-oxidation(38).

1.2.2.1.3. Stearoyl-CoA desaturase (SCD)

SCD, also known as D9D (delta9 desaturase) is essential for the synthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA) (39). The enzyme introduces the first cis-double bond at the 9,10 position from the carboxyl end of fatty acyl-CoA with 12 to 19 carbon chains. The desaturation reaction takes place in ER and involves cytochrome b₅, NADH (P)-cytochrome b₅ reductase, and molecular oxygen.

Four isoforms of SCD have been cloned and characterized in mice (SCD1-4), whereas only two human SCD genes have been identified(40-45). In adult mice, SCD1 is the predominant isoform in most lipogenic tissues and is markedly induced
in liver in response to high carbohydrate diet(40, 41, 46). SCD2, was recently shown to be required for adipogenesis in 3T3-L1 cells due to a requirement for adequate levels of peroxisome proliferator-activated receptor (PPAR)-γ(47). Human SCD1 shares 85% sequence identity with mouse SCD1 genes, while human SCD5 shares limited identity to mouse SCD genes and is unique to primates(45, 48). The main products of SCD are oleic acid (C16:1) and palmitoleic acids (C18:1), which are the major MUFA in fat depots and membrane phospholipids.

SCD1 deficient mice (asebia mice, homozygous for a natural mutation of SCD1 gene) display low levels of hepatic cholesterol esters and triglycerides, have loss of body weight, hypoglycemia and hypercholesterolemia in response to high-carbohydrate feeding(49-51). SCD is required for cholesteryl ester (CE) synthesis in liver, as evidenced by the fact that dietary supplementation of C18:1 or C16:1 to SCD1−/− mice failed to restore the C18:1 and C16:1 levels of cholesterol ester despite the presence of normal activities of acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme responsible for cholesterol ester synthesis in liver (49). SCD1 deficient mice also have increased energy expenditure, enhanced fatty acid oxidation and thermogenesis in liver, muscle, and brown adipose tissue(52-54). SCD1−/− mice are thus protected from both high-fat diet and leptin deficiency-induced obesity and hepatic steatosis(55, 56). However, SCD1 deficiency exacerbates diabetes(55).

SCD1 is considered as one of the main factors preventing the progression of simple steatosis to steatohepatitis(57). The mechanism of action may be based on the fact that monounsaturated free fatty acids are necessary for normal rates of synthesis of TAG and esterification of free cholesterol (58). MUFA are more easily incorporated into triglycerides and then safely stored in lipid droplets than saturated free fatty acid, thus reducing the risk of lipotoxicity(59-61). Moreover, endogenously synthesized MUFA, which are the preferred substrates for ACAT, are utilized to esterify free cholesterol, thus reducing its lipotoxicity and increase the availability of cholesterol esters for secretion in the form of VLDL(49).
SCD1 gene expression is regulated by number of nutrients (i.e. glucose and fatty acids) and hormones (i.e. insulin, leptin, thyroid hormone). SCD1 expression is positively regulated by LXR by directly binding an LXR response element in the SCD1 promoter, and indirectly via LXR-mediated activation of SREBP-1c(62-64). Cholesterol supplementation in the diet can induce SCD1 mRNA levels and enzyme activity(39). Dietary carbohydrates increase hepatic SCD1 through ChREBP and insulin stimulated activation of SREBP1c (21, 65). Dietary n-3 and n-6 PUFA suppress SCD1 gene expression by decreasing the binding of SREBP-1 with the SREBP response element of SCD1 promoter and repression of SREBP-1c maturation(39). In contrast, saturated fatty acids can induce SCD1 expression, in a mechanism that may involve fatty acid upregulation of PPARγ coactivator 1β and subsequent coactivation of SREBP-1c and LXR(39, 66, 67). Leptin, a key regulator of energy homeostasis and satiety, represses the expression of the SCD1 gene(56).

1.2.2.2. Biosynthesis of polyunsaturated fatty acids (PUFA)

In the eukaryotic cell, fatty acids synthesized from DNL in the cytosol and absorbed from the diet can be further desaturated and/or elongated into long-chain (C16, 18) and very-long-chain fatty acids (C≥20, VLCFA) with different of degrees of saturation. PUFA, such as arachidonic acid (AA, C20:4, n-6) and docosahexaenoic acid (DHA, C22:6, n-3) are important fatty acids because they are required for various physiological functions such as maintenance of membrane fluidity, regulation of lipid metabolism and eicosanoid signaling(68-70). Due to the lack of either delta12 desaturase or delta15 desaturase, mammals cannot synthesize PUFAs from malonyl CoA. Thus, C18:2 (n-6) and C18:3 (n-3) are essential fatty acids that must be supplied from the diet to serve as precursors for PUFA biosynthesis. The synthesis of PUFA requires various desaturases and elongases in the endoplasmic reticulum (ER) and β-oxidation enzymes in peroxisomes (71).

D6D is the rate-determining enzyme in LCPUFAs synthesis, catalyzing the first step in the pathway for both n-3 and n-6 families and the final desaturation step of DHA synthesis (Figure 1.2-4). Dietary linoleic acid (9, 12-cis-C18:2) is desaturated
by D6D at delta 6 position, sequentially elongated by two carbons to become 8, 11, 14-\textit{cis}-C20:3. C20:3 is then desaturated by D5D, to generate AA (5, 8, 11, 14-\textit{cis}-C20:4, n-6).

The synthesis of DHA (4, 7, 10, 13, 16, 19-\textit{cis}-C22:6) shares a pathway with 20:4, n-6 up to the 20:5 n-3 step\cite{72}. After two cycles of elongation, D6D converts 9, 12, 15, 18, 21-C24:5 to 6, 9, 12, 15, 18, 21-C24:6. C24:6 is then shorted by peroxisomal \(\beta\)-oxidation to yield DHA (4, 7, 10, 13, 16, 19-\textit{cis}-C22:6, n-3).

1.2.2.2.1. Desaturases

Delta-6 desaturase (D6D, FADS2: fatty acid desaturase 2) and delta-5 desaturase (D5D, FADS1: fatty acid desaturase 1) are required for PUFA synthesis in mammals. Both D6D and D5D are membrane-bound, front-end desaturase because it introduces a double bond between the pre-existing double bond and the carboxyl (front) end of the fatty acid. Human D5D gene shares 61% amino acid identity and 75% similarity with the human D6D\cite{73, 74}. Human D5D and D6D genes are localized in chromosome 11 (11q12-q13.1) as a cluster in a head-to-head orientation\cite{18}. Both desaturase are widely expressed in human tissues, with the highest levels in liver.

PUFA levels in serum phospholipids in healthy adults are maintained in a narrow range despite differences in precursor PUFA intake\cite{75}. Animal studies yielded similar results, suggesting that the activity of desaturases is tightly regulated\cite{76, 77}. Indeed, desaturases are primarily regulated at transcription level by SREBP-1c by binding with SRE in their promoters\cite{20, 78}. Both n-3 and n-6 PUFAs can suppress D6D and D5D through inhibition of SREBP-1c. Dietary PUFAs can reduce the active, nuclear form SREBP-1c and reduce SREBP-1c mRNA stability\cite{79-82}. Both precursors and products of D5D and D6D are able to suppress SREBP-1c. The potency of unsaturated fatty acids increases with increasing chain length and degree of unsaturation. Therefore, the products of desaturases, known as highly unsaturated fatty acids (HUFA), are more potent than the precursors \cite{79}. 
Desaturase activity is also suppressed by low levels of circulating insulin\(^{18, 83}\) and excess of steroids\(^{84}\), both of which are also mediated by inhibition of SREBP-1c.

D6D is also controlled by PPAR\(\alpha\). D6D expression in rat liver can be induced by PPAR\(\alpha\) ligands such as fibrates and Wy14643\(^{85-87}\). Similarly, long-chain NEFA are considered endogenous ligands for PPAR\(\alpha\) and can induce D6D\(^{88, 89}\). It has been shown that D6D was not induced in PPAR\(\alpha\)^{−/−} mice by essential fatty acid deficient diet, indicating the requirement of PPAR\(\alpha\) for PUFA feedback regulation of D6D\(^{90}\).

1.2.2.2.2. Elongases

PUFA synthesis requires elongation of C18 fatty acids by elongases localized in ER\(^{91}\). Microsomal fatty acid elongation adds two-carbon units to fatty acyl-CoA using malonyl-CoA as the donor and NADPH as the reducing agent\(^{92}\). Fatty acid elongation is achieved with four sequential reactions (Figure 1.2-4)\(^{93}\):

- condensation between fatty acyl-CoA and malonyl-CoA to generate 3-ketoacyl-CoA;
- reduction of 3-ketoacyl-CoA using NADPH to form 3-hydroxyacyl-CoA;
- dehydration of 3-hydroxyacyl-CoA to \textit{trans}-2-enoyl-CoA;
- and reduction of \textit{trans}-2-enoyl-CoA to fully elongated \(n+2\) fatty acyl-CoA. Each reaction is catalyzed by a different enzyme that is encoded by separate gene.
Elongation of very long chain fatty acids is catalyzed by elongases (ELOVLs) (92, 94, 95). Seven ELOVL enzymes (ELOVL1-7) have been identified in mammals(96-100). Each ELOVL has a distinct tissue distribution and exhibits different substrate preferences. ELOVL1 and ELOVL3 catalyze the elongation of very long chain (C>20) saturated and monounsaturated fatty acids(101). ELOVL2 elongates C20 and C22 PUFAs(98, 102) and ELOVL4 elongates fatty acids with chain longer than C26(103, 104). ELOVL6 catalyzes the elongation of long chain (C12-C16) saturated and monounsaturated fatty acids. ELOVL5 is involved in the elongation of C18 and C20 PUFAs(105-107).
PUFA synthesis requires ELOVL5 and ELOVL2 (92). However, ELOVL2 is less abundant, shows very little sensitivity to metabolic mediators and increases its transcription level only as a result of the overexpression of SREBP-1c (107). ELOVL5 is expressed in all tissues tested to date, with the highest levels in liver, testis and adrenal gland (96). Overexpression of ELOVL5 in rat primary hepatocytes promotes elongation of C20:4, n-6 and C20:5, n-3 to C22:4, n-6 and C22:5, n-3, respectively (108). ELOVL5 transcription is regulated by SREBP-1c and LXRα. Similar to the regulation of D5D and D6D, ELOVL5 is transcriptionally activated by LXRα through SREBP-1c. PUFAs are also able to suppress ELOVL5 expression by suppressing SREBP-1c (80, 109). ELOVL5−/− mice have elevated TAG level in liver and develop hepatic steatosis (110). This is due to the impaired synthesis of PUFA and loss of PUFA mediated inhibition on SREBP-1c activation and its target genes. Dietary supplementation of AA plus DHA reversed the phenotypes in these mice, highlighting the importance of maintaining constant level of PUFAs (80).

1.2.2.2.3. Peroxisomal β-oxidation

The last step of DHA synthesis requires chain-shortening of C24:6 by peroxisomal β-oxidation. This will be discussed in detail in section 1.2.4.2

1.2.2.3. Regulation of de novo lipogenesis (DNL)

1.2.2.3.1. Nuclear transcription factors

1.2.2.3.1.1. SREBP

Sterol regulatory element-binding proteins (SREBPs) are a family of helix-loop-helix leucine zipper transcription factors. SREBPs regulate genes involved in fatty acids, cholesterol biosynthesis and adipocyte differentiation (20, 111-113).

Three isoforms of SREBPs have been identified: SREBP-1a and -1c and -2. SREBP-1a and -1c are encoded by a single gene, generated through alternative splicing, and only differ in the first exon (20, 113, 114). SREBP-1a and -1c promote lipogenesis by upregulating genes involved in fatty acid synthesis such as ACC, FAS and SCD-1 (115). SREBP-1a is the predominant expression form in cultured cells
and liver, while SREBP-1c is predominantly expressed in most of the tissue of mice and human, including liver, adipose tissue, adrenal gland and brain. SREBP-2 is encoded by different gene and regulates cholesterol synthesis genes including HMG-CoA reductase and HMG-CoA synthase.

The precursor SREBPs contain three domains: 1) NH$_2$ terminal of about 480 amino acids (function as a transcriptional factor that can bind with DNA in nucleus when released); 2) two transmembrane domain connected by short loop of 30 amino acids; and 3) COOH terminal of about 590 amino acids which is involved in its regulation.

The precursor of SREBPs binds with SCAP (SREBP cleavage activating protein) and Insig and is trapped in ER membrane(113). SCAP functions as a sensor for cholesterol concentration, when cholesterol concentration is low, Insig dissociates from SCAP and SREBPs, SCAP then escorts SREBPs to Golgi, where it can be sequentially cleaved by protease (site-1 protease, S1P and site-2 protease, S2P) and releases the active NH$_2$ terminal of the protein. The nuclear SREBPs (nSREBPs) translocates to nucleus, binds with sterol response element (SRE) in the promoter/enhancer regions of its target genes and activates their transcription. This regulation mechanism only occurs to SREBP-1a and SREBP2, while SREBP-1c is regulated differently.

SREBP-1c is induced by LXR$\alpha$. LXR$\alpha$ is a transcription factor that is highly expressed in liver and activated endogenously by oxysterol to induce expression of genes involved in cholesterol efflux and clearance(116, 117). Animals lacking LXR$\alpha$ showed a reduced expression of SREBP-1c and lipogenic genes like FAS (115), ACC and SCD-1(116, 118). Animals fed with high cholesterol diet or LXR$\alpha$ agonist showed enhanced SREBP-1c transcription and nSREBP-1c processing, as well as increased lipogenic gene expression(117, 119). This effect of LXR$\alpha$ is mediated by binding of LXR/RXR with LXR response element in promoter region of SREBP-1c and other genes(120).
SREBP-1c expression is also sensitive to energy status. Its mRNA is suppressed during the fasted stage and upregulated when animals are refeed\(^{(121, 122)}\). This induction of SREBP-1c after refeeding is mediated by insulin. In rat primary hepatocytes, expression of a dominant negative SREBP-1c blocked the effect of insulin on transcriptional activation of genes involved in fatty acid synthesis, whereas expression of dominant positive SREBP-1c mimicked the insulin effect\(^{(123)}\).

Insulin is secreted by pancreas in response to elevated plasma glucose, to stimulate glucose uptake, lipogenesis and suppress gluconeogenesis. The insulin signaling pathway is initiated by binding of insulin with its receptor, followed by receptor-mediated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and/or IRS-2. IRS-1/2 can then activate PI3K, which activates Akt. The inhibition effect of insulin on gluconeogenesis is mediated through IRS-2, while the lipogenic effect is mediated through IRS-1. The induction of SREBP-1c transcription by insulin is dependent on the integrity of two LXR\(\alpha\) binding sites located in the promoter of SREBP-1c\(^{(23, 124, 125)}\). In insulin resistant mice, despite the disinhibition of glucose production by downregulated IRS-2, Induction of SREBP-1c and its target lipogenic genes is still active, promotes fatty acid synthesis and accelerates TAG accumulation. The combination of these two actions leads to hyperglycemia and hypertriglyceridemia which characterizes fatty liver and diabetic states\(^{(126, 127)}\).

Studies of knockout and transgenic mice of each SREBP isoform have provided information of their function. Knockout of SREBP-1a or SREBP-2 is embryonic lethal, whereas knockout of SREBP-1c is not\(^{(128)}\). When both SREBP-1a and -1c are eliminated, SREBP-2 mRNA and protein is overexpressed to compensate the loss of SREBP-1a and -1c\(^{(64, 129)}\).

Overexpression of SREBP-1a in mice leads to hepatic steatosis characterized by a 26-fold increase in triglyceride and a 5-fold increase in cholesterol synthesis rates. The massive accumulation of TAG and cholesterol in these mice indicates that SREBP-1a is able to regulate genes controlling both TAG and cholesterol synthesis,
but favors TAG synthesis(114, 130). SREBP-1c transgenic mice also develop fatty liver disease, but with only increase in TAG and no changes in cholesterol synthesis, suggesting that, as the predominant isoform of SREBP in liver of mice and human, SREBP-1c exclusively controls TAG synthesis(131, 132). SREBP-1c is elevated in the fatty liver of insulin resistant, ob/ob mice(126, 133). Knockout of SREBP-1c in ob/ob mice markedly attenuates the fatty liver disease by reducing approximately 50% of hepatic triglycerides(134). SREBP-2 transgenic mice only show upregulation of the cholesterol biosynthesis pathway (75 fold increase) and much less upregulation in fatty acid synthesis (4 fold increase), suggesting that SREBP-2 is predominantly a regulator of cholesterol biosynthesis rather than TAG(135).

1.2.2.3.1.2. ChREBP

Dietary carbohydrate consumption results in elevated blood glucose levels which stimulates insulin secretion from pancreas β-cells. Glucose and insulin synergistically induce glycolytic and lipogenic gene expression. Liang et al, observed that in SREBP-1c deficient mice fed with carbohydrate diet, which fail to upregulate lipogenic genes in response to insulin, there was only 50% reduction in fatty acid synthesis rate, suggesting the existence of another insulin independent, glucose triggered regulatory pathway(64). This led to the discovery of a basic/helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor named ChREBP (carbohydrate responsive element binding protein)(136).

ChREBP is a large protein (864 amino acids) that contains several domains including nuclear export and import signals, a DNA binding motif of the bHLH/LZ type, and proline-rich regions implicated in protein-protein interactions. ChREBP is predominantly expressed in liver, kidney, and both white and brown adipose tissue(137, 138). ChREBP stimulates the expression of most lipogenic genes and L-PK (liver-type pyruvate kinase), a key regulator in glycolysis that catalyzes the conversion of PEP (phosphoenolpyruvate) to pyruvate.
ChREBP is regulated in a reciprocal manner by glucose and cAMP\(^{(139)}\). At low glucose concentrations, the inactive phosphorylated ChREBP is localized in the cytosol, when stimulated by high glucose concentration, it interacts with Mlx(Max-like protein) to form a heterotetramer, translocates to the nucleus, binds to ChoRE (carbohydrate response element) elements and promotes transcription of its target genes\(^{(136)}\).

Both liver and primary hepatocyte from ChREBP\(^{-/-}\) mice showed decreased lipogenic gene expression and reduced fatty acid synthesis rates, suggesting the essential role of ChREBP in liver to utilize glucose for DNL\(^{(21, 140)}\). ChREBP gene expression and nuclear protein content were markedly increased in the liver of ob/ob mice under both fasted and fed conditions. Liver specific inhibition of ChREBP in ob/ob mice markedly improved hepatic steatosis by decreasing lipogenic rates, decreasing plasma TAG and NEFA levels after 7 days of adenoviral delivery of a shRNA against ChREBP. Insulin signaling in liver, skeletal muscle and white adipose tissue and overall glucose tolerance were also restored\(^{(141)}\).

1.2.2.3.1.3. PPARs

Peroxisome proliferator-activated receptors (PPARs) were initially characterized for their capacity to increase peroxisome numbers in the livers of mice and rats in response to fibrates or xenobiotics. Three isoforms of PPAR have been cloned: 1) PPAR\(\alpha\) is mainly expressed in liver, digestive tract and kidney; 2) PPAR\(\beta\), \(\delta\) or NUC-1 (respectively cloned in Xenopus, mouse and human) are ubiquitously expressed and often at higher levels than PPAR\(\alpha\) and \(\gamma\); and 3) PPAR\(\gamma\) (\(\gamma 1, \gamma 2, \gamma 3\) arising from an alternative splicing of a single gene) is mainly expressed in adipose tissue and in macrophages.

Like LXR\(\text{s}\), PPAR\(\text{s}\) are part of the nuclear receptor superfamily. PPAR\(\text{s}\) form heterodimers with the retinoid X receptor (RXR). The PPAR/RXR heterodimers, when bound to a ligand, change conformation and bind to specific response elements located in the 5’ end region of their target genes resulting in gene transcription\(^{(142)}\).
PPARα is expressed in the liver and other metabolically active tissues including striated muscle, kidney and pancreas(143, 144). Endogenous PPARα ligands include n-3 PUFAs and eicosanoids. PPARα regulates both mitochondrial and peroxisomal fatty acid oxidation by directly controlling the expression of key enzymes. In mitochondria, its target genes include acyl-CoA synthetase, the carnitine palmitoyltransferase I (CPT-1), the very long-chain acyl-CoA dehydrogenase and the tri-functional protein encoding genes(145-148). In peroxisomes, PPARα activates the transcription of acyl-CoA synthetase, the straight-chain acyl-CoA oxidase, the L-bifunctional protein and the 3-ketoacyl-CoA thiolase(147-150).

Transgenic mice over-expressing PPARα in the muscle exhibit increased fatty acid oxidation(151). Bezafibrate, a potent PPARα agonist, prevents liver TAG accumulation and improves hepatic steatosis in mice fed a methionine- and choline-deficient diet(152). PPARα−/− mice develop hepatic steatosis during fasting or high fat diet feeding due to the insufficient fatty acid oxidation accompanied by increased lipolysis and fatty acid delivery to liver(153, 154).

There are important differences in PPARα activity between rodents and humans. PPARα DNA binding activity and PPARα expression in human hepatocytes is less than 10-fold that observed in mice(155, 156). Despite the beneficial effects of PPARα activation on hepatic lipid homeostasis in rodent models, the similar effects of PPARα in human NAFLD patients are either minimal or not seen(157-159).

n-3 PUFAs present in fish oil, and their metabolites are potent endogenous ligands of PPARα. PUFAs inhibit de novo lipogenesis by antagonizing activation of LXR, thus reducing expression of SREBP-1c. n-3 PUFA supplementation in mice improves hepatic steatosis and insulin sensitivity, decreases fasting NEFA concentrations and serum TAG levels(160). Similarly, a number of pre-clinical and clinical studies have demonstrated an ameliorative effect of supplemental fish oil, seal oil and purified LC-n-3 FAs in reducing hepatic lipid content in NAFLD(161, 162).
PPARγ is highly expressed in adipose tissue and to a lesser degree in hepatocytes. PPARγ is involved in adipocyte differentiation, lipid storage in adipocytes and insulin sensitivity regulation.

PPARγ promotes adipocyte differentiation and expression of proteins in adipocytes involved in fatty acid uptake, fatty acid transport and fatty acid synthesis(160). PPARγ promotes lipid storage in adipocytes by inducing the expression of lipogenic genes such as fatty acid synthase(163). PPARγ also promotes the release of fatty acids from lipoproteins and their uptake into adipocytes(164). PPARγ increases insulin sensitivity by upregulating GLUT4, an insulin dependent glucose transporter in adipose tissue and skeletal muscle(165).

Patients with dominant negative mutations in PPARγ have NAFLD and lipodystrophy, suggesting increased lipid delivery to the liver(166). Liver specific PPARγ deficient mice are protected against the development of steatosis suggesting a critical role for hepatic PPARγ in liver triglyceride accumulation(167, 168).

Thiazolidinediones (TZD), ligands for PPARγ have been shown to improve glycemic control in patients with type 2 diabetes mellitus by increasing insulin sensitivity in adipose tissue, liver and skeletal muscle(169-171). Rosiglitazone and pioglitazone treatment are associated with reduction in aminotransferase levels and improvement in liver histology in patients with NAFLD(169, 172-175).

PPARδ has been implicated in energy consumption in peripheral tissues by controlling β-oxidation. Skeletal muscle cells are an important site of action where PPARδ regulates genes such as FABP3, lipoprotein lipase, FAT, CPT-1, long-chain acyl-CoA dehydrogenase and uncoupling proteins (UCPs)(176). Activation of PPARδ by fatty acids enhances fatty acid transport and oxidation, improves glucose homeostasis via inhibition of hepatic glucose output, reduces macrophage inflammatory responses, and dramatically increases circulating high density lipoprotein levels(10). Several studies have demonstrated the potential of agonists
to target multiple components of the metabolic syndrome including obesity, dyslipidemia, hyperglycemia, insulin resistance, and NAFLD(152, 177).

1.2.2.3.1.4. LXR

LXRs are nuclear transcriptional factors that can sense elevated intracellular cholesterol levels in the form of oxysterols (22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol and 24(S), 25-epoxycholesterol) in hepatocytes and other cell types(178). When activated, LXR forms heterodimers with the RXR, which bind to LXR-responsive elements (LXREs) in DNA consisting of direct repeats (DRs) of the core sequence AGGTCA separated by 4 nucleotides. (DR4)(179).

There are two LXRs: LXRα and LXRβ, which share considerable sequence homology and respond to the same ligands(180). LXRα is highly expressed in the liver, adipose tissue and macrophages. Whereas LXRβ is ubiquitously expressed(143).

LXRs play a critical role in cholesterol homeostasis by regulating a set of genes that participate in reverse cholesterol transport, the process transports excess cholesterol from peripheral tissue to the liver for elimination(181). This effect of LXRs is dependent on the ability of LXRs to induce the expression of members of the ABC superfamily of membrane transporters that are involved in the efflux of cholesterol, including ABCA1, ABCG5 & G8, and ABCG1(182-188). Activation of LXRs with a synthetic ligand increases HDL levels and net cholesterol secretion in vivo (182).

In addition to their ability to modulate cholesterol metabolism, LXRs are also key regulators of hepatic lipogenesis. LXRα directly activates ACC, FAS, SCD-1 and SREBP-1c transcription(22, 117, 120). Administration of synthetic LXR agonists in mice elevates triglyceride levels in the liver as well as transiently in the plasma(22, 117). Activation of LXRα is associated with massive liver steatosis and larger VLDL with a 2.5-fold increase in their serum levels(189). These effects pose a significant
obstacle for the development of LXR agonists as human therapeutics. LXRα also induces several enzymes involved in lipoprotein remodeling, including lipoprotein lipase, human cholesterol ester transport protein (CETP), and the phospholipid transfer protein (PLTP)\(^\text{190}\).

LXRα\(^/-\) mice display marked cholesterol ester accumulation in livers when challenged with a cholesterol-rich diet\(^\text{118}\). LXR deficient mice are resistant to obesity when challenged with a high-fat, high-cholesterol diet. Remarkably, this phenotype was dependent on the presence of cholesterol in the diet and was largely attributed to increased peripheral utilization of dietary fat as manifested by a marked enhance metabolic rate\(^\text{190, 191}\).

1.2.2.3.2. Long chain PUFAs and nonalcoholic fatty liver disease (NAFLD)

1.2.2.3.2.1. Decreased bioavailability of PUFAs in NAFLD

A growing body of evidence has demonstrated the importance of long-chain PUFAs (LCPUFAs) in the regulation of energy metabolism\(^\text{192, 193}\). Reduced bioavailability of LCPUFAS have been associated with pathologies of many metabolic diseases, such as obesity\(^\text{194}\), type II diabetes\(^\text{195}\) and NAFLD\(^\text{196, 197}\). Of particular biological interest are three specific LCPUFAs, arachidonic acid, an omega-6 (n-6) fatty acid and omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and DHA\(^\text{198}\).

It has been reported that hepatic TAG accumulation resulting from steatosis is associated with a decrease of EPA in liver\(^\text{199}\). Other studies also supported the notion that NAFLD patients have significant decreases in hepatic levels of n-3 and n-6 LCPUFAs, highlighting the importance of maintaining adequate levels of PUFAs\(^\text{57, 196, 200, 201}\).

Araya J. et al. analyzed fatty acid composition of liver and adipose tissue from 11 control subjects and 19 NAFLD patients, within which 10 patients were grouped as steatosis with the presence of macrovesicular steatosis alone in liver histology, and
9 patients are grouped as steatohepatitis with steatosis and lobular inflammation with hepatocyte ballooning in liver histology (184). They reported a depletion in the level of n-6 (32% decrease in steatosis patients and 36% decrease in steatohepatitis patients) and n-3 (76 and 86% decrease) LCPUFAs in liver total lipids, with decreased C20:4, n-6/C18:2, n-6 and (C20:5, n-3 + C22:6, n-3)/18:3, n-3 ratios, indicating the impairment in PUFA synthesis. An elevation in MUFA but not SFA was also observed in NAFLD patients. Consistently, Puri et al reported a decrease in 20:4, n-6 in FFA, TAG and PC, C20:5 n-3 and C22:6 n-3 in TAG of NAFLD patients(188). The decrease in levels of PUFAs could be due to several reasons: 1) decrease in ingestion of essential fatty acid precursors, 2) impaired PUFA biosynthesis and 3) increased degradation of PUFA.

As discussed in section 4.2, biosynthesis of PUFA depends on the enzyme activity of desaturases, elongases and peroxisomal β-oxidation. In obese mice (lep ob/ob) with NAFLD, the activity of both elongases is not impaired. This suggests that the elongation continues to occur during NAFLD and that elongases are not responsible for the inhibition of biosynthesis of LCPUFAs(93).

In addition, hepatic desaturases play an important role in the decline of PUFAs and progression of NAFLD. Animal studies have demonstrated an increase in desaturases transcription in mice with type II diabetes and, occasionally, associated with NAFLD(107, 202, 203). Wang et al have studied the regulation of desaturases and elongases in different models of metabolic diseases, including diabetes, obesity and NAFLD(93). Streptozotocin-induced diabetes (a model of type I diabete) showed decline in SCD1 mRNA abundance, but does not affect D5D and D6D expression. Consistently, PUFA levels were not altered in this model. High fat diet induced obesity and NAFLD model correlates with decreased C20:4, n-6 synthesis, but does not affect D5D or D6D expression. In leptin deficiency induced obesity and NAFLD, PUFA content (C18:2, n-6, C20:4, n-6 and C22:6 n-3) was significantly decreased; major desaturases (D5D, D6D and D9D) and elongases (ELOVL-5 and ELOVL6) expression were significantly elevated. These studies directly
demonstrated that alterations in liver fatty acid composition due to changed desaturase and elongase activity greatly contribute to the development of NAFLD.

1.2.2.3.2.2. Dietary PUFAs supplementation improves NAFLD

Dietary LCPUFAs can improve NAFLD(57). Delarue et al. have reported that the ingestion of fish oil rich in n-3 LCPUFAs (1.1 g of EPA + 0.7 g of DHA daily) for 3 weeks stimulated an increase of almost 35% in fatty acid oxidation(204). Moreover, Capanni et al. observed significant improvements in hepatic steatosis and inflammatory parameters in patients treated with EPA plus DHA (1 g, 0.9/1.5 EPA/DHA ratio) daily during 12 months(205). Spadaro et al. also demonstrated that LCPUFAs (1g capsule twice a day) during a 6-month period reduced liver fat content and inflammatory markers and improved IR in NAFLD patients(206).

The possible explanations for the benefits of PUFAs in NAFLD contain both genomic and non-genomic mechanisms(207). For non-genomic mechanisms, incorporation of LCPUFAs into biological membranes increases membrane fluidity(198, 208). An adequately fluid membrane affects the activity of transmembrane enzymes(209, 210) and improves the function of cell surface receptors and signals transduction(211). Accordingly, LCPUFAs increase the number of insulin receptors in the cell membrane in a fluidity dependent manner(212), and enhance the translocation of glucose transporter type 4 (GLUT4) to the cell surface(213-215).

Through genomic mechanisms, PUFAs function as feed-forward activators of fatty acid oxidation through PPARα and feedback inhibitors of DNL through SREBP-1c and ChREBP. This regulatory pattern not only reduces overall hepatic lipid content and VLDL secretion by inhibiting lipogenic genes, but also eliminates excessive very long chain PUFA that may cause oxidative stress or impair membrane integrity by enhancing their oxidation.

The PUFA mediated suppression of SREBP-1c occurs at three different levels (Figure 1.2-6)(79-82). First, PUFAs decrease the transcription of SREBP-1c by
antagonizing ligand-dependent activation of the LXR, a positive regulator of SREBP-1 gene\(216\); second, LCPUFAs reduce the transcriptionally active nuclear form of SREBP-1c by preventing proteolytic processing; third, LCPUFAs reduce SREBP-1c mRNA stability. Similarly, LCPUFAs inhibit ChREBP by promoting ChREBP mRNA decay and decreasing the nuclear active form of ChREBP\(217\). Known as potent endogenous ligands for PPAR\(\alpha\), LCPUFAs directly bind and activate PPAR\(\alpha\), leading to increased rate of hepatic \(\beta\)-oxidation of fatty acids\(218\).

\[
\text{Figure 1.2-6. (n-3) PUFA suppress nSREBP-1c by different mechanisms (207)}
\]

(n-3) PUFA suppress SREBP-1c gene transcription and proteolytic processing. It also enhance mRNA SREBP-1c decay and nSREBP-1c degradation in the proteasome. A decline in nSREBP-1c results in reduced DNL, fatty acid desaturation and elongation, and elevated expression of MTP and PEPCK.
1.2.3. Lipid storage in adipose tissue

TAGs stored in fat cells come from two major routes: lipids synthesized in the liver by DNL and delivered by VLDL and dietary lipids absorbed by small intestine and delivered by chylomicrons (CM) through lymph prior to entering the systemic circulation.

When VLDL and CM travel to adipose tissue capillaries, lipoprotein lipase (LPL) located on luminal face of the capillary endothelium hydrolyzes TAGs and releases NEFA(3). The NEFA then diffuse through endothelial lining to the adipocytes(3).

NEFA enter cells through fatty acid transporters: fatty acid transport protein (FATP), CD36 or diffusion. Once inside, NEFA are rapidly converted to fatty acyl CoA by FATP(219) or fatty acyl CoA synthetases to trap NEFA inside of cells and also maintain concentration gradient for diffusion(220). NEFA and fatty acyl CoA are bound to fatty acid binding protein (FABP) and acyl CoA binding protein (ACBP), proteins that transport fatty acids among intracellular compartments for metabolism(221) or to the nucleus to interact with transcription factors(222). These binding proteins and pathways keep intracellular NEFA and fatty acyl CoA very low(207).

NEFA are highly toxic to cells. Therefore they are esterified to TAGs and packaged into lipid droplets to protect fat cells from the detrimental effects of NEFA (Figure 1.2-7). The lipid droplet is coated with protein such as perilipins to protect esterified FA from lipases(223).

1.2.4. Lipid mobilization and utilization

1.2.4.1. Lipid mobilization from adipose tissue

1.2.4.1.1. Adipose tissue lipolysis

TAGs can be hydrolyzed in a stepwise fashion to diacylglycerol (224) and monoacylglycerol (MAG) (Figure 1.2-7). Complete break down of 1 mole of TAG produces 3 mole of NEFA and 1 mole glycerol.
Figure 1.2-7. Synthesis and lipolysis pathway of DAG and TAG

MGL, monoacylglycerol lipase; MOGAT, monoacylglycerol acyltransferase; HSL, hormone-sensitive lipase; DGATs, diacylglycerol acyltransferase; ATGL, adipose triglyceride lipase.

Lipolysis in adipose tissue is through three major lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Figure 1.2-8).

Figure 1.2-8. Regulation of adipocyte lipolysis

Figure is adapted from (225)
HSL can hydrolyze both TAG and DAG, however, its activity for DAG is 10-fold greater than the activity for TAG in vitro\(^{(226-229)}\). HSL activity is modulated by cAMP levels, catecholamines and insulin through phosphorylation (Figure 1.2-8 & 9). In the resting (i.e., unstimulated) state, HSL is dispersed in the cytoplasm, whereas perilipin coats the lipid droplets and binds to \(\beta\)-hydrolase domain-containing protein 5 (ABHD5 also known as CGI-58), a key co-activator of ATGL. Catecholamines promote cAMP production while natriuretic peptides stimulate cGMP production. cAMP activates PKA while cGMP activates PKG (cGK-I), both leading to phosphorylation of perilipin and HSL. In the activated state, perilipin phosphorylation induces a physical alteration of the lipid droplet surface, which facilitates the action of phosphorylated HSL on TAG hydrolysis.

ATGL is another TAG hydrolase discovered in 2004. It was discovered because lipolysis was not completely abolished in HSL-deficient mice. ATGL shows 10-fold higher affinity for TAG than DAG\(^{(230)}\). However, HSL has higher capacity to break down TAG than ATGL in vitro\(^{(231)}\). ABHD5 is the co-activator of ATGL and essential for ATGL activity. ABHD5 is tightly bound with perilipin A in the basal state. When lipolysis is stimulated, perilipin A is phosphorylated by PKA, ABHD5 disassociates from perilipin A, translocates to the cytosol, and activates ATGL by directly interacting with ATGL\(^{(232-234)}\) (Figure 1.2-9). An inhibitor of ATGL, named as G0S2 (G0/G1 switch gene 2), has been recently identified by Liu et al\(^{(235)}\). G0S2 is co-localized with ATGL in hela cells and inhibits ATGL activity by directly interacting with the patatin-like domain of ATGL.
Figure 1.2-9. Activation of ATGL and HSL

Figure is from (3). ATGL, adipose triglyceride lipase; ABHD5, α/β-hydrolase domain-containing protein 5; GC, guanylyl cyclase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; NEFA, nonesterified fatty acid; NPR-A, natriuretic peptide receptor-A;

MGL is required for the last step of TAG hydrolysis. MGL hydrolyzes the 1or 2-monoacylglycerols with the same specificity, but has no activity on TAG, DAG or cholesteryl esters hydrolysis. Concomitant enhancement of ATGL, HSL and MGL activities is necessary for full hydrolysis of TAG and release of NEFAs and glycerol.

1.2.4.1.2. Regulation of lipolysis

Fasting stimulates lipolysis in adipose tissue to release NEFAs and provide fuel that can be oxidized to generate energy in other metabolic tissues. The most potent lipolysis activator is catecholamines. Catecholamines bind with β-AR (β-adrenergic receptors) in adipocytes, which are coupled to the G-α subunit of the Gs protein. The activated Gs protein stimulates adenylyl cyclase to produce cAMP, and activate PKA.
HSL and perilipin are then phosphorylated by PKA to induce lipolysis (237-239) (Figure 1.2-8 & 9).

Conversely, lipolysis is inhibited during the fed state by insulin, the most important inhibitor of lipolysis. When insulin binds with insulin receptor (IR) (Figure 1.2-8), it causes tyrosine phosphorylation of IR, and activates PI3-K (phosphatidylinositol kinase-3)- PKB/Akt- PDE3B (phosphodiesterase 3B) cascade. PDE3B can degrade cAMP (cyclic AMP) to 5'AMP, terminate cAMP dependent activation of PKA. This results in reduced phosphorylation of HSL and perilipins, and thus decreased lipolysis.

Lipolysis can also be stimulated by inflammatory cytokines secreted from dysfunctional adipose tissue, including IL-6 (interleukin 6) and TNFα (tumor necrosis factor by a paracrine/autocrine mechanism. IL-6 and TNFα production are increased in adipocyte from obese patients and may contribute to increase lipolysis in obesity. IL-6 stimulates lipolysis in human adipocytes and exerts anti-insulin actions (240). TNFα stimulates lipolysis and inhibits insulin-induced glucose transport (225, 241, 242). By binding with TNFα receptor 1, TNFα activates MAP kinases p44/42 and JNK (Jun kinase), which in turn decreases PDE3B, increases phosphorylation of perilipin and induces lipolysis. TNFα also reduces the effect of insulin by inactivating IRS-1 through p42/44 (243).

Insulin resistance is closely related to NAFLD (244-250). Studies have been conducted to identify which insulin-resistance tissues are most critical for the development of this disease. It was logical to assume that insulin resistance in liver plays a major role in the development of NAFLD since this is a liver disease; however, liver-specific insulin receptor knockout (LIRKO) does not cause NAFLD in mice (251). It has now been clearly shown that insulin resistance in adipose tissue is the major contributor for development of NAFLD (244, 250, 252). Failure of insulin to inhibit lipolysis releases excess amounts of NEFA, which overloads the capacity of liver to handle and eventually leads to lipid accumulation.
1.2.4.1.3. Selective mobilization of fatty acids

The hydrophobic NEFA generated by lipolysis must be trafficked from the site of lipolysis (lipid droplet) to the plasma membrane. This process is mainly mediated by FABP4 (fatty acid binding protein 4)(253). A variety of saturated and unsaturated NEFA binds FABP4 at 1:1 ratio with affinities that range from 0.1 to 5.0 µM(254). FABP4 is associated with phosphorylated, activated hormone-sensitive lipase (HSL) on the surface of lipid droplet. The FABP4-FA-HSL complex then translocates to plasma membrane. In FABP4 deficient mice, there is a ~35% reduction in the level of glycerol released from isoproterenol-stimulate lipolysis and a nearly 3-fold increase in NEFA content of adipocytes compared to wild-type mice(255).

The selectivity of fatty acids mobilized from adipose tissue has been studied during fasting in vivo(256, 257) and during stimulated lipolysis in isolated adipocytes(258, 259). The relative mobilization rate for essential fatty acids decreased in the following order: C20:5, n-3>C20:4, n-6>C18:3, n-3>C18:2, n-6>C22:6, n-3. Interestingly, C20:5, n-3 and C20:4, n-6, which are respectively precursors of the 3- and 2-series of prostaglandins, were preferentially mobilized. As a rule, the relative mobilization decreases with increasing chain length for a given degree of unsaturation and increases with increasing desaturation for a given chain length.(3)

1.2.4.2. Lipid oxidation

Most fatty acyl-CoAs are metabolized through β-oxidation, which occurs mainly in mitochondria, but also in peroxisomes. A third of fatty acyl CoAs are metabolized by ω-oxidation by cytochrome P450 in microsomes. In higher eukaryotes, including humans, rats and mice, both mitochondria and peroxisomes are capable of fatty acid β-oxidation, whereas in lower eukaryotic organisms, including yeasts and plants, fatty acid β-oxidation is confined to peroxisomes(260).
1.2.4.2.1. Fatty acid β-oxidation

Mitochondrial and peroxisomal β-oxidation share common mechanism comprised of four steps: dehydrogenation, hydration, dehydrogenation again and finally thiolytic cleavage. Although similar in mechanism, mitochondrial and peroxisomal β-oxidation differ in substrate, function and enzymatic machinery (Figure 1.2-10).

Figure 1.2-10. Comparison of peroxisomal β-oxidation to mitochondrial β-oxidation(261)

NEFAs are transported into the peroxisome via the ABC transporters (ABCD), by contrast, they enter the mitochondria in a carnitine-dependent manner catalyzed by carnitine palmitoyltransferase (CPT). The electrons in the first reaction of peroxisomal β-oxidation are transferred by acyl-CoA oxidases (ACOXs) to molecular oxygen, whereas in the mitochondria the acyl-CoA dehydrogenases reduce FAD to FADH₂, which is regenerated in the respiratory chain (RC). The subsequent
reactions are similar in both compartments; they are catalysed by different enzymes with distinct substrate specificities.

Short-chain (<C8), medium-chain (C8-12), and long-chain (C12-20) fatty acids are oxidized in mitochondria. In mitochondria, β-oxidation is tightly coupled to the production of ATP. The resulting NADH and FADH2 and the following oxidation of acetyl-CoA in the citric acid cycle, are used for ATP production in the respiratory chain(262).

Very long chain saturated and polyunsaturated fatty acids (>C20) and branched chain fatty acids are firstly metabolized in peroxisomes, chain shortened, and then imported to mitochondria for further oxidation(263). Other peroxisomal metabolism substrates are: 1) pristanic acid (2,4,6,19-tetramethylpentadecanoic acid, which is converted by α-oxidation; 2) di- and trihydroxycholestanolic acid; 3) long chain dicarboxylic acids; 4) certain LCPUFAs, including C24:6, which undergoes one cycle of β-oxidation in peroxisomes to produce C22:6 and 5) prostaglandins and leukotrienes(264-266).

The oxidation rate of specific substrates is partially controlled by the rate of entry into oxidative compartments. The entry of substrates across membranes of mitochondria and peroxisome is mediated by different mechanisms. Long-chain fatty acids enter mitochondria by the CPT cycle. Short- and medium- chain fatty acids enter mitochondria directly in their protonated form. Entry of fatty acyl-CoAs into peroxisomes is mediated by ABCD transporters (ATP-binding cassette, D subfamily) (D1-D4)(260). In the peroxisomal membrane, two specific acyl-CoA synthetases were identified that are responsible for the esterification of fatty acids(267).

The first reaction in mitochondria β-oxidation is the dehydrogenation of the acyl-CoA ester by a family of four chain length-specific straight-chain acyl-CoA dehydrogenases. These include very long chain, long chain, medium chain and short
chain enzymes. The electrons of this reaction are transferred to FAD and are used in the respiratory chain for ATP production.

In the second reaction, the enzyme enoyl-CoA hydratase adds water to trans-delta2-enoyl-CoA. The production 3-L-hydroxyacyl-CoA is then oxidized by dehydrogenase, which transfer two electrons to NAD⁺. In the last step, the acyl-thioester is hydrolyzed and shortens the chain by two carbon atoms. The resulting n-2 fatty acyl-CoA can be further degraded by another β-oxidation cycle and the generated acetyl-CoA can be metabolized in the citric acid cycle(262).

Similarly, peroxisomal-oxidation system consists of four steps with each metabolic reaction carried out by at least two different enzymes. In the first step, fatty acyl-CoA is dehydrated to produce trans-2-enoyl-CoA. This reaction is catalyzed by specific peroxisomal acyl-CoA oxidase (ACOX) isoforms. In rats and mice, three acyl-CoA oxidases isoforms have been identified. In humans, two acyl-CoA oxidases have been identified: ACOX1 is specific for the CoA esters of straight-chain fatty acids. Whereas ACOX2 can react with both straight-chain and 2-methyl branched-chain fatty acids, including pristanoyl-CoA and di- and trihydroxycholestanoyl-CoA(268). This FAD-containing oxidase transfers the generated electrons directly to oxygen, leading to the formation of hydrogen peroxide (H₂O₂). Catalase then converts hydrogen peroxide into water and oxygen to reduce oxidative stress generated in this step. This reaction is unique to peroxisomes.

The second and third steps: hydration and dehydrogenation of enoyl-CoA esters to 3-ketoacyl-CoA are catalyzed by a single enzyme, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-bi/multifunctional enzyme, L-PBE/MFP1).

In the fourth reaction of β-oxidation, 3-ketoacyl-CoAs are thiolytically cleaved to yield acetyl-CoA and n-2 acyl-CoAs(269). This reaction is catalyzed by 3-ketoacyl-CoA thiolases. The two human peroxisomal thiolases comprise (1) peroxisomal 3-ketoacyl-CoA thiolases 2, better known as sterol carrier protein X (SCPX), which catalyzes the thiolytic cleavage of the 3-ketoacyl-CoA esters of both straight-chain
and branched-chain FAs, and (2) peroxisomal 3-ketoacyl-CoA thiolases 1 (pTH1), which resembles the clofibrate-inducible thiolases and is only reactive with the 3-ketoacyl-CoA esters of straight-chain FAs(270).

The shortened acyl-CoA reenters the β-oxidation cycle, and this process repeats until the appropriately chain-shortened acyl-CoAs that can be oxidized in mitochondria are generated. The products of peroxisomal β-oxidation are exported from the peroxisome as carnitine esters and acyl moiety.

1.2.4.2.2. Anabolic aspects of peroxisomal fatty acid β-oxidation

As discussed in section 1.2.2.2, peroxisomal β-oxidation is also involved in biosynthesis of ether lipids and the last step of 22:6, n-3 and 22:5, n-6 synthesis(72). Tissue 22:6, n-3 was decreased in peroxisomal disorders that affect β-oxidation pathway(271). Human skin fibroblast with deficiency of either straight-chain ACOX or MFP1 showed reduction in 22:6, n-3 synthesis, although it is not completely abolished(272, 273). The same results were also observed in ACOX−/− mice(274). These data demonstrated an essential role of peroxisomal β-oxidation in the biosynthesis of n-3 LCPUFAs.

1.2.4.2.3. Fatty acid β-oxidation and NAFLD

Impairments in mitochondrial or peroxisomal oxidation pathway would contribute to the progression of NAFLD. Medium-chain acyl-CoA dehydrogenase deficiency is the most common inherited disorder of mitochondrial fatty acid oxidation in humans(10). Mice with disrupted medium-chain and very-long-chain acyl-CoA dehydrogenase genes showed defects in fatty acid oxidation and developed micro- and macrovascular hepatic steatosis(275).

Essentiality of peroxisomal β-oxidation was demonstrated by studies of disorders related to peroxisomal β-oxidation. Today, more than 25 proteins are known to be involved in peroxisomal β-oxidation, but only six disorders with a single protein deficiency have been clearly diagnosed(276). The best characterized, also the most common peroxisomal β-oxidation disease, is X-linked
adrenoleukodystrophy (X-ALD). Others include ACOX1, MFP2, AMACR and SCPX deficiencies. ACOX1−/− mice have high levels of very long chain fatty acids (>C22) in serum, growth retardation, severe hepatic steatosis and steatohepatitis(277).

1.3. Peroxisomes

1.3.1. ABCD transporters

The entry of substrates into peroxisomes is dependent on the D class of peroxisomal ABC transporters. ABC transporters constitute one of the largest families of proteins, with 48 members identified so far in humans(260). ABC transporters are membrane proteins that can transport variety of substrates ranging from ions to proteins. Most eukaryotic ABC transporters are full transporters comprised of two homologous halves. Each half is made up of a hydrophobic transmembrane domain containing multiple alpha helices and a hydrophilic nucleotide-binding fold with Walker A and B consensus motifs. ABC transporters also exit as half transporters, which only contain a single hydrophobic and hydrophilic domain and function as homo- or heterodimers. All peroxisomal ABC transporters are half transporters(270).

There are three currently known peroxisomal transporters in mammalian: ABCD1 (adrenoleukodystrophy protein, ALDP), ABCD2 (adrenoleukodystrophy related protein ALDRP) and ABCD3 (peroxisomal membrane protein, PMP70). A fourth half transporter, ABCD4 (70-kDa peroxisomal membrane protein related protein, P70R), initially reported as peroxisomal was recently shown to be localized to the ER(278). The four members of the D-subfamily share high sequence homology. D2 shares 66%, D3 shares 60%, and D4 shares 46% of homology of D1(279-281).

ABCD transporters are half transporters that are only functional as hetero- or homodimers. However, it is still controversial whether the different half ABCD transporters actually form homo- or heterodimers. On one hand, in vitro methods (yeast two-hybrid and immunoprecipitation experiments) showed that ABCD1 (D1) could form homodimers as well as heterodimers with PMP70 and D2(282, 283). On
the other hand, most physiological approaches suggest that D1 and D3 would form homodimers in vivo(284, 285).

The relative mRNA expression levels of ABCD transporters in tissues are very different. The expression profile of ABCD mRNA in human and mice tissue was summarized by Ronald J.A. et al (Table 1.3-1)(260). D1 mRNA was abundant in the heart, testis, lung, and intestine, whereas D2 mRNA was enriched in the brain and skeletal muscle. D3 was most abundant in the liver and kidney, where as D4 was abundant in the lung and testis.

In addition to mRNA expression, Troffer Charlier et al. also examined the protein abundance of D1 and D2 in different mouse tissues. This study revealed the mirror expression pattern of D1 and D2 within the same tissue (Table 1.3-2)(260, 286). This study suggested that D1 and D2 might perform similar functions in different cells. It also provided evidence that D1 and D2 are unlikely to function as a heterodimer. A similar expression pattern of D1 and D2 was also found in adrenal gland. In this organ, D1 in mainly expressed in the adrenal cortex whereas D2 expression is found in the medulla(287).

Table 1.3-1. Comparison of mRNA expression profiles in different mouse and human tissues

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<th>ALD mRNA Human</th>
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Data obtained from Berger et al. and Langmann et al.

- F Control diet; + F fosfomate-containing diet; mouse: /- Only detectable by RT-PCR; + low expression but detectable (by Northern blotting); ++ good expression; +++ high expression; ≈ no change; ↑ two- to fivefold induction; ↑↑ five- to tenfold induction; ↑↑↑ >tenfold induction; human: ● low expression; ●● moderate expression; ●●● good expression; ●●●● high expression; ●●●●● very high expression
Table 1.3-2. Results of immunohistochemical studies in different mouse tissues

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</tr>
<tr>
<td>Cortex</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>Medulla</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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</tr>
<tr>
<td>Hepatocytes</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data taken from Troffer-Charlier. A mosaic pattern was observed in oligodendrocytes with no expression (−) in some oligodendrocytes but abundant (+++) expression in others.

− Absent; + present; ++ moderately abundant; +++ abundant

So far, the only known human disease associated with peroxisomal ABCD transporters is X-ALD. X-ALD presents as a severe neurodegenerative disease with widely varying clinical phenotypes ranging from childhood cerebral ALD to adrenomyeloneuropathy (AMN) Currently, 1058 mutations of ABCD1 have been reported, 519 of which are unique (see www.X-ald.nl)(288). Biochemically, X-ALD is characterized by the accumulation of saturated VLCFAs, in plasma and tissues. Oxidation of the VLCFA C26:0 and C24:0 is impaired in X-ALD fibroblasts, suggesting that ABCD1 is involved in the import of VLCFAs into the peroxisome for degradation(289).

1.3.2. ABCD2

1.3.2.1. Function of D2

Overexpression of D2 in human X-ALD fibroblasts and mouse D1−/− cells increased the oxidation of C24:0 and C26:0 and normalized the VLCFAs levels(287,
This indicates that D1 and D2 are functionally redundant and likely have overlapping substrate specificities. Overexpression of D2 in vivo compensated for the loss of D1, prevented VLCFAs accumulation and several neurodegenerative features in mice, further demonstrating the functional redundancy between D1 and D2. Therefore, expression of the D2 gene constitutes a potential therapeutic target for X-ALD in a strategy aimed at inducing their expression through pharmacological treatments.

However, D2 has preferred substrates despite overlapping functions with D1. By modifying D2 expression in hepatoma cells, Genin EC et. al, demonstrated that D2 is involved in the β-oxidation of C24:0, C26:0 and C22:6, n-3 (198, 292). Wanders RJ et. al, used yesast S. cerevisiae as a model system to study D2 substrate specificity, since peroxisomes are the only organelles in which β-oxidation of fatty acids take place(293). By transforming human D1 and/or D2 cDNA in yeast, which has its own peroxisomal ABC transporters (Pxa1p and Pxa2p) mutated, they demonstrated that D2 is largely responsible for facilitating the β-oxidation of C22:0 and exclusively responsible for oxidation of unsaturated VLCFAs including C24:6, n-3 and especially C22:6, n-3. In contrast, D1 is responsible for the import of C24:0 and C26:0.

Pujol et al, analyzed the fatty acid profile in tissues from D2−/− mice(101). Consistent with in vitro studies, they observed impaired β-oxidation of C24:6, n-3 and C26:0, and impaired synthesis of C22:6, n-3 and C22:5, n-6. In addition to that, they also observed the accumulation of C20:0, C20:1, n-9 and C22:1, n-9 in adrenal gland and sciatic nerve. C20:0 and C22:1, n-9 levels in serum were also elevated in D2−/− mice fasted for 48 hours. Accumulation of C20:0 and C22:0 in liver reaches statistical significance at 12 months in D2−/− mice. These data indicated that D2 plays a role in the degradation of long-chain saturated and n-9 monounsaturated fatty acids, as well as degradation and synthesis of DHA.

D2−/− mice exhibit a late onset cerebellar and sensory ataxia, loss of cerebellar Purkinje cells, and dorsal root ganglia cell degeneration together with oxidative
damage in adrenal gland\((294, 295)\). To date, human D2 deficiency has not been reported.

### 1.3.2.2. Regulation of D2 expression

Regulation of D2 gene expression in rodents and humans has been intensively investigated because of the potential therapeutic effect of D2 in X-ALD. D2 expression is regulated by various transcriptional factors (PPAR\(\alpha\), SREBP and LXR), thyroid hormone, energy status (fasting and refeeding), and nutrients such as cholesterol and PUFAs. D2 can also be induced by the pro-drug 4-phenylbutyrate, a histone deacetylase (HDAC) inhibitor.

PPAR\(\alpha\) agonists, such as fenofibrate, can strongly induce ABCD2 mRNA levels in liver and intestine (but not brain) of rats and mice\((296-299)\). Another PPAR\(\alpha\) agonist: GW7647 can also upregulate D2 expression in mouse liver and adrenals, but not brain and testis\((300)\). The induction of D2 by fibrates was abolished in PPAR\(\alpha\) knockout mice. PPAR\(\alpha\) is a nuclear receptor that activates transcription of genes participating in lipid metabolism, including peroxisomal \(\beta\)-oxidation of fatty acids. No functional PPRE can be identified in the human or rodent D2 promoter, suggesting that the effect of PPAR\(\alpha\)-agonist on D2 expression might be indirect\((296, 298, 300)\). Since SREBP2 was induced by fenofibrate through PPAR\(\alpha\), it was assumed then that SREBP2 mediated the indirect induction of D2 by PPAR\(\alpha\) agonist\((300)\).

Later, by using reporter gene studies, site directed mutagenesis and gel shift assays, a functional sterol regulatory element (SRE) has been identified in the human D2 promoter region, thus providing a link between peroxisomes, cholesterol and fatty acid metabolism\((301, 302)\). Activated SREBP1c directly binds with the SRE and induces D2 expression\((302)\).

Interestingly, the SRE motif in the promoter region of human D2 overlaps with a direct repeat (DR4) element by 5 bp, which is capable of binding a retinoid X receptor \(\alpha\) (RXR), thyroid hormone receptor \(\alpha\) and \(\beta\) heterodimer and
LXRα (Figure 1.3-1) (302). LXRα is a negative modulator of D2 gene expression. In cultured cells, ligand-activated LXRα can directly bind the DR4 element in the promoter of D2, antagonize the access of SREBP1c to the SRE, and therefore suppress the SREBP-1c-mediated induction of D2.

Figure 1.3-1. The SRE and DR-4 motifs of the human ABCD2 promoter

Figure is from (302). An SRE is located at nucleotide position -401 to -391 with respect to the translation start site of the human ABCD2 gene. The SRE sequence overlaps by 5 bp with a direct repeat hexameric sequence of the DR-4 type (in the opposite orientation) that serves as binding site for both TRβ/RXRα and LXRα/RXRα heterodimers.

T₃ induces D2 expression in murine D1⁻/⁻ fibroblasts (but not in primary rat astrocytes), and in the liver but not in the brain of rats (303, 304). The upregulation of D2 in response to T₃ is TRα dependent and requires intact SRE/DR-4 motifs (304). In contrast, unliganded TRβ represses D2 expression without binding with D2 promoter. This effect was relieved by hormone binding, especially in the context of SREBP-activated D2 expression.
mRNA levels of D2 in mouse adipose tissue and liver are sensitive to fasting and refeeding (302). In white adipose tissue (WAT), response of D2 mRNA to energy status correlates with SREBP-1c target genes (Figure 1.3-2). D2 expression in WAT was downregulated following a 24 hour fast and restored by refeeding of a high carbohydrate/low fat diet for 12 hours. Liver D2 mRNA levels exhibit the opposite pattern in the same experiment. D2 expression in liver was induced during fasting stage and suppressed during refeeding stage, which is due to the concurrent activation of LXRα and SREBP-1c.

**Figure 1.3-2. ABCD2 mRNA expression in liver and white adipose tissue is sensitive to fasting-refeeding.**

Fasting-refeeding of mice reveals that Abcd2 expression in the liver is regulated by PPARα, whereas Abcd2 mRNA levels in white adipose tissue correlate with SREBP1c-stimulated genes. Wild type C57BL/6 mice were fasted for 24 h and then refeed a high carbohydrate/low fat diet for 12 h. Total RNA was isolated from adipose tissue and liver and Abcd2 mRNA levels were evaluated by (a, b) QRT-PCR performed in triplicate wells and normalized to the number of hydroxyphosphoribosyl transferase mRNA copies (results are shown as mean ± S.E. with the number of treated mice in parentheses and statistically significant differences by Student’s t test are indicated by asterisks (p < 0.05))

D2 expression can be induced under cholesterol deprivation condition in cultured human and murine monocytes as well as in human primary fibroblasts.
Cholesterol-lowering compounds such as lovastatin also induce D2 expression\(^{(301)}\). Dietary cholesterol regulates D2 expression in liver\(^{(302)}\). Feeding of a high cholesterol diet (2% cholesterol) for 1 week results in the activation of LXR\(\alpha\) and SREBP-1c, but significant suppression of liver D2 expression.

D2 is also regulated by dietary PUFAs. Leclercq S et al. reported that when rats are fed with \(\alpha\)-linolenic acid, 18:3, n-3 deficient diet, D2 expression in liver was found to be significantly higher than the rats fed with \(\alpha\)-linolenic acid containing diet or the DHA supplemented diets\(^{(305)}\).

### 1.4. Erucic acid

C22:1, n-9, also known as erucic acid, accumulates in the serum and liver of D2\(^{-/-}\) mice, indicating that it is a substrate for D2. We therefore used this fatty acid to explore D2 mediated peroxisomal oxidation of unusual fatty acids. Erucic acid is naturally enriched in rapeseed and mustard oil. Erucic acid was associated with transient cardiac lipidosis when fed to rats\(^{(306)}\). In isolated liver cells, both the rate of esterification and mitochondrial oxidation of erucic acid is distinctly slower, which is calculated to be about 1/3 of the rate of palmitic acid\(^{(307-310)}\). In perfused heart, erucic acid is esterified as rapidly as palmitic acid, whereas the rate of mitochondrial oxidation of erucic acid is comparable to its rate in liver\(^{(307, 311, 312)}\). In liver and heart, erucic acid is shortened to C18:1, n-9, and smaller amounts of C20:1, n-9 and C16:, n-9 fatty acids, by peroxisomal \(\beta\)-oxidation.

In adipocytes isolated from rat epididymal fat, erucic acid can either be incorporated into triglycerides, diacylglycerol and phospholipids or be oxidized\(^{(313)}\). The rate of esterification of erucic acid in TAG, DAG or phospholipids occurred at 1/3 of the rate with palmitic acid as substrate. The incorporation in DAG was 10% and the incorporation in the phospholipids was 1% of the rate of esterification in TAG. The rate of oxidation of erucic acid to CO\(_2\) and acid-soluble intermediates was about 1/3 of the rate of palmitic acid. However, no significant
chain-shortening of erucic acid to shorter monounsaturated fatty acid was identified in the isolate adipocytes.

In rats, long term feeding of erucic acid enriched diet (5 month) results in a significant elevation of liver triglyceride levels (314). However, mice are resistant to the lipotoxicity of erucic acid.

1.5. Summary

Peroxisomes are sites of both n-3 PUFAs synthesis and degradation, therefore, peroxisomes play an important role in maintaining proper levels of PUFAs in TAG, CE and phospholipids and regulating energy homeostasis. Among the four members of ABCD transport, D2 is uniquely regulated by key transcriptional factors that control lipogenesis, sterol metabolism and β-oxidation, which allows D2 to serve as an important regulator of peroxisomal lipid metabolism by importing very-long-chain MUFA and PUFA into peroxisomes. However, the physiological role of D2 remains unclear.
2. Chapter 2: The role of ABCD2 in adipogenesis and metabolism of VLCFA C22:1

2.1. INTRODUCTION

Peroxisomes play essential roles in the metabolism of both dietary and endogenously synthesized lipids (315, 316). The synthesis of etherphospholipids (platelet activating factor and plasmalogens), partial β-oxidation of very long chain fatty acids (VLCFA, C>22), and the α-oxidation of phytanic acid occur within this organelle. A comprehensive integrative map of peroxisomal metabolic pathways can be found in http:www.peroxisomedb.org (317). The importance of the organelle is best exemplified by the occurrence of peroxisome biogenesis disorders and single enzyme deficiencies that result in severe metabolic diseases which are often lethal in early childhood. X-linked Adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder and is characterized by the accumulation of VLCFA in tissues, progressive demyelination and adrenocortical dysfunction (318). X-ALD is caused by mutations in ABCD1 (D1), an ATP binding cassette (ABC) half transporter that presumably mediates the transport of very long chain acyl-CoAs into peroxisomes (319). D1 is the founding member of a quartet of ABC half-transporters that contain peroxisomal targeting sequences and form heterodimers in vitro (282). However, homodimers appear to be preferred in vivo and each subfamily member has a unique tissue distribution (320, 321).

The closest parologue to D1 is D2, which shares 66% amino acid identity and is expressed in the adrenal, brain and liver (322). Expression of a D2 transgene in D1 deficient mice partially corrected the phenotype in this mouse model of X-ALD indicating that these two transporters have some degree of overlapping substrate specificity (323, 324).

In liver, D2 mRNA levels are typically less than 10-20% of those observed in brain or adrenal (322, 325). However its expression is induced by fasting, feeding fibrates and statin treatment, while expression levels in brain remain constant (302,
More recently, D2 mRNA was shown to be expressed in adipose tissue where its mRNA levels are highest in the fed state, decline in the fasted state and return during re-feeding (302). The promoter of D2 contains a functional sterol response element that interacts with both SREBP-1a and -1c, suggesting it is a component of the lipogenic program (302, 330, 331).

Mice deficient in D2 (D2−/−) are characterized by late-onset cerebellar and sensory ataxia, degeneration of dorsal root ganglia (DRG), and accumulation of VLCFA in DRG (332). They show evidence of disrupted mitochondrial membranes, consistent with increased oxidative stress. In addition, the adrenal gland of D2−/− mice shows morphological signs of oxidative stress and has increased expression of manganese superoxide dismutase (SOD2) (295). Collectively, the data suggest that D2 opposes the accumulation of VLCFA and oxidative stress in adrenal and brain. A recent report suggests that D2 may play a role in fatty acid metabolism in adipose tissue since VLCFA increased in plasma following a 48 hr fast in mice lacking D2 but not in wild-type controls (333). However, the role of D2 in adipocytes and adipose tissue remains unexplored. In the present study we determined the relative abundance of D2 protein in adipose tissue, its role in adipogenesis and the clearance of dietary erucic acid in adipose tissue. Our results indicate that D2 expression is robust in adipose tissue and upregulated during adipogenesis, but is not essential for adipogenesis or lipid storage in vitro. Conversely, erucic acid accumulated in the adipose tissue of mice challenged with a diet enriched in this fatty acid in a gene-dosage dependent manner, suggesting that the role of D2 in fat is to facilitate the clearance of this, and presumably other, dietary VLCFA that are generally very low or absent in adipose tissue but present in significant quantities in the diet.

2.2. MATERIALS AND METHODS

2.2.1. Development of ABCD2 antibody

The cDNA encoding the cytoplasmic domain c-terminal of the ATP binding cassette of murine D2 (amino acids 366-711) was fused to the large T-antigen of
tetanus toxin and a six-histidine tag and cloned into pET28a (+) bacterial expression vector. Expression in the BL21 strain of E. coli cells was induced by IPTG and the 6-His fusion protein isolated under denaturing conditions using NiNTA agarose beads. The purified peptide was dialyzed against phosphate buffered 4M urea and quantified. The immunization of rabbits was outsourced to ProSci (Poway, CA). Antiserum was screened for immunoreactivity to D2 by immunoblotting of the antigen. Its utility immunoblotting and immunofluorescence applications was determined in CHO-K1 cells transfected with plasmid harboring cDNA encoding full length murine ABCD2 and total membrane preparations from adipose tissue of wild-type and D2 deficient mice (Figure 2.4-1).

### 2.2.2. Animal husbandry

Mice lacking Abcd2 and their wild-type littermates maintained on the C57BL/6J background were examined at 8 weeks of age. Genotyping experiments to differentiate Abcd2 knockouts from heterozygotes and wild types was done as described(332). Animals were housed in a temperature-controlled room with 12:12 light:dark cycle (6:00 am to 6:00 pm). All mice were maintained on standard rodent chow (Harlan Teklad 2014S). The erucic acid enriched diet was made by mixing 50g (37°C) of erucic acid with 1000g of powdered diet (Tables 2.4-3 and 2.4-4; #D1001, Research Diets, New Brunswick, NJ) in a standard mixer equipped with a wire wisk. The diet was vacuum packaged after preparation and stored at 4°C to prevent oxidation.

Body composition of mice was measured using Echo MRI. Following a 4 hr fast beginning shortly after lights-on, mice were killed by exsanguination under ketamine/xylazine anesthesia. Blood was collected from the right ventricle with a 1 cc syringe fitted with a 20ga hypodermic needle. Serum was separated by centrifugation and stored at -20°C until analysis. Tissues were excised, rinsed with PBS to remove blood and snap frozen in liquid nitrogen. Tissue samples were stored at -80°C until analyzed.
2.2.3. **Blood analysis**

Blood glucose levels were measured using a standard glucometer from a drop of blood obtained by tail-vein prick following a 4 hr fast beginning at lights-on. Total serum cholesterol and triglyceride concentrations were determined by colorimetric-enzymatic assays (Wako Chemicals, Richmond, VA.)

2.2.4. **Lipid analysis**

Total fatty acids in adipose tissue were extracted by chloroform-BHT (50mg/ml) and C17:0 was added after extraction as internal standard. The total fatty acids were methyl esterified with BF3/Methanol (10%, Supelco, Bellefonte, PA) by incubating the mixture at 60°C for 16 hours. The fatty acid esters were extracted with chloroform. The BF3 and methanol was removed by 3X water wash and the samples were ready for GC-MS analysis. Serum fatty acid was extracted by Folch reagent after adding C17:0 as an internal standard. The lipids were esterified following the same procedure as adipose tissue lipid extraction. 1µl of the sample was injected onto a gas chromatography system (Agilent 6890 GC G2579A system, Palo Alto, CA) equipped with an OMEGAWAXTM 250 capillary column (Supelco, Bellefonte, PA) and a FID detector. Agilent 5973 network mass selective detector (Agilent, Palo Alto, CA) was used to identify target peaks. The GC program was: Injector: 1µl at 10:1 split, 250°C; Detector: FID, 260°C; oven: 160°C (5min) to 220°C at 4°C/min; Carrier: helium, 1.2 ml/min.

2.2.5. **Cell culture**

3T3-L1 cells were propagated as fibroblasts in sub-confluent cultures. Two days post-confluence, the cells were differentiated into adipocytes using a standard protocol (1.7 µM insulin, 0.5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 µM Rosiglitizone). Mouse embryonic fibroblasts (MEF) were isolated from Day 13.5 embryos and differentiated exactly as 3T3-L1 cells at between passage 0 and 3.
2.2.6. **Protein and RNA analysis**

Membrane proteins were prepared and analyzed by SDS-PAGE and immunoblot analysis as previously described\(^{334}\). The isolation of total RNA and the determination of relative transcript abundance by quantitative Real-Time PCR (qRT-PCR) for both tissues and cells was conducted as previously described\(^{334}\).

2.2.7. **Statistical analysis**

Data were analyzed by ANOVA. Bonferroni posts tests were employed where indicated. All statistical analyses were conducted using GraphPad Prism statistical analysis software.

2.3. **RESULTS**

Previous reports indicated that D2 mRNA is present in adipose tissue and is regulated by fasting and re-feeding in mice; however, the presence and relative abundance of D2 protein in adipose tissue is unknown \(^{302}\). In order to determine the relative abundance of D2 protein in fat with respect to other tissues, we developed a polyclonal antibody to mouse ABCD2 (Figure 2.4-1). We then prepared total membranes from tissue homogenates and compared the relative levels of immunoreactive D2 by immunoblot analysis (Figure 2.4-2).
Figure 2.3-1 Validation of D2 antibody

(A) Immunofluorescence microscopy and immunoblot analysis of D2 protein in cultured cells. Cells were transfected with a plasmid containing a cDNA encoding mouse D2. Cells were processed for indirect immunofluorescence microscopy using a polyclonal D2 antibody. Inset: lysates from cells transfected with control plasmid (V) and D2 were analyzed by immunoblotting using a polyclonal antibody directed against D2. Results from cells infected with adenovirus encoding D2 were essentially identical. (B) Total membranes were prepared from wild-type and D2−/− mice and analyzed by immunoblotting with the D2 antibody.
Figure 2.3-2 D2 protein expression among different tissues

Relative expression of immunoreactive D2 in selected mouse tissues. Total membrane proteins were prepared from tissues of male C57Bl6/J mice. Equal amounts of proteins were pooled from each mouse and analyzed by immunoblotting using the antibody directed against D2. Specificity of the immunoreactive D2 bands was confirmed using tissues from D2-deficient mice (D2−/−) mice. Epi., epididymal; NSB, nonspecific band.

Total membrane preparations were pooled from four male C57BL6/J mice. Epididymal fat from a D2 deficient mouse (D2−/−) was used as a negative control. The antibody detected a 75kDa band in brain, skeletal muscle, lung, liver, testis, brown fat and epididymal fat. With longer exposures, D2 was also detected in adrenal (not shown). Our antibody also cross-reacted with a 65 kDa band present in several tissues that was most prominent in lung, liver, kidney, testis and brown fat. This band was judged to be non-specific (251) based on its persistence in blots in each of these tissues in D2−/− mice (not shown).

The 75 kDa band was most prominent in adipose tissue. In addition, much of the immunoreactive D2 migrated as a prominent “smear” in our SDS-PAGE gels ranging from 120 kDa to greater than 220 kDa, the largest of our molecular weight markers.
We initially presumed these forms to represent protein aggregates of D2 since a number of ABC transporters are prone to aggregation upon boiling in SDS. However, efforts to resolve this material using gentle heating in urea buffer or increasing the relative amount of SDS and β-ME to membrane proteins failed to resolve this protein into a single 75kDa band (Figure 2.4-3).

Figure 2.3-3 High molecular weight signal of D2 protein

(A) To increase the SDS/protein ratio, adipose membrane proteins were diluted in a common volume of Laemmli Buffer (3% SDS final) and 50, 20 and 10 mg of total protein were loaded on an SDS-PAGE gel and analyzed by western blotting. (B) Signal intensities were analyzed by densitometry. The sum intensity of the high molecular weight (HMW) signal was divided by the total sum intensity of the lane and expressed as percent. These results indicate that increasing the relative amount of detergent did not denature the HMW forms to D2 monomers. (C) Many ABC transporters aggregate upon boiling in SDS. To determine if gentle heating in urea buffer (37ºC for 60 min) would resolve the HMW forms of D2, we compared this method with our standard procedure. ABCA1 was used as a positive control for protein aggregation in boiling SDS. These results indicate that the HMW forms of D2 are unlikely to be protein aggregates.
Given the prominent signal observed for D2 in adipose tissue, we next determined the relative abundance of D2 among mouse fat depots by qRT-PCR and immunoblotting (Figure 2.4-4). Whereas the mRNAs for D2 were similar among the epididymal, inguinal and retroperitoneal fat pads, the expression was low in mesenteric and brown fat (Figure 2.4-4A). For comparative purposes, we evaluated the expression of the related family member, D1. Whereas D1 was similar among each of the white fat pads, its expression was approximately 4 fold greater in brown adipose tissue when compared to epididymal fat. It should be noted that the relative abundance of D1 mRNA among the white fat depots was similar to that of liver. Conversely, the abundance of D2 mRNA in liver was less than 5% of epididymal fat (not shown).
Figure 2.3-4 Relative expression of D2 mRNA and protein among selected mouse fat depots

(A) The relative levels of D2 and D1 mRNA among fat pads were measured by qRT-PCR and normalized to cyclophilin and epididymal fat using the CT method. (B) Total membrane proteins were prepared from epididymal (Epi.), inguinal, retroperitoneal, mesenteric, and brown fat pads and analyzed by immunoblotting. Epididymal and brown fat pads from D2−/− mice were also analyzed to ensure specificity of immunoreactive proteins.

Immunoblotting results were consistent with mRNA data indicating abundant expression of D2 in epididymal, inguinal and retroperitoneal fat pads and much less
expression in mesenteric and brown fat (Figure 2.4-4B). As in Figure 2.4-2, visualization of immunoreactive D2 in membrane preparations from mesenteric and brown adipose tissue resulted in over-exposure of films for epididymal and other fat pads.

Although the adipocyte is the predominant cell type present in adipose tissue, other cell types are also present and could be the source of immunoreactive D2 in fat. To test the hypothesis that D2 was present in adipocytes within adipose tissue, we compared the expression between adipocytes and the stromal vascular fraction that contains a mixture of pre-adipocytes, fibroblasts, macrophages, endothelial cells, and others. Following collagenase digestion of adipose tissue, adipocytes were separated from stromal vascular cells by low-speed centrifugation. Whole adipose, adipocytes and stromal vascular cells were evaluated for abundance of D2 by quantitative PCR and immunoblotting (Figure 2.4-5). The relative abundance of adipocyte markers (fatty acid synthase, FAS; acetyl CoA carboxylase, ACC), macrophages (CD68) and endothelial cells (CD31) were used as controls in this experiment. Whereas mRNAs for FAS and ACC are enriched in adipocytes, CD68 and CD31 are enriched in the stromal vascular fraction (Figure 2.4-5A). D2 and D1 were enriched in the adipocyte and stromal vascular fractions, respectively. These results were confirmed by immunoblotting (Figure 2.4-5B), which show that virtually all of the immunoreactive D2 was confined to the adipocyte fraction within adipose tissue. CD36, a protein expressed in adipocytes, macrophages and endothelial cells were used as a loading control, although it is slightly enriched in adipocytes.
Figure 2.3-5 Relative expression of D2 mRNA and protein among adipose tissue fractions

(A) Adipocytes were separated from stromal vascular (SV) cells by collagenase digestion and low-speed centrifugation. The mRNA levels of D2 were measured by qRT-PCR as described in methods section. FAS and ACC were used as positive controls for adipocytes. Macrophage (CD68) and endothelial cells (CD31) were used as controls for separation of stromal vascular cells from adipocytes. (B) Total membranes were prepared and analyzed by immunoblotting. CD36 was used as a control, although it is somewhat enriched in adipocytes.
Next we determined if D2 was upregulated during adipogenesis. For these experiments, we utilized murine NIH3T3-L1 cells (Figure 2.4-6). Cells were cultured to confluence (Day 0) and treated with differentiation cocktail beginning on Day 2 (see methods). Neutral lipids were stained with Oil-Red O on even days of the differentiation protocol (Figure 2.4-6A). Neutral lipid is detectible by Day 4, accumulates in a linear fashion until Day 8 and remains constant through Day 10 (not shown). The expression of aP2 was used to assess expression of adipocyte markers (Figure 2.4-6B). Although not nearly as robust as the upregulation of fatty acid binding protein 4 (aP2), D2 mRNA increased four-fold between Day 2 and Day 4 and remained elevated throughout the differentiation protocol. In contrast, the expression of D1 declined between days 0 and 2 and never returned to pre-differentiation levels. We also evaluated immunoreactive levels of D2 in cell lysates on even days of the differentiation protocol. Similar to other tissues, a non-specific band was observed for D2 in NIH3T3-L1 cells. Immunoreactive D2 was first visible on Day 4 and accumulated throughout the differentiation protocol. The high molecular weight forms of D2 were only observed with long exposure in these immunoblots, indicating that they are either poorly soluble in our lysis buffer or are far less abundant in 3T3-L1 adipocytes compared to adipocytes in vivo.
Figure 2.3-6 Expression of D2 mRNA and protein during adipogenesis in 3T3-L1 cells

(A) 3T3-L1 cells were differentiated to adipocytes and stained with Oil-Red O. (B) D2 mRNA levels were determined on even days of differentiation by qRT-PCR. Fatty acid binding protein 4 (aP2) was examined as positive control for differentiation. D1 was examined as negative control. (C) D2 protein expression during differentiation was analyzed by immunoblotting. Actin was blotted as loading control. Asterisk denotes the nonspecific band.

Next, we evaluated mice (n=10) deficient in D2 for adipose-related phenotypes. Male wild-type and D2 deficient mice were weaned at 3 weeks of age and
maintained on standard rodent chow. Mice were evaluated at 8 weeks of age for differences in body weight, adiposity, blood lipids and glucose, and adipocyte diameter (Table 2.4-1). We observed no statistically significant differences due to genotype in these parameters although there was a tendency for increased adiposity in D2⁻/⁻ mice. We also evaluated a smaller cohort of female mice and obtained similar results (not shown).

Table 2.3-1 Parameters of wild-type and D2⁻/⁻ mice at 8 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>D2⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>25.6 ± 0.74</td>
<td>25.9 ± 0.99</td>
</tr>
<tr>
<td>Adiposity (%)</td>
<td>4.3 ± 0.62</td>
<td>5.4 ± 0.45</td>
</tr>
<tr>
<td>Adipocyte diameter (mM)</td>
<td>44.1 ± 13.6</td>
<td>48.6 ± 14.5</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dl)</td>
<td>109 ± 8</td>
<td>114 ± 9</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>110.1 ± 16.7</td>
<td>113.6 ± 7.6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>78.61 ± 20.52</td>
<td>69.43 ± 6.02</td>
</tr>
</tbody>
</table>

GC-MS analysis of adipose tissue revealed significant differences in the fatty acid profiles of these mice (Table 2.4-2). We observed an accumulation of C20 and C22 fatty acids in adipose tissue, but not C24 or C26. These results are largely consistent with those observed in neural tissues of D2 deficient mice (332) and suggest that although D2 is a target of SREBP-1a/c and upregulated during adipogenesis, it is not essential to adipogenesis or lipid accumulation in mice maintained on standard rodent chow.
Table 2.3-2 Fatty acid profile in adipose tissue of wild-type and D2⁻/⁻ mice

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wild Type</th>
<th>D2⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>0.1347 ± 0.0196</td>
<td>0.1271 ± 0.0183</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.0297 ± 0.0015</td>
<td>0.0226 ± 0.0055</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.0192 ± 0.0037</td>
<td>0.0224 ± 0.0023</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.1594 ± 0.0255</td>
<td>0.1780 ± 0.0128</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.2293 ± 0.0285</td>
<td>0.2329 ± 0.0185</td>
</tr>
<tr>
<td>18:3, n-6</td>
<td>0.0034 ± 0.0008</td>
<td>0.0038 ± 0.0010</td>
</tr>
<tr>
<td>18:3, n-3</td>
<td>0.0193 ± 0.0026</td>
<td>0.0147 ± 0.0030</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.0021 ± 0.0008</td>
<td>0.0033 ± 0.0005 *</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.0027 ± 0.0024</td>
<td>0.0118 ± 0.0011 *</td>
</tr>
<tr>
<td>C22:1</td>
<td>nd</td>
<td>0.0054 ± 0.0011 *</td>
</tr>
</tbody>
</table>

One potential explanation for this result is that adipose tissue is not the predominant lipogenic organ *in vivo* and that the absence of D2 in adipose tissue is compensated by other mechanisms, perhaps the presence of the related family member D1, in the liver where the majority of endogenous lipids are synthesized. To test the hypothesis that D2 expression is critical for adipogenesis in cultured adipocytes, we isolated Day 13.5 mouse embryonic fibroblasts (MEF) from wild-type and D2⁻/⁻ mice and differentiated them to adipocytes. Total lipid accumulation was measured by oil-red-O staining (Figure 2.4-7). Consistent with our *in vivo* observations, the absence of D2 did not prevent adipogenesis or suppress lipid accumulation in adipocytes. In fact, there was a significant increase in lipid accumulation in MEF cells from D2⁻/⁻ mice compared to cells from wild-type controls. These results indicate that although D2 is upregulated during adipogenesis and is
regulated by SREBP, it is not essential for adipocyte differentiation or lipid accumulation in vitro or in vivo.

Figure 2.3-7 Lipid accumulation in wild-type and D2⁻/⁻ MEF cells

(A) Mouse embryonic fibroblasts were isolate from wild-type and D2⁻/⁻ mice (Day13.5) and differentiated into adipocytes. Neutral lipids were stained with Oil-Red-O. Stained adipocytes were imaged in bright field. (B) Oil-Red-O was extracted with isopropanol throughout the differentiation protocol and the amount of lipid accumulation was quantified by absorbance (560 nM). Data were analyzed by two-way ANOVA (genotype x time). Main effects of genotype and time were detected (p < 0.05). Interaction not significant.

These findings suggest that the role of D2 in adipose is not to facilitate bulk lipid storage, but rather to mediate the clearance of fatty acids not typically found in the triglyceride storage droplets of adipocytes, but may be present in the diet. To determine if D2 mediated the clearance of such fatty acids in adipose, we evaluated
the effect of a diet enriched in erucic acid (C22:1, n-9; Table 2.4 & 4). Although it is unlikely that the substrates of D2 are limited to erucic acid, this fatty acid was selected as a model lipid since it accumulates to the greatest extent in adipose and neural tissues of mice maintained on standard rodent chow.

Table 2.3-3 Composition of erucic acid enriched diet

<table>
<thead>
<tr>
<th></th>
<th>D10001 (AIN-76-A)</th>
<th>Erucic Enriched D10001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm%</td>
<td>kcal%</td>
</tr>
<tr>
<td>Protein</td>
<td>20.3</td>
<td>20.8</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>66</td>
<td>67.7</td>
</tr>
<tr>
<td>Fat</td>
<td>5</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Table 2.3-4 Fatty acid profile of erucic acid enriched diet

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>D10001 (AIN-76-A)</th>
<th>Erucic Enriched D10001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% by label</td>
<td>% by GC</td>
</tr>
<tr>
<td>16:0</td>
<td>11.1</td>
<td>4.2</td>
</tr>
<tr>
<td>18:0</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>18:1</td>
<td>25.2</td>
<td>12.7</td>
</tr>
<tr>
<td>18:2</td>
<td>60.7</td>
<td>22.1</td>
</tr>
<tr>
<td>22:1</td>
<td>* nd</td>
<td>55.6</td>
</tr>
</tbody>
</table>
Wild-type, heterozygous and D2/− mice were challenged with a diet containing 50% erucic acid for 4 weeks. The fatty acid profile of serum and adipose tissue was examined by GC-MS. Our results indicate that in adipose tissue, the deficiency of D2 has a gene-dosage dependent effect on the accumulation of erucic acid (Figure 2.4-8A). The increase in erucic acid occurred at the expense of 18:1 and 18:2. In D2 deficient mice, the relative abundance of erucic acid among adipose tissue fatty acids mirrored that of the diet, suggesting that there is neither preferential metabolism nor storage in the absence of D2.
Figure 2.3-8 Fatty acid profile in adipose tissue and serum from wild-type and D2-/- fed with an erucic acid enriched diet.
(A) Total lipids were extracted from adipose and analyzed by GC-MS-FID. Fatty acid abundance was normalized to C17 internal standard and expressed milligram/milligram of white adipose tissue mass (WAT). (B) Fatty acid profile of serum from mice maintained on an erucic acid diet and fasted for 24 h. Total serum lipids were extracted with Folch reagent and prepared for GC-MS-FID as described for adipose tissue. Inset: total free fatty acid in serum following a 24 h fast. Asterisks denote significant differences from the wild type (P < 0.05).

Consistent with the observations in adipose tissue, erucic acid accumulated to a greater extent in serum from D2+/- and D2-/- compared to wild-type (D2+/+) mice after 24 hours fasting (Figure 2.4-8B). However, erucic acid was not detected in serum from mice fasted for 4 hours (not shown). These data suggest that in the absence of D2, erucic acid is efficiently stored in adipose tissue and is mobilized from TG stores during fasting. Interestingly, total free fatty acids were greater in fasted D2 deficient mice compared to wild-type controls (Figure 2.4-8B inset). The significance of this observation awaits further investigation.

2.4. DISCUSSION

The major findings of this study are: 1) expression of ABCD2 protein is much more abundant in adipose tissue than in other tissues in which it is expressed. 2) Consistent with previous reports of its regulation by SREBP, it is upregulated during adipogenesis. 3) Although D2 is a component of the adipogenic program, its expression is not required for adipogenesis or lipid storage in vitro or in vivo. 4) Erucic acid accumulates in adipose of D2-/- and D2+/-, when mice were challenged with an erucic acid enriched diet suggesting that D2 facilitates metabolism of this dietary fatty acid in adipose tissue. To the best of our knowledge, this is the first report that establishes a role for D2 in VLCFA metabolism in non-neuronal tissues.

In addition, our results suggest a novel role for peroxisomes within adipose tissue in the clearance of “atypical” dietary lipids.

The majority of lipids stored in adipose tissue triglycerides is comprised of the fatty acids palmitate, oleate and linoleate (16:0, 18:1 & 18:2, respectively). However,
dietary lipids can contain many “atypical” fatty acids which may require remodeling to one of these forms prior to storage. The term “atypical” is used to describe fatty acids that are not generally stored in triglyceride pools. To the best of our knowledge, a role for peroxisomal metabolism of dietary fatty acids in adipose tissue has not been described. However, peroxisomes harbor a unique set of enzymes that are capable of both α- and β-oxidation of fatty acids, removing double bonds from unsaturated fatty acids, and metabolizing 2-hydroxy fatty acids (reviewed in (335)). Therefore, peroxisomes may play a significant role in this putative remodeling process by allowing for incomplete oxidation of fatty acyl CoA esters and the release of chain-shortened, saturated fatty acids.

Unlike storage, the role of peroxisomal metabolism in the utilization of fatty acids for energy has been extensively studied. Generally, fatty acyl-CoA esters are transported into mitochondria through CPT for complete oxidation to provide energy. However, acyl-CoA esters containing atypical fatty acids are not the substrates for CPT (336). These include VLCFA, branched chain fatty acids, highly unsaturated fatty acids (HUFA), some polyunsaturated fatty acids, each of which have been shown to be metabolized in peroxisomes (336-338).

Peroxisomal oxidation of fatty acids yields acetyl-CoA and chain-shortened fatty acids (C8-C20). To exit peroxisomes, medium and long chain fatty acids require esterification to carnitine by one of two peroxisomal acyl-carnitine transferase (335). Acyl-carnitine esters can be transported into mitochondria independently of carnitine palmitoyltransferases, effectively bypassing the rate-limiting step in mitochondrial oxidation of fatty acids (335). Alternatively, removal of the carnitine moiety allows for re-activation of fatty acids which can then be elongated and desaturated to generate the more common species stored in triglycerides. Consequently, the transport of atypical fatty acyl-CoAs into adipose tissue peroxisomes would allow for “remodeling” of dietary fatty acids and facilitate energy storage. A limitation of the present study is that the fate of dietary erucic acid in wild-type mice is not known. While it may have been “remodeled” and stored in adipose tissue, this has not formally been demonstrated and will require
additional studies. In addition, the present study cannot exclude a role for D2 in non-adipose tissues in this process.

The entry of atypical fatty acyl CoAs into peroxisomes is thought to be dependent upon the transporters on the peroxisomal membrane, principally ABCD transporters. Unlike other classes of transport proteins, ABC transporters generally mediate the transmembrane movement of a variety of substrates. Consequently, D2 may facilitate the metabolism of a number of fatty acyl CoAs. The type and number of atypical lipids dependent upon D2 for adipose tissue metabolism and the consequences of allowing atypical lipid accumulation within the adipose triglyceride storage pool awaits further investigation.
3. Chapter 3: The role of D2 in opposing dietary C22:1 induced metabolic diseases

3.1. INTRODUCTION

Peroxisomes are essential organelles that play critical roles in lipid metabolism, biosynthesis of ether phospholipids and bile acids and detoxification of hydrogen peroxide. Peroxisomes contain enzymatic machinery for β-oxidation of VLCFAs and branched chain fatty acids. However, peroxisomal β-oxidation is incomplete and produces C16~C18 acyl-CoA, which are then shuttled into mitochondria for further oxidation or ER for de novo lipid synthesis. Peroxisomal β-oxidation is also involved in the last step of polyunsaturated fatty acids (PUFA) synthesis such as docosapentaenoic acid (4,7,10,14,16-C22:5, n-6) and docosahexaenoic acid (DHA, 4,7,10,13,16,19-C22:6, n-3). Due to the limited types of desaturase that exist in mammals (delta 5, 6 and 9 desaturase), biosynthesis of C22:5 and C22:6 begins with the essential fatty acids linoleate (9,12-C18:2) and linolenate (9,12,15-C18:3). Linoleate and linolenate undergo a series of elongation and desaturation steps in the ER to generate 6,9,12,15,18,21-C24:6 (n-3) and 6,9,12,15,18-C24:5 (n-6) respectively, but must be transported into peroxisomes and chain shortened to yield 22 carbon HUFAs via β-oxidation.

The entry of fatty acids into peroxisome is dependent on ABCD transporters. There are four members in this subfamily: D1 (ALDP), D2 (ALDRP, adrenoleukodystrophy-related protein), D3 (PMP70, peroxisomal membrane protein 70KDa) and D4 (PMP69). ABCD transporters are half transporters that function as homodimers in vivo. However, when these proteins are overexpressed in vitro, heterodimers can be formed and are functional. The only known human disease associated with this class of transporters is X-linked adrenoleukodystrophy (X-ALD), a demyelinating disorder of the central nervous system that is caused by mutations in the D1 gene. X-ALD patients accumulate very long chain fatty acids (VLCFA) in plasma, adrenal cortex and the nervous system, presumably due to the lack transport into peroxisomes. The closet paralog of D1 is D2, which share 66%
amino acid sequence similarity and some overlapping function. Studies have shown that the overexpression of D2 is able to compensate for the loss of D1 and correct VLCFA accumulation in D1−/− mice and X-ALD fibroblast.

D2 mRNA has been detected in brain, liver, lung, and adipose tissue. mRNA levels of D2 in liver and adipose tissue respond differently to fasting and refeeding. In adipose tissue, D2 mRNA level significantly declines during fasting and restores when mice are refed with a high carbohydrate/low fat diet, which shows the same regulation pattern as other SREBP-1c target genes, indicating the possible role of D2 in lipid synthesis and storage in adipose tissue. However, the opposite pattern of D2 mRNA regulation was seen in liver, where expression induced by fasting but suppressed during refeeding. This pattern resembles the one of PPARα target gene acyl-CoA oxidase, which suggests that D2 might play a role in lipid degradation in the liver in energy depleted states. Moreover, active SREBP-1c can turn on D2 transcription by binding with SRE in D2 promoter, but LXR can block the binding of SREBP-1 with the SRE through a overlapping response element. The significance of this unique regulation pattern remains unexplored.

It has been previously shown in section 2.4 that D2 protein is most abundant in white adipose tissue (~50 fold compare to its level in liver). Among different fat depots, D2 is enriched in epididymal, inguinal and retroperitoneal fat. Unlike other srebp-1 target genes, D2 is not essential for lipid storage or adipogenesis in vitro and in vivo. We have shown that D2 is a major player in the metabolism of very long chain fatty acids (VLCFA), especially erucic acid (EA, C22:1, n-9). The absence of D2 leads to accumulation of erucic acid in both differentiated 3T3-L1 cells in vivo and in mice fed with an EA enriched diet.

Erucic acid is naturally enriched in rapeseed and mustard seed. It is well characterized that erucic acid can cause transient triglyceride accumulation (lipidosis) and dyslipidemia when fed to rats. However, mice are resistant to this effect. Due to health concerns in humans, EA was virtually eliminated from rapeseed oil in the development of canola oil. However, EA is used therapeutically as a
component of lorenzo's oil for treatment of X-ALD due to its inhibition of fatty acid elongation(341).

Our studies use erucic acid as a tool to understand the role of D2 in the clearance of very long chain fatty acid and its effect on lipotoxicity. We showed that deficiency of D2 sensitizes mice to the effect of erucic acid. We established a novel protective role of D2 against the EA induced obesity, dyslipidemia and hepatic steatosis.

3.2. MATERIALS AND METHODS

3.2.1. Animal husbandry

Mice harboring the mutant Abcd2 allele are maintained on the C57BL/6J background as heterozygotes. Strain refreshing is conducted every five generations using C57BL6/J females obtained from The Jackson Laboratory (Bar Harbor, ME). Genotyping experiments to differentiate Abcd2-deficient (D2−/−) from heterozygous and wild-type (WT) mice was conducted as previously described(332). Animals were housed in a temperature-controlled room with 12:12 light:dark cycle (6:00 am to 6:00 pm). All mice were maintained on standard rodent chow (Harlan Teklad 2014S). The erucic acid enriched diet was custom made by adding 50g/kg of purified erucic acid to standard rodent chow (#D1001, Research Diets, New Brunswick, NJ, Tables 2.4-3 and 2.4-4). The diet was provided in pelleted form and stored at 4°C. The diet was provided ad libitum and was replaced twice weekly.

Body composition of mice was measured using Echo MRI. Mice were killed by exsanguination under ketamine/xylazine anesthesia following a 4 hr fast beginning shortly after lights-on. Blood was collected from the right ventricle with a 1 cc syringe fitted with a 20ga hypodermic needle. Serum was separated by centrifugation and stored at -20°C until analysis. Tissues were excised, rinsed with PBS to remove blood and snap frozen in liquid nitrogen. Tissue samples were stored at -80°C until analyzed.
3.2.1. Blood analysis

Blood glucose levels were measured using a standard glucometer from a drop of blood obtained by tail-vein prick following a 4 hr fast beginning at lights-on. For glucose tolerance test, mice were intraperitoneally injected with sterilized 20% glucose solution (10 µl/ gram of body weight), blood glucose levels were measured prior to and 30, 60, 90 and 120 min time following glucose injection. Total serum cholesterol and triglyceride concentrations were determined by colorimetric-enzymatic assays (Wako Chemicals, Richmond, VA.) Plasma adipokine concentrations were determined using a mouse adipokine lincoPlex multiplex immunoassay kit, insulin levels was measured using ELISA.

3.2.2. Tissue histological analysis

Small pieces of liver and epididymal adipose tissue were excised and fixed overnight in 4% (w/v) paraformaldehyde (PFA) in PBS, pH7.4 and stored in 70% ethanol at 4°C until further use. Tissue was processed in a dehydrating ethanol gradient, followed by xylene incubation and paraffin embedding. Paraffin blocks of tissue were cut into sections of 1-3 µm thickness. Haematoxylin and eosin (H&E) stain of tissue sections was performed by COBRE facility.

Small pieces of tissue were directly embedded into a cryo-preservative solution (Optimal Cutting Temperature, OCT) in freezing molds and placed on dry ice. The OCT-embedded tissue was stored at -80°C prior to use. Frozen tissue were cut at 10µm thickness and subjected to Oil-Red-O staining.

3.2.3. Lipid analyses

Total lipids from individual mice were extracted by chloroform-BHT (50mg/ml) and fractionated into cholesteryl esters, triglycerides, and phospholipids using 500-mg silica columns (varian, 1211-3036) as previous described(110). C17:0 was added after extraction as internal standard. After methyl esterification with BF₃/Methanol (10%, Supelco, Bellefonte, PA) by incubating the mixture at 60°C for 16 hours (110, 131, 339), the fatty acid methyl esters were extracted by chloroform. Fatty acid composition was analyzed by GC-MS as described in section 2.3.4.
3.2.4. Fatty acid elongation assay

Fatty acid elongase activity was conducted as previously published\(^{(110)}\). Microsomes were prepared from wild-type and D2\(^{-/-}\) mice fed with erucic acid enriched diet. Microsomal proteins (30 \(\mu\)g) were incubated with a reaction mixture containing 150 \(\mu\)M \([^{14}\text{C}]\) malonyl-CoA (American Radiolabeled Chemicals, Inc., St. Louis, MO) and 20 \(\mu\)M of the indicated fatty acids in the presence of 100 \(\mu\)M CoA, 1mM ATP, and 1 mM NADPH at 37°C for 10 min. Fatty acids were extracted from the reaction mixture and the total incorporation of \([^{14}\text{C}]\) malonyl-CoA to fatty acid substrates was measured.

3.2.5. Protein and RNA analysis

Proteins were prepared and analyzed by SDS-PAGE and immunoblot analysis as previously described\(^{(334)}\). The isolation of total RNA and the determination of relative transcript abundance by quantitative Real-Time PCR (qRT-PCR) for both tissues and cells was conducted as previously described\(^{(334)}\).

3.2.6. Statistical analysis

Data were analyzed by ANOVA. Bonferroni posts tests were employed where indicated. All statistical analyses were conducted using GraphPad Prism statistical analysis software.

3.3. RESULTS

We previously published that WT and D2\(^{-/-}\) mice were indistinguishable with respect to body weight, adiposity and plasma lipids at 8 weeks of age\(^{(340)}\). Similarly, we observed no discernable differences between genotypes at the initiation of EA feeding (Figure 3.4-1a, week 1). Following 8 weeks of a diet enriched in EA feeding, we observed increased body weight in D2\(^{-/-}\) mice as compare to WT controls. Body composition analysis by MRI indicated a significant increase in fat mass in D2\(^{-/-}\) mice, but not WT controls. Following EA feeding, lean mass was lower in D2\(^{-/-}\) mice compared to WT controls, but due to the lack of an increase in D2\(^{-/-}\) mice as opposed to a decline. The accumulation of excess adipose tissue occurred primarily in epididymal and retroperitoneal depots, with a tendency towards an
increase in the inguinal fat pad. (Figure 3.4-1b). The expansion of adipose in the absence of an increase in lean mass produced a substantial increase in adiposity in D2−/− mice (Figure 3.4-1c). H&E staining of adipose tissue revealed that the expansion of adipose tissue was associated with the enlargement of adipocytes in D2−/− mice (Figure 3.4-1d). Analysis of fasting levels of serum lipids showed an elevation in cholesterol, but no change in triglycerides (Figure 3.4-2a). Fasting glucose levels were increased by 75% in D2−/− mice (Figure 3.4-2b). A glucose tolerance test revealed a decrease in glucose disposal (Figure 3.4-2c, d).

**Figure 3.3-1** EA feeding induces obesity in the absence of D2

(A) Body composition was measured by Echo MRI at the beginning and end of study (n=7). (B) Different fat pads were dissected and weighted at the end of study (n=4).
(C) Adiposity was calculated by ratio of fat mass/ body weight (n=4). (D) H&E staining of paraffin embedded epididymal fat. * p < 0.05, **p < 0.01, ***p < 0.001. Two way ANOVA
Figure 3.3-2 Dyslipidemia and glucose intolerance in D2⁻/⁻ mice fed with EA diet

(A) Serum was collected at the end of study, total cholesterol (TC) and triglycerides (TG) level in serum were measured. (B) Fasting glucose levels measured following 8 weeks of EA diet. (C) Glucose tolerance test was performed at week 8. Both real value and percentage value (D) Area under the curve of GTT were calculated. Data are shown as mean ± SD (n=7 unless indicated otherwise). * p < 0.05, **p < 0.01, ***p < 0.001, t test
The expansion of adipose and the loss of glycemic control is generally associated with inflammation of the adipose tissue. We measured serum levels of adipocytokines using a LINCOplex panel (Tabel 3.4-1). Consistent with the expansion of adipose tissue, leptin levels were elevated in D2⁻/⁻ mice. However, we failed to detect any differences in other adipocytokines typically associated with inflammation including adiponectin, Il-6 and TNF-α. We also stained adipose tissue sections for CD68 and did not observe an accumulation of crown macrophages or the presence of crown-like structures (data not shown). These data suggest the absence of inflammation in D2⁻/⁻ adipose tissue following EA feeding despite the increase in adipose mass, adipocyte hypertrophy loss of glycemic control.

**Table 3.3-1 Adipokine and insulin levels mice fed with EA diet**

<table>
<thead>
<tr>
<th></th>
<th>Erucic acid enriched Diet (22% kCal fat)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>D2⁻/⁻</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>2.704 ± 1.18</td>
<td>20.236 ± 6.53 *</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7.998 ± 0.89</td>
<td>10.30 ± 2.31</td>
</tr>
<tr>
<td>Resistin (pg/mL)</td>
<td>2991 ± 187</td>
<td>2570 ± 245</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>3104 ± 276</td>
<td>2806 ± 348</td>
</tr>
<tr>
<td>PAI-1(pg/mL)</td>
<td>3104 ± 276</td>
<td>2806 ± 348</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.30 ± 0.06</td>
<td>0.299 ± 0.13</td>
</tr>
</tbody>
</table>

GC-MS analysis of the fatty acid profile of epididymal adipose tissue showed that the relative abundance of EA was similar in WT and D2⁻/⁻ mice and comprised less
than 7% of the total fatty acids within adipose tissue (Figure 3.4-3a). The relative abundance of C18 and C20 monounsaturated fatty acids (MUFA) was lower in D2-deficient mice, which was offset by an increase in C14 and C16 saturated fatty acids (SFA). The reduction in 20:1 and 18:1 in D2−/− mice is consistent with a reduction in β-oxidation and shortening of dietary EA prior to storage in adipose tissue TAGs. However, it should be noted that given the increase in adipose tissue mass, the total amount of all fatty acids is greater in D2−/− compared to WT mice. On the whole, there was an increase in SFA and decrease in MUFA in adipose tissue, while the levels of polyunsaturated fatty acids were unaffected (Figure 3.4-3b).

To explore potential mechanism for increased adiposity as well as alterations in the lipid profile in adipose tissue, we analyzed the mRNA levels of several key genes involved in the regulation of adipose function and lipid and carbohydrate metabolism. Although the mRNA level of the lipogenic transcription factor, SREBP-1, was not altered, several SREBP-1 target genes were selectively downregulated in adipose tissue of D2−/− mice fed EA, including ACC1, FAS and SCD-1 (Figure 3.4-3c). In addition, transcription factors that regulate cholesterol, fatty acid and glucose homeostasis were also downregulated in D2−/− mice (LXR, ChREBP). Consistent with the development of obesity and insulin resistance, Insig-1 was also significantly suppressed in D2−/− mice. Consequently, it is difficult to discern if the alterations in gene expression in adipose tissue of D2−/− mice are causative or secondary to obesity and impaired insulin signaling.
Figure 3.3-3 Adipose tissue lipid profile and gene expression

(A) Lipid was extracted and methyl esterified, then analyzed using GC-MS for fatty acids profile. Fatty acid levels were normalized to internal standard C17 and expressed as percentage of total fatty acids. (B) Lipids are classified by degree of

---

83
saturation. (C) mRNA levels of lipogenic genes in adipose tissue were measured by qRT-PCR. Data are shown as mean ± SD (n=7 unless indicated otherwise). * p < 0.05, **p < 0.01, ***p < 0.001, t test

The development of obesity is often associated with ectopic accumulation of lipids. Therefore, we measured total lipids in liver. Indeed, there was an accumulation of both cholesterol and triglycerides (Figure 3.4-4a). H&E staining of liver sections revealed vacuolar structures in liver sections that stained positively with Oil-Red-O in D2−/− mice (Figure 3.4-4b). Whereas the alteration in the fatty acid profile in adipose tissue was limited to a few specific fatty acids, the hepatic fatty acid profile revealed significant differences for many fatty acid species (Figure 3.4-4c). There is dramatic shift from wild-type to D2−/− mice toward more MUFA (C18-22) and less PUFA (C18-22) (Figure 3.4-4c and inset). Levels of C22:6 and C20:4, as well as intermediates of PUFA synthesis such as C18:2 and C20:3, were significantly lower in D2−/− mice. Unlike adipose tissue, the absence of D2 leads to greater accumulation of C22:1 as well as C20:1 in liver, indicating the important role of D2 in the clearance of dietary C22:1. Another significant change is the dramatic increase in the level of C18:1 in D2−/− mice which constitutes nearly 50% of the total fatty acid pool in liver as compared to 30% in wild-type controls.
Figure 3.3-4 Liver histology and lipid profile

(A) Paraffin embedded liver sections from wild-type and D2-/- mice were stained with H&E. Cryo-sections of liver are stained with Oil-Red-O. (B) TC and TG levels in total hepatic lipid extraction were measured. (C) Fatty acid profile of total hepatic lipid were measured by GC-MS. Fatty acid levels were normalized to internal standard C17 and expressed as percentage of total fatty acids. Data are shown as
mean ± SD (n=7 unless indicated otherwise). Insert showed the summary of lipid profile by degree of saturation. * p < 0.05, **p < 0.01, ***p < 0.001, t test

EA is used to treat X-ALD patient due to its ability to inhibit elongase activity (341). Moreover, deficiency of elongase, especially ELOVL5, which is essential for PUFA biosynthesis is associated with hepatic steatosis (110). Therefore, we hypothesized that the reduction of PUFA synthesis could be due to inhibition of elongase activity by EA. Assays for measuring elongase activity were conducted. The results showed that the elongation of PUFA precursors were elevated in D2-/ mice compare to wild-type mice (Figure 3.4-5).

![Figure 3.3-5 Elongase activity in liver microsomes from mice fed with EA diet](image)

Figure 3.3-5 Elongase activity in liver microsomes from mice fed with EA diet

Elongase activity is measured as the incorporation of [14C] malonyl-CoA to fatty acid substrates. Each bar represents the mean ± SE of the values (n=4 unless indicated otherwise). * p < 0.05, ***p < 0.001, t test.
To explore other potential mechanisms for reduced PUFA levels. We measured expression of genes involved in PUFA and MUFA synthesis in liver. The results showed significant upregulation of SCD-1, D6D and SREBP-1(Figure 3.4-6a). SCD-1 is a important desaturase that introduces a double bond at the n-9 position of C16 and C18 to generate C16:1 and C18:1, respectively. The selective upregulation of SCD-1 among other SREBP-1 target genes reflected the increase in MUFA, in this case C18:1 in hepatic lipid profile (30% in wild-type vs 50% in D2⁻/⁻ mice). The ratio of C18:1/C18 was a standard method used to estimate SCD-1 activity. By using this method, we detected a 123% increase in SCD-1 activity in D2⁻/⁻ mice compare to wild-type mice. (Figure 3.4-6b).

It has also been reported that endogenously synthesized C18:1 is the preferred substrate for acyl-CoA: cholesterol acyltransferase 1 (SOAT1), which catalyze the esterification of fatty acid acyl-CoA and free cholesterol. To understand if the accumulation of C18:1 in D2⁻/⁻ mice liver contributed to the increase in hepatic cholesterol ester, total hepatic lipid extraction was separated into cholesterol ester (CE), triglycerides (TG), unesterified fatty acid (NEFA) and phospholipid (PL) by silica column(58). The fatty acid profile of CE but not TG showed significant elevation of C18:1 and reduction in C18 in D2⁻/⁻ mice (Figure 3.4-6c&d). In addition, free cholesterol detected in unesterified fatty acid fractions from D2⁻/⁻ mice were significantly decreased, indicating that more free cholesterol had been actively esterified to C18:1 cholesterol esters because of the excessive availability of C18:1 in D2⁻/⁻ mice liver (Table 3.4-2). The elevation in MUFA content in total hepatic lipid extract was only reflected in CE lipid pool, which also suggested that the accumulation of C18:1 is driving cholesterol esterification in liver.
Figure 3.3-6 liver gene expression and lipid profile

(A) mRNA levels of liver lipogenic genes were measured by qRT-PCR. (B) Desaturase index calculated by ratio of product/precursor of specific desaturase (Δ9: C16:1/C16:0, Δ9: C18:1/C18:0, Δ6: C18:3/C18:2, Δ5: C20:4/C20:3). (C) Hepatic lipid isolated from mice was separated into different lipid pools, lipid profile of each fraction were analyzed using GC-MS (n=3). Ratio of saturated/monounsaturated
fatty acids was calculated for each lipid fraction. (E) Lipid profile of hepatic cholesterol ester pool. * p < 0.05, **p < 0.01, ***p < 0.001, t test
Table 3.3-2 Lipid profile of major hepatic lipid classes

<table>
<thead>
<tr>
<th></th>
<th>CE (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>FFA (mg/dL)</th>
<th>PL (mg/dL)</th>
<th>TFA (mg/dL)</th>
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<tr>
<td></td>
<td>++</td>
<td>+/−</td>
<td>++</td>
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<tr>
<td>14:0</td>
<td>1.2±2.1</td>
<td>0.82±1.0</td>
<td>2.3±0.4</td>
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<tr>
<td>16:0</td>
<td>36.8±5.1</td>
<td>32.1±2.5</td>
<td>28.0±4.8</td>
<td>3.8±6.7</td>
<td>23.1±1.0</td>
</tr>
<tr>
<td></td>
<td>4.0±3.9</td>
<td>3.7±1.8</td>
<td>5.0±0.02</td>
<td>0.4±0.7</td>
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<td>18:0</td>
<td>26.8±4.0</td>
<td>8.9±6.0*</td>
<td>2.9±2.3</td>
<td>9.3±0.5</td>
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<td>27.4±5.0</td>
<td>47.3±4.0**</td>
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<td>14.9±3.9</td>
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<td>18:2</td>
<td>7.8±3.3</td>
<td>6.5±0.1</td>
<td>14.7±2.9</td>
<td>11.2±2.9</td>
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</tr>
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<td>0.07±0.1</td>
<td>0.2±0.4</td>
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<td>20:1</td>
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<td>0.6±1.1</td>
<td>1.2±1.0</td>
<td>1.8±0.1</td>
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<tr>
<td>20:2</td>
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<td>0.7±0.2</td>
<td>7.0±12.1</td>
<td>12.7±11.0</td>
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<td>20:3</td>
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<td>2.2±2.5</td>
<td>0.9±0.2</td>
<td>1.3±0.2*</td>
</tr>
<tr>
<td>22:6</td>
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<td>2.3±2.6</td>
<td>4.6±1.5</td>
<td>2.2±0.5*</td>
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<tr>
<td>cholesterol</td>
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<td>18.2±1.8</td>
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</tbody>
</table>
3.4. DISCUSSION

It has been shown in the previous chapter that D2 promotes the clearance of dietary EA after short term feeding the EA enriched diet (4 weeks). In this chapter, our studies demonstrated that D2 protects mice from EA induced metabolic syndrome following long term feeding.

EA causes transient lipidosis and dilated cardiomyopathy in mice and other species. EA is also associated with an elevation in liver triglycerides after long term feeding of rats (306). However, mice are resistant to the deleterious effects of EA. In the absence of D2, the phenotype is revealed in mice and is no longer transient. This suggests that mice have sufficient D2 present at all times or can upregulate D2 very rapidly to compensate for the presence of dietary EAs. Interestingly, D2 expression is highly regulated during 3T3-L1 adipogenesis as described in aim I, whether this provides mice with protection against EA toxicity is still unknown. Unlike the rat phenotype, we did not observe evidence of dilated cardiomyopathy. While the species difference is interesting, it is beyond the scope of these studies.

EA leads to obesity and insulin resistance in the absence of D2. However, the expanded adipose tissue does not show signs of inflammation, which implies that the obesity phenotype is not cause of insulin resistance (IR) but secondary.

Although D2 opposes the transient accumulation of EA in adipose tissue, EA levels in wild-type and D2⁻/⁻ adipose tissue reach the same amount after long term feeding. The level of EA detected in adipose tissue (around 5%) is much lower than its level in the diet (50%), suggesting that EA is not a good substrate for esterification and storage as TAG. Indeed, its rate of esterification to TG is about 1/3 of the rate of palmitic acid in rat liver and epididymal fat (307-310), (313). Failure of the proper storing of EA in the form of TAG increases the demand for β-oxidation in peroxisomes in order to eliminate its toxic effects. In the absence of D2, import of EA into peroxisomes for β-oxidation in liver and adipose tissue is compromised, therefore exposing liver to more EA. Consistently, EA level is higher in liver of D2⁻/⁻ mice compare to wild-type mice. However, the immediate β-oxidation products of
EA: C20:1 and C18:1 levels are also higher in D2−/− mice, indicating that there is compensatory pathway responsible for β-oxidation of EA in the liver in the absence of D2. ABCD1 expression in liver is higher than D2. Substrate specificity studies of D1 and D2 showed that C22 are good substrate for both D1 and D2 facilitated peroxisomal import (198, 289). Therefore, it is possible that D1 is compensating the loss of D2 and promoting the oxidation of EA in liver.

In addition, EA disrupted the ratio of MUFA to PUFA in liver of D2 deficient mice. Low PUFA bioavailability is associated with increased SREBP1 activity and SREBP-1 dependent gene expression. Indeed, we observed increased SREBP and elevated expression of some target genes, but not others. However, liver tissues from these mice are collected during the fasting stage, when SREBP1 pathway is suppressed. So the results could under-represent the true gene expression levels. This suggests that in the absence of D2, EA bypassed the regulatory machinery to detect lipid overload in liver, resulting in accumulation of C18:1, either from endogenous synthesis or shortening of EA via ABCD1.

The precise biochemical mechanism by which EA disrupts lipid metabolism remains unresolved. We hypothesized that it may be related to inhibition of elongase activity, but in fact, the opposite phenotype was observed. This results were consistent with other literature, which reports increased elongase activity in fibroblasts from X-ALD patients. Further studies are needed to measure the desaturase activity, which might explain the increased MUFA/MUFA ratio.
4. Chapter 4: Determine the subcellular localization of D2 in adipose tissue

4.1. INTRODUCTION

Peroxisomes are ubiquitous subcellular organelles present in most eukaryotic cells. General biochemical functions of peroxisomes include β-oxidation of fatty acids, biosynthesis of plasmalogens (ether lipids) and bile acids, and the detoxification of hydrogen peroxide by catalase (315, 316). Peroxisomes, often described as multipurpose organelles, exhibit significant functional differences not only between species but between different tissues within single organisms. This functional diversity is achieved by modulating their protein composition (342).

Peroxisomes are best characterized in liver due to their high abundance and induction by peroxisome proliferators. In addition to peroxisomal fatty acid β-oxidation, liver peroxisomes contain unique enzymatic machinery for bile acid synthesis. Bile acid intermediates DHCA (dihydroxycholestanoic acid) and THCA (trihydroxycholestanoic acid) are synthesized from cholesterol and imported into peroxisomes, where their side chains are shortened by the enzymatic machinery required for degradation of methyl-branched fatty acids. Peroxisomal modified bile acids are subsequent conjugated to taurine or glycine (343). Since conversion of cholesterol into bile acids is the predominant route for its removal from the human body, peroxisomes have been regarded as important for maintenance of cholesterol hemeostasis.

Peroxisomes isolated from different tissues including kidney, intestine, heart, lung, brain and testis are heterogeneous and contribute to varying pathways in each tissue. For example, testicular peroxisomes are enriched in Leydig cells, proliferate in response to luteinizing hormone (LH), and are associated with free cholesterol staining (224, 344). However, the exact physiological role of peroxisomes in testis is still unknown. In contrast, peroxisome abundance in sertoli cells are very low.

AB. Novikoff et. al, have reported the presence of microperoxisomes in differentiating 3T3-L1 cells (345, 346). These microperoxisomes lack nucleoids, are...
small (ranging from 0.05 to 0.25 µm in length and are ~0.1 µm wide) and may be contiguous with the ER. These organelles are more numerous in differentiating 3T3-L1 cells than in any other cell types studied at that time. These studies imply that peroxisomes could play important role in lipid synthesis during adipogenesis. Indeed, up to 40% of glycerolphosphate backbone synthesis for TAG synthesis in 3T3-L1 cells is thought to occur in the alternative pathway from peroxisomal acyl-DHAP (acyl dihydroxyacetone phosphate)(347).

Import of fatty acyl-CoAs into peroxisomes is dependent on ABC transporters. All peroxisomal ABC transporters are half-transporters and belong to D subclass of the ABC protein family. There are four members in this family: D1 (ALDP, adrenoleukodystrophy protein), D2 (ALDRP, adrenoleukodystrophy-related protein), D3 (PMP70, peroxisomal membrane protein 70KDa) and D4 (PMP69). The primary structure of D2 contains a peroxisomal targeting signal (PTS) and is thought to be a peroxisomal membrane protein like D1 and PMP70, whereas D4 has recently been reassigned to the ER(278). D2 has been reported as peroxisomal membrane protein in transiently transfected CHO cells in vitro(348). However, the localization of D2 to peroxisomes has not been formally demonstrated.

Expressions of ABCD transporters among different tissues are also distinct. D1 and D2 mRNA and protein expression levels display a mirror pattern in most mouse tissues. In adrenal gland, D1 in mainly expressed in the adrenal cortex whereas D2 expression is found in the adrenal medulla(287). In testis, D1 highly expressed in Sertoli cells, whereas D2 shows highest concentrations in Leydig cells(224). In our studies, we have demonstrated that within adipose tissue, D1 is enriched in stromal vascular fraction and D2 is exclusively expressed in adipocytes(340). However, subclass of peroxisome within cell-type has not been reported.

D2 mRNA has been detected in brain, liver, lung, and is highly abundant in adipose tissue. Similar to other SREBP-1 target genes, D2 mRNA in adipose tissue is suppressed during fasting and restored when mice are refed with a high carbohydrate/low fat diet, indicating the possible role of D2 in lipid synthesis and
storage in adipose tissue. However, peroxisomes are not major component of adipocytes and the abundance and role of peroxisomes in adipose tissue has never been carefully characterized. To explore the role of D2 and peroxisome, we examined the subcellular localization of D2 in adipose tissue of mouse using a variety of peroxisomal markers with morphological and biochemical approaches.

4.2. MATERIALS AND METHODS

4.2.1. Isolation of peroxisome

A protocol was adapted from published methods for peroxisome isolation from liver (349-351). Adipose tissue was dissected from mice, placed in ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA, 50 mM MOPS, pH 7.4) and minced into small pieces using scissors. The tissue was homogenized using Potter-Elvehjem homogenizer for 5 strokes at 500 rev./min at 4°C. The homogenate was centrifuged at 750 x g, 4°C for 10 min to generate post-nuclear supernatant (named nuclear supernatant, NS). The NS fraction was then centrifuged at 8500 xg for 10 min to generate mitochondria enriched pellet (named heavy mitochondria pellet, HM). The resulting supernatant (named heavy mitochondria supernatant, HMS) was then centrifuged at 27,000 xg for 20 min to generate peroxisome enriched pellet (named light mitochondria pellet, LM). The resulting supernatant (light mitochondria supernatant, LMS) was centrifuged at 100,000 xg, for 45 min to precipitate all the membrane proteins. All the pellet fractions generated were resuspended in 1X protein sample buffer. Protein concentration of each fraction was determined.

4.2.2. Immunofluorescence:

CHO-K1 cells were transiently transfected with plasmids containing myc-tagged D2. Cells are fixed in methanol (-20°C) for 5 min, blocked in 0.1% BSA in PBS for 1 hour and then incubated with primary antibody in blocking solution overnight at 4°C with gentle rocking. Following wash with PBS, samples incubated with fluorescent conjugated secondary antibody for 1 hour. After being washed with PBS for 3 times, the cells are mounted on #1.0 borosilicate coverglass, covered with
~150 µl of Vectashield mounting medium and imaged under fluorescence microscope.

4.2.3. Fixation and EM

Biochemically generated fractions (HMP or LMP) from adipose tissue were fixed in 4% paraformaldehyde and washed 3 times for 5 min with 0.1 M cacodylate buffer. Samples were dehydrated in a series of graded ethanol (70% to absolute) and embedded in Epoxy resin. Ultrathin sections were inspected after contrasting with electron microscope.

4.2.4. Immunoelectron microscopy

Wild-type and D2⁻/⁻ mice were anaesthetized by intraperitoneal injection and perfused via the left ventricle with a mixture of 4% paraformaldehyde, 2% glutaraldehyde in 0.01M cacodylate buffer (pH7.4). After fixation, adipose tissue were dissected, cut in slices with razor blades, post-fixed in 2% glutaraldehyde in cacodylate buffer (pH7.4) for 45 min, and washed 3 times for 5 min with 0.1 M cacodylate buffer. Fixed adipose tissue were embedded into LR White resin (medium grade). LR White-filled gelatin capsules were polymerized at 50°C for three days. After preparation of semithin sections blocks, ultrathin sections of 80nm were cub, collected on 100 mesh nickel grids and therefore coated on the back side with a 1% formvar film. The grids were dried at 37°C overnight prior to immunostaining. The sections were incubated with blocking solution (1% BSA in TBST) for 30 min at room temperature. Incubation with the primary antibodies was performed on droplets with antibodies (anti-D2 and anti-pex19) in 0.5% BSA in TBST overnight at room temperature. Thereafter the sections were incubated with a secondary antibodies conjugated with gold particles (12nm particles conjugated with anti-rabbit antibody and 6nm particles conjugated with anti-mouse antibody) in 0.5% BSA in TBST for 1 hour at room temperature. Negative controls were processed in parallel. The grids were rinsed on droplets of TBST and subsequently contrasted with uranyl acetate for 2 min and lead citrate for 45 sec. The sections were examined using electron microscope.
4.3. RESULTS

We first examined the subcellular localization of D2 in CHO-K1 cells transfected with plasmid harboring a cDNA encoding full length murine ABCD2 engineered with a COOH terminal myc tag. Cells transfected with G5-myc containing plasmid were used as negative control.

CHO-K1 cells were immunostained with anti-myc and anti-pex19 antibodies, and imaged using fluorescent microscopy. D2-myc protein showed a punctuate staining pattern consistent with peroxisomal proteins (Figure 4.4-1). A similar pattern was seen in the pex19 signals. However, the D2 signals do not overlap with pex19 signals in the merged image.

![Figure 4.3-1 Localization of D2 and Pex19 in transfected CHO-K1 cells](image URL)

CHO-K1 cells are transfected with a plasmid containing a cDNA encoding mouse D2 and myc tag. Cells were processed for indirect immunofluorescence microscopy using monoclonal myc antibody and polyclonal pex19 antibody.
Another peroxisomal marker PMP70 was used to confirm our finding. In the merged image, yellow signals are visible, representing the overlapping of D2 (red signals) and PMP70 (green signals) (Figure 4.4-2). Stacks of images were taken using a confocal microscope. A 3D cut view of stacked images is shown in the boxes located on the top and right side of main image (Figure 4.4-3). Red and green signals are clearly identifiable and do not co-localize with each other. This suggests that the yellow signals seen in the 2D images were artifactual. Our studies suggested that although D2 protein displays classical punctuate pattern of peroxisomes, it does not co-localize with pex19 or PMP70 in transfected CHO-K1 cells.

Figure 4.3-2 Localization of D2 and PMP70 in transfected CHO-K1 cells

CHO-K1 cells are transfected with a plasmid containing a cDNA encoding mouse D2 and myc tag. Cells were processed for indirect immunofluorescence microscopy using monoclonal myc antibody and polyclonal PMP70 antibody.
Figure 4.3-3 Confocal imaging of D2 and PMP70 in transfected CHO-K1 cells
Images were taken under confocal microscope at apotome mode. Cut view images were generated using Zeiss AxioVision software.

To confirm the results in vivo, adipose tissue was fractionated using a protocol adapted from liver that generates a peroxisome enriched fraction that also contains light mitochondria (LM). A flowchart of the protocol and designation for each fraction is shown in Figure 4.4-4. To confirm the successful isolation of peroxisomes, LM fractions from liver and adipose tissue were fixed and imaged using electron microscope (Figure 4.4-5). Hepatic peroxisomes are clearly identifiable as single membrane organelle with dense matrix. However, peroxisomes found in adipose tissue (0.2 ~ 0.25 µm in diameter) are smaller compare to the ones found in liver. This finding is consistent with the microperoxisomes reported in differentiating 3T3-L1 cells. HM fractions that contain heavy mitochondria as also imaged as control. In these HM images, the mitochondria are recognizable by their cristae and outer tripartite membrane. Our results confirmed the enrichment of peroxisomes in LM fraction in both liver and adipose tissue.
Figure 4.3-4 Flowchart of method used to isolate peroxisomes from adipose tissue
Figure 4.3-5 EM imaging of mitochondria and peroxisomes isolated from liver and adipose tissue

HM and LM pellet isolated from liver and adipose tissue of wild-type mice (n=4) were fixed using 4% paraformaldehyde and processed for EM imaging.

To determine the subcellular localization of D2 in adipose tissue, 2.3% of each fraction was loaded on an SDS-PAGE gel and immunoblotted for D2 (Figure 4.4-6). As we expected, D2 is abundant in the LM fraction. To our surprise, other peroxisomal markers including pex19, D3 (PMP70) and catalase were absent in LM fraction, but present in the LM supernatant. However, another peroxisomal marker pex14 was present in the LM fraction, which indicates that D2 resides in a distinct compartment that does not contain catalase, pex19 or PMP70. However, the LM fraction contained pex14, suggesting that D2 is in a distinct subclass of peroxisome, rather than other organelle.
Figure 4.3-6 Abundance of D2 and other peroxisomal markers in biochemically generated fractions from adipose tissue

2.3% of each fraction generated from adipose tissue was loaded onto SDS-PAGE. Abundance of D2 and other peroxisomal markers were examined (n=4).

To determine the localization of D2 within adipose tissue, LM fractions were isolated from adipose tissue of wild-type and D2−/− mice, immunolabeled with D2 antibody and imaged with an electron microscope. Results demonstrated that D2 protein is specifically associated with organelles that are similar to microperoxisomes observed in the LM fractions from adipose tissue in figure 4.4-5 (Figure 4.4-7). The absence of D2 signals in the LM fraction from D2−/− mice demonstrated the specificity of D2 antibody used in this experiment.
Figure 4.3-7 Immunogold labeling of peroxisomes isolated from wild-type and D2/- adipose tissue

Peroxisomes were isolated from adipose tissue of wild-type and D2/- mice (n=4). The resulting pellet were fixed and immunostained using a D2 antibody. Pellet were then embedded and imaged under electron microscope.

In addition to biochemically generated fractions, we also examined the localization of D2 in whole adipose tissue. Adipose tissue was immunostained with D2 and pex19 antibody and processed for EM imaging (Figure 4.4-8). Pex19 antibody was labeled with secondary antibody conjugated with 12 nm gold particles (as indicated by red arrows). D2 antibody was recognized by secondary antibody conjugated with 6 nm gold particles (as indicated by black arrows). Results clearly show that D2 dose not co-localize with pex19 in adipose tissue. However, we could not use pex14 antibody to confirm the co-localization of D2 with pex14 because both antibodies are generated in rabbit.
Mice were perfused with a mixture of 4% paraformaldehyde, 2% glutaraldehyde in 0.01M cacodylate buffer (pH7.4). After fixation, epididymal fat were dissected and embedded. The embedded samples are cut into ultrathin sections of 80nm and immunostained with D2 and pex19 antibody.

**4.4. DISCUSSION**

Our studies demonstrate the subcellular localization of D2 in a compartment consistent with peroxisomes within adipose tissue. However, D2 resides in a distinct subclass of peroxisomes that does not contain classical peroxisomal markers such as catalase, pex19 or PMP70. Catalase is a peroxisomal matrix protein. The absence of catalase in LM fraction could not fully exclude the existence of catalase in D2 containing peroxisomes if the integrity of peroxisomes is destroyed in the preparation and peroxisomal matrix proteins have been released. Therefore, it is
necessary to determine the abundance of catalase in whole adipose tissue using Immunoelectron microscopy.

D2 containing peroxisomes in adipose tissue has not been reported previously. The physiological role of D2 in adipose tissue is still not completely understood. Peroxisomes are heterogeneous organelle that can modulate their protein composition to meet the specific metabolic needs of particular tissue. To investigate the role of D2 containing peroxisomes in adipose tissue, protein composition of this organelle needs to be identified. Peroxisomal isolation methods that allow for purification of the D2 containing compartment without losing the structural or functional integrity of the complex will be critical for the purpose of proteomic analysis. Generally, peroxisomes are purified by combining the classical differential centrifugation and density gradient centrifugation. These fractions still contain other contaminating organelles such as mitochondria. By using a specific antibody against peroxisomal membrane protein D3, Miki Kikuchi et al. were able to immunoisolate peroxisomes and perform proteomic analysis (352). We have adapted this method and successfully immunoisolated D2 containing compartment in CHO-K1 cells transfected with D2-myc plasmid (Figure 2.4-1c). In future experiments, D2 containing peroxisomes will be immunoisolated from adipose tissue and analyzed by proteomics, which will reveal the function of this unique organelle and D2 in adipose tissue. This experiment may also provide us with information about how D2 is distinguished from some classic peroxisomal markers such D3 and targeted to this unique subclass of peroxisomes.

In addition, our data suggest that the peroxisomes found in adipose tissue are consistent with the microperoxisomes found in differentiating 3T3-L1 cells. Interestingly, D2 is also highly upregulated in differentiating 3T3-L1 cells, indicating that this novel organelle plays a role in adipogenesis. However, we have demonstrated in aim I that D2 is not required for either adipocytes differentiation or lipid storage. The role of this D2 containing compartment needs further investigation.

Moreover, the function of classic peroxisomes (D3 containing) in adipose tissue is also a mystery. Therefore, immunoisolation of D3 containing peroxisomes and
analysis of its protein composition is necessary. These future experiments would greatly increase our knowledge on peroxisomes in adipose tissue.
5. Chapter 5: General Discussion

The goal of my studies was to determine the role of D2 in adipose tissue with regards to adipocyte differentiation, lipid accumulation, promoting metabolism of VLCFA, the subcellular localization of D2 within adipose tissue, and the role of peroxisome in adipose tissue.

First, we determined the abundance of D2 in adipose tissue. Our studies showed that the expression of ABCD2 protein is much more abundant in adipose tissue than in other tissues in which it is expressed. D2 is exclusively enriched in adipocytes within adipose tissue. This is the first report on the relative high abundance of D2 in adipose tissue. We also observed a high molecular weight immuno-reactive form of D2 in adipose tissue. Although the nature of this D2 protein is still unknown, it is the major form of D2 in adipose tissue. We also demonstrated that D2 is upregulated during adipogenesis. Although D2 is a component of the adipogenic program, its expression is not required for adipogenesis or lipid storage in vitro or in vivo. This finding is unusual for a SREBP target gene. However, the regulation of D2 by both LXR and SREBP in an opposite direction represents a novel regulation pattern which distinguishes D2 from classical lipogenic genes regulated by SREBP.

Next, we studied the effect of D2 on the clearance of the atypical fatty acid C22:1. The majority of lipids stored in adipose tissue triglycerides is comprised of the fatty acids palmitate, oleate and linoleate (16:0, 18:1 & 18:2, respectively). However, dietary lipids can contain many “atypical” fatty acids that may require remodeling to one of these forms prior to storage. The term “atypical” is used to describe fatty acids that are not generally stored in triglyceride pools. Peroxisomes harbor a unique set of enzymes that are capable of β-oxidation of very long chain, polyunsaturated and branched chain fatty acids. The entry of atypical fatty acyl CoAs into peroxisomes is thought to be dependent upon the transporters on the peroxisomal membrane, principally ABCD transporters. Unlike other classes of transport proteins, ABC transporters generally mediate the transmembrane
movement of a variety of substrates. As the most abundant peroxisomal ABC transporter in adipose tissue, we hypothesized that D2 could facilitate the import of its substrates such as very long chain fatty acyl-CoA into peroxisomes for clearance. Our preliminary data revealed the accumulation of erucic acid in adipose tissue of D2\(^{-/-}\) mice. Therefore we tested erucic acid as a potential substrate of D2. Erucic acid accumulates in adipose of D2\(^{-/-}\) and D2\(^{+/-}\) when mice were acutely challenged with an erucic acid enriched diet, suggesting that D2 facilitates metabolism of this dietary fatty acid in adipose tissue. To the best of our knowledge, this is the first report that establishes a role for D2 in VLCFA metabolism in non-neuronal tissues. In addition, our results suggest a novel role for peroxisomes within adipose tissue in the clearance of “atypical” dietary lipids. The type and number of atypical lipids dependent upon D2 for adipose tissue metabolism awaits further investigation.

In the second part of my studies, we examined the consequences of allowing atypical lipid accumulation within the adipose triglycerides. EA causes transient lipidosis and dilated cardiomyopathy in rats and other species. EA is also associated with elevation in liver triglycerides after long term feeding of rat(306). However, mice are resistant to the deleterious effects of EA. In the absence of D2, mice are sensitized to the deleterious effects of EA and the phenotypes are no longer transient.

EA leads to obesity and insulin resistance in the absence of D2. However, the expanded adipose tissue does not show signs of inflammation, which implies that the obesity phenotype is not cause of insulin resistance but secondary to expanded adipose tissue. Although D2 opposes the transient accumulation of EA in adipose tissue, EA levels in wild-type and D2\(^{-/-}\) adipose tissue reach the same amount after long term feeding. The level of EA detected in adipose tissue (around 5 %) is much lower than its level in the diet (50%), suggesting that EA is not a good substrate for esterification. Fat absorbed from diet is first delivered to adipose tissue by chylomicrons for storage in TAGs before it reaches liver. If a fatty acid is not a good substrate for esterification, the fatty acids will not be efficiently packaged into TAGs and stored in adipose tissue. Indeed, its rate of esterification to TAG is about 1/3 of
the rate of palmitic acid in rat liver and epididymal fat (307-310), (313). Failure of the proper storing of EA in TAGs exposes both adipose tissue and liver to EA and increases the demand for its clearance through peroxisomal β-oxidation in order to eliminate its toxic effects. In the absence of D2, import of EA into peroxisomes for β-oxidation is compromised, therefore exposing liver to more EA, which is the major organ for lipid metabolism. Consistently, EA level is higher in liver of D2−/− mice compare to wild-type controls. However, the immediate β-oxidation products of EA: C20:1 and C18:1 levels are also higher in D2−/− mice, indicating that there are compensatory pathway responsible for β-oxidation of EA in the absence of D2. The abundance of D1 in liver is higher compare to D2, therefore D1 could be responsible for the oxidation of EA. So the question remains: what does D2 do with EA in adipose tissue?

In addition, EA disrupted the ratio of MUFA to PUFA in liver of D2 deficient mice. Low PUFA bioavailability is associated with increased SREBP1 activity and SREBP-1 dependent gene expression. Indeed, we observed increased SREBP and elevated expression of some target genes (SCD1 and D6D), but not others. We also observed an elevation in C18:1 in liver that is associated with increased C18:1-cholesterol esters. Consistent with other reports on C18:1 being a driver of cholesterol esterification, the increased MUFA contributes to this unique CE enriched hepatic steatosis phenotype. These results suggested a novel role for peroxisomes in the maintenance of MUFA/PUFA ratios in liver, which is known to be essential to avoid steatosis.

Peroxisomes are poorly understood organelle outside of the liver and perhaps the brain. Previous studies showed the presence of microperoxisomes in 3T3-L1 cells. However, no study has been conducted to determine the role of microperoxisomes in adipocyte differentiation. Interestingly, D2 protein is highly regulated during 3T3-L1 differentiation, whether D2 is associated with the microperoxisomes needs further investigation.
D2 contains a peroxisomal targeting signal (PTS) in its primary structure and is thought to be a peroxisomal membrane protein like D1 and D3. However, its localization to peroxisomes has not been formally demonstrated. Since only sparse information is available on D2 or peroxisomes in the adipose tissue, we have characterized the subcellular localization of D2 in adipose tissue of mouse using a variety of peroxisomal markers with morphological, biochemical techniques.

Our studies have clearly demonstrated the subcellular localization of D2 in a compartment consistent with peroxisomes within adipose tissue. However, D2 resides in a distinct subclass of peroxisomes that do not contain classical peroxisomal markers such as catalase, pex19 or PMP70. Our results revealed a novel subpopulation of D2 containing peroxisomes in adipose tissue has never been reported before. The physiological role of D2 containing peroxisome in adipose tissue needs further investigation.

In conclusion, this research demonstrated a novel role of D2 and peroxisomes in opposing the accumulation of erucic acid and protecting mice from the deleterious effects associated with this atypical fatty acids. The studies also revealed a novel subclass of peroxisomes that contains D2, but not classical peroxisomal markers. Further studies are required to identify the protein composition of this organelle and provide deeper insight into its role in adipose tissue.

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6. Chapter 6: Future Directions

Peroxisomes are heterogenous organelles that can modulate their protein composition to meet the specific metabolic needs of particular tissue. To investigate the role of D2 containing peroxisomes in adipose tissue, protein composition of this organelle needs to be identified. Proper peroxisomal isolation method that allows purification of this D2 containing compartment without losing the structural or functional integrity of the complex is critical for the purpose of proteomic analysis. Generally, peroxisomes are purified by combining the classical differential centrifugation and density gradient centrifugation. These fractions still contain other contaminating organelles such as mitochondria. By using a specific antibody against peroxisomal membrane protein D3, Miki Kikuchi et al. was able to immunoisolating peroxisomes and perform proteomic analysis (352). We have adapted this method and successfully immunoisolated D2 containing compartment in CHO-K1 cells transfected with D2-myc plasmid (Figure 2.4-1c). In the future experiment, D2 containing peroxisomes will be immunoisolated from adipose tissue and analyzed by proteomic, which will provide information for the function of this unique organelle and D2 in adipose tissue.

Moreover, peroxisomal protein content is distinct within cell-types. How this diversity is achieved is poorly understood. How is D2 get separated from other peroxisomal proteins and incorporated into this distinct subclass of peroxisomes is still a myth. Understanding the protein targeting of D2, and identify possible novel regulator for peroxisomal protein targeting by proteomic of D2 containing peroxisomes will benefit such studies.

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### 7. Appendices

Appendix I: Buffers and solutions

Table 0-1 Solutions, buffers and medium used in this dissertation

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solutions for Biochemistry</strong></td>
<td></td>
</tr>
<tr>
<td>50X TAE Buffer</td>
<td>121.1g of Tris-base, 28ml of Glacial acetic acid, 50ml of 0.5M EDTA, adjust pH to 8.0, qs to 500ml with H₂O</td>
</tr>
<tr>
<td>4X Laemmli Buffer</td>
<td>120 mM Tris base, 40 mM EDTA, 20% glycerol, 0.025% bromophenol blue, pH 6.8</td>
</tr>
<tr>
<td>Lower Tris Buffer</td>
<td>1.5 M Tris base, 0.4% w/v SDS, pH 8.8</td>
</tr>
<tr>
<td>Upper Tris Buffer</td>
<td>0.5 M Tris base, 0.4% w/v SDS, pH 6.8</td>
</tr>
<tr>
<td>10X Running Buffer</td>
<td>250 mM Tris base, 1.92 M Glycine, 1% w/v SDS</td>
</tr>
<tr>
<td>10X Transfer Buffer</td>
<td>250 mM Tris base, 1.92 M Glycine</td>
</tr>
<tr>
<td>10X TBS</td>
<td>200 mM Tris-base, 1.37 M NaCl, pH 7.6</td>
</tr>
<tr>
<td>TBST (Wash Buffer)</td>
<td>0.2% Tween 20, 1X TBS</td>
</tr>
<tr>
<td>Blotting Buffer</td>
<td>5% Carnation dry milk, 0.2% Tween 20, 1X TBS</td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>62.5 mM Tris-base, 2.0 % SDS, 100 mM β-mercaptoethanol, pH 6.7</td>
</tr>
<tr>
<td>Triton Lysis Buffer</td>
<td>80 mM NaCl, 50 mM Tris (pH 8.0), 2 mM CaCl₂, 1% Triton, supplemented with 10% Protease Inhibitors</td>
</tr>
<tr>
<td>Membrane Protein Buffer</td>
<td>20 mM Tris-Cl (pH7.5), 2 mM MgCl₂, 0.25 M sucrose, pH 7.5, qs to 500 ml</td>
</tr>
<tr>
<td>Homogenization Buffer for Tissue Fractions (SEM Buffer)</td>
<td>250 mM Sucrose, 1 mM EDTA, 50 mM MOPS, supplemented with 10% Protease Inhibitors, pH7.4</td>
</tr>
<tr>
<td><strong>SDS-PAGE Solutions:</strong></td>
<td></td>
</tr>
<tr>
<td>10% resolving gel for 2 SDS-PAGE gels</td>
<td>4.5 ml of lower tris, 4.5 ml of 40% Acyl-bis, 9 ml of ddH₂O, 180 ml of 10% aps, 20 ml of Temed</td>
</tr>
<tr>
<td>4% stacking gel for 2 SDS-PAGE gels</td>
<td>1.5 ml of upper tris, 600 µl of 40% Acyl-bis, 3.9 ml of ddH₂O, 60 µl of 10% APS, 20 µl of Temed</td>
</tr>
<tr>
<td><strong>Fixation Solution (for Paraffin Embedding)</strong></td>
<td>4% (w/v) Paraformaldehyde in 1X PBS, pH 7.4</td>
</tr>
<tr>
<td>Fixation Solution (for Electron Microscope)</td>
<td>4% depolymerized Paraformaldehyde, 0.1-2% Glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4).</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>0.2M Sorensen's phosphate buffer</td>
<td>4.03 g potassium phosphate (KH₂PO₄), 18.87 g sodium phosphate heptahydrate (Na₂HPO₄ 7H₂O), qs to 500ml, pH 7.2</td>
</tr>
<tr>
<td>IF Blocking Solution (Buffer A)</td>
<td>1% BSA in 1X PBS</td>
</tr>
<tr>
<td>IF Washing Solution (Buffer B)</td>
<td>0.1% BSA in 1X PBS</td>
</tr>
<tr>
<td>Hematoxyling and Eosin Staining</td>
<td>Xylene, 100%, 96%, 80%, 70%, and aqua dest, 10% Mayer's Hematoxyling, 1% Acetic Acid Eosin</td>
</tr>
<tr>
<td>Oil Red O Stock Solution</td>
<td>0.5% Oil red O stock solution in 100% Isopropanol</td>
</tr>
<tr>
<td>Oil Red O Working Solution</td>
<td>0.3% Oil red O stock solution (30 ml stock and 20 ml distilled water)</td>
</tr>
<tr>
<td><strong>Solutions for Molecular Biology</strong></td>
<td></td>
</tr>
<tr>
<td>2% agarose gel, 50 ml</td>
<td>1 g of Agarose, 50 ml of 1X TAE, 1 ml of Ethidium Bromide (10 mg/ml)</td>
</tr>
<tr>
<td><strong>Solutions for Lipid Extraction</strong></td>
<td></td>
</tr>
<tr>
<td>Folch/BHT Reagent</td>
<td>Chloroform: Methanol (2:1), BHT (to 100ug/ml)</td>
</tr>
<tr>
<td>2X MTSP/OG (100ml)</td>
<td>0.97 g of MES (pH 6.4), 1.75 g of NaCl, 2 ml of Triton X-100, 3.51 g of Octylglucopyranosid, qs to 100 ml with ddH₂O, supplemented with 10% Protease Inhibitors</td>
</tr>
<tr>
<td>Fatty acid separation solvents</td>
<td></td>
</tr>
<tr>
<td>Cholesteryl Ester solvent (Solvent I)</td>
<td>Hexane/MTBE (Methyl-tert-butyl ether) (200:3)</td>
</tr>
<tr>
<td>Triglycerides Solvent (Solvent III)</td>
<td>Hexane/MTBE (96:4)</td>
</tr>
<tr>
<td>Phospholipid Solvent (Solvent IV)</td>
<td>MTBE/Methanol/Ammonium Acetate (pH 8.6) (5:8:2)</td>
</tr>
<tr>
<td>Ammonium Acetate</td>
<td>0.001 M Ammonium Hydroxide/0.001 M Acetic Acid (2:1)</td>
</tr>
<tr>
<td>Free Cholesterol (Solvent II)</td>
<td>MTBE/Glacial Acetic Acid (500:1)</td>
</tr>
<tr>
<td>Fatty Acid (Solvent VI)</td>
<td>Hexane/MTBE/Acetic Acid (100:2:0.2)</td>
</tr>
<tr>
<td>Column Acidification (Solvent V)</td>
<td>Hexane/Acetic Acid (100:0.2)</td>
</tr>
</tbody>
</table>
Solutions for Cell Culture

<table>
<thead>
<tr>
<th>3T3 Cell Culture medium</th>
<th>13.4 g of D-MEM powder, 3.7 g of sodium bicarbonate, qs to 1 L with ddH2O, pH to 7.3. Filter to sterilize. Supplement with 10% NCS and 1% P/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 medium (1 L)</td>
<td>13.4 g of D-MEM powder, 3.7 g of sodium bicarbonate, qs to 1 L with ddH2O, pH to 7.3. Filter to sterilize. Supplement with 10% NCS and 1% P/S</td>
</tr>
<tr>
<td>3T3 Adipocyte medium (1 L)</td>
<td>13.4 g of D-MEM powder, 3.7 g of sodium bicarbonate, qs to 1 L with ddH2O, pH to 7.3. Filter to sterilize. Supplement with 10% FBS and 1% P/S</td>
</tr>
<tr>
<td>Initiation medium</td>
<td>3T3 Adipocyte Medium supplement with 100 nM Insulin, 500 μM IBMX, 250 nM Dexamethasone, 1 μM Rosiglitizone</td>
</tr>
<tr>
<td>Progression medium</td>
<td>3T3 Adipocyte Medium supplement with 1 μg/mL Insulin</td>
</tr>
<tr>
<td>CHO-K1 medium</td>
<td>13.4 g of D-MEM/F12 powder, 3.7 g of sodium bicarbonate, qs to 1 L with ddH2O, pH to 7.3. Filter to sterilize. Supplement with 10% FBS and 1% P/S</td>
</tr>
</tbody>
</table>

Appendix II: Reagents

Table 0-1 Primary antibodies used in this dissertation

<table>
<thead>
<tr>
<th>Host</th>
<th>Primary Antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>ABCA1</td>
<td>WB 1:1000</td>
<td>Gift from Dr. Mason Freeman (Harvard Medical School, Boston, MA, USA)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>ABCD2</td>
<td>WB 1:1000</td>
<td>Made in our lab</td>
</tr>
<tr>
<td>Goat</td>
<td>AP2/ FABP4</td>
<td>WB 1:250</td>
<td>Abcam Inc., Cambridge, MA 02139, USA Cat. No: ab23693</td>
</tr>
<tr>
<td>Mouse</td>
<td>β-actin</td>
<td>WB 1:1000</td>
<td>Sigma, St Louis, MO 63103, USA Cat. No: A1978</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Calnexin</td>
<td>WB 1:5000</td>
<td>Stressgen, Victoria, BC Canada Cat. No: SPA-860</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Catalas</td>
<td>WB 1:5000</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>CD36</td>
<td>WB 1:500</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>MTCO1</td>
<td>WB 1: 18000</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>myc (polyclonal)</td>
<td>WB 1:500</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>Pex 14</td>
<td>WB 1:500</td>
</tr>
<tr>
<td></td>
<td>Chicken</td>
<td>Pex 19</td>
<td>WB 1:500 IF 1:600</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>PMP 70</td>
<td>WB 1:500 IF 1:600</td>
</tr>
</tbody>
</table>

**Table 0-2 Primers sequence for gene expression RT-PCR**

<table>
<thead>
<tr>
<th>Abreviation</th>
<th>Gene Name</th>
<th>Also known as</th>
<th>Accession Numb.</th>
<th>Forward/reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCD1</td>
<td>ATP-binding cassette, sub-family D</td>
<td>ALDP</td>
<td>NM_007435.1</td>
<td>For CATCGACCCTCCCTATGGAAGTAC; Rev CATGGCTTCTCCTTCAGTCAGGC</td>
</tr>
<tr>
<td>ABCD2</td>
<td>ATP-binding cassette, sub-family D (ALD), member 2</td>
<td>ALDR; ALDL1</td>
<td>NM_011994.2</td>
<td>For TTACACCAGCGGGCGAAGTG; Rev GCCACACGGCCCGCTTAAGAT</td>
</tr>
<tr>
<td>ACC1</td>
<td>acetyl-coa carboxylase</td>
<td>Acaca</td>
<td>NM_133360.2</td>
<td>For TGGACAGACTGATCGCAGAGAAA</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>GenBank ID</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------</td>
<td>------------</td>
<td>-----------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>AP2</td>
<td>fatty acid binding protein 4, adipocyte</td>
<td>FABP4</td>
<td>For CACCATCCGGTCAGAGAGTACTT</td>
<td>Rev CGGTGATTTCATCGAATTTCCA</td>
</tr>
<tr>
<td>ChREBP</td>
<td>carbohydrate response element binding protein</td>
<td>NM_021455.3</td>
<td>For CCTTCGCCAAACTCACGACTT</td>
<td></td>
</tr>
<tr>
<td>D5D</td>
<td>delta-5 fatty acid desaturase</td>
<td>NM_146094.1</td>
<td>For CACCCCTTGGATCTTTTGAA</td>
<td>Rev GTGCCCAAAGTCTGATGCTGA</td>
</tr>
<tr>
<td>D6D</td>
<td>delta-6 fatty acid desaturase</td>
<td>NM_019699.1</td>
<td>For ACCGTGTCATCGGACACTATT</td>
<td>Rev AAGAATGGCCCCACAGGACT</td>
</tr>
<tr>
<td>ELOVL2</td>
<td>elongation of long chain fatty acids 2</td>
<td>NM_019423.1</td>
<td>For TCAATGCTTTTCTTGGACACATG</td>
<td>Rev GGTAAGAGTCCAGGAACACC</td>
</tr>
<tr>
<td>ELOVL4</td>
<td>elongation of long chain fatty acids 4</td>
<td>NM_148941.1</td>
<td>For CAATGAGCGCAAGCAGTCAA</td>
<td>Rev TCACGCGTTCTGAGATGACA</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>elongation of long chain fatty acids 5</td>
<td>NM_134255.2</td>
<td>For ATGGACACCTTTTTTCCATGCTT</td>
<td>Rev ATGGTAGCGTGTTGGTGTAGACATG</td>
</tr>
<tr>
<td>ELOVL6</td>
<td>elongation of long chain fatty acids 6</td>
<td>NM_130450.2</td>
<td>For TGTACGCTGCTTTATCTTGG</td>
<td>Rev GCGGCTTCCGAAGTTCAA</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
<td>NM_007988.3</td>
<td>For GCTGCGGAAACTTCAGGAAAT</td>
<td>Rev AGAGACGTGGTCACTCCTGGACTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>XR_035503.1</td>
<td>For TGTGTCGCTGTTGATCTGA</td>
<td>Rev CCGCTTCCACCACCTTTCTTGAT</td>
</tr>
</tbody>
</table>

Rev TGGAGAGCCCCACACACACA
Appendix III: Protocols

7.1.1. Protein analysis

7.1.1.1. Membrane protein preparation

- Weigh out tissue (pulverized) 0.2 g for everything except fat (for fat use 0.4 g) into 14 ml Falcon tubes containing 1.2 ml of membrane buffer.
- Homogenize approx. 1 min and put on ice (Tekmar SDT-25 Homogenizer, probe SDT-080 EN)
- Centrifuge for 10 min, 3500 rpm at 4 °C (~2000 xg)
- Remove 1 ml of supernatant. Do not take any of the pellet or fat/junk that may be on top of supernatant.
- Place in centrifuge tube for either 100.2 (1.5 ml) or 120.2 (3 ml) rotor.
- Centrifuge at 100,000 x g (55000 rpm), 45 min, 4°C.
- Decant supernatant. Leave tube upside down to drain for 1 min.
- Suspend pellet in 1X sample buffer (100 µl - 250 µl).
7.1.1.2. SDS-PAGE

- Wash glass spacers and combs in EtOH, and assemble plates.
- Pour resolving gel to 0.5 cm below comb.
- Overlay with water saturated isobutanol and allow to polymerize.
- Rinse top of gel with distilled H₂O. Remove remaining water with filter paper.
- Insert comb and pour stacking gel.
- Allow to polymerize and submerge in 1X Running Buffer until use.
- Heat samples to 95°C for 5 min. and quick spin. While samples are heating, remove comb and rinse wells with running buffer.
- Run at 50 mAmps (constant current)
- Transfer to nitrocellulose membrane for western blot
- Remove stacking gel and soak in 1X Transfer Buffer for 5 min.
- Cut two pieces of filter paper and 1 piece of nitrocellulose membrane for each gel.
- Wet in 1X Transfer Buffer for 5 min.
- Build sandwich in cold (4°C) Transfer Buffer, and run at 200 mAmps (constant current) for 2hrs.
- After transfer, trim excess membrane and do not allow nitrocellulose to dry

7.1.1.3. Western blotting

- Block membrane with Blotting Buffer for 30 min at room temp.
- Pour off Blotting Buffer and incubate with 1° antibody for 1 hr at room temp., or 4°C overnight.
- Remove 1° antibody and wash 15 min at room temp. Change Wash Buffer every 5 min.
- Incubate with 2° antibody for 30 min at room temp.
- Remove 2° antibody and wash 15 min at room temp. Change Wash Buffer every 5 min.
- Wash with TBS for 5 min.
- Develop with ECL reagent
- Immediately prior to use combine 3 ml of reagent A with 3 ml reagent B for each membrane and vortex.
- Place membrane in a clean dish, add ECL reagent and incubate for 5 min. at room temp.
- Wrap membrane in food service film and immediately expose to film (typically 30 s, 60 s, 3 min. and 5 min.; however, some reactions may take longer to develop).
7.1.1.4. Immunoprecipitation

- Isolate and weight 5 g of adipose tissue and place it in a chilled beaker containing 10 ml of ice-cold SEM buffer (Add protease inhibitors before experiment)
- Decant the medium and finely mince the liver using scissors
- Suspend the minced tissue in 20 ml SEM buffer and transfer half of this suspension of to the glass vessel of the Potter-Elvehjem homogenizer. (ice cold)
- The motor should be mounted either to a wall, via a G-clamp to the bench, or in a floor-standing cradle. Attachment to a free-standing retort stand is not adequate.
- Attach the cold pestle to the electric motor and homogenize the adipose tissue mince using 4 to 5-up- and-down strokes of the pestle, rotating at ~500 rpm.
- Decant the homogenate into a beaker on ice, and after rinsing the homogenizer with medium and wiping the pestle to remove any connective tissue that may be adhering, repeat steps 5 and 6 with the other half of the mince and combine the homogenates.
- Centrifuge the homogenate 10 min at 750 xg, 0 to 4 °C, in the low-speed centrifuge.
- Decant and retain the post nuclear supernatant (PNF)

Prebind protein A-agrose beads with PI serum

- Add 20 µl of protein A–agrose beads to 200 µl of PI (pre-immu) serum and incubate for 20 minutes at 4°C
- Centrifuge beads 2000 rpm on microfuge for 0.5 min and retain beads
- Wash the beads 2 times with 1 ml of SEM buffer and resuspend beads in 1 ml of SEM buffer

Pre-clear of PNF

- Add beads to PNF and rotate at room temperature for 30 min (while waiting, prepare the binding of magnetic beads with D2 antibody)
- Centrifuge beads at 2000 rpm for 0.5 min
- Transfer supernatant to a new tube.

Pre-bind magnetic beads with D2 antibody

- 200 µl of the Magna TM bind beads are washed with 1 ml of SEM buffer for 3 times. Between each wash, perform magnetic separation. Resuspend the beads in 200 ml of SEM buffer.
- The beads are incubated with 5 µl of D2 antibody for 20 min at 4°C
- Magnetically separate the beads/antibody complex and wash 3 times with 1 ml SEM buffer and resuspended in 1 ml of SEM buffer.
Immuno-isolation of D2 containing compartments

- The PNF will be added to the beads/antibody complex and incubated for overnight at 4°C, agitating the suspension every 10 min to promote attachment.
- The beads will be magnetically separated for 10 min.
- The magnetic pellet as well as the supernatant will be collected and stored at -20°C for later assay.

7.1.2. Cell biology

7.1.2.1. Mitochondria and peroxisome isolation

- Dissect adipose tissue, minced with fine scissors.
- The minced tissue (2.5 g of brown adipose tissue/ liver and 4~5 g of white adipose tissue) is transferred to 10ml homogenize buffer and homogenized with two or three strokes in a Potter-Elvehjem glass-Teflon homogenizer to prepare homogenate.
- The homogenate is filtered through gauze, and diluted to a final volume of 20 ml with SEM buffer.
- The homogenates are centrifuged at 750 xg, 4°C for 10 min to remove the nuclear pellet.
- Record the volume of post-nuclear supernatant (PNS). The PNS is centrifuged at 4°C, 8500 g for 10 min to remove the heavy mitochondria pellet (HMP).
- The heavy mitochondria supernatant (HMS) is centrifuged at 27,000 g for 20 min at 4°C, to obtain the light mitochondria pellet (LMP).
- Pellets are suspended using 1X protein sample buffer (PSB), supernatants are diluted using 4X PSB. Equal amount of protein (50 µg) from each fraction was loaded to SDS-PAGE to examine the enrichment of interested protein in different fractions. While equal percentage of each fraction (2%) was loaded on SDS-PAGE to examine the distribution of interested protein in each fraction.

7.1.2.2. Immunofluorescence

- Cells are cultured on the coverslips placed in 6-well plate.
- After transfection, coverslips are rinsed 2 x with PBS (4°C) quickly.
- Add 2 ml MeOH (-20°C) and incubate at -20°C for 10 min.
- Rinse cells 3 - 5 min in PBS (4°C).
- Block in 2 ml PBS contain 1% BSA (Buffer A) for 30 min (4°C).
• Blot edge of coverslip on a paper towel to remove excess Buffer and place in a humidified chamber
• Quickly add 150 µl of primary antibody diluted in Buffer A
• Incubate at room temp for 1 hr
• Transfer coverslips to 6-well plates containing 3 ml of PBS containing 0.1% BSA (Buffer B)
• Rinse 3 - 5 min in 3 ml of Buffer B (RT)
• Transfer coverslips to a humidified chamber
• Quickly add 150-200 µl of secondary antibody diluted in Buffer A
• Incubate at room temp for 1 hr
• Transfer coverslips to 6-well plates containing 3 ml of Buffer B
• Rinse 3 - 5 min in 3 ml of Buffer B (RT)
• Rinse 2 x quickly with PBS
• Rinse 2 x quickly in water
• Blot edge of coverslip on a paper towel and mount on glass slides using 40 µl (for 24 mm2 coverslips) of mounting medium
• Allow mounting medium to set at least 30 min and seal coverslip to slides with nail polish

7.1.2.3. Electron microscope

• Biochemically generated fractions (HMP or LMP) from adipose tissue was fixed in 4% paraformaldehyde for 45 min
• After fixation, the pellets were washed 3 times for 10 min with 0.1 M Sorenson's buffer
• Samples were dehydrated in a series of graded ethanol (70% to absolute) and embedded in Epoxy resin
• Ultrathin sections were inspected after contrasting with electron microscope

7.1.2.4. Immunoelectron microscopy

• Wild-type and D2-/- mice were anaesthetized by intraperitoneal injection and perfused via the left ventricle with a mixture of 4% paraformaldehyde, 2% glutaraldehyde in 0.1M Sorenson’s buffer (pH7.4)
• Adipose tissue were dissected, cut in slices with razor blades and post-fixed in 2% glutaraldehyde in Sorenson’s buffer (pH7.4) for 45 min
• Samples were washed 3 times for 10 min with 0.1 M Sorenson’s buffer
• Fixed adipose tissue was dehydrated in a graded series of alcohols (50% and 70% ethanol) and embedded into LR White resin (medium grade)
• LR White-filled gelatin capsules were polymerized at 50°C for 24 hours
• After preparation of semithin sections blocks, ultrathin sections of 80nm were cut, collected on 100 mesh nickel grids and therefore coated on the back side with a 1% formvar film
• The grids were dried at 37°C overnight prior to immunostaining
• The sections were incubated with blocking solution (1% BSA in 0.1M phosphate buffer, pH7.4) for 30 min at room temperature
• Incubation with the primary antibodies was performed on droplets with antibodies (anti-D2 and anti-pex19) in 1% BSA in 0.1M phosphate buffer, pH 7.4 overnight at 4°C.
• Sections were rinsed in 0.1 M phosphate buffer containing 10 mg/20 ml of polyethylene glycol 3 times and rinsed in phosphate buffer
• Sections were then incubated in colloidal gold secondary antibody (12nm particles conjugated with anti-rabbit antibody and 6nm particles conjugated with anti-mouse antibody) in 1% BSA in 0.1 M phosphate buffer, pH 7.4 for 1-2 hours. Negative controls were processed in parallel.
• The grids were rinsed on droplets of 0.1 M phosphate buffer containing 10 mg/20 ml of polyethylene glycol 3 times and rinsed in phosphate buffer
• Sections were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer for 5 min
• Sections were rinsed in 0.1 M phosphate buffer
• Sections were subsequently contrasted with uranyl acetate for 2 min and lead citrate for 45 sec. The sections were examined using electron microscope.

7.1.3. Molecular biology

7.1.3.1. RNA isolation

• Weight 40mg tissue and put the tissue into the tube containing 0.8 ml RNA STAT-60TM Reagent
• Homogenize tissue with homogenizer machine.
• Store the homogenate for 5 min at room temperature
• Add 160 μl of chloroform to each tube, and Vortex 15 sec
• Let the samples stay at room temperature for 2 min
• Centrifuge the homogenate at 12,000g (353) for 15 minutes at 4°C
• Transfer the aqueous phase (about 455μl) to a new tube
• Prepare labeled spin column
• Add 245 μl 100% EtOH, pipette immediately to mix
• Transfer 750 μl of liquid into spin column, centrifuge for 30 S at 12,000 rpm. Discard the flow-through
• Add 700 μl Buffer RW1 and centrifuge for 30 S at 12,000 rpm. Discard the flow-through
• Add 500 μl Buffer RPE and centrifuge for 30 S at 12,000 rpm. Discard the flow-through
• Add 500 μl Buffer RPE and centrifuge for 2 min at 12,000 rpm.
• Place the RNeasy spin column in a new 2 ml collection tube, and centrifuge at full speed for 1 min
• Place the RNeasy spin column in a new 1.5 ml collection tube (supplied).
• Add 40 μl RNase-free water directly to the spin column membrane.
• Centrifuge for 1 min at 12,000 rpm to elute the RNA

7.1.3.2. RT-PCR

7.1.3.2.1. Primer Design
• Investigator provides 500-1500 bp of sequence, preferably covering at least one intron-exon boundary within the target gene/cDNA or spanning a region in the 3'-untranslated sequence.
• Primer Express oligonucleotide prediction program is used to determine possible primer pairs. The primers are designed around a probe that is noted but not ordered. All parameters have been preset within the software. Four pairs are chosen for synthesis.
• Primers are stored at stock concentration of 100μM and working concentration of 2.5μM at -20°C.

7.1.3.2.2. Reverse Transcription
• Isolated RNA is also unstable and must be stored at -80°C in RNAse free water or as a precipitate in 70% ethanol; freeze thaw cycles should be kept to a minimum. In order to analyze levels of mRNAs in samples, we first convert (reverse transcribe) them to cDNAs since these are much more stable. The following is an example using iScript cDNA Synthesis Kit. Once synthesized, cDNAs should be stored at -20°C.

Each RT cocktail:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Iscript reaction mix</td>
<td>4 μl</td>
</tr>
<tr>
<td>Iscript reverse transcriptase</td>
<td>1 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>X μl</td>
</tr>
<tr>
<td>RNA template (200ng to 10000ng total RNA)</td>
<td>Y μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

Reaction protocol
Step 1: 5 minutes at 25°C
Step 2: 30 minutes at 42°C
Step 3: 5 minutes at 85°C
Step 4: Hold at 4°C

7.1.3.2.3. RT-PCR reaction

- Program the robotic pipettor according to specific experiment plate design
- Pipette a 1:10 serial dilution of target gene cDNA in 0.5 ml tubes.
- Dissolve primers in Nuclease Free Water (5 mM stock)

- Determine PCR conditions for each reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (1 Reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x SYBR Green PCR Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>4.2 µl</td>
</tr>
<tr>
<td>Forward Primer (100 nM final)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>Reverse Primer (100 nM final)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>*cDNA template</td>
<td>*5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
• Prepare each gene specific Sub-Master
• The table below shows our calculations. Additional volumes are present to allow for the robotic pipettor to operate properly.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>Note</td>
</tr>
<tr>
<td>1</td>
<td>Number of samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>cDNAs</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>replicates</td>
<td>3</td>
<td>Total=5*3=15 Rxns</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Gene specific Submaster</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2X SYBRGreen (354)</td>
<td>165</td>
<td>C6=10*15 Rxns *1.1</td>
</tr>
<tr>
<td>7</td>
<td>Nuclease Free water</td>
<td>69.3</td>
<td>C7=4.2*15 Rxns *1.1</td>
</tr>
<tr>
<td>8</td>
<td>Forward primer (5μM stock; 100 nM final)</td>
<td>6.6</td>
<td>C8=0.4*15 Rxns *1.1</td>
</tr>
<tr>
<td>9</td>
<td>Reverse primer (5μM stock; 100 nM final)</td>
<td>6.6</td>
<td>C9=0.4*15 Rxns *1.1</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><strong>Mr Neo</strong> Pipettes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>cDNA</td>
<td>5</td>
<td>There should be at least 20-25μl solution left in each tube after the robot pipettes.</td>
</tr>
<tr>
<td>13</td>
<td>Gene-specific Submaster</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

• Run the program
• After finishing, seal the plate thoroughly to prevent evaporation
• Centrifuge for 3 min at 1,200 x g
• Program 7900HT, specify the samples, target genes detectors and run rt-PCR

**Reaction protocol**

Step 1: 2 minutes at 50°C
Step 2: 10 minutes at 95°C
Step 3: 15 seconds at 95°C followed by 1 minute at 60°C (40 cycles)

7.1.3.2.4. Results analysis

• Calculate ΔCt: Ct of target gene subtract corresponding points of GAPDH
• Calculate slope with dilution point and ΔCt
• Choose relative standard curve method if the slope is > 0.1. If the slope is ≤ 0.1, choose comparative Ct (ΔΔCt) method.
7.1.3.3. Transfection

- Allow FuGENE HD transfection reagent, DNA, and medium to warm up at room temperature. Vortex for one second.
- Dilute DNA with serum-free medium to a concentration of 2μg plasmid DNA/100μl SF medium
  Tube I (labeled as Control): 12μg plasmid DNA (β-Gal) + 600μl of SF medium
  Tube II (labeled as D2): 12μg plasmid DNA (D2) + 600μl of SF medium
- Form the transfection complex (FuGENE HD : cDNA = 6 μl : 2 μg)
- Pipet the FuGENE HD transfection reagent 30μl directly into the medium containing the diluted DNA without allowing contact with the walls of the plastic tubes
- Mix and incubate the transfection complex
- Vigorously tap the tube or vortex for one to two seconds to mix the contents. Incubate the transfection reagent:DNA complex for 15 minutes at room temperature.
- Add the transfection complex to cells
- Remove the old medium. Add the transfection complex 500μl to the cells in a drop-wise manner. Swirl the wells to ensure distribution over the entire plate surface. Use of a rotating platform shaker for 30 seconds at low speed to shake.
- Incubate the transfected cells for 3 hours and add serum-containing medium and return the cells back to incubator.
- Incubate the cells for 24 hours before determining the transfection efficiency

7.1.4. Cell culture

7.1.4.1. CHO/HEK culture

- Thawing and freezing protocol are the same as described below for 3T3 cell culture
- Cells are ready for passage when they are between 80-95% confluent. Cells that are 100% confluent will have lower viability.
- Pre-warm CHO medium to 37°C
- Aspirate medium and wash cells with 2 ml Trypsin-EDTA.
- Add 1 ml of Trypsin-EDTA and incubate at room temperature until cells dissociate from dish and from one another (1-3 min). Tap the side of the dish repeatedly to release cells; verify single cell suspension by microscope.
- Bring volume up to 10 ml with culture medium, run cell suspension up and down 10 ml, pipette 3-5 times to assure single cell suspension.
- Remove a small amount of suspended cells and place in the hemocytometer
- Count the number of cells in 1-4 of the four, 4x4 grids and use the following formula to determine the cell density:
Cells/ml = \[ \text{Total number of cells \times 10}^4 \] 
\# of 4x4 grids counted

- Seed two, 10 cm dishes of cells at 2.5 and 5 x 10^5 cells/dish in 10 ml of CHO medium in order to maintain the line.
- Seed cells at the desired density for experiments. The table below is only a guide.

<table>
<thead>
<tr>
<th>Dish (Volume)</th>
<th>Cells/dish</th>
<th>~D1 Confluence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 cm (10 ml)</td>
<td>1 \times 10^6</td>
<td>50%</td>
</tr>
<tr>
<td>60 mm (4 ml)</td>
<td>5 \times 10^5</td>
<td>50%</td>
</tr>
<tr>
<td>35 mm (3 ml)</td>
<td>2 \times 10^5</td>
<td>50%</td>
</tr>
</tbody>
</table>

7.1.4.2. 3T3-L1 and MEF cells differentiation

**Thawing:**
- Pre-warm 3T3 Medium to 37°C
- Remove vial from nitrogen storage tank and thaw rapidly by holding in a 37°C water bath (Do not drop the vial into the bath)
- Once the cells have thawed, rinse the vial with 70% ethanol and decant the cells into a 10 cm dish.
- Add 10 ml 3T3 Medium, drop-wise, over a 1-2 min period (this improves viability by allowing the cells to equilibrate to the DMSO free medium).
- Culture the cells in a humidified incubator (5% CO₂) for 12-16h.
- Aspirate medium and feed 10 ml of 3T3 medium. Do not allow the cells to reach greater than 90% confluence prior to passage.

**Passage:** Cells are ready for passage when they are between 70-90% confluent. Do not allow the cells to reach confluence. They will not differentiate into adipocytes with subsequent passage.
- Pre-warm 3T3 Medium to 37°C
- Aspirate medium and wash cells with 2 ml Trypsin-EDTA.
- Add 2 ml of Trypsin-EDTA and incubate at room temperature until cells dissociate from dish and from one another (1-3 min). Tap the side of the dish repeatedly to release cells; verify single cell suspension by microscope.
- Bring volume up to 10 ml with culture medium, run cell suspension up and down 10 ml pipette 3-5 times to assure single cell suspension.
- Remove a small amount of suspended cells and place in the hemocytometer
- Count the number of cells in 1-4 of the four, 4 x 4 grids and use the following formula to determine the cell density:
• Cells/ml = Total number of cells x 10^4  
• # of 4 x 4 grids counted  
• Seed two, 10 cm dishes of cells at 2.5 and 5 x 10^5 cells/dish in 10 ml of 3T3 medium in order to maintain the line.

**Freezing:** Cells should be frozen when they are actively dividing in order to optimize viability (70-90% confluence)

• Pre-warm 3T3 Medium to 37°C  
• Aspirate medium and wash cells with 2 ml Trypsin-EDTA.  
• Add 2 ml of Trypsin-EDTA and incubate at room temperature until cells dissociate from dish and from one another (1-3 min). Tap the side of the dish repeatedly to release cells; verify single cell suspension by microscope.  
• Bring volume up to 10 ml with culture medium, run cell suspension up and down 10 ml pipette 3-5 times to assure single cell suspension.  
• Remove a small amount of suspended cells and count on hemocytometer  
• Centrifuge cells at 500 x g for 5 min (22°C)  
• Add 90% of the calculated volume of 3T3 Medium to achieve a final suspension of 5 x 10^6 cells/ml and suspend the cells by gently pipetting up and down 3-5 times.  
• Slowly add DMSO, drop-wise, to a final concentration of 10%.  
• Aliquot the 1 ml of cells into an appropriately labeled cryo-vial and place in a room temperature Mr. Frosty® freezing container that contains isopropanol.  
• Place in the -80°C freezer overnight and transfer into the liquid nitrogen container the following day. Do not forget to enter the vials into Cell Culture Database

**Differentiation into Adipocytes:**

• Day -X  
  - Trypsinize sub-confluent cells and count on hemocytometer.  
  - Centrifuge cells at 500 x g for 5 min (22°C)  
  - Suspend cells (1 x 10^4/ml) in 3T3 Medium  
  - Aliquot 2 ml/well in a 24-well plate (seed at 2 x 104 in 2 ml 3T3 Medium)  
  - Allow cells to grow until confluent, aspirate and feed 2 ml of 3T3 medium, and define as Day 0

• Day 2  
  - Aspirate medium from confluent cells  
  - Add 2 ml of Initiation Medium to each well

• Day 4  
  - Aspirate medium from cells  
  - Add 2 ml of Progression Medium to each well

• Day 6  
  - Aspirate medium from cells
- Add 2 ml of 3T3 Adipocyte Medium to each well
- **Day 8-14**
  - Feed cells 2 ml of 3T3 Adipocyte Medium every 48h.
  - Cells should contain lipid droplets and are suitable for lipolysis assays from Days 8-14.

**Isolation of Mouse Embryonic Fibroblasts (MEF)**

- **Timed Pregnancy**
  - Place male with female and monitor female for appearance of vaginal plug shortly after lights-on the following and subsequent mornings. The presence of a plug is defined as Day 1.
  - Separate and weigh the female on Day 1
  - Weigh the female on Day 14.5. If pregnant with a sizeable litter, she should gain 2.5-3g.

- **Isolation of embryonic fibroblasts (MEFs)**
  - Administer a lethal dose of ketamine/xylazine to the pregnant female
  - Carefully lift the abdominal wall away from the body. Cut the skin and peritoneum at the midline just proximal to the hind legs. Cut along the midline to the sternum. Make two lateral incisions away from the midline at the proximal and distal end of the first incision.
  - Lift the uterus containing the pups away from the abdomen and cut near the cervix. Place the uterus into a 10 cm dish and transport to the tissue culture hood for immediate dissection.
  - Cut along the surface of the uterus and release the pups into the dish. Carefully dissent the pups away from the placenta and transfer them to a new dish.
  - Remove the head and abdominal organs of each pup. Transfer the rest of the pup to a new dish.
  - Once all the pups have been dissected, finely mince the tissue with a sterile razor.
  - Digest the tissue in 10 ml of Trypsin-EDTA (0.5%). for up to 30 min at 37°C.
  - Pipette the cells, trypsin and tissue clumps up and down 2-3X every 3-5 min. (solution will be viscous).
  - Seed the MEFs in 10 ml of DMEM (10% FBS, 1% P/S, 1% Fungizone) at a density of 1 pup/dish.
  - Culture overnight in a humidified CO₂ (5%) incubator.
  - Remove unattached cells by aspiration.
  - Cells are generally very near confluence the following morning. They can be passed and propagated or frozen for future use.
  - Differentiation of MEF cells follows similar protocol for 3T3-L1 cells differentiation with few differences described below.
- Plate MEF cells on Day 1 in 10 cm dish to reach 50~60% confluence on next day.
- Digest cells and plate the cells into 1 well of 6-well plate to reach high density of cells.
- Start differentiation on Day 3 by adding Initiation medium to cells
- Maintain cells in Initiation medium for 4 days, change medium every other day
- Switch to progression medium and follow the 3T3-L1 cells protocol

### 7.1.4.3. FA containing medium preparation

- Make the FA solution of required concentration (eg., 100 μM) using serum free medium supplemented with 0.2% essential fatty acid free-bovine serum albumin (FAF-BSA).
- Depending on the number of culture plates, prepare required amount of fatty acid-enriched medium.
- Take out stock solution from the freezer, open cap, pass nitrogen gas through it for 10 sec.
- Take out about 0.5 ml of stock FA solution using a pasteur pipette (previously cleaned with chloroform:methanol).
- Pass N₂ gas through the flask containing stock FA for 10 sec again, recap it and put it back in freezer immediately.
- Calculate how much of stock is needed to make 100 μM conc. Measure the amount of stock and add it to a glass tube. Cover the mouth of the tube to prevent oxidation.
- Calculate the amount of 5N NaOH needed. Add NaOH to FA, mix thoroughly-vortex.
- Pass N₂ gas through the tube until the solution dries up into a salt of FA.
- Boil distilled water in a small beaker.
- Add about 1 ml of hot water into dried down FA + NaOH, and mix well. Dip the tube in boiling water for a few minutes so that all the FA + NaOH goes into solution.
- Add the fatty acid solution to required amount of medium (5%) and adjust the pH with 2N HCl.
- Filter the fatty acid containing media through a 0.45 μm filter into a 50 ml falcon tube under hood.

### FAs solution preparation:
- The pure fatty acids are purchased from NuCheck (http://www.nuchekprep.com/home.htm).
- First make the solution in Hexane. Record the weight of fatty acid and the molecular weight. Make a 5 ml solution in graduated flask and then calculate the final concentration: N1
\[ X \text{ mg} \times 1000 \]
\[ 5 \text{ ml} \times \text{MW} \]

- Result is in unit as mM.
- Fill with gas N\(_2\) and carefully sealed with parafilm.

**FA Media preparation:**
- For 10ml plate, if the final conc. of fatty acid is 20mM, we need to calculate how much fatty acid solution in hexane we need. \( V_1 \)
- 1: stock solution, 2: experimental solution.
  \[ V_1 N_1 = V_2 N_2 \]
  \[ X \times N_1 = 10 \times 0.02 \]
  \[ 10 \times 0.02 \]
  \[ X = N_1 \]

- \( X \) is in the unit of ml.
- For 12 plates, we will need 120ml media. Always prepare extra 5 ml as later we will need to filter the media and lost some volume.
- Fatty acid will be prepared in serum free medium enriched with 0.2% (v/v) FAF-BSA. The ratio of free fatty acids to albumin should not exceed 3 to 1, otherwise fatty acid is toxic to cells.

**NaOH 30 times exceed of FA concentration.**
- Volume of 5 N NaOH: \( V_3 \)
  \[ \frac{\text{FA conc.} \times 30 \times V_2}{5} \]
- Put NaOH (V3) in a culture glass tube, then add FA (V1).
- Carefully blow in the gas N\(_2\) for 15 min to 30 min until it totally dried (white powder).
- Wash out FA from glass tubes into the media (V2)
- Adjust pH of the media to 7.4 with HCl.

### 7.1.5. Lipid analysis

#### 7.1.5.1. Lipid extraction

7.1.5.1.1. Lipid extraction from cells
- Rinse plates 2X with 3 mL PBS (4\(^\circ\)C).
- Scrape dishes in 3 mL of PBS and transfer to a 12x75mm glass tube.
- Vortex and take a 100 \( \mu \)L aliquot for protein determination.
- Centrifuge at 1000 xg for 5 min. (4\(^\circ\)C), and aspirate PBS.
• Add 2 mL 3:2 hexane:isopropanol. Vortex.
• Incubate and for 10 min. (20°C).
• Centrifuge at 1000 xg for 5 min. (4°C), and transfer supernatant to a fresh 12x75 mm tube.
• To pellet, add 2 mL 3:2 hexane:isopropanol. Vortex.
• Incubate and for 10 min. (20°C).
• Centrifuge at 1000 xg for 5 min. (4°C), and transfer supernatant to 12x75 mm tube (7).
• Evaporate to dryness under N\textsubscript{2}(g).
• Solubilize the extracted lipids in a suitable solvent.
• For Storage: Suspend in 500 \(\mu\)L of CHCl\textsubscript{3}, flush with N\textsubscript{2}(g) and store at -20°C.
• For TLC: Suspend in 200 \(\mu\)L of 1:1 CHCl\textsubscript{3}:CH\textsubscript{3}OH (must be used immediately).
• For Enzymatic Cholesterol Assays: Suspend in 2 mL 1\% Triton in CHCl\textsubscript{3}. Solution can be stored at -20°C.

7.1.5.1.2. Lipid extraction from tissue

• Cut 100 mg of tissue (0.1 g) and put in dounce test tube. Homogenize using dounce pestle.
• After three/four ups and downs, insert 1 ml of MTSP/OG and homogenize a little more. Put the dounce test tube in ice and wait 30 min for extraction.
• Add 50 \(\mu\)l of C17:0 standard (5 mg/ml in CHCl\textsubscript{3}) in another test tube. Then move the liver soln to the test tube by using a glass pipette.
• Add 2 ml of Folch/BHT reagent(355). Vortex for 3 x 10 sec (vortex all samples once, then twice, then three times. Leave 5 min. waiting time between each vortex).
• Soln should look like floating milky-powders right now. Centrifuge at 2000 rpm for 10 min at RT. (RPM = 2000, RCF = 850, Rotor (the big wheel and its name) = S-5.1, Time = 10 min, Temp = 20°C) After centrifuge, soln should have three phases (methanol phase, protein & nucleic acid phase, then chloroform and FA phase at bottom).
• Move majority of lower phase to reaction tube with glass pipette, dry under N\textsubscript{2} to about 50 \(\mu\)l.
• Add 1 ml BF3/methanol, cap, 55 °C for overnight.
• Move to a glass tube, + 2 ml chloroform, mix well, + 2 ml water, vortex vigorously for 3x 10 sec.
• Remove upper phase. + 2 ml water, vortex vigorously for 3x 10 sec.
• Move majority of lower phase to sample vial, inject 1ul at 1:10 to 1:100 to GC-MS analysis.

7.1.5.1.3. Lipid extraction from serum

- In a glass tube, + 50 µl of serum + 1 ml Folch/BHT + 5 µl of C17:0 standard, vertex vigorously, + 1 ml of water, vertex vigorously for 3x 10 sec. stand for 5 min until two phase separated.
- Remove upper phase, + 1 ml of water, vertex vigorously for 3x 10 sec.
- Centrifuge at 2000 rpm for 5 min at room temperature
- Move majority of lower phase to a reaction vial, dry under N₂ to about 50 µl.
- Add 1 ml BF₃/methanol, cap, 55°C for overnight (16h).
- Follow the rest steps described in tissue lipid extraction.

7.1.5.2. Lipid fractionation

- Take approximate 400 mg of liver and place in a fatty acid tube containing 20 ml Folch/BHT reagent.
- Homogenize liver and filter into a new fatty acid tube using a No. 2 Whatman Filter.
- Rinse fatty acid tube with 20 ml folch and pour through same filter.
- Remove 2 ml and place in screw top glass tube for total fatty acid determination. Evaporate under nitrogen and proceed with methyl-esterification protocol.
- Evaporate the remaining folch under nitrogen.
- Set up silica columns (one for each sample) with collection tubes in vacuum apparatus.
- Equilibrate silica column with 1 column full (approx. 10 ml) of cholesteryl ester solvent (solvent I).
- Resuspend dried samples with 2 ml of cholesteryl ester solvent by vortexing and transfer each to its own silica column. Let solvent go completely through column by slow dripping (vacuum applied).
- Add additional 8 ml of cholesteryl ester solvent to the fatty acid tube and vortex to ensure complete removal from fatty acid tube. Transfer solvent to the appropriate silica column and collect flow through in test tube and transfer to new fatty acid tube. MUST use vacuum for each of the remaining steps.
- Rinse column again with 1 column full of cholesteryl ester solvent and collect flow-thorugh. Combine with previous flow-through (contains cholesteryl esters) and evaporate under nitrogen in fatty acid tube.
- Change collection test tubes in rack and fill column with triglyceride solvent (solvent III) and collect flow-through in a new fatty acid tube/
- Fill column a second time with triglyceride solvent and collect flow-through and combine with first TG flow (contains triglycerides). Dry samples down under nitrogen.
- Fill column with 10 ml acidification solvent (solvent V) and 10 ml again.
- Discard the flow-through.
• Fill column with 10 ml of fatty acid solvent (solvent VI) and collect flow-through.
• Repeat last step and combine the two flow-through.
• Fill column with phospholipid solvent (solvent IV) and collect flow-through to a new collection test tube. Transfer flow through to a new fatty acid tube.
• Fill column a second time with phospholipid solvent and collect flow-through. Combine with first flow-through in fatty acid tube and evaporate under nitrogen (will take over-night).
• Proceed with methyl-esterification with each fraction as per protocol.

7.1.6. Animal experiments

7.1.6.1. Genotyping

7.1.6.1.1. DNA isolation
• Small piece of ear was collected from each mouse by ear punching
• The tissue was lysed in 200µl of DirectPCR® lysis reagent (Viagen Biotech Inc., Los Angeles, CA) at 55°C overnight
• DNA was purified using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following manufacture’s instruction

7.1.6.1.2. PCR amplification of DNA

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (1 Reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Thermo Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>GC rich</td>
<td>4 µl</td>
</tr>
<tr>
<td>Primer D2 WT</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer D2 Common</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer D2 KO</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>10.2 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Reaction Protocol

<table>
<thead>
<tr>
<th>Cycle Protocol</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>20</td>
<td>5:00</td>
</tr>
<tr>
<td>1X</td>
<td>95</td>
<td>4:00</td>
</tr>
</tbody>
</table>
7.1.6.1.3. DNA Electrophoresis

**Sample Preparation:**
- DNA samples can be dissolved in water or TE buffer. For most applications, simply add appropriate amount of 6X dye to the samples, and they are ready to load.

**Electrophoresis:**
- Rinse Horizontal Gel Apparatus with Nanopure water.
- Assemble gel casting tray by the manufacturer’s instructions.
- Add appropriate amount of 1X TAE and agarose to Erlenmeyer flask.
- Heat to a slow boil in microwave oven, hot plate or autoclave. Agarose should be completely dissolved, and solution should be clear. Do not overheat as agarose will begin to break down.
- Allow solution to cool to 50°C. Solution must be stirred frequently to prevent localized gelling of the agarose.
- Add 10 mg/mL Ethidium Bromide (EtBr) at a rate of 1µL/10 mL gel. Note: EtBr is a powerful mutagen (cancerogen) and gloves should be worn any time EtBr is used.
- Carefully pour the gel into the gel casting tray such that it is free of bubbles.
- After the gel has solidified, carefully remove the comb and place the gel in the gel box.
- Add 1X TAE such that the gel is completely submerged with ~1mm of buffer above the surface of the gel.
- Load samples and Marker (If using lambda DNA EcoRI/HindIII fragments, it must be heated to 42°C for 5 min. prior to loading)
- Run the gel at a voltage of 1-5 V/cm (distance between electrodes) for an adequate time as judged by migration of bromophenol blue and xylene cyanol.
- Visualize separated DNA by ultraviolet light on a transilluminator.

### 7.1.6.2. Fasting glucose and glucose tolerance test

- Glucometer & Strips: FreeStyle with Test Strips or other commercially available device
Transfer mice to individual cages that contain water, but no food, and fast for four hours beginning shortly after “lights-on”

- Fasting glucose on each mouse (Time 0)
- Place boxes under hood with a wire rack on the cage
- Carefully remove the mouse, weigh and place on the wire rack.
- Hold the mouse by the tail and prick the tail vein with a 26 ga needle.
- Touch the glucometer strip to the drop of blood that forms on the tail.
- Record the result
- Inject with 10 ml of glucose per gram body weight (leave at least 2 min between mouse injections)
- Record glucose levels at 30, 60, 90, and 120 min after injections
- Calculate Means and Standard Deviations for fasting glucose levels
- Calculate % change in blood glucose for each time point
- Divide each value by Time 0 and multiply by 100

7.1.7. Histology analysis

7.1.7.1. Hematoxylin and Eosin (H&E) staining

Deparafinization
- Xylene 3 min (2 X)
- 100% EtOH (2 X)
- 95% EtOH (1 X)
- 80% EtOH (1 X)
- H2O (2 X)
- Replace the dirty xylene #1, above with fresh xylene.
- Replace the dirty 100% EtOH #1, above with fresh 100% EtOH.

Staining
- (Notes: Unless otherwise specified, slides should be dipped 10 times in each solution)
- Hematoxylin, 2 min (x1)
- Running H2O x 2 min
- Acid Alcohol x1 ("Differentiation" lightens the staining, especially outside the nucleus) Skip this differentiation step for progressive staining with the weaker Mayer’s Hematoxylin stain.
- H2O x1
- Ammonia solution 10 dips (x1) (changes the stain from purple to blue)
- Running H2O 5 min
- 80% EtOH x1
• Eosin 15"  
• 95% EtOH x2  
• 100% EtOH  
• Xylene 3 min x2

**Mounting**

• Wipe off the xylene off the back of a slide on a paper towel  
• Using a clean glass rod add a drop of permount to the slide  
• Cover with a glass slip (use 2 drops of permount for large slips)  
• Tilt the slide on edge on a paper towel to remove extra xylene or permount  
• If bubbles are present gently squeeze them out by pressing on slip with a pencil eraser  
• Place slides on a paper towel to cure overnight

### 7.1.7.2. Oil-Red-O staining

• Wash: cells 1ml/well PBS. Aspirate  
• Dehydrate: Add 1ml/well 60% Isopropyl alcohol,  
• Let sit for 5 minutes, aspirate  
• Stain: Add 1ml/well Oil Red O Working solution, (cell plate +1 blank well of empty plate)  
• Let sit for 15 minutes. Aspirate.  
• Rinse: both plate samples with 60% Isopropyl alcohol, swish, and aspirate  
• Wash: both with PBS.  
• Leave in PBS for viewing under scope at RT (stable for several weeks and possibly longer).  
• Extract the red color:  
• Add 200µl/well of 100% isopropyl alcohol (cell plate +1 blank well of empty plate)  
• Rotate slowly for 15 min.  
• Remove liquid from sample and blank wells, and place in empty 24 well plate  
• Measure at O.D. of 540 nM. (Tecan Safire equipment)  
• Data/graph: subtract blank values from each experimental (cell) data point, take average and plot  
• Record visual estimate of differentiation, along with passage number and dates.

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