

The role of skeletal muscle PLIN proteins at rest and following lipolytic stimulation

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Abstract

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This thesis investigated the subcellular location of skeletal muscle PLIN proteins (PLIN2, PLIN3, and PLIN5) as well as protein interactions with ATGL and HSL at rest and following lipolytic stimulation. In addition, the serine phosphorylation state of PLIN2, PLIN3, and PLIN5 was determined at rest and following lipolytic stimulation.

An isolated whole muscle technique was used to study the effects of contraction and epinephrine-induced lipolysis. This method allowed for the examination of the effects of contraction and epinephrine alone and in combination. Further, the soleus was chosen for investigating the role of PLIN proteins in skeletal muscle lipolysis due to its suitability for isolated incubation, and the fact that it is primarily oxidative in nature (~80% type I fibres). It has also been previously shown to have the greatest reliance on lipid metabolism and for this reason is ideal for investigating the role of PLIN proteins in lipolysis.

Immunofluorescence microscopy revealed that skeletal muscle lipid droplets are partially co-localized to both PLIN2 and PLIN5 and that contraction does not affect the amount of colocalization, indicating that PLIN5 is not recruited to lipid droplets with contraction (PLIN2 ~65%; PLIN5 ~56%).

Results from the immunoprecipitation studies revealed that with lipolysis in skeletal muscle the interaction between ATGL and CGI-58 is increased (study 2: 128% with contraction, $p < 0.05$; study 3: 50% with contraction, 25% epinephrine, 80% contraction + epinephrine, $p > 0.05$). Further PLIN2, PLIN3, and PLIN5 all interact with ATGL and HSL, while only PLIN3 and PLIN5 interact with CGI-58. Among these interactions, the association between PLIN2 and ATGL decreases with lipolytic stimulation (study 2: 21% with contraction, $p < 0.05$). Finally our results demonstrate that PLIN3 and PLIN5 are serine phosphorylated at rest and that the level of phosphorylation remains unchanged in the face of either contractile or adrenergic stimulation.

In summary, the regulation of skeletal muscle lipolysis is a complex process involving multiple proteins and enzymes. The skeletal muscle PLIN proteins likely play a role in skeletal muscle lipid droplet dynamics, and the data from this thesis indicate that these proteins may work together in regulating lipolysis by interaction with both ATGL and HSL.

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List of Abbreviations

ACAT	acyl coenzyme A:cholesterol acyltransferase
ADRP	adipose differentiation related protein (also adipophilin, PLIN2)
AMPK	AMP activated protein kinase
ATGL	adipose triglyceride lipase
CaMK	calcium calmodulin dependent kinase
cAMP	cyclic AMP
CGI-58	comparative gene identification-58
DG	diglyceride; diacylglycerol
DGAT	diacylglycerol acyl transferase
ERK	extracellular signal regulated kinase
G0S2	G(0)/G(1) switch gene 2
HSL	hormone sensitive lipase
IMTG	intramuscular triglyceride
LSDP5	lipid storage droplet protein 5 (also OXPAT, MLDP or PLIN5)
MG	monoglyceride; monoacylglycerol
MGL	monoglyceride lipase
MLDP	myocardial lipid droplet protein (also LSDP5 or OXPAT or PLIN5)
PKA	protein kinase A
PKC	protein kinase C
PLIN	perilipin
PPAR	peroxisomal proliferator-activated receptors
TG	triglyceride; triacylglycerol

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Chapter 1

Introduction

The discovery of a family of lipid droplet proteins known as the perilipin (PLIN) family has led to a resurgence in research in the area of lipid metabolism. Due to the structural location of PLIN proteins on lipid droplets, they have gained increasing attention in the past decade with regard to potential roles in lipogenesis and lipolysis. The PLIN family of proteins consists of five members, all of which have a unique tissue distribution and likely a unique function in lipid droplet dynamics. Specifically in adipose tissue, PLIN1 plays a key role in regulating basal and stimulated lipolysis by directly controlling the activity and access of lipases at the lipid droplet surface. However, PLIN1 is not expressed in skeletal muscle and the roles of the PLIN proteins that are expressed in this tissue (PLIN2, PLIN3, and PLIN5) remain unknown.

In skeletal muscle triglycerides are stored inside dynamic organelles known as lipid droplets. It is on the surface of these lipid droplets that the PLIN proteins are thought to play a role in regulating both storage and use of intramuscular triglycerides (IMTG). In times of increased energy demand, such as exercise, fatty acids released from triglycerides stored in lipid droplets can provide a significant amount of energy.

Our understanding of the regulation of triglyceride lipolysis in skeletal muscle is continuously growing, and the exact mechanisms that regulate skeletal muscle lipolysis remain largely unknown. While research investigating the regulation of hormone sensitive lipase (HSL) in skeletal muscle is abundant, there are few papers investigating the regulation of the recently discovered rate limiting lipase, ATGL. Further, given the large role that PLIN1 plays in regulating adipose

tissue lipolysis by interacting with the lipases, it seems likely that the skeletal muscle PLIN proteins play a similar role in exercise induced lipolysis.

This thesis examines the effects that skeletal muscle lipolysis has on PLIN2, PLIN3, and PLIN5. Specifically, this thesis examines subcellular location, protein interactions with lipases, as well as serine phosphorylation status at rest and following lipolysis.

Chapter 2

Literature Review

Overview of Skeletal Muscle Lipolysis

Intramuscular triglycerides (IMTGs) represent an important energy source that can be mobilized during exercise. While research investigating the use of IMTGs during exercise began in 1958 (30, 39, 47, 52) a complete understanding of the mechanisms regulating this process remain largely unknown. It is now known that IMTGs are stored in metabolically active organelles, known as lipid droplets. These lipid droplets are encased by a phospholipid monolayer coated with a number of different proteins. Growing evidence indicates that skeletal muscle lipolysis is regulated at the surface of these triglyceride storing lipid droplets by protein-protein interactions (16, 99, 116, 122). However, the exact mechanisms regulating the protein interactions are relatively unknown in skeletal muscle.

Recently, a family of lipid droplet associated proteins, known as the PLIN family of proteins, have gained increasingly more attention with regards to a role in lipid droplet dynamics and metabolism, specifically to a role in regulating lipolysis. Our understanding of PLIN proteins in skeletal muscle is limited, however studies in other tissues and in cell culture indicate that PLIN proteins are key regulators of lipid metabolism as they appear to be directly involved with how cells and tissues store, mobilize, and utilize fatty acids (22, 27, 43, 97, 100, 146). PLIN1 is the only member of this family for which a specific role in regulating lipolysis through interactions with the lipases has been identified, however this member is only expressed in adipose tissue and a similar role for the skeletal muscle PLINs has not

been determined. In general, mobilization of fatty acids from triglycerides stored in lipid droplets is mediated by three lipases: 1) adipose triglyceride lipase (ATGL); 2) hormone sensitive lipase (HSL); and 3) monoglyceride lipase (MGL). These lipases work in a sequential fashion, where ATGL releases the first fatty acid from the triglyceride (TG) leaving a diglyceride (DG) (180, 187). HSL acts predominantly on diglycerides but can also hydrolyze triglycerides, monoglycerides (MG), and cholesterol esters. Finally, MGL completes the hydrolysis by releasing the last fatty acid and a glycerol backbone (Figure 1). In adipose tissue, PLIN1 directly controls the activity of the rate limiting lipase, ATGL, as well as the access of HSL to the lipid droplet at rest and with adrenergic stimulation.

To date a similar role for the remaining PLIN proteins expressed in skeletal muscle has not been identified, therefore the focus of this thesis is the skeletal muscle PLIN proteins and their potential role in skeletal muscle lipolysis.

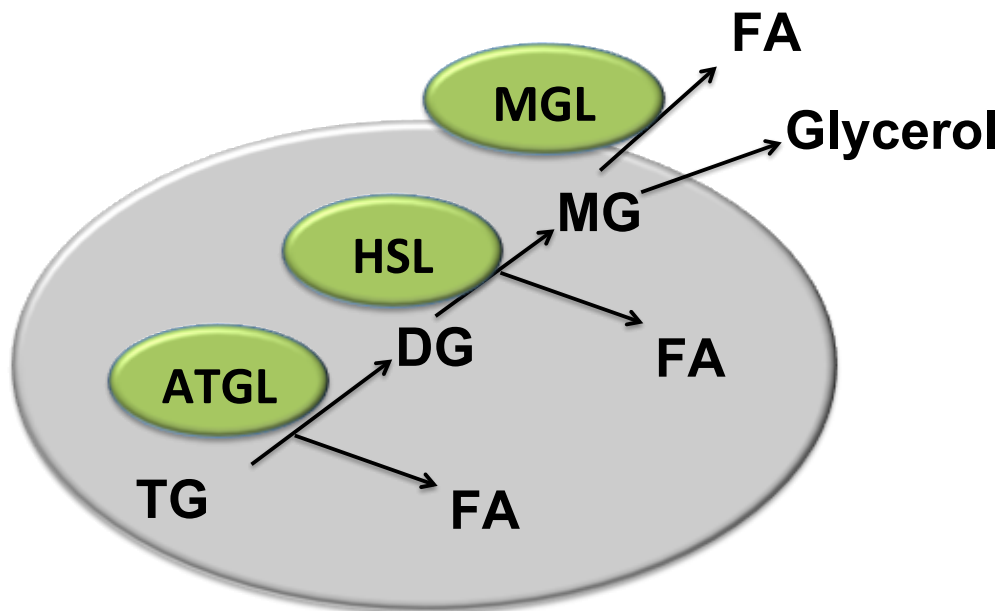


Figure 1. Sequential hydrolysis of TG by ATGL, HSL, and MGL, resulting in the release of three fatty acids and a glycerol backbone (TG=triglyceride; DG=diglyceride, MG=monoglyceride; ATGL= adipose triglyceride lipase; HSL= hormone sensitive lipase; MGL monoglyceride lipase).

Lipid droplets

Structure and function

Although adipose tissue is the predominant site for triglyceride storage, nearly all cell types possess the capacity to store fatty acids as triglycerides in intracellular lipid droplets. Lipid droplets were initially considered inert storage depots for intracellular fats, and consequently little was known about the exact regulation of lipid droplet formation, growth, or degradation. Today, lipid droplets are recognized as highly dynamic organelles that play critical roles in lipid

metabolism, cell signaling, vesicle trafficking, and energy homeostasis (13). Due to these dynamic roles, lipid droplets are continuously being synthesized and degraded as they traffic within the cell to interact with other organelles such as the endoplasmic reticulum (site of triglyceride synthesis) and the mitochondria (site of fatty acid oxidation) (127) [reviewed in (13, 155, 162)] (Figure 2a).

Lipid droplets have a unique structure, as they are the only cellular organelle to be surrounded by a single phospholipid monolayer and not a bilayer. This monolayer encases a hydrophobic core consisting of neutral lipids (triglycerides, diglycerides, monoglycerides, cholesterol esters, and free cholesterol) (11, 114, 116, 149) (Figure 2b). Lipid droplets are formed as small (0.2 μm diameter) spherical structures (103), and can vary in size (1-200 μm) as well as in function depending on the tissue and the cellular condition (116). For example, in adipocytes one large lipid droplet can occupy up to 90% of the total cell volume (54), whereas skeletal muscle contains several small lipid droplets located in close proximity to mitochondria and sarcoplasmic reticulum (33, 127, 148). In skeletal muscle, transmission electron microscopy (TEM) images show that lipid droplets occupy the gaps between adjacent mitochondria in the A band region on both sides of the sarcomere (Figure 2c). It has been hypothesized that the close proximity of lipid droplets to mitochondria has a functional role that allows for the fatty acids released from the droplet to be oxidized efficiently during exercise (148).

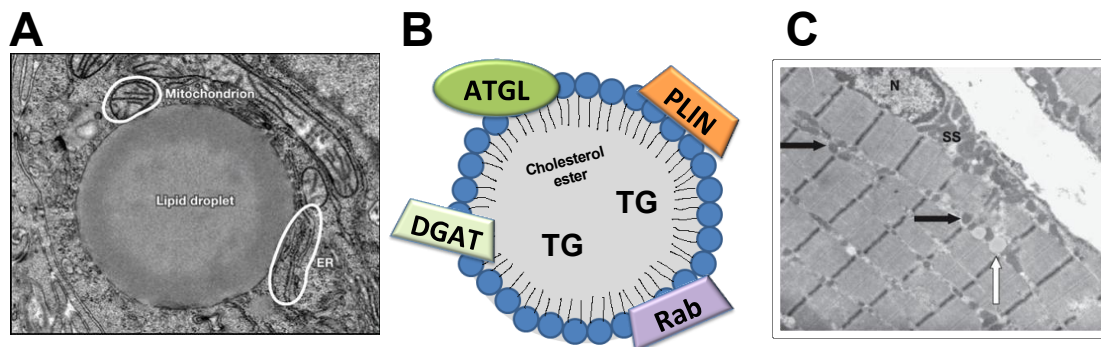


Figure 2

A: Electron micrograph showing a lipid droplet in a cultured hepatoma cell. The phospholipid monolayer surrounding the lipid droplet is visible, as are close associations with mitochondria and ER membranes (Adapted from Walter and RV Farese 2009). B: Structural features of a lipid droplet. Showing lipid droplet phospholipid monolayer surrounding neutral lipid core containing triglycerides and cholesterol esters. The surface of the lipid droplet is coated with a variety of proteins (ATGL: adipose triglyceride lipase; DGAT: diglyceride acyltransferase, Rab, PLINs: perilipins, etc). C: Transmission electron micrograph (TEM) of human skeletal muscle. Image from a type IIX or IIA fiber (note the thin Z-line), with light arrow, indicating an intramyocellular lipid droplet and dark arrows, indicating mitochondrial fragments in cross section, notice close proximity of each organelle (Adapted from Tarnopolsky et al. 2007).

In times of excess fatty acid delivery to the tissue, lipid droplets will increase in volume and number in order to store the energy for a later time or to be used as precursors for membrane or hormone synthesis (95, 183). This protects cells from lipotoxicity and is essential for survival in cases where food is not always in abundance. Conversely, at times of increased energy needs (such as during exercise), the cell can mobilize the stored lipids to produce energy (21, 43, 182). What is currently known about the processes of lipid droplet synthesis and growth as well as degradation will be covered in the following sections.

Lipid Droplet Proteome

Studies of the lipid droplet proteome have found more than 300 different proteins on the surface of the droplet (168, 184), yet little is known about the function of any of these proteins. A few of these proteins are known to be involved in triglyceride synthesis (diacylglycerol acyl transferase; DGAT2) and breakdown (ATGL), vesicle trafficking (Rab proteins), intracellular signaling, and lipid droplet fusion and fission events (SNAREs) (16, 99, 65, 117). But the majority of the protein functions remain to be determined. The lipid droplet protein coat is highly heterogeneous depending on the subcellular location and the tissue that it is found in, therefore it has been hypothesized that lipid droplet function is mediated by the proteins coating the phospholipid monolayer. Among the many proteins found on lipid droplets the perilipin or PLIN family of proteins (formerly known as PAT proteins) have emerged as essential in regulating lipid droplet hydrolysis (14), however information on the role of PLIN proteins in skeletal muscle is still limited.

Lipid droplet synthesis

The exact mechanism of lipid droplet formation is not fully understood, particularly for skeletal muscle; consequently information must be extrapolated from in vitro cell studies or other tissues. The current model of lipid droplet formation suggests that lipid droplets originate from the endoplasmic reticulum (16). It is hypothesized that once synthesized, neutral lipids accumulated between the lipid bilayer of the endoplasmic reticulum. In support of this theory, the

endoplasmic reticulum contains many of the enzymes that are involved in the synthesis of triglycerides (DGAT) and cholesterol esters (acyl coenzyme A:cholesterol acyltransferase, ACAT) (28, 49). Eventually a lipid lens is formed in the lipid bilayer of the endoplasmic reticulum which then buds completely off of the endoplasmic reticulum forming a lipid droplet (reviewed by (13, 26, 43, 155)) (Figure 3).

These newly formed small lipid droplets can continue to grow either by local synthesis of neutral lipids or by fusing with other droplets. The protein catalyzing the final step in triglyceride synthesis (DGAT2) is found on lipid droplets, suggesting that triglycerides can also be made on the droplet surface (86, 138). However, once it is formed, it is not clear how the newly synthesized neutral lipids are delivered to the core of a growing lipid droplet. Lipid droplets can also increase in size by fusing with other lipid droplets. Again the mechanisms remain largely unknown however it is known that the proteins that mediate vesicle fusion (SNAREs) are found on the lipid droplet surface (20).

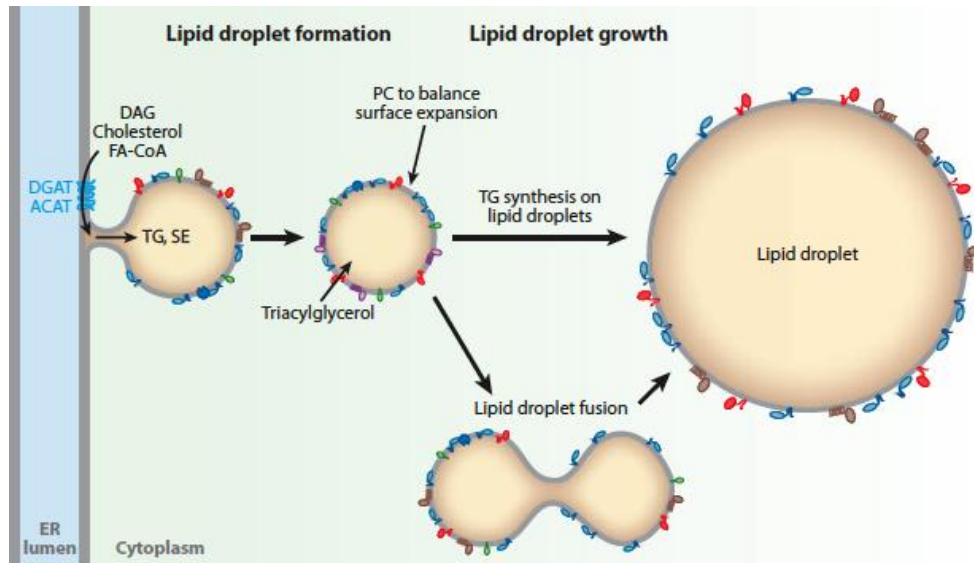


Figure 3

Model of lipid droplet formation and expansion. For formation, the lipid droplet buds and detaches from the endoplasmic reticulum. The neutral lipid synthesis enzymes, ACAT or DGAT, catalyze the formation of lipids that fill the core. For lipid droplet expansion, the models of local synthesis of triacylglycerol at the lipid droplet and of lipid droplet fusion are shown. (DAG=diacylglycerol; FA-CoA=fatty acyl-coenzyme A; SE=sterol ester; PC=phosphatidylcholine; DGAT=diacylglycerol acyl transferase; ACAT=acyl coenzyme A:cholesterol acyltransferase) (Adapted from Walther et al 2012).

The PLIN family of proteins

The PLIN family includes PLIN1 (perilipin), PLIN2 (adipocyte differentiation-related protein, ADRP), PLIN3 (tail interacting protein 47, TIP47), PLIN4 (S3-12), and PLIN5 (lipid storage droplet protein 5, LSDP5, also known as myocardial lipid droplet protein, MLDP, or OXPAT) (21, 82). In general the members of the PLIN family are characterized as being localized to lipid droplets, and having a similarity to either PLIN1 or PLIN2 at either the amino- or the carboxy-terminal of the protein (21). With the exception of PLIN4, the PLIN proteins share a 100 amino acid sequence at the amino-terminal known as the PAT1 domain, and have weaker

homology in the central region (21, 101, 111). The function of this well conserved PAT domain has not yet been identified. The middle portion of PLIN1 contains three sequences of hydrophobic amino acids that are required to direct and anchor the protein into the lipid droplet (59, 140). Of the five proteins PLIN1 is, thus far, the only one for which six protein kinase A (PKA) phosphorylation sites have been identified (21, 101). PLIN2 and PLIN4 are expressed in the majority of cells and share a high identity (43%) between amino acid sequences (21). PLIN5 is similar to PLIN2 and PLIN3 (55% and 51% homology over the entire protein), with highest homology at the PAT1 domain (36, 125). PLIN2, 3, 4, and 5 all share a hydrophobic cleft at the carboxy-terminal that is thought to position these proteins at the surface of lipid droplets to optimally serve their function (21) (Figure 4).

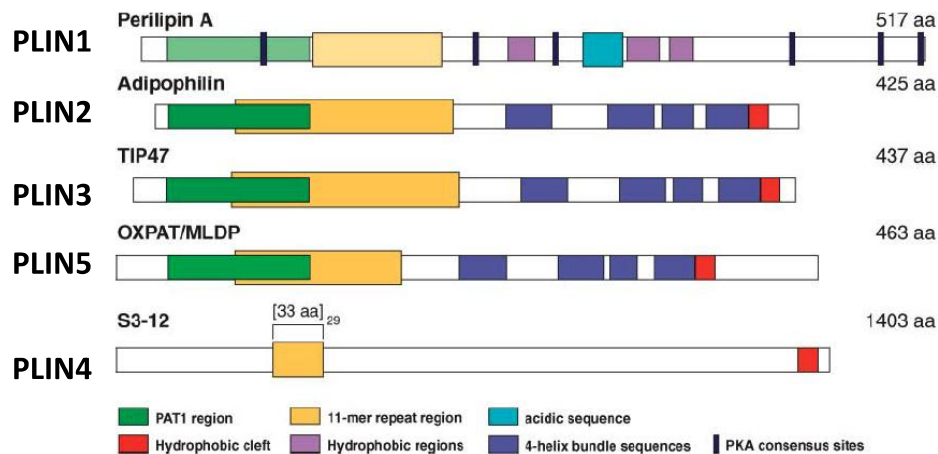


Figure 4

Schematic diagram of the structural features of PLIN family proteins (mouse sequences are depicted); greater intensity of color represents higher similarity of the sequences between family members. (Adapted from Brasaemle et al. 2007).

Transcriptionally, four of the five PLIN proteins are regulated by members of the peroxisomal proliferator-activated receptors (PPARs). The PPAR family consists of three isoforms: PPAR α , PPAR β/δ , and PPAR γ , each having a different tissue distribution. PPAR γ is highly expressed in white adipose tissue and macrophages, PPAR α is expressed in the liver, muscle, and kidney, and PPAR β/δ is more ubiquitously expressed (reviewed in (3)). PPAR γ regulates PLIN1 (8), PLIN4(36), and PLIN5 (171) and PPAR α regulates PLIN2 (37, 45, 147) and PLIN5 (176), whereas PLIN3 expression does not appear to be regulated by PPARs. Interestingly, it has recently been shown that PLIN3 mRNA is upregulated by high glucose and insulin concentrations (48).

Each PLIN family member has a unique tissue distribution and therefore likely a unique role and function in regulating lipid droplet dynamics (171) (Figure 5). Further, within tissues, lipid droplets have a heterogeneous coat of PLIN proteins indicating specific roles for these proteins depending on the size or location of the droplet. In fact, in cultured adipocytes (170) and hepatocytes (12), PLIN3 and PLIN4 are generally associated with smaller nascent lipid droplets, whereas increased association of PLIN1 and PLIN2 is observed as the droplets mature, suggesting that there are differential roles for these proteins during the different stages of a lipid droplet development. The following sections will discuss the role of PLIN1 in regulating basal and stimulated lipolysis in adipocytes as well as speculate on the potential roles of the remaining PLIN proteins in regulating skeletal muscle lipolysis.

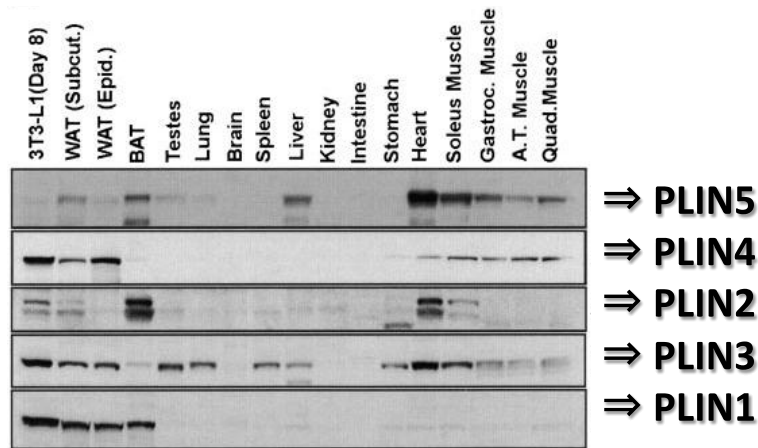


Figure 5
 PLIN family protein expression in 3T3-L1 adipocytes and mouse tissues. PLIN5 is expressed in tissues with high oxidative capacity. PLIN1 is only expressed in adipose tissue (adapted from Wolins et al. 2006).

PLIN1

PLIN1 or perilipin was the first of the PLIN family to be identified, and the only member for which a specific role in regulating lipolysis has been determined. PLIN1 is located on lipid droplets, and is only expressed in adipocytes and steroidogenic cells (98). There are three PLIN1 splice variants: 1A, 1B and 1C; however PLIN1A is the most abundant and by far the most studied of the three (99, 101). The past two decades of research has determined a specific role for PLIN1A in the turnover of adipocyte triglycerides and will be the focus of the following section.

PLIN1 was initially identified as a protein phosphorylated by PKA in lipolytically stimulated adipocytes (65). It wasn't until a decade later that the role of PLIN1 in regulating adipocyte lipolysis came into light. Overexpression of PLIN1 in 3T3-L1 pre-adipocytes resulted in a dramatic reduction in lipolytic activity and a

significant increase in stored triglycerides, suggesting that PLIN1 limited the access of lipases to lipid droplets thus protecting the triglyceride core (25). This role for PLIN1 was further confirmed in PLIN1 knockout mice that demonstrated a substantial increase in basal lipolysis, a 75% reduction in adipose tissue mass, and a resistance to diet-induced obesity. Further, these mice did not respond to beta-adrenergic stimulation of lipolysis, and cells derived from these mice failed to show translocation of HSL to the lipid droplet (105, 145). It was later determined that PLIN1 restrains basal lipolysis and facilitates hormone-stimulated lipolysis (25, 57, 62, 65, 113). This facilitation of lipolysis by PLIN1 under stimulated conditions is controlled by the phosphorylation of its six PKA sites in response to PKA activation (25, 57, 62, 65, 113).

The currently understood role of PLIN1 in regulating adipose tissue lipolysis will be discussed in terms of a basal state and a stimulated state (Figure 6). The basal state in adipocytes and adipose tissue refers to a resting state where circulating catecholamines (epinephrine and norepinephrine) are low. Under basal conditions, PLIN1 is found on the lipid droplet surface preventing the full activation of ATGL and the access of HSL to the lipid core (21, 145). HSL is in the cytosol and cannot access the PLIN1-coated lipid droplets and therefore its substrates stored in the core (21, 24, 143). ATGL is found both in the cytosol and on the surface of lipid droplets. The ATGL that is associated with lipid droplets during the basal state is thought to be responsible for a low level of triglyceride hydrolysis (102, 180). Comparative gene identification-58 (CGI-58), a potent co-activator of ATGL (92), is bound to the carboxyl-terminus of PLIN1, thus limiting the availability of CGI-58 to

interact with ATGL (62, 141, 178). As a result of these conditions there is low lipolytic activity; triglycerides are hydrolyzed to diglycerides and most of the released fatty acids are re-esterified (for review (88)). Due to this continuous recycling, triglyceride levels remain relatively constant.

The stimulated state refers to times of energy demand such as exercise and fasting where stored fats are mobilized and used to produce energy in peripheral tissues. Lipolysis in adipose tissue is initiated by catecholamines (epinephrine and norepinephrine) binding to β -adrenergic receptors on the plasma membranes (31, 150). This stimulates a G_s -protein-mediated signaling cascade activating adenylate cyclase, which converts ATP to cyclic AMP (cAMP), increasing intracellular cAMP levels (136, 150). The increased cAMP levels then activate PKA, which can then phosphorylate both PLIN1 (65, 185) and HSL (7, 143, 146, 157)

Phosphorylation of PLIN1 leads to the activation of ATGL as well as allows access of both ATGL and HSL to the lipid droplet. Therefore PLIN1 phosphorylation is essential in assembling the required machinery on lipid droplets in a phosphorylation dependent manner (21, 145). PLIN1 contains six PKA phosphorylation sites (Ser81, Ser222, Ser276, Ser433, Ser492, and Ser517 in the murine sequence) (65, 185) (Figure 4). In order for HSL to interact with PLIN1, both proteins must be phosphorylated by PKA (139, 143, 157). Mutagenesis studies of the six PKA sites of PLIN1 have found that the three N-terminal PKA sites (serine 81, 222, 276) are critical for HSL catalyzed lipolysis (134, 144, 185). All three of these sites must be phosphorylated for HSL to bind to PLIN1 (157). Once phosphorylated, PLIN1 changes its conformation from basal conditions, providing a docking site for

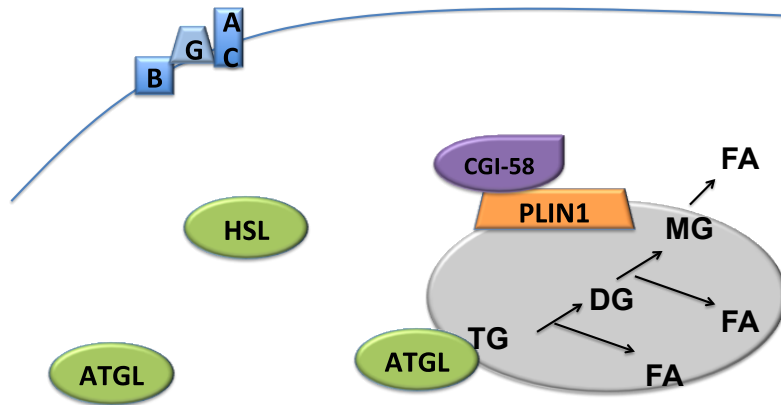
phosphorylated HSL on lipid droplets (21) (139). Once HSL is phosphorylated, it translocates to the lipid droplet surface where it binds to phosphorylated PLIN1, gaining access to substrate lipids within the droplet (21, 24, 143, 157).

PLIN1 contains three additional C-terminal PKA sites (Ser433, Ser492, and Ser517) (66). A definitive function for each of these sites has yet to be determined, but serine 517 is known of as the “master regulator” of PKA stimulated lipolysis (112). Overexpression of PLIN1 with an alanine substitution of serine 517 eliminated 95% of PKA-stimulated release of fatty acids and glycerol relative to control cells. When ATGL was present, phosphorylation of the serine 517 site contributed to the control of lipolysis catalyzed by ATGL with CGI-58. These findings suggest that phosphorylation of serine 517 is somehow required in ATGL-mediated lipolysis (66, 112, 185). It is now known that with phosphorylation, PLIN1 releases CGI-58 allowing it to interact with ATGL (62, 141, 178). This interaction leads to a fully activated ATGL (62, 92, 141, 178, 186). With stimulation, ATGL moves from the cytosol to the lipid droplet through a PLIN1-independent mechanism that is yet to be determined (180). It is unclear if ATGL binds to lipid droplets through interactions with CGI-58 or if it binds directly to the lipid droplet surface. One possibility is that a direct physical interaction between ATGL and CGI-58 is not actually necessary to increase the lipolytic activity of ATGL (102). Gruber et al. (68) demonstrated that CGI-58 is able to bind directly to the phospholipid monolayer of lipid droplets and that without this there is a complete loss of the ability of CGI-58 to activate ATGL, regardless of their interaction. In addition, a single mutation of the PKA site at serine 492 of PLIN1 caused ~30% inhibition of PKA-stimulated lipolysis,

indicating that the phosphorylation of serine 492 plays a role in the PKA-stimulated lipolysis, together with serine 517. The accessory role of serine 492 of PLIN1 in PKA-stimulated lipolysis may be due to its contribution to lipid droplet fragmentation and dispersion (104). Fragmentation of larger lipid droplets into smaller ones during lipolysis would be advantageous as it provides a larger surface area for lipases to work.

With ATGL and HSL now on the surfaces of the lipid droplets, triglycerides (TG) are hydrolyzed to diglycerides via ATGL working in partnership with CGI-58. Diglycerides (DG) are hydrolyzed by HSL to monoglycerides (MG). The remaining MG is cleaved by monoglyceride lipase (MGL) to release the final fatty acid. In total, one molecule of TG is hydrolyzed to three molecules of fatty acid and one molecule of glycerol. Fatty acids and glycerol are exported from adipocytes for use by other tissues. Overall, PLIN1 is considered a scaffolding protein on the surface of lipid droplets that interacts with both HSL and the ATGL co-activator CGI-58, thus providing an essential role in regulating lipolysis in adipocytes under both basal and stimulated conditions.

A. Basal



B. Adrenergic Stimulation

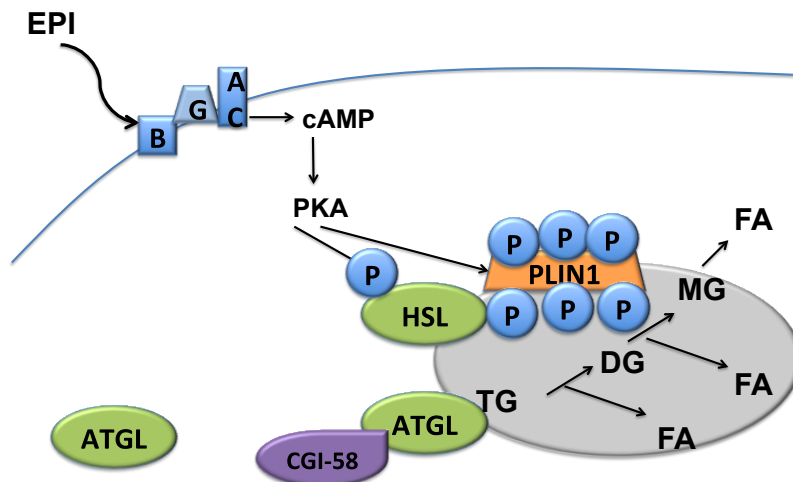


Figure 6

A. Basal lipolysis: PLIN1 on the lipid droplet is bound to CGI-58. ATGL is found both in the cytosol and on the lipid droplet and HSL is found in the cytosol. There is a low rate of lipolysis occurring in this situation. **B.** Stimulated lipolysis: Adrenergic stimulation by epinephrine and norepinephrine increases intracellular cAMP levels and activates PKA. PKA activation phosphorylates HSL and PLIN1 at six serine residues (denoted by P). Phosphorylation of PLIN1 releases CGI-58, which binds ATGL to initiate lipolysis. HSL translocates to the lipid droplet and binds to phosphorylated PLIN1. HSL then degrades DG. MG lipase cleaves the final FA to produce glycerol. (ATGL=adipose triglyceride lipase; HSL=hormone sensitive lipase; B=beta-adrenergic receptor; G= stimulatory G protein; AC=adenylyl cyclase; TG=triglyceride; DG=diglyceride; MG=monoglyceride; FA=fatty acid; cAMP=cyclic AMP; PKA=protein kinase A).

PLIN2

The second PLIN protein to be discovered was PLIN2 (adipophilin or ADRP). It was identified as a fatty acid binding protein implicated in adipocyte differentiation, and was found to have a striking homology with PLIN1 at the amino-terminal end (78). PLIN2 coats the lipid droplet membrane and is stable in the presence of neutral lipids but is otherwise believed to be targeted for degradation by proteasomes (174, 175). It is for this reason that PLIN2 has been considered a marker of lipid droplets. PLIN2 is ubiquitously expressed (14, 23, 71, 78) and is one of the most predominant PLIN proteins in skeletal muscle (23, 71, 110, 120, 122, 129-131). To date, PLIN2 has mainly been studied in fibroblasts, macrophages and the liver, with only a few studies in skeletal muscle (17, 38, 120, 129-131). Therefore it has been difficult to determine a role for PLIN2 in skeletal muscle. PLIN2 is thought of as a marker of lipid droplets, although in skeletal muscle information regarding PLIN2 distribution and its association with lipid droplets is limited and contradictory. One study determined that PLIN2 is highly co-localized (80%) to the lipid droplet surface in isolated rat soleus fibres, making it an ideal marker of lipid droplets (122), while another study has found that this is not the case with only 64% of PLIN2 associated with lipid droplets in human vastus lateralis (129). A specific function for PLIN2 has yet to be identified, however it is considered to be involved in both lipid droplet biogenesis as well as regulation of lipolysis (32, 76, 96, 123).

The role of PLIN2 in neutral lipid storage has been established in a range of in vivo and in vitro studies (6, 32, 55, 76). Originally, PLIN2 was identified as a

protein likely to be involved in lipid droplet synthesis because PLIN2 is expressed early during adipocyte differentiation and localizes to lipid droplet surfaces (23). Wolins et al. later observed that the addition of fatty acids to cultures of fully differentiated adipocytes drives PLIN2 onto the surfaces of both small and large lipid droplets (170). In most cell types and animal models, PLIN2 protein expression is increased with treatments that induce lipid droplet formation (6, 107, 175). These studies provide evidence for a role of PLIN2 in lipid droplet growth. Further, PLIN2 overexpression was shown to be associated with increased fatty acid uptake (58). In studies where the expression of PLIN2 is increased there is also an increased triglyceride content. This effect was seen in fibroblasts (76), HEK 293 cells (94), hepatic stellate cells (55), and COS-7 cells (58). Together, these studies suggest that PLIN2 has a role in both the development and growth of lipid droplets.

Interestingly, PLIN2 interacts with DGAT2 (138), providing more evidence for a role in lipid droplet synthesis and growth.

However, PLIN2 knockout mice indicate a role for PLIN2 in regulating lipid droplet stability and lipolysis. Interestingly, knocking out PLIN2 does not result in any dramatic changes in phenotype or effects on adipocyte differentiation or lipolysis (32), however there is a major effect in the liver where there is a significantly reduced triglyceride content and lipid droplet number (12, 32). Furthermore, PLIN2 knockout mice were found to be resistant to diet-induced hepatosteatosis. In skeletal muscle knockdown of PLIN2 prevented oleate-induced IMTG accumulation and lipid droplet storage. In the absence of PLIN2, lipid droplet accumulation was restricted to a few lipid droplets per myotube (17). These

knockout mice provide evidence in support of PLIN2 somehow preventing high rates of lipolysis and also for a role of PLIN2 in lipid droplet synthesis. In further studies using cells from PLIN2 knockout mice, PLIN3 was directed to the droplets replacing PLIN2 (142), and perhaps compensating for the loss. When PLIN3 function was also ablated, the ability to store neutral lipids as lipid droplets was diminished, and exogenously added fatty acids were directed to phospholipid biosynthesis (142). Together, these data indicate that the role of PLIN2 may be tissue specific and that PLIN2 is not only involved in lipid droplet synthesis but also in lipid droplet stability or basal lipolysis.

In general, there is a high correlation between PLIN2 protein content and the amount of neutral lipid within a cell indicating that the relative expression of PLIN2 may determine the capacity for intracellular triglyceride storage. This is especially true in skeletal muscle. For example, PLIN2 content is greater in type I fibres compared to type II fibres (129), it is higher in endurance trained athletes in comparison to sedentary controls (5, 110, 120, 128, 131), is higher in women compared to men (120), and is closely associated with triglyceride levels in skeletal muscle of Zucker diabetic fatty rats (110). These are all cases in which IMTG content is higher suggesting that PLIN2 may either promote triglyceride synthesis or reduce lipolysis. With interventions that increase IMTG content, such as fatty acid loading of myotubes in culture or fasting and high fat diets in vivo, PLIN2 protein content increases (17). In human skeletal muscle, higher PLIN2 content has been found in muscle from insulin-resistant subjects that have undergone therapeutic weight loss or metformin treatment to increase muscle insulin sensitivity,

suggesting that it might play a role in decreasing intramuscular lipid toxicity by promoting either lipid synthesis or oxidation (121). At this point it is unclear whether PLIN2 increases the amount of lipid or whether lipid droplet formation stabilizes PLIN2 and increases protein expression. However, overexpression of PLIN2 in vitro and in vivo resulted in elevated neutral IMCL levels as well as increased expression profiles of genes involved in lipid synthesis (17), indicating that PLIN2 helps increase lipid storage.

Recent work in human skeletal muscle found that PLIN2-associated lipid droplets are preferentially depleted over those lipid droplets not associated with PLIN2, strongly supporting a role for PLIN2 in exercise induced lipolysis (130). It has been suggested that PLIN2 acts in a similar fashion to PLIN1 as a scaffold protein regulating stimulated lipolysis through interactions with lipolytic enzymes (12, 94, 96, 122, 129, 153). In agreement with this, CGI-58 has been identified as an interaction partner for PLIN2 (179). Further, overexpression of PLIN2 in human embryonic kidney cells leads to a reduced association of ATGL at the lipid droplet and reduced lipolysis (96, 156). Examination of interactions between PLIN2, CGI-58, and ATGL has yet to be investigated in skeletal muscle. Thus far, it has been shown that in isolated soleus muscle, HSL translocates to PLIN2-coated lipid droplets with either β -adrenergic stimulation or electrically stimulated contraction (122), however a direct physical interaction between the two proteins was not determined. Research into the role of PLIN2 in skeletal muscle lipolysis should focus on interactions with the lipases.

PLIN3

PLIN3 (TIP47) was the third PLIN protein to be identified and is one of the least studied of this family of proteins. PLIN3 was initially described as a ubiquitously expressed cytosolic and endosomal 47-kDa protein involved in the intracellular transport of mannose-6-phosphate receptors between the trans-Golgi apparatus and endosomes (40, 85). However, in 2001, PLIN3 was reported to be present on lipid droplets in HeLa cells and has an amino acid sequence 43% similar to PLIN1 and PLIN2 (172). A functional role in skeletal muscle has not been confirmed, but studies in other cell types indicate that PLIN3 is involved in lipid synthesis (29).

In response to lipid loading in both HeLa cells (human cell line derived from cervical cancer cells) and adipocytes, PLIN3 appeared to move from the cytosol and onto lipid droplets (170, 172). Thus, PLIN3 was the first PLIN family protein discovered that had the ability to translocate between the cytosol and the lipid droplet surface (170, 172). Upon lipid loading of cultured differentiated 3T3-L1 adipocytes, PLIN3 localized to smaller nascent lipid droplets, providing evidence that PLIN3 might support the rapid formation of new lipid droplets (170). Further, the homology of PLIN3 is similar to that of PLIN2 (117), and, as described earlier, PLIN3 compensates for the loss of PLIN2 in PLIN2 knockout mice (142). When PLIN3 function was also ablated in these cells, the ability to store neutral lipids as lipid droplets was diminished, and exogenously added fatty acids were directed to phospholipid biosynthesis (142). These findings define PLIN3 as playing an important role in the formation of new lipid droplets.

PLIN3 is expressed in skeletal muscle (120, 171), however few studies have investigated a role for PLIN3 in this tissue. In human skeletal muscle PLIN3 expression is higher in women but does not increase with training (120). Interestingly, in a human study in which no differences were found regarding the protein expression of enzymes/proteins involved in triglyceride synthesis or lipolysis, the protein abundance of PLIN3 was lower in muscle of obese versus lean controls when expressed relative to IMTG content (93). The cellular localization of PLIN3 is not altered with contraction or epinephrine administration in isolated rat soleus fibres (122). PLIN3 was also shown to associate with mitochondria in NIH3T3 cells (fibroblasts) under conditions that induce oxidative stress and it is speculated that PLIN3 prevents cell death by maintaining mitochondrial membrane potential, although the mechanisms are not fully understood (73). It is possible that through interactions with skeletal muscle lipases, PLIN3 regulates the delivery of fatty acids to the mitochondria, thus preventing a lipid overload. However, it is not presently known whether CGI-58 or ATGL bind to PLIN3 in skeletal muscle.

PLIN4

PLIN4 (S3-12) was identified in a screen for adipocyte-specific secretory or plasma membrane proteins (125). To date, only a small number of reports have investigated PLIN4 as a lipid droplet protein. PLIN4 is ubiquitously expressed (14), however PLIN4 expression is low in heart and skeletal muscle, and is higher in white adipose tissue (125, 173). Little is known about PLIN4 function, but it does

appear to function in a manner similar to PLIN3, translocating to newly formed lipid droplets in response to fatty acid stimulation (173). In 2003, Wolins et al. used immunofluorescence microscopy to show that PLIN4 localized to tiny lipid droplets in cultured 3T3-L1 adipocytes (142). When adipocytes were incubated with oleate over a time-course of four hours, PLIN4 localized to the smallest, newly formed lipid droplets (170). PLIN4 has not been defined as having a role in coordinating lipase interaction with lipid droplets. Due to the low expression of PLIN4 in skeletal muscle it will not be discussed in detail in this thesis.

PLIN5

In 2006, PLIN5 was identified and described by three independent groups within a year, and therefore received three different names: lipid droplet storage protein 5 (LSDP5), OXPAT, and myocardial lipid droplet protein (MLDP) (34, 171, 176). This PLIN protein is mainly expressed in tissues with high rates of lipolysis and beta-oxidation such as muscle, heart, liver, and brown adipose tissue (8, 44, 47, 110, 120, 129-131, 171). PLIN5 shares high homology with the other PLIN proteins (especially PLIN2), and is transcriptionally regulated by PPAR α in muscle and liver, and by PPAR γ in white adipose tissue (although expression is generally low in this tissue) (34, 171, 176). Like PLIN3, PLIN5 is found at the lipid droplet surface as well as in the cytosol (34, 35, 171, 177). Due to its unique tissue distribution, it has been hypothesized that PLIN5 is a key regulator of lipolysis in skeletal muscle. PLIN5 might translocate to the lipid droplet surface in circumstances that promote

lipolysis, such as muscle contraction. If this is true, PLIN5 may function by directly regulating lipase action at the lipid droplet surface.

Similar to PLIN2, PLIN5 protein expression correlates well with the neutral lipid content of the cell. In skeletal muscle PLIN5 content is higher in obesity or animal models of type 2 diabetes (110), in women compared to men (120), and it increases with training (120, 131), while high fat feeding increases PLIN5 expression only modestly in skeletal muscle (171). Cardiac PLIN5 expression is also induced in situations that would increase fat oxidation (e.g., fasting (34)). It appears from cell culture studies that PLIN5 may be important for the accumulation of triglycerides, since expression of PLIN5 in Chinese hamster ovary cells (34) or COS-7 cells (171) increases triglyceride concentration. In skeletal muscle, PLIN5 overexpression resulted in increased lipid droplet size and elevated neutral lipid content mainly consisting of triglycerides (19), it was hypothesized from these results that PLIN5 overexpression may serve as an indication for inhibition of lipolysis (19). These observations suggest that PLIN5 may either promote triglyceride synthesis or act as a scaffold protein regulating the rate of lipolysis and delivery of fatty acids to the mitochondria for oxidation.

Recently, Kuramoto et al. described the phenotype of PLIN5 knockout mice. These mice lacked detectable lipid droplets in the heart and lipid droplets in skeletal muscle were smaller and less abundant. This provides evidence in support of a role for PLIN5 in inhibiting lipolysis and stabilizing lipid droplets. Further work using cultured cardiomyocytes from these PLIN5 knockout mice showed a higher rate of fatty acid oxidation, potentially reflecting a compensation for a lack of lipid droplet

stability and storage capacity as a result of increased lipase activity. They next perfused the PLIN5 knockout hearts with an acute inhibitor of ATGL and found that the lipid droplet content was recovered, suggesting an interaction between PLIN5 and ATGL in this tissue (87). Therefore in tissues that lack PLIN1, PLIN5 is thought to be an important scaffold protein regulating triglyceride storage and mobilization by controlling the availability lipases on the lipid droplet surface.

Studies in 3T3-L1, Chinese hamster ovary (CHO), COS-7, H4IIE, and human embryonic kidney-293T cells show that PLIN5 binds both CGI-58 and ATGL (but not both at the same time) to negatively regulate basal and PKA-activated lipolysis (63, 64, 156). PLIN1, 2, 3, and 5 contain a conserved amino terminal sequence allowing them to bind to HSL (7) and the carboxyl terminal region of PLIN5 is able to bind either ATGL or CGI-58 (63, 64, 156). In CHO cells when PLIN5 is bound to CGI-58 or ATGL there is a reduction in triglyceride hydrolysis in the absence of PKA stimulation (156). However, activation of PKA in cells with ectopic expression of PLIN5 and ATGL resulted in increased lipolysis (156). Further, with PKA stimulation there is a 2-fold increase in [³²P]-orthophosphate incorporation into PLIN5 (156). It is possible that phosphorylation of PLIN5 releases CGI-58 and/or ATGL, allowing the two proteins to interact at the surfaces of lipid droplets, thus increasing hydrolysis of triglycerides (156). It is not known if PLIN5 interacts with either of these proteins in skeletal muscle and future studies will be required to unravel the molecular mechanisms regulating the interaction between ATGL, CGI-58, and PLIN5 in the control of lipolysis in skeletal muscle. Subsequent studies identifying the phosphorylation site(s) of PLIN5 as well as any relevant kinase

would also help to identify the mechanism by which phosphorylation of PLIN5 affects lipolysis.

Given that PLIN5 is expressed in highly oxidative tissues, PLIN5 has been hypothesized to play a role in channeling fatty acids towards mitochondria for β -oxidation (18, 19, 158, 171). In support of this hypothesis, PLIN5 expression is induced by physiological, pharmacological, and genetic perturbations that increase utilization of fatty acids for oxidative phosphorylation (35, 171, 177). For example, PLIN5 mRNA and protein content is increased in both the liver and the heart in response to fasting (35, 171, 177). In skeletal muscle, PLIN5 protein expression is increased under conditions that increase fatty acid oxidation, such as fasting and insulin deficiency (34, 171). Further, PLIN5 expression is markedly higher in endurance-trained athletes in comparison to sedentary BMI-matched controls (5, 84), and increases in response to both endurance and interval training (120, 131). Further, lipid droplets containing PLIN5 are preferentially broken down during an acute bout of exercise, compared to those lipid droplets not containing PLIN5 (131). These findings provide evidence for a role of PLIN5 in regulating stimulated lipolysis in skeletal muscle.

Due to the tissue expression of PLIN5, it has also been associated with regulating fatty acid oxidation. Recently, PLIN5 has been shown to localize to mitochondria in smooth and skeletal muscle cells (18) as well as link lipid droplets to mitochondria (158). Overexpression of PLIN5 results in a more intimate interaction between the lipid droplets and mitochondria, where almost all lipid droplets in PLIN5 overexpressing muscle were in contact with mitochondria (18).

Peters et al. showed that increased PLIN5 is associated with improved oxidative capacity (120) and PLIN5 correlates with markers for oxidative capacity (18). Further, PLIN5 overexpression in vitro (171) and in tibialis anterior was associated with increased fatty acid oxidation (13) and increased expression of genes under control of PGC1 α suggesting improved oxidative capacity (19).

Collectively, these studies suggest an important role for PLIN5 in lipid droplet stability by directly interacting with both HSL and ATGL as well as ATGLs co-activator, CGI-58. Further, PLIN5 appears to play an important role in fatty acid oxidation and perhaps delivery of fatty acids to the mitochondria. PLIN5 may be a key player in regulating cellular metabolism upon energy demand by regulating lipolysis and interactions between lipid droplets and mitochondria.

Lipolysis in Skeletal Muscle

The use of IMTGs as an important substrate contribution to fat oxidation during exercise has been demonstrated by the use of several different techniques (biochemical extraction (137, 160), tracer methodology (152), immunohistochemistry (153) and magnetic resonance spectroscopy (126)). It is now known that during exercise IMTGs are mobilized through a combination of external hormonal (increased epinephrine) and internal metabolic signals (intracellular Ca²⁺, and metabolite concentrations). However, the exact mechanisms regulating IMTG hydrolysis at the lipid droplet during exercise are poorly understood. Previous studies in skeletal muscle have focused on the role of HSL in

regulating lipolysis and there are only a handful of studies investigating ATGL regulation or the role of skeletal muscle PLIN proteins.

As outlined above, most research in the field of lipid droplet coat proteins has focused on adipose tissue and PLIN1. While PLIN1 is not expressed in skeletal muscle, evidence suggests that skeletal muscle lipolysis is regulated by PLIN protein interactions occurring on the lipid droplet surface in a manner similar to that observed in adipose tissue (15, 22, 43, 120, 122). Skeletal muscle is very distinct from adipose tissue given its higher metabolic rate, which is accelerated during exercise. Adipose tissue lipolysis aims to export and deliver fatty acids to other tissues for use, but the goal of skeletal muscle lipolysis is to provide fatty acids directly to the mitochondria for oxidation and energy production. Exercise leads to the activation of several skeletal muscle kinases, each of which may play into regulating the rate of lipolysis. Hormonal regulation of skeletal muscle lipolysis is similar to adipose tissue lipolysis, but the full activation of ATGL and HSL in skeletal muscle is likely more complex involving contraction-mediated routes (108). Increased circulating epinephrine concentrations during dynamic exercise will lead to the activation of PKA, but at the same time contraction increases intramuscular calcium levels which stimulate calcium calmodulin kinase (CaMK) and extracellular signal regulated kinase (ERK) activation. Further, the mismatch between ATP utilization and production during contraction also leads to increased levels of AMP, which activates AMP-activated kinase (AMPK). The following sections will focus on what is currently known about the regulation of lipolysis in skeletal muscle, with a

focus on how the above-mentioned kinases are currently known to regulate HSL and ATGL.

Skeletal Muscle Hormone Sensitive Lipase Regulation

Until 2005 HSL was considered to be the only triglyceride lipase and therefore the function of HSL has been extensively studied and reviewed (163, 164, 181). In skeletal muscle, HSL protein content is highly correlated with IMTG concentration and oxidative capacity and therefore has a higher expression in type I fibres compared to type II fibres (91, 119). HSL has a high substrate specificity for diglycerides (50, 51) but is also active against a variety of lipid substrates, including triglycerides, monoglycerides, and cholesteryl esters (181). Ablation of the HSL gene in mice results in a significant accumulation of diglycerides, this finding highlights the importance of HSL in complete triglyceride hydrolysis (70). In skeletal muscle, HSL activity increases in response to exercise at varying intensities but is not proportional to exercise intensity (159, 160). During exercise skeletal muscle HSL can be activated by both catecholamines (91, 167) and muscle contraction (44, 74, 90, 135), and these effects on HSL activity have been shown to be partially additive (89). Together this indicates that epinephrine and contraction activate HSL through different signaling mechanisms. HSL contains three domains: a catalytic domain, a regulatory domain containing several phosphorylation sites, and an NH₂-terminal domain involved in protein-protein and protein-lipid interactions (163). Studies using isolated HSL protein have demonstrated that HSL is phosphorylated on five

serine residues (563, 565, 600, 659, 660; rat sequence) in vitro (7). It is now known that HSL phosphorylation and activity during exercise is regulated by multiple kinases; PKA, ERK, CaMK, and AMPK (124, 161). The regulation of skeletal muscle HSL phosphorylation and activity through adrenergic and contractile stimulation will be discussed further in the following sections.

Epinephrine

During exercise circulating epinephrine concentrations are increased leading to increased HSL activity in skeletal muscle in both isolated muscle and in vivo (53, 91). Similar to adipose tissue, in skeletal muscle epinephrine binds beta-adrenergic receptors on the cell membrane leading to subsequent PKA and HSL activation (91, 160, 161) (Figure 7). Mutagenesis experiments demonstrate that Ser563, Ser659, and Ser660 are the major PKA phosphorylation sites responsible for stimulating HSL (7, 42), although Ser563 may not affect catalytic activity directly (7). In skeletal muscle, PKA activation leads to HSL phosphorylation at serine 563 and 660 (161).

Overall, PKA phosphorylation of HSL results in modest activation of the enzyme (2-to 3-fold) and promotes the translocation of HSL to the lipid droplet (46). The recruitment of HSL to the lipid droplet appears to be crucial for complete hydrolase activity of HSL (122, 157), and has been shown to occur in isolated rat soleus muscle following epinephrine incubation or electrically-stimulated muscle contraction (122). This indicates that PKA phosphorylation is not the only effect that can cause HSL translocation to the lipid droplet. In isolated soleus muscle

incubated with epinephrine, HSL colocalization with PLIN2 and lipid droplets increased significantly indicating that there may be an essential role for the association of HSL with PLIN2 in lipolytic control (122). However direct protein-protein interactions between PLIN2 and HSL have yet to be investigated in skeletal muscle. Thus, phosphorylation of HSL by PKA stimulates lipolysis by increasing lipase activity and moving HSL to its substrates, however this may not be essential for HSL translocation as HSL also moves to the lipid droplet with contraction (122).

In human experiments, skeletal muscle samples obtained before, 15 and 90 min during a 120 min steady state exercise showed that HSL activity was increased by ~80% at 15 min compared with rest and returned to resting rates at the cessation of and 120 min after exercise. Consistent with changes in plasma epinephrine, skeletal muscle HSL Ser563 and Ser660 phosphorylation were increased by 27% at 15 min, remained elevated at 90 min, and returned to resting values post-exercise. Skeletal muscle phosphorylation of the inhibitory site of HSL (Ser565) as well as AMPK signaling were increased at 90 min during, and after, exercise (161). These findings show that increased HSL activity in skeletal muscle occurs at the onset of exercise due to phosphorylation of the PKA sites (Ser563 , Ser659 , and Ser660), but HSL activity is reduced later on in prolonged exercise and recovery likely due to phosphorylation of HSL Ser565 by AMPK (124, 161). Therefore, epinephrine and PKA play a large role in activating HSL during exercise but other contractile mechanisms (e.g. AMPK) also appear to regulate the rate of lipolysis through HSL activity.

Adrenergic Stimulation

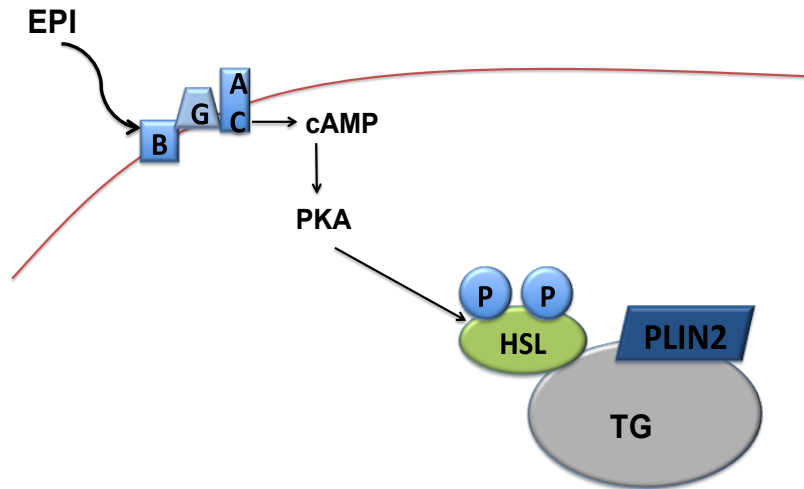


Figure 7

Skeletal muscle adrenergic stimulation by epinephrine increases intracellular cAMP levels and activates PKA. PKA activation phosphorylates HSL at Ser563 and Ser660 (denoted by P). Phosphorylation of HSL causes translocation to the lipid droplet where HSL can hydrolyze diglycerides. (EPI=epinephrine; B=beta-adrenergic receptor; G= stimulatory G protein; AC= adenylyl cyclase; cAMP= cyclic AMP; PKA= protein kinase A; ATGL= adipose triglyceride lipase; HSL= hormone sensitive lipase; TG=triglyceride).

Contraction

In the absence of adrenergic stimulation, contraction alone has been shown to increase skeletal muscle triglyceride utilization, suggesting that kinases other than PKA are important for stimulating IMTG hydrolysis and fatty acid oxidation (44). Contraction leads to increased intracellular calcium and AMP concentrations, both of which are important signals activating different kinases (CaMK, protein kinase C: PKC, and AMPK) (161). The roles of these kinases on HSL activity will be discussed in the following sections.

Calcium ion and HSL activity

During muscle contraction, calcium ions are released from the sarcoplasmic reticulum and acts as a critical second messenger involved in changing metabolism (166, 109). Increased skeletal muscle calcium concentrations lead to the activation of both PKC and CaMK. This is interesting, as these kinases have been shown to have opposing effects on HSL activity (60, 67). For example, isolated skeletal muscle incubated with caffeine in a concentration known to increase intracellular calcium without causing contraction has resulted in both an increase (41) and a decrease (159) in neutral lipase activity.

The effect of PKC on stimulated HSL activity seems to be mediated via extracellular signal-regulated kinase (ERK) (41, 67). Contraction increases ERK activity in skeletal muscle, while epinephrine does not influence this activity (118, 169). In adipose tissue, ERK phosphorylates HSL at Ser660 and increases activity (67). It seems as though ERK plays a similar role in regulating skeletal muscle HSL activity. Using PKC inhibitors, Donsmark et al. were able to abolish the contraction-mediated HSL activation in muscle without effecting epinephrine-stimulated HSL activation (41). Also complete blockade of ERK activation in contracting muscle reduces the increase in HSL activity by 50% (41). Further, direct pharmacological activation of PKC in resting muscle is accompanied by ERK phosphorylation and increased HSL activity (41). Therefore, it has been determined that ERK increases HSL activity in isolated rodent skeletal muscle (41), likely by phosphorylation at Ser660.

From studies in adipose tissue it is known that CaMK phosphorylates the

“inactivating” Ser565 site on HSL preventing further phosphorylation and activation (60). The role of CaMK in skeletal muscle seems to be similar. To investigate if this occurs in skeletal muscle, Watt et al. incubated isolated soleus muscles with cyclopiazonic acid, a sarcoplasmic reticulum calcium-ATPase inhibitor, or caffeine, both of which increase intracellular calcium. Both experimental conditions lead to a significant decrease in HSL activity. They then used an inhibitor of CaMK (KN-93) in these incubations and saw that HSL activity was returned back to base values, thus indicating that CaMK is also an inhibitor of HSL in skeletal muscle (166).

It seems perplexing that in skeletal muscle calcium ion leads to the activation of two kinases that have opposing effects on HSL activity, but perhaps as the calcium concentration changes with exercise duration or intensity one kinase could overrule the other. Because both kinases are activated by calcium, it has been difficult to determine the overall effect of calcium in regulating HSL activity (Figure 8).

AMP and HSL activity

Due to the high ATP turnover rate during exercise, there is an increase in AMP levels, which leads to the activation of AMPK. In adipocytes, AMPK phosphorylates HSL at Ser565, preventing HSL activation by PKA (61). Similarly, in both resting (115) and contracting (133) skeletal muscle, triglyceride hydrolysis is suppressed in response to AMPK activation by the adenosine analogue AICAR (5-amino-4-imidazolecarboxamide-1- β -D-ribofuranoside). These studies have found that AMPK attenuates HSL activity during exercise (159, 160, 165). AMPK

phosphorylation of HSL also prevents activation by PKA, as demonstrated in L6 myotubes treated with AICAR which result in reduced PKA stimulated HSL activity (165). It has been suggested that increased activation of AMPK is capable of overriding elevations in adrenaline and PKA stimulation of HSL, this is consistent with a reduction in fatty acid oxidation during intense exercise.

In support of this a study involving 90min of steady state cycling found that HSL Ser565, which is proposed to prevent the increase in HSL activity (61), was phosphorylated at 90 min of exercise as well as in recovery (161). This finding was consistent with the significant activation of AMPK signaling at these times points. The increased AMPK phosphorylation and HSL Ser565 phosphorylation at 90 min corresponded to decreased HSL activity, consistent with previous results suggesting that phosphorylation of HSL by AMPK prevents activation of this enzyme (61, 165). HSL activity declined to resting rates late in prolonged exercise (90 min) despite high circulating epinephrine concentrations and further increases in the phosphorylation on the PKA site Ser563 (161), which would be expected to increase HSL activity (83, 91, 167). In addition to increased AMPK activity during exercise, the observation that fatty acyl-CoA accumulation occurs at high exercise intensities may also allosterically inhibit HSL activity (81). Consistent with the *in vivo* findings, results from *in vitro* studies found that AMPK inhibited epinephrine-induced HSL activity in L6 myotubes and was associated with reduced HSL Ser660 but not Ser563 phosphorylation. HSL activity was reduced in L6 myotubes expressing constitutively active AMPK, confirming the inhibitory effects of AMPK on HSL activity (161) (Figure 8).

Contractile Stimulation

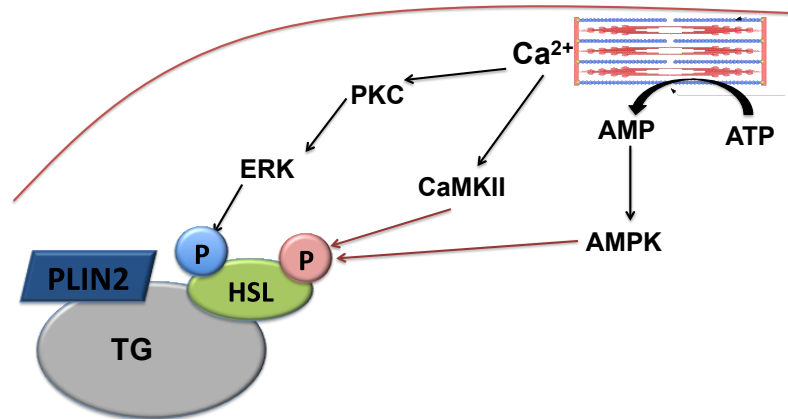


Figure 8

Contraction stimulated lipolysis. Increased calcium concentrations activate both PKC and CaMK. PKC activates ERK which phosphorylates HSL at Ser660 resulting in increased activation. CaMK phosphorylates the inhibitory Ser565 site of HSL. High ATP turnover during exercise leads to increased AMP and AMPK activity. AMPK phosphorylates Ser565 on HSL and inhibits its activity. (Ca²⁺=calcium; PKC=protein kinase C; ERK=extracellular signal regulated kinase; CaMK=calcium calmodulin kinase; ATGL=adipose triglyceride lipase; HSL=hormone sensitive lipase).

Summary of HSL regulation

The regulation of HSL activity in skeletal muscle involves multiple kinases and seems to be exercise intensity and duration dependent. HSL activity in skeletal muscle is increased during exercise due to phosphorylation of the PKA sites (Ser563, Ser659, and Ser660), increased calcium leads to ERK activation which phosphorylates and activates HSL Ser660, while CaMK and AMPK phosphorylate and inhibit HSL Ser565 (124, 161). These opposing pathways appear to indicate the

presence of a mechanism by which HSL activity can be adjusted relative to exercise intensity and duration (161).

Skeletal Muscle ATGL Regulation

ATGL, also known as desnutrin and calcium-independent phospholipase A2 ζ , was recently identified by three separate laboratories (77, 154, 187) and is now known as the predominant triacylglycerol lipase. ATGL is critical for efficient triacylglycerol lipolysis in all tissues assessed to date (2, 69, 151), including skeletal muscle (10, 75). Unlike the wide substrate specificity of HSL, ATGL exhibits a strong specificity for triglycerides (77, 187, 154, 187), has very low diglyceride-hydrolase activity (92), and has no cholesteryl or retinyl esterases activities (187). Interestingly, ATGL also possess transacylase activity (77), but the importance of this has yet to be determined. Although most abundantly expressed in adipose tissue, ATGL is also expressed in cardiac tissue and skeletal muscle (187), and like HSL, appears to be most abundant in type I compared to type II muscle fibres (80).

The regulation of ATGL activity is poorly understood in skeletal muscle. In adipose tissue the activity of ATGL is enhanced upon interaction with its co-activator, comparative gene identification 58 (CGI-58) (92), and reduced by G(0)/G(1) switch gene 2 (G0S2) (180). Both CGI-58 and G0S2 are expressed in skeletal muscle (9, 92, 180), although their role here has is yet to be established. The importance of CGI-58 in ATGL activation in skeletal muscle is demonstrated by the fact that individuals who have a mutation of CGI-58 have an increased IMTG content

(92). Further, overexpression of ATGL in non-adipocyte cells (Hela cells and myotubes) enhances the degradation of triglycerides (10, 132), whereas knockout of ATGL in mice results in IMTG accumulation (69). Along with increased IMTG content, ATGL knockout mice also demonstrate an increased reliance on extracellular fatty acids for oxidation indicating that the ability to mobilize IMTGs is impaired (75). Finally, in human skeletal muscle ATGL protein content was significantly increased after 8 wk of endurance exercise training, consistent with increased IMTG stores and an enhanced capacity to utilize IMTGs (4).

ATGL Phosphorylation

It has been known for some time that ATGL can be phosphorylated on Ser404 (corresponding to Ser406 in mice) (11), however the upstream kinases have remained elusive and thus far there is only one study in skeletal muscle. Ahmadian et al. (1) showed that AMPK phosphorylates ATGL at Ser406 in HEK293 (Human Embryonic Kidney) cells and that lipolysis is decreased in murine adipocytes expressing an ATGL Ser406A mutation, suggesting that AMPK phosphorylates and activates ATGL. The finding that AMPK phosphorylation enhances ATGL activity and lipolysis is interesting because it contrasts other studies demonstrating decreased HSL activity and lipolysis with AMPK activation (56). This indicates that the triacylglycerol (ATGL) and diacylglycerol (HSL) lipases, which normally act in unison to completely degrade triacylglycerol, are oppositely regulated by AMPK

phosphorylation. However, few studies have investigated the role of AMPK on ATGL phosphorylation in skeletal muscle.

A recent study investigated the phosphorylation state of Ser404 in human skeletal muscle during 90min of steady state cycling (106). They found that ATGL Ser404 phosphorylation did not increase from rest during the 90min of exercise, indicating the ATGL phosphorylation may not be an important regulator of skeletal muscle lipolysis. In addition, there was no evidence of a physical interaction between ATGL and AMPK after immunoprecipitation. To further investigate a role for AMPK in ATGL phosphorylation, this group used cultured myotubes and pharmacologically activated AMPK which resulted in no change in ATGL Ser404 phosphorylation (106). Due to the importance of PKA stimulation in activating lipolysis in skeletal muscle (79, 91, 167), this group also hypothesized that PKA might phosphorylate ATGL. Using forskolin to activate PKA in cultured myotubes they found no effect of on ATGL Ser406 phosphorylation. Together, these studies suggest that neither AMPK nor PKA appear to be an important upstream kinase of ATGL in skeletal muscle (106). Therefore, further work is necessary to determine the importance of ATGL phosphorylation and to determine if phosphorylation of ATGL is required to modulate its interaction with its co-activator, CGI-58, or for ATGL's translocation and interaction with the lipid droplet.

Skeletal Muscle PLINs

Due to the fact that PLIN1 is not expressed in skeletal muscle (171), it is likely that other PLIN proteins may be required to regulate lipolysis in this tissue. It has been suggested that PLIN2, PLIN3, and PLIN5 play a large role in regulating lipolysis in skeletal muscle (120, 128, 130, 131). As a result these are the PLIN members studied in this thesis. Given that the location of a protein is generally related to its physiological function, the myocellular locations of both PLIN2 and PLIN5 are crucial in understanding their roles. PLIN5 has the ability to translocate on and off of the lipid droplet and has also been found located on mitochondria, therefore an understanding of its subcellular location with contraction is important. Further, interactions between these PLIN proteins and both ATGL and HSL have not been investigated in skeletal muscle. In addition, the reversible phosphorylation of PLIN1 is necessary for lipolytic activation in adipose tissue (104, 113, 139, 143), however the phosphorylation state of the remaining PLINs has yet to be investigated in skeletal muscle. A phosphorylation site has been identified on PLIN2 (Ser 291) (11), and PLIN3 (Ser 245) (72), and some evidence indicates that PLIN5 is a substrate for PKA phosphorylation (156). It is possible that the phosphorylation of PLIN2, PLIN3, or PLIN5 may be required to optimally position ATGL, CGI-58, and/or HSL for activation of lipolysis in skeletal muscle. While the importance of PLIN1 in adipose tissue lipolysis has been established, the role of the remaining PLIN family proteins in skeletal muscle remains unknown.

Chapter 3

Statement of the problem

The importance of proper storage and use of IMTGs has become increasingly more evident in the past decade. Identifying the underlying mechanisms of how IMTG are mobilized from lipid droplets may provide key information in regards to the development of metabolic diseases caused by lipotoxicity. At the time that this thesis was undertaken (2009), growing evidence pointed toward the PLIN proteins in regulating lipid storage and use. However, there were only a handful of studies investigating PLIN proteins in skeletal muscle and our initial studies were based on what little information was known at that time. Therefore the primary objective of this thesis was to expand our knowledge of the role of PLIN proteins in relation to skeletal muscle lipolysis.

The first study investigates the subcellular distribution of PLIN2, PLIN5, and lipid droplets at rest and following 30min of stimulated contraction. The second study describes the skeletal muscle PLIN interactions with to ATGL and CGI-58 at rest and following contraction. Finally, due to the importance of PLIN1 phosphorylation in initiating lipolysis in adipose tissue it was important to determine if this occurred with any of the skeletal muscle PLIN proteins. Thus, the third study investigated skeletal muscle PLIN phosphorylation at rest, following epinephrine incubation, and following stimulated contraction. The isolated muscle technique utilized in all three studies provided a well-suited model to investigate lipolysis in a highly oxidative muscle (soleus).

Purposes and Hypotheses

Study 1 – Chapter 4

The purpose of this study was two-fold, first to investigate the myocellular location and co-localization of lipid droplets, PLIN2, and PLIN5 and second to determine if PLIN5 is recruited to lipid droplets with contraction. It was hypothesized that contraction would recruit a cytosolic pool of PLIN5 to the lipid droplet surface allowing fatty acids to be oxidized and used for energy.

Study 2 – Chapter 5

The purpose of this study was to first determine if there were any interactions between PLIN2, 3, and 5 with ATGL and/or CGI-58 at rest, and second, to evaluate the effects of lipolytic muscle contraction on these interactions in an isolated muscle preparation. We hypothesized that PLIN proteins would contribute to the regulation of skeletal muscle lipolysis by preventing the interaction of ATGL and CGI-58 at rest.

Study 3 – Chapter 6

The purpose of this study was to examine the isolated and additive effects of epinephrine and contraction on skeletal muscle PLIN protein to ATGL and HSL interactions as well as phosphorylation status. A major objective of this study was to determine if PLIN2, PLIN3, and/or PLIN5 are phosphorylated and whether this changes during lipolysis. If so, this study aimed to separate adrenergic and/or contractile stimulation. A second objective of this study was to investigate the role

of PLIN2, PLIN3 and PLIN5 in governing the accessibility of ATGL and HSL via direct protein-protein interactions during these perturbations. We hypothesized that skeletal muscle PLIN to lipase interactions are governed by PLIN phosphorylation status.

References

1. **Ahmadian M, Abbott MJ, Tang T, Hudak CS, Kim Y, Bruss M, Hellerstein MK, Lee HY, Samuel VT, Shulman GI, Wang Y, Duncan RE, Kang C, and Sul HS.** Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell metabolism* 13: 739-748, 2011.
2. **Ahmadian M, Duncan RE, Varady KA, Frasson D, Hellerstein MK, Birkenfeld AL, Samuel VT, Shulman GI, Wang Y, Kang C, and Sul HS.** Adipose overexpression of desnutrin promotes fatty acid use and attenuates diet-induced obesity. *Diabetes* 58: 855-866, 2009.
3. **Ahmed W, Ziouzenkova O, Brown J, Devchand P, Francis S, Kadakia M, Kanda T, Orasanu G, Sharlach M, Zandbergen F, and Plutzky J.** PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? *Journal of internal medicine* 262: 184-198, 2007.
4. **Alsted TJ, Nybo L, Schweiger M, Fledelius C, Jacobsen P, Zimmermann R, Zechner R, and Kiens B.** Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. *American journal of physiology Endocrinology and metabolism* 296: E445-453, 2009.
5. **Amati F, Dube JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, Switzer GE, Bickel PE, Stefanovic-Racic M, Toledo FG, and Goodpaster BH.** Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* 60: 2588-2597, 2011.
6. **Andersson L, Bostrom P, Ericson J, Rutberg M, Magnusson B, Marchesan D, Ruiz M, Asp L, Huang P, Frohman MA, Boren J, and Olofsson SO.** PLD1 and ERK2 regulate cytosolic lipid droplet formation. *Journal of cell science* 119: 2246-2257, 2006.
7. **Anthonsen MW, Ronnstrand L, Wernstedt C, Degerman E, and Holm C.** Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *The Journal of biological chemistry* 273: 215-221, 1998.
8. **Arimura N, Horiba T, Imagawa M, Shimizu M, and Sato R.** The peroxisome proliferator-activated receptor gamma regulates expression of the perilipin gene in adipocytes. *The Journal of biological chemistry* 279: 10070-10076, 2004.
9. **Badin PM, Loubiere C, Coonen M, Louche K, Tavernier G, Bourlier V, Mairal A, Rustan AC, Smith SR, Langin D, and Moro C.** Regulation of skeletal muscle lipolysis and oxidative metabolism by the co-lipase CGI-58. *Journal of lipid research* 53: 839-848, 2012.
10. **Badin PM, Louche K, Mairal A, Liebisch G, Schmitz G, Rustan AC, Smith SR, Langin D, and Moro C.** Altered skeletal muscle lipase expression and activity contribute to insulin resistance in humans. *Diabetes* 60: 1734-1742, 2011.
11. **Bartz R, Zehmer JK, Zhu M, Chen Y, Serrero G, Zhao Y, and Liu P.** Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation. *Journal of proteome research* 6: 3256-3265, 2007.

12. **Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, and Sztalryd C.** Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* 57: 2037-2045, 2008.
13. **Beller M, Thiel K, Thul PJ, and Jackle H.** Lipid droplets: a dynamic organelle moves into focus. *FEBS letters* 584: 2176-2182, 2010.
14. **Bickel PE, Tansey JT, and Welte MA.** PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et biophysica acta* 1791: 419-440, 2009.
15. **Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondinone CM, Theodorakis JL, Greenberg AS, and Londos C.** Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *Journal of lipid research* 36: 1211-1226, 1995.
16. **Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondinone CM, Theodorakis JL, Greenberg AS, and Londos C.** Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *Journal of lipid research* 36: 1211-1226, 1995.
17. **Bosma M, Hesselink MK, Sparks LM, Timmers S, Ferraz MJ, Mattijssen F, van Beurden D, Schaart G, de Baets MH, Verheyen FK, Kersten S, and Schrauwen P.** Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes* 61: 2679-2690, 2012.
18. **Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P, and Hesselink MK.** The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochemistry and cell biology* 137: 205-216, 2012.
19. **Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S, and Hesselink MK.** Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochimica et biophysica acta* 1831: 844-852, 2013.
20. **Bostrom P, Rutberg M, Ericsson J, Holmdahl P, Andersson L, Frohman MA, Boren J, and Olofsson SO.** Cytosolic lipid droplets increase in size by microtubule-dependent complex formation. *Arteriosclerosis, thrombosis, and vascular biology* 25: 1945-1951, 2005.
21. **Brasaemle DL.** Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of lipid research* 48: 2547-2559, 2007.
22. **Brasaemle DL.** Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of lipid research* 48: 2547-2559, 2007.
23. **Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, and Londos C.** Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of lipid research* 38: 2249-2263, 1997.
24. **Brasaemle DL, Levin DM, Adler-Wailes DC, and Londos C.** The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive

- lipase to the surfaces of lipid storage droplets. *Biochimica et biophysica acta* 1483: 251-262, 2000.
25. **Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, and Londos C.** Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *The Journal of biological chemistry* 275: 38486-38493, 2000.
26. **Brasaemle DL and Wolins NE.** Packaging of fat: an evolving model of lipid droplet assembly and expansion. *The Journal of biological chemistry* 287: 2273-2279, 2012.
27. **Brown DA.** Lipid droplets: proteins floating on a pool of fat. *Current biology : CB* 11: R446-449, 2001.
28. **Buhman KF, Accad M, and Farese RV.** Mammalian acyl-CoA:cholesterol acyltransferases. *Biochimica et biophysica acta* 1529: 142-154, 2000.
29. **Bulankina AV, Deggerich A, Wenzel D, Mutenda K, Wittmann JG, Rudolph MG, Burger KN, and Honing S.** TIP47 functions in the biogenesis of lipid droplets. *The Journal of cell biology* 185: 641-655, 2009.
30. **Carlson LA, Ekelund LG, and Froberg SO.** Concentration of triglycerides, phospholipids and glycogen in skeletal muscle and of free fatty acids and beta-hydroxybutyric acid in blood in man in response to exercise. *European journal of clinical investigation* 1: 248-254, 1971.
31. **Carmen GY and Victor SM.** Signalling mechanisms regulating lipolysis. *Cellular signalling* 18: 401-408, 2006.
32. **Chang BH, Li L, Paul A, Taniguchi S, Nannegari V, Heird WC, and Chan L.** Protection against fatty liver but normal adipogenesis in mice lacking adipose differentiation-related protein. *Molecular and cellular biology* 26: 1063-1076, 2006.
33. **Coen PM, Dube JJ, Amati F, Stefanovic-Racic M, Ferrell RE, Toledo FG, and Goodpaster BH.** Insulin resistance is associated with higher intramyocellular triglycerides in type I but not type II myocytes concomitant with higher ceramide content. *Diabetes* 59: 80-88, 2010.
34. **Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, and Nebb HI.** LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochimica et biophysica acta* 1771: 210-227, 2007.
35. **Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, and Nebb HI.** LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochimica et biophysica acta* 1771: 210-227, 2007.
36. **Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, and Nebb HI.** Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-gamma. *Diabetes* 53: 1243-1252, 2004.
37. **De Feyter HM, Schaart G, Hesselink MK, Schrauwen P, Nicolay K, and Prompers JJ.** Regional variations in intramyocellular lipid concentration correlate with muscle fiber type distribution in rat tibialis anterior muscle. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine* 56: 19-25, 2006.

38. **de Wilde J, Smit E, Snepvangers FJ, de Wit NW, Mohren R, Hulshof MF, and Mariman EC.** Adipophilin protein expression in muscle--a possible protective role against insulin resistance. *The FEBS journal* 277: 761-773, 2010.
39. **Denton RM and Randle PJ.** Concentrations of glycerides and phospholipids in rat heart and gastrocnemius muscles. Effects of alloxan-diabetes and perfusion. *The Biochemical journal* 104: 416-422, 1967.
40. **Diaz E and Pfeffer SR.** TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell* 93: 433-443, 1998.
41. **Donsmark M, Langfort J, Holm C, Ploug T, and Galbo H.** Contractions activate hormone-sensitive lipase in rat muscle by protein kinase C and mitogen-activated protein kinase. *The Journal of physiology* 550: 845-854, 2003.
42. **Donsmark M, Langfort J, Holm C, Ploug T, and Galbo H.** Regulation and role of hormone-sensitive lipase in rat skeletal muscle. *The Proceedings of the Nutrition Society* 63: 309-314, 2004.
43. **Ducharme NA and Bickel PE.** Lipid droplets in lipogenesis and lipolysis. *Endocrinology* 149: 942-949, 2008.
44. **Dyck DJ and Bonen A.** Muscle contraction increases palmitate esterification and oxidation and triacylglycerol oxidation. *The American journal of physiology* 275: E888-896, 1998.
45. **Edvardsson U, Ljungberg A, Linden D, William-Olsson L, Peilot-Sjogren H, Ahnmark A, and Oscarsson J.** PPARalpha activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes. *Journal of lipid research* 47: 329-340, 2006.
46. **Egan JJ, Greenberg AS, Chang MK, Wek SA, Moos MC, Jr., and Londos C.** Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. *Proceedings of the National Academy of Sciences of the United States of America* 89: 8537-8541, 1992.
47. **Essen B, Hagenfeldt L, and Kaijser L.** Utilization of blood-borne and intramuscular substrates during continuous and intermittent exercise in man. *The Journal of physiology* 265: 489-506, 1977.
48. **Fan B, Gu JQ, Yan R, Zhang H, Feng J, and Ikuyama S.** High glucose, insulin and free fatty acid concentrations synergistically enhance perilipin 3 expression and lipid accumulation in macrophages. *Metabolism: clinical and experimental* 62: 1168-1179, 2013.
49. **Farese RV, Jr., Cases S, and Smith SJ.** Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase. *Current opinion in lipidology* 11: 229-234, 2000.
50. **Fredrikson G, Stralfors P, Nilsson NO, and Belfrage P.** Hormone-sensitive lipase from adipose tissue of rat. *Methods in enzymology* 71 Pt C: 636-646, 1981.
51. **Fredrikson G, Tornqvist H, and Belfrage P.** Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol. *Biochimica et biophysica acta* 876: 288-293, 1986.
52. **Fritz IB, Davis DG, Holtrop RH, and Dundee H.** Fatty acid oxidation by skeletal muscle during rest and activity. *The American journal of physiology* 194: 379-386, 1958.

53. **Froberg SO, Hultman E, and Nilsson LH.** Effect of noradrenaline on triglyceride and glycogen concentrations in liver and muscle from man. *Metabolism: clinical and experimental* 24: 119-126, 1975.
54. **Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, and Burrell MA.** The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *American journal of physiology Endocrinology and metabolism* 280: E827-847, 2001.
55. **Fukushima M, Enjoji M, Kohjima M, Sugimoto R, Ohta S, Kotoh K, Kuniyoshi M, Kobayashi K, Imamura M, Inoguchi T, Nakamuta M, and Nawata H.** Adipose differentiation related protein induces lipid accumulation and lipid droplet formation in hepatic stellate cells. *In vitro cellular & developmental biology Animal* 41: 321-324, 2005.
56. **Gaidhu MP, Fediuc S, Anthony NM, So M, Mirpourian M, Perry RL, and Ceddia RB.** Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. *Journal of lipid research* 50: 704-715, 2009.
57. **Gandotra S, Lim K, Girousse A, Saudek V, O'Rahilly S, and Savage DB.** Human frame shift mutations affecting the carboxyl terminus of perilipin increase lipolysis by failing to sequester the adipose triglyceride lipase (ATGL) coactivator AB-hydrolase-containing 5 (ABHD5). *The Journal of biological chemistry* 286: 34998-35006, 2011.
58. **Gao J and Serrero G.** Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. *The Journal of biological chemistry* 274: 16825-16830, 1999.
59. **Garcia A, Sekowski A, Subramanian V, and Brasaemle DL.** The central domain is required to target and anchor perilipin A to lipid droplets. *The Journal of biological chemistry* 278: 625-635, 2003.
60. **Garton AJ, Campbell DG, Carling D, Hardie DG, Colbran RJ, and Yeaman SJ.** Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. *European journal of biochemistry / FEBS* 179: 249-254, 1989.
61. **Garton AJ and Yeaman SJ.** Identification and role of the basal phosphorylation site on hormone-sensitive lipase. *European journal of biochemistry / FEBS* 191: 245-250, 1990.
62. **Granneman JG, Moore HP, Krishnamoorthy R, and Rathod M.** Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *The Journal of biological chemistry* 284: 34538-34544, 2009.
63. **Granneman JG, Moore HP, Mottillo EP, and Zhu Z.** Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *The Journal of biological chemistry* 284: 3049-3057, 2009.
64. **Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L.** Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
65. **Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, and Londos C.** Perilipin, a major hormonally regulated adipocyte-specific

- phosphoprotein associated with the periphery of lipid storage droplets. *The Journal of biological chemistry* 266: 11341-11346, 1991.
66. **Greenberg AS, Egan JJ, Wek SA, Moos MC, Jr., Londos C, and Kimmel AR.** Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proceedings of the National Academy of Sciences of the United States of America* 90: 12035-12039, 1993.
67. **Greenberg AS, Shen WJ, Muliro K, Patel S, Souza SC, Roth RA, and Kraemer FB.** Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. *The Journal of biological chemistry* 276: 45456-45461, 2001.
68. **Gruber A, Cornaciu I, Lass A, Schweiger M, Poeschl M, Eder C, Kumari M, Schoiswohl G, Wolinski H, Kohlwein SD, Zechner R, Zimmermann R, and Oberer M.** The N-terminal region of comparative gene identification-58 (CGI-58) is important for lipid droplet binding and activation of adipose triglyceride lipase. *The Journal of biological chemistry* 285: 12289-12298, 2010.
69. **Haemmerle G, Lass A, Zimmermann R, Gorkiewicz G, Meyer C, Rozman J, Heldmaier G, Maier R, Theussl C, Eder S, Kratky D, Wagner EF, Klingenspor M, Hoefler G, and Zechner R.** Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312: 734-737, 2006.
70. **Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, and Zechner R.** Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. *The Journal of biological chemistry* 277: 4806-4815, 2002.
71. **Heid HW, Moll R, Schwetlick I, Rackwitz HR, and Keenan TW.** Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell and tissue research* 294: 309-321, 1998.
72. **Hickenbottom SJ, Kimmel AR, Londos C, and Hurley JH.** Structure of a lipid droplet protein; the PAT family member TIP47. *Structure* 12: 1199-1207, 2004.
73. **Hocsak E, Racz B, Szabo A, Mester L, Rapolti E, Pozsgai E, Javor S, Bellyei S, Gallyas F, Jr., Sumegi B, and Szigeti A.** TIP47 protects mitochondrial membrane integrity and inhibits oxidative-stress-induced cell death. *FEBS letters*, 2010.
74. **Hopp JF and Palmer WK.** Electrical stimulation alters fatty acid metabolism in isolated skeletal muscle. *J Appl Physiol* 68: 2473-2481, 1990.
75. **Huijsman E, van de Par C, Economou C, van der Poel C, Lynch GS, Schoiswohl G, Haemmerle G, Zechner R, and Watt MJ.** Adipose triacylglycerol lipase deletion alters whole body energy metabolism and impairs exercise performance in mice. *American journal of physiology Endocrinology and metabolism* 297: E505-513, 2009.
76. **Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, and Nawata H.** ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American journal of physiology Endocrinology and metabolism* 283: E775-783, 2002.
77. **Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, and Gross RW.** Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase

- and acylglycerol transacylase activities. *The Journal of biological chemistry* 279: 48968-48975, 2004.
78. **Jiang HP and Serrero G.** Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proceedings of the National Academy of Sciences of the United States of America* 89: 7856-7860, 1992.
79. **Jocken JW, Roepstorff C, Goossens GH, van der Baan P, van Baak M, Saris WH, Kiens B, and Blaak EE.** Hormone-sensitive lipase serine phosphorylation and glycerol exchange across skeletal muscle in lean and obese subjects: effect of beta-adrenergic stimulation. *Diabetes* 57: 1834-1841, 2008.
80. **Jocken JW, Smit E, Goossens GH, Essers YP, van Baak MA, Mensink M, Saris WH, and Blaak EE.** Adipose triglyceride lipase (ATGL) expression in human skeletal muscle is type I (oxidative) fiber specific. *Histochemistry and cell biology* 129: 535-538, 2008.
81. **Kiens B, Roemen TH, and van der Vusse GJ.** Muscular long-chain fatty acid content during graded exercise in humans. *The American journal of physiology* 276: E352-357, 1999.
82. **Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, and Londos C.** Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *Journal of lipid research* 51: 468-471, 2010.
83. **Kjaer M, Howlett K, Langfort J, Zimmerman-Belsing T, Lorentsen J, Bulow J, Ihlemann J, Feldt-Rasmussen U, and Galbo H.** Adrenaline and glycogenolysis in skeletal muscle during exercise: a study in adrenalectomised humans. *The Journal of physiology* 528 Pt 2: 371-378, 2000.
84. **Koves TR, Sparks LM, Kovalik JP, Mosedale M, Arumugam R, DeBalsi KL, Everingham K, Thorne L, Phielix E, Meex RC, Kien CL, Hesselink MK, Schrauwen P, and Muoio DM.** PPARgamma coactivator-1alpha contributes to exercise-induced regulation of intramuscular lipid droplet programming in mice and humans. *Journal of lipid research* 54: 522-534, 2013.
85. **Krise JP, Sincock PM, Orsel JG, and Pfeffer SR.** Quantitative analysis of TIP47-receptor cytoplasmic domain interactions: implications for endosome-to-trans Golgi network trafficking. *The Journal of biological chemistry* 275: 25188-25193, 2000.
86. **Kuerschner L, Moessinger C, and Thiele C.** Imaging of lipid biosynthesis: how a neutral lipid enters lipid droplets. *Traffic* 9: 338-352, 2008.
87. **Kuramoto K, Okamura T, Yamaguchi T, Nakamura TY, Wakabayashi S, Morinaga H, Nomura M, Yanase T, Otsu K, Usuda N, Matsumura S, Inoue K, Fushiki T, Kojima Y, Hashimoto T, Sakai F, Hirose F, and Osumi T.** Perilipin 5, a lipid droplet-binding protein, protects heart from oxidative burden by sequestering fatty acid from excessive oxidation. *The Journal of biological chemistry* 287: 23852-23863, 2012.
88. **Lafontan M and Langin D.** Lipolysis and lipid mobilization in human adipose tissue. *Progress in lipid research* 48: 275-297, 2009.
89. **Langfort J, Ploug T, Ihlemann J, Baranczuk E, Donsmark M, Gorski J, and Galbo H.** Additivity of adrenaline and contractions on hormone-sensitive lipase, but

not on glycogen phosphorylase, in rat muscle. *Acta physiologica Scandinavica* 178: 51-60, 2003.

90. **Langfort J, Ploug T, Ihlemann J, Holm C, and Galbo H.** Stimulation of hormone-sensitive lipase activity by contractions in rat skeletal muscle. *The Biochemical journal* 351: 207-214, 2000.
91. **Langfort J, Ploug T, Ihlemann J, Saldo M, Holm C, and Galbo H.** Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle. *The Biochemical journal* 340 (Pt 2): 459-465, 1999.
92. **Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, Kienesberger P, Strauss JG, Gorkiewicz G, and Zechner R.** Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chananin-Dorfman Syndrome. *Cell metabolism* 3: 309-319, 2006.
93. **Li M, Paran C, Wolins NE, and Horowitz JF.** High muscle lipid content in obesity is not due to enhanced activation of key triglyceride esterification enzymes or the suppression of lipolytic proteins. *American journal of physiology Endocrinology and metabolism* 300: E699-707, 2011.
94. **Listenberger LL and Brown DA.** Fluorescent detection of lipid droplets and associated proteins. *Current protocols in cell biology / editorial board, Juan S Bonifacino [et al]* Chapter 24: Unit 24 22, 2007.
95. **Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS, and Schaffer JE.** Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* 100: 3077-3082, 2003.
96. **Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, and Brown DA.** Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *Journal of lipid research* 48: 2751-2761, 2007.
97. **Liu P, Ying Y, Zhao Y, Mundy DI, Zhu M, and Anderson RG.** Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *The Journal of biological chemistry* 279: 3787-3792, 2004.
98. **Londos C, Brasaemle DL, Gruia-Gray J, Servetnick DA, Schultz CJ, Levin DM, and Kimmel AR.** Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochemical Society transactions* 23: 611-615, 1995.
99. **Londos C, Brasaemle DL, Schultz CJ, Segrest JP, and Kimmel AR.** Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Seminars in cell & developmental biology* 10: 51-58, 1999.
100. **Londos C, Sztalryd C, Tansey JT, and Kimmel AR.** Role of PAT proteins in lipid metabolism. *Biochimie* 87: 45-49, 2005.
101. **Lu X, Gruia-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, Londos C, and Kimmel AR.** The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mammalian genome : official journal of the International Mammalian Genome Society* 12: 741-749, 2001.
102. **Lu X, Yang X, and Liu J.** Differential control of ATGL-mediated lipid droplet degradation by CGI-58 and GOS2. *Cell Cycle* 9: 2719-2725, 2010.

103. **Marchesan D, Rutberg M, Andersson L, Asp L, Larsson T, Boren J, Johansson BR, and Olofsson SO.** A phospholipase D-dependent process forms lipid droplets containing caveolin, adipocyte differentiation-related protein, and vimentin in a cell-free system. *The Journal of biological chemistry* 278: 27293-27300, 2003.
104. **Marcinkiewicz A, Gauthier D, Garcia A, and Brasaemle DL.** The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. *The Journal of biological chemistry* 281: 11901-11909, 2006.
105. **Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, and Chan L.** Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nature genetics* 26: 474-479, 2000.
106. **Mason RR, Meex RC, Lee-Young R, Canny BJ, and Watt MJ.** Phosphorylation of adipose triglyceride lipase Ser(404) is not related to 5'-AMPK activation during moderate-intensity exercise in humans. *American journal of physiology Endocrinology and metabolism* 303: E534-541, 2012.
107. **Masuda Y, Itabe H, Odaki M, Hama K, Fujimoto Y, Mori M, Sasabe N, Aoki J, Arai H, and Takano T.** ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. *Journal of lipid research* 47: 87-98, 2006.
108. **Meex RC, Schrauwen P, and Hesselink MK.** Modulation of myocellular fat stores: lipid droplet dynamics in health and disease. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R913-924, 2009.
109. **Melzer W, Herrmann-Frank A, and Luttgau HC.** The role of Ca²⁺ ions in excitation-contraction coupling of skeletal muscle fibres. *Biochimica et biophysica acta* 1241: 59-116, 1995.
110. **Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MK.** Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *The Journal of clinical endocrinology and metabolism* 94: 4077-4085, 2009.
111. **Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B, and Kimmel AR.** Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. *The Journal of biological chemistry* 277: 32253-32257, 2002.
112. **Miyoshi H, Perfield JW, 2nd, Souza SC, Shen WJ, Zhang HH, Stancheva ZS, Kraemer FB, Obin MS, and Greenberg AS.** Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis in adipocytes. *The Journal of biological chemistry* 282: 996-1002, 2007.
113. **Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS.** Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
114. **Monetti M, Levin MC, Watt MJ, Sajan MP, Marmor S, Hubbard BK, Stevens RD, Bain JR, Newgard CB, Farese RV, Sr., Hevener AL, and Farese RV, Jr.** Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell metabolism* 6: 69-78, 2007.

115. **Muoio DM, Seefeld K, Witters LA, and Coleman RA.** AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *The Biochemical journal* 338 (Pt 3): 783-791, 1999.
116. **Murphy DJ.** The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progress in lipid research* 40: 325-438, 2001.
117. **Murphy S, Martin S, and Parton RG.** Lipid droplet-organelle interactions; sharing the fats. *Biochimica et biophysica acta* 1791: 441-447, 2009.
118. **Napoli R, Gibson L, Hirshman MF, Boppart MD, Dufresne SD, Horton ES, and Goodyear LJ.** Epinephrine and insulin stimulate different mitogen-activated protein kinase signaling pathways in rat skeletal muscle. *Diabetes* 47: 1549-1554, 1998.
119. **Peters SJ, Dyck DJ, Bonen A, and Spriet LL.** Effects of epinephrine on lipid metabolism in resting skeletal muscle. *The American journal of physiology* 275: E300-309, 1998.
120. **Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA, and Tarnopolsky MA.** Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 37: 724-735, 2012.
121. **Phillips SA, Choe CC, Ciaraldi TP, Greenberg AS, Kong AP, Baxi SC, Christiansen L, Mudaliar SR, and Henry RR.** Adipocyte differentiation-related protein in human skeletal muscle: relationship to insulin sensitivity. *Obesity research* 13: 1321-1329, 2005.
122. **Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, and Ploug T.** Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *Journal of lipid research* 47: 2392-2399, 2006.
123. **Robenek H, Hofnagel O, Buers I, Robenek MJ, Troyer D, and Severs NJ.** Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. *Journal of cell science* 119: 4215-4224, 2006.
124. **Roepstorff C, Donsmark M, Thiele M, Vistisen B, Stewart G, Vissing K, Schjerling P, Hardie DG, Galbo H, and Kiens B.** Sex differences in hormone-sensitive lipase expression, activity, and phosphorylation in skeletal muscle at rest and during exercise. *American journal of physiology Endocrinology and metabolism* 291: E1106-1114, 2006.
125. **Scherer PE, Bickel PE, Kotler M, and Lodish HF.** Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nature biotechnology* 16: 581-586, 1998.
126. **Schrauwen-Hinderling VB, Schrauwen P, Hesselink MK, van Engelshoven JM, Nicolay K, Saris WH, Kessels AG, and Kooi ME.** The increase in intramyocellular lipid content is a very early response to training. *The Journal of clinical endocrinology and metabolism* 88: 1610-1616, 2003.
127. **Shaw CS, Jones DA, and Wagenmakers AJ.** Network distribution of mitochondria and lipid droplets in human muscle fibres. *Histochemistry and cell biology* 129: 65-72, 2008.

128. **Shaw CS, Shepherd SO, Wagenmakers AJ, Hansen D, Dendale P, and van Loon LJ.** Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibres of patients with type 2 diabetes. *American journal of physiology Endocrinology and metabolism*, 2012.
129. **Shaw CS, Sherlock M, Stewart PM, and Wagenmakers AJ.** Adipophilin distribution and colocalization with lipid droplets in skeletal muscle. *Histochemistry and cell biology* 131: 575-581, 2009.
130. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS.** Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Experimental physiology* 97: 970-980, 2012.
131. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS.** Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *The Journal of physiology* 591: 657-675, 2013.
132. **Smirnova E, Goldberg EB, Makarova KS, Lin L, Brown WJ, and Jackson CL.** ATGL has a key role in lipid droplet/adiposome degradation in mammalian cells. *EMBO reports* 7: 106-113, 2006.
133. **Smith AC, Bruce CR, and Dyck DJ.** AMP kinase activation with AICAR further increases fatty acid oxidation and blunts triacylglycerol hydrolysis in contracting rat soleus muscle. *The Journal of physiology* 565: 547-553, 2005.
134. **Souza SC, Muliro KV, Liscum L, Lien P, Yamamoto MT, Schaffer JE, Dallal GE, Wang X, Kraemer FB, Obin M, and Greenberg AS.** Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. *The Journal of biological chemistry* 277: 8267-8272, 2002.
135. **Spriet LL, Heigenhauser GJ, and Jones NL.** Endogenous triacylglycerol utilization by rat skeletal muscle during tetanic stimulation. *J Appl Physiol* 60: 410-415, 1986.
136. **Steinberg D and Huttunen JK.** The role of cyclic AMP in activation of hormone-sensitive lipase of adipose tissue. *Advances in cyclic nucleotide research* 1: 47-62, 1972.
137. **Stellingwerff T, Boon H, Jonkers RA, Senden JM, Spriet LL, Koopman R, and van Loon LJ.** Significant intramyocellular lipid use during prolonged cycling in endurance-trained males as assessed by three different methodologies. *American journal of physiology Endocrinology and metabolism* 292: E1715-1723, 2007.
138. **Stone SJ, Levin MC, Zhou P, Han J, Walther TC, and Farese RV, Jr.** The endoplasmic reticulum enzyme DGAT2 is found in mitochondria-associated membranes and has a mitochondrial targeting signal that promotes its association with mitochondria. *The Journal of biological chemistry* 284: 5352-5361, 2009.
139. **Su CL, Sztalryd C, Contreras JA, Holm C, Kimmel AR, and Londos C.** Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. *The Journal of biological chemistry* 278: 43615-43619, 2003.
140. **Subramanian V, Garcia A, Sekowski A, and Brasaemle DL.** Hydrophobic sequences target and anchor perilipin A to lipid droplets. *Journal of lipid research* 45: 1983-1991, 2004.

141. **Subramanian V, Rothenberg A, Gomez C, Cohen AW, Garcia A, Bhattacharyya S, Shapiro L, Dolios G, Wang R, Lisanti MP, and Brasaemle DL.** Perilipin A mediates the reversible binding of CGI-58 to lipid droplets in 3T3-L1 adipocytes. *The Journal of biological chemistry* 279: 42062-42071, 2004.
142. **Sztalryd C, Bell M, Lu X, Mertz P, Hickenbottom S, Chang BH, Chan L, Kimmel AR, and Londos C.** Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line. *The Journal of biological chemistry* 281: 34341-34348, 2006.
143. **Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C.** Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
144. **Tansey JT, Huml AM, Vogt R, Davis KE, Jones JM, Fraser KA, Brasaemle DL, Kimmel AR, and Londos C.** Functional studies on native and mutated forms of perilipins. A role in protein kinase A-mediated lipolysis of triacylglycerols. *The Journal of biological chemistry* 278: 8401-8406, 2003.
145. **Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, and Londos C.** Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 98: 6494-6499, 2001.
146. **Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR, and Londos C.** The central role of perilipin a in lipid metabolism and adipocyte lipolysis. *IUBMB life* 56: 379-385, 2004.
147. **Targett-Adams P, McElwee MJ, Ehrenborg E, Gustafsson MC, Palmer CN, and McLauchlan J.** A PPAR response element regulates transcription of the gene for human adipose differentiation-related protein. *Biochimica et biophysica acta* 1728: 95-104, 2005.
148. **Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, and Hamadeh MJ.** Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *American journal of physiology Regulatory, integrative and comparative physiology* 292: R1271-1278, 2007.
149. **Tauchi-Sato K, Ozeki S, Houjou T, Taguchi R, and Fujimoto T.** The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. *The Journal of biological chemistry* 277: 44507-44512, 2002.
150. **Taylor SS, Yang J, Wu J, Haste NM, Radzio-Andzelm E, and Anand G.** PKA: a portrait of protein kinase dynamics. *Biochimica et biophysica acta* 1697: 259-269, 2004.
151. **Turpin SM, Hoy AJ, Brown RD, Rudaz CG, Honeyman J, Matzaris M, and Watt MJ.** Adipose triacylglycerol lipase is a major regulator of hepatic lipid metabolism but not insulin sensitivity in mice. *Diabetologia* 54: 146-156, 2011.
152. **van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, and Saris WH.** Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *The Journal of physiology* 553: 611-625, 2003.

153. **Varela GM, Antwi DA, Dhir R, Yin X, Singhal NS, Graham MJ, Crooke RM, and Ahima RS.** Inhibition of ADRP prevents diet-induced insulin resistance. *American journal of physiology Gastrointestinal and liver physiology* 295: G621-628, 2008.
154. **Villena JA, Roy S, Sarkadi-Nagy E, Kim KH, and Sul HS.** Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *The Journal of biological chemistry* 279: 47066-47075, 2004.
155. **Walther TC and Farese RV, Jr.** Lipid droplets and cellular lipid metabolism. *Annual review of biochemistry* 81: 687-714, 2012.
156. **Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman R, Gong D, Brasaemle D, and Sztalryd C.** Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *The Journal of biological chemistry* 286: 15707-15715, 2011.
157. **Wang H, Hu L, Dalen K, Dorward H, Marcinkiewicz A, Russell D, Gong D, Londos C, Yamaguchi T, Holm C, Rizzo MA, Brasaemle D, and Sztalryd C.** Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. *The Journal of biological chemistry* 284: 32116-32125, 2009.
158. **Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley WC, and Sztalryd C.** Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *Journal of lipid research* 52: 2159-2168, 2011.
159. **Watt MJ, Heigenhauser GJ, O'Neill M, and Spriet LL.** Hormone-sensitive lipase activity and fatty acyl-CoA content in human skeletal muscle during prolonged exercise. *J Appl Physiol* 95: 314-321, 2003.
160. **Watt MJ, Heigenhauser GJ, and Spriet LL.** Effects of dynamic exercise intensity on the activation of hormone-sensitive lipase in human skeletal muscle. *The Journal of physiology* 547: 301-308, 2003.
161. **Watt MJ, Holmes AG, Pinnamaneni SK, Garnham AP, Steinberg GR, Kemp BE, and Febbraio MA.** Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *American journal of physiology Endocrinology and metabolism* 290: E500-508, 2006.
162. **Watt MJ and Hoy AJ.** Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *American journal of physiology Endocrinology and metabolism*, 2011.
163. **Watt MJ and Spriet LL.** Triacylglycerol lipases and metabolic control: implications for health and disease. *American journal of physiology Endocrinology and metabolism* 299: E162-168, 2010.
164. **Watt MJ and Steinberg GR.** Regulation and function of triacylglycerol lipases in cellular metabolism. *The Biochemical journal* 414: 313-325, 2008.
165. **Watt MJ, Steinberg GR, Chan S, Garnham A, Kemp BE, and Febbraio MA.** Beta-adrenergic stimulation of skeletal muscle HSL can be overridden by AMPK signaling. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 18: 1445-1446, 2004.

166. **Watt MJ, Steinberg GR, Heigenhauser GJ, Spriet LL, and Dyck DJ.** Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid. *American journal of physiology Endocrinology and metabolism* 285: E412-419, 2003.
167. **Watt MJ, Stellingwerff T, Heigenhauser GJ, and Spriet LL.** Effects of plasma adrenaline on hormone-sensitive lipase at rest and during moderate exercise in human skeletal muscle. *The Journal of physiology* 550: 325-332, 2003.
168. **Welte MA.** Proteins under new management: lipid droplets deliver. *Trends in cell biology* 17: 363-369, 2007.
169. **Widegren U, Ryder JW, and Zierath JR.** Mitogen-activated protein kinase signal transduction in skeletal muscle: effects of exercise and muscle contraction. *Acta physiologica Scandinavica* 172: 227-238, 2001.
170. **Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel PE.** S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *The Journal of biological chemistry* 280: 19146-19155, 2005.
171. **Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, and Bickel PE.** OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55: 3418-3428, 2006.
172. **Wolins NE, Rubin B, and Brasaemle DL.** TIP47 associates with lipid droplets. *The Journal of biological chemistry* 276: 5101-5108, 2001.
173. **Wolins NE, Skinner JR, Schoenfish MJ, Tzekov A, Bensch KG, and Bickel PE.** Adipocyte protein S3-12 coats nascent lipid droplets. *The Journal of biological chemistry* 278: 37713-37721, 2003.
174. **Xu G, Sztalryd C, and Londos C.** Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochimica et biophysica acta* 1761: 83-90, 2006.
175. **Xu G, Sztalryd C, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, and Londos C.** Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *The Journal of biological chemistry* 280: 42841-42847, 2005.
176. **Yamaguchi T, Matsushita S, Motojima K, Hirose F, and Osumi T.** MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. *The Journal of biological chemistry* 281: 14232-14240, 2006.
177. **Yamaguchi T, Matsushita S, Motojima K, Hirose F, and Osumi T.** MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. *The Journal of biological chemistry* 281: 14232-14240, 2006.
178. **Yamaguchi T, Omatsu N, Matsushita S, and Osumi T.** CGI-58 interacts with perilipin and is localized to lipid droplets. Possible involvement of CGI-58 mislocalization in Chanarin-Dorfman syndrome. *The Journal of biological chemistry* 279: 30490-30497, 2004.
179. **Yamaguchi T, Omatsu N, Omukae A, and Osumi T.** Analysis of interaction partners for perilipin and ADRP on lipid droplets. *Molecular and cellular biochemistry* 284: 167-173, 2006.

180. **Yang X, Lu X, Lombes M, Rha GB, Chi YI, Guerin TM, Smart EJ, and Liu J.** The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell metabolism* 11: 194-205, 2010.
181. **Yeaman SJ.** Hormone-sensitive lipase--new roles for an old enzyme. *The Biochemical journal* 379: 11-22, 2004.
182. **Zechner R, Strauss JG, Haemmerle G, Lass A, and Zimmermann R.** Lipolysis: pathway under construction. *Current opinion in lipidology* 16: 333-340, 2005.
183. **Zehmer JK, Huang Y, Peng G, Pu J, Anderson RG, and Liu P.** A role for lipid droplets in inter-membrane lipid traffic. *Proteomics* 9: 914-921, 2009.
184. **Zhang H, Wang Y, Li J, Yu J, Pu J, Li L, Zhang H, Zhang S, Peng G, Yang F, and Liu P.** Proteome of skeletal muscle lipid droplet reveals association with mitochondria and apolipoprotein a-I. *Journal of proteome research* 10: 4757-4768, 2011.
185. **Zhang HH, Souza SC, Muliro KV, Kraemer FB, Obin MS, and Greenberg AS.** Lipase-selective functional domains of perilipin A differentially regulate constitutive and protein kinase A-stimulated lipolysis. *The Journal of biological chemistry* 278: 51535-51542, 2003.
186. **Zimmermann R, Lass A, Haemmerle G, and Zechner R.** Fate of fat: the role of adipose triglyceride lipase in lipolysis. *Biochimica et biophysica acta* 1791: 494-500, 2009.
187. **Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, and Zechner R.** Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306: 1383-1386, 2004.

Chapter 4

Study 1

Subcellular localization of skeletal muscle lipid droplets and PLIN family proteins OXPAT and ADRP at rest and following contraction in rat soleus muscle

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Rebecca MacPherson: designed the study, performed the experiments and wrote the manuscript

Eric Herbst: metabolite assays (ATP, phosphocreatine), reviewed and critiqued the manuscript

Erica Reynolds: some of the image analysis quantification, reviewed and critiqued the manuscript

Dr. Rene Vandenboom: gave input to the study design, assisted with methods related to measuring muscle contraction and reviewed and critiqued the manuscript

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Dr. Sandra Peters: conceived the idea, supervised the work, provided funding through NSERC Discovery Grant, assisted with writing the manuscript.

ABSTRACT

Skeletal muscle lipid droplet associated proteins (PLINs) are thought to regulate lipolysis through protein-protein interactions on the lipid droplet surface. In adipocytes, PLIN2 (ADRP) is found only on lipid droplets, while PLIN5 (OXPAT, expressed only in oxidative tissues) is found both on and off the lipid droplet, and may be recruited to lipid droplet membranes when needed. Our purpose was to determine if PLIN5 is recruited to lipid droplets with contraction, and to investigate the myocellular location and co-localization of lipid droplets, PLIN2, and PLIN5. Rat solei were isolated and following a 30min equilibration period were assigned to one of two groups: 1) 30min resting incubation; 2) 30min stimulation (n=10 each). Immunofluorescence microscopy was used to determine subcellular content, distribution, and co-localization of lipid droplets, PLIN2, and PLIN5. There was a main effect for lower lipid and PLIN2 content in stimulated compared to rested muscles ($p < 0.05$). Lipid droplet distribution declined exponentially from the sarcolemma to the fibre centre in the rested muscles ($p = 0.001$, $r^2 = 0.99$) and linearly in stimulated muscles (slope = -0.0023 ± 0.0006 , $p < 0.001$, $r^2 = 0.93$). PLIN2 distribution declined exponentially from the sarcolemma to the fibre centre in both rested and stimulated muscles ($p < 0.0001$, $r^2 = 0.99$ rest; $p = 0.0004$, $r^2 = 0.98$ stimulated) while PLIN5 distribution declined linearly (slope = -0.0085 ± 0.0009 , $p < 0.0001$, $r^2 = 0.94$ rest; slope = -0.0078 ± 0.0010 , $p = 0.0003$, $r^2 = 0.91$ stimulated). PLIN5-lipid droplets co-localized at rest with no difference post stimulation ($p = 0.47$; rest $r^2 = 0.55 \pm 0.02$, stimulated $r^2 = 0.58 \pm 0.03$). PLIN2-lipid droplets co-localized at rest with no difference post stimulation ($p = 0.48$; rest $r^2 = 0.66 \pm 0.02$, stimulated $r^2 = 0.65 \pm 0.02$). Contrary to our hypothesis these results show that PLIN5 is not recruited to lipid droplets with contraction in isolated skeletal muscle.

Keywords: intramuscular triglycerides, contraction, adipophilin, MLDP, OXPAT

Introduction

Growing evidence indicates that skeletal muscle lipolysis is regulated by protein-protein interactions occurring on the surface of triacylglycerol (TAG) storing lipid droplets (22, 31). The most abundant proteins found on the lipid droplet surface are the PLIN family of proteins, of which there are five members. Each member of the family is named after the founding protein of the PAT family (*perilipin*, *PLIN*), with the different proteins numbered sequentially (PLIN1-5)(19). The PLIN family consists of five members: perilipin (PLIN1), adipocyte differentiation-related protein (ADRP, adipophilin, PLIN2), Tail-interacting protein of 47 kDa (TIP47, PLIN3), S3-12 (PLIN4) and OXPAT (MLDP, LSD5, PLIN5) (28). In general, lipid droplets are coated with one or more members of this protein family each serving important roles in regulating lipolysis. It is thought that each PLIN protein plays a unique role in regulating lipid metabolism due to distinct tissue distributions; however the mechanisms regulating lipid droplet dynamics in skeletal muscle are largely unknown.

To date PLIN1 is the only PAT protein for which a distinct role has been established in regulating both TAG storage and lipolysis in adipocytes. At rest in adipocytes PLIN1 coats the lipid droplet regulating the activities of the lipases (adipose triglyceride lipase (ATGL) and hormone Sensitive Lipase (HSL)) to the triglycerides (TAGs) (5, 29). ATGL activity requires activation by comparative gene identification-58 (CGI-58) (34). At rest, CGI-58 is prevented from interacting with ATGL because it is co-localized with PLIN1 on the surface of lipid droplets. HSL is found in the cytoplasm and has several serine residues that when phosphorylated

result in activation and translocation to the lipid droplet (4, 6). In adipocytes, activation of the lipases occurs via a hormonal route. During lipolytic stimulation catecholamines bind to β -adrenergic receptors on the cell surface. Adenylate cyclase is then activated through the action of a stimulatory G-protein, the intracellular level of cAMP is elevated, and cAMP-dependent protein kinase (PKA) is activated. PKA phosphorylates both perilipin and HSL (5, 15, 29). The phosphorylated perilipin releases CGI-58, which is now free to interact and activate ATGL, promoting degradation of TAGs to DAGs (11). Perilipin phosphorylation also permits the phosphorylated HSL to access the lipid droplet (29, 30, 38). This may be due to phosphorylated perilipin leaving the lipid droplet or causing the large droplets to fragment into smaller ones, thereby increasing the accessible droplet surface area which may enhance the activity of ATGL and HSL at the lipid droplets (25).

However, information on lipid droplet dynamics in skeletal muscle is scarce and researchers have extrapolated current knowledge from other tissues to make conclusions/hypotheses about skeletal muscle (26). Given that PLIN1 is not present in skeletal muscle and the tissue distributions of both PLIN2 and PLIN5, these two PAT proteins are the most likely candidates to be involved in regulating muscular lipid storage and use (27). PLIN2 is ubiquitously expressed and is one of the predominant PAT proteins found in skeletal muscle (3, 32). The exact role of PLIN2 is currently unknown, however due to its position on the lipid droplet membrane it may regulate lipolysis through interactions with lipolytic enzymes at the lipid droplet surface (21, 32). Studies in cell culture (Chinese hamster ovary

cells and human embryonic kidney 293 cells) have shown that PLIN2 coats the lipid droplet membrane and is stable in the presence of neutral lipids but is otherwise targeted for degradation by proteasomes (21, 45, 46). Information in skeletal muscle regarding PLIN2 distribution and its association with lipid droplets is limited and contradictory. One study determined that PLIN2 is highly co-localized (80%) to the lipid droplet surface, making it an ideal marker of lipid droplets (32), while a recent study has found that this is not the case with only 64% of PLIN2 associated with lipid droplets (36).

PLIN5 is unique in that its distribution is restricted to tissues that undergo high rates of lipolysis, such as skeletal muscle (specifically type 1 fibers), cardiac muscle, liver, and brown adipose tissue (8, 44, 47). Due to this unique distribution it has been hypothesized that PLIN5 facilitates the oxidation of intracellular lipids in these tissues. Further, PLIN5 expression is induced by physiological, pharmacological, and genetic perturbations that increase utilization of fatty acids for oxidative phosphorylation (8, 44, 47). For example, PLIN5 mRNA and protein content is increased in both the liver and the heart in response to fasting (8, 44, 47). Recent work indicates that PLIN5 may regulate intramuscular TAG storage and mobilization by controlling the availability of CGI-58, an activator of adipose triglyceride lipase (ATGL), on the lipid droplet surface (12). PLIN5 is found associated with the lipid droplets as well as in a cytosolic form (8, 44, 47). Lipolysis occurs on the surface of intracellular lipid droplets, therefore it is possible that PLIN5 is translocated to the lipid droplet surface in circumstances that promote

lipolysis, such as muscle contraction. If this is true, PLIN5 may function by directly regulating lipase action at the lipid droplet surface.

Given that the location of a protein is generally related to its physiological function, the myocellular locations of both PLIN2 and PLIN5 are crucial in understanding their roles. Therefore, to fully understand how PLIN5 may regulate skeletal muscle lipolysis, it is important to first determine the location of this protein at rest and following contraction. The purpose of this study is two-fold, we plan first to investigate the myocellular location and co-localization of lipid droplets, PLIN2, and PLIN5 and second to determine if PLIN5 is recruited to lipid droplets with contraction. It was hypothesized that contraction would recruit a cytosolic pool of PLIN5 to the lipid droplet surface allowing fatty acids to be oxidized and used for energy.

Materials and Methods

Animals.

A total of 10 male Long Evans rats (~ 4-6 weeks old, body mass 163 ± 13 g) were used in this study. Animals were housed in groups within the Brock University Animal Facility, where they were maintained on a 12:12 light-dark cycle at $\sim 22^{\circ}\text{C}$. The rats were fed a standard rodent diet and had ad libitum access to food and water. All experimental procedures and protocols were approved by the Brock University research subcommittee on animal care and conform to all Canadian Council on Animal Care guidelines.

Muscle Preparation.

Animals were anaesthetized via intraperitoneal injection of sodium pentobarbital (6 mg/100g body wt), and then the left and right soleus muscles were removed and placed in an organ bath where they were assigned to one of two experimental groups: 1) rest or 2) stimulated. To briefly summarize the preparation, each soleus muscle was dissected from tendon-to-tendon, sutures were tied in-situ, the muscle was removed and immediately placed in an organ bath (Radnoti Glass Technology, Monrovia, CA), which contained 15 ml of fully oxygenated liquid Sigma medium 199 and suspended at a resting tension of 1 g force. The incubation medium was continuously gassed with 95% O₂ 5% CO₂, and temperature was maintained at 25°C by circulating distilled water from a bath through an outer water jacket of the incubation reservoir (1). Muscles were incubated at rest for 30 min to equilibrate. After the initial incubation, the muscles were assigned to either the rest or stimulated group.

Stimulation Protocol.

Following the equilibration period the muscles remained at rest or were stimulated to contract for 30 min. Initially, optimal stimulus voltage was determined by assessing force responses (Grass Telefactor force transducer, West Warwick, RI) to single electrical pulses (Grass Model FT03 with P11T amplifier). Stimulus intensity was increased from 10 volts in 10 volt increments, until a plateau

in twitch force was reached, after which stimulus voltage was increased to ~ 1.25 of this level. During the 30 minute stimulus protocol, muscles received repeated volleys of brief (150 ms) but high frequency (60 Hz) trains at a train rate of 20 tetani/min (muscles were suspended at 1 g of resting tension throughout). This protocol has previously proven to elicit maximal rates of triglyceride pool turnover and rates of TAG oxidation without the development of fatigue (9). Throughout this period, muscle force production was recorded using Grass Polyview Data Acquisition and Analysis System (West-Warwick, RI) and analyzed using the Polyview Reviewer (Grass Polyview Data Acquisition and Analysis System; Astro-Med Inc., West-Warwick, RI).

Metabolite analysis.

Following the 60-min incubations, soleus muscles were removed from the bath and cut into two pieces, with one piece being snap frozen in liquid nitrogen for metabolite analysis and the other piece mounted for histochemical analysis (see below). The extraction process was completed after the frozen soleus sections were freeze dried (Labconco Corporation; Kansas City; MO, USA). Muscles were powdered and any visible blood and connective tissue were removed and then acid extracted for measurement of muscle metabolites (ATP, PCr, and lactate). Muscle metabolite content was determined by fluorometric techniques according to the procedures described by Harris et al. (16) and modified by Green et al. (14). Each sample was analyzed in triplicate during the same analytical session for each of the measured metabolites.

Immunohistochemical Analysis.

The muscle section used for histochemical analysis was oriented for transverse sections and mounted, in embedding medium (Cryomatrix, Pittsburgh, PA), on a piece of cork which was plunged into 2-methylbutane cooled in liquid nitrogen. Following rapid freezing the samples were stored at -80°C until sectioning. Sectioning was completed with a cryotome (ThermoShandon, Runcorn, Cheshire, UK) optimally set at -20°C. Sections were (10 µm thick) thaw mounted onto slides and stored at -80°C until immunohistochemical staining.

To permit the examination of lipid droplets stained by oil red O (ORO; 00625; Sigma-Aldrich, USA) together with immunolabelled PLIN5 or PLIN2, the same protocol developed by Koopman et al. (20) was utilized (20, 37, 41, 42). Phosphate buffered saline (PBS) was used for dilution of antibodies, reagents, and for use in the washing steps of the protocol. Briefly, cryosections were fixed in 3.7% formaldehyde for 1 h. Slides were then be rinsed three times in deionised water for 30 s then treated with 0.5% Triton X-100 in PBS for 5 min and washed three times with PBS for 5 min. Sections where then incubated for 1 hour at room temperature with a primary antibody against PLIN5 (#GP31; Guinea pig polyclonal; Progen, Heidelberg, Germany) or PLIN2 (#GP40; Guinea pig polyclonal; Progen, Heidelberg, Germany) in the appropriate dilution (1:50 and 1:100 respectively). Incubation was followed by three 5 min washes in PBS. Then the appropriate fluorescent secondary antibody Alexa488 (1:200 in PBS; A-11073; Goat anti-guinea pig; Molecular probes, Invitrogen, Breda, Netherlands) was applied for 60 min at room temperature. This

was followed by a 15 min wash with three exchanges of PBS. The slides were then immersed in the working solution of oil red O for 30 min. Slides were rinsed three times in deionised water. Cover slips were mounted with a prolonging agent (#P36930; Prolong Gold antifade reagent; Invitrogen; Breda, Netherlands).

Double staining of PLIN5 and PLIN2 together was performed by simultaneous incubation with primary antibodies against PLIN5 (#GP31; Guinea pig polyclonal; Progen, Heidelberg, Germany) and PLIN2 (ab37516; Chicken polyclonal; abcam, Cambridge, MA, USA). Visualization of primary antibody binding was achieved with the following secondary antibodies: Alexa488 (1:200 in PBS; A-11073; Goat anti-guinea pig; Molecular probes, Invitrogen, Breda, The Netherlands), and Alexa Fluor 555 (1:200 in PBS; A-21437; Goat anti-chicken Molecular probes, Invitrogen, Breda, The Netherlands). Control steps were performed to eliminate the chance of cross-reaction between the oil red O and one of the antibodies, or between the pair of antibodies, where either oil red O or one of the antibodies were omitted, resulting in no signal for all cases (data not shown).

Image capturing and analyses.

All sections were examined using a Nikon Eclipse 80i fluorescence microscope (Nikon Eclipse 80i, Chiyoda-ku, Tokyo). Digital images of the slides were captured with a digital camera (Retiga 1300, QImaging, Burnaby, B.C., Canada) attached to the microscope. In order to visualize the oil red O stain and the 488 and 550 fluophores the FITC UV (450-490 nm) and TRITC (510-560 nm) excitation filters were used. Digitally captured images (40x magnification), four fields of

view/muscle cross section (17.9 ± 1.1 fibers/field of view), were processed and analysed using imaging software (NIS-Elements AR 3.00, Nikon Instruments Inc., Melville, N.Y., USA). An intensity threshold representing minimal values corresponding to lipid droplets, PLIN2, and PLIN5 was set manually and applied uniformly in all images.

The lipid droplets, PLIN5, and PLIN2 fluorescent signals were quantified for each muscle fiber, resulting in a total of 4315 muscle fibers analyzed for each muscle cross section. Fiber area as well as the number and area of objects emitting a fluorescent signal were recorded. Muscle fiber lipid droplet, PLIN2, and PLIN5 content was expressed as the fraction of the measured area that was stained (41).

Within each separate muscle cross section, 67 ± 5 muscle fibers were selected to further investigate the lipid droplet, PLIN2, and PLIN5 distribution patterns. To determine distribution, the area of the objects within eight successive bands of $2 \mu\text{m}$ in width from the sarcolemma toward the central region ($16 \mu\text{m}$ from sarcolemma) of each muscle fiber was recorded. Lipid and protein content of these bands were recorded as area fraction (area stained divided by total area measured in each band/central region)(41).

Colocalization of the fluorescent signals was determined in four fields of view per muscle cross section. Pearson's linear regression analyses were performed for each field of view, and coefficients of determination (r^2) were averaged for each cell.

All measures were manually outlined and traced by investigators for each individual myocyte. The immunofluorescence method described here covers numerous fibers per muscle cross section and therefore gives a good representation

of the entire muscle. To test the reliability of the method in our hands, both intra- and inter-observer reliability were evaluated by two investigators. The intra-observer reliability involved the two investigators performing analysis of 1 image three times, at least 1 week apart. The inter-observer reliability involved two independent investigators performing analysis for 3 separate images. These tests proved to be reliable with a coefficient of variation <5% for both intra- and inter-observer reliability.

Statistics

Differences in total content and co-localization coefficients were evaluated by t-tests. Comparisons of lipid droplet and protein content in successive 2 um bands from the sarcolemma was performed using a 2 way ANOVA (rest or stimulated, level of band) and a best fit multiple regression analysis. Tukey post hoc tests were performed when significance was detected. Statistical significance was set at $p < 0.05$. All data are expressed as mean \pm standard error of the mean (SEM).

Results

Muscle Viability and Force

The viability of incubating muscles at 25°C was verified by the maintenance of muscle ATP, phosphocreatine and lactate concentrations (Table 1). Further, adequate oxygenation and muscle viability were assessed by the ability to maintain force production over the duration of contractions (30 min) as previously

demonstrated (9, 33). The initial isometric force normalized to soleus mass was 106.8 ± 11.4 g/g wet wt (n=10). During stimulation force output was recorded at 5 min intervals and this was noted not to vary by more than 5% of initial at any point during the protocol. Accordingly, little or no fatigue or muscle degradation was evident in our results indicating that muscle remained stable and viable during the entire protocol.

Table 1. Muscle Metabolite Concentrations

Experimental Group	ATP	PCr	Lactate
Rest	21.27 ± 1.4	56.49 ± 4.2	12.57 ± 1.8
Stimulated	20.09 ± 0.9	45.18 ± 3.1†	25.05 ± 3.0*

Values are means ± SE (mmol/kg dry wt). PCr, phosphocreatine; *Significant difference between experimental groups ($p < 0.05$). † trend ($p = 0.053$).

Lipid droplet, PLIN2, and PLIN5 content

The average muscle fiber area was $2631 \pm 87 \mu\text{m}^2$. Figure 1 shows representative images of rat skeletal muscle cross sections viewed with a fluorescence microscope following incubation with lipid droplet protein antibodies (PLIN2 and PLIN5) combined with oil red O staining. The antibodies directed against PLIN2 (Fig. 1a: green, 1c: red) and PLIN5 (Fig. 1b: green) successfully stained the proteins with visually distinct dots in the central area of the muscle fibers. In addition, areas of intense fluorescence signal can be seen in the subsarcolemma region most likely reflecting accumulations of densely packed lipid droplets. Fiber lipid droplet content was greater in the resting muscle group compared to the stimulated group ($0.030 \pm 0.009\%$ rest vs. $0.012 \pm 0.006\%$ area lipid stained contraction, $p = 0.029$; Fig. 2a). Fiber PLIN2 content did not differ between resting and stimulated muscles ($0.22 \pm 0.05\%$ rest and $0.14 \pm 0.04\%$ area PLIN2 stained contraction, $p = 0.23$; Fig. 2b). Fiber PLIN5 content did not differ between resting and stimulated muscle groups ($0.035 \pm 0.004\%$ rest and $0.038 \pm 0.009\%$ area PLIN5 stained stimulated, $p = 0.081$; Fig. 2c).

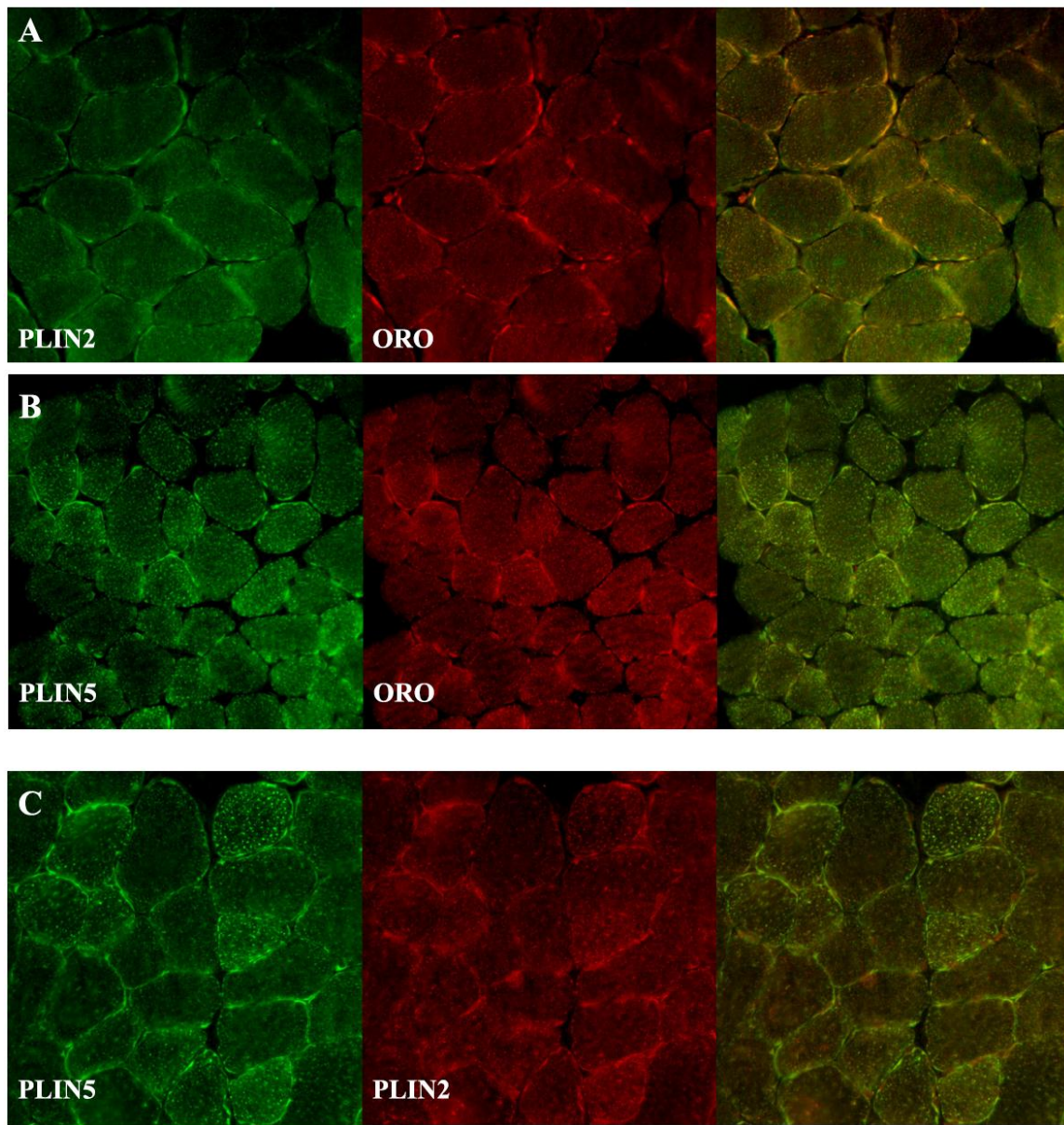


Figure 9

Figure 1: Digitally captured images of one single field of view (40x magnification) taken from a soleus muscle cross section. Oil red O (ORO), PLIN5, and PLIN2 immunofluorescent staining. A) PLIN2 (green), Oil Red O (red), and combined staining (3rd panel) showing higher levels of PLIN2 and lipid droplets closer to the sarcolemmal region. B) PLIN5 (green), Oil Red O (red), and combined staining (3rd panel) showing higher levels of PLIN5 and lipid droplets closer to the sarcolemmal region. C) PLIN5 (green), PLIN2 (red), and combined staining (3rd panel).

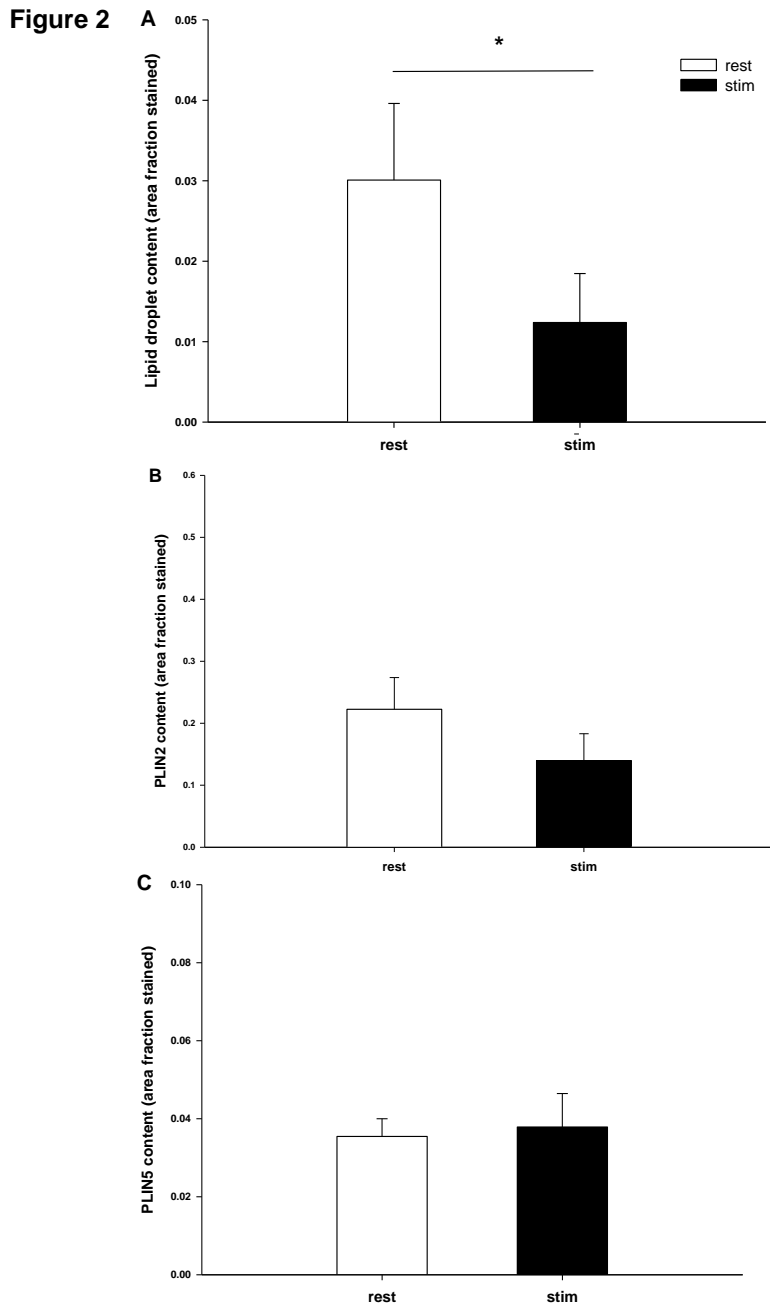


Figure 10

Figure 2:

Lipid droplet (A), PLIN2 (B), and PLIN5 (C) content (expressed as area fraction stained) in rested and stimulated rat soleus muscle (n=10). Values are means \pm SEM. *Significantly lower compared to values observed in rest muscle ($p < 0.05$).

Lipid droplet, PLIN2, and PLIN5 distribution

Lipid droplet distribution is presented as lipid content in 2 μm wide bands from the sarcolemma toward the central region (16 μm) of the fiber and is expressed as the area fraction lipid stained (Fig. 3a). There was a greater lipid content in resting muscles when compared to stimulated muscles at all band levels ($p = 0.001$). The concentration of the lipid droplets appeared to be greater closer to the sarcolemma, however no significant difference was found for lipid droplet content between the different band levels ($p = 0.23$). Regression analysis demonstrates that there was an exponential decline in lipid content from the sarcolemma to the centre of the fibre in the resting muscles ($p=0.001$, $r^2=0.99$). Regression analysis of the stimulated muscles revealed that there was a linear decline in lipid droplet content starting from the sarcolemma (slope= -0.0023 ± 0.0006 , $p < 0.001$, $r^2=0.93$).

Both PLIN5 and PLIN2 distribution mirror the lipid droplet distribution showing distinct intracellular dots and a higher concentration closer to the sarcolemma (Fig. 1a and 1b). There was a main effect for PLIN2 content between rest and stimulated muscles regardless of band level ($p = 0.004$, Fig. 3b). PLIN2 content was significantly greater in the 2 μm region compared to the 6, 8, 10, 12, 14, and centre regions ($p < 0.05$). Regression analysis demonstrated that in both resting and stimulated muscles there was an exponential decline in PLIN2 content from the sarcolemma to the central region of the fibre ($p < 0.0001$, $r^2=0.99$ rest; $p=0.0004$, $r^2=0.98$ stimulated). PLIN5 content did not differ between resting and stimulated

muscles when analysed for content in 2 μm wide bands from the sarcolemma toward the onset of the central region of the fiber ($p = 0.34$, Fig. 3c). PLIN5 content was significantly greater in the 2 and 4 μm regions when compared to the 8, 10, 12, 14, and central regions ($p < 0.05$). PLIN5 content in the 6 μm region was significantly greater when compared to the 14 and centre region ($p < 0.05$). Regression analysis demonstrated that in both resting and stimulated muscles there was a linear decline in PLIN5 content from the sarcolemma to the central region of the fibre (slope= -0.0085 ± 0.0009 , $p < 0.0001$, $r^2 = 0.94$ rest; slope= -0.0078 ± 0.0010 , $p = 0.0003$, $r^2 = 0.91$ stimulated).

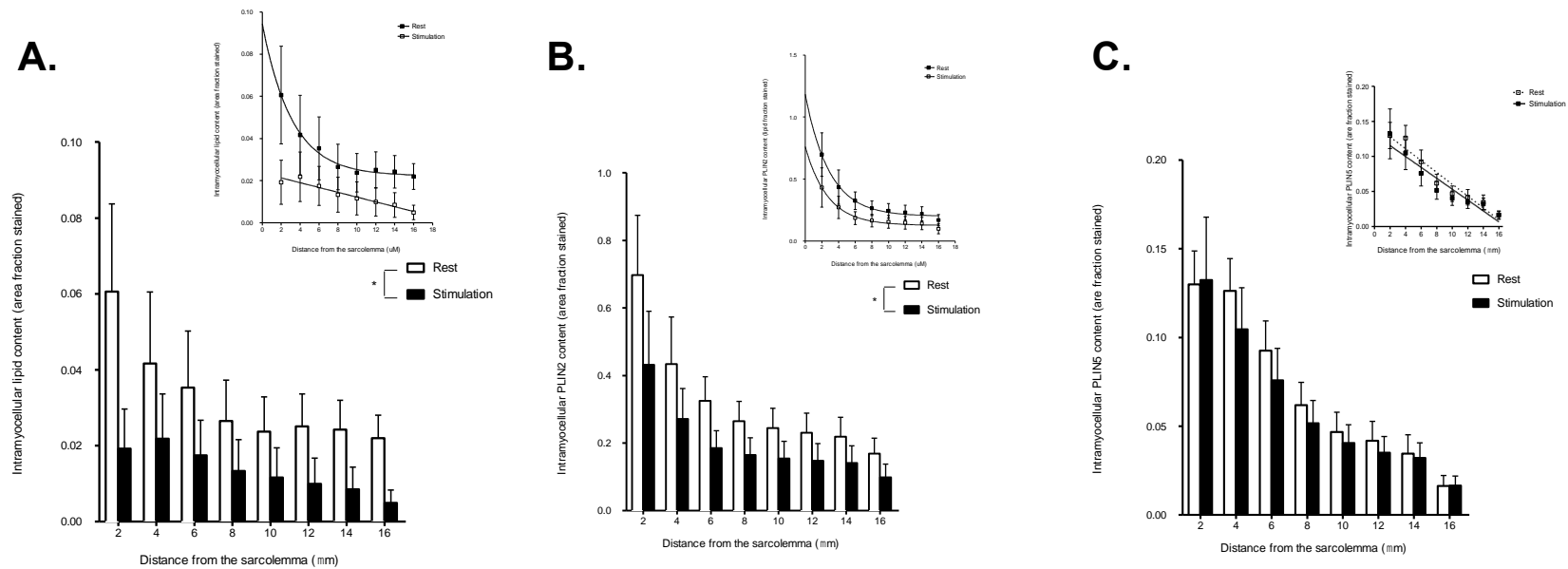


Figure 11

Figure 3: Lipid droplet (A), PLIN2 (B), and PLIN5 (C) distribution presented in 2 μm wide bands from the sarcolemma toward the central region of the fiber ($n=10$). *Main effect for lower lipid and PLIN2 content in stimulated muscles compared to rested muscles ($p < 0.05$). Insets show best fit regression analysis for lipid content, PLIN2, and PLIN5. Analysis demonstrates an exponential decline in lipid content from the sarcolemma to the centre of the fibre in the resting muscles ($p=0.001$, $r^2=0.99$). Analysis of stimulated muscles reveals a linear decline in lipid droplet content starting from the sarcolemma (slope= -0.0023 ± 0.0006 , $p < 0.001$, $r^2=0.93$). PLIN2 content demonstrates an exponential decline in resting and stimulated muscles from the sarcolemma to the central region of the fibre ($p < 0.0001$, $r^2=0.99$ rest; $p=0.0004$, $r^2=0.98$ stimulated). PLIN5 content demonstrates a linear decline in both resting and stimulated muscles from the sarcolemma to the central region of the fibre (slope= -0.0085 ± 0.0009 , $p < 0.0001$, $r^2=0.94$ rest; slope= -0.0078 ± 0.0010 , $p=0.0003$, $r^2=0.91$ stimulated). Values are means \pm SEM.

Co-localization of lipid droplets and PLIN2, and lipid droplets and PLIN5

Combined oil red O staining of lipid droplets with PLIN2 or PLIN5 allowed the position of the lipid droplets to be observed in relation to each of these PAT proteins. All images were quantified by Pearson's correlation coefficient (r^2). Immunostaining of PLIN2 was combined with oil red O staining on muscle sections and viewed with wide field fluorescence microscopy (Fig. 4a). The Pearson's correlation coefficient demonstrated that $66.4 \pm 0.02\%$ (rest) and $65.5 \pm 0.02\%$ (stimulated) of PLIN2 co-localized with lipid droplets with no significant difference between experimental groups ($p = 0.48$). Co-localization of PLIN5 and lipid droplets demonstrated that $55.0 \pm 0.02\%$ (rest) and $57.7 \pm 0.03\%$ (stimulated) of PLIN5 co-localized with lipid droplets with no significant difference between experimental groups ($p = 0.47$, Fig. 4b). Combined staining of PLIN5 and PLIN2 reveals that $67.2 \pm 0.02\%$ (rest) and $66.5 \pm 0.02\%$ (stimulated) of PLIN5 and PLIN2 are co-localized with no significant difference between rest and stimulated muscles ($p=0.792$, Fig. 4c).

Figure 4

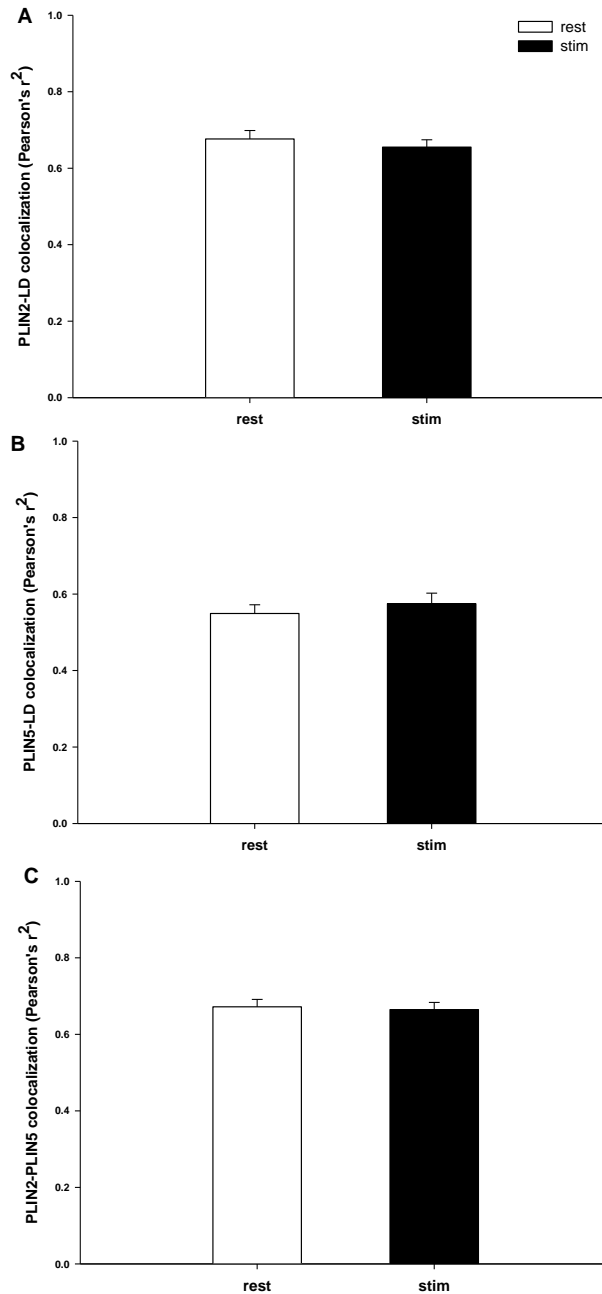


Figure 12

Figure 4: Co-localization of lipid droplets, PLIN2 and PLIN5 (n=10). A) PLIN5-lipid droplet co-localization expressed as average coefficients of determination (Pearson's r^2) indicating the magnitude of co-localization. B) PLIN2-lipid droplet co-localization expressed as average coefficients of determination (Pearson's r^2) indicating the magnitude of co-localization. C) PLIN5-PLIN2 co-localization expressed as average coefficients of determination (Pearson's r^2) indicating the magnitude of co-localization. Values are means \pm SEM.

Discussion

This is the first study to investigate the content, distribution, and co-localization of lipid droplets, PLIN2, and PLIN5 in contracted skeletal muscle. The main findings from this study are that PLIN5 is not recruited to lipid droplets with muscle contraction and that both PLIN2 and PLIN5 are partially co-localized to lipid droplets at any given time, indicating that skeletal muscle lipid droplets are differentially coated with PAT proteins.

This stimulation protocol was chosen to maximize intramuscular TG lipolysis and was effective as evidenced by the significant reduction in lipid droplet content in the stimulated group as compared to the rested group (Fig 2a)(9). This decline in lipid content in the stimulated group was significant regardless of sub-cellular location as demonstrated when fibers were analysed using successive 2 μm bands from the sarcolemma to the fiber centre. Further, regression analysis reveals that with stimulation there is a greater decline in lipid content in the subsarcolemmal region as the relationship changes from an exponential decline to a linear decline with stimulation (Fig 3a). Overall, total PLIN2 content was not significantly different between experimental groups, however there was a significantly lower PLIN2 content as a main effect when analysed by regional band content (Fig 2b and 3b). The absence of a significant difference in overall PLIN2 content may have been due to the large variance in muscle fiber content from the subsarcolemmal region to the centre of the muscle fibers. Nonetheless, the significant drop in PLIN2 content with contraction in the present study is in agreement with previous data in cultured

human embryonic kidney 293 and hamster ovary fibroblastic cells demonstrating that PLIN2 is targeted for degradation if it is not bound to the lipid droplet membrane (21, 46). Consequently as the lipid droplet volume decreased during the contraction protocol, the PLIN2 bound to those lipid droplets may have been degraded in the cytosol. However, in the present study only ~66% of PLIN2 content was found co-localized to lipid droplets suggesting that 34% of the PLIN2 protein is located in the cytosol. There are only two previous studies in skeletal muscle with varying results describing the co-localization of PLIN2 and lipid droplets. One study in rat soleus demonstrated a strong ~80% association between PLIN2 and lipid droplets, with no difference in this association in response to epinephrine or contraction (32). While the other study in rested human vastus lateralis muscle demonstrated a partial, ~64%, association between PLIN2 and lipid droplets (36). Together these results suggest that there is a consistent proportion of PLIN2 associated with lipid droplets, with some found free in the cytosol. Further research is needed to determine the physiological role that PLIN2 plays both at the lipid droplet surface and in the cytosol in skeletal muscle. Total PLIN5 content did not change with stimulation and analyses of PLIN5 distribution further reveals no difference post-stimulation (Fig 2c and 3c).

A novel finding of this study is that both PLIN2 and PLIN5 mirror the myocellular distribution of the lipid droplets. Lipid content as well as PLIN2 and PLIN5 content is found in high density at the subsarcolemmal region with a progressive decline towards the central regions of the muscle fiber (Fig. 3a, b, and c). This lipid droplet distribution is similar to previous studies, where the amount of

lipid was higher in the periphery of the myofibers in comparison to the fibers central region (7, 35, 41). Interestingly, the lipid droplet and PAT protein distribution pattern observed in the present study is similar to the distribution pattern that has also been reported for mitochondria (7, 10, 35, 39). Shaw et al. (35) were the first to show that intramuscular TAG content and mitochondria have a similar pattern of distribution in human muscle with the highest density in subsarcolemmal regions with a progressive decline in the deeper regions of the muscle cell. This is in accord with previous reports in human skeletal muscle showing 2-3 fold greater mitochondrial density in the subsarcolemmal region compared to the inter-myofibrillar area (7, 10, 24, 35, 40). It is possible that the similar distribution and close proximity of lipid droplets, PLIN2, PLIN5, and mitochondria may indicate a functional relationship allowing the lipids to be hydrolysed and oxidised efficiently during exercise (17, 35, 36, 39).

Lipid droplets appear to be differentially coated with PLIN2 and PLIN5. Both PLIN2 and PLIN5 were found to be partially co-localized with lipid droplets (~66% and 55% respectively) and no significant changes in co-localization to lipid droplets in response to contraction was observed. It is not possible to determine the physiological significance of the partial co-localization of PLIN2 or PLIN5 with lipid droplets; however it is hypothesized that subpopulations of lipid droplets presenting different PAT proteins have specific functions within skeletal muscle (36, 43, 44). Cell culture studies (murine fibroblasts, human embryonic kidney cells, McA-RH7777 cells, and primary rat hepatocytes) have demonstrated that overexpression of PLIN2 encourages lipid accumulation (18, 21, 23), whereas down

regulation of PLIN2 results in elevated rates of basal lipolysis (2). Thus PLIN2 is thought to form a protective coat restricting lipolysis and it may be possible that ~66% of the lipid droplets are protected from lipolysis. We believe that PLIN5 may play a role in regulating the interaction of this lipase and the lipase co-activator. Very recently, it was demonstrated that PLIN5 facilitates lipolysis by promoting the co-localization and functional interaction of CGI-58 and ATGL in the basal state (12, 13). Interestingly, while PLIN5 binds both CGI-58 and ATGL, the same PLIN5 molecule does not bind both at the same time (13). Moreover, ATGL interacts with PLIN5 in the cytoplasm and on intracellular structures lacking neutral lipid, indicating that the interaction does not require lipid droplets (13). Perhaps the portion of PLIN5 that is not co-localized to lipid droplets regulates how ATGL interacts with CGI-58 on the lipid droplet surface. Further study is needed to determine how PLIN5 regulates the interaction of ATGL and CGI-58 on the lipid droplet surface promoting lipolysis.

Perspectives

In summary, this study directly analysed the spatial organization of lipid droplets, PLIN5 and PLIN2 in skeletal muscle in terms of content, distribution and co-localisation in response to contraction. The primary finding of this study is that PLIN5 is not recruited to lipid droplets with muscle contraction. Lipid droplets were found to be heterogeneous in nature in terms of their PAT protein content, and both PLIN2 and PLIN5 mirror the distribution of lipid droplets. Further

investigation is needed to establish the exact roles that these proteins play in skeletal muscle lipid metabolism and should focus on the interactions that these proteins may have with lipases and lipase co-activators. Understanding the intricate mechanisms by which PAT proteins contribute to lipid storage and use will help to clarify both the physiology of healthy cells and tissues as well as the basis of some important metabolic diseases.

Grants

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

References

1. **Antolic A, Harrison R, Farlinger C, Cermak NM, Peters SJ, LeBlanc P, and Roy BD.** Effect of extracellular osmolality on cell volume and resting metabolism in mammalian skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 292: R1994-2000, 2007.
2. **Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, and Sztalryd C.** Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* 57: 2037-2045, 2008.
3. **Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, and Londos C.** Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res* 38: 2249-2263, 1997.
4. **Brasaemle DL, Levin DM, Adler-Wailes DC, and Londos C.** The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta* 1483: 251-262, 2000.
5. **Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, and Londos C.** Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem* 275: 38486-38493, 2000.
6. **Clifford GM, Londos C, Kraemer FB, Vernon RG, and Yeaman SJ.** Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. *J Biol Chem* 275: 5011-5015, 2000.
7. **Cogswell AM, Stevens RJ, and Hood DA.** Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions. *Am J Physiol* 264: C383-389, 1993.
8. **Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, and Nebb HI.** LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochimica et biophysica acta* 1771: 210-227, 2007.
9. **Dyck DJ, and Bonen A.** Muscle contraction increases palmitate esterification and oxidation and triacylglycerol oxidation. *Am J Physiol* 275: E888-896, 1998.
10. **Elander A, Sjostrom M, Lundgren F, Schersten T, and Bylund-Fellenius AC.** Biochemical and morphometric properties of mitochondrial populations in human muscle fibres. *Clin Sci (Lond)* 69: 153-164, 1985.
11. **Granneman JG, Moore HP, Granneman RL, Greenberg AS, Obin MS, and Zhu Z.** Analysis of lipolytic protein trafficking and interactions in adipocytes. *J Biol Chem* 282: 5726-5735, 2007.
12. **Granneman JG, Moore HP, Mottillo EP, and Zhu Z.** Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *J Biol Chem* 284: 3049-3057, 2009.
13. **Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L.** Interactions of perilipin-5 (PLIN5) with adipose triglyceride lipase (ATGL). *J Biol Chem* 2010.
14. **Green HJ, Thomson JA, and Houston ME.** Supramaximal exercise after training-induced hypervolemia. II. Blood/muscle substrates and metabolites. *J Appl Physiol* 62: 1954-1961, 1987.

15. **Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, and Londos C.** Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem* 266: 11341-11346, 1991.
16. **Harris RC, Hultman E, and Nordesjo LO.** Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest* 33: 109-120, 1974.
17. **Hoppeler H, Luthi P, Claassen H, Weibel ER, and Howald H.** The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. *Pflugers Arch* 344: 217-232, 1973.
18. **Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, and Nawata H.** ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am J Physiol Endocrinol Metab* 283: E775-783, 2002.
19. **Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, and Londos C.** Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *J Lipid Res* 51: 468-471, 2010.
20. **Koopman R, Schaart G, and Hesselink MK.** Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* 116: 63-68, 2001.
21. **Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, and Brown DA.** Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J Lipid Res* 48: 2751-2761, 2007.
22. **Londos C, Brasaemle DL, Schultz CJ, Segrest JP, and Kimmel AR.** Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin Cell Dev Biol* 10: 51-58, 1999.
23. **Magnusson B, Asp L, Bostrom P, Ruiz M, Stillemark-Billton P, Linden D, Boren J, and Olofsson SO.** Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arterioscler Thromb Vasc Biol* 26: 1566-1571, 2006.
24. **Malenfant P, Joanisse DR, Theriault R, Goodpaster BH, Kelley DE, and Simoneau JA.** Fat content in individual muscle fibers of lean and obese subjects. *Int J Obes Relat Metab Disord* 25: 1316-1321, 2001.
25. **Marcinkiewicz A, Gauthier D, Garcia A, and Brasaemle DL.** The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. *J Biol Chem* 281: 11901-11909, 2006.
26. **Meex RC, Schrauwen P, and Hesselink MK.** Modulation of myocellular fat stores: lipid droplet dynamics in health and disease. *Am J Physiol Regul Integr Comp Physiol* 297: R913-924, 2009.
27. **Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MK.** Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *J Clin Endocrinol Metab* 94: 4077-4085, 2009.

28. **Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B, and Kimmel AR.** Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *J Biol Chem* 277: 32253-32257, 2002.
29. **Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS.** Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
30. **Mottagui-Tabar S, Ryden M, Lofgren P, Faulds G, Hoffstedt J, Brookes AJ, Andersson I, and Arner P.** Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. *Diabetologia* 46: 789-797, 2003.
31. **Murphy DJ.** The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog Lipid Res* 40: 325-438, 2001.
32. **Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, and Ploug T.** Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *J Lipid Res* 47: 2392-2399, 2006.
33. **Rennie MJ, and Holloszy JO.** Inhibition of glucose uptake and glycogenolysis by availability of oleate in well-oxygenated perfused skeletal muscle. *Biochem J* 168: 161-170, 1977.
34. **Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, Tornqvist H, Zechner R, and Zimmermann R.** Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *J Biol Chem* 281: 40236-40241, 2006.
35. **Shaw CS, Jones DA, and Wagenmakers AJ.** Network distribution of mitochondria and lipid droplets in human muscle fibres. *Histochem Cell Biol* 129: 65-72, 2008.
36. **Shaw CS, Sherlock M, Stewart PM, and Wagenmakers AJ.** Adipophilin distribution and colocalization with lipid droplets in skeletal muscle. *Histochem Cell Biol* 131: 575-581, 2009.
37. **Stellingwerff T, Boon H, Jonkers RA, Senden JM, Spriet LL, Koopman R, and van Loon LJ.** Significant intramyocellular lipid use during prolonged cycling in endurance-trained males as assessed by three different methodologies. *Am J Physiol Endocrinol Metab* 292: E1715-1723, 2007.
38. **Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C.** Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
39. **Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, and Hamadeh MJ.** Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol Regul Integr Comp Physiol* 292: R1271-1278, 2007.
40. **van Loon LJ.** Use of intramuscular triacylglycerol as a substrate source during exercise in humans. *J Appl Physiol* 97: 1170-1187, 2004.
41. **van Loon LJ, Koopman R, Manders R, van der Weegen W, van Kranenburg GP, and Keizer HA.** Intramyocellular lipid content in type 2 diabetes

patients compared with overweight sedentary men and highly trained endurance athletes. *Am J Physiol Endocrinol Metab* 287: E558-565, 2004.

42. **van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, and Saris WH.** Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *J Physiol* 553: 611-625, 2003.

43. **Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel PE.** S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *J Biol Chem* 280: 19146-19155, 2005.

44. **Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, and Bickel PE.** OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55: 3418-3428, 2006.

45. **Xu G, Sztalryd C, and Londos C.** Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochimica et biophysica acta* 1761: 83-90, 2006.

46. **Xu G, Sztalryd C, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, and Londos C.** Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *J Biol Chem* 280: 42841-42847, 2005.

47. **Yamaguchi T, Matsushita S, Motojima K, Hirose F, and Osumi T.** MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. *The Journal of biological chemistry* 281: 14232-14240, 2006.

Chapter 5

Study 2

Skeletal muscle PLIN proteins, ATGL and CGI-58, interactions at rest and following stimulated contraction

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Rebecca MacPherson: helped to conceive the idea, designed the study, did the experiments, wrote the manuscript

Sofhia Ramos: PLIN3 Western blots, reviewed and critiqued the manuscript

Dr. Rene Vandenboom: gave input to the study design, and reviewed and critiqued the manuscript

Dr. Brian Roy: gave input to the study design, and reviewed and critiqued the manuscript

Dr. Sandra Peters: helped to conceive the idea, gave input into the study design, supervised the work, provided funding through NSERC Discovery Grant, assisted with writing the manuscript.

Running Head: Interactions between skeletal muscle PLINs, ATGL, and CGI-58

Abstract

Evidence indicates that skeletal muscle lipid droplet proteins (PLINs) regulate lipolysis through protein-protein interactions on the lipid droplet surface. In adipocytes, PLIN1 is thought to regulate lipolysis by directly interacting with CGI-58, an activator of adipose triglyceride lipase (ATGL). Upon lipolytic stimulation PLIN1 is phosphorylated releasing CGI-58 to fully activate ATGL and initiate triglyceride breakdown. The absence of PLIN1 in skeletal muscle leads us to believe that other PLIN family members undertake this role. Our purpose was to examine interactions between PLIN2, PLIN3, and PLIN5, with ATGL and its co-activator CGI-58 at rest and following contraction. Isolated rat solei were incubated for 30 min at rest or during 30 min of intermittent tetanic stimulation (150 ms volleys at 60 Hz with a train rate of 20 tetani per min (25°C) to maximally stimulate intramuscular lipid breakdown. Results show that the interaction between ATGL and CGI-58 increased 128% following contraction ($p=0.041$). Further, ATGL interacts with PLIN2, PLIN3, and PLIN5 at rest and following contraction. The PLIN2-ATGL interaction decreased significantly by 21% following stimulation ($p=0.013$). Both PLIN3 and PLIN5 co-precipitated with CGI-58 at rest and following contraction, while there was no detectable interaction between PLIN2 and CGI-58 in either condition. Therefore, our findings indicate that in skeletal muscle during contraction-induced muscle lipolysis ATGL and CGI-58 strongly associate and that the PLIN proteins work together to regulate lipolysis in part by preventing ATGL and CGI-58 interactions at rest.

Keywords: ADRP, adipophilin, OXPAT, MLDP, TIP47, Abhd5

Introduction

Fatty acids (FA) released from intramuscular triglycerides (IMTG) during lipolysis provide an important source of energy during muscle contraction. In skeletal muscle IMTGs are packaged into lipid droplets that possess a unique coat of proteins associated with the surrounding phospholipid monolayer. This protein coat provides an interface for specific processes, such as transport, lipogenesis and lipolysis (10, 34). Perilipins or PLIN proteins are the most recognized family of lipid droplet proteins and are the most likely to be involved in the regulation of lipogenesis and lipolysis in skeletal muscle (31).

Our understanding of PLIN proteins in skeletal muscle is limited, however studies in other tissues and in cell culture indicate that PLIN proteins are key regulators of lipid metabolism as they appear to be directly involved with how cells and tissues store, mobilize, and utilize fatty acids (8, 12, 15, 34, 35, 62). The PLIN family consists of five members, PLIN1 (perilipin), PLIN2 (adipocyte differentiation-related protein; ADRP), PLIN3 (Tail-interacting protein of 47 kDa; TIP47), PLIN4 (S3-12) and PLIN5 (OXPAT, MLDP, LSDP5) (41). Each PLIN has a unique tissue distribution and possibly a unique physiological function. To date PLIN1 is the only protein of this family for which a distinct role has been established; regulating triglyceride storage and lipolysis (for review see (4, 69, 74)). PLIN1 is primarily expressed in adipose tissue and controls adipocyte lipolysis by directly regulating the activity of the lipases surrounding the droplet (11, 42, 55, 60). Lipolysis is regulated by three lipases, adipose triglyceride lipase (ATGL), the rate limiting lipase that initiates lipolysis by hydrolysing the first ester bond releasing the first

fatty acid, hormone sensitive lipase (HSL), which has a high affinity for diacylglycerol as a substrate, and finally monoacylglycerol lipase (MAG). In order to fully activate ATGL in adipose tissue it must be associated with the protein comparative gene identification-58 (CGI-58) (51). However, in adipocytes, under basal conditions CGI-58 is bound to PLIN1 thus preventing full ATGL activity (56, 72). Adipocyte lipolysis is initiated when catecholamines bind to β -adrenergic receptors on the cell surface. Through the action of a stimulatory G-protein, adenylate cyclase is activated leading to increased intracellular cAMP and activation of cAMP-dependent protein kinase (PKA). PKA phosphorylates both PLIN1 and HSL (11, 18, 26, 42, 59, 60). It is the PKA dependent phosphorylation of PLIN1 that causes the release of CGI-58, leading to the initiation of lipolysis by activating ATGL (4, 18-21). Phosphorylation of PLIN1 and HSL promotes the translocation and docking of HSL to PLIN1 on the lipid droplet surface (42, 43, 53, 58, 66).

Evidence suggests that skeletal muscle lipolysis is regulated by PLIN protein interactions occurring on the lipid droplet surface in a manner similar to that observed in adipose tissue (6, 8, 15). However, there are a few distinct differences between adipose tissue and skeletal muscle lipolysis. While adipose tissue lipolysis aims to deliver FAs to other tissues for use, the goal of skeletal muscle lipolysis is to directly provide FAs to the mitochondria for oxidation and energy production. Further, while hormonal regulation of skeletal muscle lipolysis is similar to adipose tissue lipolysis, the full activation of ATGL and HSL in skeletal muscle is likely more complex involving contraction-mediated routes (39). PLIN1 is not expressed in skeletal muscle, but PLIN2, PLIN3, and PLIN5 are all expressed and may be involved

in regulating contraction-induced lipolysis (26, 71). Previous studies in skeletal muscle have focused on the role of HSL in regulating lipolysis and there have been few studies investigating PLIN proteins in skeletal muscle. Interestingly, there have been no studies to date investigating the interactions between PLIN proteins, ATGL and/or CGI-58 in response to contraction in skeletal muscle.

PLIN2 is one of the predominant PLIN proteins found in skeletal muscle (9, 27, 48). PLIN2 is believed to form a protective coat restricting lipolysis through interactions with lipolytic enzymes (3, 33, 48, 52). Other studies have supported a role for PLIN2 in limiting the interaction of the lipases with the triglycerides within the lipid droplet (as reviewed by (5)), but this has yet to be investigated in whole skeletal muscle. In support of this theory, recent work in human skeletal muscle found that PLIN2-associated lipid droplets are preferentially depleted over those lipid droplets not associated with PLIN2 (54). Further, work in isolated rat soleus indicates that HSL is recruited to lipid droplets and PLIN2 with β -adrenergic stimulation or electrically stimulated contraction (48). Together, these results suggest that PLIN2 expression in skeletal muscle may be related to an enhanced IMTG utilization during contraction.

PLIN3 is one of the least studied of this family of proteins, but the homology of PLIN3 is similar to PLIN2 (44) and PLIN3 compensates for the loss of PLIN2 in *Plin2*^{-/-} mice (57). It is speculated that PLIN3 prevents cell death by maintaining mitochondrial membrane potential although the mechanisms are not fully understood (29). It is possible that through interactions with skeletal muscle lipases, PLIN3 regulates the delivery of FAs to the mitochondria, thus preventing an

overload. It is not presently known whether CGI-58 or ATGL bind to PLIN3 in skeletal muscle.

PLIN5 is unique in that its distribution is restricted to tissues that undergo high rates of lipid oxidation, such as skeletal muscle (specifically type I fibers), cardiac muscle, liver, and brown adipose tissue (8, 44, 47). Due to this unique distribution, it has been hypothesized that PLIN5 facilitates lipolysis and the oxidation of intracellular lipids in these tissues. In support of this hypothesis skeletal muscle PLIN5 protein expression is increased under conditions that increase FA oxidation, such as fasting and insulin deficiency (13, 71). Further, PLIN5 content increases in response to endurance training (47). Recent work in *Plin5*^{-/-} mice found that the hearts lacked detectable lipid droplets and contain less TG and FA as compared to wild type controls and perfusion of these hearts with an inhibitor of ATGL recovers the lipid droplets content, suggesting an interaction between PLIN5 and ATGL in this tissue (32). These findings indicate that PLIN5 limits lipase actions, however if PLIN5 interacts with either ATGL and/or CGI-58 in whole skeletal muscle has yet to be determined.

The precise mechanisms regulating contraction-induced lipolysis in skeletal muscle are poorly understood. Therefore the purpose of this study was to first determine if there are any interactions between PLIN2, 3, and 5 with ATGL and/or CGI-58 at rest, and second, to evaluate the effects of lipolytic muscle contraction on these interactions in an isolated muscle preparation. We hypothesized that PLIN proteins would contribute to the regulation of skeletal muscle lipolysis by preventing the interaction of ATGL and CGI-58 at rest.

Methods

Animals.

A total of 20 male Long-Evans rats (4–6 wk old, body mass 101 ± 7 g) were used in this study. Animals were housed in groups within the Brock University Animal Facility, where they were maintained on a 12:12-h light-dark cycle at 22°C. The rats were fed a standard rodent diet and had ad libitum access to food and water. All experimental procedures and protocols were approved by the Brock University Animal Care and Utilization Committee and conformed to all Canadian Council on Animal Care guidelines.

Muscle preparation.

Animals were anesthetized via intraperitoneal injection of pentobarbital sodium (6 mg/100g body wt), and then the left and right soleus muscles were removed and placed in organ baths, where they were assigned to one of two experimental groups: 1) rest or 2) electrically stimulated contraction (36). To briefly summarize the preparation, each soleus muscle was dissected from tendon-to-tendon, sutures were tied in-situ, the muscle was then removed and immediately placed in an organ bath (Radnoti Glass Technology, Monrovia, CA), which contained 15 ml of fully oxygenated liquid Sigma medium 199 (M 4530, Sigma-Aldrich, Canada) and suspended at a resting tension of 1 g. The incubation medium was continuously gassed with 95% O₂ 5% CO₂, and temperature was maintained at 25°C (2). All muscles were allowed to equilibrate at rest for 30 min. After the initial incubation, the muscles were assigned to either the rest or stimulated group. The soleus was

chosen for this set of experiments because it is primarily oxidative in nature (~80% type I fibres) and has previously shown the greatest reliance on lipid metabolism (14, 17, 46). Further, while PLIN5 is expressed in all skeletal muscles, its expression is highest in more oxidative muscles containing more type I fibres, such as the soleus (71). These characteristics make the soleus an ideal muscle for investigating the role of PLIN proteins in skeletal muscle lipolysis. Further, the stimulation protocol that utilized in this study and a previously published study (36) was designed and developed to elicit maximal rates of triglyceride use in the soleus muscle (16).

Stimulation protocol.

Following the equilibration period the muscles remained at rest or were stimulated to contract for 30 min as previously reported by our lab (36). Initially, optimal stimulus voltage was determined by assessing force responses (Grass Telefactor force transducer, West Warwick, RI) to single electrical pulses (Grass Model FT03 with P11T amplifier). Stimulus intensity was increased from 10 V in 10-V increments, until a plateau in twitch force was reached, after which stimulus voltage was increased to ~1.25 of this level. During the 30-min stimulus protocol, muscles received repeated volleys of brief (150 ms) but high-frequency (60 Hz) trains at a train rate of 20 tetani/min (muscles were suspended at 1 g of resting tension throughout). This protocol was previously proven to elicit maximal rates of triglyceride pool turnover and rates of TAG oxidation without the development of fatigue (16). Throughout this period, muscle force was recorded using Grass

Polyview Data Acquisition and Analysis System (West-Warwick, RI) and analyzed using the Polyview Reviewer (Grass Polyview Data Acquisition and Analysis System; Astro-Med, West-Warwick, RI).

Co-Immunoprecipitation

Soleus muscles were homogenized in Griffin lysis buffer (150 mM NaCl, 50 mM Tris HCl, 1 mM EGTA) using a 1:25 dilution of muscle to buffer with added protease (11836170001, Roche, QC) and phosphatase inhibitor tablets (04906845001, Roach, QC). Protein concentration of the total homogenates was determined using a Bradford Assay. Sample homogenates were immunoprecipitated with 5 ul of the appropriate antibody and then immunoblotted for the corresponding protein. Specifically, 500-1000 ug of protein from each sample was incubated for 2 h with 5ul of the desired primary antibody at 4 °C. Following this 20 ul of Protein G or A-Agarose beads (sc-2001, sc-2002; Santa Cruz Biotechnology Inc) was added to each sample for overnight incubation at 4 °C. The pellet of each sample was then collected by centrifugation at 130 rpm for 5-10 seconds. Pellets were washed three times in phosphate buffered saline (PBS) and re-suspended in 40 µl of 2x sample buffer. To test for antibody interference in the samples a blank sample containing only the precipitating antibody and lysis buffer was prepared in exactly the same manner as the experimental samples. For interactions where antibody interference occurred a secondary that only detects native antibodies was used (Clean Blot IP Detection Reagent; Thermo Scientific). All samples were then boiled and separated using 8 or 10% SDS-polyacrylimide gel electrophoresis. As validation that all of our

protein of interest precipitated from the sample, pilot work was done where the supernatant leftover from the IP procedure was Western blotted for the protein of interest.

Western blotting

SDS-polyacrylamide gel electrophoresis (8 or 10% separating; 4% stacking) was used to separate proteins (CGI-58, PLIN2, PLIN3, and PLIN5) at 120 V for 1.5 h, and proteins were electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, New Jersey) for 1 h at 100 V followed by blocking in 2, 3, or 5% fat-free milk in TBST. Primary antibodies for co-precipitated proteins were diluted 1:1000 in 2 or 3% fat-free milk in TBST and incubated overnight at 4 °C. Secondary antibodies were diluted 1:10000-20000 in 2 or 3% milk and incubated for 1 hour. Blots of specific proteins were visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, New Jersey). The densities of the individual bands were integrated using Image J software (<http://rsbweb.nih.gov/ij/>). Each blot had loaded whole soleus homogenate as a positive control for the co-precipitated protein. Blots were normalized to total protein loaded determined by Ponceau S staining (M530; Sigma-Aldrich) and results are reported as the ratio of the density of the target protein to the density of the loaded protein in arbitrary units (50).

Antibodies

The following antibodies were used and have been used previously: PLIN2 (52 kDa) mouse monoclonal antibody (Progen Biotechnik, Heidelberg, Cat. No. 610102) (47), PLIN3 (47 kDa) (ProSci Inc., Poway, California, Cat. No. 3883)(47), PLIN5 (52 kDa) guinea pig polyclonal antibody (Progen Biotechnik, Heidelberg, Cat. Nos. GP34 and GP31) (7, 40, 47), ATGL (54 kDa) rabbit monoclonal antibody (Cell signalling technology, #2439, Danvers, MA) (1), and CGI-58 (42 kDa) rabbit polyclonal antibody (Novus Biologicals, NB110-41576, Oakville, ON) (1, 63).

Statistics

Differences in protein interactions between rest and stimulated muscles were evaluated using two-tailed unpaired t-tests. Statistical significance was set at $p < 0.05$. All data are expressed as mean \pm standard error of the mean (SEM).

Results

Muscle force

Adequate oxygenation and muscle viability were assessed by the ability to maintain force production over the duration of the stimulation protocol (30 min), as previously demonstrated (16, 36, 49). In our hands this type of non-fatiguing contraction did not deplete muscle ATP concentrations (36). The initial isometric force normalized to soleus mass was 86.8 ± 4.1 g/g wet wt (n=19). During stimulation, force output was recorded at 5-min intervals, and this was noted not to

vary by more than 5% of initial isometric force at any point during the protocol. Accordingly, little or no fatigue or muscle degradation was evident in our results, indicating that the muscles remained stable and viable during the entire protocol.

Association between ATGL and CGI-58

ATGL co-immunoprecipitated with CGI-58 at rest and following stimulated contraction. However, following stimulated contraction the amount of CGI-58 protein that co-immunoprecipitated with ATGL significantly increased by 128% ($p=0.041$) (Figure 1).

Figure 1

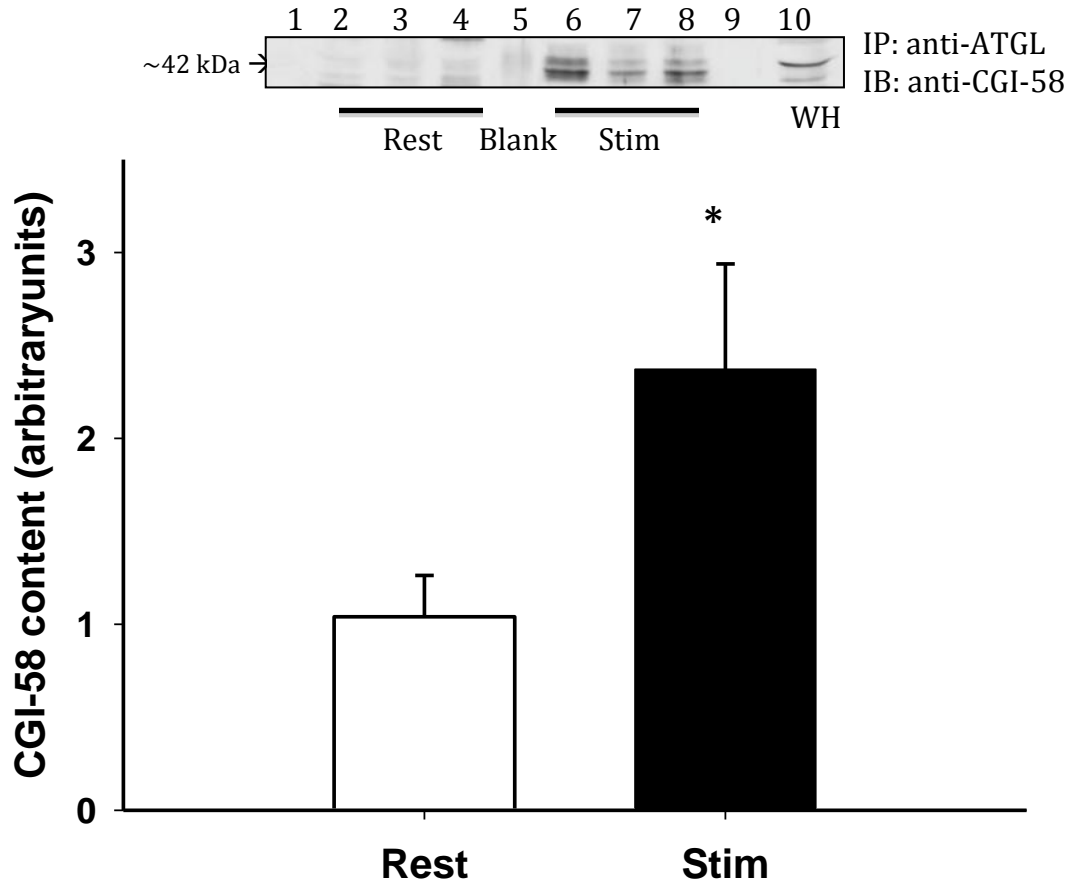


Figure 13

Figure 1. ATGL-CGI-58 interaction at rest and following stimulated contraction. CGI-58 protein content (arbitrary units relative to total protein measured by ponceau staining) in ATGL immunoprecipitated samples at rest and following contraction (n=12). Representative Western blot for ATGL-CGI-58 protein interaction, shown as IP ATGL and Western blot for CGI-58. Lane 1 standard, 2-4 rest samples, 5 blank (absent antibody interference), 6-8 stimulated samples, 9 empty, 10 soleus whole homogenate used as a positive control. * Significant increase in ATGL-CGI-58 interaction following stimulated contraction (p=0.041).

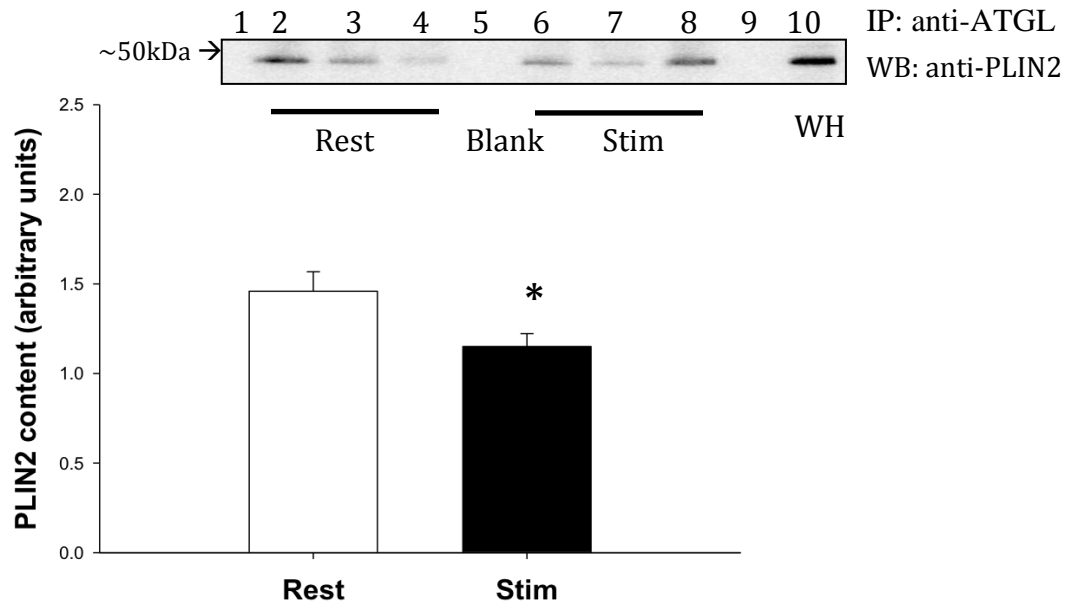
Association between PLIN2, 3, and 5 with ATGL

PLIN2, 3 and 5 all co-immunoprecipitated with ATGL at rest and following stimulated contraction. PLIN2 protein content in ATGL immunoprecipitated samples significantly decreased by 21% following stimulated contraction ($p=0.028$) (Figure 2a).

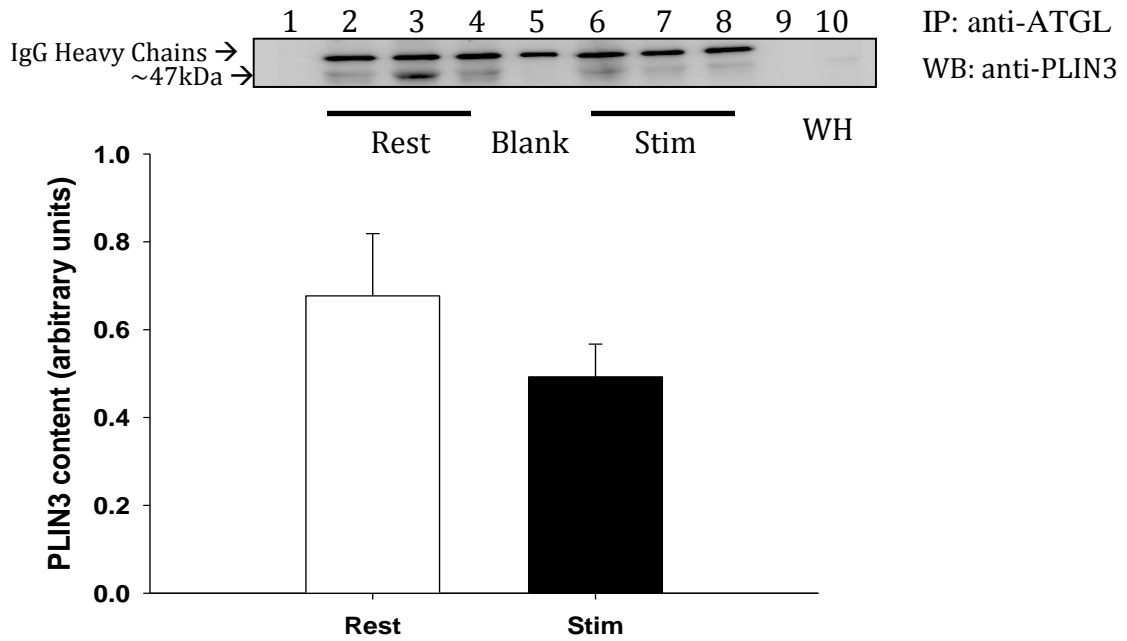
Similarly, PLIN3 protein content in ATGL immunoprecipitated samples decreased by 27% following stimulated contraction however this decrease was not significant ($p=0.266$) (Figure 2b). Finally, PLIN5 protein content in ATGL immunoprecipitated samples also decreased by 19% following stimulated contraction however this decrease was not significant ($p=0.59$) (Figure 2c).

Figure 2

a.



b.



c.

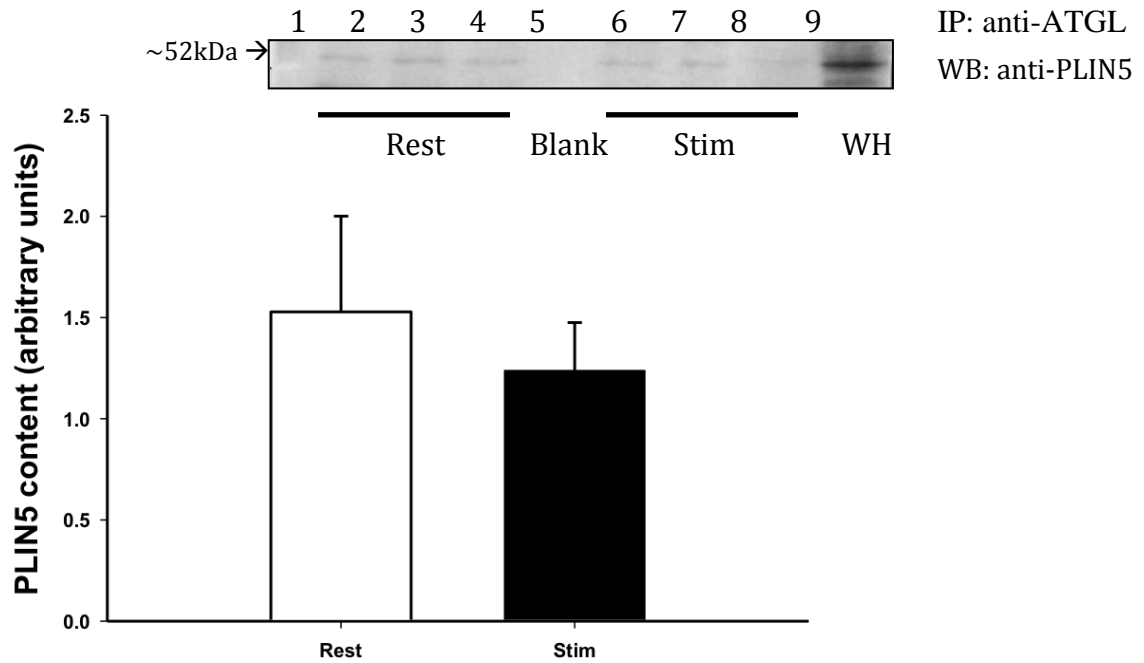


Figure 14

Figure 2. ATGL-PLIN interactions at rest and following stimulated contraction

a. PLIN2 protein content (arbitrary units relative to total protein measured by ponceau staining) in ATGL immunoprecipitated samples at rest and following contraction (n=9). Representative Western blot for ATGL-PLIN2 protein interaction, shown as IP ATGL and Western blot for PLIN2. Lane 1 standard, 2-4 rest samples, 5 blank, 6-8 stimulated samples, 9 empty, 10 soleus whole homogenate used as a positive control. * Significant decrease in ATGL-PLIN2 association following stimulated contraction (p=0.028).

b. PLIN3 protein content (arbitrary units relative to total protein measured by ponceau staining) in ATGL immunoprecipitated samples at rest and following contraction (n=9). Representative Western blot for ATGL-PLIN3 protein interaction, shown as IP ATGL and Western blot for PLIN3. Lane 1 standard, 2-4 rest samples, 5 blank (Note IgG band at ~50kDa), 6-8 stimulated samples, 9 empty, 10 whole soleus homogenate No significant difference in ATGL-PLIN3 association following stimulated contraction (p=0.266).

c. PLIN5 protein content (arbitrary units relative to total protein measured by ponceau staining) in ATGL immunoprecipitated samples at rest and following contraction (n=9). Representative Western blot for ATGL-PLIN5 protein interaction, shown as IP ATGL and Western blot for PLIN5. Lane 1 standard, 2-4 rest samples, 5 blank (absent antibody interference), 6-8 stimulated samples, 9 soleus whole homogenate used as positive control. No significant difference in ATGL-PLIN5 association following stimulated contraction (p=0.591).

Association between PLIN2, 3, and 5 with CGI-58

There was no detectable interaction found between PLIN2 and CGI-58 in PLIN2 immunoprecipitated samples blotted for CGI-58 (Figure 3a).

CGI-58 immunoprecipitated with both PLIN3 and PLIN5 at rest and following stimulated contraction. However, there was no change following contraction for either PLIN proteins ($p=0.08$ and $p=0.42$ respectively) (Figure 3b and 3c).

Figure 3

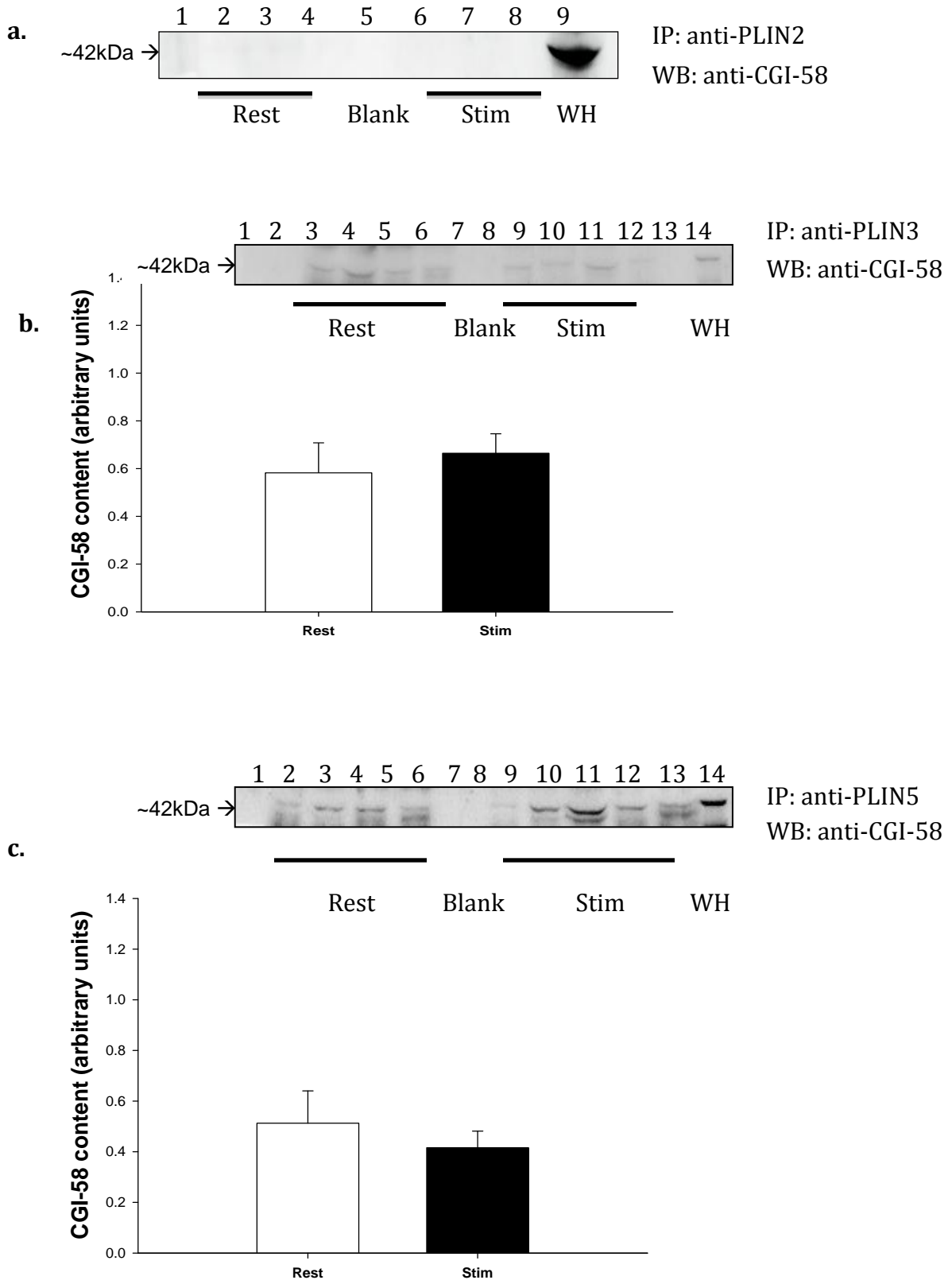


Figure 15

Figure 3. PLIN-CGI-58 at rest and following stimulated contraction

a. Representative western blot of CGI-58 in immunoprecipitated PLIN2 samples. Lane 1 standard, 2-4 rest samples, 5 blank (absent antibody interference), 6-8 stimulated samples, 9 soleus whole homogenate used as positive control. No detectable interaction between PLIN2 and CGI-58.

b. CGI-58 protein content (arbitrary units relative to total protein measured by ponceau staining) in PLIN3 immunoprecipitated samples at rest and following contraction (n=9). Representative western blot of CGI-58 in IP PLIN3 samples. Lanes 1 standard, 2-5 rest samples, 6 blank (absent antibody interference), 7-10 stimulated samples, 12 soleus whole homogenate used as positive control. No significant difference in PLIN3-CGI-58 association following stimulated contraction (p=0.58).

c. CGI-58 protein content (arbitrary units relative to total protein measured by ponceau staining) in PLIN5 immunoprecipitated samples at rest and following contraction (n=9). Representative western blot of CGI-58 in IP PLIN5 samples. Lanes 1 standard, 2-5 rest samples, 6 blank (absent antibody interference), 7-10 stimulated samples, 11 soleus whole homogenate used as positive control. No significant difference in PLIN5-CGI-58 association following stimulated contraction (p=0.50).

Discussion

This is the first study to examine the interactions between PLIN proteins, ATGL and CGI-58 in whole skeletal muscle at rest and following stimulated contraction in the absence of adrenergic stimulation. Using an isolated muscle technique we examined interactions of ATGL and its co-activator, CGI-58, with each other as well as PLIN2, PLIN3, and PLIN5. We found that in skeletal muscle the interaction between ATGL and CGI-58 increases ~2-fold (128%) after 30min of stimulating contraction. Further novel results from this study show that PLIN2, 3, and 5 all interact with ATGL at rest and following contraction, and that only PLIN3 and 5 interact with CGI-58. Further, the interaction between PLIN2 and ATGL is significantly decreased following stimulated contraction, although similar decreases in the interaction of PLIN3 and PLIN5 with ATGL were not significant. These findings support the hypothesis that PLIN proteins may regulate skeletal muscle lipolysis by sequestering ATGL and CGI-58, and it is possible that in skeletal muscle these PLIN proteins work together in concert to regulate lipolysis.

The stimulation protocol used in this study was chosen because it elicits maximal rates of lipolysis in isolated soleus muscle and we have previously shown this method leads to a significant decline in IMTGs (16, 36). This is the first study to show that during lipolytic stimulating contraction in skeletal muscle ATGL and CGI-58 interact. This interaction increased 128% post contraction consistent with an increased rate of lipolysis during this period (16). We found that PLIN2, 3, and 5 all interact with ATGL at rest and following stimulated contraction, but the PLIN2-

ATGL interaction was the only one to show a significant decrease post-stimulation. However, this decrease in the association between PLIN2 and ATGL (21%) does not fully account for the large increase in the association between ATGL and CGI-58 (128%) following contraction. With more than one PLIN protein interacting with ATGL and/or CGI-58, perhaps in skeletal muscle the PLIN proteins work together as a complex. The results of this study indicate that the role(s) of PLIN proteins in regulating skeletal muscle lipolysis are much more complex than what is currently understood in adipose tissue.

Cell culture studies provide evidence that both PLIN2 and PLIN5 compete with lipases at the lipid droplet surface to lower basal lipolytic rates (3, 33, 65, 67). Specifically, increasing the expression of PLIN2 and/or PLIN5 in human embryonic kidney cells or AML12 liver cells reduces the rate of basal and PKA-stimulated lipolysis, potentially by interacting with ATGL (3, 33, 65). Our finding that PLIN2 is associated with ATGL at rest is consistent with previous work indicating that PLIN2 encourages lipid accumulation by inhibiting lipolysis. In general, PLIN2 is found on the lipid droplet surface from the beginning of synthesis and is up regulated in parallel with stored lipid during lipid droplet formation (9, 30, 68). Cell culture studies (murine fibroblasts, human embryonic kidney cells, McA-RH7777 cells, and primary rat hepatocytes) have also demonstrated that overexpression of PLIN2 encourages lipid accumulation (30, 33, 37), whereas down regulation of PLIN2 results in elevated rates of basal lipolysis (3). Further, in PLIN1-null mice PLIN2 replaces PLIN1 on the lipid droplets (61), but PLIN2 appears to be a less robust barrier to lipases than PLIN1(38, 61). Although PLIN2 is similar to PLIN1 in

sequence homology is has been demonstrated that, unlike PLIN1, PLIN2 is not phosphorylated by protein kinase A (59, 61). Since PLIN2 was the only PLIN protein in the present study to show a significant decline in ATGL association following contraction it is possible that this interaction is regulated by a contraction mediated route rather than a hormonal one. The exact mechanisms regulating the interaction between PLIN2 and ATGL in skeletal muscle needs further study. Our finding that PLIN2 does not interact with CGI-58 is in agreement with a previous study using CHO cells that found that lipid droplets coated with PLIN2 did not recruit CGI-58 to the lipid droplets as effectively as either PLIN1 or PLIN5 (64). However, there are other studies with conflicting results. Yamaguchi et al. (2006) investigated the functions of PLIN2 and performed yeast two-hybrid screening to find any functional partners, finding that CGI-58 can interact with PLIN2 (73). Therefore, it is possible that the lack of interaction may be cell/tissue-specific.

Due to PLIN5 being highly expressed in oxidative tissues it seems likely that this PLIN protein is involved in the regulation of skeletal muscle lipid droplet dynamics. In PLIN5 null mice lipid droplets are undetectable in hearts, however lipid droplets are observed in other tissues including skeletal muscle suggesting that the role(s) of PLIN5 may differ depending on the individual cell/tissue type. There was no significant difference in TG content in soleus muscle from PLIN5 null mice compared to wild type controls (small decrease), however this could be due to the other skeletal muscle PLIN proteins compensating for the loss, or the possibility that more than one PLIN protein is required to fully regulate skeletal muscle lipolysis. Previous work investigating possible interaction partners for PLIN5 has been done

with a cell culture model. Using CHO cells, Wang et al. 2011, determined that PLIN5 directly binds to ATGL and that PLIN5 is a substrate for PKA, suggesting that phosphorylation of PLIN5 enables lipolysis (65). The lack of a significant decline in the PLIN5-ATGL interaction seen in the present study may be due to our stimulation protocol, which did not include any adrenergic stimulation to activate PKA.

Recently, it was demonstrated that PLIN5 facilitates lipolysis by promoting the co-localization and functional interaction of CGI-58 and ATGL (23, 25). The same PLIN5 molecule does not bind both at the same time (24) and appears to be responsible for directing CGI-58 to the droplet surface to increase ATGL activity (22). In the present study we found that PLIN5 interacts with both ATGL and CGI-58, however it is not known what proportion of PLIN5 is bound to either ATGL or CGI-58, or if PLIN5 interacts with both ATGL and CGI-58 at the same time.

Previous to the current work, it was unknown if PLIN3 interacted with either ATGL or CGI-58. Our results suggest that PLIN3 is also involved in reducing lipolysis through interactions with both ATGL and CGI-58. It has been suggested that PLIN3 plays a role in lipid droplet formation/growth synthesis by inhibiting lipolysis. This is supported by work using immunofluorescence microscopy in 3T3-L1 cells treated with oleate, glucose and insulin (designed to increase TG synthesis) finding that PLIN3 moved from the cytosol to the lipid droplet (70). Moreover, in PLIN2 null mice, PLIN2 on the LD is replaced with PLIN3 (57). To further support a role for PLIN3 in preventing lipolysis and promoting TG synthesis, siRNA knockdown of PLIN3 in PLIN2 knockout mice resulted in reduced number of lipid droplets, reduced incorporation of oleate into TG, and increased incorporation of oleate to

phospholipids, where knockout of PLIN2 alone resulted in no change (Ducharme & Bickel, 2008). Interestingly, the sequence of amino acids 191-437 of PLIN3 is similar to the amino acid sequence 200-463 of PLIN5 which has been found to be the binding site for both ATGL and CGI-58 on PLIN5 (28). Our finding that both PLIN3 and PLIN5 interact with ATGL and CGI-58 in skeletal muscle is consistent with these findings. However, as PLIN3 and 5 are found in the cytosol as well as on the lipid droplet it is unknown which population of these proteins is bound to either ATGL and/or CGI-58. Further study in this area is needed to determine these interactions.

Perspectives and Significance

This study examined the interactions of the rate limiting lipase, ATGL, its co-activator, CGI-58, and three skeletal muscle PLIN proteins at rest and following contraction. This is the first study to demonstrate that in isolated skeletal muscle the interaction between ATGL and CGI-58 is significantly increased following a contraction protocol that lacks adrenergic stimulation. Further, this study provides evidence that in skeletal muscle both ATGL and CGI-58 are potentially regulated by more than one protein of the PLIN family. Taken together, these data suggest that, the skeletal muscle PLIN proteins, ATGL, and CGI-58 may not ever exist in a single complex at rest or during contraction but may instead represent a network of proteins interacting with one another at different times for different purposes. Evidence indicates that all three of these PLIN proteins are associated with

mitochondria (7, 29, 45, 67). It is possible that in skeletal muscle these PLIN proteins work together to control the rate of lipolysis and thus of FA entry into the mitochondria during lipolysis. Understanding the mechanisms by which PLIN proteins contribute to these processes will help to clarify both the physiology of healthy cells and tissues, as well as the pathophysiological basis of some important metabolic diseases. Further research is needed to elucidate the specific roles of skeletal muscle PLIN proteins in regulating lipolysis and FA entry into the mitochondria.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

References

1. **Alsted TJ, Nybo L, Schweiger M, Fledelius C, Jacobsen P, Zimmermann R, Zechner R, and Kiens B.** Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. *American journal of physiology Endocrinology and metabolism* 296: E445-453, 2009.
2. **Antolic A, Harrison R, Farlinger C, Cermak NM, Peters SJ, LeBlanc P, and Roy BD.** Effect of extracellular osmolality on cell volume and resting metabolism in mammalian skeletal muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 292: R1994-2000, 2007.
3. **Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, and Sztalryd C.** Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* 57: 2037-2045, 2008.
4. **Bezaire V, and Langin D.** Regulation of adipose tissue lipolysis revisited. *Proc Nutr Soc* 68: 350-360, 2009.
5. **Bickel PE, Tansey JT, and Welte MA.** PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica et biophysica acta* 1791: 419-440, 2009.
6. **Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondinone CM, Theodorakis JL, Greenberg AS, and Londos C.** Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *Journal of lipid research* 36: 1211-1226, 1995.
7. **Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P, and Hesselink MK.** The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochemistry and cell biology* 137: 205-216, 2012.
8. **Brasaemle DL.** Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of lipid research* 48: 2547-2559, 2007.
9. **Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, and Londos C.** Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of lipid research* 38: 2249-2263, 1997.
10. **Brasaemle DL, Dolios G, Shapiro L, and Wang R.** Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *The Journal of biological chemistry* 279: 46835-46842, 2004.
11. **Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, and Londos C.** Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *The Journal of biological chemistry* 275: 38486-38493, 2000.
12. **Brown DA.** Lipid droplets: proteins floating on a pool of fat. *Current biology : CB* 11: R446-449, 2001.

13. **Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, and Nebb HI.** LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochimica et biophysica acta* 1771: 210-227, 2007.
14. **Delp MD, and Duan C.** Composition and size of type I, IIA, IID/X, and IIB fibers and citrate synthase activity of rat muscle. *J Appl Physiol* 80: 261-270, 1996.
15. **Ducharme NA, and Bickel PE.** Lipid droplets in lipogenesis and lipolysis. *Endocrinology* 149: 942-949, 2008.
16. **Dyck DJ, and Bonen A.** Muscle contraction increases palmitate esterification and oxidation and triacylglycerol oxidation. *The American journal of physiology* 275: E888-896, 1998.
17. **Dyck DJ, Peters SJ, Glatz J, Gorski J, Keizer H, Kiens B, Liu S, Richter EA, Spriet LL, van der Vusse GJ, and Bonen A.** Functional differences in lipid metabolism in resting skeletal muscle of various fiber types. *The American journal of physiology* 272: E340-351, 1997.
18. **Egan JJ, Greenberg AS, Chang MK, and Londos C.** Control of endogenous phosphorylation of the major cAMP-dependent protein kinase substrate in adipocytes by insulin and beta-adrenergic stimulation. *The Journal of biological chemistry* 265: 18769-18775, 1990.
19. **Granneman JG, and Moore HP.** Location, location: protein trafficking and lipolysis in adipocytes. *Trends in endocrinology and metabolism: TEM* 19: 3-9, 2008.
20. **Granneman JG, Moore HP, Granneman RL, Greenberg AS, Obin MS, and Zhu Z.** Analysis of lipolytic protein trafficking and interactions in adipocytes. *The Journal of biological chemistry* 282: 5726-5735, 2007.
21. **Granneman JG, Moore HP, Krishnamoorthy R, and Rathod M.** Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *The Journal of biological chemistry* 284: 34538-34544, 2009.
22. **Granneman JG, Moore HP, Mottillo EP, and Zhu Z.** Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *The Journal of biological chemistry* 284: 3049-3057, 2009.
23. **Granneman JG, Moore HP, Mottillo EP, and Zhu Z.** Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *The Journal of biological chemistry* 284: 3049-3057, 2009.
24. **Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L.** Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
25. **Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L.** Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
26. **Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, and Londos C.** Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *The Journal of biological chemistry* 266: 11341-11346, 1991.
27. **Heid HW, Moll R, Schwetlick I, Rackwitz HR, and Keenan TW.** Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell and tissue research* 294: 309-321, 1998.

28. **Hickenbottom SJ, Kimmel AR, Londos C, and Hurley JH.** Structure of a lipid droplet protein; the PAT family member TIP47. *Structure* 12: 1199-1207, 2004.
29. **Hocsak E, Racz B, Szabo A, Mester L, Rapolti E, Pozsgai E, Javor S, Bellyei S, Gallyas F, Jr., Sumegi B, and Szigeti A.** TIP47 protects mitochondrial membrane integrity and inhibits oxidative-stress-induced cell death. *FEBS letters* 2010.
30. **Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, and Nawata H.** ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American journal of physiology Endocrinology and metabolism* 283: E775-783, 2002.
31. **Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, and Londos C.** Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *Journal of lipid research* 51: 468-471, 2010.
32. **Kuramoto K, Okamura T, Yamaguchi T, Nakamura TY, Wakabayashi S, Morinaga H, Nomura M, Yanase T, Otsu K, Usuda N, Matsumura S, Inoue K, Fushiki T, Kojima Y, Hashimoto T, Sakai F, Hirose F, and Osumi T.** Perilipin 5, a Lipid Droplet-binding Protein, Protects Heart from Oxidative Burden by Sequestering Fatty Acid from Excessive Oxidation. *The Journal of biological chemistry* 287: 23852-23863, 2012.
33. **Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, and Brown DA.** Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *Journal of lipid research* 48: 2751-2761, 2007.
34. **Liu P, Ying Y, Zhao Y, Mundy DI, Zhu M, and Anderson RG.** Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *The Journal of biological chemistry* 279: 3787-3792, 2004.
35. **Londos C, Sztalryd C, Tansey JT, and Kimmel AR.** Role of PAT proteins in lipid metabolism. *Biochimie* 87: 45-49, 2005.
36. **Macpherson RE, Herbst EA, Reynolds EJ, Vandenboom R, Roy BD, and Peters SJ.** Subcellular localization of skeletal muscle lipid droplets and PLIN family proteins OXPAT and ADRP at rest and following contraction in rat soleus muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 302: R29-36, 2012.
37. **Magnusson B, Asp L, Bostrom P, Ruiz M, Stillemark-Billton P, Linden D, Boren J, and Olofsson SO.** Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arteriosclerosis, thrombosis, and vascular biology* 26: 1566-1571, 2006.
38. **Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, and Chan L.** Absence of perilipin results in leanness and reverses obesity in *Lepr*(db/db) mice. *Nature genetics* 26: 474-479, 2000.
39. **Meex RC, Schrauwen P, and Hesselink MK.** Modulation of myocellular fat stores: lipid droplet dynamics in health and disease. *American journal of physiology Regulatory, integrative and comparative physiology* 297: R913-924, 2009.
40. **Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MK.** Adipocyte differentiation-related protein and OXPAT in rat

- and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *The Journal of clinical endocrinology and metabolism* 94: 4077-4085, 2009.
41. **Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B, and Kimmel AR.** Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, *Drosophila*, and *Dictyostelium*. *The Journal of biological chemistry* 277: 32253-32257, 2002.
 42. **Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS.** Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
 43. **Mottagui-Tabar S, Ryden M, Lofgren P, Faulds G, Hoffstedt J, Brookes AJ, Andersson I, and Arner P.** Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. *Diabetologia* 46: 789-797, 2003.
 44. **Murphy S, Martin S, and Parton RG.** Lipid droplet-organelle interactions; sharing the fats. *Biochimica et biophysica acta* 1791: 441-447, 2009.
 45. **Nakamura N, and Fujimoto T.** Adipose differentiation-related protein has two independent domains for targeting to lipid droplets. *Biochemical and biophysical research communications* 306: 333-338, 2003.
 46. **Peters SJ, Dyck DJ, Bonen A, and Spriet LL.** Effects of epinephrine on lipid metabolism in resting skeletal muscle. *The American journal of physiology* 275: E300-309, 1998.
 47. **Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA, and Tarnopolsky MA.** Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 37: 724-735, 2012.
 48. **Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, and Ploug T.** Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *Journal of lipid research* 47: 2392-2399, 2006.
 49. **Rennie MJ, and Holloszy JO.** Inhibition of glucose uptake and glycogenolysis by availability of oleate in well-oxygenated perfused skeletal muscle. *The Biochemical journal* 168: 161-170, 1977.
 50. **Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, and de Medina FS.** Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Analytical biochemistry* 401: 318-320, 2010.
 51. **Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, Tornqvist H, Zechner R, and Zimmermann R.** Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *The Journal of biological chemistry* 281: 40236-40241, 2006.
 52. **Shaw CS, Sherlock M, Stewart PM, and Wagenmakers AJ.** Adipophilin distribution and colocalization with lipid droplets in skeletal muscle. *Histochemistry and cell biology* 131: 575-581, 2009.

53. **Shen WJ, Patel S, Miyoshi H, Greenberg AS, and Kraemer FB.** Functional interaction of hormone-sensitive lipase and perilipin in lipolysis. *Journal of lipid research* 50: 2306-2313, 2009.
54. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS.** Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Experimental physiology* 97: 970-980, 2012.
55. **Souza SC, Muliro KV, Liscum L, Lien P, Yamamoto MT, Schaffer JE, Dallal GE, Wang X, Kraemer FB, Obin M, and Greenberg AS.** Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. *The Journal of biological chemistry* 277: 8267-8272, 2002.
56. **Subramanian V, Rothenberg A, Gomez C, Cohen AW, Garcia A, Bhattacharyya S, Shapiro L, Dolios G, Wang R, Lisanti MP, and Brasaemle DL.** Perilipin A mediates the reversible binding of CGI-58 to lipid droplets in 3T3-L1 adipocytes. *The Journal of biological chemistry* 279: 42062-42071, 2004.
57. **Sztalryd C, Bell M, Lu X, Mertz P, Hickenbottom S, Chang BH, Chan L, Kimmel AR, and Londos C.** Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line. *The Journal of biological chemistry* 281: 34341-34348, 2006.
58. **Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C.** Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
59. **Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C.** Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
60. **Tansey JT, Huml AM, Vogt R, Davis KE, Jones JM, Fraser KA, Brasaemle DL, Kimmel AR, and Londos C.** Functional studies on native and mutated forms of perilipins. A role in protein kinase A-mediated lipolysis of triacylglycerols. *The Journal of biological chemistry* 278: 8401-8406, 2003.
61. **Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, and Londos C.** Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 98: 6494-6499, 2001.
62. **Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR, and Londos C.** The central role of perilipin a in lipid metabolism and adipocyte lipolysis. *IUBMB life* 56: 379-385, 2004.
63. **Timmers S, de Vogel-van den Bosch J, Hesselink MK, van Beurden D, Schaart G, Ferraz MJ, Losen M, Martinez-Martinez P, De Baets MH, Aerts JM, and Schrauwen P.** Paradoxical increase in TAG and DAG content parallel the insulin sensitizing effect of unilateral DGAT1 overexpression in rat skeletal muscle. *PloS one* 6: e14503, 2011.
64. **Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman R, Gong D, Brasaemle D, and Sztalryd C.** Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid

- droplet-associated protein. *The Journal of biological chemistry* 286: 15707-15715, 2011.
65. **Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman R, Gong D, Brasaemle D, and Sztalryd C.** Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *The Journal of biological chemistry* 286: 15707-15715, 2011.
66. **Wang H, Hu L, Dalen K, Dorward H, Marcinkiewicz A, Russell D, Gong D, Londos C, Yamaguchi T, Holm C, Rizzo MA, Brasaemle D, and Sztalryd C.** Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. *The Journal of biological chemistry* 284: 32116-32125, 2009.
67. **Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley WC, and Sztalryd C.** Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *Journal of lipid research* 52: 2159-2168, 2011.
68. **Wang SM, Hwang RD, Greenberg AS, and Yeo HL.** Temporal and spatial assembly of lipid droplet-associated proteins in 3T3-L1 preadipocytes. *Histochemistry and cell biology* 120: 285-292, 2003.
69. **Watt MJ, and Hoy AJ.** Lipid metabolism in skeletal muscle: generation of adaptive and maladaptive intracellular signals for cellular function. *American journal of physiology Endocrinology and metabolism* 2011.
70. **Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel PE.** S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *The Journal of biological chemistry* 280: 19146-19155, 2005.
71. **Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, and Bickel PE.** OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55: 3418-3428, 2006.
72. **Yamaguchi T, Omatsu N, Matsushita S, and Osumi T.** CGI-58 interacts with perilipin and is localized to lipid droplets. Possible involvement of CGI-58 mislocalization in Chanarin-Dorfman syndrome. *The Journal of biological chemistry* 279: 30490-30497, 2004.
73. **Yamaguchi T, Omatsu N, Omukae A, and Osumi T.** Analysis of interaction partners for perilipin and ADRP on lipid droplets. *Molecular and cellular biochemistry* 284: 167-173, 2006.
74. **Zechner R, Kienesberger PC, Haemmerle G, Zimmermann R, and Lass A.** Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *Journal of lipid research* 50: 3-21, 2009.

Chapter 6

Study 3

Skeletal muscle PLIN3 and PLIN5 are serine phosphorylated at rest and following lipolysis during adrenergic or contractile stimulation

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Rebecca MacPherson: conceived the idea, designed the study, did the experiments, wrote the manuscript

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Abstract

In adipose tissue, access of adipose triglyceride and hormone sensitive lipases (ATGL and HSL) to the lipid droplet depends on PLIN1 phosphorylation, however PLIN1 is not expressed in skeletal muscle and the phosphorylation of the expressed PLINs has yet to be investigated. Further, direct interactions between skeletal muscle PLINs and HSL are unknown. We investigated the isolated and combined effects of epinephrine and contraction on PLIN-to-lipase interactions as well as phosphorylation. Isolated rat solei were assigned to one of four 30min *in-vitro* conditions (25°C): 1) rest; 2) intermittent tetanic stimulation (60Hz for 150ms; train rate 20/min); 3) 5nM epinephrine; 4) intermittent tetanic stimulation and 5nM epinephrine. Immunoprecipitation of serine-phosphorylated proteins followed by Western blotting for PLIN2, PLIN3, PLIN5, revealed that only PLIN2 is not phosphorylated under any of the experimental conditions. This is the first study to show that in whole rat skeletal muscle PLIN3 and PLIN5 are serine phosphorylated. The degree of serine phosphorylation remained unchanged following adrenergic and/or contractile stimulation. Oil red O staining of muscle sections for lipid content shows a significant decrease following each condition, confirming lipolysis occurred ($P < 0.05$). PLIN2, 3, and 5 all interact with HSL and ATGL, but these interactions were unchanged following treatments. Our results show that in skeletal muscle, PLIN2 is not serine phosphorylated at rest or with lipolytic stimulation and that while PLIN3, PLIN5 are serine phosphorylated at rest, the degree of phosphorylation does not change with lipolytic stimulation.

Keywords: exercise, lipolysis, OXPAT, ADRP, TIP47

Introduction

Intramuscular triglycerides (IMTGs) represent an important energy source that can be mobilized during exercise through a combination of external hormonal (increased epinephrine) and internal metabolic signals (intracellular Ca^{2+} , and metabolite concentrations). However, the exact mechanisms regulating IMTG breakdown during exercise are poorly understood. IMTGs are stored in metabolically active organelles known as lipid droplets that are encased by a phospholipid monolayer coated with a variety of proteins (4, 33). Evidence indicates that the regulation of skeletal muscle lipolysis is mediated by protein-protein interactions occurring on the lipid droplet surface (37, 48). Specifically, a family of lipid droplet proteins, known as PLIN proteins, have emerged as likely candidates in mediating the hydrolysis of IMTGs (10, 37). To date, work investigating the role(s) of PLINs has focused on adipose tissue, however recent investigations in skeletal muscle support a role for PLIN proteins in the regulation of IMTG degradation (36, 37, 51-53).

The PLIN family is composed of five members (PLIN1 through PLIN5) (29, 41), each with a unique tissue distribution and potentially a unique role in cellular lipid metabolism (26, 68). PLIN1 is the only member of this family for which a specific role in regulating lipolysis has been determined, however it is only expressed in adipose tissue. More specifically, in a basal state PLIN1 limits the activity of the rate limiting lipase, ATGL, by directly binding to its co-activator, CGI-

58 (11, 42, 54, 60). Under lipolytic stimulation, initiated by catecholamines, it is believed that the PKA-dependent serine phosphorylation of PLIN1 initiates lipolysis by releasing CGI-58 and allowing it to bind to and activate ATGL (6, 16, 18-20). Further, phosphorylation of PLIN1 is required for HSL recruitment to the lipid droplet through binding to PLIN1 (65). Skeletal muscle does not express PLIN1 and, thus far, similar roles for skeletal muscle PLIN proteins have yet to be determined.

It has been suggested that PLIN2, PLIN3, and PLIN5 play a large role in regulating lipolysis in skeletal muscle (36, 37, 46, 51-53). PLIN2 is the predominant lipid droplet associated protein in skeletal muscle (47) and PLIN5 is unique in that it is highly expressed in oxidative tissues (68). Although knowledge of PLIN3 in skeletal muscle is scarce, recent work from our laboratory showed that ATGL interacts with PLIN2, PLIN3, and PLIN5 in isolated rat soleus muscle (37). These protein interactions suggest that PLIN2, PLIN3, and PLIN5 may have a role in the regulation of ATGL activity and therefore the initiation of skeletal muscle lipolysis. In adipose tissue, the reversible phosphorylation of PLIN1 is necessary for lipolytic activation (38, 43, 57, 59), however the phosphorylation state of the remaining PLINs has yet to be investigated in skeletal muscle. A phosphorylation site has been identified on PLIN2 (serine 291) (4), PLIN3 (serine 245) (24), and some evidence indicates that PLIN5 is a substrate for PKA phosphorylation (64). It is possible that the phosphorylation of PLIN2, PLIN3, or PLIN5 may be required to optimally position ATGL, CGI-58, and/or HSL for activation of lipolysis in skeletal muscle.

Exercise leads to the activation of several skeletal muscle kinases, all of which may play into regulating the rate of lipolysis. Increased circulating

epinephrine concentrations leads to the activation of PKA, while at the same time contraction increases intramuscular calcium levels and CaMK and ERK activation. The use of ATP during contraction also leads to increased levels of AMP, which activates AMPK. It is known that epinephrine and contraction activate skeletal muscle hormone sensitive lipase (HSL) with additive effects, thus indicating that epinephrine and contraction activate HSL through different signaling mechanisms (13-15, 25, 31, 55, 66). Therefore the purpose of this study was to examine the isolated and additive effects of epinephrine and contraction on skeletal muscle PLIN protein to ATGL and HSL interactions as well as phosphorylation status. A major objective of this study was to determine if PLIN2, PLIN3, and/or PLIN5 are phosphorylated and whether this changes during lipolysis. If so, this study aimed to separate adrenergic and/or contractile stimulation. A second objective of this study was to investigate the role of PLIN2, PLIN3 and PLIN5 in governing the accessibility of ATGL and HSL via direct protein-protein interactions during these perturbations. We hypothesized that skeletal muscle PLIN to lipase interactions are governed by PLIN phosphorylation status.

Methods

Animals

A total of 48 male Long-Evans rats (4–6 wk old) were used in this study. Animals were housed in groups within the Brock University Animal Facility, where they were maintained on a 12:12-h light-dark cycle at 22°C. The rats were fed a

standard rodent diet with ad libitum access to food and water. All experimental procedures and protocols were approved by the Brock University Animal Care and Utilization Committee and conformed to all Canadian Council on Animal Care guidelines.

Muscle preparation

Animals were anesthetized via intraperitoneal injection of pentobarbital sodium (6 mg/100g body wt). The left and right solei were removed and placed in organ baths, where they were assigned to one of four experimental conditions: 1) rest; 2) electrically stimulated contraction (15, 36); 3) 5nM epinephrine (45); and 4) epinephrine and electrical stimulation. To briefly summarize the preparation, each soleus muscle was dissected tendon-to-tendon, sutures tied in-situ, the muscle was then removed and immediately placed in an organ bath (Radnoti Glass Technology, Monrovia, CA), which contained 7-8 ml of fully oxygenated liquid Sigma medium 199 (M 4530, Aigma-Aldrich, Canada) and suspended at a resting tension of 1 g. The incubation medium was continuously gassed with 95% O₂ 5% CO₂, and temperature was maintained at 25°C (3). All muscles were allowed to equilibrate at rest for 30 min. This isolated muscle preparation allows for the examination of the isolated effects of epinephrine, contraction, as well as epinephrine and contraction together on muscle lipid metabolism in isolation, absent other systemic perturbations.

Perturbation

1. *Epinephrine Incubation:* Soleus muscles were incubated with 5nM epinephrine for 30 min. This concentration of epinephrine has previously proven to maximally promote triglyceride breakdown in isolated soleus muscles (45).

2. *Stimulated Contraction:* Soleus muscles were stimulated to contract for 30 min as previously reported by our lab and others (15, 36, 37). Initially, optimal stimulus voltage was determined by assessing force responses (Grass Telefactor force transducer, West Warwick, RI) to single electrical pulses (Grass Model FT03 with P11T amplifier). Stimulus intensity was increased from 10 V in 10-V increments, until a plateau in twitch force was reached, after which stimulus voltage was increased to ~1.25 of this level. During the 30-min stimulus protocol, muscles received repeated volleys of brief (150 ms) but high-frequency (60 Hz) trains at a train rate of 20 tetani/min whilst muscles were suspended at 1 g of resting tension. This protocol was previously proven to elicit maximal rates of triglyceride pool turnover and rates of IMTG oxidation without the development of fatigue (15, 36). Throughout this period, muscle force was recorded using Grass Polyview Data Acquisition and Analysis System (West-Warwick, RI) and analyzed using the Polyview Reviewer (Grass Polyview Data Acquisition and Analysis System; Astro-Med, West-Warwick, RI).

3. *Epinephrine and Electrically Stimulated Contraction.* Soleus muscles were incubated with 5.0 nM epinephrine as well as electrically stimulated to contract, describe above, for 30 min.

Sample Preparation

Following the incubations, solei were removed from the baths and cut into two pieces. One piece was snap frozen in liquid nitrogen for Western blotting analysis and the other piece was mounted for histochemical analysis (see below). Soleus muscles were homogenized in Griffin lysis buffer (150 mM NaCl, 50 mM Tris HCl, 1 mM EGTA) using a 1:25 dilution of muscle to buffer with added protease (11836170001, Roach, QC), and phosphatase inhibitor tablets (04906845001, Roach, QC). For measures of protein phosphorylation a kinase inhibitor (7,8-Dihydroxycoumarin, 1001251297, Sigma Aldrich) was added to the homogenization buffer. Protein concentration of the total homogenates was determined using a Bradford Assay (Bio-Rad Protein Assay Dye Reagent Concentrate; #500-0006, Bio-Rad, USA).

Protein Interactions (co-immunoprecipitation)

Sample homogenates were immunoprecipitated with 5 μ l of the appropriate primary antibody (ATGL or HSL) and then immunoblotted for the corresponding protein (PLIN2; PLIN3; PLIN5; CGI-58). Specifically, 500-1000 μ g of protein from each sample were incubated for 2 h with the antibody at 4 °C. Pilot work was done in order to determine the appropriate amount of whole homogenate to incubate with the antibody in order to fully isolate the protein of interest. Following this 20 μ l of Protein G Agarose beads (sc-2001, Santa Cruz Biotechnology Inc) were added to each sample for overnight incubation at 4 °C. The pellet of each sample was

collected by centrifugation at 130 rpm for 5-10 seconds. Pellets were washed three times in phosphate buffered saline (PBS) and re-suspended in 40 μ l of 2x sample buffer. To test for antibody interference in the samples a blank sample containing only the precipitating antibody and lysis buffer were prepared in exactly the same manner as the experimental samples. For interactions where antibody interference occurred, a secondary antibody that only detects native (i.e., not denatured) antibodies was used (Clean Blot IP Detection Reagent; Thermo Scientific). All samples were boiled and separated using 8 or 10% SDS-polyacrylimide gel electrophoresis.

Protein Phosphorylation (immunoprecipitation of P_{Ser} proteins)

To ensure immunoprecipitation of all serine phosphorylated proteins sample homogenates were immunoprecipitated with 40 μ l of anti-phosphoserine antibody (Millipore AB1603) and then immunoblotted for the corresponding protein (ATGL; PLIN2; PLIN3; PLIN5). Specifically, 500 μ g of protein from each sample were used in order to isolate P_{Ser} proteins. In order to avoid antibody interference as well as save sample these samples were prepared using a Pierce[®] immunoprecipitation kit (#26149, Thermo Scientific, Rockford, IL, USA). A phosphoserine antibody was used based on previous work indicating that PLIN2, PLIN3, and ATGL contain serine phosphorylation sites (4, 24, 39), and that PLIN5 may be substrate for PKA (64).

Western blotting

SDS-polyacrylamide gel electrophoresis (8 or 10% separating; 4% stacking) was used to separate proteins (ATGL, CGI-58, PLIN2, PLIN3, and PLIN5) at 120 V for 1.5 h, and proteins were electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, New Jersey) for 1 h at 100 V followed by blocking in 2 or 5% fat-free milk in TBST, or 5% BSA in TBST. Primary antibodies for co-precipitated proteins were diluted 1:1000 in 2 or 3% fat-free milk, or 1 or 5% BSA in TBST and incubated overnight at 4 °C. Secondary antibodies were diluted 1:10000-20000 in 2 or 3% milk, or 1 or 5% BSA and incubated for 1 hour. Blots of specific proteins were visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, New Jersey). The densities of the individual bands were integrated using Image J software (<http://rsbweb.nih.gov/ij/>). Each blot had loaded whole soleus homogenate as a positive control for the co-precipitated protein. Blots were normalized to total protein loaded determined by Ponceau S staining (M530; Sigma-Aldrich) and results are reported as the ratio of the density of the target protein to the density of the loaded protein in arbitrary units (37, 49).

Antibodies

The following antibodies were used and have been used previously: anti-phosphoserine antibody (Millipore AB1603), PLIN2 (52 kDa) mouse monoclonal antibody (Progen Biotechnik, Heidelberg, Cat. No. 610102) (37, 46), PLIN3 (47 kDa) (ProSci Inc., Poway, California, Cat. No. 3883)(37, 46), PLIN5 (52 kDa) guinea pig

polyclonal antibody (Progen Biotechnik, Heidelberg, Cat. Nos. GP34 and GP31) (8, 37, 40, 46), ATGL (54 kDa) rabbit monoclonal antibody (Cell Signalling Technology, #2439, Danvers, MA) (2, 37), CGI-58 (42 kDa) rabbit polyclonal antibody (Novus Biologicals, NB110-41576, Oakville, ON) (2, 37, 61), and HSL (Cell Signalling Technology, 4107S, Danvers, MA).

Histochemical Analysis

The muscle section used for histochemical analysis was oriented for cross sections and mounted, in embedding medium (Cryomatrix, Pittsburgh, PA), on a piece of cork, which was plunged into 2-methylbutane cooled in liquid nitrogen. Following rapid freezing, the samples were stored at -80°C until sectioning. Sectioning was completed with a cryotome (ThermoShandon, Runcorn, Cheshire, UK) optimally set at -20°C. Sections were (10 µm thick) thaw mounted onto slides and stored at -80°C until histochemical staining. To permit the examination of lipid droplets, oil red O (ORO; O0625; Sigma-Aldrich, St. Louis, MO) (30) was utilized (30, 56, 62, 63). Briefly, cryosections were fixed in 3.7% formaldehyde for 1h. Slides were then rinsed 3 times in deionized water for 30 s, and then immersed in the working solution of ORO for 30 min. Slides were rinsed three times in deionized water and cover slips were mounted with a prolonging agent (no. P36930; Prolong Gold anti-fade reagent; Invitrogen).

Image capturing and analyses

All sections were examined using a Nikon Eclipse 80i fluorescence microscope (Nikon Eclipse 80i; Chiyoda-ku, Tokyo, Japan). Digital images of the slides were captured with a digital camera (Retiga 1300, QImaging, Burnaby, BC, Canada) attached to the microscope. To visualize the ORO stain a TRITC (510–560 nm) excitation filter was used. Digitally captured images (x40 magnification), four fields of view/muscle cross section (23.1 ± 0.6 fibers/field of view), were processed and analyzed using imaging software (NIS-Elements AR 3.00; Nikon Instruments, Melville, NY). An intensity threshold representing minimal values corresponding to lipid droplets was set manually and applied uniformly in all images. The lipid droplet fluorescent signals were quantified for each muscle fiber, resulting in a total of 92.3 ± 2.6 fibers analyzed for each muscle cross section. Fiber area, as well as number and area of objects emitting a fluorescent signal, were recorded. Muscle fiber lipid droplet content was expressed as the fraction of the measured area that was stained per fibre (36, 62). All measures were manually outlined and traced by investigators for each individual myocyte. The immunofluorescence method described here covers numerous fibers per muscle cross section and, therefore, gives a good representation of the entire muscle. To test the reliability of the method in our hands, both intra-observer and inter-observer reliability were evaluated by two investigators. The intra-observer reliability involved the two investigators performing analysis of one image three times, at least 1 wk apart. The inter-observer reliability involved two independent investigators performing analysis for

three separate images. These tests proved to be reliable with a coefficient of variation <5% for both intra-observer and inter-observer reliability.

Statistics

Comparisons of mean lipid content, protein interactions and phosphorylation between groups were performed using a one-way ANOVA (incubation condition). Tukey post hoc tests were performed when significance was detected. Statistical significance was set at $P < 0.05$. All data are expressed as means \pm SEM.

Results

Lipid droplet content

The average muscle fiber area was $2604 \pm 532 \mu\text{m}^2$. Figure 1 shows representative images of rat skeletal muscle cross sections viewed with an immunofluorescence microscope following incubation with ORO. Fiber lipid droplet content (area lipid stained) decreased to ~30% of resting values in all groups ($p=0.001$; Figure 1).

Figure 1

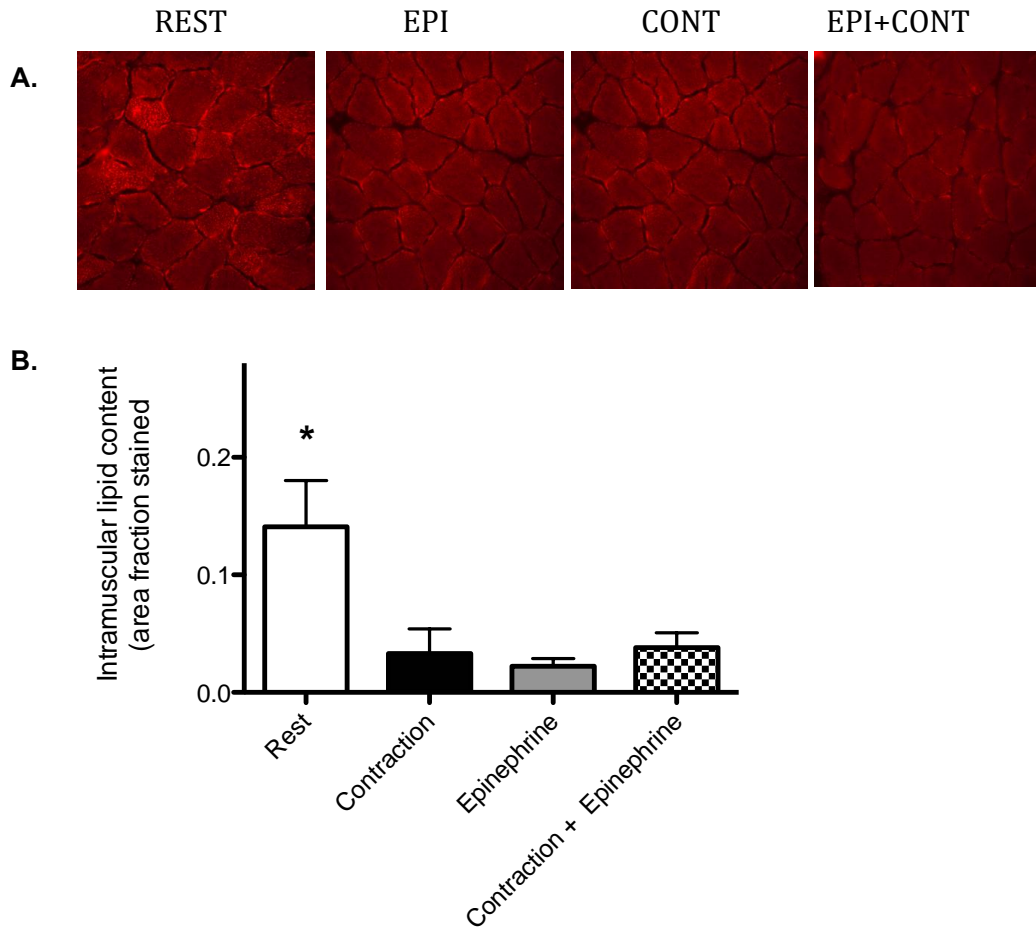


Figure 16

Figure 1. Digitally captured images of one single field of view ($\times 40$ magnification) taken from a soleus muscle cross section. **A.** Oil red O (ORO) staining. EPI, epinephrine stimulate 5 nM/L; CONT, electrically stimulated contractions for 30 min; EPI+CONT, combination of 5 nM/L epinephrine and electrically stimulated contractions for 30 min.

B. Lipid content (expressed as area fraction stained) in rested and stimulated rat soleus muscle. Values are expressed as means \pm SE. *Significantly different from experimental conditions ($P < 0.05$) ($n=9$ for EPI and CONT; $n=10$ for REST and EPI+CONT).

Protein interactions

ATGL co-immunoprecipitated with CGI-58 at rest and following each perturbation. Contraction, epinephrine, and the combination appeared to increase this interaction ~50%, 25%, and 80% respectively, although this was not statistically significant ($p=0.25$; Figure 2a).

PLIN2, 3 and 5 all co-immunoprecipitated with ATGL at rest and following each perturbation. There were no significant differences in PLIN protein content in ATGL immunoprecipitated samples following any of the perturbations ($p>0.05$) (Figure 3 and 4).

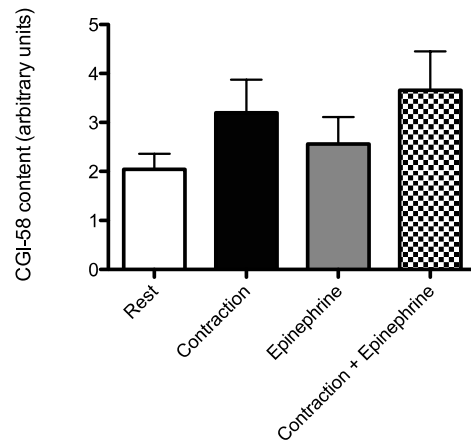
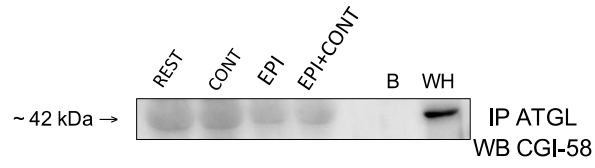
PLIN2, 3 and 5 all co-immunoprecipitated with HSL at rest and following each perturbation. There were no significant differences in PLIN protein content in HSL immunoprecipitated samples following any of the perturbations ($p>0.05$) (Figure 3 and 4).

Protein Serine Phosphorylation

Western blotting for PLIN2 protein content in immunoprecipitated serine phosphorylated proteins resulted in no detectable PLIN2 protein at rest or with any of the perturbations (Figure 3). Phosphorylation of PLIN3, PLIN5, and ATGL was detectable at rest with no significant difference under any of the perturbations ($p>0.05$) (Figure 4).

Figure 2

A.



B.

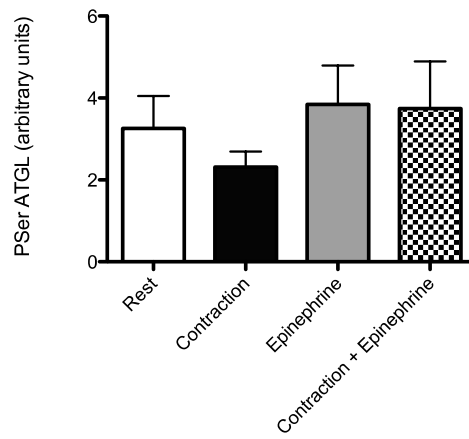
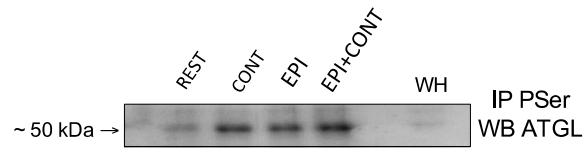


Figure 17

Figure 2. A. ATGL-CGI-58 interaction: CGI-58 protein content (arbitrary units) in ATGL immunoprecipitated samples at rest and following stimulation with representative Western blot (EPI, epinephrine stimulate 5 nM/L; CONT, electrically stimulated contractions for 30 min; EPI+CONT, combination of 5 nM/L epinephrine and electrically stimulated contractions for 30 min; B negative control containing just the IP antibody; WH whole homogenate) (n=12 for REST and CONT+EPI; n=11 for CONT and EPI). CGI-58 content is relative to total protein content as measured by ponceau staining **B.** ATGL serine phosphorylation: ATGL protein content (arbitrary units) in phosphoserine immunoprecipitated samples at rest and following stimulation with representative Western blots (n=8). ATGL serine phosphorylation is relative to total protein content as measured by ponceau staining

Figure 3

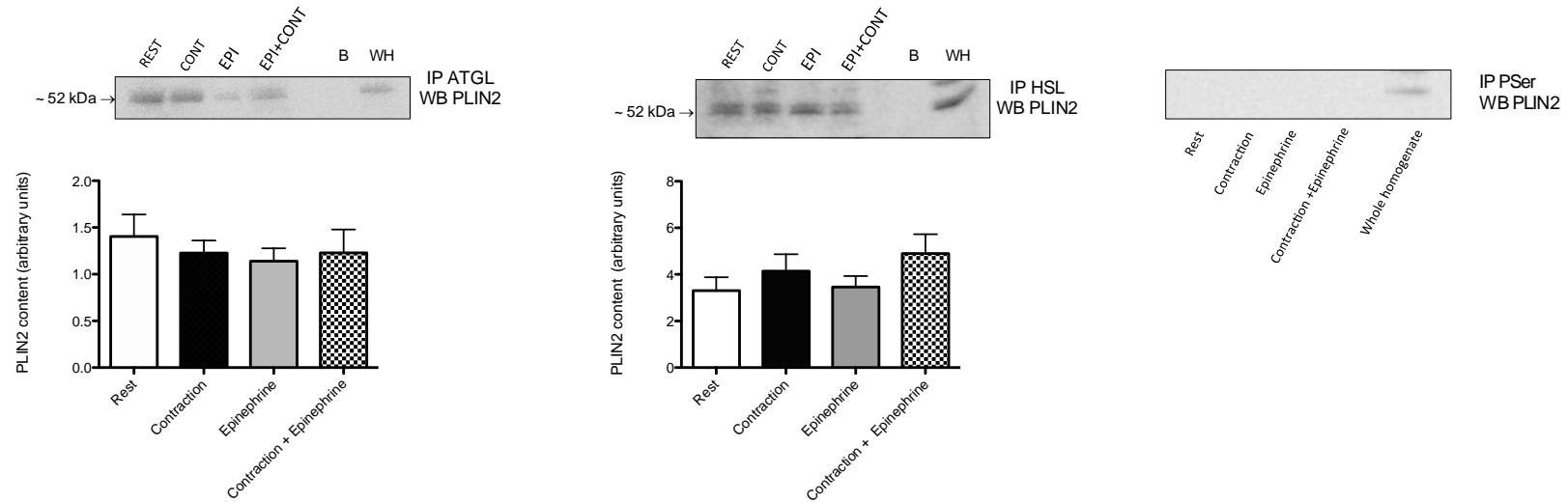
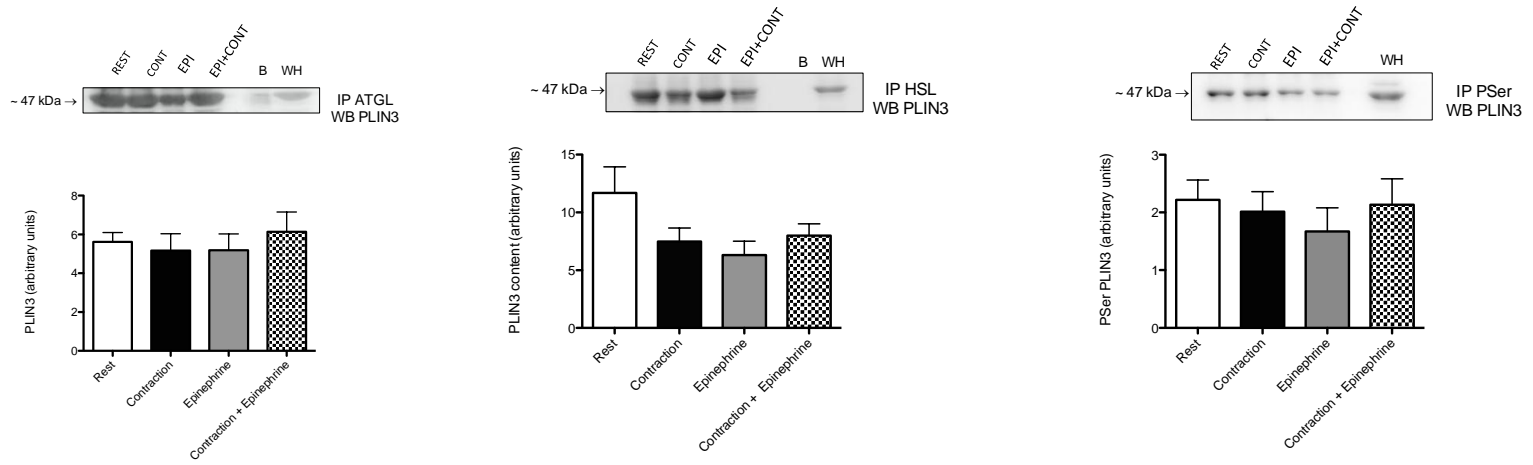


Figure 18

Figure 3. ATGL/HSL-PLIN2 protein interactions and PLIN2 serine phosphorylation at rest and following stimulation shown as PLIN protein content (arbitrary units) in immunoprecipitated samples with representative Western blots (EPI, epinephrine stimulate 5 nM/L; CONT, electrically stimulated contractions for 30 min; EPI+CONT, combination of 5 nM/L epinephrine and electrically stimulated contractions for 30 min; B negative control containing just the IP antibody; WH whole homogenate) (n=10). PLIN2, Western blotting for PLIN2 in phosphoserine immunoprecipitated samples resulted in no detectable signal under any condition. PLIN2 content is relative to total protein content as measured by ponceau staining.

Figure 4.

A. PLIN3



B. PLIN5

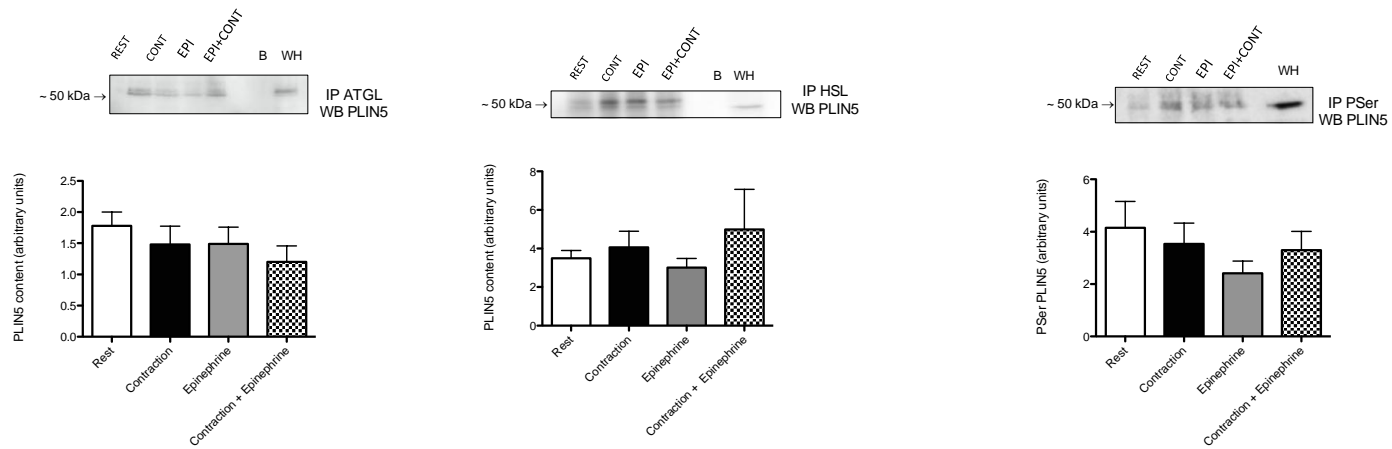


Figure 19

Figure 4. ATGL/HSL-PLIN protein interactions and PLIN serine phosphorylation at rest and following stimulation shown as PLIN protein content (arbitrary units relative to total protein measured by ponceau staining) in immunoprecipitated samples with representative Western blots (EPI, epinephrine stimulate 5 nM/L; CONT, electrically stimulated contractions for 30 min; EPI+CONT, combination of 5 nM/L epinephrine and electrically stimulated contractions for 30 min; B negative control containing just the IP antibody; WH whole homogenate) (n=10). **A.** PLIN3; PLIN3 **B.** PLIN5.

Discussion

Skeletal muscle PLIN proteins are believed to play a critical role in regulating IMTG turnover, however the exact mechanisms regulating lipolysis within skeletal muscle during exercise remain unknown. This study investigated the effects of contraction and epinephrine alone and in combination on the phosphorylation state of PLIN2, PLIN3, PLIN5, and ATGL, as well as the PLIN interactions with ATGL and HSL. This is the first study to show in intact rat skeletal muscle that PLIN2, PLIN3, and PLIN5 interact with HSL and that these interactions are unchanged following either contractile or adrenergic stimulation. Further findings from this work demonstrate that both PLIN3 and PLIN5 are serine phosphorylated while PLIN2 is not serine phosphorylated. Contrary to our hypothesis there was no significant change in PLIN3 or PLIN5 phosphorylation with contraction or epinephrine alone or in combination. These novel findings indicate that, unlike adipose tissue where PLIN1 phosphorylation is required for lipolysis, skeletal muscle PLIN proteins are phosphorylated in a basal state and that overall this total phosphorylation does not change with lipolysis under either adrenergic or contractile stimulation. Further work is necessary to investigate the site specific phosphorylation of these two proteins to determine if there are other sites that may have been affected.

Our experimental conditions were chosen for their ability to elicit large levels of intramuscular triglyceride turnover. The stimulated contraction protocol used in this study has been shown to elicit maximal rates of lipolysis in isolated soleus muscle and we have previously shown that this method leads to a significant decline

in IMTGs (15, 36). Moreover, the epinephrine concentration used (5nM) was previously shown to increase IMTG hydrolysis in isolated rat soleus (45). In the present study we found a significant reduction in intramuscular lipids stained by oil red O following each experimental condition (Figure 1; electrically stimulated contraction 68% decrease, epinephrine stimulation 65% decrease, and combination of both 73% decrease), thus confirming that our model elicited high rates of lipolysis in all experimental conditions.

We show that the interactions of PLIN2, PLIN3, and PLIN5 with ATGL and HSL are unchanged following contraction or epinephrine stimulation alone or in combination. PLIN2 was the only skeletal muscle PLIN protein not to be serine phosphorylated under any of the experimental conditions (rest, contraction, epinephrine, or both). This finding is interesting, as PLIN2 has been suggested to function similarly to PLIN1 by increasing triglyceride storage and decreasing triglyceride turnover (5, 12, 17, 27, 28). In skeletal muscle, some evidence indicates that PLIN2 is involved in regulating lipolysis, however other research points towards an essential role of PLIN2 in IMTG synthesis. Recently, Shepherd et al. (52) found that in human skeletal muscle PLIN2-associated lipid droplets are preferentially used with an acute bout of exercise, supporting a role for PLIN2 in regulating muscle lipolysis. Interestingly, PLIN2 protein content is higher in cases where there is increased IMTG content, such as in females, type II diabetics, and with endurance training (40, 46, 51). Therefore, a higher PLIN2 content could simply be a consequence of increased PLIN2 protein stability through increased lipid droplet size and number. Previous work with skeletal muscle contraction has

shown a translocation of HSL towards PLIN2-coated lipid droplets (48), however this study did not determine if there was a direct physical interaction between these two proteins. Our work demonstrates an interaction between PLIN2 and ATGL (37) as well as HSL, however this interaction remains unchanged following contraction and/or epinephrine stimulation *in vitro*. Previously, our group found that the interaction between PLIN2 and ATGL is significantly decreased (21%) following electrically stimulated contraction (37). The present study shows a trend for a decline in this interaction following each of the experimental conditions however this did not meet statistical significance likely due to the small sample size and the addition of two groups to the statistical analysis. Future work should investigate the importance of this interaction in skeletal muscle. It is possible that PLIN2 may not be directly involved in stimulated skeletal muscle lipolysis but is more involved with IMTG synthesis and lipid droplet growth. PLIN2 content increases with fatty acid loading of myotubes and with *in vivo* interventions that lead to an increased muscle lipid content (high fat diet and fasting) (7). Additionally, overexpression of PLIN2 *in vitro* and *in vivo* results in increased intramyocellular lipid content, while knockdown of PLIN2 in cultured myotubes prevents fatty acid induced IMTG accumulation and lipid droplet storage (7). Due to the association of PLIN2 with both ATGL and HSL it is possible that PLIN2 is important for regulating basal lipolysis however future work may want to investigate a role for PLIN2 in lipid droplet development and its relationship to proteins involved in IMTG synthesis.

In this study both PLIN3 and PLIN5 were found to be serine phosphorylated and this did not change following either adrenergic or contractile stimulation. Also,

both PLIN3 and PLIN5 interacted with ATGL and HSL but this was unchanged following lipolytic stimulation. Therefore, like PLIN2, the roles of PLIN3 and PLIN5 in skeletal muscle lipid dynamics remain unclear. Among the PLIN family, PLIN3 has a high degree of sequence similarity with PLIN2 and also shows a ubiquitous tissue expression (34, 69), therefore PLIN3 may play a similar role as PLIN2 in lipid droplet dynamics. Association of PLIN3 with lipid droplets increases in cells grown in media supplemented with exogenous fatty acids or glucose and insulin (designed to increase TG synthesis) (67, 69). Moreover, in PLIN2 null mice, PLIN2 on the LD is replaced with PLIN3 (58). This research suggests that PLIN3, like PLIN2, plays a role in lipid droplet formation and triglyceride synthesis and less of a role in stimulated lipolysis. PLIN5 has previously been shown to be essential for ATGL-mediated lipolysis (21, 22) and was found to be a substrate for PKA in AML12 mouse liver cells, suggesting that PLIN5 phosphorylation may be important for lipolysis (64). However our results show that in skeletal muscle, PKA stimulation through epinephrine does not change the serine phosphorylation state of PLIN5 or the association with either ATGL or HSL. PKA is both a serine and threonine kinase and therefore it is possible that PLIN5 is also phosphorylated on a threonine site. This highlights the importance of determine the specific sites on which PLIN5 may be phosphorylated. Further, we show that contraction-induced kinases did not result in changes in the overall serine phosphorylation state of PLIN5 or change the interactions with lipases. Due to the oxidative tissue expression of PLIN5 (68), as well as its association with mitochondria (8), it is likely that PLIN5 plays a role in facilitating fatty acid oxidation and not IMTG hydrolysis per se. In support of this

theory, Peters et al. (46) found PLIN5 protein content increased with endurance training and this increase was associated with improved muscle oxidative capacity. Other work has demonstrated that overexpression of PLIN5 *in-vitro* increased fatty acid oxidation (68), providing evidence for a larger role of PLIN5 in enhancing the oxidation of the fatty acids released from the lipid droplets over IMTG synthesis or lipolysis. Overexpression of PLIN5 in skeletal muscle also promotes expression of several genes, regulated by PPAR α and PGC1 α , involved in fatty acid catabolism and oxidation (9). The recent discovery that PLIN5 is located in direct contact with mitochondria and that PLIN5 overexpression results in more intimate interactions between these two organelles provides more evidence in support of a strong role in oxidation (8). Our findings that PLIN5 is associated with both ATGL and HSL with no change under any condition indicate that PLIN5 may be involved in regulating basal lipolysis but not stimulated lipolysis. Future work should investigate a role for PLIN5 in fatty acid oxidation and mitochondrial association. The mechanistic function of interactions between PLIN2, PLIN3, and PLIN5 with both ATGL and HSL remains to be investigated. These interactions might be essential for maintaining a stable rate of basal lipolysis or ensuring that the lipases are targeted to the right subcellular location.

ATGL is now known as the rate-limiting lipase in lipolysis and its activity is enhanced in the presence of CGI-58 (32, 70). We have previously shown that 30 min of isolated soleus contraction leads to a significant increase in the interaction between ATGL and CGI-58, consistent with the increased rates of lipolysis observed in our study, as well as being consistent with the activation of lipolysis in adipose

tissue (37). However, in the present study we found that the increased ATGL and CGI-58 interaction did not reach significance with any of our experimental conditions. It has been suggested that a direct physical interaction between ATGL and CGI-58 is not actually necessary to increase the lipolytic activity of ATGL (35). Gruber et al. (23) demonstrated that CGI-58 is able to bind directly to the phospholipid monolayer of lipid droplets and that without this there is a complete loss of the ability of CGI-58 to activate ATGL, regardless of their interaction. These results may explain why in the present study there was no significant increase in protein-protein interaction between ATGL and CGI-58. It may be more important for CGI-58 to bind to the lipid droplet membrane and somehow allow ATGL to access the triglyceride core (35).

Previous studies have identified serine phosphorylation sites on ATGL (Ser 406/404 and 428/430) (4, 70), however functional roles of phosphorylation and the kinases involved remain unknown. While work in other cell types (HEK293 cells and adipocytes) indicates that AMPK (1) and PKA (44) can phosphorylate ATGL, our results show that ATGL serine phosphorylation remains unchanged in the face of contractile and/or adrenergic stimulation. This finding is in agreement with Mason et al. (39) who demonstrated that ATGL Ser404 phosphorylation is not increased in human skeletal muscle during moderate-intensity cycling exercise. In addition, that study demonstrated that there was no evidence of a physical interaction between ATGL and AMPK after immunoprecipitation, and pharmacological activation of AMPK did not affect ATGL Ser404 phosphorylation in cultured myotubes (39). Further, direct studies in C2C12 myotubes showed no effect of forskolin (PKA

activation) on ATGL Ser406 phosphorylation (39). From this, Mason et al. concluded that neither AMPK nor PKA phosphorylate skeletal muscle ATGL Ser404 in intact cell systems or whole organisms. Taken together with the results from the present study it seems as though there is a tissue-specific regulation of ATGL, potentially explaining the differences seen in skeletal muscle compared to HEK cells and adipocytes.

Perspectives and Significance

This study examined the serine phosphorylation states of skeletal muscle PLIN proteins (PLIN2, PLIN3, and PLIN5) as well as their interactions with ATGL and HSL at rest and following contraction and epinephrine stimulation in isolation as well as in combination. This is the first study to show that both PLIN3 and PLIN5 are serine phosphorylated under all conditions and that PLIN2 is not. Further this is the first study to determine that PLIN2, PLIN3, and PLIN5 all interact with HSL and that this relationship remains unchanged following stimulation. Together, the above-mentioned data suggest that, unlike in adipose tissue where PLIN1 is unphosphorylated at rest, in skeletal muscle PLIN3 and 5 are serine phosphorylated at rest and this is unchanged with lipolysis. The physiological importance of skeletal muscle PLIN3, PLIN5, and ATGL phosphorylation as well as the PLIN-to-lipase interactions remains unknown. A role for skeletal muscle PLIN phosphorylation in lipid droplet dynamics should be further examined with the development of phospho-specific antibodies. In comparison to adipose tissue skeletal muscle has a

much higher triglyceride turnover (50). Therefore, it is possible that the PLIN proteins are important for basal muscle lipolysis rather than epinephrine or contraction mediated IMTG breakdown. Future work should examine the roles that skeletal muscle PLINs play on lipogenesis as well as fatty acid oxidation.

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No conflicts of interest, financial or otherwise, are declared by the authors.

References

1. **Ahmadian M, Abbott MJ, Tang T, Hudak CS, Kim Y, Bruss M, Hellerstein MK, Lee HY, Samuel VT, Shulman GI, Wang Y, Duncan RE, Kang C, and Sul HS.** Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell metabolism* 13: 739-748, 2011.
2. **Alsted TJ, Nybo L, Schweiger M, Fledelius C, Jacobsen P, Zimmermann R, Zechner R, and Kiens B.** Adipose triglyceride lipase in human skeletal muscle is upregulated by exercise training. *American journal of physiology Endocrinology and metabolism* 296: E445-453, 2009.
3. **Antolic A, Harrison R, Farlinger C, Cermak NM, Peters SJ, LeBlanc P, and Roy BD.** Effect of extracellular osmolality on cell volume and resting metabolism in mammalian skeletal muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 292: R1994-2000, 2007.
4. **Bartz R, Zehmer JK, Zhu M, Chen Y, Serrero G, Zhao Y, and Liu P.** Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation. *Journal of proteome research* 6: 3256-3265, 2007.
5. **Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, and Sztalryd C.** Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* 57: 2037-2045, 2008.
6. **Bezaire V, and Langin D.** Regulation of adipose tissue lipolysis revisited. *Proc Nutr Soc* 68: 350-360, 2009.
7. **Bosma M, Hesselink MK, Sparks LM, Timmers S, Ferraz MJ, Mattijssen F, van Beurden D, Schaart G, de Baets MH, Verheyen FK, Kersten S, and Schrauwen P.** Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes* 61: 2679-2690, 2012.
8. **Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P, and Hesselink MK.** The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochemistry and cell biology* 137: 205-216, 2012.
9. **Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S, and Hesselink MK.** Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochimica et biophysica acta* 1831: 844-852, 2013.
10. **Brasaemle DL, Dolios G, Shapiro L, and Wang R.** Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *The Journal of biological chemistry* 279: 46835-46842, 2004.
11. **Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, and Londos C.** Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *The Journal of biological chemistry* 275: 38486-38493, 2000.

12. **Chang BH, Li L, Paul A, Taniguchi S, Nannegari V, Heird WC, and Chan L.** Protection against fatty liver but normal adipogenesis in mice lacking adipose differentiation-related protein. *Molecular and cellular biology* 26: 1063-1076, 2006.
13. **Donsmark M, Langfort J, Holm C, Ploug T, and Galbo H.** Contractions activate hormone-sensitive lipase in rat muscle by protein kinase C and mitogen-activated protein kinase. *The Journal of physiology* 550: 845-854, 2003.
14. **Donsmark M, Langfort J, Holm C, Ploug T, and Galbo H.** Hormone-sensitive lipase as mediator of lipolysis in contracting skeletal muscle. *Exercise and sport sciences reviews* 33: 127-133, 2005.
15. **Dyck DJ, and Bonen A.** Muscle contraction increases palmitate esterification and oxidation and triacylglycerol oxidation. *The American journal of physiology* 275: E888-896, 1998.
16. **Egan JJ, Greenberg AS, Chang MK, and Londos C.** Control of endogenous phosphorylation of the major cAMP-dependent protein kinase substrate in adipocytes by insulin and beta-adrenergic stimulation. *The Journal of biological chemistry* 265: 18769-18775, 1990.
17. **Fukushima M, Enjoji M, Kohjima M, Sugimoto R, Ohta S, Kotoh K, Kuniyoshi M, Kobayashi K, Imamura M, Inoguchi T, Nakamuta M, and Nawata H.** Adipose differentiation related protein induces lipid accumulation and lipid droplet formation in hepatic stellate cells. *In vitro cellular & developmental biology Animal* 41: 321-324, 2005.
18. **Granneman JG, and Moore HP.** Location, location: protein trafficking and lipolysis in adipocytes. *Trends in endocrinology and metabolism: TEM* 19: 3-9, 2008.
19. **Granneman JG, Moore HP, Granneman RL, Greenberg AS, Obin MS, and Zhu Z.** Analysis of lipolytic protein trafficking and interactions in adipocytes. *The Journal of biological chemistry* 282: 5726-5735, 2007.
20. **Granneman JG, Moore HP, Krishnamoorthy R, and Rathod M.** Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *The Journal of biological chemistry* 284: 34538-34544, 2009.
21. **Granneman JG, Moore HP, Krishnamoorthy R, and Rathod M.** Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *The Journal of biological chemistry* 284: 34538-34544, 2009.
22. **Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L.** Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
23. **Gruber A, Cornaciu I, Lass A, Schweiger M, Poeschl M, Eder C, Kumari M, Schoiswohl G, Wolinski H, Kohlwein SD, Zechner R, Zimmermann R, and Oberer M.** The N-terminal region of comparative gene identification-58 (CGI-58) is important for lipid droplet binding and activation of adipose triglyceride lipase. *The Journal of biological chemistry* 285: 12289-12298, 2010.
24. **Hickenbottom SJ, Kimmel AR, Londos C, and Hurley JH.** Structure of a lipid droplet protein; the PAT family member TIP47. *Structure* 12: 1199-1207, 2004.
25. **Hopp JF, and Palmer WK.** Effect of electrical stimulation on intracellular triacylglycerol in isolated skeletal muscle. *J Appl Physiol* 68: 348-354, 1990.

26. **Hsieh K, Lee YK, Londos C, Raaka BM, Dalen KT, and Kimmel AR.** Perilipin family members preferentially sequester to either triacylglycerol-specific or cholesteryl-ester-specific intracellular lipid storage droplets. *Journal of cell science* 125: 4067-4076, 2012.
27. **Imai Y, Varela GM, Jackson MB, Graham MJ, Crooke RM, and Ahima RS.** Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. *Gastroenterology* 132: 1947-1954, 2007.
28. **Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, and Nawata H.** ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American journal of physiology Endocrinology and metabolism* 283: E775-783, 2002.
29. **Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, and Londos C.** Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. *Journal of lipid research* 51: 468-471, 2010.
30. **Koopman R, Schaart G, and Hesselink MK.** Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochemistry and cell biology* 116: 63-68, 2001.
31. **Langfort J, Ploug T, Ihlemann J, Baranczuk E, Donsmark M, Gorski J, and Galbo H.** Additivity of adrenaline and contractions on hormone-sensitive lipase, but not on glycogen phosphorylase, in rat muscle. *Acta physiologica Scandinavica* 178: 51-60, 2003.
32. **Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, Kienesberger P, Strauss JG, Gorkiewicz G, and Zechner R.** Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell metabolism* 3: 309-319, 2006.
33. **Londos C, Brasaemle DL, Schultz CJ, Segrest JP, and Kimmel AR.** Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Seminars in cell & developmental biology* 10: 51-58, 1999.
34. **Lu X, Gruia-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, Londos C, and Kimmel AR.** The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mammalian genome : official journal of the International Mammalian Genome Society* 12: 741-749, 2001.
35. **Lu X, Yang X, and Liu J.** Differential control of ATGL-mediated lipid droplet degradation by CGI-58 and GOS2. *Cell Cycle* 9: 2719-2725, 2010.
36. **Macpherson RE, Herbst EA, Reynolds EJ, Vandenboom R, Roy BD, and Peters SJ.** Subcellular localization of skeletal muscle lipid droplets and PLIN family proteins OXPAT and ADRP at rest and following contraction in rat soleus muscle. *American journal of physiology Regulatory, integrative and comparative physiology* 302: R29-36, 2012.
37. **Macpherson RE, Ramos S, Vandenboom R, Roy BD, and Peters SJ.** Skeletal muscle PLIN proteins, ATGL and CGI-58, interactions at rest and following stimulated contraction. *American journal of physiology Regulatory, integrative and comparative physiology* 2013.

38. **Marcinkiewicz A, Gauthier D, Garcia A, and Brasaemle DL.** The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. *The Journal of biological chemistry* 281: 11901-11909, 2006.
39. **Mason RR, Meex RC, Lee-Young R, Canny BJ, and Watt MJ.** Phosphorylation of adipose triglyceride lipase Ser(404) is not related to 5'-AMPK activation during moderate-intensity exercise in humans. *American journal of physiology Endocrinology and metabolism* 303: E534-541, 2012.
40. **Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MK.** Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *The Journal of clinical endocrinology and metabolism* 94: 4077-4085, 2009.
41. **Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C, Oliver B, and Kimmel AR.** Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. *The Journal of biological chemistry* 277: 32253-32257, 2002.
42. **Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS.** Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
43. **Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS.** Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
44. **Pagnon J, Matzaris M, Stark R, Meex RC, Macaulay SL, Brown W, O'Brien PE, Tiganis T, and Watt MJ.** Identification and functional characterization of protein kinase A phosphorylation sites in the major lipolytic protein, adipose triglyceride lipase. *Endocrinology* 153: 4278-4289, 2012.
45. **Peters SJ, Dyck DJ, Bonen A, and Spriet LL.** Effects of epinephrine on lipid metabolism in resting skeletal muscle. *The American journal of physiology* 275: E300-309, 1998.
46. **Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA, and Tarnopolsky MA.** Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 37: 724-735, 2012.
47. **Phillips SA, Choe CC, Ciaraldi TP, Greenberg AS, Kong AP, Baxi SC, Christiansen L, Mudaliar SR, and Henry RR.** Adipocyte differentiation-related protein in human skeletal muscle: relationship to insulin sensitivity. *Obesity research* 13: 1321-1329, 2005.
48. **Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, and Ploug T.** Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *Journal of lipid research* 47: 2392-2399, 2006.
49. **Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, and de Medina FS.** Reversible Ponceau staining as a loading

- control alternative to actin in Western blots. *Analytical biochemistry* 401: 318-320, 2010.
50. **Sacchetti M, Saltin B, Olsen DB, and van Hall G.** High triacylglycerol turnover rate in human skeletal muscle. *The Journal of physiology* 561: 883-891, 2004.
51. **Shaw CS, Shepherd SO, Wagenmakers AJ, Hansen D, Dendale P, and van Loon LJ.** Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibres of patients with type 2 diabetes. *American journal of physiology Endocrinology and metabolism* 2012.
52. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS.** Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Experimental physiology* 97: 970-980, 2012.
53. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS.** Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *The Journal of physiology* 591: 657-675, 2013.
54. **Souza SC, Muliro KV, Liscum L, Lien P, Yamamoto MT, Schaffer JE, Dallal GE, Wang X, Kraemer FB, Obin M, and Greenberg AS.** Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. *The Journal of biological chemistry* 277: 8267-8272, 2002.
55. **Spriet LL, Heigenhauser GJ, and Jones NL.** Endogenous triacylglycerol utilization by rat skeletal muscle during tetanic stimulation. *J Appl Physiol* 60: 410-415, 1986.
56. **Stellingwerff T, Boon H, Jonkers RA, Senden JM, Spriet LL, Koopman R, and van Loon LJ.** Significant intramyocellular lipid use during prolonged cycling in endurance-trained males as assessed by three different methodologies. *American journal of physiology Endocrinology and metabolism* 292: E1715-1723, 2007.
57. **Su CL, Sztalryd C, Contreras JA, Holm C, Kimmel AR, and Londos C.** Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. *The Journal of biological chemistry* 278: 43615-43619, 2003.
58. **Sztalryd C, Bell M, Lu X, Mertz P, Hickenbottom S, Chang BH, Chan L, Kimmel AR, and Londos C.** Functional compensation for adipose differentiation-related protein (ADFP) by Tip47 in an ADFP null embryonic cell line. *The Journal of biological chemistry* 281: 34341-34348, 2006.
59. **Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C.** Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
60. **Tansey JT, Huml AM, Vogt R, Davis KE, Jones JM, Fraser KA, Brasaemle DL, Kimmel AR, and Londos C.** Functional studies on native and mutated forms of perilipins. A role in protein kinase A-mediated lipolysis of triacylglycerols. *The Journal of biological chemistry* 278: 8401-8406, 2003.
61. **Timmers S, de Vogel-van den Bosch J, Hesselink MK, van Beurden D, Schaart G, Ferraz MJ, Losen M, Martinez-Martinez P, De Baets MH, Aerts JM, and Schrauwen P.** Paradoxical increase in TAG and DAG content parallel the insulin

sensitizing effect of unilateral DGAT1 overexpression in rat skeletal muscle. *PLoS one* 6: e14503, 2011.

62. **van Loon LJ, Koopman R, Manders R, van der Weegen W, van Kranenburg GP, and Keizer HA.** Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *American journal of physiology Endocrinology and metabolism* 287: E558-565, 2004.

63. **van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, and Saris WH.** Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. *The Journal of physiology* 553: 611-625, 2003.

64. **Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman R, Gong D, Brasaemle D, and Sztalryd C.** Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *The Journal of biological chemistry* 286: 15707-15715, 2011.

65. **Wang H, Hu L, Dalen K, Dorward H, Marcinkiewicz A, Russell D, Gong D, Londos C, Yamaguchi T, Holm C, Rizzo MA, Brasaemle D, and Sztalryd C.** Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. *The Journal of biological chemistry* 284: 32116-32125, 2009.

66. **Watt MJ, Heigenhauser GJ, and Spriet LL.** Effects of dynamic exercise intensity on the activation of hormone-sensitive lipase in human skeletal muscle. *The Journal of physiology* 547: 301-308, 2003.

67. **Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel PE.** S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *The Journal of biological chemistry* 280: 19146-19155, 2005.

68. **Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, and Bickel PE.** OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55: 3418-3428, 2006.

69. **Wolins NE, Rubin B, and Brasaemle DL.** TIP47 associates with lipid droplets. *The Journal of biological chemistry* 276: 5101-5108, 2001.

70. **Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, and Zechner R.** Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306: 1383-1386, 2004.

Chapter 7

General Discussion

Research in the last 20 years has made significant progress in identifying lipid droplet associated proteins and developing an understanding of the role(s) that they may play in regulating intracellular lipid homeostasis. Specifically, the discovery of PLIN proteins has led to great advancements in the understanding of adipose tissue lipolysis and a specific role for PLIN1 has now been established in this tissue. PLIN1 is only expressed in adipose tissue and while research in the area continues to expand; the role(s) of the remaining PLIN proteins remains unknown. Among the PLIN family, PLIN2, PLIN3, and PLIN5 are all expressed in skeletal muscle and are therefore likely targets in regulating skeletal muscle lipid dynamics.

A proper balance between fatty acid uptake, storage, and fat oxidation in skeletal muscle is important when considering metabolic diseases that are believed to be related to intracellular lipotoxicity (52). Therefore, a full understanding of the metabolic processes regulating how IMTGs are stored and mobilized is crucial. This thesis describes three studies that contribute significantly to the current knowledge of skeletal muscle PLIN proteins and their potential roles in regulating exercise-induced lipolysis. Since this thesis began in 2009, the number of publications in this area has highlighted the importance that the skeletal muscle PLIN proteins may have in intracellular lipid dynamics. Thus far, most studies in skeletal muscle have examined changes in PLIN protein content following interventions such as exercise training. However, an understanding of the skeletal muscle PLIN protein intramyocellular location, interaction with lipases, and post-translational modification (e.g., phosphorylation) had not been investigated. Therefore, a specific

aim of this thesis was to provide information about the roles of skeletal muscle PLIN proteins in relation to the above listed items at rest as well as following lipolytic stimulation.

Summary of findings

Results from Chapter 4 demonstrated that PLIN5 is not recruited to lipid droplets with muscle contraction and that both PLIN2 and PLIN5 are partially co-localized to lipid droplets at any given time, indicating that skeletal muscle lipid droplets differentially express PLIN proteins. This was the first study to investigate the content, distribution, and co-localization of lipid droplets, PLIN2, and PLIN5 in contracted skeletal muscle.

From Chapter 5 and 6 it was demonstrated that PLIN2, 3, and 5 all interact with ATGL and HSL at rest and following contraction. PLIN2 was the only skeletal muscle PLIN protein that did not interact with CGI-58. The fact that all three skeletal muscle PLIN proteins interact with the rate limiting lipase, ATGL, as well as HSL, indicates a role for all of these PLINs in regulating lipolysis, potentially by working together as a complex. Further, PLIN2 was the only PLIN that was not serine phosphorylated at rest or with either adrenergic or contractile stimulation. The results from Chapter 6 show that PLIN3 and PLIN5 appear to be serine phosphorylated at all times with no changes under lipolytic stimulation. While the specific serine phosphorylation site(s) remain unknown, this study demonstrates

that both PLIN3 and PLIN5 are serine phosphorylated and that this level of phosphorylation is unchanged with lipolytic stimulation.

A developing model for the role of skeletal muscle PLIN proteins

Results from the studies presented in this thesis provide novel information in regards to the roles that the skeletal muscle PLIN proteins may have at rest as well as under contractile and adrenergic stimulation. By coupling these results with the information that has emerged from recent publications in this area, we may be able to develop a working model of skeletal muscle lipid droplet metabolism at rest and during lipolysis.

Skeletal muscle PLINs and the basal state

Information presented in this thesis provides evidence that the skeletal muscle PLIN proteins may be involved in the regulation of basal lipolysis. It is important to understand the subcellular location of each of the skeletal muscle PLIN proteins as well as the protein interactions between these proteins in this state. The following sections aim to develop an outline of where the skeletal muscle PLIN proteins (PLIN2, PLIN3, and PLIN5) are found at rest as well as their protein interactions and post-translational state in the basal state.

In skeletal muscle, PLIN2, PLIN3, and PLIN5 all appear to play a role in limiting basal rates of lipolysis by interacting with both HSL and ATGL. Only PLIN3

and PLIN5 interact with CGI-58 at rest. These interactions might be essential for maintaining a stable rate of basal lipolysis by ensuring that the lipases are targeted to the right subcellular location or by controlling ATGL activity by sequestering CGI-58.

In the basal state, it is now known that approximately 60% of PLIN2 and PLIN5 are found co-localized to the lipid droplets (Chapter 1), while the rest of the proteins are found off of the lipid droplet. These findings are consistent with other recent work in human skeletal muscle showing that lipid droplets are differentially coated with PLIN2 and PLIN5 (43, 45, 46). It is known that PLIN5 is found associated with the lipid droplets as well as in a cytosolic form (10, 62, 66) therefore the finding that PLIN5 is partially co-localized to lipid droplets was not surprising. However, the finding that PLIN2 is found off of the lipid droplet seems odd as previous studies in cell culture (Chinese hamster ovary cells and human embryonic kidney 293 cells) have shown that PLIN2 coats the lipid droplet membrane and is stable in the presence of neutral lipids but is otherwise targeted for degradation by proteasomes and ubiquitination (29, 64, 65). It was due to this cytosolic instability that PLIN2 was originally considered to be a lipid droplet marker (29, 64, 65). However, it is possible that this partial co-localization of PLIN2 to lipid droplets is due to methodological limitations; the oil red O stain may not be sensitive enough to detect the smallest of lipid droplets. Therefore, future work should take caution in utilizing PLIN2 as a marker of lipid droplets until a more conclusive method demonstrates this.

If we consider that the majority of PLIN2 is located on the lipid droplet surface and is interacting with both HSL and ATGL but not CGI-58, then PLIN2 may promote lipid accumulation at the lipid droplet by sequestering the lipases and inhibiting lipolysis. In support of this, it is generally accepted that PLIN2 is found on the lipid droplet surface from the beginning of synthesis and is up regulated in parallel with stored lipid during lipid droplet formation (8, 26, 56). Cell culture studies (murine fibroblasts, human embryonic kidney cells, McA-RH7777 cells, and primary rat hepatocytes) have also demonstrated that overexpression of PLIN2 encourages lipid accumulation (26, 29, 31). Further, in PLIN1-null mice PLIN2 replaces PLIN1 on lipid droplets (50), although PLIN2 appears to be a less robust barrier to lipases than PLIN1(33, 50). Since PLIN2 is mostly found co-localized with lipid droplets surface, it is possible that the interactions with ATGL and HSL are necessary for regulating basal lipolysis. This theory is supported by work done in cultured AML12 (liver) cells lacking PLIN2, where an increase in ATGL at the lipid droplet surface was observed in conjunction with elevated rates of basal lipolysis (4). Taken together, it seems as though PLIN2 may be involved in both lipolysis and lipogenesis, perhaps through interactions with lipases and maybe even through interactions with enzymes/proteins involved with IMTG synthesis.

The partial co-localization of PLIN5 to the lipid droplets described in this thesis is consistent with the ability of PLIN5 to translocate to and from the lipid droplet surface and also indicates that PLIN5 may be found in other subcellular locations. It has very recently been demonstrated that PLIN5 is not only found in the cytosol and the lipid droplet surface but that it is also in contact with mitochondria

(6, 55). Using immunogold electron microscopy and Western blots in isolated mitochondria, Bosma et al. (6) found that PLIN5 in muscle is found at the lipid droplet surface as well as inside the mitochondria. This finding was consistent across various muscle types as PLIN5 was found in mitochondria in oxidative (soleus), glycolytic (EDL), and mixed (tibialis anterior) muscles as well as in cardiac muscle (6). An exact role for PLIN5 at the mitochondria has not been determined, however it is hypothesized that PLIN5 may play a role in directing fatty acids from the lipid droplet to the mitochondria for oxidation.

The proportion of PLIN3 co-localized to lipid droplets in skeletal muscle was not examined in this thesis, but it is expected that PLIN3 would show a similar partial co-localization as is seen with PLIN5. PLIN3 may be found in various cellular locations and, like PLIN5, has the ability to translocate between the cytosol and the lipid droplet surface (61, 63). Prats et al. (41) found that approximately 76% of the lipid droplet surfaces co-localized with PLIN3 at rest and remained unchanged following electrical or epinephrine stimulation. Interestingly, PLIN3 was shown to associate with mitochondria under conditions that would induce oxidative stress, and it is speculated that PLIN3 prevents cell death by maintaining mitochondrial membrane potential (24). However, an investigation of basal mitochondrial PLIN3 content has not yet been done in skeletal muscle.

It is not possible to determine the physiological significance of the partial co-localization of these three PLIN proteins with lipid droplets; however it was hypothesized that subpopulations of lipid droplets presenting different PLIN proteins have specific functions within skeletal muscle (43, 61, 62). For example,

PLIN5 is associated with distinct, high-density lipid droplets with a low size limit (~15 nm in diameter) (2). Recent work has demonstrated that different PLINs sequester to different types of lipid droplets that are composed of either triglycerides or cholesterol esters. Using cells cultured (Y1 adrenal cells and McARH7777 rat liver cells) in the absence or presence of fatty acid and/or cholesterol it was demonstrated that PLIN proteins differentially sequester to either triglycerides or cholesterol ester containing lipid droplets. PLIN2 and PLIN3 interact with both triglycerides and cholesterol esters lipid droplets, and PLIN5 primarily targets triglyceride-storing lipid droplets (25). Very recently, Shepherd et al. (45, 46) have found that in human vastus lateralis there are lipid droplets that are not associated with PLIN2 (PLIN2-null lipid droplets) or PLIN5 (PLIN5-null lipid droplets). This study only assessed the co-localization of either PLIN2 or PLIN5 with the lipid droplets, and therefore cannot discriminate lipid droplets containing both PLIN2 and PLIN5 from those lipid droplets containing only PLIN2 or PLIN5. Together these findings emphasize the diversity of function of these PLIN proteins and indicate that the lipid droplet protein coat is dictated by the size of the lipid droplet as well as its contents.

With the ability to translocate to and from the lipid droplet surface, both PLIN3 and PLIN5 may function by directing ATGL, HSL, and CGI-58 to the right subcellular location, or by regulating the activity of ATGL by also interacting with CGI-58. However, as PLIN3 and PLIN5 are found in the cytosol as well as on the lipid droplet it is unknown which population of these proteins is bound to either ATGL and/or CGI-58. Cell culture studies have determined that PLIN5 can directly bind to

both ATGL and CGI-58 but not to both at the same time (20, 53). In this thesis, it was found that PLIN5 interacts with both ATGL and CGI-58, however it is not known what proportion of PLIN5 is bound to either ATGL or CGI-58, or if PLIN5 interacts with both ATGL and CGI-58 at the same time. Interestingly, a sequence of amino acids in PLIN3 is similar to the sequence of amino acids that is the binding site for both ATGL and CGI-58 on PLIN5 (23). Therefore, it is also possible that PLIN3, like PLIN5, can also bind to both ATGL and CGI-58 but not at the same time. Working together, PLIN3 and PLIN5 may be able to regulate lipase activity by sequestering both ATGL and CGI-58. Further, Chapter 6 of this thesis demonstrated that both PLIN3 and PLIN5 are serine phosphorylated in the basal state, however the importance of this phosphorylation remains unknown. It is possible that these PLIN proteins are phosphorylated in order to maintain certain protein interactions or subcellular locations and future work should investigate the exact residues at which they are phosphorylated and determine if this changes with physiological perturbations.

Skeletal muscle PLINs and the lipolytically stimulated state

During exercise, skeletal muscle lipolysis can be initiated by either circulating epinephrine or contraction therefore it was possible that these separate pathways may lead to different changes in PLIN location, protein interactions, and phosphorylation. Results from the studies presented in this thesis demonstrate that our experimental conditions (stimulated contraction and epinephrine incubation)

result in a significant amount of lipolysis. Both Chapter 4 and 6 demonstrate lipolysis has occurred through a significant decrease in neutral lipid content measured by Oil Red O staining, and Chapter 5 shows a significant increase in ATGL to CGI-58 association, a known stimulus for enhanced ATGL activity in adipose tissue, cultured adipocytes, and fibroblasts (28, 68). The question remains as to how the PLIN proteins are involved and what is turning on lipolysis under these conditions. The following section describes our findings and discusses the potential roles that the PLIN proteins may have in regulating both ATGL and HSL under these lipolytic conditions.

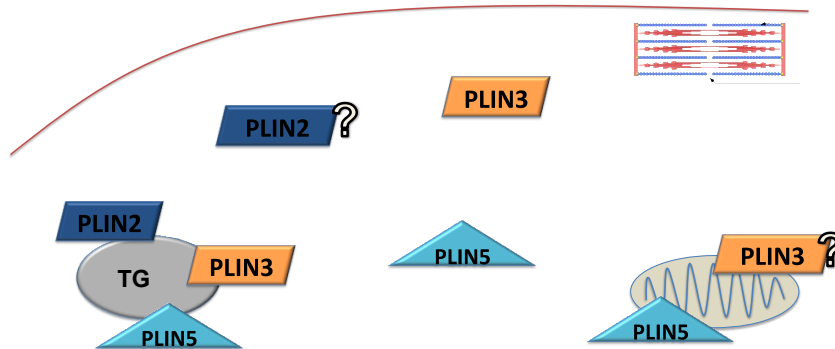
PLIN location with lipolysis

Due to the ability of PLIN5 to translocate from the cytosol to the lipid droplet (10, 62, 66) we had hypothesized that PLIN5 may be recruited to the lipid droplet surface with contraction in order to mediate lipolysis in skeletal muscle. However, we found that the fraction of PLIN5 co-localized to the lipid droplets remained unchanged following contraction. This indicates that PLIN5 is not recruited to lipid droplets to assist with lipolysis and that there is a constant proportion of PLIN5 in association with lipid droplets as well as elsewhere in the cell. This thesis did not investigate the association of PLIN3 with lipid droplets however previous work has shown that the cellular localization of PLIN3 is not altered with contraction or epinephrine administration (41), indicating similar findings as found with PLIN5. The recent findings that PLIN5 is also located at the mitochondria (6, 55) and that

PLIN3 may prevent oxidative stress at the mitochondria (24), make it conceivable that these two PLIN proteins may be recruited to the mitochondrial membrane in skeletal muscle during exercise to assist with the transfer of fatty acids from the lipid droplets, however this has yet to be investigated and is a possibility for future studies.

The portion of PLIN proteins co-localized with lipid droplets does not seem to change with lipolysis, however recent work indicates that both PLIN2 and PLIN5 play an important role at the lipid droplet surface. In the past year, Shepherd et al. (45, 46) have demonstrated that in human skeletal muscle, lipid droplets associated with either PLIN2 or PLIN5 are preferentially utilized during exercise. The findings from this thesis show that the skeletal muscle PLIN proteins interact with both HSL and ATGL. Pairing these results with the recent discovery that lipid droplets coated with either PLIN2 or PLIN5 are preferentially utilized indicates that the skeletal muscle PLIN proteins may be important for regulating lipase access to the lipid droplets.

A. Basal



B. Stimulated

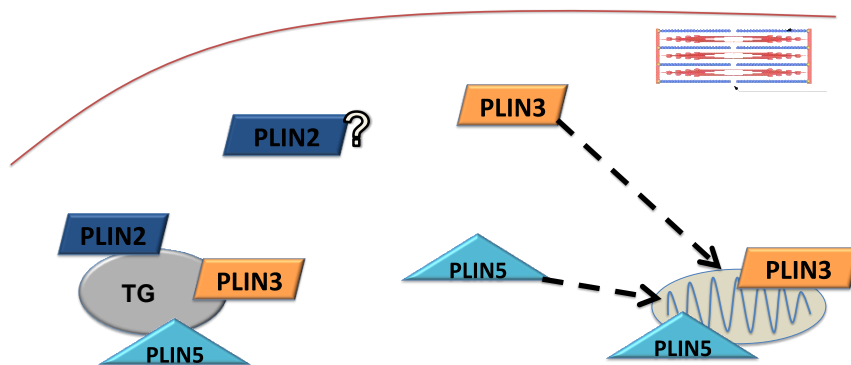


Figure 20

Figure 1. Representative figures of skeletal muscle PLIN protein locations in a basal and stimulated state. **A.** Basal state; showing PLIN2, PLIN3, and PLIN5 on the lipid droplet surface. PLIN5 is also located in the cytosol and in association with mitochondria. PLIN3 is also located in the cytosol and potentially in association with mitochondria. PLIN2 may also be found in the cytosol. **B.** Stimulated state; dashed lines represent possible movement of PLIN5 and PLIN3 to the mitochondria.

PLIN regulation of ATGL

As ATGL is now considered to be the rate limiting lipase in lipolysis it is important to examine changes in its phosphorylation state as well as any changes in protein interactions that might occur with lipolytic stimulation in skeletal muscle. Chapter 6 demonstrated that ATGL is serine phosphorylated at rest and that this level of phosphorylation remains unchanged following either contractile or adrenergic stimulation. Previous studies have identified two serine phosphorylation sites on ATGL (serine 404/406 and 428/430; variation in location is humans vs. rodent) (3, 68) and the results from our study can not discount the possibility that while we observed no overall change in phosphorylation there could have been changes at the individual sites.

However, recent work in human skeletal muscle found similar results in ATGL Ser404 phosphorylation. Mason et al. (34) demonstrated that ATGL Ser404 phosphorylation is not increased in human skeletal muscle during moderate-intensity cycling exercise. In addition, that study demonstrated that there was no evidence of a physical interaction between ATGL and AMPK after immunoprecipitation, and pharmacological activation of AMPK did not affect ATGL Ser404 phosphorylation in cultured myotubes (34). Further, direct studies in C2C12 myotubes showed no effect of forskolin (i.e., PKA activation) on ATGL Ser406 phosphorylation (34). From this, Mason et al. concluded that neither AMPK nor PKA phosphorylate skeletal muscle ATGL Ser404 in intact cell systems or whole organisms. Mason et al. did not investigate changes at the other serine phosphorylation site, however with our results showing no change in overall serine

phosphorylation it is unlikely that there are any changes in Ser430 phosphorylation of ATGL. Overall, the evidence from this thesis and from Mason et al. would indicate that ATGL activity in skeletal muscle does not seem to be regulated by phosphorylation. A role for the observed basal ATGL phosphorylation remains to be determined, however it is possible that it may regulate basal lipolysis.

As mentioned previously the association between ATGL and CGI-58 is a known stimulus for enhanced ATGL activity (28, 68). Chapter 4 of this thesis provided the first evidence of this interaction in skeletal muscle. We found that in skeletal muscle the interaction between ATGL and CGI-58 increases more than 2-fold (~128%) after 30min of electrically stimulated contraction, consistent with an increased rate of lipolysis during this period (12). In Chapter 6, we found that the increased ATGL and CGI-58 interaction did not reach statistical significance with any of our experimental conditions (contraction or epinephrine or the combination), however there was a tendency for an increased interaction in all cases (contraction 56%; epinephrine 25%; contraction + epinephrine 79%). The lack of statistical significance may be due to a lack in power when comparing four group means rather than two, or to the large variability in the values. While we cannot definitely say that the ATGL-CGI-58 interaction is increased with skeletal muscle lipolysis, we should not discount these findings as they may be physiologically relevant even though they are statistically insignificant. Another possible explanation for the variability seen with the ATGL-CGI-58 interaction is that a direct physical interaction between ATGL and CGI-58 may not actually be necessary to increase the lipolytic activity of ATGL (30). Gruber et al. (22)

demonstrated that CGI-58 is able to bind directly to the phospholipid monolayer of lipid droplets and that without this there is a complete loss of the ability of CGI-58 to activate ATGL, regardless of their interaction. It may be more important for CGI-58 to bind to the lipid droplet membrane and somehow allow ATGL to access the triglyceride core (30).

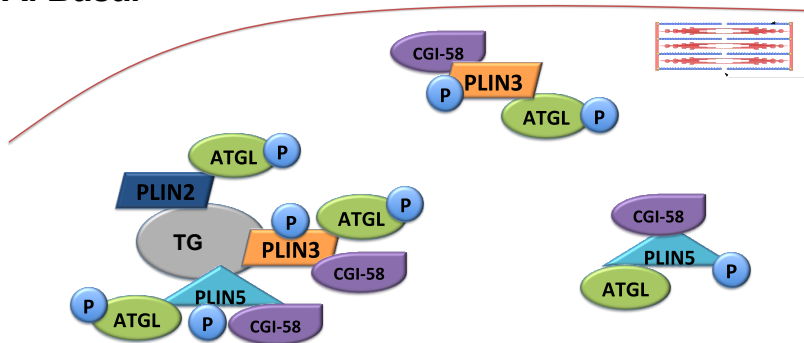
ATGL may also be regulated by the protein interactions that were demonstrated in Chapters 5 and 6. In skeletal muscle we showed that PLIN2, PLIN3, and PLIN5 all interact with ATGL, and that PLIN3 and PLIN5 interact with CGI-58. Therefore it may be possible that these PLINs work together as scaffolding proteins sequestering ATGL and CGI-58 until there is a lipolytic stimulus. In Chapter 5 the interaction between PLIN2 and ATGL was significantly decreased following stimulated contraction, however, in Chapter 6 a significant decline in the PLIN2-ATGL interaction did not occur with either contractile or adrenergic stimulation, but there was a tendency towards a decrease (contraction 12%; epinephrine 18%). Further, similar decreases in the interaction between PLIN5 and ATGL were found in both Chapter 5 and 6 with contractile and adrenergic stimulation although this did not reach significant. Alone, the decline in the association between either PLIN2 or PLIN5 and ATGL likely do not fully account for the increased association between ATGL and CGI-58, but together they may explain it. Interestingly, the interaction between PLIN3 and ATGL does not seem to change with either contraction or epinephrine-induced lipolysis.

From Chapter 5 we have shown that in skeletal muscle both PLIN3 and PLIN5 interact with CGI-58 and that these interactions do not change following

stimulated contraction. It is thought that PLIN5 facilitates lipolysis by promoting the co-localization and functional interaction of CGI-58 and ATGL (18, 19, 21), however the mechanisms are unknown. Given that PLIN5 can bind to both ATGL and CGI-58 but not both at the same time (20) and that this amino acid sequence is similar to one in PLIN3, it is possible that PLIN3 and PLIN5 play similar roles in regulating the location and activation of ATGL and CGI-58. Future studies should investigate proportions of PLIN3 and PLIN5 that are bound to CGI-58 or ATGL as well as their subcellular locations.

In adipose tissue, it is the PKA-dependent phosphorylation of PLIN1 that causes the release of CGI-58, leading to the initiation of lipolysis by activating ATGL (5, 14-17) (32, 36, 47, 48). This thesis presents novel findings that PLIN3 and PLIN5 are serine phosphorylated, however the level of phosphorylation does not change with either contractile or adrenergic stimulation. This indicates that serine phosphorylation of these two proteins may not be important in regulating lipolysis. However, we cannot conclude that phosphorylation does not play a role, as we do not know if there are multiple serine phosphorylation sites that could potentially be altered or if there are sequences other than serine phosphorylation sites. Therefore, the exact role of skeletal muscle PLIN3 and PLIN5 phosphorylation remains unknown and warrants further investigation. Further, we found that PLIN2 is the only PLIN protein that is not serine phosphorylated at rest or following lipolytic stimulation.

A. Basal



B. Stimulated

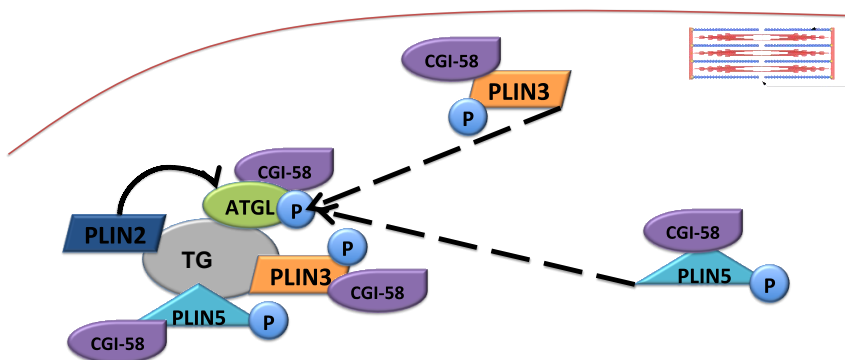


Figure 21

Figure 2. Representative figures for ATGL regulation by skeletal muscle PLIN proteins. **A.** Basal state; PLIN2, PLIN3, and PLIN5 interact with ATGL while only PLIN3 and PLIN5 interact with CGI-58. It is currently unknown if PLIN3 and PLIN5 can interact with both CGI-58 and ATGL at the same time or if these interactions are occurring at the lipid droplet or in the cytosol. **B.** Stimulated state; the interaction between ATGL and CGI-58 is increased at the lipid droplet. The association between PLIN2 and ATGL has decreased. It is currently unknown if the associations between PLIN3 and PLIN5 with ATGL are also decreased, allowing for the large increase in ATGL to CGI-58 interactions.

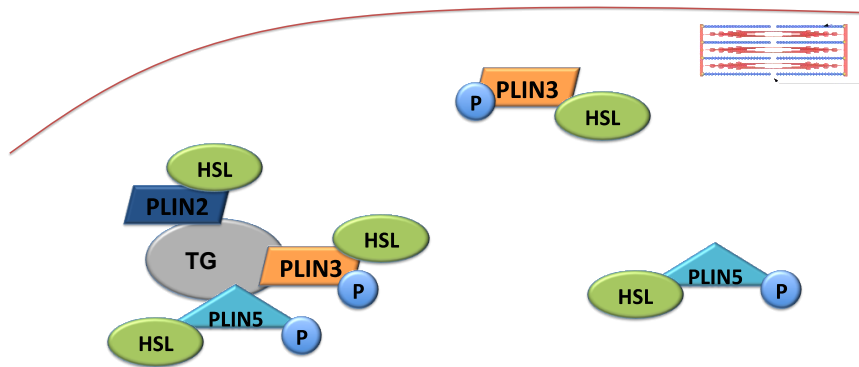
PLIN regulation of HSL

As discussed in Chapter 1, the function of HSL in skeletal muscle has been extensively studied and reviewed (58, 59, 67). It is now known that HSL activity in skeletal muscle is increased during exercise due to phosphorylation by PKA at Ser563, Ser659, and Ser660, and by ERK at Ser660 (11), and that HSL activity is reduced later on in prolonged exercise and recovery likely due to phosphorylation of HSL Ser565 by AMPK and/or CaMK (42, 57, 60). Prior to this thesis, a role for the skeletal muscle PLIN proteins in regulating HSL activity was unknown and we can now add to the HSL story.

In adipose tissue, phosphorylation of PLIN1 and HSL promotes the translocation and docking of HSL to PLIN1 on the lipid droplet surface (35, 37, 44, 49, 54). Chapter 6 of this thesis aimed to determine if a similar series of events occurs in skeletal muscle during lipolysis. Previous work with skeletal muscle contraction has shown a translocation of HSL towards PLIN2-coated lipid droplets (41), however this study did not determine if there was a direct physical interaction between these two proteins. The results from Chapter 6 provide the first evidence of a physical interaction between PLIN2 and HSL, however this interaction remains unchanged following contraction or epinephrine stimulation alone or in combination. This indicates that PLIN2 may not be involved in recruiting HSL to the lipid droplet surface, or that once HSL has translocated to the lipid droplet it does not interact with PLIN2.

Chapter 6 also demonstrated that both PLIN3 and PLIN5 interact with HSL. Statistical analysis revealed that the interaction between PLIN3 and PLIN5 with HSL is unchanged following contraction and epinephrine alone as well as in combination. From Chapter 4 we know that PLIN5 is not recruited towards lipid droplets with contraction, and results from Prats et al. (41) show the same for PLIN3, therefore it seems unlikely that they are acting as a chaperone for HSL with lipolytic stimulation. A closer look at the results from Chapter 6 indicates that the association between PLIN3 and HSL may be decreased with lipolysis (even though this was not statistically significant), and this association should be investigated further. If this is true, it may indicate that PLIN3 acts to sequester HSL in the basal state and releases it with lipolytic stimulation, allowing HSL to translocate to the lipid droplet. Regardless, the finding that all three skeletal muscle PLIN proteins interact with HSL indicates a potential role for these proteins in regulating either the location of HSL or its activity at the lipid droplet surface.

A. Basal



B. Stimulated

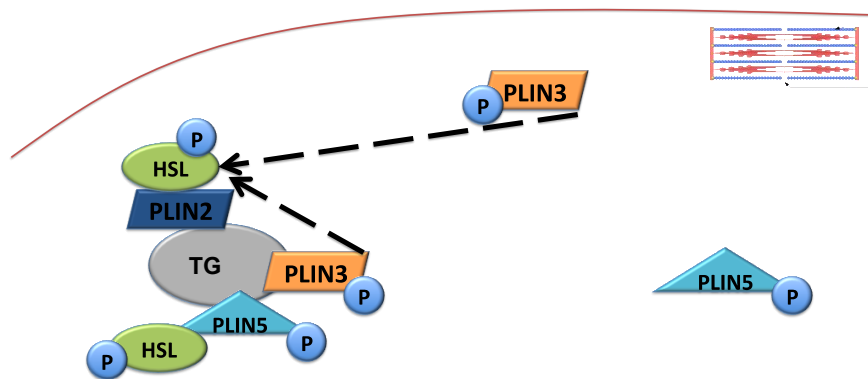


Figure 22

Figure 3. Representative figure of HSL regulation by skeletal muscle PLIN proteins. **A.** Basal state; PLIN2, PLIN3, and PLIN5 interact with HSL. **B.** Stimulated state; HSL is phosphorylated on multiple serine sites (shown as P) and has translocated to the lipid droplet. It is not known if HSL is interacting with PLIN2 at the lipid droplet surface or if PLIN2 is released from either PLIN3 or PLIN5.

Summary

A full understanding of the roles that the skeletal muscle PLINs (PLIN2, PLIN3, and PLIN5) play in lipid droplet dynamics is still in its infancy. The discovery of the PLIN proteins, their tissue distributions and the important role that PLIN1 plays in regulating adipose tissue lipolysis has provided an excellent starting point. Study of the potential roles of the skeletal muscle PLINs has only evolved in the past few years and should continue to piece together the role(s) that these proteins may have in lipid storage and use. This thesis has provided novel information to the ever-expanding model of skeletal muscle lipolysis. It is now known that ATGL, CGI-58 and HSL are all likely regulated by all three of these PLIN proteins. Results from this thesis support a role for the PLIN proteins in sequestering the lipases during basal conditions and potentially working together to allow for lipase translocation and activity during lipolysis. The results of this thesis have laid the ground-work for examining the role(s) of PLIN2, PLIN3, and PLIN5 in skeletal muscle lipolysis. Future work using both acute and chronic perturbations in lipid metabolism will contribute to completing the overarching PLIN protein puzzle.

Future Studies

The results of the studies presented in this thesis indicate that the role(s) of PLIN proteins in regulating skeletal muscle lipolysis are much more complex than what is currently understood in adipose tissue. Future work investigating the roles

of skeletal muscle PLIN proteins can go in several directions. This thesis has focused on a role in regulating lipolysis and has presented some necessary first steps in understanding the roles of skeletal muscle PLIN proteins. Further, the recent finding that PLIN5 is located at the mitochondria warrants more studies to elucidate the role of PLIN5 at this location.

Role for skeletal muscle PLINs in lipid synthesis

To date most studies in skeletal muscle PLIN research have focused on adaptations to training or, as presented in this thesis, roles in lipolysis. Future work should explore the importance of free fatty acids and insulin in mediating the up-regulation of the PLIN proteins in skeletal muscle as well as their role in lipid droplet growth/synthesis. These investigations can use both acute and chronic approach.

Insulin has potent anti-lipolytic effects, as well as a pro-lipogenic effects in resting skeletal muscle (1, 13, 38, 39). The mechanisms underlying insulin's potential regulation of muscle lipid metabolism have remained relatively unexamined. It is possible that insulin promotes the interactions of PLIN2, PLIN3 and PLIN5 with HSL and ATGL. Further, interactions between the PLIN proteins and any of the synthetic machinery have yet to be evaluated. Initial studies should focus on an acute stimulation with insulin and/or free fatty acids to promote lipid uptake and synthesis, as well as inhibit lipolysis. Previous work using an isolated muscle incubation model (similar to the one utilized in this study) have shown that insulin

can increase fatty acid esterification in resting skeletal muscle (38, 39) while simultaneously lowering the rate of fatty acid oxidation in this tissue (1, 38, 39).

Evidence indicates that the skeletal muscle PLIN proteins are involved in both lipolysis as well as lipid droplet synthesis but it remains unclear if the PLIN proteins are present on/or interact with the cellular site of synthesis (endoplasmic reticulum) and this should be investigated. To date only PLIN3 has been suggested to be present on the endoplasmic reticulum, suggesting it might have a role in lipid droplet synthesis (9). It is also possible that the PLINs interact with enzymes involved in neutral lipid synthesis. These interactions should be investigated in the basal as well as the lipogenic or insulin-stimulated state.

Once it has been determined if the PLIN proteins also reside at the endoplasmic reticulum and do in fact interact with lipogenic enzymes studies using chronic treatments known to increase lipid storage such as high fat diets or exercise training can be undertaken to determine if there are changes in these interactions. This will provide much needed information on lipid droplet dynamics and perhaps explain differences in IMTG storage between trained vs untrained individuals.

Role for PLIN5 at the mitochondria

Due to the oxidative tissue expression of PLIN5 (62), as well as its association with mitochondria (6), it is likely that PLIN5 plays a role in facilitating fatty acid oxidation and not IMTG hydrolysis per se. In support of this theory, Peters et al. (40) found PLIN5 protein content increased with endurance training and this

increase was associated with improved muscle oxidative capacity. Other work has demonstrated that overexpression of PLIN5 *in-vitro* increased fatty acid oxidation (62), providing evidence for a larger role of PLIN5 in enhancing the oxidation of the fatty acids released from the lipid droplets over IMTG synthesis or lipolysis. Overexpression of PLIN5 in skeletal muscle also promotes expression of several genes, regulated by PPAR α and PGC1 α , involved in fatty acid catabolism and oxidation (7). The recent discovery that PLIN5 is located in direct contact with mitochondria and that PLIN5 overexpression results in more intimate interactions between these two organelles provides more evidence in support of a strong role in oxidation (6). It has been hypothesized that PLIN5 may direct fatty acids from the lipid droplet to the mitochondria for oxidation. If this were true in skeletal muscle one would expect that the content of PLIN5 interacting with the mitochondria would increase in times where the reliance on fatty acids for energy is higher. These situations can arise from acute bouts of moderate intensity exercise as well as endurance training. Initially it would be wise to investigate this theory with an acute study using either an isolated muscle contraction model, similar to what was utilized in this thesis. However in order to isolate enough mitochondria for analysis larger and/or more skeletal muscle tissue may be needed. Therefore an acute bout of hindlimb exercise via sciatic nerve stimulation or whole body exercise via treadmill running would be more appropriate.

It is known that exercise training results in an increase in the proportion of lipid droplets in contact with mitochondria (51), presumably allowing for a more efficient transfer of fatty acids to be oxidized. Further, the content of PLIN5 is

increased with training (40, 46), however the location of this increased protein content is unknown. If PLIN5 is recruited to the mitochondria with an acute bout of exercise it may also be possible that training results in an increased mitochondrial content of PLIN5.

Further, the use of the recently development PLIN5 knockout mice (27) may provide more information of the role of PLIN5 in providing fatty acids to the mitochondria for energy production. These knockout mice could be subjected to either acute or chronic exercise training, similar to what has been described above. These studies would provide details as to whether PLIN5 is essential for fatty acid provision from lipid droplets as well as fatty acid oxidation during exercise.

Limitations

This section discusses limitations of the presented studies and potential avenues by which future work may be improved. Physiological studies are frequently underpowered due to smaller sample sizes. The most straightforward consequence of underpowered studies is that effects of practical importance may not be detected. Further underpowered studies result in larger variance of the estimates of the parameter being estimated. This might explain the differences in the results for ATGL to CGI-58 protein interactions from Chapters 5 and 6. In Chapter 5 with an $n=12$ and a direct comparison between rested and contracted muscles there was a significant increase in the interactions, however these results were not repeated in Chapter 6. This could be due to the small sample size resulting in low power as the power of the performed ANOVA test (0.106) was far below the desired power of 0.80. This also may have occurred because there were multiple groups (4 groups) to compare compared to only 2 in the previous study. Using the means and standard deviations from the data, a power calculation (with an alpha level of 0.05 and power of 0.80) reveals that a sample size of 21 would be needed in order to observe significant results. However, when animal testing is involved, designing an overpowered study may be considered unethical if it wastes resources such as time and research money for materials. As well, it is necessary to limit the sample size to avoid unnecessary use of animals. While we did not observe statistically significant results in Chapter 6 these results still provide insight into the interactions between ATGL and CGI-58 and it is impossible to discount the importance of this association in accelerating lipolysis. Further studies may want to

include more than one method to determine protein-protein interactions to further strengthen these results.

In examining protein-protein interactions this thesis utilized a co-immunoprecipitation method. While this method can provide great insight into protein interactions there are inherent limitations that should be discussed. Immunoprecipitation methods (and co-IP) typically require a great deal of optimization and troubleshooting to determine the optimal conditions and methods to use. Prior to the study, pilot work was performed in order to ensure that we were pulling down all of our protein of interest and therefore we are confident that we have fully precipitated our proteins. Ideally we would have preferred to have been able to show the interactions between proteins both ways (i.e., co-IP one protein and look for the second followed by co-IP of the second to look for the first). However, with the currently available antibodies we were unable to do so. One of the most commonly encountered problems with both IP and co-IP approaches is interference from antibody bands during Western blotting. Detection of the antibody (light and heavy chain, ~25 and ~50kDa bands, in SDS-PAGE gels, respectively) used to pull out the protein of interest during the co-IP can interfere with the interpretation of the results from a Western blot. The ideal situation would be to analyze the co-IP without contamination of the eluted antigen with antibody; with this potential interference eliminated, only the co-precipitated proteins would be present and detected on the gel. In developing the methods to examine each protein interaction for these studies we tried several different approaches in order to eliminate antibody interference. Other than traditional co-IP we tried two

methods to avoid this problem: 1) using a detection reagent that does not recognize the denatured antibody fragment and 2) antibody immobilization. We used a Pierce secondary (The Clean-Blot IP Detection Reagent (Thermo Scientific, Rockford, IL, USA) that does not recognize or bind to denatured IgG that has undergone electrophoresis under reducing SDS-PAGE. However, like most methods, this was also dependent on the primary antibody used and did not work for the CGI-58 primary antibody, resulting in completely blank blots. To immobilize the antibody used for the IP we used a Pierce Co-IP Kit (Thermo Scientific, Rockford, IL, USA) to covalently link the IP antibody to the beads. However, this kit was unsuccessful at binding certain antibodies to the resin, resulting in blank blots. In developing our methods we had gone through each of these approaches with each interaction and determined the appropriate method to investigate each one. In order to be consistent with our methods we found that immunoprecipitating ATGL and HSL and then Western blotting for a protein of interest was most appropriate and saved a considerable amount of tissue. For the detection of serine phosphorylation, the traditional IP route resulted in significant antibody interference and the most appropriate approach ended up being antibody immobilization with use of the kit. Future studies may want to include another method of detecting protein interactions to strengthen the results. One potential method may be FRET (Forster Resonance Energy Transfer) analysis. This method is similar to the immunohistochemical methods describe in this thesis, but two proteins would be tagged with fluorescent antibodies that would only fluoresce if the proteins were interacting.

References

1. Alam N and Saggerson ED. Malonyl-CoA and the regulation of fatty acid oxidation in soleus muscle. *The Biochemical journal* 334 (Pt 1): 233-241, 1998.
2. Bartholomew SR, Bell EH, Summerfield T, Newman LC, Miller EL, Patterson B, Niday ZP, Ackerman W, and Tansey JT. Distinct cellular pools of perilipin 5 point to roles in lipid trafficking. *Biochimica et biophysica acta* 1821: 268-278, 2012.
3. Bartz R, Zehmer JK, Zhu M, Chen Y, Serrero G, Zhao Y, and Liu P. Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation. *Journal of proteome research* 6: 3256-3265, 2007.
4. Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, and Sztalryd C. Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* 57: 2037-2045, 2008.
5. Bezaire V and Langin D. Regulation of adipose tissue lipolysis revisited. *Proc Nutr Soc* 68: 350-360, 2009.
6. Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P, and Hesselink MK. The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochemistry and cell biology* 137: 205-216, 2012.
7. Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S, and Hesselink MK. Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochimica et biophysica acta* 1831: 844-852, 2013.
8. Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, and Londos C. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *Journal of lipid research* 38: 2249-2263, 1997.
9. Brasaemle DL and Wolins NE. Packaging of fat: an evolving model of lipid droplet assembly and expansion. *The Journal of biological chemistry* 287: 2273-2279, 2012.
10. Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, and Nebb HI. LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochimica et biophysica acta* 1771: 210-227, 2007.
11. Donsmark M, Langfort J, Holm C, Ploug T, and Galbo H. Contractions activate hormone-sensitive lipase in rat muscle by protein kinase C and mitogen-activated protein kinase. *The Journal of physiology* 550: 845-854, 2003.
12. Dyck DJ and Bonen A. Muscle contraction increases palmitate esterification and oxidation and triacylglycerol oxidation. *The American journal of physiology* 275: E888-896, 1998.

13. Dyck DJ, Steinberg G, and Bonen A. Insulin increases FA uptake and esterification but reduces lipid utilization in isolated contracting muscle. *American journal of physiology Endocrinology and metabolism* 281: E600-607, 2001.
14. Egan JJ, Greenberg AS, Chang MK, and Londos C. Control of endogenous phosphorylation of the major cAMP-dependent protein kinase substrate in adipocytes by insulin and beta-adrenergic stimulation. *The Journal of biological chemistry* 265: 18769-18775, 1990.
15. Granneman JG and Moore HP. Location, location: protein trafficking and lipolysis in adipocytes. *Trends in endocrinology and metabolism: TEM* 19: 3-9, 2008.
16. Granneman JG, Moore HP, Granneman RL, Greenberg AS, Obin MS, and Zhu Z. Analysis of lipolytic protein trafficking and interactions in adipocytes. *The Journal of biological chemistry* 282: 5726-5735, 2007.
17. Granneman JG, Moore HP, Krishnamoorthy R, and Rathod M. Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *The Journal of biological chemistry* 284: 34538-34544, 2009.
18. Granneman JG, Moore HP, Mottillo EP, and Zhu Z. Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *The Journal of biological chemistry* 284: 3049-3057, 2009.
19. Granneman JG, Moore HP, Mottillo EP, and Zhu Z. Functional interactions between Mldp (LSDP5) and Abhd5 in the control of intracellular lipid accumulation. *The Journal of biological chemistry* 284: 3049-3057, 2009.
20. Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L. Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
21. Granneman JG, Moore HP, Mottillo EP, Zhu Z, and Zhou L. Interactions of perilipin-5 (Plin5) with adipose triglyceride lipase. *The Journal of biological chemistry* 286: 5126-5135, 2011.
22. Gruber A, Cornaciu I, Lass A, Schweiger M, Poeschl M, Eder C, Kumari M, Schoiswohl G, Wolinski H, Kohlwein SD, Zechner R, Zimmermann R, and Oberer M. The N-terminal region of comparative gene identification-58 (CGI-58) is important for lipid droplet binding and activation of adipose triglyceride lipase. *The Journal of biological chemistry* 285: 12289-12298, 2010.
23. Hickenbottom SJ, Kimmel AR, Londos C, and Hurley JH. Structure of a lipid droplet protein; the PAT family member TIP47. *Structure* 12: 1199-1207, 2004.
24. Hocsak E, Racz B, Szabo A, Mester L, Rapolti E, Pozsgai E, Javor S, Bellyei S, Gallyas F, Jr., Sumegi B, and Szigeti A. TIP47 protects mitochondrial membrane integrity and inhibits oxidative-stress-induced cell death. *FEBS letters*, 2010.
25. Hsieh K, Lee YK, Londos C, Raaka BM, Dalen KT, and Kimmel AR. Perilipin family members preferentially sequester to either triacylglycerol-

- specific or cholesteryl-ester-specific intracellular lipid storage droplets. *Journal of cell science* 125: 4067-4076, 2012.
26. Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, and Nawata H. ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American journal of physiology Endocrinology and metabolism* 283: E775-783, 2002.
27. Kuramoto K, Okamura T, Yamaguchi T, Nakamura TY, Wakabayashi S, Morinaga H, Nomura M, Yanase T, Otsu K, Usuda N, Matsumura S, Inoue K, Fushiki T, Kojima Y, Hashimoto T, Sakai F, Hirose F, and Osumi T. Perilipin 5, a lipid droplet-binding protein, protects heart from oxidative burden by sequestering fatty acid from excessive oxidation. *The Journal of biological chemistry* 287: 23852-23863, 2012.
28. Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, Kienesberger P, Strauss JG, Gorkiewicz G, and Zechner R. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell metabolism* 3: 309-319, 2006.
29. Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, and Brown DA. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *Journal of lipid research* 48: 2751-2761, 2007.
30. Lu X, Yang X, and Liu J. Differential control of ATGL-mediated lipid droplet degradation by CGI-58 and G0S2. *Cell Cycle* 9: 2719-2725, 2010.
31. Magnusson B, Asp L, Bostrom P, Ruiz M, Stillemark-Billton P, Linden D, Boren J, and Olofsson SO. Adipocyte differentiation-related protein promotes fatty acid storage in cytosolic triglycerides and inhibits secretion of very low-density lipoproteins. *Arteriosclerosis, thrombosis, and vascular biology* 26: 1566-1571, 2006.
32. Marcinkiewicz A, Gauthier D, Garcia A, and Brasaemle DL. The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. *The Journal of biological chemistry* 281: 11901-11909, 2006.
33. Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, and Chan L. Absence of perilipin results in leanness and reverses obesity in *Lepr*(db/db) mice. *Nature genetics* 26: 474-479, 2000.
34. Mason RR, Meex RC, Lee-Young R, Canny BJ, and Watt MJ. Phosphorylation of adipose triglyceride lipase Ser(404) is not related to 5'-AMPK activation during moderate-intensity exercise in humans. *American journal of physiology Endocrinology and metabolism* 303: E534-541, 2012.
35. Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS. Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.
36. Miyoshi H, Souza SC, Zhang HH, Strissel KJ, Christoffolete MA, Kovsan J, Rudich A, Kraemer FB, Bianco AC, Obin MS, and Greenberg AS. Perilipin

promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *The Journal of biological chemistry* 281: 15837-15844, 2006.

37. Mottagui-Tabar S, Ryden M, Lofgren P, Faulds G, Hoffstedt J, Brookes AJ, Andersson I, and Arner P. Evidence for an important role of perilipin in the regulation of human adipocyte lipolysis. *Diabetologia* 46: 789-797, 2003.
38. Muoio DM, Dohm GL, Fiedorek FT, Jr., Tapscott EB, and Coleman RA. Leptin directly alters lipid partitioning in skeletal muscle. *Diabetes* 46: 1360-1363, 1997.
39. Muoio DM, Dohm GL, Tapscott EB, and Coleman RA. Leptin opposes insulin's effects on fatty acid partitioning in muscles isolated from obese ob/ob mice. *The American journal of physiology* 276: E913-921, 1999.
40. Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA, and Tarnopolsky MA. Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme* 37: 724-735, 2012.
41. Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, and Ploug T. Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *Journal of lipid research* 47: 2392-2399, 2006.
42. Roepstorff C, Donsmark M, Thiele M, Vistisen B, Stewart G, Vissing K, Schjerling P, Hardie DG, Galbo H, and Kiens B. Sex differences in hormone-sensitive lipase expression, activity, and phosphorylation in skeletal muscle at rest and during exercise. *American journal of physiology Endocrinology and metabolism* 291: E1106-1114, 2006.
43. Shaw CS, Sherlock M, Stewart PM, and Wagenmakers AJ. Adipophilin distribution and colocalization with lipid droplets in skeletal muscle. *Histochemistry and cell biology* 131: 575-581, 2009.
44. Shen WJ, Patel S, Miyoshi H, Greenberg AS, and Kraemer FB. Functional interaction of hormone-sensitive lipase and perilipin in lipolysis. *Journal of lipid research* 50: 2306-2313, 2009.
45. Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS. Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Experimental physiology* 97: 970-980, 2012.
46. Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ, and Shaw CS. Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *The Journal of physiology* 591: 657-675, 2013.
47. Su CL, Sztalryd C, Contreras JA, Holm C, Kimmel AR, and Londos C. Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. *The Journal of biological chemistry* 278: 43615-43619, 2003.
48. Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C. Perilipin A is essential for the translocation of hormone-sensitive

- lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
49. Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, and Londos C. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *The Journal of cell biology* 161: 1093-1103, 2003.
50. Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, and Londos C. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 98: 6494-6499, 2001.
51. Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, and Hamadeh MJ. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *American journal of physiology Regulatory, integrative and comparative physiology* 292: R1271-1278, 2007.
52. van Loon LJ, Koopman R, Manders R, van der Weegen W, van Kranenburg GP, and Keizer HA. Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *American journal of physiology Endocrinology and metabolism* 287: E558-565, 2004.
53. Wang H, Bell M, Sreenevasan U, Hu H, Liu J, Dalen K, Londos C, Yamaguchi T, Rizzo MA, Coleman R, Gong D, Brasaemle D, and Sztalryd C. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *The Journal of biological chemistry* 286: 15707-15715, 2011.
54. Wang H, Hu L, Dalen K, Dorward H, Marcinkiewicz A, Russell D, Gong D, Londos C, Yamaguchi T, Holm C, Rizzo MA, Brasaemle D, and Sztalryd C. Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. *The Journal of biological chemistry* 284: 32116-32125, 2009.
55. Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley WC, and Sztalryd C. Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *Journal of lipid research* 52: 2159-2168, 2011.
56. Wang SM, Hwang RD, Greenberg AS, and Yeo HL. Temporal and spatial assembly of lipid droplet-associated proteins in 3T3-L1 preadipocytes. *Histochemistry and cell biology* 120: 285-292, 2003.
57. Watt MJ, Holmes AG, Pinnamaneni SK, Garnham AP, Steinberg GR, Kemp BE, and Febbraio MA. Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *American journal of physiology Endocrinology and metabolism* 290: E500-508, 2006.
58. Watt MJ and Spriet LL. Triacylglycerol lipases and metabolic control: implications for health and disease. *American journal of physiology Endocrinology and metabolism* 299: E162-168, 2010.

59. Watt MJ and Steinberg GR. Regulation and function of triacylglycerol lipases in cellular metabolism. *The Biochemical journal* 414: 313-325, 2008.
60. Watt MJ, Steinberg GR, Heigenhauser GJ, Spriet LL, and Dyck DJ. Hormone-sensitive lipase activity and triacylglycerol hydrolysis are decreased in rat soleus muscle by cyclopiazonic acid. *American journal of physiology Endocrinology and metabolism* 285: E412-419, 2003.
61. Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel PE. S3-12, Adipophilin, and TIP47 package lipid in adipocytes. *The Journal of biological chemistry* 280: 19146-19155, 2005.
62. Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, and Bickel PE. OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55: 3418-3428, 2006.
63. Wolins NE, Rubin B, and Brasaemle DL. TIP47 associates with lipid droplets. *The Journal of biological chemistry* 276: 5101-5108, 2001.
64. Xu G, Sztalryd C, and Londos C. Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochimica et biophysica acta* 1761: 83-90, 2006.
65. Xu G, Sztalryd C, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, and Londos C. Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *The Journal of biological chemistry* 280: 42841-42847, 2005.
66. Yamaguchi T, Matsushita S, Motojima K, Hirose F, and Osumi T. MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. *The Journal of biological chemistry* 281: 14232-14240, 2006.
67. Yeaman SJ. Hormone-sensitive lipase--new roles for an old enzyme. *The Biochemical journal* 379: 11-22, 2004.
68. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, and Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* 306: 1383-1386, 2004.

Appendix

a. Preparation of muscle for histological analysis

Snap freezing muscle tissue

Principle:

The use of fresh unfixed snap-frozen tissue is employed to preserve muscle and cellular detail without compromising or sacrificing enzyme activity. The amount of time taken to freeze the muscle is proportional to the amount of freezing artifact observed under the microscope. (ie. if rapid freezing is not achieved, there will be a considerable amount of freezing artefact). Prior to staining, the muscle tissue must be mounted on cork chucks in an embedding medium (Cryomatrix). The fibres are aligned on the cork for either cross-sectional or longitudinal sections. The embedding medium is used to maintain the fibre arrangement prior to freezing and to present a hard surface at the time of cutting.

Equipment:

1. Liquid nitrogen
2. 2-Methyl butane
3. Long-handled forceps
4. A container for liquid nitrogen
5. A metal bowl that fits into the liquid nitrogen container
6. label cork for each specimen

Procedure:

1. Cool 2-methyl butane in a small metal container to -155°C in a liquid nitrogen bath.
2. Cool the 2-methyl butane until the outer area is frozen solid and the inner area is thick and slushy.
3. place a drop of O.C.T embedding compound in the centre of a labelled piece of cork.
4. Put the piece of cork with the O.C.T. into the isopentane container, cork first to cool the O.C.T. slightly
5. Place the specimens in the centre of the O.C.T. doughnut with the muscle fibres horizontal (or vertical) to the surface of the cork (mark the direction on the cork)
6. if the muscle sample is not completely submerged in O.C.T. place more O.C.T. on the top to cover the sample.
7. Plunge the O.C.T. covered specimen into the 2-methyl butane for 30 seconds.
8. Rapid freezing prevents ice crystal formation
9. Wrap in aluminium foil and label specimen.
10. Store at -80°C until sectioning.

Sectioning:

Carried out in a cryostat, optimally at -22°C

1. samples should be left for ~ 20 min to equilibrate to cryostat temperature ($\sim -22^{\circ}\text{C}$)

2. re-orientate the muscle if need be
3. 3 transverse serial sections per slide at a thickness of 10um are sufficient from each specimen to carry out one required staining protocol
4. allow 30 min or more for samples to dry before storing samples at -80 C

b. Oil Red O Staining

Principle: Histology uses specific dyes and reagents to stain for properties of cellular components so that these cellular features and structures can be seen.

Histochemistry uses substrates reacting with tissues enzymes to form colour precipitates at the site of the reaction. The physical properties of the muscle should be closely linked to the enzymes present in the tissue. Histochemical staining using Oil Red O can be used to stain neutral lipids (mainly triglycerides) with an orange-red tint (Lillie and Ashburn 1943). The histological mechanism of the staining of lipids is invariably a function of the physical properties of the dye being more soluble in the lipid to be demonstrated than in the vehicular solvent.

Muscle samples

1. sections 10 um
2. thaw mounted
3. air dry samples for 15 min
4. stored at -80°C

Reagents:

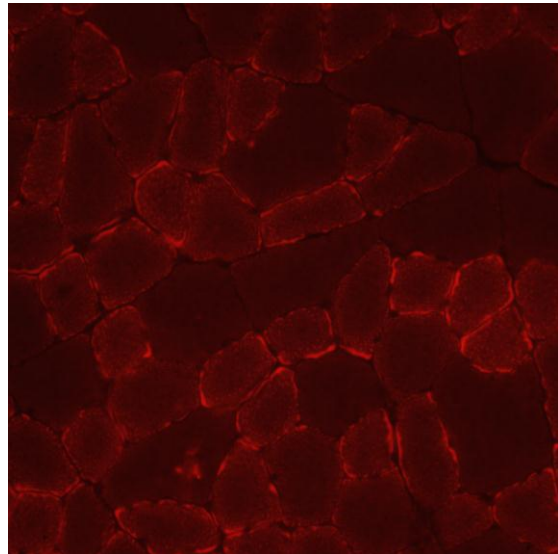
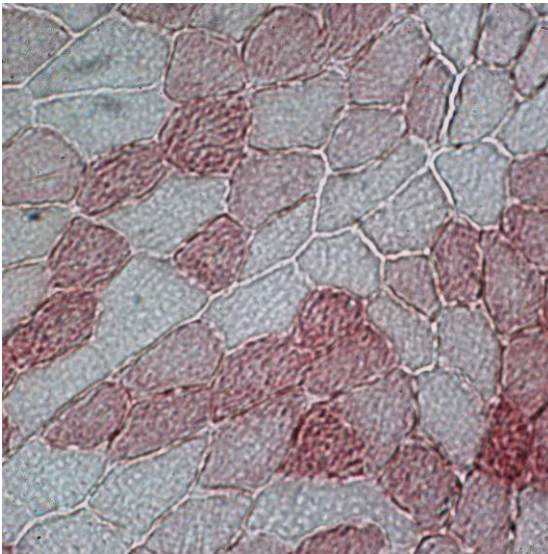
1. 3.7% formaldehyde solution:
 - 37 % stock solution
 - Deionised water
2. Oil Red O:
 - Dissolve to a stock solution*
 - oil red O (500.0 mg)
 - 60% triethyl-phosphate (C₂H₅)₃PO (100.0 ml)
 - mix on heat
 - Prior to staining (during formaldehyde incubation)*
 - 36% triethyl-phosphate working solution
 - oil red O stock solution (12.0 ml)
 - deionised water (8.0 ml)
 - mix on heat
 - filter 2x through Whatman paper number 42 to remove crystallized oil red O
3. Phosphate-buffer saline (PBS) (1x working solution)
 - Sodium chloride (NaCl) (137 mM)
 - Potassium chloride (KCl) (3 mM)
 - Disodium hydrogen phosphate (Na₂HPO₄) (8mM)
 - Potassium dihydrogen phosphate (KH₂PO₄) (3mM pH 7.4)
 - Can be used for dilution of Triton X-100, glycerol and antibodies

Note: make a 10x stock solution for long term storage

Oil Red O Staining

Procedure:

1. Staining protocol starts with 1 h fixation of the air-dried, thawed sections using 3.7% formaldehyde solution in deionised water.
2. Mix working solution of ORO on heat, double filter
3. Prepare beaker of dH₂O
4. Wash excess formaldehyde with 3 rinses in deionized water for 30 sec. (3dips in beaker; dab dry using kimwipes)
5. Immerse slides in working solution of oil red O for 30 min.
6. prepare beaker of dH₂O
7. Wash sections were washed with 3 exchanges of deionised water for 3 x 30 sec (3 dips; dab dry).
8. If desired, sections can be counter stained using Mayer's haematoxylin for 60s to visualise nuclei.
9. Sections rinsed with running tap water for 10 min and covered with a coverslip using 10% glycerol in PBS (Note: if using fluorescence mount using an antifade reagent, Prolong Gold Antifade)



c. Combined oil red O and immunofluorescence staining

Principle:

Combining conventional Oil Red O with other (immuno) histochemical stains, provides information on the exact subcellular location of intramuscular triglycerides and its potential vicinity with proteins involved in lipid metabolism.

Reagents:

4. 3.7% formaldehyde solution:
 - 37 % stock solution
 - Deionised water

5. Oil Red O:
 - Dissolve to a stock solution*
 - oil red O (500.0 mg)
 - 60% triethyl-phosphate (C₂H₅)₃PO (100.0 ml)
 - mix on heat
 - Prior to staining (during formaldehyde incubation)*
 - 36% triethyl-phosphate working solution
 - oil red O stock solution (12.0 ml)
 - deionised water (8.0 ml)
 - mix on heat
 - filter 2x through Whatman paper number 42 to remove crystallized oil red O

6. Phosphate-buffer saline (PBS) (1x working solution)
 - Sodium chloride (NaCl) (137 mM)
 - Potassium chloride (KCl) (3 mM)
 - Disodium hydrogen phosphate (Na₂HPO₄) (8mM)
 - Potassium dihydrogen phosphate (KH₂PO₄) (3mM pH 7.4)
 - Can be used for dilution of Triton X-100, glycerol and antibodies

Note: make a 10x stock solution for long term storage

7. 0.5% Triton X-100 in PBS

Procedure:

1. Staining protocol starts with 1 h fixation of the air-dried, thawed sections using 3.7% formaldehyde solution in deionised water.
2. Prepare beaker of dH₂O

3. Wash excess formaldehyde with 3 rinses in deionized water for 30 sec. (3dips in beaker; dab dry using kimwipes)
4. sections were treated with 0.5% Triton X-100 in PBS 5 min
5. washed 3 x in PBS for 5 min
6. incubate for 30 min at room temp with primary antibody in appropriate dilution (dilute in PBS)
7. 3x5 min washing with PBS
8. incubate for 30 min at room temp with secondary antibody in appropriate dilution (in PBS)

Protein	1° Ab in PBS	2° Ab
PLIN2	1:100 (Progen GP40; guinea pig polyclonal)	1:200 (anti guinea pig Alexa Fluor 488)
PLIN5	1:50 (Progen GP31; guinea pig polyclonal)	1:200 (anti guinea pig Alexa Fluor 488)

Notes:

- use ~10ul of Ab PBS solution per muscle section
- when optimizing ensure that there is no cross talk between wavelengths from the 1° Ab by testing each filter.
- also optimize with slides that have only been incubated with the 1° or 2°Ab to

9. 3x5 min washing with PBS
10. slides immersed in the working solution of oil red O for 30 min
11. slides rinsed 3x with deionised water
12. 10 min wash with running tap water
13. stained sections were embedded in 10% glycerol in 10 mM TRIS-HCl, pH 8.5 (containing DAPI to visualise nuclei if wanted) (use Prolong Gold antifade reagent to coverslip)
14. cover with coverslip

Summarized staining protocol for oil red O (from Koopman et al. 2001):

Treatment	ORO	ORO with IF
Fixation	3.7% formaldehyde for 60'	3.7% formaldehyde for 60'
Rinsing	3×30'' with deionised water	3×30'' with deionised water
Pretreatment ^a	–	0.5% Triton X-100 in PBS for 5'
Rinsing	–	3×5' with PBS
Incubation ^b	–	Primary ab in PBS for 30'
Rinsing	–	3×5' with PBS
Incubation ^b	–	Secondary ab in PBS for 30'
Rinsing	–	3×5' with PBS
Staining	30' in ORO working solution	30' in ORO working solution
Rinsing	3×30'' with deionised water	3×30'' with deionised water
Rinsing	10' with running tap water	10' with running tap water
Mounting	10% glycerol in PBS	10% glycerol in PBS with DAPI

References:

René Koopman, Gert Schaart, Matthijs K.C. Hesselink. Optimization of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* (2001) 116:63–68

Lillie RD and Ashburn LL,(1943), Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique. *Archs.Path.*,**36**,432.

Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MKC. Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes. *J Clin Endocrinol Metab* 94: 4077–4085, 2009.

d. Western Blotting

Background: Western blotting is a technique that identifies specific proteins in a given sample or extract after separation by molecular weight using polyacrylamide gel electrophoresis. The polyacrylamide gel is placed to a membrane, which is typically nitrocellulose or PVDF (polyvinylidene fluoride), and the application of an electrical current induces the proteins to migrate from the gel to the membrane on which they become immobilized. The membrane is then a replica of the gel protein and can be tagged with an antibody.

Reagents:

Homogenizing Buffer

- 250mM sucrose (21.4g)
- 100mM KCL (1.865g)
- 5mM EDTA (0.4695g)
- add 225mL dH₂O, fix pH 6.8, top up to 250mL dH₂O
- mix and store in fridge

Laemelli Buffer (2x Sample Buffer)

- 2.5ml 0.5M Tris-HCl pH 6.8
- 2ml glycerol
- 2ml 10% SDS
- 0.2mL 1% bromophenol blue
- 1ml β-mercaptoethanol
- 0.3mL dH₂O

Running Gel Buffer

- 1.5M Tris-HCl, pH 8.8

Stacking Gel Buffer

- 0.5M Tris-HCl, pH 6.8

10x Running Buffer, pH 8.3

- 250mM Tris base
- 1.92M glycine
- 1% SDS
- Store at 4°C. If precipitation occurs, warm to room temp before use.

10x Transfer Buffer (semi-dry)

- 312.5mM Tris base (3.786g)
- 2.4M glycine (18g)
- fill to 100mL with dH₂O
- 0.01% SDS
- *before use, dilute 80mL in 720 mL dH₂O and add 200mL of methanol

10x TBS

- 200mM Tris base (24.2 g/L)
- 1.37M NaCl (80.1 g/L)
- 38mL of 1M HCl/L of TBS made
- adjust to pH 7.5

TBST

- dilute 100mL of 10x TBS in 900mL dH₂O
- add 4mL of 25% Tween 20 while stirring (polyoxyethylenesorbitan monolaurate)

Sample Preparation

Homogenization:

1. Make up homogenization buffer:
 - 250mM sucrose = 21.4g
 - 100mM KCl = 1.865g
 - 5mM EDTA = 0.4695
 - add 225mL dH₂O, fix pH 6.8, top up to 250mL dH₂O and mix well, store in fridge
2. Add protease inhibitor tablet (from antibody drawer) (Roche, 1 tablet /10mL) and add phosphatase inhibitor (Roche, 1 tablet /10mL)
3. Prepare glass homogenizers with ~ 200μL of buffer, tear in scale, remove ~ 10mg chunk of muscle from sample for analysis, add muscle and weigh
 - keep on ice when possible
4. Correct buffer volume in homogenizer so that 25μL of buffer is present for every 1mg of sample
5. Homogenize well on ice (~30 plunges)

Protein Determination (Bradford Assay)

Standard Curve Preparation:

1. Retrieve all samples (whole homogenate) from -80° C storage and place on ice.
2. Remove one aliquoted sample of 1 mg/mL stock BSA solution from -20° C storage.
3. Prepare all BSA standard solutions in eppendorf tubes on ice.

Table d1. Volumes of Stock BSA Solution and dH₂O required for the preparation of Bradford protein assay standard solutions.

Protein Concentration (mg/mL)	Stock BSA Solution (μL)	dH₂O (μL)
1	1000	0
0.5	500 (from 1 mg/mL)	500
0.25	500 (from 0.5 mg/mL)	500
0.125	500 (from 0.25 mg/mL)	500
0.05	100 (from 0.5 mg/mL)	900
0	0	1000

4. Prepare 10-fold dilutions for all samples by loading individual eppendorf tubes with 10 μ L of whole homogenate and 90 μ L of DDH₂O.
4. Pipette 10ul of each sample (standard and diluted samples) into 96 well plate, do this in triplicate. (Hint: the Bradford method is a sensitive spectrophotometric procedure that is fully dependent upon samples' color perception and subsequent light absorption. Colour presents as a result of a reaction between the protein assay dye reagent and the positively charged amine groups of proteins within a sample. Consequently, any debris on the well plate could interfere with light absorption readings.)
5. Add 200 μ L of diluted buffer (1:4 with Bio-Rad Protein Assay Dye Reagent: dH₂O)
6. Let incubate for 5min and read absorbance as wavelength 595nm
7. Insert the well plate into the spectrophotometer to generate absorbance readings.
8. Open KC4™ data analysis software, and ensure that the reading parameters are set to absorbance.
9. Define the reading parameters by the following details: absorbance wavelength of 595 nanometers, shaking intensity level of 3 and shaking duration of 5 seconds
10. Proceed to read the absorbance of each standard and sample
11. Copy the absorbance readings into a Microsoft® Excel spreadsheet, and generate a standard curve by plotting known protein concentrations (BSA standard solutions) against their average absorbance readings. Make sure that you subtract the absorbance reading of water from each sample so that the curve starts at zero-zero.
12. Outfit the standard curve with a polynomial trendline, and display the quadratic equation derived from this curve.
13. Subtract the average absorbance reading of water for every sample, and calculate the unknown protein concentrations of all samples using the quadratic equation. Don't forget to multiple sample concentrations by 10.

Sample preparation for Western blotting

1. To prepare samples for blotting (best done on same day as SDS PAGE) first determine your desired total volume of sample as well as the sample concentration needed (this will vary depending on which protein you are probing for; optimization is recommended). Then using the results from the Bradford assay calculate your sample volume, sample buffer volume, water volume and DTT volume (if needed).

Total Volume (TV)	Optional 25 - 100 μ L
Sample Volume (SV)	$SV = TV \cdot \frac{[\text{Desired Final Protein}]}{[\text{Protein}]}$
Sample (Laemelli) Buffer (SB)	$SB_f = \frac{TV}{[SB_i]}$ (i.e. $\frac{TV}{5}$ for 5x SB _i)
Water (dH₂O)	$dH_2O = TV - SV - SB$
1M DTT (optional)	= 10% of total volume

References

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-54.

SDS PAGE Procedure

Electrophoresis Preparation:

1. Before using glass plates, wipe with methanol and a kimwipe
2. Assemble the glass-holding apparatus with the short plate facing front.
Ensure plates are flush and level before locking and inserting onto the gel casting stand
3. Prepare gels (makes 2):

Percent Gel	dH ₂ O (ml)	Protogel (ml) (30% acrylamide)	Gel Buffer (ml)
4 (Stacking)	6.1	1.3	2.5 <u>Stacking</u>
6	5.3	2.0	2.5 Resolving
8	4.62	2.67	2.5 Resolving
10 (Running)	3.96	3.33	2.5 <u>Resolving</u>
12	3.29	4.0	2.5 Resolving
15	2.29	5.0	2.5 Resolving

4. Before pouring the **running gel**, add 100 μ L APS (60mg/600 μ L) and 10 μ L of TEMED. Swirl and pour until reaching the depth of the comb. Use methanol to remove any bubbles and dump once hardened
5. Before pouring the **stacking gel**, add 100 μ L APS and 20 μ L TEMED. Swirl and pour until the top of the short plate is reached. Insert combs of desired size. Once hardened, remove combs and rinse with dH₂O
6. Place gel cassettes into electrode assembly with the short plates facing inwards, clamp down firmly and place into mini-tank
7. Boil samples for 5-minutes and place subsequently on ice for 5-minutes while preparing *running buffer* (50mL 10x running buffer + 450mL dH₂O)
8. Fill the inner chamber of the apparatus with diluted running buffer add ~200mL to the outside chamber
9. Load the standard (5ul) and the appropriate amount of sample into each well. (Note: to determine the appropriate amount of sample to load, perform a separate optimization with varying amounts of protein).
10. Run electrophoresis at 120-volts for 90-minutes or until the blue dye travels to the bottom edge of the plate. If tiny bubbles rise then electrophoresis is working. Prepare transfer buffer.

Transfer

1. Remove gels carefully, discard the stacking gel, and transfer to a plastic tray to equilibrate in transfer buffer
2. Soak all transfer materials in freshly prepared transfer buffer to equilibrate before electrophoresis.
3. Create the "transfer sandwich" in a shallow container filled with transfer buffer:
 - white/clear side of the holder
 - sponge/ fibre pad (presoaked in transfer buffer)
 - filter paper (presoaked in transfer buffer)
 - membrane (PVDF presoaked in methanol)
 - Gel
 - filter paper (presoaked in transfer buffer)
 - sponge/ fibre pad (presoaked in transfer buffer)
 - black side of the holder

*Be sure to roll out any air bubbles, close firmly, and place the black side of the holder to the black (/back) side of the electrophoresis apparatus.

4. Fill the tank with transfer buffer and the surrounding environment with plenty of ice to prevent from cooking the gel and add a stir-bar to help with heat loss.
5. Run the transfer at 100-volts for 60-minutes

Determining efficient transfer:

6. Place the membrane in a disposable square petri dish with the proteins facing upward.

7. Add 10 ml Ponceau S solution to stain protein bands. Rock until stained bands are visible (~10min).
8. Pour off Ponceau S solution. Rinse membrane in dH2O to wash off excess stain. Repeat as necessary.
9. Place membrane between transparent film and scan an image for future reference of total protein content in each lane

(Hint: this step is crucial towards eliminating any error that may have occurred during sample loading. At times protein of interest contents must be normalized to total protein contents in order to control for any error during sample loading.)

10. Rinse the membrane in TBST until stain has disappeared then continue to next step.

Antibodies

1. Discard the gel and filter papers and transfer the membranes to small dishes
2. Block with 20mL TBST-5% skim milk or TBST-3% BSA solution for 1-hour
3. Discard blocking solution and add 1° AB in TBST-% skim milk or TBST-% BSA, incubating for 1-hour or overnight with the appropriate dilution

Antibody	Size (kDa)	Dilution	Blocking Solution	1° AB solution
PLIN2	48 (apparent at 52)	1:1000	5% milk	3% milk
PLIN3	47	1:1000	5% BSA	1% BSA
PLIN5	50.8 (apparent 52)	1:1000	5% milk	3% milk
ATGL	50	1:1000	5% BSA	5% BSA
CGI-58	42	1:1000	2% milk	2% milk

4. Wash 3-5x 5-minutes with TBST (10mL)
5. Add 2° Ab (dependent on 1° Ab) in TBST-% skim milk or TBST-% BSA solution for 1-hour with the appropriate dilution

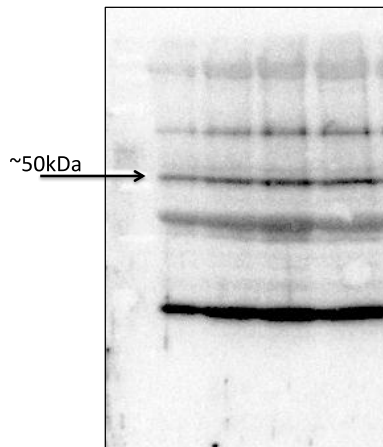
1° Ab	2° Ab	Dilution	Solution
PLIN2	Anti-mouse	1:10000	3% milk
PLIN3	Anti-rabbit	1:10000	1% BSA
PLIN5	Anti-guinea pig	1:10000	3% milk
ATGL	Anti-rabbit	1:10000	5% BSA
CGI-58	Anti-rabbit	1:20000	2% milk

Enhanced Chemiluminescence

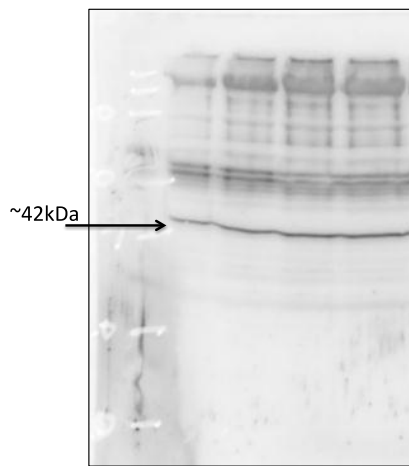
1. Wash 3x 5-minutes with TBST

2. Combine 1mL of each chemiluminescent HPR substrate (peroxide solution + luminal reagent) over membrane and continue to pipette over membrane or let rock for 5-minutes
3. Place membrane protein-side down on transparency sheet cover back with another transparency sheet
4. Take an initial photo with a pin beside the standard that represents the weight of your protein for overlapping later if need be. Take a subsequent photo with the appropriate exposure time and analyze later with "Imagej".

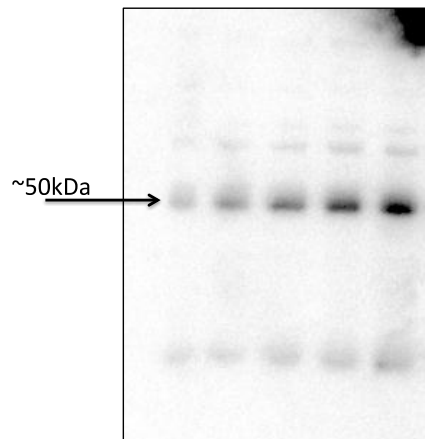
Representative blots:



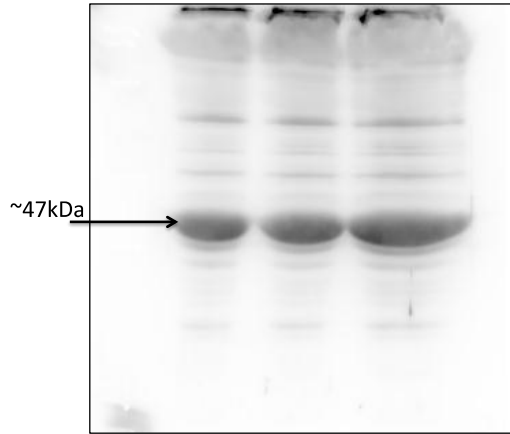
A. Representative blot for ATGL or soleus whole homogenate (10, 20 30, 37.5ug)



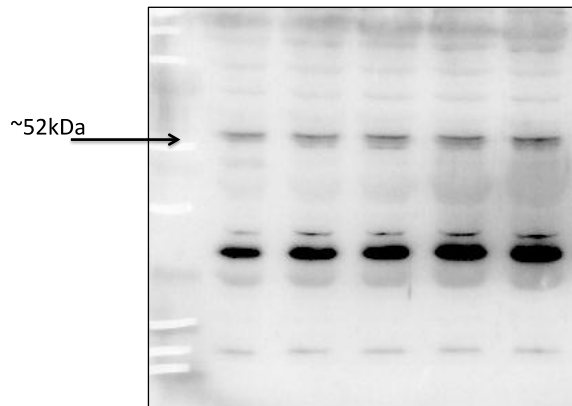
B. Representative blot for CGI-58 of soleus whole homogenate (10, 20 30, 37.5ug)



C. Representative blot for PLIN2 in soleus whole homogenate (5, 8, 10, 12, 15 ug)



D. Representative blot for PLIN3 in soleus whole homogenate (10, 15, 20 ug).



E. Representative blot for PLIN5 in soleus whole homogenate, showing the effects of boiling (Lanes: no boil, 30s, 1min, 2min, 5min).

e. Co-immunoprecipitation of proteins

Principle:

1. 10x Stock Phosphate Buffered Saline (PBS) – pH 7.4

- 1.37 M NaCl
- 30 mM KCl
- 80 mM Na₂HPO₄
- 30 mM KH₂PO₄
- ddH₂O (100 mL final volume)
- Dilute to 1x PBS as needed

2. Homogenization buffer (Griffin)

- 150 mM NaCl
- 50 mM TrisCl
- 1 mM EGTA

Sample Homogenization:

The homogenization procedure is the same as that described for standard Western blotting, however the homogenization buffer differs. Homogenization buffer components can and will influence the efficiency of the IP reaction and detergent composition is a major factor for IP reactions. The choice to use a homogenization buffer that did not contain detergents in this thesis was made because co-immunoprecipitation depends so much on antibody-antigen binding and protein-protein interactions. Further, the ability to maintain stable physiological interactions throughout the mechanical and chemical stresses of the incubation and washing steps is a critical factor when performing a co-IP reaction. Therefore for co-IP, the homogenization buffer should be relatively mild so as not to disrupt the protein-protein interactions. In our case it was important to preserve protein-protein interactions and to minimize denaturation, therefore a buffer without ionic or non-ionic detergents was used. We believe that the homogenization and disruption of the lipid droplets using with detergent-free buffer was achieved by physical disruption using a Dounce homogenizer, and freezing and thawing of the samples.

Immunoprecipitation procedure:

1. Using previously collected protein concentrations from the Bradford assay, incubate 500-1000ug or protein from whole homogenates with 5ul of primary antibody. (Note: optimal protein content for incubation will need to be optimized)
2. Incubate samples for 2 hours at 4° C with gentle agitation

3. Add 20 ul of agarose A or G beads to the samples. Incubate all samples overnight at 4° C with gentle agitation. (Note: when pipetting agarose beads cut the pipette tip).
4. Collect pellet by centrifugation at 2,500 rpm (approximately 1,000xg) for 5-10 seconds. A touch spin will work. With enough samples, gravity will pellet the beads as well.
5. Carefully aspirate and discard supernatant. The trick here is to slowly aspirate using a gel loading pipette tip (or syringe and needle) touching just the top of the liquid and slowly draw down so that you are pulling at the surface tension of the supernatant. This will ensure no loss of beads. **Note:** when optimizing keep the supernatant to ensure that all protein of interest has been collected in the pellet.

Figure a. Western blot of ATGL. Lane 1 is IP ATGL and lane 2 is the supernatant left over from the IP, showing that we have fully isolated ATGL with our IP methods

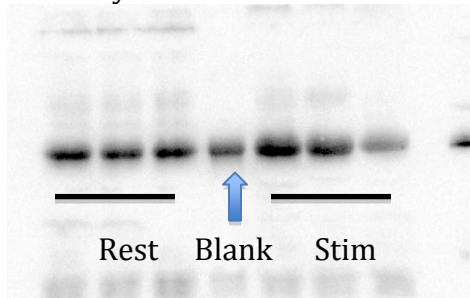


6. Wash pellet 3 times with ~500ul PBS (a more stringent buffer can be used if needed), each time repeating centrifugation step above.
7. After final wash resuspend pellet in 40ul of 2x sample buffer.
8. Boil samples in order to separate the protein of interest from the bead. Samples are now prepared for gel electrophoresis.

Note:

When doing co-immunoprecipitation one of the most commonly encountered problems with both IP and co-IP approaches is interference from antibody bands during Western blotting. Detection of the antibody (light and heavy chain, ~25 and ~50kDa bands, in SDS-PAGE gels, respectively) used to pull out the protein of interest during the co-IP can interfere with the interpretation of the results from a Western blot. The ideal situation would be to analyze the co-IP without contamination of the eluted antigen with antibody; with this potential interference eliminated, only the co-precipitated proteins would be present and detected on the gel. In developing the methods to examine each protein interaction for this study we tried several different approaches in order to eliminate antibody interference. Other than traditional co-IP we tried two methods to avoid this problem: 1) using a detection reagent that does not recognize the denatured antibody fragment (Pierce secondary, The Clean-Blot IP Detection Reagent) and 2) antibody immobilization (Pierce Co-IP Kit).

Figure b. Immunoblot of rested and stimulated muscles showing significant antibody interference in the “blank” lane that only contains buffer and the IP antibody.



References

Elion, E. A. (2006). Detection of protein–protein interactions by coprecipitation. *Current Protocols in Neuroscience*, 35, 5.25.1–5.25.10.

f. Immunoprecipitation of serine–phosphorylated proteins

Reagents

- Pierce Co-immunoprecipitation Kit (#26149)
- 7,8–dihydroxycoumarin

Note: Due to significant antibody interference when immunoprecipitating all serine phosphorylated proteins and blotting for PLIN2, PLIN3, or PLIN5, the Pierce co-immunoprecipitation kit was utilized. This kit enables isolation of native protein complexes from a lysate by directly immobilizing antibodies to an agarose support. The antibodies are covalently coupled onto an amine-reactive resin therefore resolving issues with antibody inference.

Procedure

As described by the instruction provided with the Pierce Co-immunoprecipitation (Co-IP) kit.

Anti body immobilization

1. 40 ul of anti-phosphoserine primary antibody was used when preparing the antibody resin

Sample Preparation

1. Skeletal muscle homogenization is as described in Appendix d Western Blotting, with the addition of a kinase inhibitor (200 μ m of 7,8–dihydroxycoumarin) to the buffer
2. Using previously collected protein concentrations from the Bradford assay, aliquot 500 μ g of protein per sample was added to the antibody resin.

References

1. Yang, E. B., Zhao, Y. N., Zhang, K., & Mack, P. (1999). Daphnetin, one of coumarin derivatives, is a protein kinase inhibitor. *Biochemical and Biophysical Research Communications*, 260, 682–685.

g. Sigma medium 199

Product Name Medium 199
Product Number M4530

TEST	SPECIFICATION
APPEARANCE	CLEAR SOLUTION
PH TEST	7.0-7.6
OSMOLALITY TEST	280-310 mOsm•kg ⁻¹
STERILITY BY USP	STERILE
ENDOTOXIN ASSAY	NMT 1EU/ML
GLUCOSE CONTENT	0.9-1.1 g•l ⁻¹
CELL CULTURE TEST	PASS
EXPIRATION DATE	12 MONTHS

Components **M4530**
[1X]
g•L⁻¹

Inorganic Salts

CaCl ₂ •2H ₂ O	0.265
Fe(NO ₃) ₃ •9H ₂ O	0.00072
MgSO ₄ (anhyd)	0.9767
KCl	0.4
KH ₂ PO ₄	--
Na•Acetate (anhyd)	0.05
NaHCO ₃	2.2
NaCl	6.8
Na ₂ HPO ₄ (anhyd)	--
NaH ₂ PO ₄ (anhyd)	0.122

Amino Acids

DL-Alanine	0.05
L-Arginine•HCl	0.07
DL-Aspartic Acid	0.06
L-Cysteine•HCl•H ₂ O	0.00011
L-Cystine•2HCl	0.026
DL-Glutamic Acid	0.1336
L-Glutamine	0.1
Glycine	0.05
L-Histidine•HCl•H ₂ O	0.02188
Hydroxy-L-Proline	0.01
DL-Isoleucine	0.04
DL-Leucine	0.12
L-Lysine•HCl	0.07
DL-Methionine	0.03
DL-Phenylalanine	0.05
L-Proline	0.04
DL-Serine	0.05

DL-Threonine	0.06
DL-Tryptophan	0.02
L-Tyrosine 2Na·2H ₂ O	0.05766
DL-Valine	0.05

Vitamins

Ascorbic Acid·Na	0.000056
D-Biotin	0.00001
Calciferol	0.0001
Choline Chloride	0.0005
Folic Acid	0.00001
Menadione (sodium bisulfite)	0.000016
myo-Inositol	0.00005
Niacinamide	0.000025
Nicotinic Acid	0.000025
p-Amino Benzoic Acid	0.00005
D-Pantothenic Acid·½Ca	0.00001
Pyridoxal·HCl	0.000025
Pyridoxine·HCl	0.000025
Retinol Acetate	0.00014
Riboflavin	0.00001
DL-α-Tocopherol Phosphate·Na	0.00001
Thiamine·HCl	0.00001

Other

Adenine Sulfate	0.01	
Adenosine Triphosphate·2Na	0.001	
Adenosine Monophosphate·Na	0.000238	
Cholesterol	0.0002	
Deoxyribose	0.0005	
Glucose	1.0	(5.5 mmol/L – same as blood glucose)
Glutathione (reduced)	0.00005	
Guanine·HCl	0.0003	
HEPES (synthetic buffer)		--
Hypoxanthine	0.0003	
Phenol Red·Na	0.0213	(pH indicator)
Tween 80	0.02	
Ribose	0.0005	
Thymine	0.0003	
Uracil	0.0003	
Xanthine·Na	0.000344	

References

1. Morgan, J. F., Campbell, M. E., & Morton, H. J. (1955). The nutrition of animal tissues cultivated in vitro. I. A survey of natural materials as supplements to synthetic medium 199. *Journal of the National Cancer Institute*, 16, 557–567.
2. Morgan, J. F., Morton, H. J., & Parker, R. C. (1950). Nutrition of animal cells in tissue culture; initial studies on a synthetic medium. *Proceedings of the Society for Experimental Biology and Medicine*, 73, 1–8.