Identification of transcription factors and response elements driving expression of *Plin5*

Master Thesis in nutrition

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Tone Lise Aarnes Hjørnevik

Abstract

Obesity is a major health problem in the world today, as it results in several metabolic changes leading to elevated risk of various severe conditions such as cancer, diabetes mellitus, coronary heart disease and stroke. The mechanisms leading to development of excess body fat is complex, and there are still many unanswered questions regarding the factors involved.

Lipid droplets (LDs) function as cellular fat stores, and are coated with various proteins such as members of the Perilipin family. This family consists of five members (perilipin 1-5, encoded by the genes *Plin1-5*) that differ in tissue expression, transcriptional regulation, protein stability and binding affinity to the LDs. The perilipins are thought to have unique roles in the regulation of fatty acid metabolism in various tissues. Previous studies have demonstrated that perilipin 1, 2 and 4 is transcriptionally regulated by direct binding of Peroxisome Proliferator-Activated Receptors (PPARs) to responsive elements in the promoters.

The focus in this thesis has been the transcriptional regulation of perilipin 5 in cultured mouse muscle cells. Perilipin 5 is the latest discovered member of the perilipin family. This family member has been demonstrated by our group and others to be highly expressed in tissues with active fatty acid oxidation, such as heart, liver, striated muscles and brown adipose tissue, and to be induced by PPARα. Preliminary studies in our group suggested that activation of PPARδ stimulate *Plin5* expression in muscle. Based on existing literature, we also hypothesized that the PGC1s and ERRs transcription factors could be important regulators of *Plin5*.

The main objective for this thesis was to investigate the role of the transcription factors PPARs, PGC1s and ERRs for perilipin 5 expression in cultured mouse muscle cells, and to identify any response elements used by these transcription factors in the *Plin5* promoter. Our results confirmed that activation of PPARα regulates the expression of perilipin 5 in muscle cells. However, they also demonstrated that PPARδ is an important regulator of *Plin5* expression. Furthermore, when PPARα is over expressed or PPARδ is ligand-activated, the relative level of perilipin 5 increase with time, suggesting the involvement of other factors. We found that overexpression of either PGC1α or PGC1β enhanced basal expression of

perilipin 5 compared to non-transfected cells. Interestingly, cells overexpressing these co-activators, especially PGC1β, were less responsive to ligand activation of PPARs. This result suggests that overexpression of these coactivators functions to activate the PPARs similar to ligand activation. ERRs did not appear to regulate *Plin5* expression. We were also able to confirm a novel functional PPRE in the *Plin5* promoter, located downstream of the *Plin5* transcriptional start site at position +139 to +151 in intron 1 of the *Plin5* gene. This establishes *Plin5* as a novel direct PPAR target gene in mouse muscle cells.

Abbreviations

ΔCT	Delta CT
AC	Adenylyl Cyclase
ADP	Adenosine diphosphate
ADRP	Adipocyte Differentiation-Related Protein
ATP	Adenosine-5'-triphosphate
BAT	Brown Adipose Tissue
BRL-49653	Synthetic PPARγ ligand
BSA	Bovine Serum Albumin
cDNA	complementary DNA
DAG	Diacylglycerol
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DR	Direct repeat
ER	Endoplasmatic Reticulum
ERR	Estrogen Related Receptor
FBS	Fetal Bovine Serum
gDNA	genomic DNA
GW-501516	Synthetic PPARδ ligand
h	hours
LBD	Ligand Binding Domain
LD	Lipid Droplet
LSDP5	Lipid Storage Droplet Protein 5

Abbreviations

Luc	Luciferase
MAG	Monoacylglycerol
mRNA	messenger RNA
MPR	Mannose 6-Phosphate Receptor
ng	nanogram
NR	Nuclear Receptor
OA	Oleic acid
PAT	Perilipin, ADRP, TIP47
PBS	Phosphate buffered saline solution
PCR	Polymerase Chain Reaction
P/S	Penicillin/Streptomycin
PGC-1	PPARγ Coactivator-1
PKA	Protein Kinase A
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	PPAR Response Element
PUFA	Polyunsaturated Fatty Acids
qRT-PCR	quantitative Real-Time PCR
RAR	Retinoic Acid Receptor
RE	Response Element
RNA	Ribonucleic Acid
Rnase	Ribonuclease
Rt	Room temperature
RT	Reverse Transcription
RT-PCR	Reverse Transcription PCR
RXR	Retinoid X Receptor
TAG	Triglyceride (TAG, Triacylglycerol)
TIP47	Tail-interacting Protein of 47kDa
VI	

TF   Transcription Factor

WAT   White Adipose Tissue

WT   Wild Type

WY-14643   Synthetic PPARα ligand

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1 Introduction

1.1 Lifestyle diseases and energy balance

The World Health Organization has referred to obesity as *"the most blatantly visible, yet most neglected public health problem"* (1). Overweight and obesity origin from excessive neutral lipid storage, and are important determinants of health and lead to adverse metabolic changes, including unfavorable cholesterol levels, increases in blood pressure and increased resistance to insulin. They result in elevated risk of coronary heart disease, stroke, diabetes mellitus, and many forms of cancer (2). On the other hand, lack of neutral lipid storage is associated with lipodystrophies (3). Understanding the molecular mechanisms that regulate neutral lipid storage holds the key to develop therapeutic treatments for these metabolic diseases.

1.2 Nutrition and gene regulation

The effects of nutrition on health and disease cannot be understood without a profound understanding of how nutrients act at the molecular level (4). From a molecular standpoint, nutrients are considered to be "signaling molecules" that, through appropriate cellular sensing mechanisms, result in translation of these dietary signals into changes in gene, protein, and metabolite expression (5). A gene is "expressed" when its code is produced into a protein (or RNA). For this to happen several steps are required, such as transcription and translation (Figure 2). This information flow from DNA to RNA to protein was named of Francis Crick as "the central dogma" of molecular biology already in 1958 (6): *"once information has got into a protein it can never get out again"*. The "information" he refers to is the amino acid residues defining the protein, the product of the translation process. This was thought to apply for all organisms, but later it was found that for RNA viruses the flow of genetic information is starting from RNA.

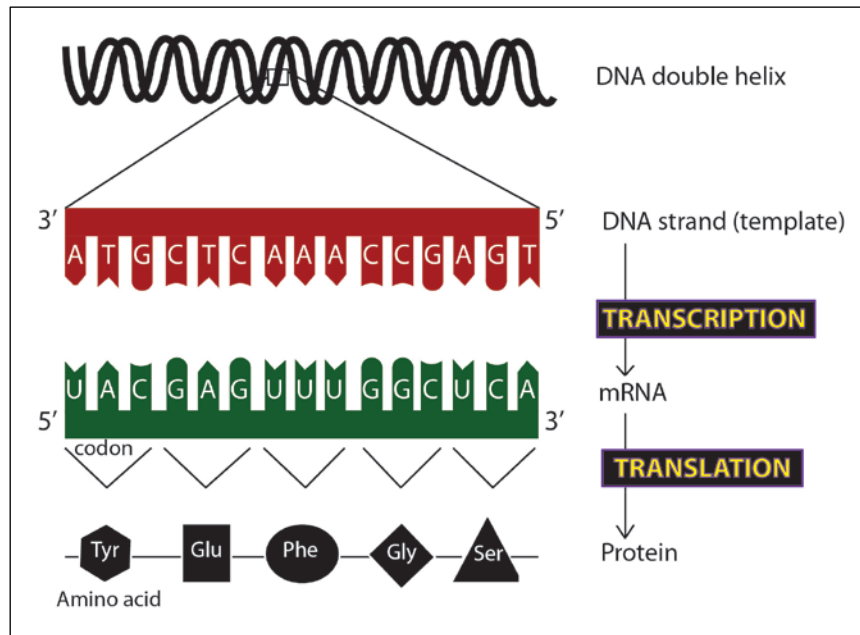**Figure 1: The central dogma of molecular biology.**

Figure 2: From DNA to mRNA to protein. During the transcription, which occurs in the nucleus, the information from a gene in the DNA double helix is copied into a preRNA that is further processed into messenger RNA (mRNA). One strand of the DNA function as a template, and the preRNA sequence forms a complementary strand where thymine (T) is substituted with uracil (U). The preRNA is then modified in several ways before it travels to the cytosol as an mRNA used for translation. During the translation, three nucleotides (codon) from the mature mRNA direct the assembly of amino acids that fold into a protein. Modified from (7).

Gene regulation controls wether a gene product will be produced, its level of production, and the timing of its production. Gene regulation is essential to control processes like cell differentiation and morphogenesis, with different cells expressing different genes despite having the same gene sequences (8). The regulation can be transcriptional, post-transcriptional and post-translational. The regulation can also occur by regulating the access to the necessary areas of the DNA.

The primary route nutrients regulate gene expression is through transcriptional regulation (see section 1.3), and the main agents through which nutrients influence gene expression are transcription factors (9).

1.3 Transcriptional regulation

The most common mechanism for regulation of gene expression is at the transcriptional level. Transcriptional regulation is the change in gene expression levels by altering transcriptional rates (10). In eukaryotic transcription, all RNA transcripts are generated from genomic DNA. To begin the process, the basic transcriptional machinery (see next section) assembles onto

DNA. This formation is under control of numerous gene-specific transcription factors and the polymerase machinery. In addition, because eukaryotic genes are wrapped around histones, the modification of histones also has a central part in the regulation of gene expression. Both the time for the transcription and the amount of RNA to be produced, are subjected to transcriptional regulation. There are three different classifications of RNAs; messengerRNA (mRNA), transferRNA (tRNA) and ribosomalRNA (rRNA). RNA polymerase I is responsible for generating rRNA, RNA polymerase II for mRNA, and RNA polymerase III for tRNA. This thesis focuses on regulation of mRNAs, which are the type of RNA that encodes for synthesis of proteins.

1.3.1   The basic transcriptional machinery

The basic transcriptional machinery contains the TATA binding protein (TBP) and general transcription factors (GTFs) that form a complex with RNA polymerase II. These factors bind to a DNA sequence called the TATA-box (see Figure 3), a short double-helical DNA sequence mainly composed of T and A nucleotides typically located 25 nucleotides upstream from the transcription start site. GTFs are proteins that assemble on all promoters transcribed by RNA polymerase II. Additional transcription factors that are ubiquitous to all promoters bind upstream at different sites in the promoter region, thereby increasing the transcription frequency and being necessary for a promoter to function at an adequate level. Genes that are regulated by these consensus elements in the promoter alone are *constitutively* expressed. But there are also gene-specific DNA regulatory sequences that are used to alter the gene transcription 1000-fold or more. Gene-specific transcription factors bind to these unique sequences often referred to as enhancers, that can be located at some distance from the core promoter, either upstream or downstream of the transcriptional initiation site. The binding of gene-specific transcription factors to enhancer regions interacts with coregulator proteins, which again interacts with the basic transcriptional machinery by forming a loop in the DNA. Depending on the coregulator proteins bound, the enhancer complex activates or represses assembly of GFT at the transcription start site on the promoter (11).
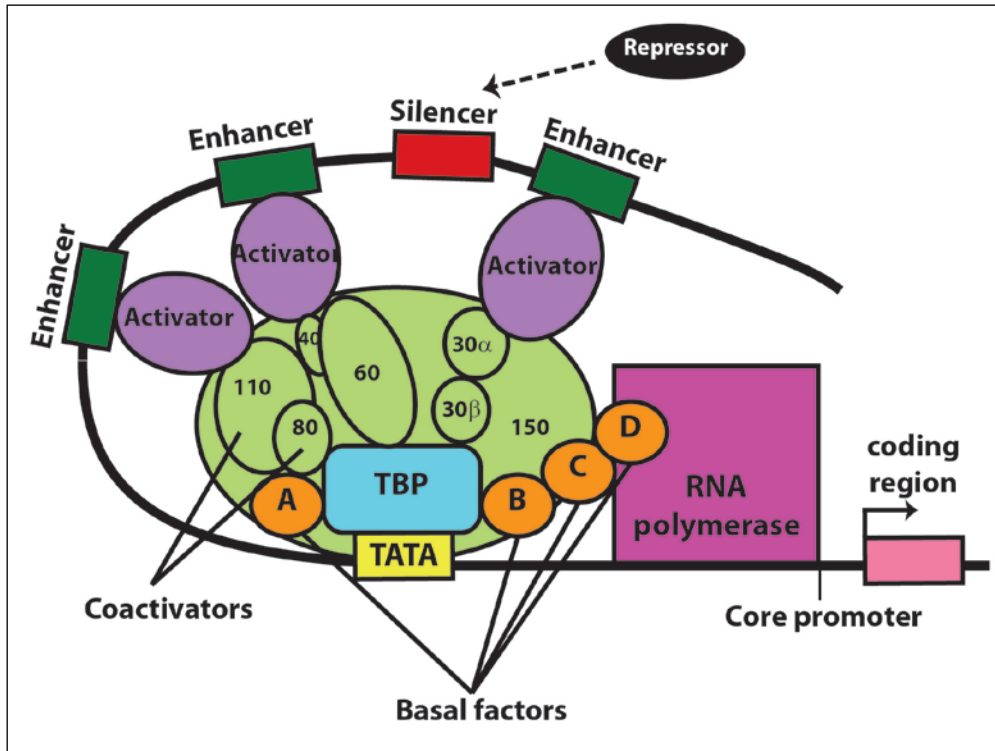
Figure 3: The gene regulatory control region. Specific transcription factors are shown as activators, but may also be repressors. First the TATA-binding protein (TBP) and some of the basal factors (A-D) bind to the TATA-box in the promoter. When the rest of the basal factors bind, a complete transcription factor complex is formed that RNA polymerase can bind to. The basal factors are needed for the transcription, but do not influence the transcription rate. Coactivators combine the basal factors with the activators, which bind to enhancers distant located from the promoter. The interaction between activators and coactivators increases the transcription rate. If a repressor binds to a "silencer" region adjacent to or overlapping the enhancer sequence, the corresponding activator can no longer bind to the DNA. TBP: Tata binding protein. Modified from (12).

Once RNA polymerase II is bound to the promoter, the double helix of DNA is opened, and the polymerase starts to synthesize a single-stranded RNA (premature mRNA) that is complementary to the DNA template strand. Unlike DNA polymerase, RNA polymerase II can start synthesizing new strands in the absence of a primer. The RNA polymerase and the newly-made RNA strand dissociate from the DNA at a termination signal which ends the transcription. After completed transcription, the pre-RNA is processed into mRNA and transported out of the nucleus. The mRNA is used in the translation process, where its information is translated from "RNA-language" to "protein-language", in other words from nucleotides to amino acids.

1.4 Nuclear receptors

Various transcription factor families alter basal transcription rate in response to change in nutrients. One large group of nutrient sensors is the nuclear receptor superfamily (see Figure 4) of transcription factors (TFs), with 48 functional members in the human genome, and 49 in the mouse genome (13). Nuclear receptors (NRs) function as ligand-activated transcription factors that regulate gene-specific expression of target genes and affect processes as diverse as nutrient metabolism, embryonic development, cell proliferation, and cellular differentiation (14). Numerous receptors in this superfamily bind nutrients or their metabolites (9;15). The NRs play a central role in the body's ability to transduce steroid, retinoid, thyroid, and lipophilic endocrine hormones (16). Together their target genes make a network with coordinated activity that defines the physiological response.

Figure 4: The nuclear receptor superfamily. Classification of NRs according to physiological properties, and source and type of ligand. Adapted from (14).

Despite having wide variation in ligand sensitivity, all NRs share a similar structural organization, see Figure 5. Almost everyone of them contains a ligand-independent transcriptional activation function (AF-1) in the NH_2-terminal region; two highly conserved zinc finger motifs targeting the NR to hormone response elements in a core DNA-binding domain; a hinge region that allows for simultaneous receptor dimerization and DNA binding through protein flexibility; and a large COOH-terminal region that contains the ligand-binding domain (LBD), dimerization interface, and a ligand-dependent activation function domain (AF-2) (14). In some nuclear receptors (e.g. PPAR, LXR, PXR) the ligand-binding

pocket in LBD is relatively large and is not fully occupied by the known ligands. In contrast to these, nuclear receptors with high-affinity ligands, like RXR (retinoid X receptor), have a tighter fit of the ligand in the ligand binding domain.
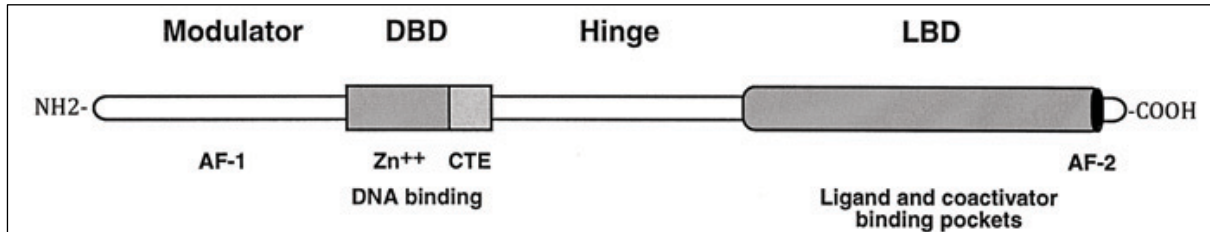
Figure 5: Anatomy of nuclear receptors and typical gene structure. Modified from (17).

A subclass of the nuclear receptors bind as homo- or heterodimers with RXR to specific nucleotide sequences (response elements) in the promoter regions of a large number of genes. These are classified as RXR heterodimers, which typically binds to two direct repeats (DR) of the core sequence AGGTCA separated by N number of nucleotides. Upon ligand binding, nuclear receptors undergo a conformational change that results in coordinated dissociation of corepressors and recruitment of coactivators to enable transcriptional activation.
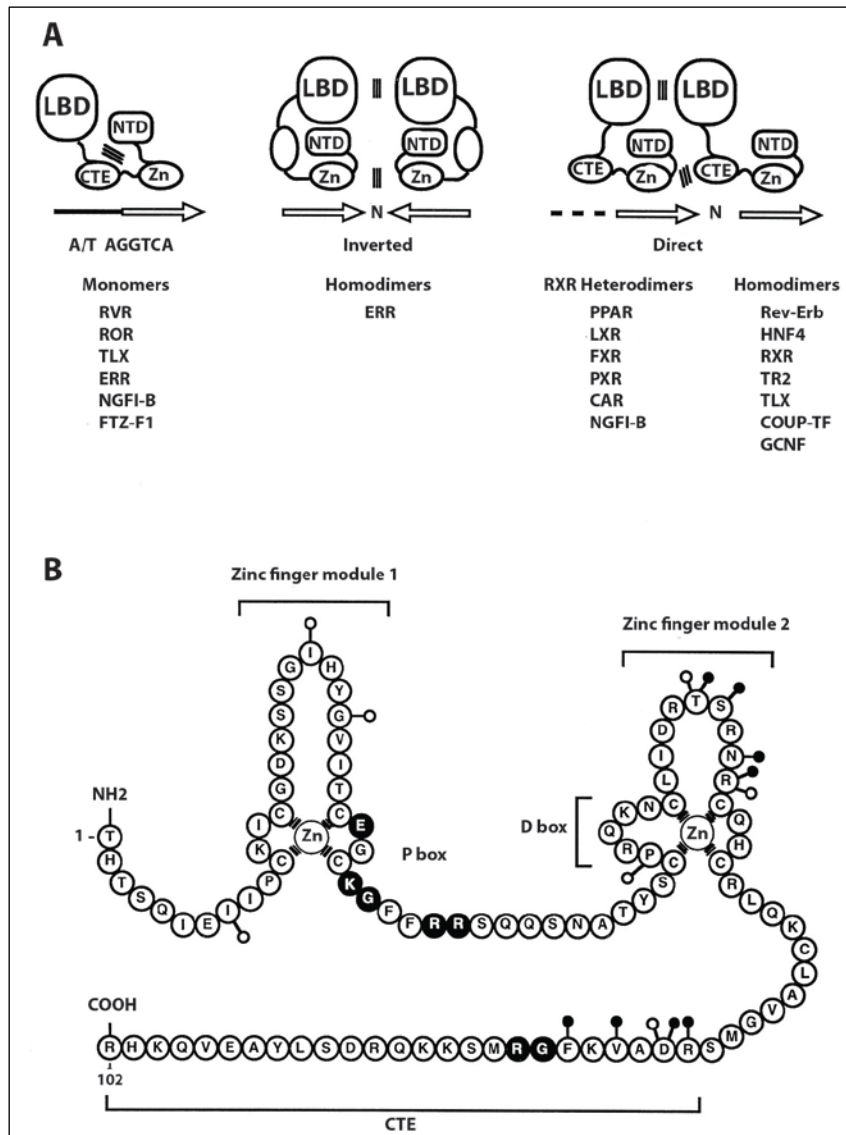
Figure 6: DNA binding by nuclear receptors. A: Nuclear receptors can bind DNA as monomers, homodimers, and RXR heterodimers. The latter binds to response elements consisting of direct repeats of the sequence AGGTCA separated by N number of nucleotides. B: Schematic structure of a nuclear receptor DBD. Adapted from (17).

1.4.1 Retinoid X Receptors

The retinoid X receptors (RXRs) are unique among the members of the nuclear receptor family as they are able to form heterodimers with many other nuclear receptors (18-21). Heterodimerization is a general and simple mechanism by which a linear signaling pathway can be converted into a highly complex network, acting in a cell-specific manner in response to physiological or pathological signals. Because of their heterodimerization with other NRs, which occurs either spontaneously (22) or is stimulated upon ligand binding (23), RXRs are often considered to reduce the specificity of the signaling pathway.

RXRs were first identified as heterodimeric partners of the retinoic acid (RAR), thyroid hormone and vitamin D nuclear receptors and are required for high affinity binding of these and other RXR heterodimers to DNA (24). Later it was discovered that the natural molecules docosahexanoic acid (DHA) (25) and β-apo14'-carotenal (26), which regulate the transcriptional activity of other receptors including PPARs, were able to activate or repress RXR transcriptional activity, yet they are not RXR-specific ligands.

There are three different RXR isotypes (RXRα, RXRβ, RXRγ) with distinct properties that thereby modulate the transcriptional activity of RXR-containing heterodimers. The homology of the three RXR subtypes indicates that these receptors regulate common target sequences and respond to common ligands (27). The expression pattern of the subtypes is rather different. RXR and RXRβ are expressed in many tissues while RXRγ expression is mainly restricted to the muscle and brain (27).

By being essential binding partners to a number of other NRs, RXRs play critical roles in a wide range of developmental processes, from embryo implantation to organogenesis, as well as in the regulation of adult physiology and metabolic processes.

1.4.2   Peroxisome Proliferator Activated Receptors (PPAR)

Peroxisome proliferator-activated receptors (PPARs) are fatty acid-activated transcription factors belonging to the nuclear hormone receptor family (28-30), thereby influencing expression of specific genes (9). One of the main functions of PPARs is that they are able to respond to dietary stimuli to maintain energy homeostasis. Three isoforms have been identified in mammals, with different tissue expression, functions and ligand specificities; PPARα, PPARβ/δ (hereafter referred to as PPARδ) and PPARγ (29-32). The PPARs were initially described as orphan NRs, but several potential endogenous ligands were later described, as reviewed in (33). In contrast to other NRs, which only bind a few specific ligands at high affinity, a ligand that is able to activate one of the PPAR subtypes can often bind to or activate the other subtypes as well. This non-strict ligand specificity is a key feature of the PPARs, which enable these TFs to act as receptors for several ligands binding to the receptors with relatively low affinity. A broad ligand-specificity is possible due to the significantly large ligand-binding pocket in the PPARs, compared to other NRs (33). Several endogenous ligands are shown to bind to PPARs, such as the polyunsaturated fatty acids (PUFAs), α-linolenic acid and linoleic acid, and conversion products of essential fatty acids

synthesized by lipoxygenases or cyclooxygenases. However, despite the demonstration that the ligands may bind to PPARs, it's not given that any of them are *bona fide* endogenous ligands; the ligands have to be present inside the cells in concentrations high enough to actually activate the PPARs. To study the transcriptional regulation of PPARs it's possible to use synthetic ligands that have been developed as drugs for different lifestyle diseases. Most of these developed synthetic ligands have specific preference for only one of the PPAR members.
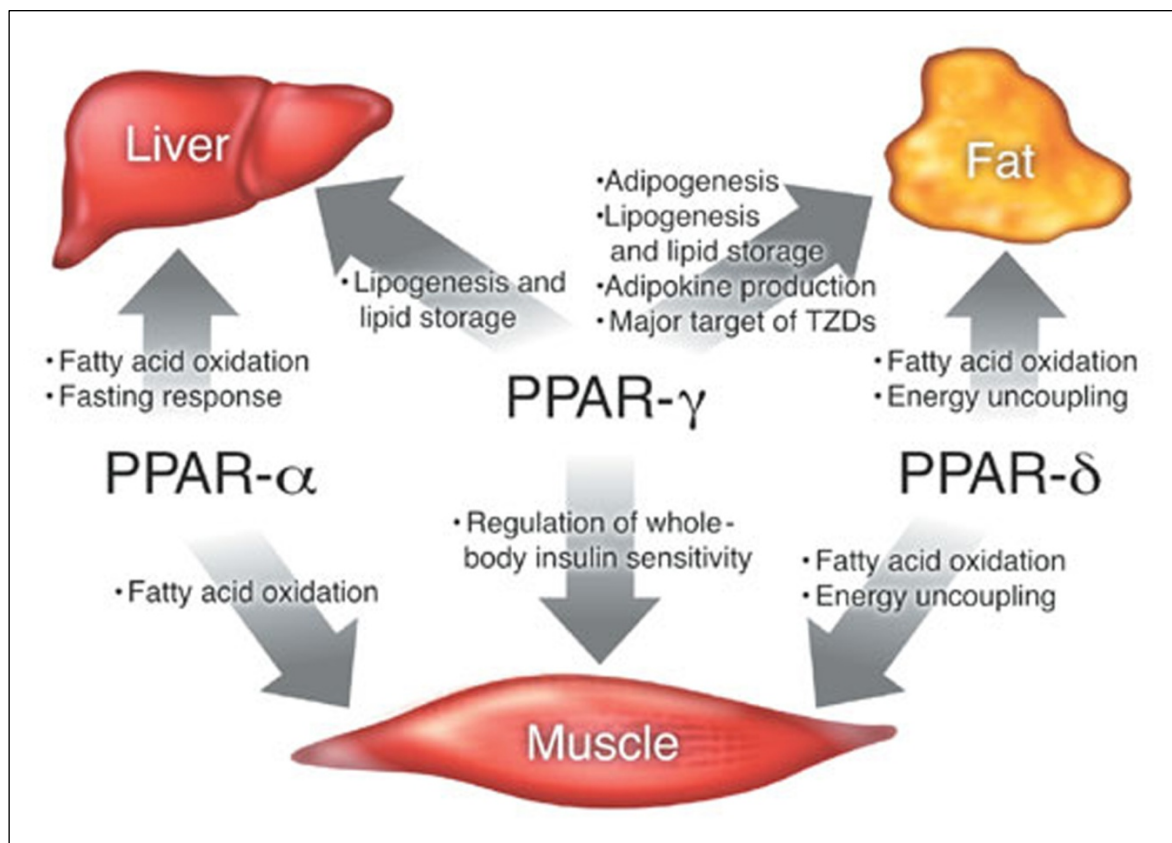
Figure 7: **Metabolic integration by PPARs. The three PPAR isoforms regulate lipid and glucose homeostasis through their coordinated activities in liver, muscle and adipose tissue. Adopted from (34).**

The PPARs bind as obligate heterodimers with RXR to PPAR response elements (PPREs) in target genes and change the dynamics between coregulators (section 1.4.4) and hence modulate transcription of the target genes (Figure 8). Because of this heterodimerization, in which both partners contact the DNA, two copies of the core motif are necessary to constitute a functional hormone response element (HRE). The usual PPREs consist of a direct repeat hexameric DNA recognition motif (AGGTCA) separated by one nucleotide (DR1) (35). However, binding of PPARs to DR2 sequences has also been reported (36-40).
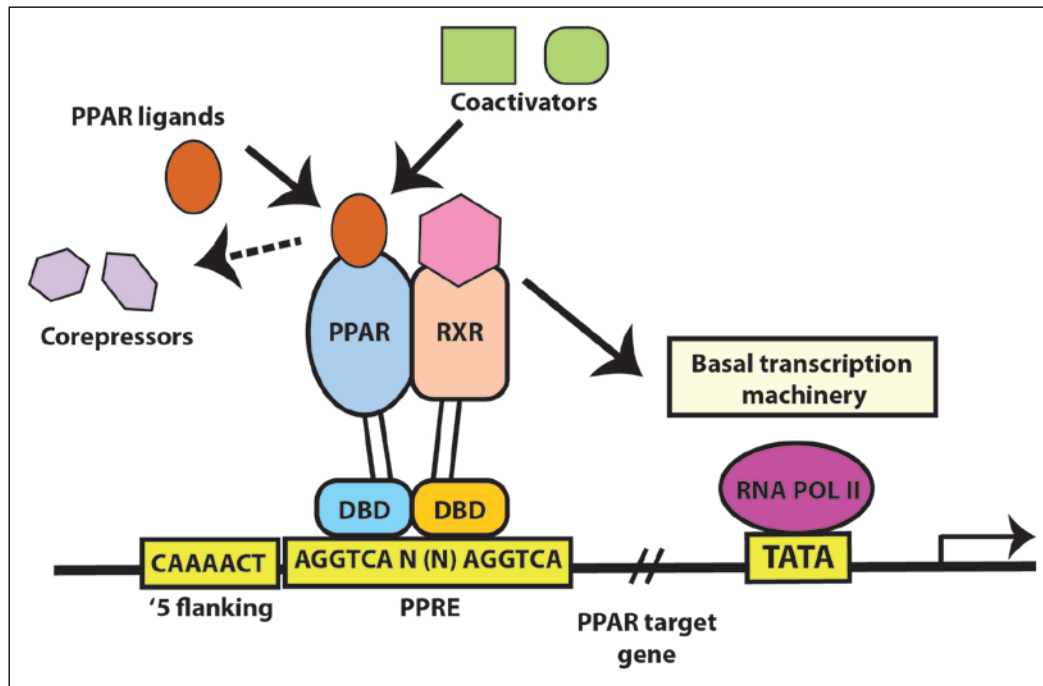
Figure 8: Schematic diagram of PPAR activation. **PPAR: Peroxisome proliferator-activated receptor; RXR: Retinoid X receptor; DBD: DNA binding domain; PPRE: Peroxisome proliferator-activated receptor response element; RNA POL II: RNA polymerase II. Modified from (41).**

The abundance of NRs, their corepressors and coactivators, activity of the pathways that produce PPAR and RXR ligands, and physiological and pathophysiological conditions affecting lipid signals are examples of different levels at which the regulatory action of the PPAR:RXR heterodimer can be exerted (42). The fact that RXR also is a NR with its own ligand, makes it possible that a functional PPAR-RXR heterodimer can bind to DR1-elements and activate transcription even in the absence of PPAR-ligand, if a RXR-ligand is present (43).

PPARα

PPARα is expressed in tissues with high fatty acid catabolism, such as brown adipose tissue, liver, heart, kidney, and intestine, where it promotes fatty acid oxidation, ketogenesis, lipid transport, and gluconeogenesis (44;45). It is also expressed in immune cells like macrophages, lymphocytes and granulocytes (46-50). During starvation and cold acclimatization, the receptor is activated and stimulates expression of genes important to provide energy from fatty acid catabolism (33), such as genes involved in stimulating fatty acid uptake into the cell and subsequent fatty acid transport into mitochondria for oxidation. As a consequent, the incorporation of fatty acids into plasma lipoproteins carrying

triglycerides (TAGs) is reduced. PPARα also lowers TAGs by increasing lipoprotein lipase and apolipoprotein A-V, and by repressing the expression of apoC-III which is an inhibitor of TAG hydrolysis (51;52). This explains why fibrate drugs, which act as synthetic ligands of PPARα, can be used in the treatment of hypertriglyceridemia by lowering triglycerides and raising HDL (53).

Dietary fatty acids can bind to and activate PPARα, which makes our diet directly linked to the regulation of genes. PPARα is the only subtype of PPARs that was thought to have an endogenous *bona fide* fatty acid ligand (33). The work of Chakravarthy et al (54) suggests that fatty acid synthase (FAS) is involved in generation of a PPARα-ligand, and recently the same group found that the FAS-product phospholipid 1-palmitoyl-2oleoyl-*sn*-glycerol-3-phosphocholine (16:0/18:1-GPC) activates transcription in a PPARα-dependent manner (55), making it an endogenous ligand that is influenced by the diet (see Figure 9). A diet with high fat content activates PPARα, while a diet with low fat and high sugar content will upregulate FAS and produce the ligand that activate PPARα. This way the PPARα functions both as a lipid sensor and a carbohydrate sensor. When PPARα is activated, numerous genes are transcriptionally regulated, which contribute to maintaining energy homeostasis partly by promoting β-oxidation of fatty acids (56).
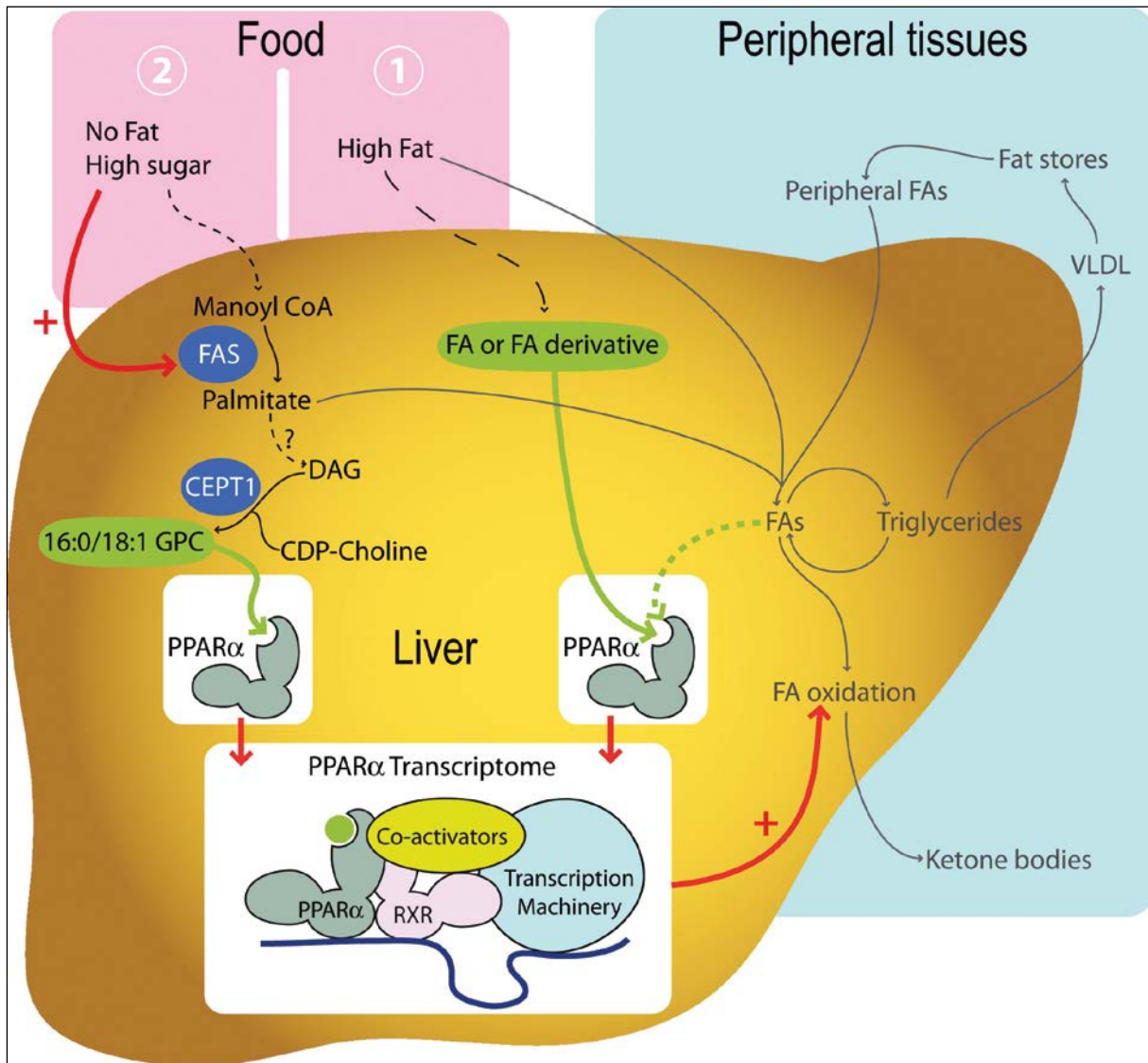
Figure 9: Routes to PPARα-activation in the liver. 1: Dietary fatty acids activate PPARα. 2: FAS and CEPT-1 control the production of 16:0/18:1-GPC when diet contains no fat. GPC activates PPARα. PPARα promotes β-oxidation of fatty acids and thereby contributes to maintaining energy balance. CEPT1: choline-ethanolamine phosphotransferase 1. DAG: diacylglycerol. FA: fatty acids. VLDL: very low density lipoprotein. Adopted from (56).

PPARβ/δ

PPARδ is expressed ubiquitously (46;57). Fatty acid metabolism, mitochondrial respiration and programming of the muscle fiber type are examples of pathways regulated by PPARδ.

PPARδ is activated by exercise (58) and regulates fatty acid oxidation in muscle (33) by stimulating transcription of genes that stimulate the conversion of fatty acids into energy for working muscles. Its role in lipid metabolism is well established based on knockout and overexpression studies in transgenic mice (59-61). The PPARδ knockout mice are smaller

than wild type animals, both before and after birth. Overexpression of a constitutively active PPARδ in WAT reduces adiposity, most likely because of enhanced level of fatty acid oxidation. Ligand activation of PPARδ delays weight gain by increasing fatty acid catabolism in adipose tissue and skeletal muscle (62). This process depends on both PPARδ and AMPK, which also is activated by the PPARδ-ligand (GW501516) through decreased ATP levels and a lower ATP:AMP ratio (63). Like PPARδ, AMPK is also activated by exercise (since this process consumes ATP). PPARδ and AMPK seem to activate a program that increases the coupling of glycolysis to glucose oxidation in muscle (64).

Muscle-specific overexpression of PPARδ in mice changes fiber composition to more oxidative fibers (58), whereas muscle-specific knockdown of PPARδ results in a fiber-type switch toward lower oxidative capacity (65), development of obesity, and diabetes type 2 (66). However, the same effects have not been shown in primates, which implies that weight reduction caused by PPARβ/δ ligands in mice might depend on their effects on thermogenesis, which is a minor mechanism of energy expenditure in humans and primates (67).

PPARγ

PPARγ is activated in the fed state (postprandial), and is especially abundantly expressed in white and brown adipose tissue where it promotes lipid storage and adipocyte differentiation and maintenance (68-70). It is also shown to be expressed in immune cells. It is a central regulator of fatty acid synthesis and insulin sensitivity for major glucose utilizing tissues. PPARγ is activated by synthetic ligands, such as the thiazolidinedione (TZD) group, which is used clinically to treat diabetes type 2.

PPARγ exists in two isoforms, transcribed from the same gene; PPARγ1 and PPARγ2. The latter is expressed highly specifically in adipose tissue, while PPARγ1is expressed at a lower level in a broader range of cell types (71-78). The receptors central role in adipocyte function has been well demonstrated. PPARγ-knockout mice fail to develop adipose tissue (79-81). Humans with different single allele dominant-negative mutations in PPARγ develop partially lipodystrophy and insulin resistance (82-84).

1.4.3 Estrogen-related receptors

The estrogen-related receptors (ERRs) are referred as "estrogen-related" as they share high degree of sequence homology with estrogen receptor α (ERα) and can be activated by non-hormonal signals (85). But they do apparently not bind estrogens or other known natural ERα agonists (86), and the three family members therefore belongs to the orphan family of NR. ERRα and ERRβ were the first orphan NRs identified during a search for genes related to the estrogen receptors (86). ERRγ was discovered a decade later (85;87;88).

All three ERR isoforms are widely expressed in the central nervous system (89). ERRα is widely expressed in adult tissues, especially in tissues that utilize or can utilize fatty-acid β-oxidation (86;90-92). It is also expressed throughout the adipocyte differentiation program (93) and in bone-derived macrophages activated by lipopolysaccharide or interferon γ (IFN-γ) (94;95). ERRα is involved in many aspects of lipid metabolism. Some of these effects are driven by coactivation by peroxisome proliferator-activated receptor gamma coactivator 1α and β (PGC1α and PGC1β) (96-100). Similar to PGC1α, ERRα expression is stimulated by physiological stimuli such as exposure to cold temperatures, exercise, and fasting (91;93;98). Induction of PGC1α expression by physiological stimuli is believed to increase the transcriptional activity of the ERRs, stimulating expression of ERRα and other ERR target genes in a positive feedforward mechanism.

ERRβ and ERRγ are more restricted expressed and are generally expressed at lower levels than those of ERRα. Little is known about the physiologic role of ERRβ. Both ERRβ and ERRγ are present in abundance in the heart and kidneys, and their expression patterns segregate with tissues associated with basal metabolic functions (90). ERRγ is generally highly expressed in tissues with high metabolic activity (e.g. heart, kidney, slow-twitch muscle, BAT and the central nervous system). Loss of ERRγ appears to prevent a perinatal transition from carbohydrate-based fetal metabolism to a lipid-predominant adult metabolism (101).

1.4.4 Coregulators

In addition to the group of sensing transcription factors that directly interact with DNA by binding to specific response elements, coregulator proteins (corepressors and coactivators) direct a substantial component of gene control. Coactivators exist in multiprotein complexes that dock on transcription factors and modify chromatin, allowing transcription to take place (9). Nearly 300 distinct coregulators have been identified, revealing that a great variety of enzymatic and regulatory capabilities exist for NRs to regulate transcription and other cellular events (16).

A coactivator is a molecule that the NR directly recruits to amplify NR-mediated gene expression. The recruitment is normally, but not always, ligand dependent. A corepressor functions in the opposite manner to repress gene expression, mainly through its interactions with unliganded NRs (16).

Coregulator dysfunction occurs in numerous human diseases. Coregulators are often over- or underexpressed in a wide range of cancers. Human genetic variations are to a great degree present in coregulatory genes causing human phenotypic variations in steroid biology, cancer, and metabolic disorders (16).

Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1)

An important group of coactivators for this master thesis is the PGC1s. They coactivate avariety of transcription factors, including the majority of the NR-family such as the retinoid receptors (102;103), the ERRs (102;104;105), and not least the PPAR transcription factor family (102;103;106;107) which they coactivate to activate the expression of genes involved in mitochondrial fatty acid oxidation.
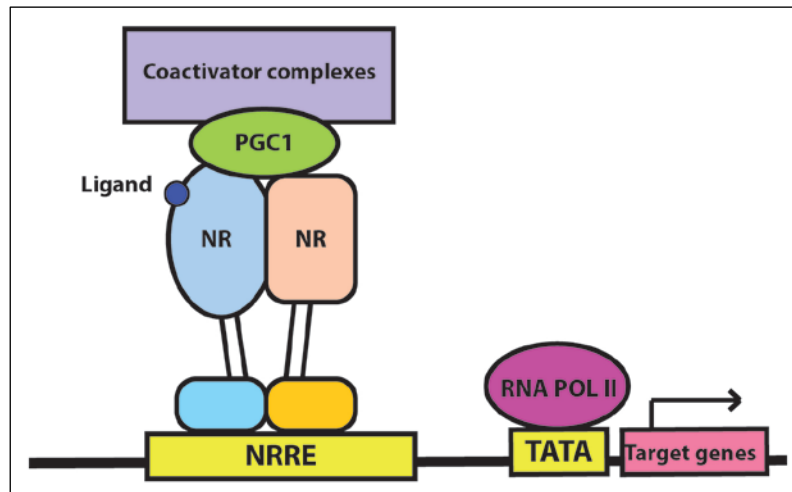
Figure 10: PGC1 coactivators dock to transcription factor targets and recruit protein complexes that activate transcription. NR: Nuclear receptor. PGC1: Peroxisome proliferator-activated receptor gamma-coactivator. RNA POL II: RNA polymerase II. NRRE: Nuclear receptor response element. Modified from (108).

PGC1α was discovered in 1998 through its functional interaction with the nuclear receptor PPARγ in brown adipose tissue (BAT) (102). PGC1β was identified a few years later in 2002 (109).

PGC1α and β are closely related and have a preferred expression in mitochondria-enriched tissues with high oxidative capacity, such as heart, slow-twitch skeletal muscle, and BAT, where they regulate mitochondrial functional capacity and cellular energy metabolism (96;102;103;109;110). PGC1α is a central coactivator in metabolic regulation (102). This coactivator is highly inducible by exercise, fasting, and cold exposure (16), all conditions that demand increased mitochondrial energy production. The same conditions also induce and activate PPARα (described in section 1.4.2). Expression of PGC1α is induced by activation of PPARα in BAT (111).

PGC1β expression is induced by fasting, but not cold exposure (103;109;112). The two PGC1s can drive different programs of fiber differentiation in skeletal muscle. Overexpression of PGC1α in transgenic mice promotes the conversion to slow, oxidative type I and IIA muscle fibers (113), while over expression of PGC1β promotes the generation of fast, oxidative type IIX (114).
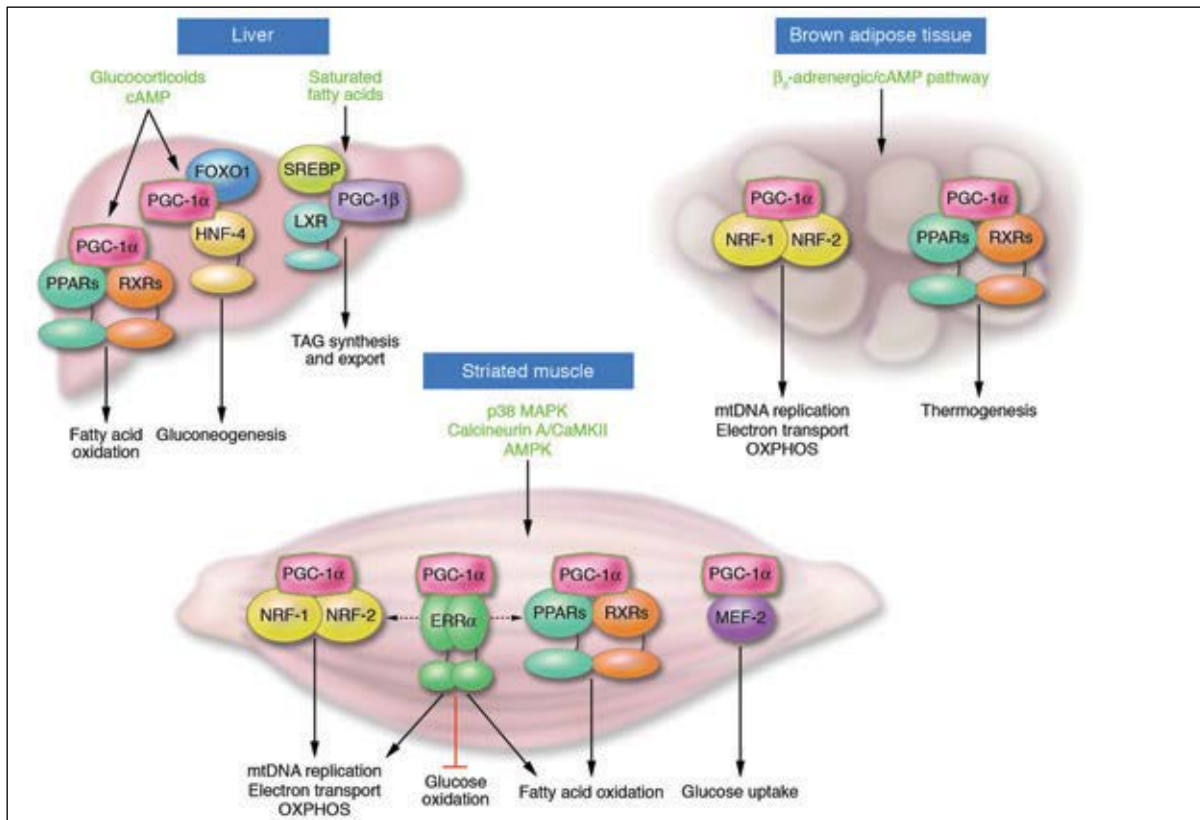
Figure 11: The PGC1 gene regulatory cascade, represented by PGC1α. The illustration indicates the upstream signaling events and downstream gene regulatory actions of the inducible PGC1 coactivators. The signaling pathways shown at the top of each organ system transduce extracellular physiologic and nutritional stimuli to the expression and/or activity of PGC1α. Adopted from (108).

1.5 Lipid droplets

Lipid droplets (LDs) were almost neglected by scientist for ~100 years due to a simplistic view of LDs as biological inert "bulbs of fat". However, research on LDs the last two decades has established LDs as a novel cellular organelle. LDs are sites of regulated release of stored fat by lipases during cell growth and fasting, thus they are central to energy balance at cellular and organismal levels. The stored lipid can also be used as substrate for synthesis of other important cellular molecules, such as membrane phospholipids and eicosanoids (115). The prevalence of life style diseases such as metabolic syndromes, obesity, steatosis and atherosclerosis, has prompted research on LDs. Furthermore, reflecting on the multifaceted nature of LD functions, the range of diseases that are linked to LD abnormality is extended to neuropathy, cardiomyopathy and other diseases that do not appear to be directly related to lipids (116).

1.5.1   Lipid droplets characteristics and function

LDs are independent organelles that are composed of a lipid ester core and a surface phospholipid monolayer coated by specific proteins (see Figure 12), including proteins of the Perilipin PAT family (see section 1.6.1). They can be observed by microscopy as round structures, but their size and number of droplets varies between cell types and within the same cell type. Their diameters range from 0.1-5 µm in nonadipocytes, but in white adipocytes they can reach more than 100 µm (117), and occupy more than 95 % of the cell volume. Most adipocytes contain only one large LD, and the LD contour is defined by the cell periphery (116). Non-adipocyte LDs are generally round and are located in the cytoplasm distant from the cell surface. The core of LDs consists of neutral lipids, such as triglycerides (TAG) and cholesteryl esters (CE) of different ratios. In white adipocytes TAG is the dominating stored lipid.
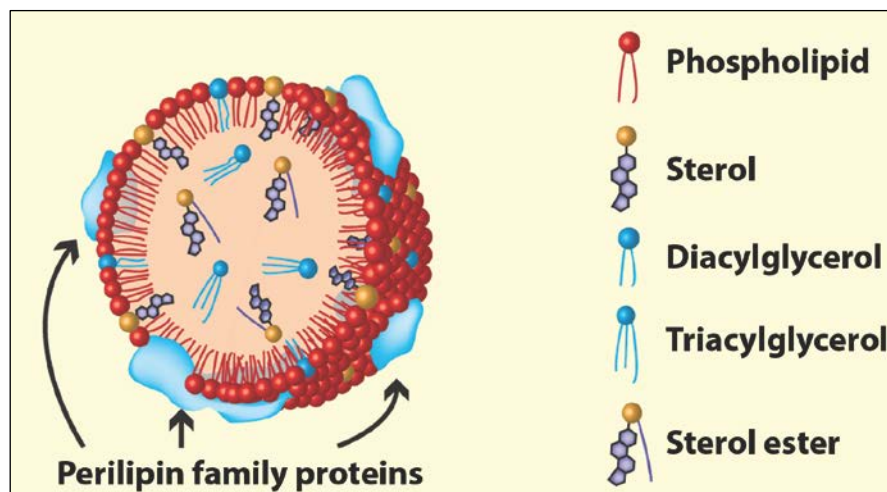
Figure 12: Lipid droplet composition. Modified from (118).

LDs are often juxtaposed to the endoplasmic reticulum (ER) (see Figure 13), mitochondria and peroxisomes, both in adipocytes and in non-adipocytes (119-123). These arrangements probably reflect active lipid transport between LDs and these organelles. Enzymes that catalyze the last step of TAG and steoryl ester synthesis, which are two isoforms of diacylglycerol acyl-CoA acyltransferases (DGAT) and cholesterol acyl-CoA acyltransferases (ACAT), respectively, harbor the ER membrane in mammalian cells (124-126). For mitochondria and peroxisomes, the close physical association with LDs is necessary for the import of fatty acids for β-oxidation (116). The molecular machinery in and around LDs regulates synthesis, utilization and trafficking of lipids and plays a crucial role in the cellular

lipid metabolism (116). Thus, in addition to being a storage organelle, LDs also function in maintaining lipid homeostasis and metabolism (127).
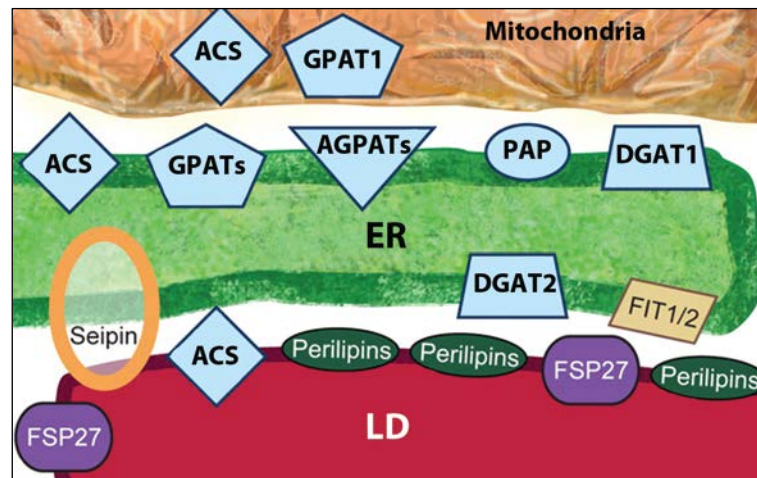
Figure 13: Lipid droplet juxtaposed to endoplasmatic reticulum. TAGs are synthesized by enzymes associated with LD, ER and mitochondria. ACS: acyl-CoA synthetase. GPATs: glycerol-3-phosphate acyltransferases. AGPATs: 1-acylglycerol-3-phosphate acyltransferases, PAP: phosphatidic acid phosphohydrolase. DGAT: diacylglycerol acyltransferase. FSP27: fat-specific protein of 27 kDa (also called CIDEC). FIT: fat storage-inducing transmembrane proteins. ER: Endoplasmatic Reticulum. LD: Lipid Droplet. Modified from (128).

1.5.2   Lipid droplet formation

The formation of LDs is highly connected to the biosynthesis of TAGs (129). Unesterified fatty acids (FA) have numerous functions, such as serving as substrates for the production of ATP and acting as ligands for nuclear receptors and thereby regulate gene expression. Nonetheless, because of their amphiphatic nature, which makes them capable of damage membranes of cells and organelles, they are toxic for the cell at higher concentrations. To avoid such toxicity, excess FAs are rapidly esterified with glycerol to form TAGs, or cholesteryl to form CEs, and stored in LDs (130;131).

The synthesis of TAG has been reviewed in (132). The first step in the dominating pathway for formation of triglycerides starts with the formation of lyso-phosphatidic acid in a reaction catalyzed by glycerol-3-phosphate acyltransferase (GPAT). In the next step, 1-acylglycerol-3phosphate O-acyltransferase (AGPAT) catalyses the formation of phosphatidic acid (PA). PA phosphohydrolase hydrolyzes PA when associated with microsomal membranes, forming diacylglycerol (DAG). Diacylglycerol acyltransferase (DGAT) then catalyzes the conversion of DAG to TAG.

Studies performed to determine the cellular location and the processes involved in LD formation are inconclusive (133-137). Increasing evidence support that the ER is tightly wrapped around LDs (138;139). Enzymes catalyzing the final steps in neutral lipid synthesis are localized to the ER membrane (124;140-143), suggesting that LDs emerge from ER when fatty acids are esterified to glycerol or cholesterol. The most accepted model for LD formation (reviewed in (144-146)) proposes that lipid esters accumulate between the two leaflets of the ER membrane, which ultimately makes the leaflets bulge in the direction to be covered by the cytoplasmic membrane leaflet (see Figure 14). Finally the droplet is pinched off from the membrane to become an independent LD.

A newly-formed LD has a diameter of only 0.1-0.4 µm, but grows as it fuses with other LDs, independently of TAG-synthesis. In order for this to happen, the LDs have to move closer to each other. This process seems facilitated by the transport of LDs on microtubuli with the help of the motor protein dynein (147;148). Fusion of individual LDs might be assisted byα-Soluble N-ethylmaleimide-sensitive factor Adaptor protein Receptor (SNARE)-proteins (149), in which different SNARE-proteins on the two fusing membranes interact with each other and form a complex that causes fusion. In this process, the LD monolayers from the two LDs will melt together, forming a new single larger LD (132;149).
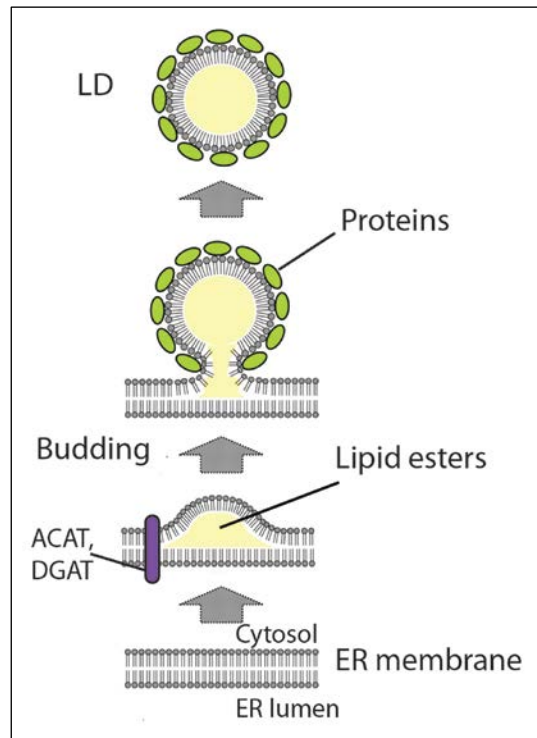
Figure 14: The leading model for LD formation. See text for details. LD: Lipid droplet. ACAT: acyl-CoA acyltransferase. DGAT: diacylglycerol acyl-CoA acyltransferase. ER: Endoplasmatic reticulum. Modified from (116) and (150).

1.5.3   Fat mobilization from lipid droplets

When the demand for energy is increased, such as during fasting, the TAGs stored in the LD can be hydrolyzed to FAs and glycerol in a process termed lipolysis. This process is best described for adipocytes, where the process is tightly regulated by hormones. Adipose tissue secretes FAs to provide them as systemic energy substrate. The liver takes up a large fraction of the FAs and converts some of these to ketone bodies. Glycerol that enters the liver is used to produce glucose through gluconeogenesis. In muscle, the majority of liberated FAs are oxidized to produce ATP.

The first and rate-limiting step of the breakdown of TAGs is performed by the enzyme adipose triglyceride lipase (ATGL), which hydrolyzes a fatty acyl chain to yield DAG (151). The activity of ATGL is stimulated by comparative gene identification-58 (CGI-58) (152). Loss of function mutations in CGI-58 severely prevents ATGL-activity and leads to massive accumulation of TAGs in affected tissues (152). On the contrary, abundance of CGI-58 increases the activity of ATGL and hydrolysis of TAG into DAG. The second fatty acyl side chain is then hydrolyzed by the enzyme hormone sensitive lipase (HSL) to yield MAG. MAG

is finally hydrolyzed by monoglycerid lipase (MGL) to yield glycerol and FA (153). In unstimulated adipocytes, CGI-58 is bound to unphosphorylated perilipin 1 at the LD-surface, ATGL is present both in the cytosol and on the LD-surface, whereas HSL is located only in the cytosol (154;155). In this basal state, re-esterification most likely also proceeds. For full hormone-activated lipolysis in WAT, β-adrenergic stimulation and PKA-activation is necessary. Briefly, phosphorylation of HSL and perilipin 1 initiates a cascade reaction. HSL translocates to the LD-surface, and CGI-58 dissociates from perilipin 1 (155;156) to interact with ATGL, mainly on the LD-surface. This process is illustrated in Figure 16 and described in detailed in the section describing the role of perilipin 1 (section 1.6.2).

Lipolysis is not limited to adipose tissue, it also occurs in other tissue like muscle, heart, and liver. In accordance with this, the lipolytic enzyme ATGL has been demonstrated to be expressed in human skeletal muscles, mainly in type I oxidative fibers (157). The coactivator of ATGL, CGI-58, is found in various tissues like adipose tissue, muscles, liver, testis, neurons and epidermis (152;158;159). It has been suggested that CGI-58 is the principal regulator of the TAG-metabolism in human skeletal muscles (160). CGI-58 is expressed at high levels in oxidative muscles, where it not only coactivates ATGL to increase the lipolytic activity, but also seems to regulate skeletal muscle gene expression through controlling the cellular levels of FAs acting as PPARδ ligands (160).

While perilipin 1 has a major role in lipolysis in adipose tissues, the other perilipin members of the perilipin family are anticipated to regulate lipolysis in nonadipose tissues (152;158). An example of this is the interaction of perilipin 5 with ATGL and CGI-58 in oxidative muscles, which will be described in section 1.6.6.

1.6 Lipid droplet associated proteins

Many proteins are associated with LDs. Proteomic studies have identified LD-associated proteins that are involved in lipid metabolism and transport, intracellular trafficking, signaling, chaperone function, RNA metabolism, and cytoskeletal organization (161-166). The protein coat of the LD can vary between droplets within a cell, between metabolic conditions, and between cell types (167;168). One of the most abundant protein family coating LDs are the perilipin family (144;169), which will be further described in the next sections (1.6.1-1.6.6). In addition to the perilipins, there are many other types of proteins on

the LD surface, such as members of the cell death-inducing DFF45-like effector (CIDE) family of proteins, putative methyltransferases METTL7A and METTL7B, and different enzymes required for lipid metabolism (170). The proteins that coat LDs within a specific cell type change during LD biogenesis and metabolic state (basal vs. lipogenic vs. lipolytic) (115).

1.6.1 Perilipins

Perilipin proteins are highly promising drug targets at the surface of LDs in mammalian tissues, as novel mechanistic targets in the treatment of obesity and secondary associated diseases. The perilipins (also known as the PAT-family, named after the three fist discovered members **P**erilipin, **A**DRP and **T**ip-47), include five members; perilipin 1 (perilipin), perilipin 2 (ADRP/adipophilin), perilipin 3 (TIP47), perilipin 4 (S3-12) and perilipin 5 (LSDP5/OxPAT/MLDP). The proteins are encoded by the *Plin 1-5* genes in mice, which share a common underlying structural organization and are acknowledged to define a novel gene family (171;172) (Table 1). The perilipin protein family is defined by primary sequence similarity across species and a common ability to target LDs.

Being at the LD surface, the perilipins direct the access of other proteins like lipases to the lipid esters in the core of the LD, suggesting a significant structural and/or regulatory role in LD maintenance. All the perilipins probably regulate the interface between LDs and their cellular environment (171).

Table 1: A unified nomenclature for the mammalian perilipin-related PAT-family (172).

			Human		Mouse	
Approved HumanSymbol	**Approved Name**	**Previous Aliases**	**Entrez GeneID**	**Chr. Location**	**Entrez GeneID**	**Chr. Location**
PLIN1	perilipin 1	perilipin, PERI, PLIN	5346	15q26	103968	7 D3
PLIN2	perilipin 2	ADRP, ADFP, adipophilin	123	9p22.1	11520	4 38.9 cM
PLIN3	perilipin 3	TIP47, PP17, M6PRBP1	10226	19p13.3	66905	17 D
PLIN4	perilipin 4	S3-12	729359	19p13.3	57435	17 D
PLIN5	perilipin 5	PAT1, LSDP5, OXPAT, MLDP	440503	19p13.3	66968	17 D

The family of perilipins is evolutionarily ancient, as members are present in several animal species. This conservation of the family indicates the relevance of its function in regulating intracellular lipid stores (171). All perilipins share primary sequence homology (see Figure 15), especially in the N-termini, and they bind to intracellular LDs, either constitutively or in response to metabolic stimuli increasing the flux of lipid in or out of LDs (167). The most highly conserved sequence called the PAT-1 domain (named after **P**erilipin, **A**drp and **T**ip47), includes ~100 amino acids located at the N-terminus of the perilipin sequence and is conserved between perilipin 1, perilipin 2, perilipin 3, and perilipin 5, but little conserved in perilipin 4 (169). Evidence suggests this domain might assist targeting of perilipins to LDs (173-175) and to be a site for interaction with lipases. Other domains shared by the perilipins are the 11-mer repeats located in the center of the proteins, which is thought to have a function in binding of the perilipins to LDs, and a hydrophobic cleft in the C-terminus with unknown function. The perilipins share considerable protein sequence identity in the C-terminus, except for perilipin 1 (176). Perilipin 2 and perilipin 3 has the greatest overall sequence homology, followed by perilipin 5. Perilipin 1 and perilipin 4 are more diverge due to their unique C- and N- termi, respectively (171).

Despite the sequence similarity and the common ability to bind to LDs, there are also important differences between the perilipins implying that each member has a special cellular function. The perilipins differ in tissue expression and transcriptional regulation, the affinity for LDs, the size of the LDs they attach to, the type of lipid filling, and protein stability when unbound to LDs (168;171). Most of the perilipins are transcriptionally regulated by PPARs, reflecting their role in lipid metabolism (171).

On the LD surface the perilipins regulate ATGL-binding and TAG-hydrolysis by distinct mechanisms. Because ATGL is expressed widely while the lipolytic activity differs among tissues, the different perilipin composition of LDs in various tissues may account for the tissue-specific regulation of lipolysis (177). A recent study by Hsideh et al (168) demonstrates that the different perilipins sequester to LDs with either TAG or CE, a finding that underline the theory that each perilipin have separate and possibly unique roles associated with their specific LD-targeting. In tissues that predominantly accumulate TAG, such as adipose tissue, heart, and oxidative muscles, the most abundant perilipins are perilipin 1(a and b) and perilipin 5 which specifically target TAG-LDs. In tissues that accumulate CE, such as steroidogenic cells, these perilipins are generally less expressed, while perilipin 1c and

perilipin 4 show a relatively higher expression. It appears that some of the perilipins have the ability to direct the type of accumulated lipids. Cells overexpressing *Plin1* have a higher content of TAG-LDs and reduced content of CE-LDs compared to control cells. The opposite is true for cells overexpressing *Plin4*.
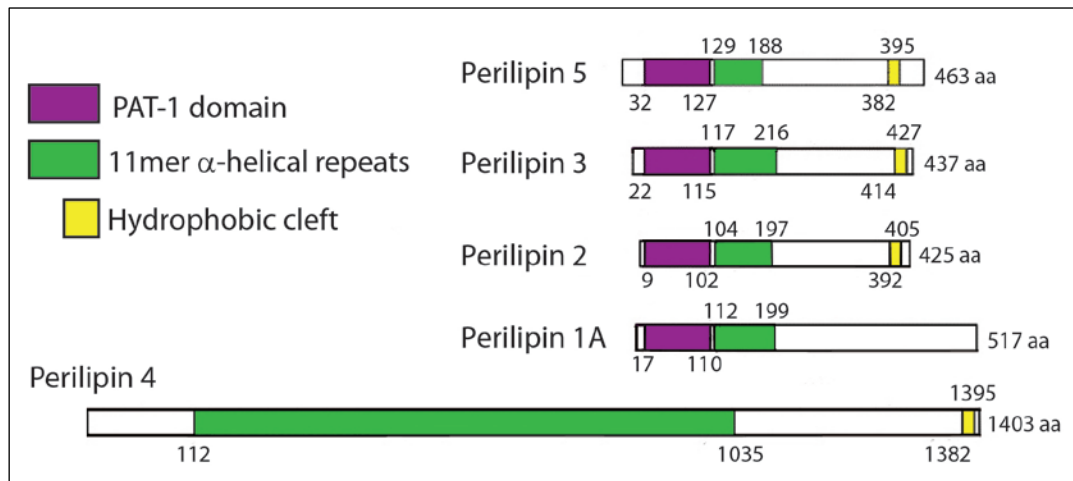
Figure 15: Schematic drawing of mouse perilipins. The locations of regions with considerable sequence identity among the family members (PAT-1 domain, 11mer α-helical repeats, hydrophobic cleft) are indicated. Modified from (171).

1.6.2   Perilipin 1

Perilipin 1 is the first identified member of the perilipin family. In mice, the single *Plin1* gene gives rise to at least four protein isoforms (perilipin 1 A, B C and D) that share a common N-terminal region but have different C-terminal tails generated through alternative mRNA splicing (178;179). Perilipin 1A is the largest protein and the most abundant protein on adipocyte LDs (179;180). The protein was originally identified as the major adipocyte protein phosphorylated in response to activation of protein kinase A (PKA) associated with the LD (181). Perilipin 1B is a less abundant protein, and found close to the plasma membrane in primary human adipocytes (178;182). Both isoforms (perilipin 1A and 1B) protect the triacylglycerols in the LDs in adipocytes from degradation by adipocyte lipases by function as inhibitors of lipolysis (183). Perilipin 1C and D are even shorter isoforms that are expressed only in steroidogenic cells (179;184). Their roles are have not been investigated.

Importantly, a unique feature of the perilipin 1 sequence that is not shared by other members of the protein family is the presence of multiple consensus sequences for the phosphorylation of serine residues by cAMP-dependent protein kinase (PKA) (185). This makes it an

important player in the control of lipolysis in adipocytes, where TAG stored within LDs is catabolized into free fatty acids (FFA) and glycerol during times of nutrient scarcity.

The mobilization of TAG stores is tightly regulated by hormones, and requires the activation of lipolytic enzymes, such as adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Perilipin 1 serves a critical role in regulating basal and stimulated lipolysis by co-coordinating the recruitment of proteins to the LD (169). There are two main signaling pathways that control the breakdown of fat in adipocytes. The inhibitory insulin-signaling pathway, where activation of protein kinase B (PKB) results in inhibition of cellular cAMP production and inhibition of lipolysis, and the stimulating pathway where catecholamines and their interaction with β-adrenergic receptors results in increased cellular cAMP levels and activation of lipolysis. An alternative stimulating pathway is activation of guanylyl cyclase with natriuretic peptides, which results in increased cellular cGMP levels and a following activation of lipolysis (186).

The classical pathway, where perilipin 1 has a crucial role, starts with catecholamines and their interaction with β-adrenergic receptors. β-adrenergic stimulation of the G-protein coupled receptor activates adenylate cyclase which increases cellular cAMP levels (187). cAMP binds and activates PKA, and perilipin 1 is phosphorylated by PKA at up to six serine residues (Ser 81, 222, 276, 433, 492, and 517 of the mouse protein). The phosphorylation of perilipin 1 is required for stimulation of ATGL and HSL activity but not for HSL translocation (188). In the basal state, CGI-58 resides on the surface of LDs and interacts with perilipin 1. ATGL is localized in part to the LD and HSL mostly in the cytoplasm, leading to incomplete or absent activation of ATGL and HSL (154;189;190). Released fatty acids are rapidly resynthesized back into TAG (futile cycling) or directed towards oxidation in mitochondria, making the rate of rate of released FAs extremely low under these conditions. After β-adrenergic stimulation PKA phosphorylates both HSL and perilipin 1. HSL translocates to the LD, and perilipin 1 releases CGI-58, which disperses into the cytoplasm within minutes, thereby permitting binding of CGI-58 with ATGL (154;158;163;190). ATGL initiate lipolysis and the breakdown of TAG to DAG (see Figure 16). HSL associates with the phosphorylated perilipin 1 and degrades DAG to MAG. The final FA is cleaved by MAG lipase (MGL) to produce glycerol (191). Lipolysis is inhibited by the anabolic hormone insulin which stimulates a phosphodiesterase that breaks down cAMP and dephosphorylates perilipin (192;193).

To summarize, the two opposing signaling pathways converge in the regulation of TAG hydrolyzing enzymes and the control of FA reesterification to TAG, resulting in a several hundredfold difference in the production of FAs and glycerol between the basal and stimulated state (191).

Perilipin 1 is used as a marker of adipocyte differentiation as the expression is tightly correlated to the expression and activation of PPAR-γ in these cells. The *Plin1* promoter contains one evolutionary conserved PPAR response element, which makes perilipin 1 a direct PPARγ target gene (176).
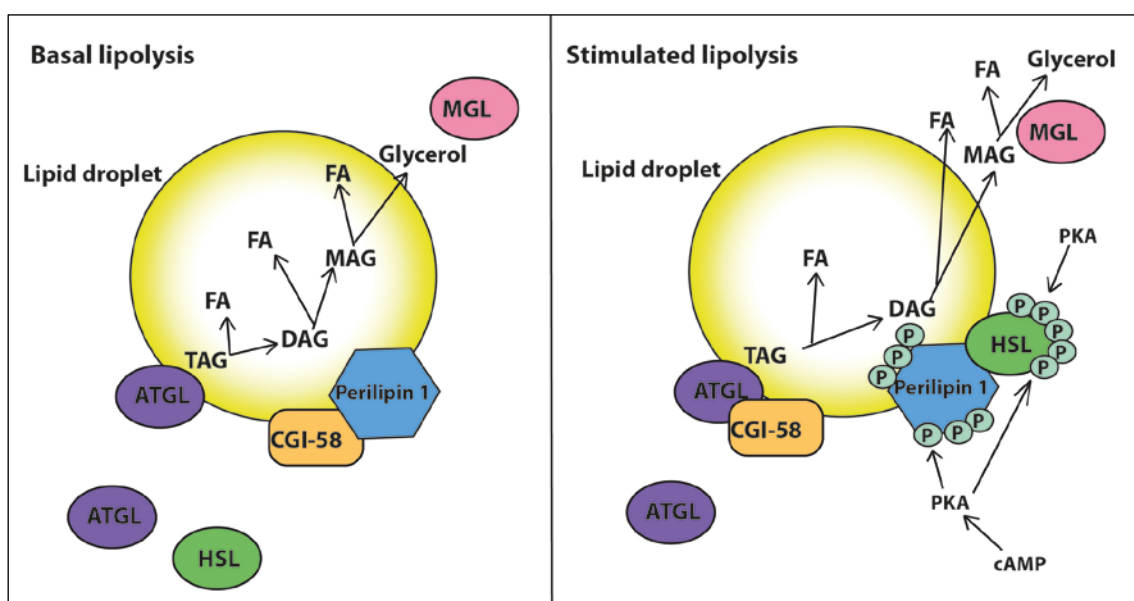
Figure 16: The emerging view of regulated lipolysis. ATGL: Adipose triglyceride lipase. HSL: Hormone sensitive lipase. CGI-58: comparative gene identification protein 58. TAG: triacylglycerol. DAG: diacylglycerol. MAG: Monoacylglycerol. FA: Fatty acids. MGL: Monoacylglycerol lipase. PKA: Protein kinase A. cAMP: cyclic adenosine monophosphate. Modified from (191). See text for details.

1.6.3   Perilipin 2 (ADRP)

Perilipin 2 was originally identified as an RNA transcript significantly induced during differentiation of cultured adipocytes (194;195). Later it was discovered that it coats small LDs in a variety of cell lines, including early differentiating 3T3-L1 adipocytes. Perilipin 2 is thus ubiquitously expressed (196), but not present in mature adipocytes where the perilipin 2 protein is replaced by perilipin 1 (196).

The mouse (197) and human (198) *Plin2* genes contain PPAR response elements, and perilipin 2 is regulated by PPARα (199-201). The protein is also regulated through post-

translational degradation by the proteasomal system (202-204). The amount of lipids inside the cell thereby directly affects the level of accumulated perilipin 2 proteins, by stabilizing it and inhibit its degradation. Perilipin 2 knock-out mice have reduced amount of TAG in the liver, while the differentiation of adipocytes and adipose lipolysis are unchanged (205). A likely reason for the modest phenotypic change in the perilipin 2 knock-out mouse is that in the absent of perilipin 2, perilipin 3 is directed to the LDs and likely substitutes for most of its functions (206). When both perilipin 2 and perilipin 3 is knocked-out in cultured liver cells, the LDs turn larger with a higher turnover of TAGs resulting in cellular insulin resistence (207).

1.6.4   Perilipin 3 (Tip 47)

Perilipin 3, originally named tail-interacting protein of 47kD (TIP47) is ubiquitously expressed with tissue specific enrichment in muscle and placenta (176;208;209). It only seems to bind to LDs under specific metabolic conditions (when the level of FAs rise), but is stable both as a soluble cytosolic protein and when associated with LDs (208-211).

Perilipin 3 was first described in a yeast two-hybrid screen for proteins that interact with the cytoplasmic tail of the mannose 6-phosphate receptor (MPR) (212), but it is now classified as a LD-associated protein (208). It was identified as a cargo selection device for MPRs, directing their transport from endosomes to the trans-Golgi network (213), but recent knockdown studies performed by Bulankina et al (214) showed no effect on MPR distribution or trafficking and did not affect lysosomal enzyme sorting. Instead they found that perilipin 3 is recruited to LDs by an amino-terminal sequence comprising 11-mer repeats, and that it reorganizes liposomes into small lipid discs. They also showed that suppression of perilipin 3 blocked maturation of LD and reduced the incorporation of TAG into LDs, and concluded that it functions in the biogenesis of LDs (214).

Unlike the other perilipins, perilipin 3 does not seem to be regulated by PPARs (176;199).

1.6.5   Perilipin 4 (S3-12)

Perilipin 4 is mainly expressed in white adipose tissue (WAT), but it is also weakly expressed in brown adipose tissue, heart, and skeletal muscle (215). In fact, Gjelstad et al (216) found that skeletal muscle, in addition to WAT, was among the tissues with the highest

expression of perilipin 4, together with heart, placenta, testis and liver. Perilipin 4 was originally identified as a plasma membrane-associated protein induced during adipocyte differentiation, containing 29 tandem repeats of a 33-residue motif that is rich in threonine and glycine (217). The *Plin4* promoter contains three evolutionarily conserved PPAR response elements, and the protein has been identified as a direct PPAR-γ target gene (176).

1.6.6   Perilipin 5 (LDSP 5)

Perilipin 5, originally named LSDP5 (lipid storage droplet protein 5) is the last member of the perilipin protein family, and was discovered by our group and others (171;218;219). The tissue distribution of perilipin 5 is limited to tissues with active fatty acid oxidation, such as the heart, striated muscle, brown adipose tissue and liver (116;171). Several studies have demonstrated that activation of PPARα induce expression of perilipin 5, suggesting an important role for perilipin 5 in FA utilization and lipid metabolism (171;218;219).

In accordance with this theory, perilipin 5 has an essential part in regulating LD accumulation (171;218-220) and LD hydrolysis (171). Perilipin 5 is a scaffolding protein for key lipolytic players, such as ATGL (177;221), HSL (222), and CGI-58 (177;223). In addition, perilipin 5 has the distinctive property of recruiting mitochondria to the LD surface (220). Evidence of a physical and metabolic link between LDs and mitochondria was first described in the work of Wang et al (220), where they suggest that perilipin 5 regulates LD hydrolysis and controls local FA flux to protect mitochondria against an FA surge. Their studies suggest that perilipin 5 has a substantial part in regulating LD hydrolysis in oxidative mammalian tissues and is a putative key player in LD function in oxidative tissues. The signal for mitochondrial interaction reside in the last 20 amino acids of its C-terminus (220), which is a domain unique to *Plin5* (171).

Perilipin 5 is the only perilipin found to bind directly to bind ATGL, which happens even in the absence of binding of CGI-58 (177). Wang et al (177) later found that in liver cells, perilipin 5 reduces the ATGL-activity if PKA is not activated. By inhibiting hydrolysis and thus stabilizing the LD, perilipin 5 helps to accumulate palmitate into triglycerides and to decrease palmitate utilization by the mitochondria in basal state. When PKA is activated, perilipin 5 is phosphorylated through an unknown mechanism which in turn increases the lipolysis, but only modestly. Others suggests that perilipin 5 recruits CGI-58 to the LD (223). Perilipin 5 seems unable of binding ATGL and CGI-58 simultaneously.

A recent study by Bosma et al (224) indicates that in order for perilipin 5 to facilitate lipolysis and fatty acids oxidation, LDs and mitochondria need to interact. Overexpression of perilipin 5 increases FA oxidation in muscle homogenates possessing both LDs and mitochondria, but not in isolated mitochondria, which suggests that perilipin 5 targets FAs from LDs towards mitochondrial oxidation. Also, the overexpression of *Plin5* led to more frequent and intimate interaction of perilipin 5-coated LDs with mitochondria (224).

The current knowledge of perilipin 5 suggests it protects against cellular lipotoxicity in oxidative cells with high energy demands by transiently entrapping bioactive lipids in LDs close to mitochondria at times of increased cellular FA influx. Also, it may facilitate the release of FA by LDs to the mitochondria in a PKA-regulated manner when LDs and mitochondria are interacting with each other.

The transcriptional mechanisms regulating perilipin 5 expression is unstudied, but activation of PPARα is know to stimulate its expression in several tissues (171;218;219).

2 Aims of the study

This work is a part of a project with the overall goal to investigate the gene regulation of perilipin 5 and the importance of lipid droplets in oxidative muscles and the heart. Expression of the *Plin5* gene encoding perilipin 5 is limited to a few tissues, including heart, brown adipose tissue, oxidative muscle and liver. The transcription factor machinery driving this tissue-selective expression pattern is not clear. We and others have previously demonstrated that expression of *Plin5* is stimulated by activation of PPARα (171;218;219). We hypothesize that transcription factors stimulating oxidative capacity play a key role in the regulation of *Plin5*. In support of this hypothesis, we found recently that overexpression of PGC-1 α in cultured muscle cells stimulated expression of the *Plin5* gene. In this master thesis we wanted to test different transcriptional factors known to stimulate oxidative capacity for their ability to induce expression of the *Plin5* gene.

2.1 Objectives

1. Identify transcription factors able to stimulate expression of *Plin5* in cultured muscle cells. Preliminary data suggest that expression of PGC1α stimulates *Plin5* expression. We want to determine if this coactivator and other transcriptional factors known to stimulate fatty acid oxidation are important for the regulation of *Plin5*.

   a. Test the ability of the transcription factors PPARs, PGC1s, and ERRs to induce transcription of the *Plin5* gene.

   b. Map the DNA binding site in the *Plin5* gene.

2.2 Hypothesis

Plin5 expression is induced by the transcription factors PPARs, PGC1s and ERRs. The *Plin5* gene promoter contains binding sites for the above transcription factors.

3 Materials

3.1 Equipment and chemicals

Product	Manufacturer
ABI Prism® Optical Adhesive sealing	ABI
ABI Prism® 96-Well Optical Reaction Plate	ABI
Aluminum Foil	Caterwrap™
Ampicillin	Sigma®
Bovine serum albumin BSA	Sigma® #A8806
BRL-49656/Rosiglitazone	Enzo Life Sciences
LB-dishes	Heger AS
Coulter Clenz cleaning agent	Beckman Coulter™
Culture plates, 6-, 12-, and 24-well	Falcon®
Culture flasks, 25, 75, and 150 cm^2	BD Falcon™
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma®
EDTA	Sigma®
Electroporation cuvette	BioRad
Eppendorf Tubes Axygen	Scientific
Ethanol	Arcus
Falcon tubes	Falcon
Fetal Bovine Serum FBS	Gibco
GW-501516	Enzo Life Sciences
Sterile filtrate filter and bottle	Corning Inc.
HEPES	Sigma®
Horse Serum	Gibco
Isopropanol	Arcus
Isoton II Beckman	Coulter™
L-Glutamine	Sigma®
Latex Gloves, SensiClean®	Ansell
Lipofectamine™ 2000	Life Technologies Corporation
Nucleic Acid Purification Elution Solution	ABI
OptiMEM-I®	Life Technologies Corporation

Oleic acid	Sigma®
Passive Lysis Buffer	Promega
Penicillin Streptomycin	Sigma®
PBS, Magnesium and Calcium free	Sigma®
PCR-water	Fluka
PfuTurbo® DNA Polymerase	Stratagene
Pipettes	Eppendorf
Pipetteboy	Integra Bioscences
Pipette-tips	Biotix, Inc.
RNase free water	Sigma®
Trypsine	Sigma®
WY-14643	Sigma, #C7081

3.2 Instruments

Product	Manufacturer
6100 Nucleid Acid PrepStation	ABI
7900HT Fast Real-Time PCR System	ABI
Centrifuge Labofuge 400e	Heraeus Instruments
Centrifuge Pico 17	Thermo Scientific
Centrifuge Avanti J-26 XP	Beckman Coulter
Coulter Counter® Z1™	Beckman Coulter
Freezer, -20°C	Electrolux
Freezer, -80°C	Forma Scientific
GeneAmp® PCR System 9700	ABI
Ice-machine, AF80	Scotsman®
Incubator-shaker	New Brunswick Scientific
Waterbath M12	LAUDA
Micro-wave oven, Talent	Whirlpool
Microscope	Leica
MilliQ-synthesis	Millipore
NanoDrop® ND-1000 Spectrophotometer	Saveen Werner AB
Pipetteboy Comfort	Integra Biosciences

Refrigerator	Electrolux
Synergy2	BioTek
Vortexer	Scientific Industries, Inc.

3.3 Kits

Product	Manufacturer
Dual Luciferase Reporter Assay™	Promega
High Capacity cDNA Transcription Kit	ABI
JETSTAR 2.0 Plasmid Midiprep kit / 20	Genomed
Plasmid DNA Purification NucleoSpin® Plasmid (NoLid)	MACHEREY-NAGEL
TaqMan® Universal PCR Master Mix	ABI
TaqMan®Gene Expression Assay	ABI
Isolation of total RNA	ABI
QuikChange® II Site-Directed Mutagenesis Kit	Stratagene

3.4 Software and internet resources

Product	Manufacturer
Adobe Illustrator CS5.1	Adobe Inc.
Adobe Photoshop CS5.1	Adobe Inc
GraphPad InStat	GraphPad Software, Inc
Microsoft Office 2007	Microsoft® Incorporated
PubMed	National Library of Medicine
Reference Manager 12	ISI ResearchSoft
RQ Manager	ABI
SDS Software 2.3	ABI
SPSS 18	SPSS Incorporated

3.5 Cell-lines, plasmids and vectors

Product	Manufacturer
C2C12, *mus musculus*, mouse myoblast	ATCC #CRL-1772
DH5α™ *E.Coli* competent cells	Life Technologies Corporation #18258-012
DH10β™ *E.Coli* competent cells	Life Technologies Corporation
pcDNA3	Life Technologies Corporation
pcDNA3-mERRα	Cloned by Knut Tomas Dalen
pcDNA3-mERRβ	Cloned by Knut Tomas Dalen
pcDNA3-mERRγ	Cloned by Knut Tomas Dalen
pcDNA3-mPPARα	Cloned by Knut Tomas Dalen
pcDNA3-mPGC1α	Cloned by Knut Tomas Dalen
pcDNA3-mPGC1β	Cloned by Knut Tomas Dalen
pcDNA3-mPPARγ1	Cloned by Knut Tomas Dalen
pcDNA3-mPPARγ2	Cloned by Knut Tomas Dalen
pcDNA3-mPPARδ	Cloned by Knut Tomas Dalen
pcDNA3-mRXRα	Cloned by Knut Tomas Dalen
pGL3-Basic-LUC reporter vector	Promega
pGL3-Basic-Plin5-LUC	Cloned by Knut Tomas Dalen

4 Methods

A number of different molecular methods were used to conduct these studies. These include transformation, plasmid isolation, PCR, cell culturing, transfection, RNA isolation, cDNA synthesis and quantitative real time PCR.

A mouse cell line able to differentiate into glycolytic muscle fibers (C2C12) was used as a model system. These cultures were differentiated *in vitro* for 4-6 days to form multinuclear contracting myotubes that were used in the experiments. Because *Plin5* only is expressed in oxidative and not glycolytic muscle cells, and the standard culturing conditions make the cells utilize glucose as energy source, *Plin5* will normally not be expressed in the cultured C2C12 cells. This makes it possible to use this cell-line to identify factors that increase expression of *Plin5*. The same factors might drive conversion of glycolytic into oxidative muscle fibres.

4.1 Transformation

In nature, bacteria can exchange DNA with each other to ease adaptations towards different environments. The DNA they exchange is small circular DNA copies called *plasmids*. In molecular biology we can use this technique, called transformation, to make more of a certain plasmid we need for experiments. Transformation is more accurately defined as "*the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s)*" (225). In other words, introducing foreign DNA into a cell (*E.coli* bacteria), which then replicates this DNA along with its own genomic DNA. Most plasmids used in laboratories carry an antibiotic resistance gene to facilitate selection of the bacteria containing the DNA of interest. Two methods are commonly used to transform *E. coli* cells with plasmid DNA in the laboratory - heat shock transformation and electroporation. For both methods, the bacteria must be specially pre-treated to facilitate efficient uptake of the plasmid. Bacteria that have the ability to take up extracellular DNA from its environment is called competent. Competent *E.coli* cells had already been generated and were stored at -80°C in the laboratory.
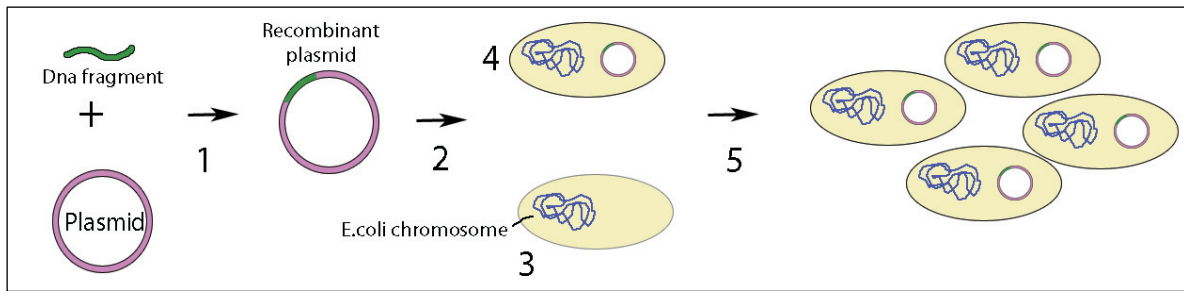
Figure 17: Transformation of *E.coli* cells. 1: A DNA fragment can be inserted into a vector (cloning) to get the insert expressed from the vector. The resulting plasmid is called a recombinant plasmid. The plasmid also carries an antibiotic resistance gene. 2: *E.coli* cells are mixed with plasmids and cultured on nutrient agar containing antibiotics. 3: *E.coli* cells that do not take up the plasmid die on antibiotic plates. 4: Transformed *E.coli* cells survive on antibiotic plates. 5: Ultimately the transformed cells multiply and form colonies. Most plasmids used in laboratories are multiplied into several hundred copies whitin each bacteria.

4.1.1   Heat shock transformation of DH5α *E.coli*

The protocol of heat-shock transformation method is based on Hanahan's protocol from 1983 (226), which still is one of the best transformation methods available. This method was worked out from a series of attempts to get maximum transformation efficiency, after the first demonstration of Ca^{2+}-dependent DNA transfer into E.coli in 1972 (227). The heat shock transformation technique is well suited for circular plasmid DNA, and was in this master thesis used to amplify all plasmids containing the different transcription factors used in the transfection experiments (pcDNA3-vectors).

Experimental procedure:

Competent cells were thawed on wet-ice. 30-50 µl competent cells were used for each transformation, and 10-50 ng DNA was added to the cells and mixed gently before incubation on ice for 30 minutes. The cells were transferred to 42°C waterbath for 90 seconds, and then back on ice for 2 minutes. 450 µl of LB-medium was added, and the cells were then incubated at 37°C for ~ 1 hour. Afterwards 50 µl cells were plated on LB-plates containing ampicillin (100 µg/ml), to allow for selective growth of transformed bacteria containing ampicillin resistant vectors, and incubated at 37°C over night.

The next day individual bacteria colonies were picked from the LB-plates and transferred to a flask with LB-medium containing ampicillin for preparation of plasmids (see section 4.2).

4.1.2   Electroporation of *E. Coli* strains

The electroporation method was developed early in the 1980s (228), and was first described for *E.coli* in 1988 (229). This is now an established method for transformation of bacteria and is used to introduce DNA into bacteteria by a brief high voltage electric discharge to render cells permeable to DNA (230). Electroporation has higher transformation efficenency than heat shock, and is the preferred method to use for transformation of low quantitites of DNA or large DNA constructs (>20 kb). This transformation method was used in this master thesis to transform the PCR products generated by site directed mutagenesis in the process where the PPRE in the Plin5-LUC reporter vector was mutated.

Experimental procedure:

Prior to electroporation, electroporation cuvettes (0.2 cM gap) were cooled in ice-water slurry for > 30 minutes. 50 µl bacteria suspension was pipetted to the pre-cooled electroporation cuvette. Then 0.1-10 µg DNA was added and mixed gently into the bacteria suspension, and the cuvette was incubated on ice for 1 minute. Electroporation was performed with a GenePulser electrophorator (Biorad) with the following condition: 1.8 kV, 25 µF with pulse controller set at 200 Ω. Time constant after electroporation should be ~ 4.2 - 4.6 with these settings. Immediately after electroporation, 1.0 ml SOC was added to the cuvette by pipetting gently up and down a few times. Afterwards the bacteria was transferred to a 15 ml tube and incubated at 37°C for 1-2 hours. Cells were plated on LB-plates containing antibiotics (100 µg/ml ampicillin).

4.2 Plasmid isolation

Bacteria can be used as factories to amplify a plasmid. After the plasmid is transformed into the bacteria, the bacteria will multiply the plasmid along with its own genomic DNA when it multiplies. By expanding a low number of bacteria in an over-night culture, a large number of plasmids can be isolated from the bacteria. An often used method to isolate DNA plasmids from bacteria is anion-columns. The midi-prep JetStar kit was used for large scale isolation of plasmids used for transfection. The NucleoSpin® Plasmid (NoLid) kit (referred to as "mini-prep") was used to isolate small quantities of plasmid used to screen mutated clones. See Appendix A protocol #1 and 2 respectively. The plasmids were stored at -20°C.

Over night culture and harvesting

Plasmid-containing bacterial colonies (4-10 for midi-prep, 1 for mini-prep) were transferred into LB-medium (50 ml for midi-prep, 5 ml for mini-prep) containing 100 μg/ml ampicillin. The LB-plates with the plasmids were stored at 4°C. Bacteria were grown overnight at 37 °C with shaking (200 rpm). The next morning, bacteria were centrifuged at 6000 g for 5 minutes to pellet the *E.coli* cells, and the supernatant discarded.

Midi-prep

Column equilibration

10 ml solution E4 was applied to the columns that were emptied by gravity flow.

Lysis of bacterial cells

Each bacterial pellet was resuspended in 4 ml cold solution E1. 4 ml of solution E2 was then added, and the solution mixed gently and then incubated at room temperature for 5 minutes.

To stop lysis, 4 ml solution E3 was added and the solution was immediately, but gently mixed until the solution was mixed.

Purification of plasmid DNA

The solution was centrifuged at 15.000 g for 10 minutes at 20°C, and the supernatant was applied to the columns and allowed to enter the resin by gravity flow. The columns were then washed twice with 10 ml solution E5, to remove unpurities from the filters, before the plasmid was eluted with 5 ml solution E6. The plasmid was precipitated with 0.7 volumes of isopropanol (3.5 ml) at room temperature, and centrifuged immediately at 15.000g for 30 minutes at 4°C. The supernatant was removed carefully without disturbing the plasmid pellet. The plasmid pellet was washed twice with 1 ml 70 % ethanol and transferred to an eppendorf tube and centrifuged at 12.000g for 2 minutes. The supernatant was removed, and the pellet air dried in room temperature for approximately 5 minutes to let ethanol evaporate. Finally, the plasmid pellet was dissolved in 100-150 μl Tris-EDTA (TE)-buffer.

Mini-prep

Lysis of bacterial cells

The bacterial pellet was resuspended in 250 µl solution A1, and 250 µl of solution A2 was then added to the solution. This was mixed gently and incubated at room temperature for 5 minutes, before adding 300 ml solution A3 and mixing immediately.

Purification of plasmid DNA

The solutions were centrifuged at 11.000 g for 5 minutes at RT. Then the supernatant was added to a NucleoSpin® Plasmid Column placed in a Collection Tube (2 ml), and centrifuged at 11.000 g for 1 minute, for binding of the plasmid to the column filter. The flow-through was discarded, and the NucleoSpin® Plasmid Column was placed back into the Collection Tube. The column was washed by adding 600 µl buffer A4, and centrifuged at 11.000 g for 1 minute. The flow-through was discarded, and the NucleoSpin® Plasmid Column was placed back into the empty collection tube. The column (in the collection tube) was then centrifuged at 11.000 g for 2 minutes to dry the silica membrane. This step was repeated once. The collection tube was then discarded, and the NucleoSpin® Plasmid Column was placed in a 1.5 ml microcentrifuge tube. To elute the plasmid, 50 µl TE-buffer was added and the column was incubated for 1 minute at RT, and then centrifuged at 11.000 g for 1 minute.

4.3 PCR mutagenesis

Polymerase Chain Reaction (PCR) is a method used to amplify segments of DNA, using the ability of DNA polymerase. DNA polymerase needs a *primer* to start attaching nucleotides (it can only add a nucleotide onto a preexisting 3'-OH group). The primers are short pieces of single-stranded DNA that are complementary to the target sequence. Because of this need for a primer, it is possible to amplify a desired region of a template sequence (Figure 18). The principles in PCR technology can also be used to generate mutations in target DNA sequences. PCR based site-directed mutagenesis can generate mutations (base substitutions, insertions, and deletions) in double-stranded plasmids.

The protocol used in this master thesis is modified from a Stratagene protocol; The QuikChange II site-directed mutagenesis method (Appendix A protocol #3).The method is

performed using PfuUltra DNA polymerase for mutagenic primer-directed replication of both plasmid strands with the highest fidelity. The process includes several steps, starting with denaturing and thus separating a supercoiled double stranded target-DNA with an insert of interest into single strands through heating. Next step is cooling the DNA-strands in the presence of a large excess of two 100% complementary oligonucleotide primers, both containing the desired mutation (Figure 19). The primers, each almost complementary to opposite strands of the target DNA, then anneal to the single-stranded DNA. To initiate the synthesis of two new strands complementary to the original DNA chains, DNA polymerase (PfuUltra HF DNA polymerase) and deoxyribonucleoside triphosphates (dNTPs) are added to the mixture. The polymerase adds nucleotides to the 3'-hydroxyl end of the primer, extending the strand across the target DNA by making complementary copies of the target sequence.

Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Two new double-stranded DNA molecules can now be denatured and copied by repeating the procedure using the original starting vector as template. Typically 20 reactions is enough to get enough produced mutated vector. The template (wild type) plasmid is dam methylated in almost all *E. coli* strain and is removed following temperature cycling by digestion with Dpn I endonuclease. The Dpn I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template to simplify selection of mutation-containing synthesized DNA. The mutated plasmid remains intact in the reaction, and this nicked vector DNA is then transformed into DH10β supercompetent cells.

Table 2: Steps in PCR-reaction.

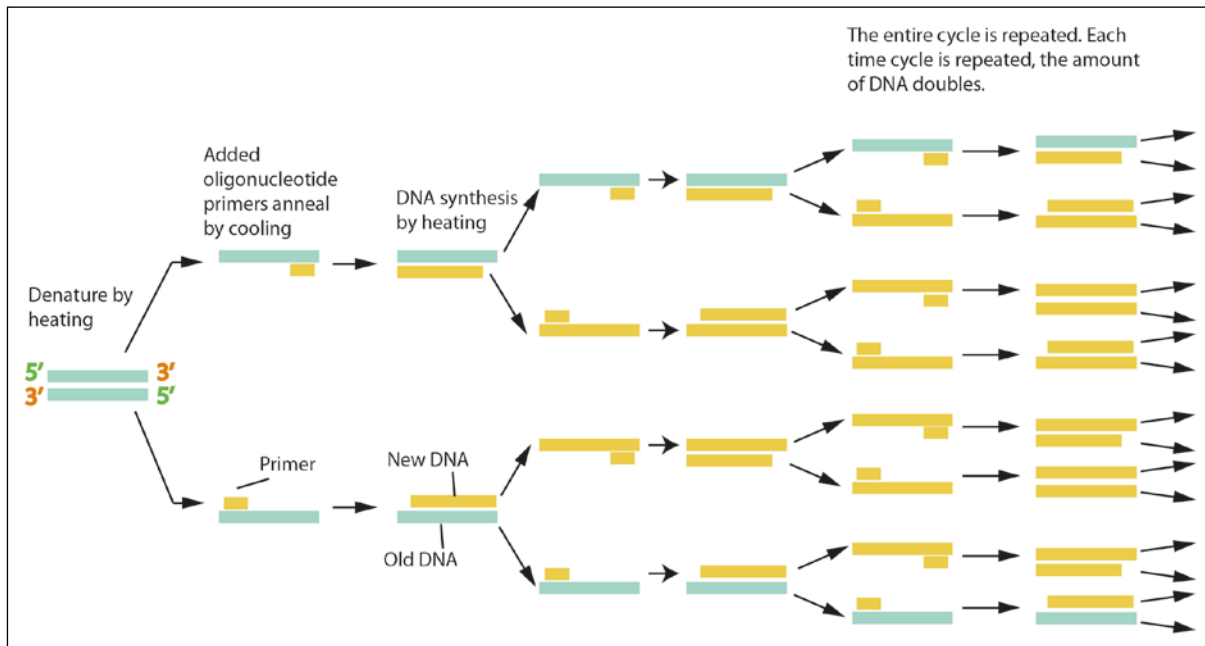|	
Denaturation	Heating of DNA (and primers) to separate it into separate strands.
Annealing	Primers bind to the single-stranded DNA through cooling.
Elongation	DNA polymerase synthesizes new DNA strands complementary to the template.

Figure 18: Steps in a normal PCR-reaction. Modified from (231).
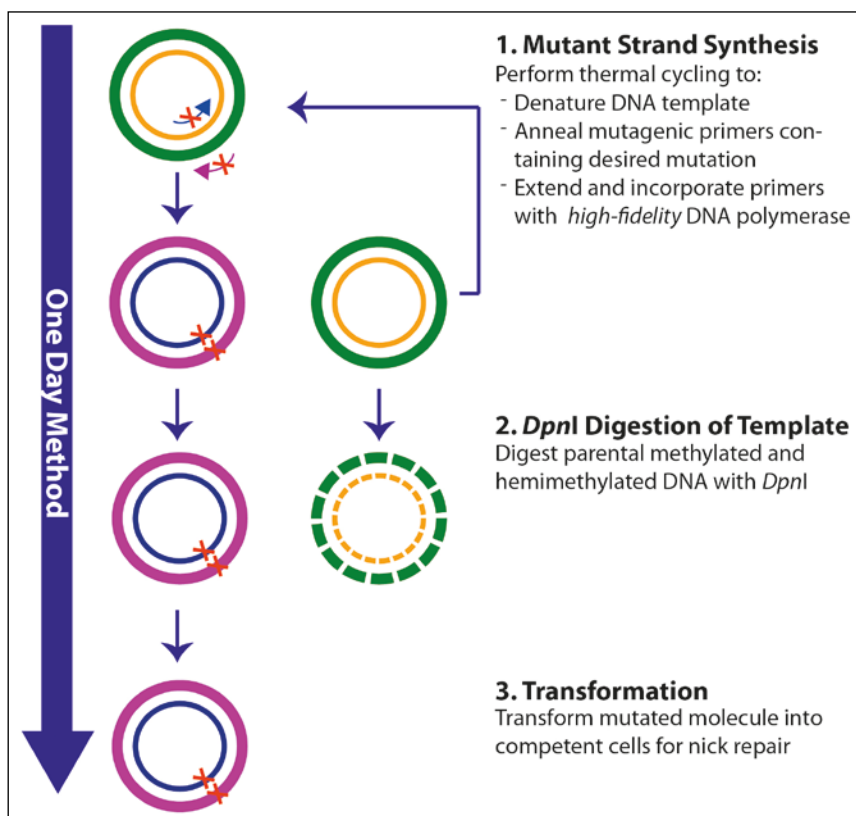

Figure 19: Overview of the QuikChange II site-directed mutagenesis method. Modified from (232).

PCR mutagenesis:

The DR-1 element in the pGL3-basic-Plin5 vector was mutated from the wild-type (Wt) sequence to mutated (Mut) using the oligos shown below as following:

Table 3: Oligos used in PCR mutagenesis of the mouse *Plin5* reporter.

Oligo name	Sequence
Wt sequence (fw)	AGCAGGTGGCA**GGACCC**T**TGGCCC**AGTGGGCCCGG
Plin5-PPRE-Mut-fw	AGCAGGTGGCA**GGA<u>GG</u>CTTGGG<u>GC</u>**AGTGGGCCCGG
Plin5-PPRE-Mut-rev	CCGGGCCCACTGCCCCAAGCCTCCTGCCACCTGCT

The mutation oligos were diluted to 10 pmol/μl, and the targeting vector to 10 ng/μl. 1 or 5 μl template (vector containing promoter) was added in the first two PCR tubes, respectively, and water was then added to the final volume of 5 μl in all three tubes (4, 0 and 5 ul). The third tube functioned as a negative control. 1.5 μl primers (10 pmol/μl each of forward and reverse mutation oligos) were added to each tube. A mixture of the components in Table 4 was made, giving 50 ul reaction volume for each sample.

Table 4: PCR mutagenesis mixture.

Volume	Component	Volume x 3
31 μl	dH$_2$O	93 μl
5 μl	10x Pfu buffer (contains MgCl$_2$)	15 μl
5 μl	dNTP-mix (2 mM of each dNTP)	15 μl
1μl	PfuTurbo 2.5 U/μl	3 μl

3 μl PfuTurbo was added as the last component to the mixture (otherwise the enzyme might degrade template or primers). The mixture was mixed well, and 42 μl was pipetted to each reaction tube. The PCR reaction (Table 5) was started as soon as possible after mixing of all reagents. Step 2-4 was repeated 22 times.

Table 5: PCR settings for site-directed mutagenesis.

Step #	Time	Temperature
1	2 minutes	95 °C
2	45 sec	95 °C
3	45 sec	55-60 °C
4	X minutes	68 °C
5	10 minutes	72 °C

Analysis of PCR reaction

Following the PCR reaction, 5 µl PCR reaction was mixed with 5 µl dH2O and 2 µl 6x loading buffer and separated on a small 0.5 % agarose 0.5 % Tris-borate-EDTA (TBE) gel (a thin comb was used). 1 kb Plus DNA ladder was used as molecular weight standard. (Standard was made by mixing 1 µl ladder with 9 µl dH2O and 2 µl loading buffer). The gel was run at 80 V for 1.5-2 hours. A clear band of the expected molecular weight (~ 7.5 kb) was seen, confirming that the vector had been copied.

Selection, transformation and verification of mutated plasmids

25 µl of the PCR mixture was mixed with 0.5 µl DpnI (10 U/µl and incubated at 37°C for 1 hour to digest the template vector. 2 µl of the DpnI treated samples was electroporated into super-competent cells (DH10β) and seeded on ampicilin (100 µg/ml) agar plates. Five independent colonies were picked for plasmid isolation to screen for a correct clone (method, see section 4.2). All clones were tested by sequencing at the ABI-lab at University of Oslo (Blindern), and three clones were found to be correct. One of these was selected for further analysis.

4.4 Measuring of DNA and RNA concentration on NanoDrop-1000

The concentration of DNA or RNA in solutions was measured using NanoDrop-1000. First, the reader was washed with MQ water. Then 1.5 µl of RNAse free water was put directly onto the bottom pedestal to initialize apparatus. The sample type was adjusted to "RNA-40", for changing the factor used for calculation of RNA concentration based on the measured absorbance. For measuring DNA the sample type was adjusted to "DNA-50". To reset background for all wavelengths (calibrate), 1.5 µl TE-buffer (for measuring plasmid-DNA) or 1.5 µl Nucleic Acid Purification Elution Solution (ABI) (for measuring isolated RNA), was applied on the NanoDrop pedestal, and measured as "Blank". The sample was mixed thoroughly to get a homogeneous sample, and 1.5 µl of each sample was used for measurement.

RNA purity is indicated by the ratio of absorption between 260 and 280 nm. A ratio below 2.0 for RNA indicates proteins and/or phenols to be present. The ratio of isolated RNA from the C2C12 cells were ~2.1 for all the experiments performed.

4.5 Cell culturing

Cell culturing is a process where cells are grown in a sterile environment without external influences. As an extra precaution they are also most often grown in medium containing antibiotic to minimize the risk of bacterial growth. When working with the cells, they are kept in a sterile cell culture hood, and all equipment being used should also be sterile.

4.5.1   Counting of cells

Cells were counted using Coulter Counter ® Z1™. Cells were trypsinated and centrifuged prior to counting. 10 ml Isoton II solution was added in two clean counting flasks, and 100 µl or 200 µl cells were pipetted to the two flask. To prepare the counter, the flask with Coulter Clenz Cleaning Agent was exchanged with a new flask containing Isoton II, before flushing the apparatus. The counter was then set to measure cells between 7 and 20 µm. Samples were mixed gently to get a homogeneous solution before counting. The number of cells counted by the apparatus is the number of cells in 0.5 ml. To determine the number of cells per ml in the original sample, the following calculations were performed:

100 µl cell sample = "count from machine" x 200 cells.
200 µl cell sample = "count from machine" x 100 cells.
The average was used to determine the cell concentration (cells/ml).

After measurements, the apparatus was flushed with Isoton II, and the flask containing Coulter Clenz Cleaning Agent was put back before turning the machine off.

4.5.2   Culturing of C2C12 cells

The cultured C2C12 cell line (ATCC #CRL-1772) resembles glycolytic fast twitch, "white" muscle cells. The cells originally derived from satellite cells isolated from a crush injured thigh muscle of a two month old female mouse donor, and have the ability to proliferate and differentiate into myogenic colonies under appropriate conditions. They proliferate as

mononucleated cells with a generation time of 24h (233). In regular growth medium they grow as undifferentiated myoblasts, while switching to differentiation medium when reaching confluence start myogenic differentiation.

Culturing of frozen cells

Cells frozen in liquid nitrogen was thawed, resuspended in 20 ml pre-warmed (37 ºC) growth media, and incubated at 37ºC until cells were attached to the culture flask (~5-6 h, or until next day). Then media was exchanged to remove DMSO.

Passage of cells (P-x)

Before the cells grew confluent they were trypsinated. Medium was removed, and the cells washed quickly with 2 ml trypsin (75 cm^2 culture flask). The fluid was removed, and 3 ml trypsin/EDTA was added. The flask was incubated at 37°C for 1-2 minutes until the cells were almost detached. To detach the rest of the cells the flask was knocked gently to the wrist. 7 ml growth media was added, and the solution was centrifuged for 3 minutes at 1300 rpm. The supernatant was removed, and the cells resuspended in 10 ml growth media. The cells were splitted 1:5-1:15 in growth media.

Seeding and differentiation of cells to myotubes

C2C12 cells were seeded at 6 x 10^4 cells/well in 1 ml medium (12 well dish) or 3 x 10^4 cells/well in 0.5 ml medium (24 well dish). Differentiation was started the next day by changing to differentiation-media (2 % HS). The cells were differentiated for 4-7 days, and medium changed each 3rd day. For transfection experiments, cells were seeded in antibiotic free medium and transfected ~24 hours later. Cells were given differentiation medium at transfection, and given fresh differentiation medium 6 hours later.

Media used

Basal media

Powdered DMEM (Sigma®, #5648) containing high glucose and L-Glutamine were solved in 980 ml MQ water. 20 ml sterile Stock mix was added and the solution sterile filtrated into 2 x 500 ml bottles. Media were stored at 4ºC until use.

The following were mixed to generate 200 ml (50 x stock):

HEPES (Sigma; #H4043):   5.958 g/L x (200 ml / 20 ml/L) = 59.58 g
NaHCO$_3$:   1.5 g/L x (200 ml / 20 ml/L) = 15 g
Sodium pyruvat: 0.11 g/L x (200 ml / 20 ml/L) = 1.1 g

The solution was heated to 37º C and pH adjusted to 7.4. Then the solution was sterile filtrated and aliquoted in 10 ml tubes.

Culture media

Basal media was supplemented with 20% Fetal Bovine Serum (Gibco #26140-079), 1 % P/S and 1% L-Gln (Sigma #G7513, 100x stock).

Differentiation media

Basal media was supplemented with 2 % horse serum (Gibco #16050-122), 1 % P/S and 1% L-Gln (Sigma #G7513, 100x stock).

4.6 Transfection of cells

Similar to transformation of bacteria, it is possible to get eukaryotic cells to take up foreign DNA. This process is called transfection. Several transfection methods exist, such as calcium-phosphate transfection, lipid-based transfection, electroporation systems, and micro-injection of DNA. The most effective regarding delivery combined with lowest toxicity is lipid-based methods in which specially designed lipids are mixed with the DNA to make the cell absorb the lipid-DNA complex. In this thesis a lipid-based method was used, with Lipofectamine™2000 (Appendix A protocol #5) as the transfection reagent. Lipofectamine™2000 contains lipid subunits that can form liposomes in an aqueous environment. The plasmid to be transfected is entrapped inside the liposomes. The lipids have a positively charged head-group, and one or two hydrocarbon chains. The cell membrane is negatively charged, and thus attracts the positive lipid-head, making a fusion between the two components. The transfection complex (the liposomes with the plasmid) is probably entering the cell through endocytosis (Figure 20). Inside the cell, the complex diffuse through the

cytoplasm to the nucleus where the genetic codes in the the transfected DNA can be transcribed and translated into functional gene products (234). .
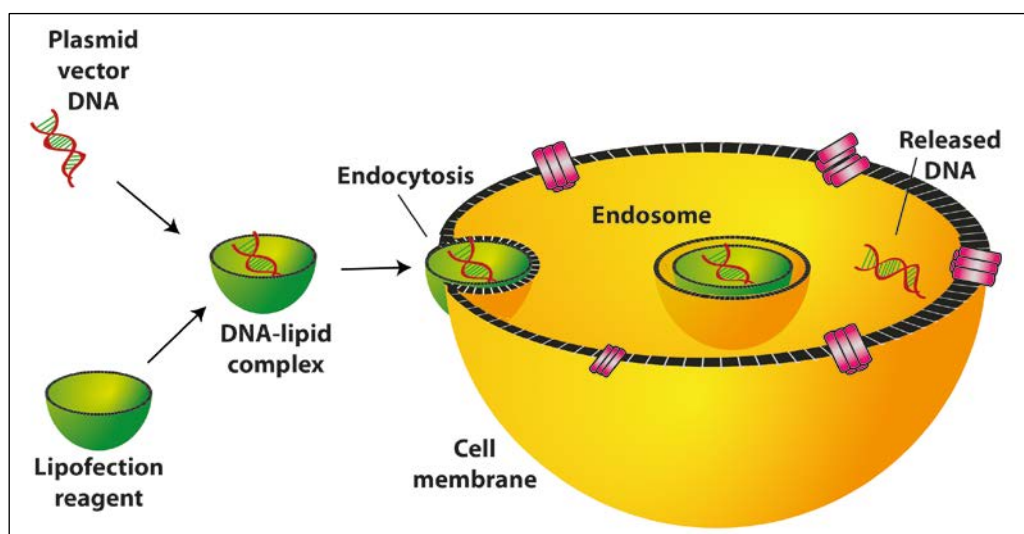

Figure 20: Lipid-mediated transfection. Modified from (235).

4.6.1   Transfection of C2C12 cells

C2C12 cells were trypsinated and seeded at 6 x 10^4 cells/well (12-well plate) in antibiotic free growth medium (20 % FCS ÷PS). The next morning the growth medium was removed from the cells, and 1 ml antibiotic free differentiation medium (2 % HS medium) was added. 2 µg DNA was mixed with 100 µl Opti-MEM I. 4 µl Lipofectamine2000 reagent was mixed with 100 µl Opti-MEM I and incubated for 5 minutes at room temperature. The diluted DNA and the diluted Lipofectamine2000 reagent were combined and mixed gently, and incubated for 20 minutes at room temperature. The DNA- Lipofectamine2000 transfection mix was spread above the cells (200 µl for each well), the plate was mixed gently, and then placed in the incubator. After 6 hours, the medium was replaced with differentiation medium (2% horse serum) containing antibiotics. For other plate-sizes see Table 6 for settings.

Adding of ligands

Cells were cultured in the presence of activators of PPARs (synthetic agonists and FAs) to determine how these agents alone or in combination altered the expression of *Plin5*. In all experiments, ligands were added 24 hours prior to harvesting.

Table 6: Transfection settings.

Dish size (# wells)	Cells (each well)	Medium (each well)	DNA (µg)	Lipofectamine 2000 (µl)	OptiMem (µl)
6	$12 \cdot 10^4$	2 ml	4	8	200
12	$6 \cdot 10^4$	1 ml	2	4	100
24	$3 \cdot 10^4$	0.5 ml	1	2	50
96 (reporter)	$5 \cdot 10^3$	75 µl	Rv: 100 ng Ic: 10 ng Ex.p: 2x 20 ng Total: 170 ng	0.5	10

Rv: reporter vector. Ic: internal control. Ex.p: Expression plasmid.

4.7 Dual Luciferase measurement

The enzyme *Firefly (*Photinus pyralis) luciferase catalyzes a two-step chemical reaction where the substrate luciferin and adenosine triphosphate (ATP) generates luciferyl adenylate, which together with oxygen results in oxyluciferin, adenosine diphosphate (ADP) and most importantly in this context – light emission (Figure 21). This principle is taken advantage of in biological research, where cells can be transfected with a construct containing the *Firefly* luciferase gene under the control of a promoter of interest. The amount of Luciferace produced and the measured light emitted will depend on the activity of the promoter, where changes in the luciferase activity is correleated with transcriptional activity of the promoter of interest.
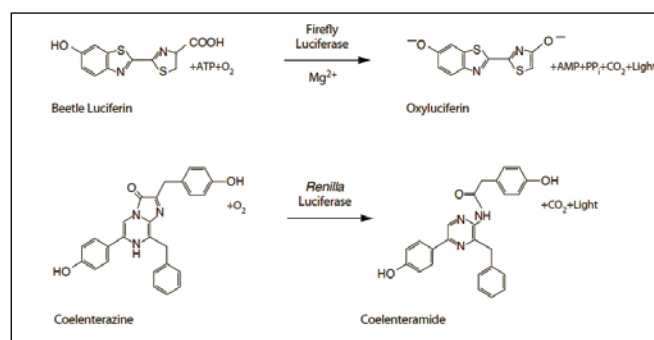
Figure 21: Firefly luciferase reaction and *Renilla* luciferase reaction. Adopted from Promega (Appendix A, Protocol #6).

Dual Luciferase measurement refers to the simultaneously expression of two luciferase enzymes in each cell sample – the main reporter *Firefly* and the co-reporter *Renilla* (Renilla reniformis) (Figure 22). The *Renilla* luciferase signal is used as an internal control to correct for experimental variance among samples (236). The use of *Renilla* as an internal control

depends on that expression of the *Renilla* luciferase is unaffected with the treatments used in the experiment. This must be experimentally determined for each cell line and experimental settings.

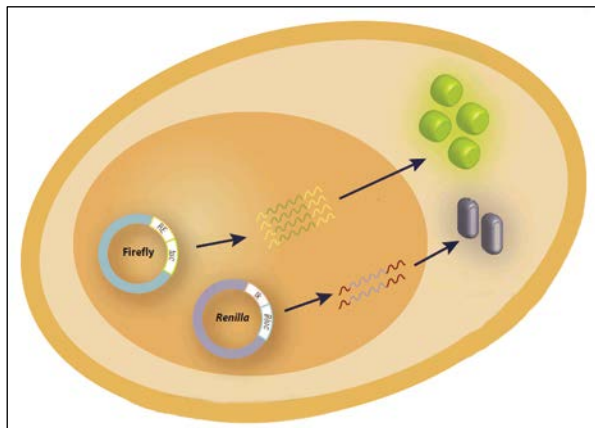**Figure 22: Dual luciferase principle. Adopted from Promega (Appendix A, Protocol #6).**

The Dual-Luciferase® Reporter Assay System (Promega) was used in this thesis (see Appendix A Protocol #6). The assay provides a fast and accurate method to determine the activity of the promoter of interest. To measure the luciferase reporter activity, Luciferase Assay Reagent II (LAR II) is added to the wells (by the Synergy2 machine) to generate a "glow-type" luminescent signal to measure the *Firefly* luciferase reporter. After measuring the *Firefly* luciferase reporter activity in all wells, the reaction is quenched by adding the Stop & Glo® Reagent. At the same time the *Renilla* luciferase reaction is initiated, and its reporter activity is measured.

Table 7: Reagents used for dual Luciferase measurement on Synergy 2.

Reagent	LARII	Stop&Glo	Lysis (PLB)
Volume (µl/well)	50	50	20
Extra volume	3 ml	3 ml	+ 10 %

Experimental procedure:

C2C12 cells were seeded in a 96-well plate and transfected, grown for 3 days, and ligands were added an additional 24 hours prior to harvesting. Cells were washed with 80 µl PBS, and lysed with 20 µl Passive Lysis Buffer (PLB). The culture plate was shaked for 15 minutes in room temperature to ensure efficient lysing of cells. Luciferase assay buffer II (LAR II) and Stop&Glo buffer was thawed, and mixed with Luciferase assay substrate and 50x Stop&Glo

substrate, respectively. Measurements were performed at the Synergy 2 machine (BioTek) with the Gen5 software and reagents as described in Table 7. Pumps and connectors were washed with ethanol and MilliQ before and after use.

4.8 Total RNA isolation of C12C12 cells

To be able to quantify changes in RNA levels in the C2C12 cells after transfection or stimulations, the RNA first have to be isolated. This is a critical step in many molecular biology experiments, including quantitative Real-Time PCR (qRT-PCR), as the RNA must be pure, intact and of high quality.

C2C12 cells differentiated into myotubes were harvested, and cells were lysed with 500 µl 1x Lysis buffer (2x Nucleic Acid Purification Lysis Solution mixed 1:1 with calcium and magnesium free PBS) per well (12-well plate). This buffer lyses the cells without physical disruption or enzymatic digestion, and it inactivates cellular RNases and separates the total RNA from genomic DNA and other cellular fragments. The RNA from the cell samples was isolated using the AB 6100 machine and the preprogrammed "RNA Cell" method described in Appendix A, protocol #4. The instrument was set up with a splashguard in the waste compartment, a 96-well archive plate in the collection compartment, and a Total RNA Purification tray in the carriage. The "RNA Cell" method was run as described in the protocol, except that cells were pre-wet with 80 µl Wash buffer I. After elution, the RNA was immediately put on ice to ensure minimal degradation. Each sample was mixed to make a homogenous RNA solution by pipetting up and down with a ~100 µl pipette prior to measurement of the RNA consentration.

4.9 cDNA synthesis by reverse transcription

After isolation of total RNA from the C2C12 cells, the next step before qRT-PCR is to convert this RNA to complementary DNA (cDNA). The total RNA was reverse transcribed into single stranded cDNA using High-Capacity cDNA Reverse Transcription kit (ABI).

Experimental procedure:

Multi-scribe enzyme was placed in mini-cooler (-20 ºC). RNA samples and the other reverse transcription (RT) Master Mix (M-Mix) components were thawed on ice. Total RNA samples

(250-600 ng) were diluted to 10 µl total volume using PCR water. The 2x RT master-mix
(recipe for one well/reaction) was prepared as following:

Table 8: Reagents used in each cDNA sample.

Reagent	Volume (µl)
RNase free H_2O	4.2
10X RT-buffer	2
10X Random Primers	2
dNTP	0.8
Multiscribe™ RT enzyme	1
Total volume	10

10 µl master-Mix and 10 µl total RNA solutions were pipetted to each well (gives a total of
20 µl reaction volume). To control for DNA contaminants (RNA quality), a negative cDNA
reaction was prepared. This contained 10 µl RNA solution from a pooled sample consisting of
4 µl from each quadruplicate, and all the reagents except for the Multiscribe RT enzyme. In
addition a control sample containing 10 µl PCR-water instead of RNA solution was made to
verify that the master-Mix was not contaminated with RNA. After pipetting, plates were
sealed with strips, vortexed, and spun down. cDNA synthesis was run on a regular PCR
machine using the settings below (Table 9).

Table 9: cDNA run setup.

Reaction	Time	Temperature
Annealing	10 min	25ºC
Reverse Transcription	120 min	37ºC
Enzyme inactivation	5 sec	95ºC

After cDNA synthesis, each cDNA reaction was diluted 5 times with water to get a larger
volume to pipette during setup of the qRT-PCR assay. Samples were stored at -20ºC.

4.10 Quantitative Real-Time PCR (qRT-PCR)

The next step was to quantify the amount of different genes such as *Plin5*. With all RNAs
copied into cDNAs, an indirect measurement of mRNA levels in the cells could be performed.
Quantitative Real-Time PCR (qRT-PCR) is a technique based on PCR, used to amplify and at
the same time quantify a targeted DNA molecule. In this project TaqMan® Gene Expression
Assays were used (Appendix A, protocol #8). In addition to the standard components used in
regular PCR, an oligonucleotide probe complementary to the target sequence of interest is

added. The probe contains a reporter dye at the 5´ end and a non-fluorescent quencher at its 3´ end. The probe anneals during extension, and the close proximity of the quencher to the fluorescent reporter represses fluorescence in the intact probe. The quencher and fluorescent reporter is separated when the Taq polymerase synthesizes the new strand, as its 5′ to 3′ nuclease activity cleaves the probe. This results in reporter fluorescence, as the reporter dye and quencher dyes are physically separated (see Figure 23).

By monitoring the increase in fluorescence of the reporter dye, accumulation of PCR products is detected directly, as the increase in signal is directly proportional to the number of molecules released during that cycle.

The detected fluorescence values are recorded during every cycle. The more template present at the start of the reaction, the less number of cycles it will take to reach a point where the signal is first recorded as statistically significant above background (237). This is the Ct-point, which always occurs during the exponential phase of amplification.
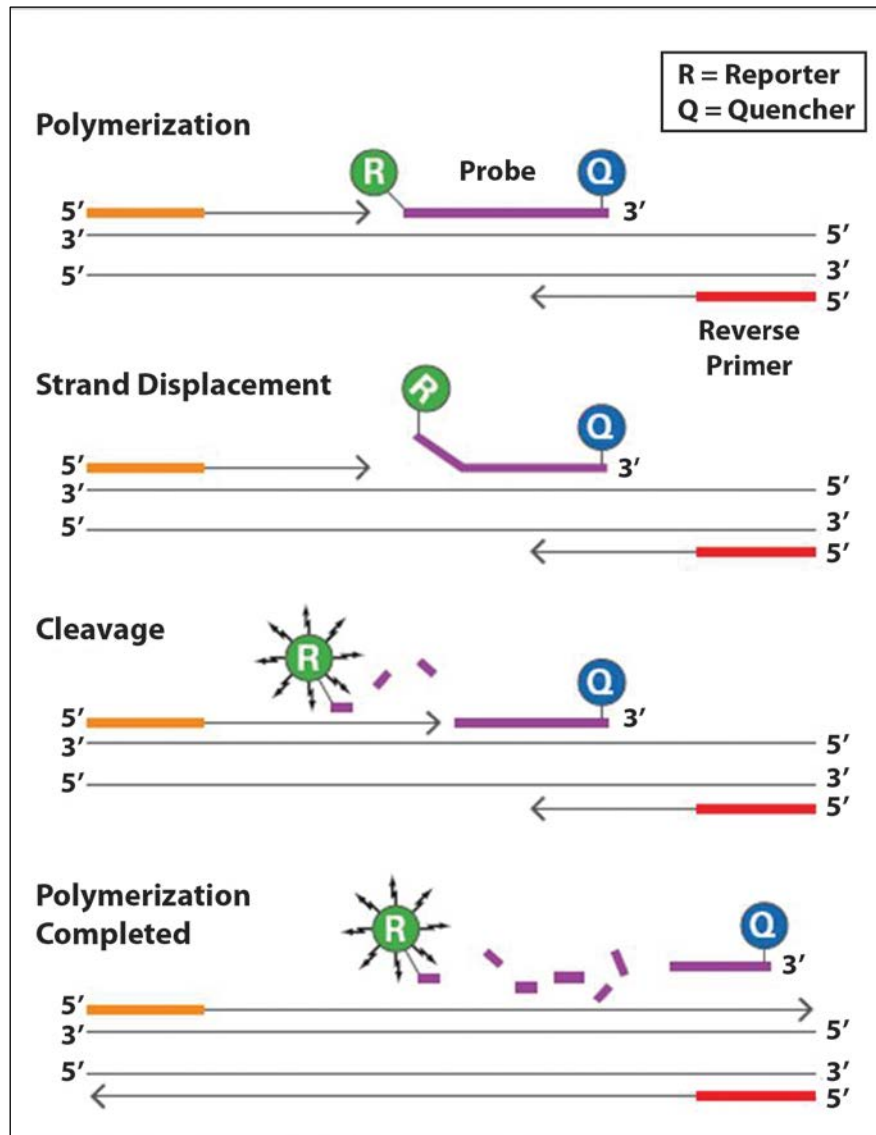
Figure 23: Principle of real time PCR by using TaqMan assay. Adopted from Appendix A, protocol #**8**.

Experimental procedure:

Real-time quantification of RNA (qRT-PCR) was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The following reagents were mixed and pipetted into selected wells on ABI Prism® 96-Well Optical Reaction Plate (15 µl/well):

Table 10: Reagents used in each qRT-PCR sample.

Reagents	Volume (µl)
2x TaqMan® Universal PCR Master Mix	10
Taqman® Gene Expression Assay	1
PCR-water	4
Total volume	15

In addition to measuring mRNA of *Plin5*, 36B4 were used as a negative control in all experiments to control for contaminants. TaqMan® Gene Expression Assays used in the experiments were as following:

Table 11: Taqman® Gene Expression Assays.

Gene name	Assay ID
36B4(Arbp)	Mm00725448_s1
Plin5	Mm00508852_m1
PPARa	Mm00440939_m1
PGC1a	Mm01208835_m1
PGC1b	Mm00504720_m1

5 µl diluted cDNA (2.5-6 ng/µl) was added to each well, and negative controls (water-samples with regular Master-mix) without cDNA were used to control the Master-mix. To control for DNA contaminants in the RNA samples, a negative cDNA control containing all reagents except the RT-enzyme was used. The top of the plate was covered by firmly putting on an ABI Prism® Optical Adhesive sealing. The plate was then vortexed and spun at 1000 rpm for 1 min and placed in the ABI 7900HT machine.

The run was set up using the SDS 2.3 Software from ABI, using standard thermal cycling conditions (Table 12) with 20 µl reaction volume and 40 cycles. After the run the results were analyzed in RQ Manager (ABI) and exported to Microsoft Office Excel 2007.

Table 12: Thermal cycling for qRT-PCR.

Initial step	PCR step (40 cycles)	
AmpliTaq® Gold DNA Polymerase Activation	Melt (denature) DNA	Anneal/Elongate
10 min at 95 °C	15 sec at 95ºC	1 min at 60ºC

4.11 Statistical analyses

Results are presented as mean ± standard deviation (SD) and are representative for one to three individual experiments. The qRT-PCR experiments were performed in triplicates or quadruplicates. Dual luciferase reporter experiments were performed in triplicates. To examine statistical significant differences between samples, the parametric t-test was performed. The Bonferroni correction for multiple comparisons was used to adjust p-values. Statistical significance was set at the 5 % ($P < 0.05$) and the 1 % ($P < 0.01$) level. Careful interpretation of the results is required due to the low number of samples. SPSS version 18.0 was used for statistical analysis.

5 Results

5.1 PPARα is an important transcription factor regulating expression of *Plin5*

To search for transcription factors being important for regulating of *Plin5* expression in muscle cells, we transiently expressed different transcription factors known to stimulate conversion of glycolytic to oxidative muscle types *in vivo* (58;64;238;239). C2C12 cells were transiently transfected to ectopically express PPARs, RXRs, ERRs and PGC1s and then subjected for myoblast to myotube differentiation for 72 hours (Figure 24). The relative levels of *Plin5* mRNA were subsequently measured by qRT-PCR analysis. A maximum 100-fold increase in relative *Plin5* mRNA was measured in cells transfected with the PPARα expression vector ($p < 0.05$). A significant increase in relative *Plin5* mNRA level was also observed for PGC1α ($P < 0.05$) and PGC1β ($P < 0.05$). Transfection with other PPARs tended to increase *Plin5* mRNA levels (ranging from 1-20 fold difference), but these inductions were evaluated as nonsignificant. Transfection with RXRs or ERRs had no affect on the expression of *Plin5* mRNA.

In addition to measure the level of *Plin5* mRNA, the levels of PPARα, PGC1α and PGC1β mRNAs were also measured to see if transfection with these transcriptional factors influenced the expression levels of other transcriptional factors affecting *Plin5* mRNA levels (Figure 24B, C and D respectively). A marginal increase in PPARα mRNA levels was observed in cells ectopically expressing PGC1α in the C2C12 cells (Figure 24B). Ectopic expression of ERRβ lowered the levels of PGC1β mRNA (Figure 24D).
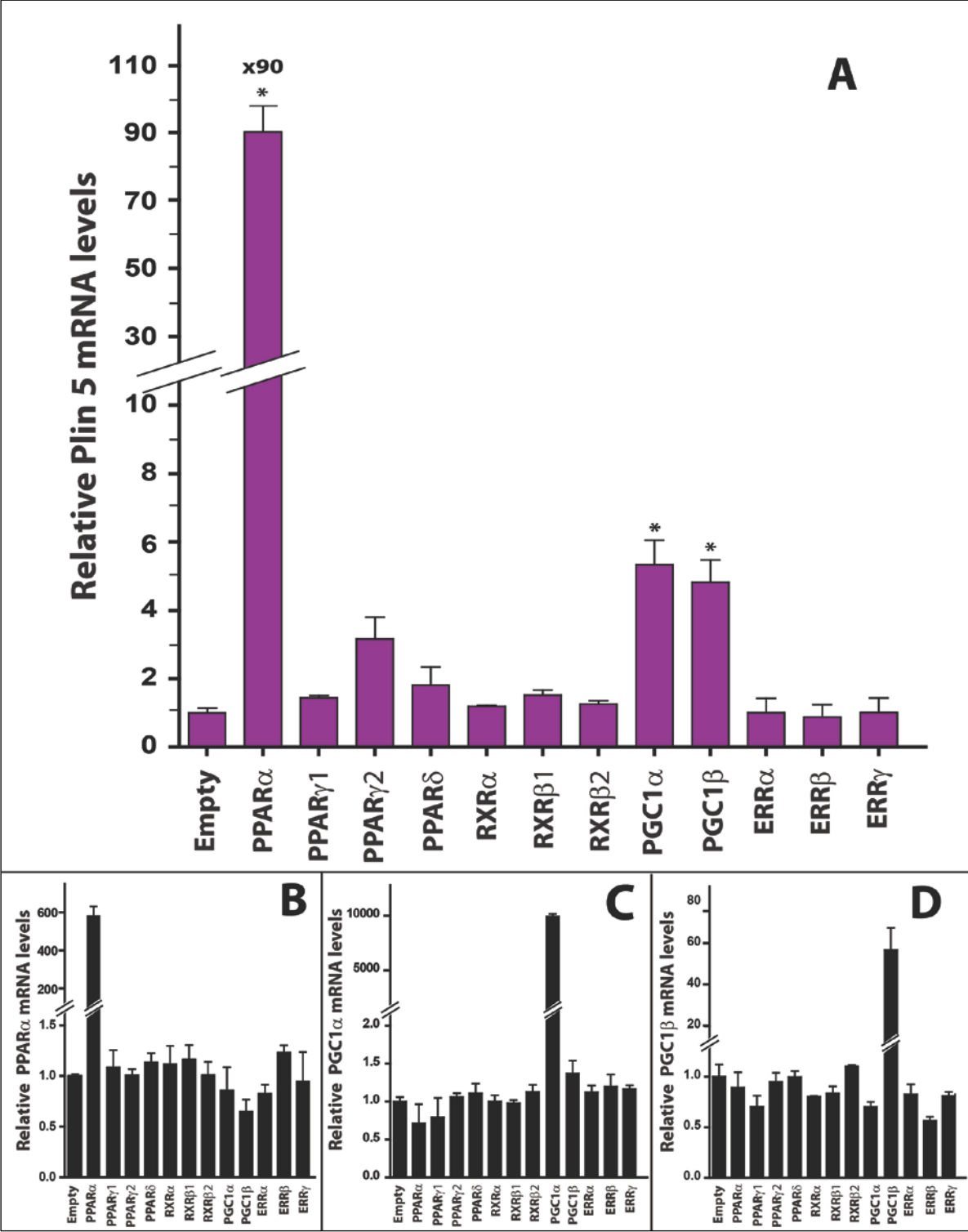
Figure 24: Relative mRNA expression of *Plin5*, PPARα, PGC1α and PGC1β in C2C12 cells ectopically expressing different NRs compared to the endogenous control 36B4. C2C12 cells were transfected with control vector (empty vector, pcDNA3) or pcDNA3-vectors expressing the indicated transcriptional factors. Myotube differentiation was initiated at start of transfections, and cells differentiated for three days before harvested. A: *Plin5* mRNA. B: PPARα mRNA. C: PGC1α mRNA. D: PGC1β mRNA. Data is presented as Mean±SD (n=3, * P < 0.05). The experiment was run in quadruplicates, and repeated three times with similar results. One representative of three experiments is shown. PPARα; peroxisome

proliferator-activated receptor α, PPARγ1; peroxisome proliferator-activated receptor γ1, PPARγ2; peroxisome proliferator-activated receptor γ2, PPARδ; peroxisome proliferator-activated receptor δ, RXRα;retinoid receptor x α, RXRβ1; retinoid receptor x β1, RXRβ2, retinoid receptor x β2, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1β; peroxisome proliferator-activated receptor gamma coactivator 1 β, ERRα; estrogen-related receptor α, ERRβ; estrogen-related receptor β, ERRγ; estrogen-related receptor γ.

5.2 Ligand stimulation of PPARδ results in a cell-line expressing *Plin5*

Based on the previous results, it seems that PPARα is the most important regulator of *Plin5* among the tested TFs. But we know from earlier work that PPARδ is an important regulator of *Plin5* expression (Berg et al, manuscript submitted). To compare the potency of PPARα and PPARδ to stimulate *Plin5* mRNA levels, C2C12 cells were transiently transfected to ectopically express PPARα or PPARδ and subsequently given selective ligands activating these PPARs. The cells were transfected, differentiated for three days, and stimulated with selective PPARα (WY-14643; 10 μM) or PPARδ (GW501516; 0.1 μM) activators for an additional 24 hours. The mRNA levels for *Plin5* were thereafter measured by qRT-PCR (Figure 25). The relative *Plin5* mRNA level increased significantly with ectopic expression of PPARα (30-fold; $P < 0.01$), ectopic expression and ligand activation of PPARα (120-fold; $P < 0.01$), or ectopic-expression and ligand activation of PPARδ (70-fold; $P < 0.05$). Interestingly, ectopic expression and ligand activation of either PPARα or PPARδ had comparable stimulating effects on *Plin5* mRNA levels. PPARδ is expressed at higher levels than PPARα in C2C12 cells (Berg et al, manuscript submitted). These results suggest that cultured C2C12 cells lack a potent ligand activating PPARδ, and that stimulating the C2C12 cells with a PPARδ-agonist results in a cell-line expressing *Plin5*. Similar results were also found when performing a reporter-assay (see Appendix B figure # 1).
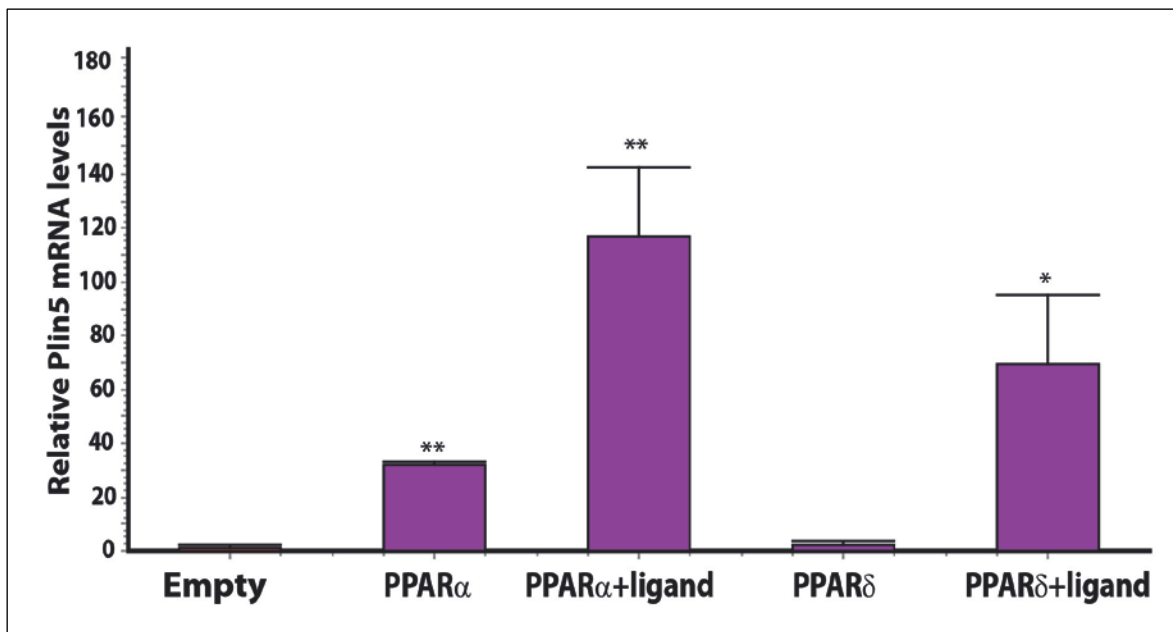
Figure 25: Relative mRNA levels of *Plin5* in C2C12 cells ectopically expressing PPARα or PPARδ and stimulated with selective ligands. C2C12 cells were transfected with control (empty vector, pcDNA3) or pcDNA3-PPARα or pcDNA3-PPARδ expression vectors at day 0 and differentiated for three days. The cells were subsequently incubated in medium containing DMSO (vehicle) or synthetic ligands activating PPARα (WY-14643;10 μM) or PPARδ (GW401516; 0.1 μM) for 24 hours. Expression of 36B4 was used as an endogenous control. The experiment was run in triplicates and performed once. Results are presented as Mean±SD. (n=3; * P < 0.05, ** P < 0.01). PPARα; peroxisome proliferator-activated receptor α, PPARδ; peroxisome proliferator-activated receptor δ.

5.3 The effect of PPARα and PPARδ on *Plin5* expression increases with time

Our results show that both PPARα and PPARδ may induce expression of *Plin5*. However, the former experiments lasted four days, and thus it is not possible to determine if the stimulation is directly or indirectly mediated. To test the responsiveness to PPARδ activation over time, cells were stimulated with vehicle or a PPARδ agonist (GW501516; 0.1 µM) at initiation of differentiation and harvested at different time points until day 3. Activation of PPARδ had no effect on *Plin5* mRNA levels the first 12 hours (Figure 26), and a significant difference in *Plin5* mRNA levels were first observed after 48 hours (7-fold; $P < 0.05$) and after 72 hours (11-fold; $P < 0.001$) of ligand activation.
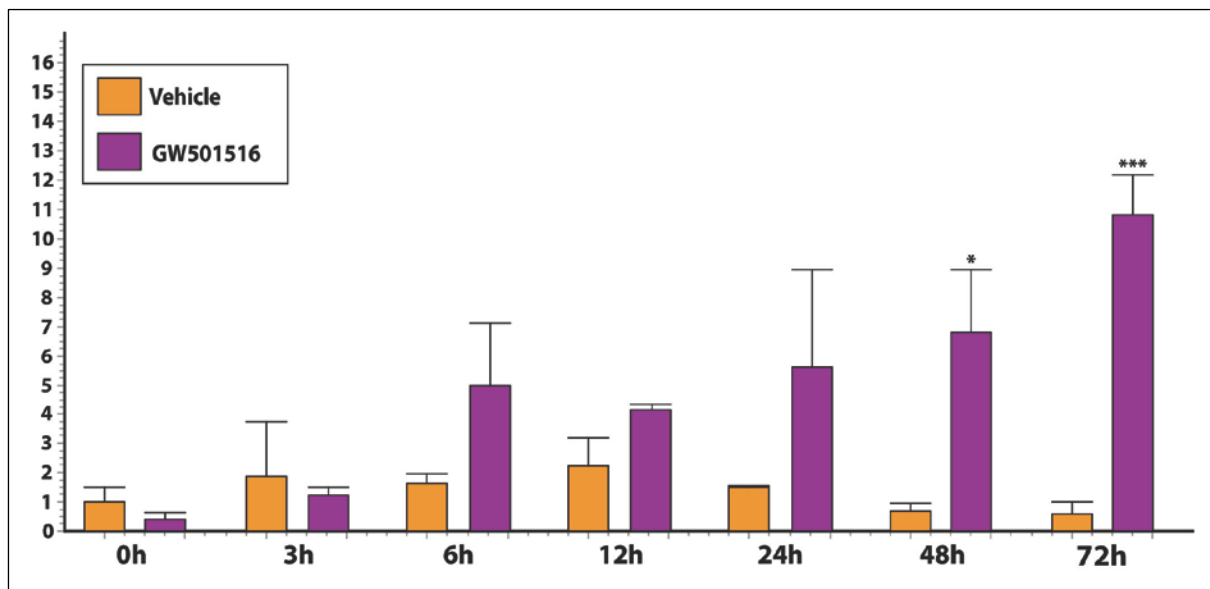
Figure 26: **Relative mRNA levels of *Plin5* in C2C12 cells stimulated with a PPARδ-activator and harvested at different time points. C2C12 cells were stimulated with vehicle (DMSO) or a PPARδ-ligand (GW-501516; 0.1µM) at day 0 and differentiated for up to three days. The cells were harvested at initiation of differentiation (0 h) or after stimulation for 3, 6, 12, 24, 48 and 72 hours respectively. 36B4 was used as an endogenous control when evaluating the qRT-PCR measurements. Results are presented as Mean±SD. (N=4; * P < 0.05, *** P < 0.001). The experiment was performed once.**

To determine how fast activation PPARα affects *Plin5* mRNA, we had to use an alternative approach. PPARα is expressed at low levels in C2C12 cells, and stimulation with a PPARα activator only result in a minor increase in *Plin5* mRNA levels in C2C12 cells (Berg et al, manuscript submitted, and see Figure 28 A). C2C12 cells were transiently transfected to overexpress PPARα (Figure 27) and harvested at different time point during differentiation.

The relative levels of *Plin5* mRNA were subsequently measured by qRT-PCR. The relative *Plin5* mRNA level in PPARα expressing cells compared to the control cells was significantly elevated after 12 hours (3-fold; $P < 0.05$), 24 hours (5-fold; $P < 0.05$) and after 72 hours (7-fold; $P < 0.05$) (Figure 27 A). The relative PPARα signals were comparable at all time points from 3 hours after transfection, demonstrating that the transfected vector was present at equal levels (Figure 27 B). In cells not ectopically expressing PPARα (control cells), the relative level of PPARα mRNA increased gradually with time, demonstrating that the level of PPARα increase during differentiation of muscle cells. However, the level of *Plin5* did not change during differentiation. The relative level of PGC1α increased with differentiation to a maximum ~8-fold increase after 72 hours (Figure 27 C). The expression level seemed to be unaffected by ectopic expression of PPARα, but there was a tendency that the relative level of PGC1α was reduced in the cells with ectopic expression of PPARα. The relative level of PGC1β mRNA seemed less influenced by differentiation, with a 2-fold increase in control cells after 72 hours of differentiation (Figure 27 D). Ectopic expression of PPARα did not seem to influence the expression of PGC1β, but like the observation for PGC1α there was a tendency of reduced expression of PGC1β in cells with ectopic expression of PPARα.
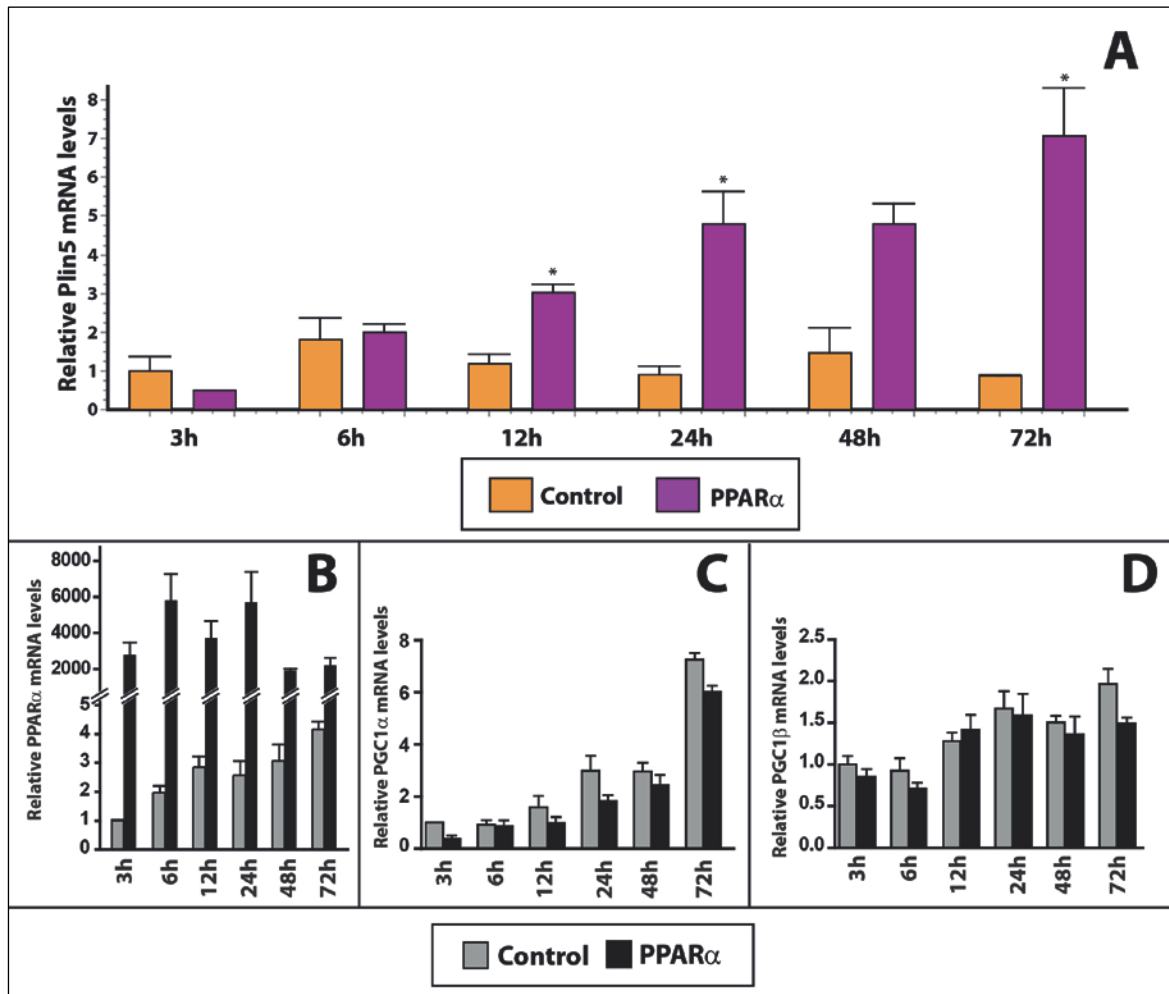
Figure 27: Relative mRNA expression of *Plin5*, PPARα, PGC1α and PGC1β in C2C12 cells overexpressed with PPARα harvested at different times. C2C12 cells were transfected with control (empty vector, pcDNA3) or pcDNA3 inserted with PPARα expression vector at day 0. The cells were harvested after 3, 6, 12, 24, 48 and 72 hours. 36B4 was used as an endogenous control when evaluating the qRT-PCR measurements. The experiment was run in quadruplicates and performed once. A: *Plin5* mRNA. B: PPARα mRNA C: PGC1α mRNA. D: PGC1β mRNA. Results are presented as Mean±SD. (* $P < 0.05$). PPARα; peroxisome proliferator-activated receptor α, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1β; peroxisome proliferator-activated receptor gamma coactivator 1 β.

The results suggest that the ectopic expression of PPARα or ligand activation of PPARδ makes the relative level of *Plin5* mRNA increase with time, suggesting both a direct and a slower indirect stimulation involving other factors in the induction of *Plin5* mRNA in C2C12 cells.

5.4 PGC1β is an important coactivator for expression of *Plin5* in muscle cells

Activation of PPARδ or ectopic expression of PPARα induces *Plin5* mRNA with time. A large number of cellular processes might be altered in the cells during the four days of incubation in the previous experiments. Theoretically, coregulators have time to be procueced and activated and thereby participate in the induction during this timeframe. The initial experiment (Figure 24) showed that ectopic expression of the coregulators PGC1α and PGC1β induced the expression of *Plin5* mRNA. It may be that these coactivators are recruited to PPARs and stimulate transcription of *Plin5* mRNA. In that case the level of *Plin5* mRNA will increase with time. To examine if this could be the case, C2C12 cells were transiently transfected to ectopically express PGC1α or PGC1β (Figure 28). The cells were differentiated for three days and then treated for 24 hours with vehicle (DMSO), PPARα activator (WY-14643;10 μM), PPARδ activator (GW501516; 0.1 μM) PPARγ activator (Rosiglitazone /BRL-49653; 1 μM), bovine serum albumin (BSA; 0.4 mM), or oleic acid (cis-C18:1 n-9) complexed to BSA (BSA-OA; 0,1 μM). After harvested, mRNA levels for *Plin5* were measured by qRT-PCR.

Consistent with the previous experiments, in cells transfected with empty vector stimulation with WY-14643 or GW-501516 increased the relative level of *Plin5* mRNA 10- and 70-fold, respectively (Figure 28 A; $P < 0.05$), confirming the previous shown PPARα and PPARδ regulation of *Plin5*. Incubation with oleic acid, which may act as an ligand for PPARs, induced *Plin5* mRNA levels ~20-fold. In cells ectopically expressing PGC1α, expression of *Plin5* was induced ~20-fold with no stimulation ($P < 0.05$). The effect of WY-14643 with ectopic expression of PGC1α was abolished, while the effect with GW persisted ($P < 0.05$). In addition, stimulation of BRL-49653 increased the relative *Plin5* mRNA level ($P < 0.01$). Ectopic expression of PGC1α resulted in increased response when stimulating the cells with BSA-OA ($P < 0.05$). The expression levels of *Plin5* was most dramatically changed in cells ectopically expressing PGC1β. Expression of *Plin5* was highly induced (~80-fold) by ectopic expression of PGC1β ($P < 0.001$), and addition of the various ligands had little further stimulating effect on *Plin5* mRNA expression levels. The only significant difference of stimulation was when stimulating the cells with BSA-OA compared to BSA, where addition of BSA-OA resulted in reduced expression of *Plin5* mRNA ($P < 0.05$). Ectopic expression of

PGC1α or PGC1β mRNAs did not influence the mRNA levels of each other (Figure 28 B and C). The different Ct-values for endogenous PGC1α (Ct ~32) and PGC1β (Ct ~28) indicate that the expression level of PGC1β is higher (~16 x) than that of PGC1α in the C2C12 cells. The lack of a strong PPAR ligand effect in cells ectopically expressing PGCβ, suggests that this cofactor may normally be recruited to PPARs when they are stimulated with their ligands.
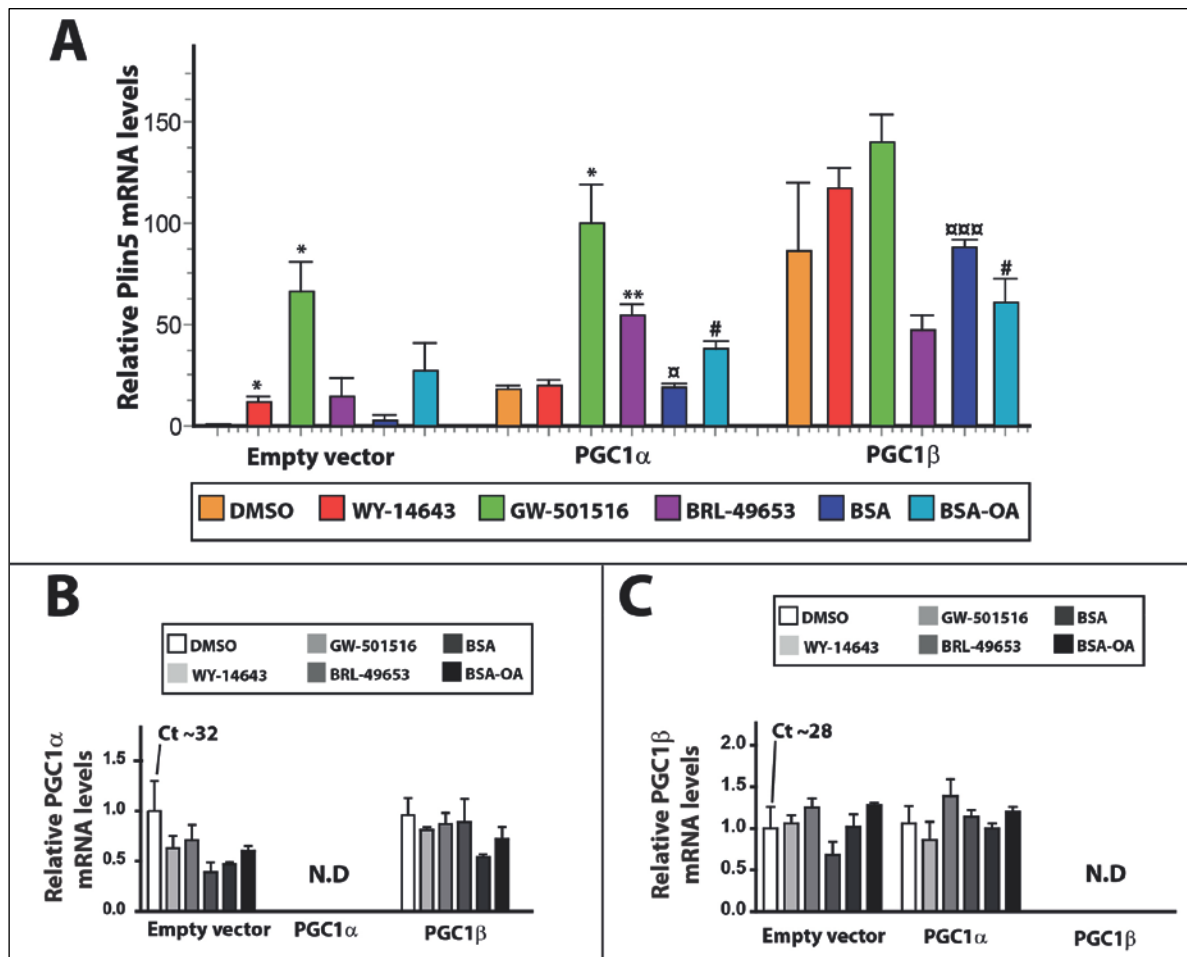

Figure 28: Relative mRNA expression of *Plin5*, PGC1α and PGC1β in C2C12 cells transfected with PGC1s and stimulated with PPAR-ligands. C2C12 cells were transfected with control (empty vector, pcDNA3) or pcDNA3 inserted with PGC1α or PGC1β expression vectors at day 0 and differentiated for three days. The cells were treated with DMSO, selective PPAR-activators, BSA, or oleic acid complexed to BSA for 24 hours. 36B4 was used as an endogenous control when evaluating the qRT-PCR measurements. The experiment was run in quadruplicates and performed once. A: *Plin5* mRNA. B: PGC1α mRNA. C: PGC1β mRNA. Results are presented as Mean±SD. Statistical differences between groups stimulated versus DMSO (* $P < 0.05$), between groups BSA (¤ $P < 0.05$, ¤¤¤ $P < 0.001$), and between BSA-OA and BSA (# $P < 0.05$), for each transfected vector group (empty, PGC1α and PGC1β respectively). PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1β; peroxisome proliferator-activated receptor gamma coactivator 1 β, N.D; Not determined. Activators of PPARα (WY-14643; 10 μM), PPARδ (GW401516; 0.1 μM), PPARγ (Rosiglitazone/BRL-49653; Rosi; 1 μM). BSA; bovine serum albumin (0,4 μM), BSA-OA; oleic acid (cis-C18:1 n-9; 0,1 μM) complexed to BSA (0,4μM.).

5.5 PPARs bind to a conserved PPRE located in *Plin5* intron 1

Earlier work by a master student suggested that the *Plin5* promoter contains a DR1-type PPAR responsive element (PPRE) located -2077 to -2064 bp upstream of the transcriptional start site. Reporter assay experiments performed in collaboration with Christina Steppeler, as teaching experiments, failed to validate these earlier observations. In our experiments, different *Plin5* Luciferase reporter constructs (full-length *Plin5* reporter [-2344 to +244], deletion constructs [-1329 to +244] and [-878 +244], or a construct with mutated PPRE) (Figure 29) were transiently transfected into C2C12 cells and co-transfected with empty vector, RXRα, or RXRα and PPARδ expression plasmids. Two days after transfection and initiation of differentiation, the medium was replaced with fresh medium containing vehicle (DMSO) or a PPARδ agonist (GW501516; 0.1 µM). There was essentially no difference in the transcriptional activity of the various transfected *Plin5* reporters (Figure 30). This implies that PPARδ regulates the expression of *Plin5* through a different PPRE, in a different region of the promoter than concluded earlier. The same effect was observed when the same experiment was performed with PPARα instead of PPARδ (Appendix B figure # 2). Conservation of the PPAR activating effect in the shortest reporter construct [-878 +244] , suggests that the element is located within this region.
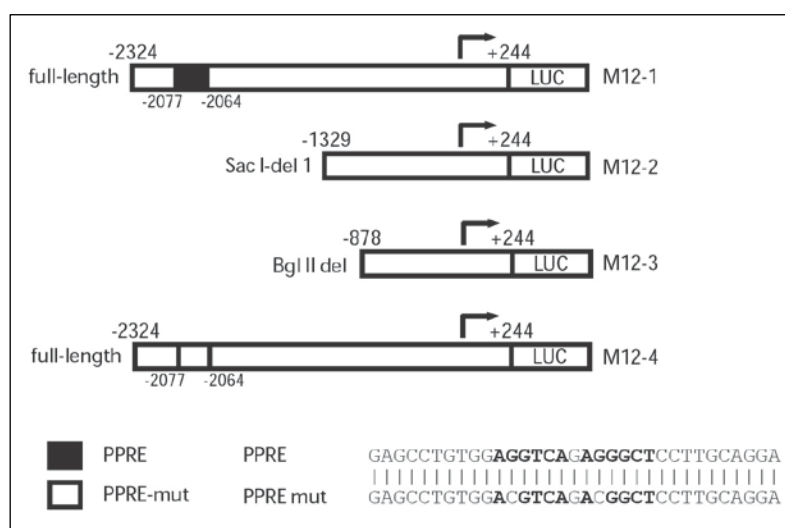
Figure 29: The *Plin5* promoter with the hypothesized DR-1 type PPRE. Schematic drawing of the Plin5 reporter constructs with the DR-1 -2077 to -2064 bp upstream for the *Plin5* promoter. The hypothesized functional DR-1 element (black rectangles), point-mutated DR-1 (white rectangles), bent arrow indicates transcription start. M12-1; full-length construct, M12-2; Sac-I deletion 1construct, M12-3; BgI II deletion construct, M12-4; mutated construct, LUC; luciferase, mut; mutated. Figure by Knut Tomas Dalen.

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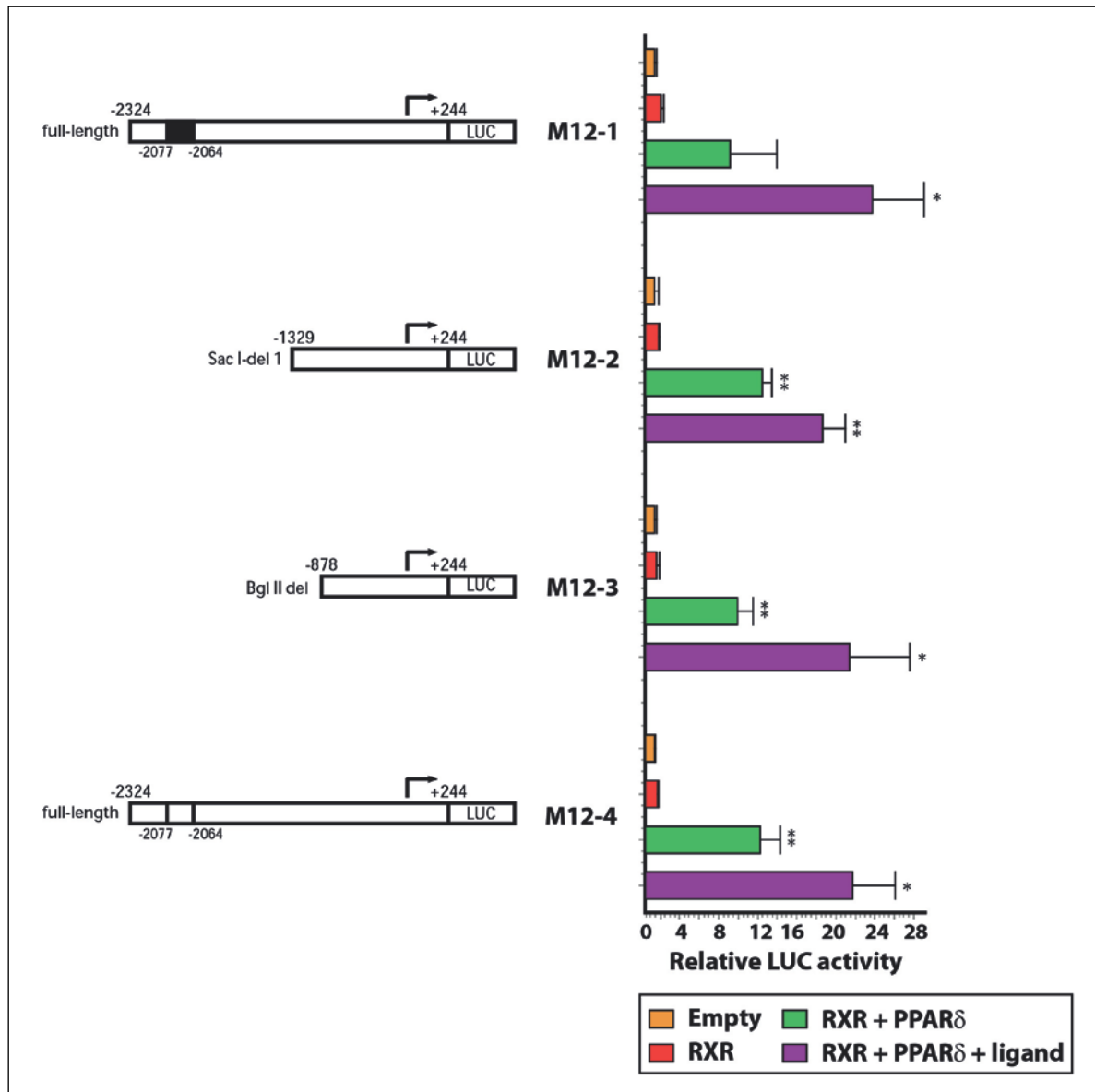
Figure 30: Transient transfection with full-length (M12-1), deletion (M12-2; m-Plin5 SacI deletion and M12-3; m-Plin5 BgI II- deletion) or mutated (M12-4) *Plin5* promoter constructs, co-transfected with no expression plasmid (Empty), RXR, or RXR and PPARδ expression vectors. Cells were stimulated with the vehicle (DMSO) or the PPARδ-ligand GW-501516 (0.1 μM). The experiment was run in triplicates and performed > 3 times. Results are presented as Mean±SD. Three statistical test were performed within each promoter group (M12-1 – M12-4); empty vector was compared to RXR, RXR+PPARδ, and RXR+PPARδ+ligand. (* $P < 0.05$, ** $P < 0.01$). PARδ; peroxisome proliferator-activated receptor δ, RXR;retinoid receptor x.

We therefore searched for an alternative PPRE in the [-878 +244] promoter region of the *Plin5* gene. With an online nuclear hormone receptor-search engine (NHR), a novel promising DR-1 type PPRE was identified, located downstream of the *Plin5* transcriptional start site at position +139 to +151 (see Figure 31) in intron 1 of the *Plin5* gene. Alignment of

the mouse and human *Plin5* promoter revealed that this DR1 sequence is highly conserved in human (87 % sequence identity).

To be able to test if this element was functionally used by PPARs, the DR-1 element was mutated by site-directed mutagenesis and positive clones sequenced to evaluate the integrity. To determine if the PPRE recruits binding of PPARs to the *Plin5* promoter, the full-length *Plin5* reporter (Plin5-Luc [-2344 to +244]) and the mutated construct (Plin5-Luc [-2344 to +244-DR1 mut]) were transiently transfected into C2C12 cells and co-transfected with empty (control), or RXRα, PPARα, PPARδ and PGC1 expression plasmids. After two days of differentiation the medium was replaced with fresh medium containing vehicle (DMSO), PPARδ agonist (GW501516; 0.1 µM) or PPARα agonist (WY-14643; 10 µM). Cells were lysed and harvested four days after transfection.
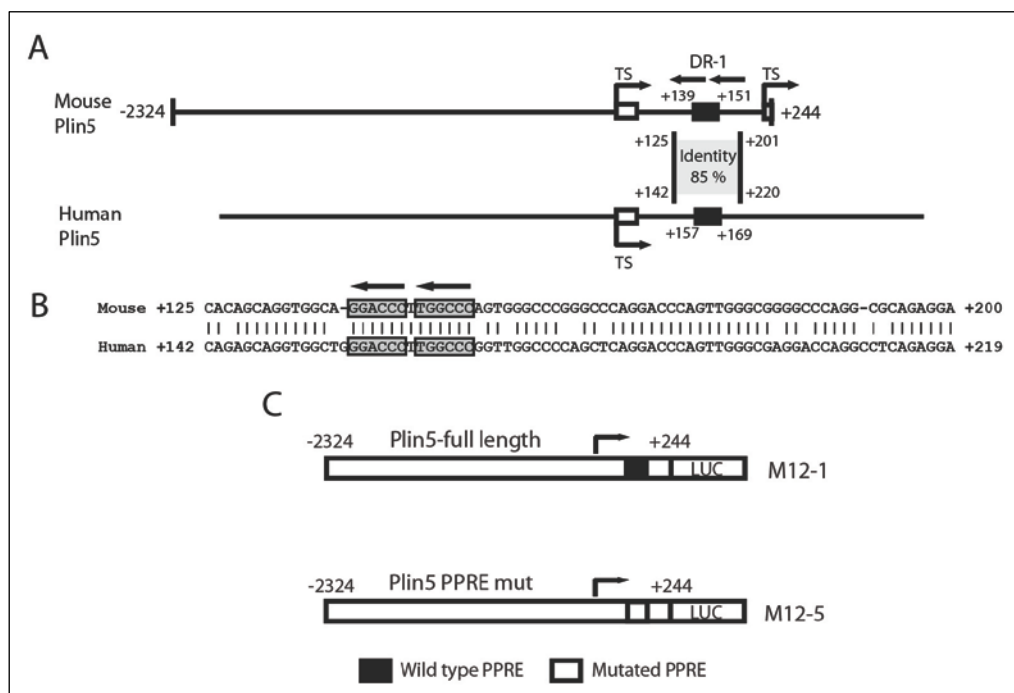
Figure 31: The *Plin5* promoter contains a conserved functional PPRE.
A: A schematic presentation of the cloned mouse *Plin5* reporter (nucleotides -2324 to +244), and the corresponding human *PLIN5* promoter. The two alternative transcriptional start sites (TS) are indicated by arrows. The localization of the conserved intronic region and percentage identity to the human intronic segment is shown. The nucleotide position of the black boxes points out the localization of the PPREs.
B: Sequence alignment and homology between nucleotides in the mouse and the human *PLIN5* intronic regions. The two half sites in the conserved PPRE are encircled by boxes, and conserved nucleotides are indicated by vertical lines.
C: Schematic drawing of the *Plin5* reporter constructs that were transfected into C2C12 cells. Functional PPREs (black rectangles), point-mutated PPREs (white rectangles), bent arrow indicates transcription start. M12-1; full-length construct; M12-5: mutated construct; LUC; luciferase, mut; mutated. Figure by Knut Tomas Dalen (Berg et al, manuscript submitted).

As shown earlier, *Plin5*-full-length reporter activity increased when cells were co-transfected with RXRα and PPARδ (P < 0.05), and when stimulated with the PPARδ-activator GW-501516 (P < 0.01), with a maximal 15-fold increase (Figure 32 A). The same response was observed when cells were co-transfected with RXRα and PPARα and stimulated with the PPARα agonist (WY-14643; 10 μM), with a maximum ~25-fold increase in the relative transcriptional activity of the *Plin5* promoter (Figure 32 B). On the contrary, the *Plin5*-mutated reporter did not respond to co-transfection or ligand stimulation of the PPARs.

Ectopic expression of PGC1s stimulates *Plin5* expression. To determine if this is caused by binding of PGC1 to PPARs on the identified PPRE, *Plin5* full-length reporter (M12-1) and *Plin5* full-length mutated reporter (M12-5) were transfected into C2C12 cells (Figure 33). Cells were co-transfected with control vector (pcDNA3; empty vector), pcDNA3-PPARδ, pcDNA3-PGC1α, or pcDNA3-PGC1β as indicated, and stimulated with vehicle or the PPARδ-activator GW-501516. When the cells were overexpressing PPARδ and were stimulated with GW-501516, overexpression of PGC1α resulted in increased *Plin5* promoter activity (P < 0.05) (Figure 33 A). Overexpression of PGC1β, on the other hand, resulted in decreased *Plin5* promoter activity when the cells were overexpressing PPARδ and were stimulated with GW-501516 (P < 0.001).

Figure 33 B shows the corresponding results for when the cells were transfected with the mutated *Plin5* promoter (M12-5). This resulted in no significant differences in the relative *Plin5* reporter activity.

For both experiments (Figure 32 and Figure 33), the same basal activity was observed for the mutated reporter and the full-length reporter, implying that the mutation did not influence basal transcriptional activity. Overall, the mutated constructs failed to induce *Plin5* reporter activity, thereby confirming that the new identified PPRE is used by both PPARδ and PPARα. Furthermore, the results suggest that the coactivator PGC1α has an enhancing role, while PGC1β has an inhibitory role when it comes to PPARδ-stimulated induction of the *Plin5* promoter.
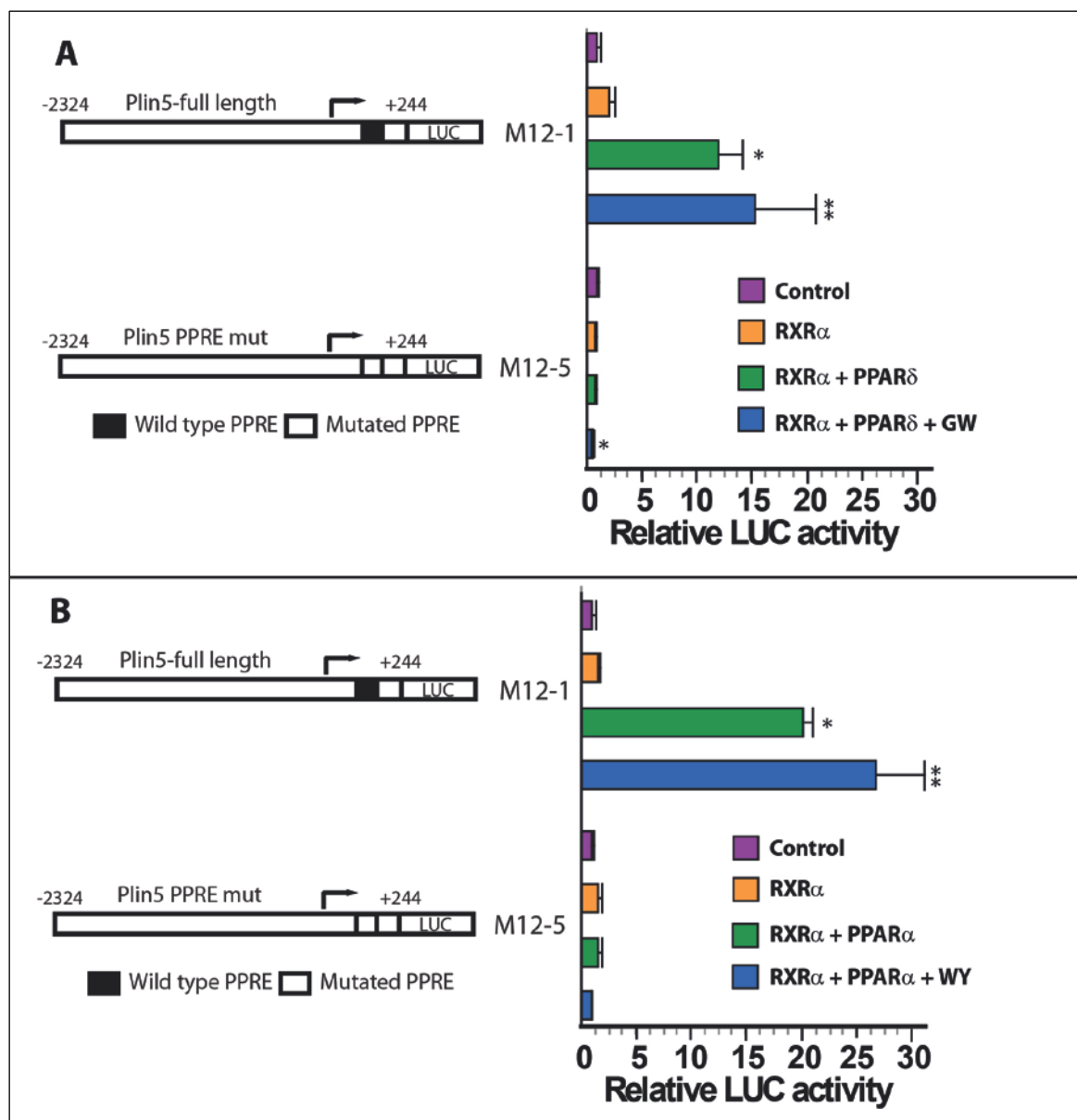
Figure 32: Transient transfection with full-length (M12-1) or mutated (M12-5) *Plin5* **promoter constructs.**
A. C2C12 cells were co-transfected with no expression plasmid (control), RXRα, RXRα and PPARδ expression vectors. Cells were stimulated with the PPARδ-ligand GW501516 (0.1 μM; GW.)
B. C2C12 cells were co-transfected with RXRα and PPARα expression vectors. Cells were stimulated with the PPARα-ligand WY- 14643 (10μM; WY).
The experiment was run in triplicates and one representative of two experiments is shown. Results are presented as Mean±SD. Three statistical test were performed within each promoter group (M12-1 and M12-5); empty vector was compared to RXRα, RXRα and PPARα or PPARδ, and RXRα and PPARα or PPARδ+ligand. (* P < 0.05, ** P < 0.01). RXRα; retinoid receptor x, PARδ; peroxisome proliferator-activated receptor δ.
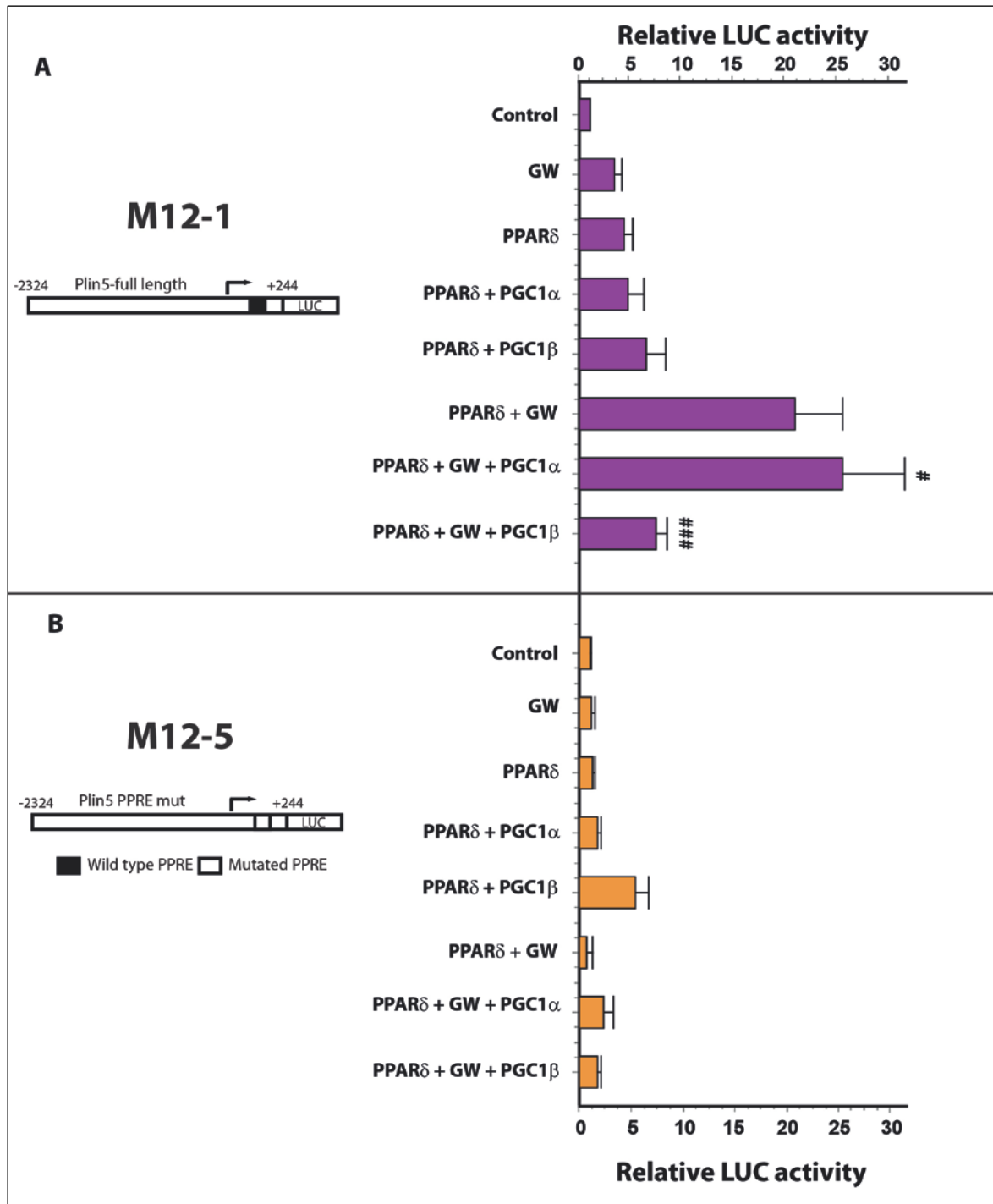
Figure 33: Transient transfection with full-length (A, M12-1) or mutated (B, M12-5) *Plin5* **promoter constructs, co-transfected with no expression plasmid (control), PPARδ, PPARδ and PGC1α, or PPARδ and PGC1β expression vectors. Cells were stimulated with the PPARδ-ligand GW501516 (0.1 μM; GW). The experiment was run in triplicates and one representative of two experiments is shown. Results are presented as Mean±SD. Statistical differences between PPARδ+GW and PPARδ+GW+PGC1α or PPARδ+GW+PGC1β. (# P < 0.05, ### P < 0.001). PARδ; peroxisome proliferator-activated receptor δ, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1β; peroxisome proliferator-activated receptor gamma coactivator 1β.**

6 Discussion

The main objective with this thesis was to investigate the transcriptional regulation of perilipin 5, which is expressed in tissues with active fatty acid oxidation such as heart, striated muscles, brown adipose tissue, and the liver. C2C12 cells were transfected with different transcription factors, differentiated into myotubes, and harvested for RNA isolation. RNA was isolated from the cell extract, and the relative amount of perilipin 5 and other relevant mRNAs in the cell samples were measured by reverse transcribing mRNA into cDNA. The cDNA abundance was analyzed by quantitative RT-PCR using TaqMan probes. The results show that PPARα and PPARδ are key players in the regulation of perilipin 5. A search for a PPRE in the *Plin5* promoter resulted in identification of a potential PPRE in the *Plin5* promoter. Luciferace reporter-assay comparing the full-length *Plin5* reporter with a reporter containing a mutated PPRE, confirmed that the PPRE discovered can be used by the PPARs to regulate the *Plin5* gene.

6.1 Methodology

Cell-line and cell culturing

The expression of perilipin 5 is previously found to be higher in oxidative, high-fat, type I fibers, compared to the glycolytic, low-fat type II-fibers (171;216;218;240;241). Cultured myotubes resemble type II fibers (242), whereas human muscles *in vivo* consist of both type I and type II fibers (243;244). To study the regulation of perilipin 5 *in vitro,* the ideal cells would be oxidative and express perilipin 5. The C2C12 cells (ATCC, #CRL-1772) use glucose as energy source under standard culturing conditions, and perilipin 5 is expressed at low levels. It might be that a transcription factor that makes the cells express perilipin 5, could also function to switch cultured cells from glycolytic (type II) to oxidative (type I). Protocols for culturing, passaging, differentiation and transfection of C2C12 cells were already established in our laboratory.

Transfection

Expression vectors that were transfected (overexpressed) in this thesis were PPARs, RXRs, PGC1s and ERRs, and also full-length and PPRE-mutated *Plin5* reporters. The level of

transcription factors differ among cell lines, and the C2C12 cells express the different PGC1s and PPARs at variable levels. In my experiments, the Ct-values for PGC1α and PGC1β imply that the cells express more PGC1β than PGC1α. Among the PPARs, PPARδ is more abundant than PPARα (Berg et al, manuscript submitted). Transfected DNA is not genomic, and therefore not subjected to (processes like) regulation of access to DNA by histone modifications. Thereby an observed response after transfection is likely due to expression of the transfected DNA.

To test if the transfected vectors regulated each other, TaqMan assays for the transfected expression vectors were also performed. The coactivators PGC1α and PGC1β did not seem to influence each other. PPARα was marginally increased by overexpression of PGC1α, while the level of PGC1α and PGC1β was unaffected or slightly reduced by overexpression of PPARα. However, the response of interest in this thesis was the amount of *Plin5*, which in the C2C12 cells will only be present as a result of transcriptional regulation processes.

Generally factors affecting transfection efficiency, apart from the method itself, are cell health, cell type, and DNA quality and quantity. The C2C12 cells used in the experiments were grown in appropriated medium with all necessary factors, and they were incubated at 37ºC under 5 % CO_2 and 100 % relative humidity. They were also maintained in exponential growth phase, and transfected at ~70-80 % confluency. The right percentage of confluency is important. If there are too many cells, it will be too "crowded" for the cells to divide (mitosis). The plasmid-DNA used was isolated on columns as described in the protocol (Appendix A Protocol # 1 and 2), and when this isolation is performed correctly, the DNA should be free of proteins, RNA, chemicals and endotoxins.

We transfected the C2C12 cells with Lipofectamine™2000, which is a lipid-based transfection method. For some cells, this is the most effective method regarding delivery, and it is also associated with the lowest toxicity. In contrast to the calcium phosphate transfection method, pH is not a problem. The method is easy to perform and involves only a few steps. The limitation of liposome-based transfection is that it is not applicable to all cell lines. However, for C2C12 cells it is an effective way of transfection. The quantity of DNA and Lipofectamine™2000 used for transfection has previously been established for the C2C12 cell line (consentrations differ slightly from the standard protocol from the supplier, Appendix A Protocol #5) .

Total RNA isolation

Only a small percentage of all RNA species in an eukaryotic cell is mRNA. To obtain high-quality RNA is a fundamental step in performing many molecular techniques such as qRT-PCR. Common problems with isolating RNA is contamination with genomic DNA (gDNA) and proteins such as inhibitors of reverse transcriptase, degradation of the RNA, and low yields of RNA. To optimize the results, all equipment (tubes, trays, pipet tips and solutions) must be RNase-free to get intact full-length RNA isolated.

The isolation of RNA was performed using ABI's Nucleic Acid Prep Station 6100, which is an established method in our laboratory. The method is a simple step-by-step procedure for applying individual samples, wash solutions and an elution solution to a 96-well total RNA purification tray placed in a vacuum carriage in the 6100 machine. The lysis solution used to lyse cells, inactivates cellular RNases and thereby prevents degradation of the isolated nucleic acid while keeping gDNA and protein in solution. As a result, the RNA is separated from the other components. The method is fast, and allows simultaneously isolating of up to 96 high quality RNA samples. Another established method for isolating high quality RNA, is the TRIzol® method. This method provides higher yields of RNA, but has several limitations. The most important one for this master thesis being the limitation of isolating samples one by one. In addition, the TRIzol® method is based on phenols, which is known to inhibit the reverse transcriptase enzyme. Inadequate removal of phenols could thereby cause less efficient cDNA synthesis. Also, the RNA is more likely to be degraded if there are phenols present in the sample. Based on these aspects, the 6100 machine from ABI was the preferred choice for isolating total RNA.

qRT-PCR

Several methods for quantifying mRNA exist, such as the traditional Northern blotting, and the more improved qRT-PCR that was used in this master thesis. Total RNA isolated from the experiments was reverse transcribed to cDNA. The amount of cDNA is directly related to the amount of the input RNA template, and the first strand of cDNA was used as a template in the PCR reaction. To measure the initial amount of a mRNA transcript, and to compare the expression levels among samples, the copy number of PCR products produced in each PCR reaction needs to be quantified (as an indirect measure of mRNA levels in the cells).

Northern blotting involves separating mRNA samples by size through gel electrophoresis, and detection of the gene of interest with hybridization with a probe complementary to the target sequence. The procedure normally takes at least three days to run, and it requires microgram quantities of RNA to detect one gene of interest. Another disadvantage is the probes used, which often are radioactively labeled. Methods to relate abundance of an individual transcript between samples are required. Northern blotting is, however, the only method revealing the size of the analyzed transcript, and gives a good indication of the integrity of the RNA sample. qRT-PCR, on the other hand, has several advantages compared to Northern blotting. An important aspect is time, as the qRT-PCR can give the results in only a couple of hours. Also, it only needs nanogram quantities of RNA, and there is no radioactivity involved. In contrast to the limited number of samples that can be detected on one membrane using Northern blotting, the number of samples to be analyzed using qRT-PCR is much higher, which makes it easy to run parallels and enable statistical analysis of changes in gene expression. A disadvantage of qRT-PCR is that contaminants can inhibit the PCR reaction, and high purity of the sample is therefore essential. In our studies, the purity depends on the protocol used for isolation of total RNA with the ABI-6100 machine. Both Northern blotting and qRT-PCR make use of control genes (house keeping genes). These genes are not affected by the treatment and are used to normalize data and determine the fold-differences in expression of the target gene among individual samples. The constitutively expressed endogenous gene 36B4 was used as a control in our experiments. This gene has been validated to have relative stable expression patterns in the cells studied and with the treatments used in this thesis.

Dual luciferase reporter assay

To see if a newly identified PPRE in the *Plin5* promoter is used by PPARs, a dual luciferase reporter assay was performed. This is an often-used method to determine the transcriptional activity of a specific promoter. The Luciferase-activity for two *Plin5* reporters (*Plin5* full-length wild type or *Plin5* mutated promoter) was normalized against *Renilla* Luciferase activity produced by the internal control (pRL) in each sample. An advantage with this method is the concept of simultaneous measuring of two different enzyme-activities in the same pipetted sample. By normalizing these activities for each sample, the experimental variation is minimized in contrast to using single a reporter in each well. The assay provides no physical evidence for a direct binding of PPARs to a PPRE in the *Plin5* promoter, but the

results can suggest it might occur, by reflecting an alteration of the transcriptional activity of the reporter. To prove a direct binding, chromatin immunoprecipitation (ChIP) assay or electromobility shift assay can be used.

Statistical analysis

The parametric independent t-test is used to examine if there is a statistically significant difference between the mean scores of two different groups. For all parametric tests, some general assumptions apply (245): the dependent variable must be continuous rather than categorical; each measurement must not be influenced by any other observation of measurement; the samples must be taken from a normal distributed group; and the samples must be obtained from groups with equal variances. Because the sample sizes in this thesis are small, a non-parametric test is more accurate to use as it does not required normal distribution. On the other hand, this type of test is less powerful than the parametric tests (246), which means that genuine differences can be undetected because it can never produce small P-values when dealing with extremely small sample size, such as comparing two groups of samples with triplicates. The variances were often unequal in the experiments performed in this thesis, as tested with Levene's test for equality of variances. When the variances were unequal, the corresponding p-value for "equal variances not assumed" was used. This way, the assumption of equal variances was not violated. The last arguable assumption concerning the statistics this thesis, is if the samples are in fact independent. However, because the independent t-test is often used in similar experiments to test for statistical differences, the test was also used in this thesis.

For each experiment, several comparisons were performed, most often between a control and several other treatment groups. When more tests are conducted, the probability that one or more are significant just by chance (type I errors) increases. To keep the familywise p-value at 0.05 and 0.01, a new p-value was set by dividing the original p-value with the number of tests performed (Bonferroni correction). Statistical significance was set at the 5 % ($P < 0.05$) and the 1 % ($P < 0.01$) level. Due to the low number of experiments and samples, careful interpretation of the results is required.

6.2 General discussion

Perilipin 5 is a member of the Perilipin-family of LD proteins (171). Evidence suggests it may have a role in protection against cellular lipotoxicity in oxidative cells with high energy demands, by transiently entrapping bioactive lipids in LDs close to mitochondria at times of increased cellular FA influx. Also, it may facilitate the release of FA by LDs to the mitochondria in a PKA-regulated manner when LDs and mitochondria are interacting with each other (177;224).

The current understanding of the transcriptional regulation of perilipin 5 is limited. Work by our group and others have found that it is regulated by ligand stimulation or ectopic expression of PPARα (171;218;219). Perilipin 5 is expressed in oxidative muscle cells (171;218;219), and based on the *in vivo* demonstration that factors like the PGC1s and the ERRs stimulate the conversion of glycolytic to oxidative muscle types (101;113;114) we wanted to test if these factors also have a role in the regulation of perilipin 5. From an ongoing study in our group, we also know that activation of PPARδ stimulate *Plin5* expression (Berg et al, manuscript submitted). However, a PPRE in the *Plin5* promoter responsible for the PPAR-effects had not yet been identified.

The main objective for this thesis was to investigate if perilipin 5 expression in cultured muscle cells is induced by the transcription factors PPARs, PGC1s and ERRs, and to identify response elements used by these transcription factors in the *Plin5* promoter. Our results confirmed that overexpression and activation of PPARα regulates the expression of perilipin 5 in muscle cells. However, they also demonstrated that another important PPAR in this regulation probably is PPARδ. Furthermore, when induced by PPARs the relative level of perilipin 5 increase with time, suggesting the involvement of other factors in the regulation of transcriptional activity. We tested if PGC1s could be such factors, and found that when cells were overexpressing PPARδ and stimulated with a PPARδ-agonist, the expression of perilipin 5 was enhanced by PGC1α and reduced by PGC1β. However, when not overexpressing PPARs, it seems like the PGC1β induce the expression of perilipin 5 suggesting a role in the basal expression of perilipin 5. ERRs did not appear to be of importance. The results also confirmed a new-located functional PPRE in the *Plin5* promoter.

PPARα induces perilipin 5 in muscle cells

The mRNA level of perilipin 5 was induced by overexpression and/or stimulation of PPARα in our cultured muscle cells. This has been demonstrated by other studies (171;218;219), both *in vitro* and *in vivo*. In skeletal muscle cells from mice, the expression of perilipin 5 was absent until stimulated with a PPARα-agonist, while cells from the PPARα-knockout mouse showed no expression of perilipin 5 even when stimulated (219). Another study (218) found the same results, with a 15-fold increase in perilipin 5 mRNA and protein levels in gastrocnemius muscle (a mix of type I and type II fibers) from mice overexpressing PPARα compared to littermate control mice. Also, a high-fat diet was demonstrated to increase the expression of perilipin 5 in gastrocnemius muscle *in vivo*, regardless of whether or not PPARα was overexpressed (218).

PPARα is activated when the cells need to use fat as energy source, such as starvation, cold exposure, or a high fat diet, and its activation induces expression of genes that contribute to maintaining energy balance (33). Also, it is activated with a low fat high sugar diet, through upregulating FAS. Both PPARα and perilipin 5 is highly expressed in tissues with active fatty acid oxidation (44;45;116;171), thereby a functional explanation for the PPARα-induced expression of perilipin 5 in our experiments might be that perilipin 5 enhances the release of FAs from LDs to provide the cell with energy.

PPARδ is an important PPAR in the transcriptional regulation of perilipin 5 in cultured muscle cells

An important result from this thesis is the demonstration that ligand stimulation of PPARδ in glycolytic muscle cells stimulates expression of perilipin 5. Perilipin 5 is normally expressed in oxidative tissues. Our result contrast to another study, which has investigated the role of PPARδ in regulation of perilipin5 in mouse Leydig cells, where no induction of *Plin5* mRNA was observed after stimulating with a PPARδ-ligand (219). Work by our group has shown that ligand stimulation of PPARδ resulted in a higher induction of perilipin 5 in muscle cells, compared to ligand stimulation of PPARα (Berg et al, manuscript submitted). This is likely due to the high expression of PPARδ compared to PPARα in muscle cells (247). However, it also demonstrates that activation of a PPARδ is important in our culture model, not only the presence of it. The present results are also in accordance with other studies demonstrating that

overexpression of PPARδ in mice resulted in a change in fiber composition to more oxidative fibers (58), which is the fiber type with the highest expression of perilipin 5.

PPARδ is ubiquitously expressed in the body, and is demonstrated to be a regulator of FA oxidation in muscle cells to provide energy for working muscles when glucose is not available (33). The induction of perilipin 5 in muscle by PPARδ suggests that this member of the perilipin family has an important role in regulating the use of energy from cellular fat stores in muscle.

Perilipin 5 is a novel direct PPAR target gene

Several experiments show a convincing increase in perilipin 5 expression after ligand stimulation or ectopic expression of PPARα or PPARδ. To determine if this is a direct regulation, we searched for potential PPREs in the *Plin5* promoter. An earlier master student had previously identified a PPRE located -2077 to -2064 bp upstream of the transcriptional start site in the *Plin5* promoter, but our experiments demonstrated that this sequence was not used by the PPARs to induce the expression of perilipin 5. Instead, we identified a new functional conserved PPRE located *downstream* of the *Plin5* transcriptional start site at position +139 to +151 (Figure 31) in intron 1 of the *Plin5* gene. Mutation of only four nucleotides within the PPRE in the *Plin5*-reporter construct abolished the induction of perilipin 5 reporter activity by PPARs compared to the full-length promoter construct in cells that was cotransfected with RXRα, PPARα, PPARδ, and PGC1s, and stimulated with PPARα- and PPARδ -ligands. Only the full-length promoter construct resulted in a significant increase in the induction of *Plin5*, implying that the novel PPRE is used by the PPARs. Perilipin 5 is thus a novel direct PPAR target gene. Yamaguchi et al (219) were unable to identify a PPRE in the 2.1-kb region upstream of the *Plin5* promoter. Instead, they suggested that the downstream region of the *Plin5* gene, which corresponds to the promoter region of the previously characterized *Plin4* gene (176), could regulate *Plin5* expression. The finding of a functional PPRE in the *Plin5* promoter in the present study supports a significant role for *Plin5* in the regulation of energy metabolism in muscle cells.

The regulation of perilipin 5 in muscle cells also involves other transcription factors than the PPARs

The induction of perilipin 5 mRNA mediated by PPARα and PPARδ increases with time. Because the endogenous expression levels of PPARα in the C2C12 cells are low, while the levels of PPARδ are high (Berg et al, manuscript submitted), the experiments with the two different PPARs were performed in different ways. When studying the time-effect of PPARα, the cells were transfected to overexpress the receptor and not stimulated, while when studying the time-effect of PPARδ the cells were only stimulated with a PPARδ-ligand without overexpressing the receptor. The results imply that perilipin 5 is induced both directly (the relative level of *Plin5* increased after 48 and 12 hours respectively) and indirectly (the relative level of *Plin5* after 72 hours was further elevated) by PPARα and PPARδ. The indirect regulation may be due to the involvement of coregulators, other PPAR target genes, or the production of endogenous PPAR-ligands, as it would take time before these factors play out their roles in the transcription.

The role of PGC1s

The PGC1s were tested for their ability to drive expression of *Plin5,* since they are known to coactivate members of the PPAR-family (102;103;106;107). In addition, studies have demonstrated that overexpression of PGC1α in transgenic mice increases the amount of type I and IIa muscle fiber (slow oxidative) (113), while overexpression of PGC1β increases the amount of type IIX muscle fiber (fast oxidative) (114). PGC1α was thus a likely candidate for coactivating the expression of perilipin 5 together with the PPARs. The findings in my experiments imply that ligand activation of PPARα has no effect on *Plin5* expression when the cells overexpress PGC1α (Figure 28), whereas ligand activation of PPARγ or PPARδ, or stimulation with fatty acids (BSA-OA) (Figure 28 and Figure 33) increases *Plin5* expression also when PGC1α is overexpressed. The enhancing effect of PGC1α when stimulating PPARδ and PPARγ may occur as PGC1α coactivates PPARδ or PPARγ which then bind to the identified PPRE in the *Plin5* promoter and induces the transcription of perilipin 5. The effect of PGC1β was different from that of PGC1α. PGC1β was in my experiments demonstrated to be present at higher levels than PGC1α in the C2C12 cells, and it seemed to be more important regulatior of *Plin5* as ectopic expression of PGC1β resulted in highly increased *Plin5* mRNA levels. When the cells were stimulated with PPAR-agonists or oleic

acid there was no further induction of relative *Plin5* mRNA, suggesting that the overexpressed PGC1β already has bound to the PPARs and activated them. These results imply that the PPARs prefer to bind PGC1β rather than PGC1α. The involvement of coregulators in the transcriptional regulation of perilipin 5 has not previously been described, but work by an earlier master student in our group investigated the role of PGC1α in this regulation process. In contrast to my results, she found that expression of PGC1α stimulated expression of perilipin 5 mediated by PPARα. However, her experiments were performed on Cos-1 cells, which are a fibroblast-like cell line derived from monkey kidney, which might explain why they are contradictory to my results in C2C12 cells.

Generation of PPAR ligands

The grade of activation of PPARs depends on several factors, as described in section 1.4.2, including not only the abundance of the receptors, but also the level of ligands and the dynamics of coactivators and corepressors. Another potential reason for the time-increasing levels of perilipin 5 mRNA mediated by the PPARs, may thus be the production of PPAR ligands. By such a mechanism, the first induction of perilipin 5 could lead to the release of FAs from the LDs, which might be responsible for the subsequent increase of perilipin 5 by acting as ligands for the PPARs. Alternatively, PPAR target genes could induce PPAR further in a positive feed forward mechanism. A combination of these mechanisms could be the production of PPAR-ligands by PPAR target genes. Interestingly, Wollins et al (218) found that overexpression of the perilipin 5 activated the expression of other known PPAR target genes involved in mitochondrial FA oxidation, suggesting that perilipin 5 may itself drive import of FAs that function as PPAR ligands. If this is the case, perilipin 5 will be placed both upstream and downstream of the PPARs, and it might be responsible for a positive feed forward regulation of its own expression. This would be beneficial when the cells require increased FA oxidation, under conditions such as fasting and when excess FAs need to be degraded.

ERRs do not stimulate *Plin5* expression

Another candidate group of factors which could have a role in the indirect induction of perilipin 5 by the PPARs, was the ERRs, that have crucial roles in the regulation of energy metabolism (248) and is coactivated by PGC1s (102;104;105). ERRα was particularly

interesting because its tissue-expression correlates to that of perilipin 5 (86;90-92). Both PGC1s and ERRα regulate genes involved in lipid metabolism, and are highly expressed in tissues with active fatty acid oxidation. PGC1α and ERRα are furthermore activated by the same physiologic stimuli, like exercise, fasting and exposure to cold temperatures. ERRα is a target gene of PGC1α, and induction of PGC1α increase expression of ERRα and its target genes in a positive feed forward mechanism (105). PGC1α is demonstrated to actually be induced by PPARδ in muscle (111;249). This means that when the muscle is exposed to exercise, PPARδ, PGC1α and ERRα are induced, leading to further activation and/or increased transcription of all three proteins by a feedforward mechanism, with the ultimate result manifested as increased oxidation of FAs in the muscle cells. Surprisingly to us, overexpressing the ERRs resulted in no induction of perilipin 5 in the cultured muscle cells.

The role of potential corepressors

Although the PGC1β coactivator was found to stimulate *Plin5* expression with highest efficiency, the expression of *Plin5* mRNA was reduced when the cells were ectopically expressing PGC1β and stimulated with oleic acid (Figure 28). *Plin5* reporter activity was also reuced when the cells were ectopically expressing both PPARδ and PGC1β *and* stimulated with a PPARδ agonist (GW501516) (Figure 33). A physiological explanation for this could be the activation of repressing mechanisms and corepressors that repress the coactivating function of PGC1β on PPARs with high activation. In glycolytic muscle cells the natural level of the corepressor RIP140 is high (250), and this has been demonstrated to interact with PGC1s to repress their activation of NRs (251). In fact, this interaction was found to regulate the expression of another protein on the surface of LDs; the CIDEA. It is possible that RIP140 has counteracted the PGC1β and reduced the PPAR-activation and thereby lowered the level of perilipin 5 induction observed when cells were overexpressing PGC1β and were stimulated with oleic acid, and when the cells were overexpressing both PPARδ and PGC1β and were stimulated with a PPARδ-agonist. However, the knowledge of what regulates RIP140 is not fully understood. It can function both as a corepressor and a coactivator, but if environmental stimuli such as fasting, exercise and a high fat diet have a role is unclear (252). Another corepressor that could be of importance in the regulation of perilipin 5 is nuclear receptor corepressor 1 (NCoR1). A muscle-specific NCoR1 knockout mouse model has been demonstrated to have better exercise capacity, due to increased muscle mass and more

oxidative muscle fibers in addition to upregulation of genes involved in mitochondrial biogenesis and function, such as PPARδ and ERRs (253). The phenotype of these mice resembles that of mouse overexpressing PGC1α (113;254). The level of NCoR1 in the nucleus is upregulated by insulin, while the mRNA and protein level is reduced when the cells are low in glucose and/or high in fat. Other conditions that demand increased FA oxidation also reduce the NCoR1 mRNA, such as exercise, fasting, high-fat diet and aging (253). If corepressors like RIP140 or NCoR1 repress the coactivation by PGC1s, such a mechanism would perhaps protect the cells against an overload of FAs in the stimulated state. The proposed mechanisms of *Plin5* regulation in muscle cells are illustrated in Figure 34.
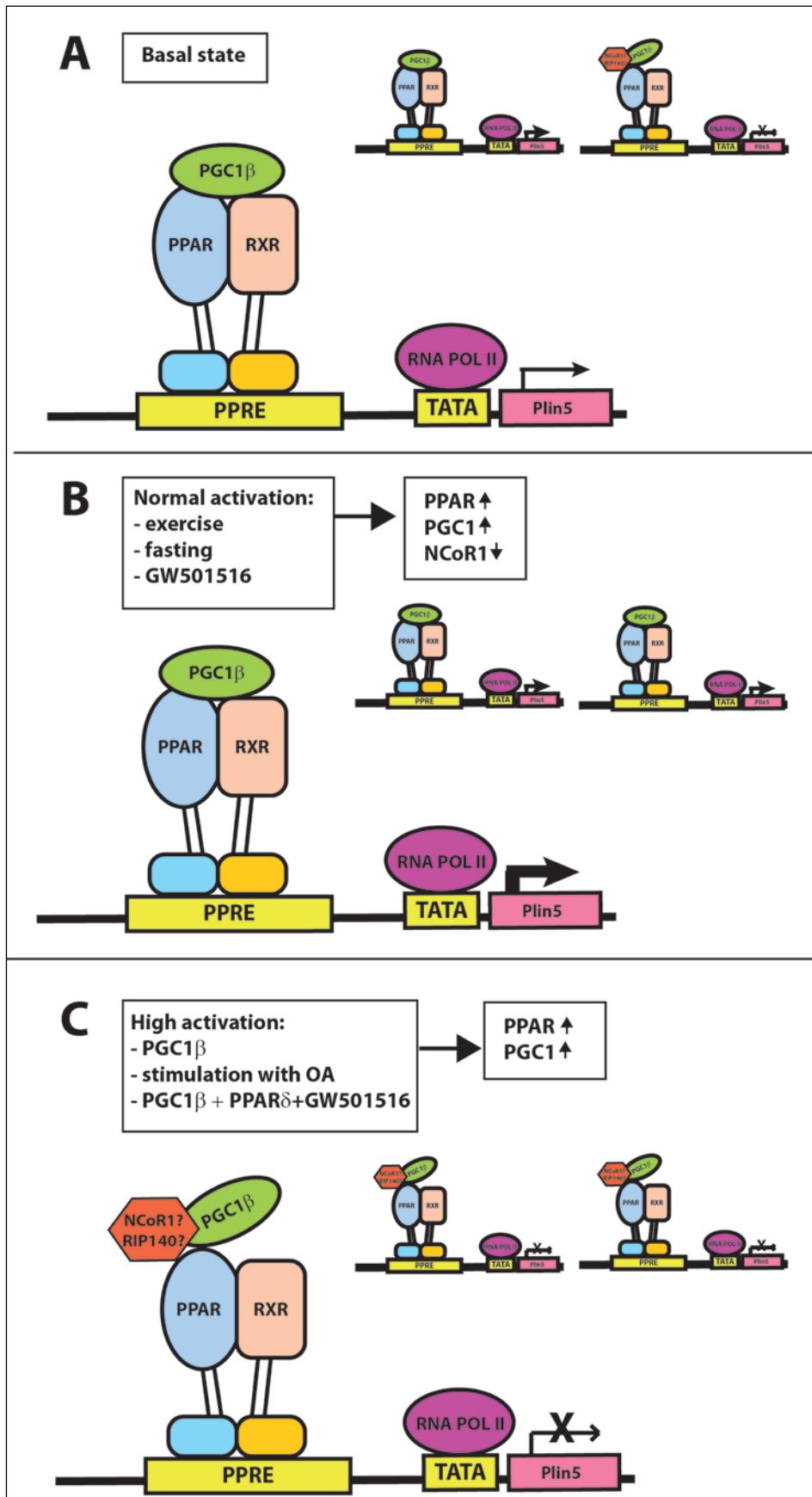
Figure 34: A proposed model for transcriptional regulation of *Plin5* in muscle cells.
A: PGC1β induces expression of *Plin5* in the basal state. Corepressors like NCoR1 and RIP140 are also present and repress the transcriptional activity by disturbing the binding of PGC1β to PPAR.
B: Under normal activation, the level of PPARs and PGC1s increase while the level of corepressors (might) decrease, resulting in elevated induction of *Plin5* transcription.
C: Under high activation conditions, like ectopic expression of PGC1β, or PGC1β+PPARδ and stimulation with GW501516, or stimulation with OA, the level of PPARs and PGC1s increase, while corepressors are abundant. NCoR1: Nuclear receptor Corepressor 1; PPAR: Peroxisome proliferator-activated receptor; RXR: retinoid x receptor; PPRE: Peroxisome proliferator-activated receptor response element; RNA POL II: RNA polymerase II; OA: oleic acid; PGC1β: Peroxisome proliferator-activated receptor gamma-coactivator beta .

A proposed role for perilipin 5 in fatty acid metabolism

The findings that both PPARα and PPARδ induce perilipin5 in muscle cells imply that it has a major role in fatty acid metabolism. In accordance with this, perilipin 5 is previously demonstrated to recruit mitochondria to the LD surface (220) and perhaps even be present within the mitochondria (224). Overexpression of perilipin 5 in muscle cells results in increased FA oxidation, and a higher interaction between perlipin 5- coated LDs and mitochondria (224). This interaction seems reasonable in conditions when the cells need to release fat from their storage, such as starvation and exercise. While resting skeletal muscles use fatty acids as their primary fuel source (8), working muscles use glucose. When glucose is depleted, the muscles must switch back to FA oxidation

By an unclear mechanism, PKA activation in cells overexpressing *Plin5* increases lipolysis (177). The current data suggests that perilipin 5 binds ATGL and inhibits lipolysis when PKA is not activated. The catecholamine epinephrine is released from the adrenal medulla in response to fright, exercise, cold and low levels of blood glucose, and activates adenylyl cyclase (AC) in muscle cells. AC catalyzes the synthesis of cAMP, which results in the activation of PKA, to produce glucose by enhancing glycogenolyse (8). Hence, the conditions that activate PKA in muscle correlate with the conditions that induce perilipin 5 through PPARα and PPARδ to yield energy from FA oxidation. When the cells need to use energy stored in the LDs, perilipin 5 is induced by PPARα and/or PPARδ and phosphorylated by the activated PKA to release ATGL and enhance lipolysis. Conversely, when energy homeostasis is adequate, and the cells still are exposed to FA-influx (through a high fat diet, or a high sugar diet in which fatty acid synthesis is upregulated), PKA will *not* be activated and thereby perilipin 5 (induced by the activated PPARα due to high fat or high sugar) might remain *unphosphorylated* and hold on to ATGL, thereby inhibiting lipolysis. However, the evidence

of *Plin5* phosphorylation by PKA is weak. Another kinase of interest is AMPK, which is known to have a major role in the energy homeostasis in the body. Interestingly, this kinase is demonstrated to phosphorylate and thereby increase the levels of PGC1α in skeletal muscle cells (255). It is also activated by a change in the cellular AMP:ATP ratio which occurs during exercise and fasting and with GW501516 stimulation of PPARδ. AMPK then suppresses fatty acid synthesis and increases fatty acid oxidation (reviewed in (256)). It is possible that the increased rate of FA oxidation partly is due to phosphorylation perilipin 5 under these conditions.

Perilipin 5 and life style diseases

By understanding the transcriptional regulation of perilipin 5, it might be possible to develop drugs that counteract obesity by stimulating expression of perilipin 5; and thereby increase the abundance of perilipin 5 on LDs and stimulate fatty acid oxidation and lipolysis. Obesity is often the first step on the way to insulin resistance and type 2 diabetes mellitus (T2DM). The energy surplus from the diet is first stored in adipose tissues, but as the storage capacity here is reached, further fat storage will occur in other cells and tissues, like skeletal muscle and pancreas, and thereby disturb their basic functions. It seems like perilipin 5 has an important function in regulating the dynamics of storage and oxidation of fatty acids in oxidative tissue.

The basal expression of perilipin 5 has been demonstrated to facilitate TAG-storage, while the stimulated state facilitates FA-oxidation in a PKA-dependent manner. This implies that if perilipin 5 is to be upregulated through a potent drug target for life style diseases, it also has to be activated through PKA in order to increase the fatty acid oxidation instead of storing the fat.

7 Conclusion

Perilipin 5 is a member of the Perilipin family of LD proteins, which is expressed at high levels in tissues with active fatty acid oxidation such as oxidative muscles, heart, brown adipose tissue and liver. The protein is able to recruit mitochondria to the LD surface, and is proposed to regulate lipolysis in a pattern analogue to that of perilipin 1 in adipose tissue by interacting with CGI-58 and ATGL. *Plin5* is suggested to protect the cell against lipotoxicity by capturing lipids in LDs when there is a high influx of fatty acids to the cell, and facilitate the release of fatty acids from the LDs when the cell needs to use fat as energy source.

Accumulation of body fat is the first step to development of obesity and insulin resistance. Therefore, the knowledge of molecular processes utilizing fat stores is of great importance as it potentially can be used to develop drugs to prevent these life style diseases. The transcriptional regulation of perilipin 5 is little understood, but previous studies have demonstrated that overexpression or ligand activation of PPARα induces *Plin5* expression. The work in this thesis revealed that the *Plin5* promoter contains a conserved functional PPRE, which makes it a novel PPAR target gene. Moreover, the results support the existing evidence that PPARα induce the expression of perilipin 5, and in addition they imply that PPARδ might play a larger role in the transcriptional regulation of perilipin 5 in muscle cells. The induction of perilipin 5 by overexpression of PPARα or ligand activation of PPARδ increases with time, suggesting the involvement of other factors important for *Plin5* expression. The ERRs were tested, but not found to affect the expression of *Plin5*. Expression of PGC1α and β, especially PGC1β, was found to induce expression of *Plin5* mRNA. The current results suggest that the PGC1s coactivte the PPARs when they stimulate transcription of *Plin5*, but additional studies are required to fully determine the role of the PGC1s for *Plin5* expression.

8 Suggested further work

To be able to address the effects of PGC1s more accurately, the transcriptional activity of the perilipin 5 promoter should be measured when cells only overexpress the PGC1s, independently of the PPARs. Also, the experiment illustrated in Figure 33 should be performed with PPARα. To confirm that the PGC1β binds to PPARδ on the PPRE in the *Plin5* promoter, future studies should include a Chromatin Immunoprecipitation (ChIP) assay that can identify PGC1β and PPARδ in the *Plin5* gene, and an experiment with siRNA PGC1β to test if the ligand-activated induction of *Plin5* is reduced when PGC1β is not present. In addition, the level of potential corepressors should be measured. Furthermore, experiments should include more transfection studies to overexpress potential corepressors and stimulate the cells with a PPARδ-ligand, to see if the relatie level of *Plin5* mRNA is reduced compared to when not stimulating PPARδ. If so, the corepressor likely represses the PPARδ-activated induction of *Plin5* in muscle cells.

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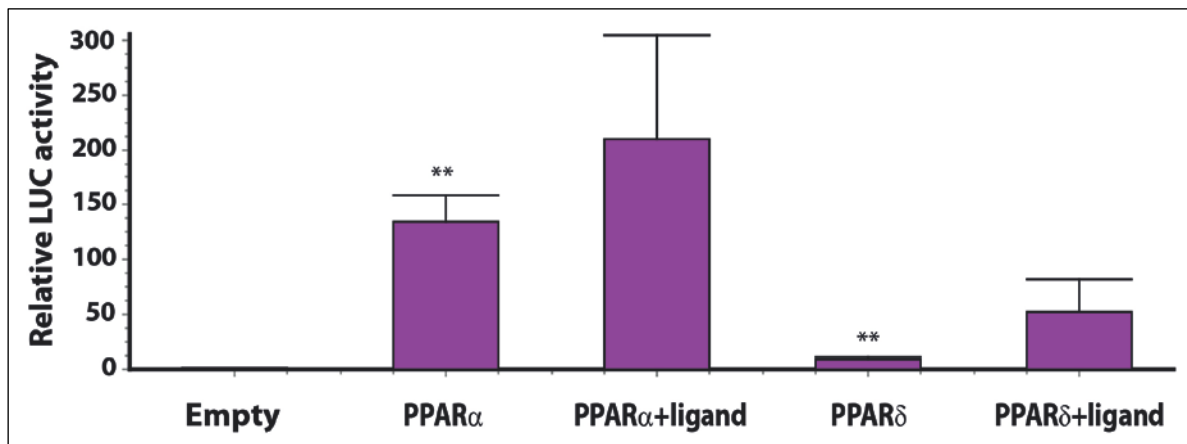
Appendix A

Table 13: Protocols used.

#	Kit	Cat.No	Link to protocol
1	JETSTAR 2.0 Plasmid Midiprep Kit	210050	http://www.genomed-dna.com/pdf/Star-PDFs/JETSTAR-Protocol.PDF
2	NucleoSpin ® Plasmid (NoLid)	740499.50	http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/Plasmid%20DNA%20Purification/UM_pDNA_NS.pdf
3	QuikChange ® II Site-Directed Mutagenesis Kit	200524	http://www.chem.uky.edu/courses/che554/quikchange.pdf
4	Isolation of Total RNA	4330254	http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041237.pdf
5	Lipofectamine™ 2000	11668-027	http://tools.invitrogen.com/content/sfs/manuals/lipofectamine2000_man.pdf
6	Dual Luciferase	E1960	http://www.promega.com/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol/
7	High cap. cDNA transcription kit	4368813	http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042557.pdf
8	qRT-PCR TaqMan Assay	4333458	http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf

Appendix B

Figure #1:

The full-length *Plin5* reporter (pcDNA3-Plin5-Luc [-2344 to +244]) was transiently transfected into C2C12 cells and co-transfected with empty vector (control), PPARα or PPARδ (Appendix B figure # 1). After two days of differentiation the medium was replaced with fresh medium containing vehicle (DMSO) or a PPAR-agonists (GW501516; 0.1 µM or WY-14643;10 µM). Cells were lysed and harvested four days after transfection. The transcriptional activity of the *Plin5* promoter was elevated when ligands were added. However, the effects of PPARδ overexpression and stimulation did not turn out to be significant in this experiment. The transcriptional activity of the *Plin5* promoter was significantly increased when overexpressing PPARα or PPARδ.
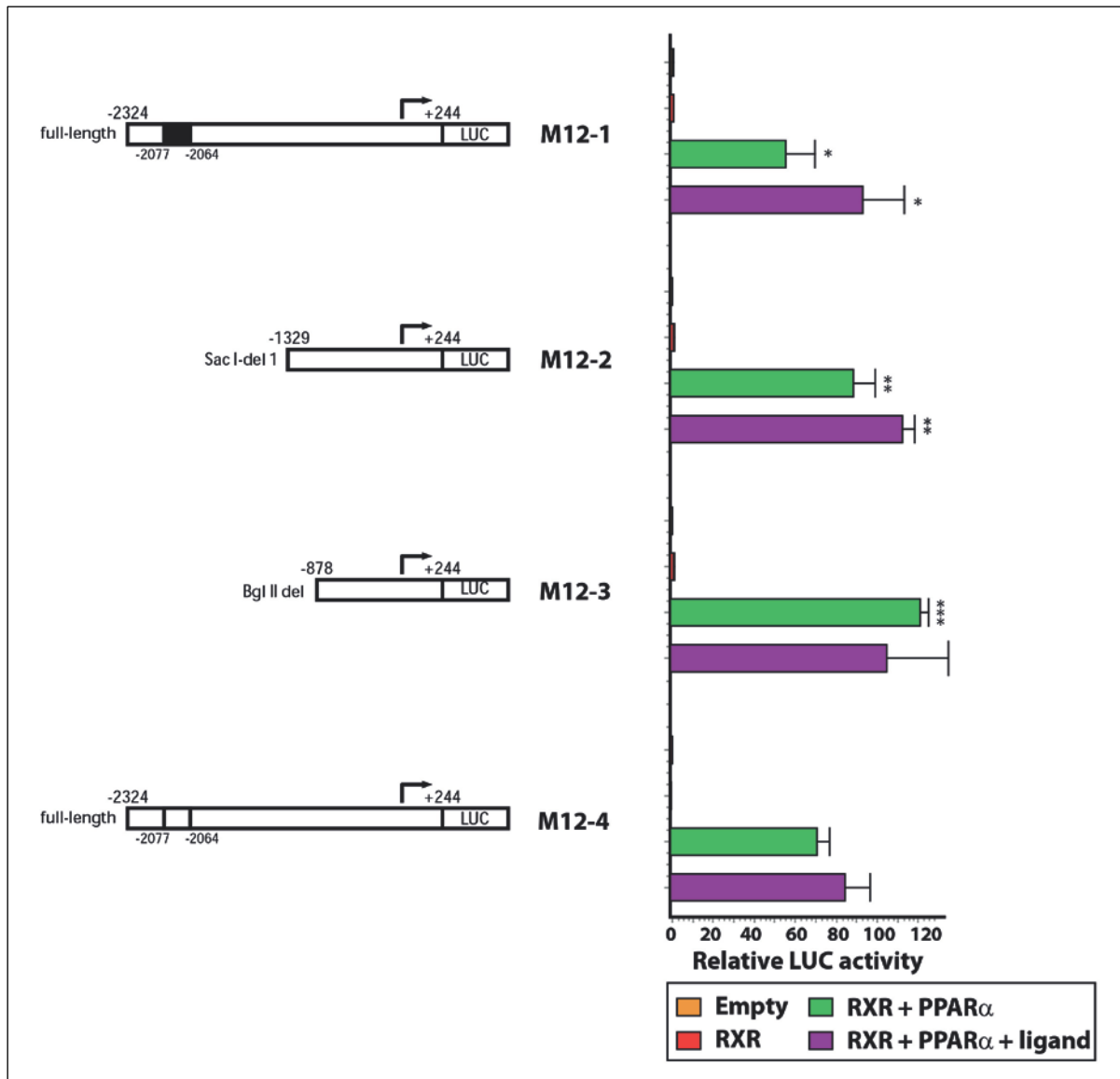

Appendix B figure # 1: Transient transfection with full-length *Plin5* promoter, co-transfected with no expression plasmid (empty), PPARα, or PPARδ expression vectors. After two days of differentiation cells were stimulated with the PPARα-ligand WY- 14643 (10µM; WY) or the PPARδ-ligand GW501516 (0.1 µM; GW) for 24 hours. The experiment was run in triplicates and performed once. PPARα; peroxisome proliferator-activated receptor α, PPARδ; peroxisome proliferator-activated receptor δ. Results are presented as Mean±SD. (** P < 0.01).

Figure # 2:

Different constructs of the *Plin5* promoter sequence (the full-length wild-type *Plin5* reporter pcDNA3-Plin5-Luc [-2344 to +244], deletion constructs or a mutated construct) (Figure 29) was transiently transfected into C2C12 cells and co-transfected with empty vector, RXR, or RXR+PPARα expression plasmids. Two days after start of differentiation, the medium was replaced with fresh medium containing vehicle (DMSO) or a PPAR agonist (WY-14643; 10 μM). There was no difference in the transcriptional activity of the *Plin5* promoter between the full-length wild-type promoter and the other promoter constructs (Appendix B figure # 2). Two statistical tests were performed within each promoter group, comparing empty vector with RXR+PPARα and empty vector with RXR+PPARα+ligand, and the significant 0.05-level was adjusted to 0.025, and the 0.01-level to 0.005 after Bonferroni-correction.

This implies that the PPARs regulate the expression of *Plin5* through a different PPRE in a different region of the promoter than the deleted and mutated areas tested in these experiments.

Appendix B figure # 2: Transient transfection with full-length (M12-1), deletion (M12-2; m-Plin5 SacI deletion and M12-3; m-Plin5 BgI II- deletion) or mutated (M12-4) *Plin5* **promoter constructs, co-transfected with no expression plasmid (Empty), RXR, or RXR and PPARδ expression vectors. Cells were stimulated with the vehicle (DMSO) or the PPARδ-ligand GW501516 (0.1 μM). The experiment was run in triplicates and performed once. Results are presented as Mean±SD. Within each promoter group three statistical tests were performed (empty versus the three different vector groups) with subsequent Bonferroni correction. (* P < 0.05, ** P < 0.01, *** P < 0.001). The P-value for the statistical difference between RXR+PPARα and empty vector for the M12-3 promoter construct, and between RXR+PPARα+ligand and empty vector for the M12-4 promoter construct, was 0.026 and 0.027 respectively. This is at the 0.05 border-line after Bonferroni-correction (p < 0.025 corresponds to P< 0.05).**