

PhD-Thesis

Regulation of intracellular lipid storage and mobilization

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Anita Sahu-Osen

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Assoc.Prof. Dr. Ruth Birner-Grünberger

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1. Zusammenfassung:

Der Fettstoffwechsel hat in den letzten Jahren zunehmend an Aufmerksamkeit gewonnen, da er mit dem metabolischen Syndrom und assoziierten pathophysiologischen Komplikationen in Verbindung gebracht wurde. Das metabolische Syndrom umfasst eine Gruppe medizinischer Zustände im Zusammenhang mit Energienutzung und -speicherung. Daher ist ein tiefgreifendes Verständnis des Lipidmetabolismus und der Mutationen/Modifikationen seiner Komponenten in den Mittelpunkt des Interesses der Forscher gerückt, um Erkrankungen wie Diabetes, Adipositas und Lipidosen zu entschlüsseln. Jüngste Arbeiten auf dem Gebiet des intrazellulären Lipidmetabolismus identifizierten mehrere neue Enzyme und deren Regulatoren im Rahmen der Regulation von Speicherung, Aufspaltung und Mobilisierung von intrazellulären Neutralfetten. Der Schwerpunkt dieser Dissertation liegt hauptsächlich auf der Untersuchung der Aufspaltung von Neutralfetten (Lipolyse).

Das Fettgewebe ist jenes Organ in dem überschüssige Neutralfette in intrazellulären Lipidtröpfchen gespeichert werden. Es wurde vor allem hinsichtlich der Lipolyse untersucht, welche eine regulierte Mobilisierung von gespeicherten Triacylglyceriden (TAG) beinhaltet. Der β -adrenerge Signalweg über Proteinkinase A ist der bedeutendste und am meisten untersuchte Signalweg, der die intrazelluläre Lipolyse in Adipozyten kontrolliert. Er spricht das lipolytische Enzym Hormone sensitive lipase (HSL) und die Lipidtröpfchen-ummantelnden Proteine namens Perilipine an, welche die Aktivität von Lipasen kontrollieren können. Allerdings wurden einige wichtige Aspekte der intrazellulären Lipolyse bislang noch nicht untersucht wie zum Beispiel: I) ob PKA ähnlich wie HSL und Perilipin auch Modulatoren der Lipolyse, z.B. Comparative gene identification 58 (CGI-58), durch Phosphorylierung regulieren kann und II) ob die An-/Abwesenheit von Perilipinen Proteinfaktoren beeinflussen kann, die an der Regulation der Größe und der strukturellen Homeostase von Lipidtröpfchen beteiligt sind.

Grundsätzlich besteht diese Dissertation aus zwei unterschiedlichen, jedoch in Verbindung stehenden Teilen, die Licht auf diese beiden fehlenden Bindeglieder in der Regulation der Lipolyse werfen.

Der erste Teil der Dissertation konzentriert sich auf die Regulation von CGI-58 durch Phosphorylierung von Serin 239, einer neu identifizierten Phosphorylierungsstelle, durch Protein kinase A (PKA), wohingegen der zweite Teil sich mit Proteinfaktoren in der Regulierung der Größe von Lipidtröpfchen befasst.

CGI-58/ABHD5 und das G0/G1 switch gene 2 (G0S2) regulieren die bedeutendste TAG-Lipase Adipose triglyceride lipase (ATGL) durch Aktivierung beziehungsweise Inhibierung. Unter basalen Bedingungen verhindert CGI-58 in Adipozyten die Interaktion von ATGL mit Perilipin 1, wodurch der Zugang zu TAG unterbunden wird. Während der PKA-aktivierten Lipolyse hingegen verteilt sich CGI-58 im Zytosol und ermöglicht dadurch ATGL den Zugang zum TAG-Speicher. Dennoch ist die Regulation von CGI-58 durch PKA nach wie vor unklar. Da die Aminosäuresequenz von CGI-58 eine PKA-Konsensussequenz RKYS²³⁹ enthält, haben wir im ersten Teil dieser Dissertation die Effekte der Phosphorylierung von CGI-58 und dessen Beitrag zum Prozess der Lipolyse erforscht.

Prof. Ronald P. Kühnleins Labor hat kürzlich die Effekte zweier unterschiedlicher Perilipine auf die Größe von Lipidtröpfchen in *Drosophila melanogaster* untersucht. Sie konnten deutlich demonstrieren, dass der Verlust von Perilipin1 (LSD1) in einer Zunahme der Lipidtröpfchengröße resultiert. Zusätzlich konnten sie zeigen, dass Perilipin 2 (Plin2/LSD2) knock-out sowie ein Perilipin-freies System (Plin1/Plin2 Doppel-knock-out) eine normale Lipidtröpfchengröße aufweisen. Diese Ergebnisse motivierten uns, im zweiten Teil dieser Dissertation das Proteom von Lipidtröpfchen aus Fettkörpern von einfachen und vollständigen Perilipin-knock-out *D. melanogaster* zu untersuchen.

Zusammenfassend bieten die Resultate dieser Dissertation erstens Einsicht in die Effekte der Phosphorylierung von CGI-58 auf dessen subzelluläre Lokalisation und zweitens identifizieren sie Cyclophilin 1 (Cyp1) als neuen, an der Regulierung der Größe von Lipidtröpfchen beteiligten Proteinfaktor.

Gemeinsam erweitern sie unsere derzeitigen Informationen über die Regulation intrazellulärer Lipidspeicherung und -mobilisierung.

2. Summary:

Lipid metabolism has gained much attention in the recent years as it has been implicated with the metabolic syndrome and associated pathophysiological complications. The metabolic syndrome is a cluster of medical conditions associated with energy utilization and storage. Therefore, an in-depth understanding of lipid metabolism and the mutations/modifications of its components has become a center of attention for researchers to resolve diseases like diabetes, obesity and lipidoses. Recent work in the intracellular lipid metabolism field has identified several new enzymes and their regulators involved in the regulation of storage, breakdown and mobilization of intracellular neutral lipids. This Ph.D. thesis is mainly focused on the investigation of breakdown of neutral lipids (lipolysis).

Adipose tissue is the organ where excess neutral lipids are stored in intracellular lipid droplets. It has been studied mostly for lipolysis, which involves mobilization of stored triacylglycerides (TAG) in a regulated manner. β -adrenergic signaling pathway via protein kinase A (PKA) is the major and most studied signaling pathway which controls intracellular lipolysis in adipocytes. It targets the lipolytic enzyme hormone sensitive lipase (HSL) and lipid droplet coating protein perilipins, which can control the activity of lipases. However, there are a few important aspects of intracellular lipolysis which have not been investigated such as whether; I) PKA can also regulate modulators of lipolysis, e.g. Comparative gene identification 58 (CGI-58), by phosphorylation similar to HSL and perilipin and II) if the presence/absence of perilipins affects protein factors which are involved in the regulation of lipid droplet size and structural homeostasis.

Fundamentally, this thesis consists of two different, yet related parts which shed light on these two missing links in the regulation of intracellular lipolysis. The first part of the thesis focuses on the regulation of CGI-58 by phosphorylation on serine 239, a newly identified phosphosite, by protein kinase A (PKA) and the second part focuses on protein factors involved in lipid droplet size regulation.

CGI-58/ABHD5 and G0/G1 switch gene 2 (G0S2) regulate the major TAG-lipase Adipose triglyceride lipase (ATGL) either by activating or inhibiting it, respectively. Under basal conditions in adipocytes CGI-58 prevents ATGL interaction with perilipin1, thus preventing access to TAG. However, during PKA activated lipolysis CGI-58 disperses into the cytosol allowing ATGL to access the TAG store. Nonetheless, CGI-58 regulation by PKA is still blurred. Since CGI-58 amino acid sequence contains a PKA consensus sequence RKYS²³⁹, we explored the effect of CGI-58 phosphorylation and its contribution in the lipolytic process in part 1 of this thesis.

Prof. Ronald P. Kühnlein's laboratory has previously investigated the effect of two different perilipins on the size of lipid droplets in *Drosophila melanogaster*. They have clearly demonstrated that loss of perilipin1 (LSD1) results in a gain in lipid droplet size. Additionally, they have shown perilipin 2 (Plin2/LSD2) knock out as well as a perilipin free system (Plin1/Plin2 double knock out) show normal lipid droplet size. These findings motivated us to study the proteome present on lipid droplets from single and complete perilipin knock out *D. melanogaster* fat bodies in part 2 of this thesis.

In summary, findings from this thesis firstly provide insight into effects of CGI-58 phosphorylation on its subcellular localization and secondly identified cyclophilin 1 (Cyp1) as a novel protein factor being involved in lipid droplet size regulation. Together all this adds information to the regulation of intracellular lipid storage and mobilization that is available to date.

3. Abbreviations

ABHD5	1-acylglycerol-3-phosphate O-acyltransferase 5
ADP	adenosine diphosphate
Akh	adipokinetic hormone
AkhR	AKH receptor
AT	adipose tissue
ATGL	adipose triglyceride lipase
ATP	adenosine triphosphate
BAT	brown adipose tissue
BDSC	Bloomington <i>Drosophila</i> Stock Center
Bmm	Brummer
bp	base pair
BSA	bovine serum albumin
cAMP	3`-5`-cyclic adenosine monophosphate
Cct1	CTP:phosphocholine cytidyltransferase
cDNA	complementary DNA
CDS	Chanarin-Dorfman syndrome
CGI-58	comparative gene identification 58
DAG	diacylglycerol
Dgat1	Diacylglycerol acyltransferase 1
Dm	<i>Drosophila melanogaster</i>
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
FA	fatty acids
FB	fat body
G0S2	G0/G1 switch protein 2
GPCR	G-protein coupled receptor
HSL	hormone sensitive lipase
IBMX	3-Isobutyl-1-methylxanthine
KO	knock-out
L3	third instar larvae

LacZ	β-galactosidase
LDAP	lipid droplet-associated proteins
LDs	lipid droplets
LPA	lysophosphatidic acid
LPAAT	lysophosphatidic acid acyltransferase
M	Molar
MAG	monoacylglycerol
mdy	midway
MGL	Monoacylglycerol lipase
miRNA	micro RNA
MOI	multiplicity of infection
mRNA	messenger RNA
MS/MS	tandem mass spectrometry
NEFA	non-esterified fatty acid
NLSD	neutral lipid storage disease
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PKA	protein kinase A
Plin1	perilipin1
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNAi	RNA interference
SDS	sodium dodecyl sulfate polyacrylamide
SDS-PAGE	SDS-gel electrophoresis
siRNA	small interfering RNA
TAG	triacylglycerol
TCEP	tris (2-carboxyethyl) phosphine
TEV	tobacco etch virus
UAS	upstream activating sequence
VDRC	Vienna <i>Drosophila</i> RNAi Center
w	white

WAT	white adipose tissue
WT	wild type
y	yellow

PART-I

CGI-58/ ABHD5 is phosphorylated by protein kinase A on Ser239: control of sub-cellular localization

4. INTRODUCTION

Lipid homeostasis is critical in many disease conditions like diabetes, cardiovascular diseases, obesity and atherosclerosis, however remains poorly understood. According to the world health organization (WHO) 2013 report cardiovascular diseases were the prominent cause of deaths in 2008. Additionally WHO stated obesity as the biggest health care challenge as its incidence has doubled since 1980. Their factsheet (N°311, 2015) claimed 39% of adults were overweight in 2014 and 13% were obese from the world's population. The association of lipid overload with the metabolic syndrome combined with the epidemic proportion of lipid related diseases raises an alarm. Therefore a thorough investigation of mechanisms involved in lipid storage and expenditure becomes crucial to provide an appropriate cure for this group of diseases.

4.1 Lipid metabolism:

Lipids are a broad group of naturally-occurring hydrophobic molecules which includes triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), phospholipids, and others. Lipids fulfil many biological functions including energy storage: being important structural components of cell membranes, as transporter and signaling molecules (Bogdanov, Mileyskaya et al. 2008, Hannun and Obeid 2008, Branicky, Desjardins et al. 2010). Therefore, digestion, transport, formation, intracellular storage and mobilization of lipids are tightly controlled processes to ensure complete energy balance. The most important lipid for energy storage in mammals is TAG, which provides energy during increased energy demands such as fasting (Wang, Soni et al. 2008). In mammals, the main sites for long and short term intracellular lipid storage are lipid droplets (LDs/adiposomes) in adipose tissue and the liver respectively.

LDs are highly dynamic organelles, which play their major role in maintaining energy homeostasis and are becoming focus of attention in lipid associated patho-

physiological conditions. Structurally, LDs consist of a neutral lipid core surrounded by a phospholipid monolayer which is covered with many LD associated proteins (Walther and Farese 2012). In adipocytes lipid droplet proteomics studies have shown a surprising complexity of LD proteome which includes protein participating in cytoskeletal organization, intracellular trafficking and lipid metabolism (Schittmayer and Birner-Gruenberger 2009). Proteins involved in lipolysis which have been discovered over the years include intracellular lipases like adipose triacylglycerol lipase (ATGL) (Zimmermann, Strauss et al. 2004), hormone sensitive lipase (HSL) (Holm, Belfrage et al. 1987), monoacylglycerol lipase (MGL) and their regulators like perilipins (Fredrikson, Tornqvist et al. 1986, Goodman 2009), G0/G1 switch gene 2 (G0S2) and comparative gene identification 58 (CGI-58/ABHD-5) (Lass, Zimmermann et al. 2006, Heckmann, Zhang et al. 2013). Hydrolysis of TAG is a highly regulated process in which ATGL catalyzes the first and rate limiting step of breakdown to DAG and non-esterified fatty acid (NEFA) (Haemmerle, Lass et al. 2006). DAG is then further hydrolyzed to MAGs and NEFA by HSL. The final step of MAG hydrolysis to glycerol and NEFA is done by MGL (Fredrikson, Tornqvist et al. 1986). Activity of ATGL is further regulated by activator CGI-58 and suppressor G0S2 by unknown mechanism which seems to involve protein interaction. Additionally under G-protein coupled receptor (GPCR) stimulated condition PKA phosphorylation of perilipin and HSL leads to recruitment and translocation of a set of proteins to the LD surface. This relocation of proteins results in a vastly increased hydrolysis of stored TAG (Fig1).

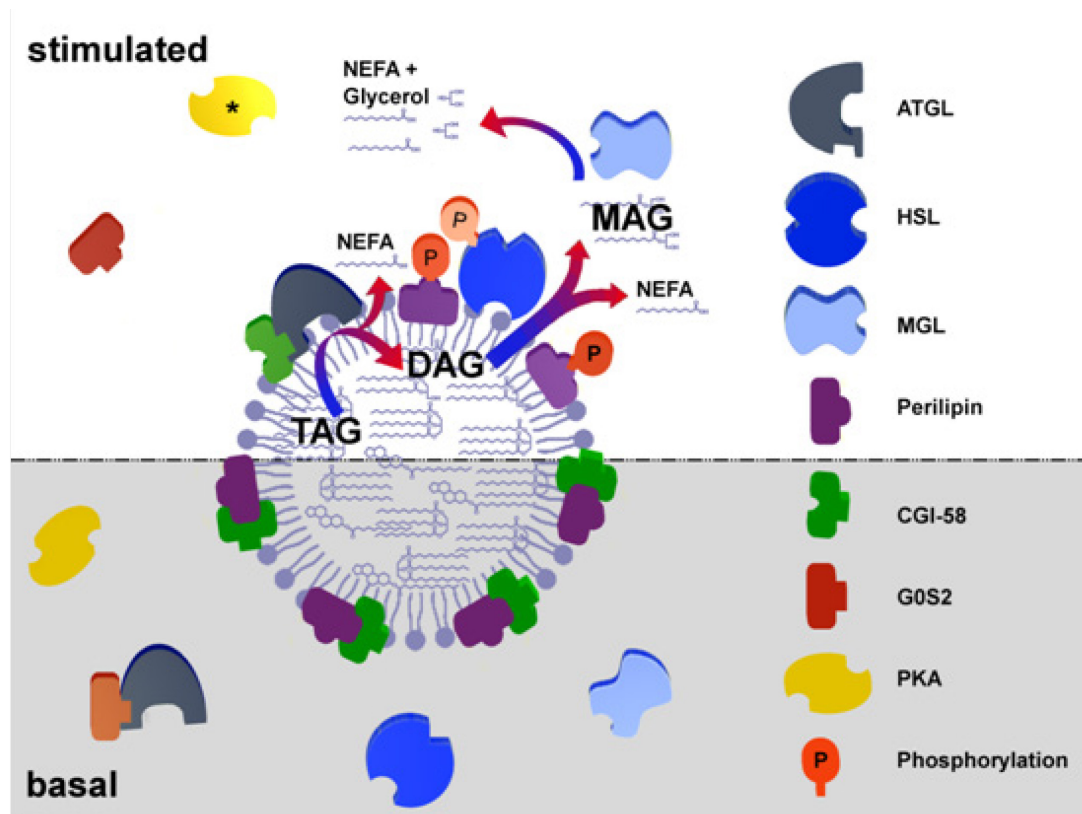


Figure 1. Triacylglycerol catabolism in adipocytes. Basal (gray field): Perilipin-1 binds comparative gene identification 58 (CGI-58) while G0/G1 switch gene 2 (G0S2) binds ATGL resulting in low basal lipolysis. Stimulated (white field): protein kinase A (PKA) gets activated (star), phosphorylates hormone sensitive lipase (HSL) and perilipin-1, which releases CGI-58. TAG: triacylglycerol; DAG: diacylglycerol; MAG: monoacylglycerol; NEFA: non-esterified fatty acid (Schittmayer and Birner-Gruenberger 2012).

4.2 Intracellular Lipases:

The chemical structure of TAG involves three fatty acid molecules bound to one molecule of glycerol; each step of hydrolysis is accompanied by the release of one free fatty acid molecule by ester bond hydrolysis. As stated above, enzymatic sequential degradation of LD TAG involves the sequential formation of DAG, then MAG and finally glycerol. The ratio of these sequential degradation products depends on the substrate specificity of the responsible lipases, which have to localize at the LD to be able to access their substrates. Three central intracellular li-

pases appear to be completing hydrolysis of TAG of LDs to NEFA and glycerol: ATGL, HSL and MGL.

4.2.1 Adipose triglyceride lipase (ATGL): The first step of lipolysis

ATGL was discovered in 2004 by several groups as a lipid droplet associated lipase (also known as desnutrin, PNPLA2, TTS2.2, and iPLA2 ζ) (Lass 2011). ATGL transcript levels in mouse adipose tissue change with respect to different metabolic states of the organism. Furthermore, in ATGL knock out mice reduced lipolysis is associated with massive fat accumulation in multiple tissues. In contrast, ATGL over-expression in mice leads to leaner animals with low TAG content and improved insulin sensitivity. Altogether, studies in various cell culture and genetically modified animal models have shown that ATGL plays a rate limiting role in both basal and stimulated lipolysis (Haemmerle, Lass et al. 2006). ATGL is highly expressed in adipose tissue, but it has been also observed in other tissues, like cardiac muscle, skeletal muscle, testis and liver. The property of ATGL to hydrolyze triacylglycerol is evolutionary preserved between humans, mice, *Drosophila*, *Saccharomyces cerevisiae* and *Arabidopsis* (Gronke, Mildner et al. 2005, Rajakumari and Daum 2010). The ATGL protein is encoded by *PNPLA2* in humans and mice, and has a molecular weight of approximately 56 kDa and shares 86% amino acid identity between mouse and human. The N-terminal half of ATGL is comprised of an α/β hydrolase fold and an overlapping patatin-like domain (Zimmermann, Strauss et al. 2004). It acts through a catalytic dyad consisting of Ser-47 present in a GXSXG motif and Asp-166 within a DXG motif (Rydel, Williams et al. 2003). Additionally, a hydrophobic C-terminal region of 45 amino acids is predicted to mediate ATGL localization to lipid droplets (Schweiger, Schoiswohl et al. 2008). Activity of ATGL has been shown to be modulated by various hormones like catecholamine and insulin (Kershaw, Hamm et al. 2006). In addition, there are effector proteins like CGI-58 which is an ATGL activator and G0S2 which represses ATGL activity. Even though much evidence supports the role of CGI-58 in ATGL activation, a defined mechanism for ATGL activation by CGI-58 remains to be clarified. Furthermore, phosphorylation of murine ATGL has been shown at S406 and S430 (Bartz, Zehmer et al. 2007) but significance of these phosphorylations in regulation of ATGL is not clear.

4.2.2 CGI-58 (ABHD5): Activator of ATGL

ATGL requires comparative gene identification 58 (CGI-58), also known as α/β hydrolase domain-containing protein 5 (ABHD5) activator to accomplish full hydrolase activity. CGI-58 belongs to an α/β hydrolase domain-containing subfamily containing 15 members to date (ABHD1-15) (Lord, Thomas et al. 2013). Most members containing an α/β hydrolase domain show enzymatic functions due the presence of a catalytic triad (Ollis, Cheah et al. 1992). Interestingly, the acyltransferase specific HX4D motif, present in most ABHD variants, is conserved in CGI-58 as well (Oberer, Boeszoermenyi et al. 2011). However, the putative active site serine of the shared α/β hydrolase fold is replaced by an asparagine (N-153) in mammalian CGI-58. Predictably, no esterase or amidase activity was ever reported for mammalian CGI-58, while other homologous mammalian enzymes such as ABHD4 retain their active site serine and show hydrolytic activity (Simon and Cravatt 2006, Lee, Simon et al. 2015). Recently, CGI-58 has been shown to facilitate the use of lipid intermediates derived from the lipolysis of stored TAG for the assembly of lipoproteins (Caviglia, Sparks et al. 2009, Brown, Betters et al. 2010). Although CGI-58 is abundantly expressed in the liver the level of ATGL is very low. Thus, further studies are required to determine whether the function of CGI-58 in lipoprotein assembly in hepatocytes is related to its putative function as a cofactor of ATGL (Lass, Zimmermann et al. 2006) or another lipase or to its reported acyltransferase activity. CGI-58 has been suggested to possess lysophosphatidic acid acyltransferase (LPAAT) activity (Ghosh, Ramakrishnan et al. 2008, Montero-Moran, Caviglia et al. 2010) and to produce phosphatidic acid (PA), which is a well know signaling molecule. However, this described potential LPAAT activity remains controversial since the reported activities are very low and may be an artifact. Additionally, CGI-58 has recently been implicated in hepatic TH1 cytokine signaling (Lord, Betters et al. 2012), which may explain the retained insulin sensitivity despite hepatic steatosis in CGI-58 knock down mice (Brown, Betters et al. 2010). It is unclear, however, whether the role of CGI-58 as a coactivator of ATGL requires acyltransferase activity, or whether CGI-58 has a separate function mediated by a protein–protein interaction between CGI-58 and lipase.

CGI-58 gene mutations have been associated with a human disease named Chanarin-Dorfman syndrome (CDS), a rare autosomal recessive disease in which excess TAG accumulates in multiple human tissues (Lefevre, Jobard et al. 2001). CDS is also referred to as NLSDi (Neutral Lipid Storage Disease with ichthyosis) due to the presence of skin disorder ichthyosis unlike NLSDm (ATGL variant of NLSD with myopathy). The difference between the two phenotypes is even more pronounced in mouse models. While ATGL knock out mice have a defective cold adaption system and accumulate excessive amounts of TAG in their cardiac tissue which leads to premature death, CGI-58 knock out mice die only a few hours after birth due to a severe impairment of the skin barrier function (Fischer, Lefevre et al. 2007, Radner, Streith et al. 2010). These data indicate an ATGL independent function of CGI-58, however its ATGL independent function remains in debate. Some studies have proposed the possibility of CGI-58 activating enzymes other than ATGL. Others have reported its *in vitro* acyl CoA-dependent LPAAT activity. Thus the ATGL independent function of CGI-58 remains to be defined and explored for its physiological relevance.

Despite playing an important role in lipid metabolism and having an indication towards additional important functions, little is known about the regulation of CGI-58. While phosphorylation of HSL, PLIN1 and more recently ATGL has also been described on various sometimes controversial sites by different known and unknown kinases, no information on phosphorylation of CGI-58 has been documented. This part of the thesis mainly focuses on demonstrating that CGI-58 is a substrate of PKA and its phosphorylation is important for subcellular trafficking of CGI-58.

4.2.3 G0S2: Selective inhibitor of ATGL:

G0/G1 switch protein 2 (G0S2) has been newly recognized as a selective negative regulator of ATGL. This 103 amino acid long protein was originally discovered during the cell cycle switch from G0 to G1 phase in blood mononuclear cells, and therefore named G0S2. The expression pattern of G0S2 shows highest levels in adipose tissue and is up-regulated in differentiating adipocytes. Additionally, its

expression in adipocytes is induced in response to lipolysis stimulation. G0S2 overexpression leads to increased size and number of lipid droplets (Yang, Lu et al. 2010). G0S2 has been shown to play multiple functions in cellular processes; apart from lipolysis and cell cycle, for example it has been documented to regulate apoptosis by interacting with Bcl-2, an anti-apoptotic factor (Welch, Santra et al. 2009). Additionally, the activity of yeast ATGL orthologue TGL4 has been shown to be changed during the entry into the S phase of the cell cycle linking lipolysis with the cell cycle. (Kurat, Wolinski et al. 2009). In *in-vitro* lipolysis assays, G0S2 inhibits ATGL by directly interacting with its N-terminal patatin domain. This interaction can be restricted/inhibited by deleting amino acids 27-42 of ATGL (Yang, Lu et al. 2010). However, G0S2 interaction with ATGL does not compete with CGI-58, which also interacts with a similar region of ATGL (Schweiger, Schoiswohl et al. 2008). Future studies are required to investigate CGI-58 and G0S2 interaction with ATGL and its roles in different (patho-)physiological conditions. Additionally, it has been speculated that G0S2 has potential phosphorylation sites targeted by protein kinase C and casein kinase II (Russell and Forsdyke 1991). However, little is known about the regulatory impact of the phosphorylation and whether other kinases are also involved in this process.

4.2.4 Hormone sensitive lipase (HSL): Main DAG hydrolase

Before the discovery of ATGL, HSL was thought to be the rate limiting enzyme in lipolysis. It was the first enzyme discovered in WAT to facilitate stimulated lipolysis, i.e. hormone induced catabolism of lipids (Vaughan, Berger et al. 1964). HSL has emerged as a major enzyme responsible for intracellular DAG hydrolysis, but it also hydrolyzes TAG, MAG, cholesteryl esters and retinyl esters (Fredrikson, Tornqvist et al. 1986, Yeaman, Smith et al. 1994). This substrate specificity of HSL towards DAG became clearer when HSL-deficient mice showed excess accumulation of DAG in multiple tissues, but no TAG accumulation (Haemmerle, Zimmermann et al. 2002). HSL is mainly expressed in adipose tissue, but is also present at low levels in many other tissues including heart, skeletal muscle, macrophages, testis and pancreatic β -cells (Kraemer, Patel et al. 1993). Structurally HSL possesses three domains: the N-terminal variable domain involved in lipid

binding and protein–protein interaction, a C-terminal catalytic domain containing the catalytic triad, and a regulatory domain containing important regulatory phosphorylation sites (Yeaman 2004). The general structure and function of HSL is conserved between many mammalian species. The amino acid sequence of human and rat HSL is 83% identical and rat and mouse share even higher (94%) amino acid sequence identity. HSL mediated lipolysis is strictly controlled by lipolytic (e.g. catecholamine, glucagon) (Stralfors and Belfrage 1983) and antilipolytic (e.g. insulin) hormones. The regulation of HSL activity in adipose tissue is controlled by two major mechanisms: first phosphorylation by PKA and secondly by interacting with secondary proteins, which affect its subcellular localization. The molecular mechanism to activate HSL by PKA phosphorylation is well established and explained in much detail in the following PKA section of this thesis. Involvement of other kinases like glycogen synthase kinase (GSK), AMP-dependent protein kinase (AMPK) and Ca²⁺/calmodulin-dependent protein kinase II (CaMK-II) in modulating HSL activity by phosphorylation is well known (Garton, Campbell et al. 1989, Watt, Steinberg et al. 2004).

4.2.5 Monoglyceride lipase (MGL): Final step of lipolysis

The last step of intracellular triacylglycerol hydrolysis is the breakdown of MAG by MGL. In absence of MGL, MAG accumulate in adipose and non-adipose tissue (Tornqvist and Belfrage 1976). MGL is ubiquitously expressed with highest expression in adipose tissue (Karlsson, Contreras et al. 1997). The human MGL protein is built of 313 amino acid residues with a molecular weight of 34kD. Recently, the 3D structure of human MGL was solved by X-ray crystallography (Bertrand, Auge et al. 2010, Labar, Bauvois et al. 2010). MGL is a member of the α/β hydrolase family and contains the classical GX SXG consensus motif consisting of Ser-122, Asp-239 and His-269. Apart from taking care of the last step of intracellular TAG breakdown, MGL plays a critical role in several other physiological processes like pain nociception through hydrolysis of the endocannabinoid 2-arachidonoylglycerol (2-AG) (Schlosburg, Blankman et al. 2010). The generation of a MGL-deficient mouse model (Schlosburg, Blankman et al. 2010) has under-

lined the role of MGL in 2-AG breakdown. However, MGL regulation in adipose tissue remains still to be investigated.

4.3 Phosphorylation, Kinases and lipid metabolism

Most of the proteins that are translated from mRNA undergo biochemical modifications before becoming functional (Nesterchuk, Sergiev et al. 2011, Beltrao, Albanese et al. 2012). These modifications on amino acids extend the range of functional diversity of a protein by attaching biochemically functional small groups such as acetate, phosphate, various lipids and carbohydrates to proteins. This changes the chemical nature of an amino acid or leads to conformational changes of the protein. These modifications are collectively known as post-translational modifications (PTMs). These PTMs can be either permanent or reversible and play a crucial role in generating protein heterogeneity to utilizing the same protein for different cellular functions in different subcellular locations and cell types. Most therapeutic proteins show one or a combination of two or more PTMs (Walsh 2010); therefore identifying and understanding PTMs is critical in the study of cell biology and disease treatment and prevention.

Phosphorylation is one of the most frequently occurring PTMs (Khoury, Baliban et al. 2011), which involves the addition of a phosphate group to certain amino acids by protein kinase enzymes. The addition of a phosphate group modifies the function, activity and localization of proteins. The major sites of protein phosphorylation are serine, threonine and tyrosine (Khoury, Baliban et al. 2011). Phosphorylation processes are especially involved in the regulation of multiple cellular processes including energy balance (Johnson 2009). Therefore the identification and characterization of new phosphorylation sites of regulatory proteins is critical for understanding various signaling events and interactions.

Protein kinases belong to a large family of phosphotransferases and are highly dynamic molecular key factors for regulating cellular behavior (Taylor, Keshwani et al. 2012) under different conditions. To date PKA, PKC and AMPK are the most

studied kinases involved in energy/lipid metabolism, yet some of their substrates and phosphorylation sites remain to be revealed.

4.3.1 Protein Kinase-A (PKA) and β -adrenergic signaling

PKA is one of the best described members of the kinase family as recently reviewed (Taylor, Ilouz et al. 2012). It is an evolutionarily conserved serine threonine kinase, which contains catalytic and regulatory subunits. The unique characteristic of PKA, also known as the cyclic AMP-dependent protein kinase, is that its activity is regulated by fluctuating levels of cyclic AMP (cAMP) within cells. This kinase thus functions as the final effector for a variety of hormones that work through cyclic AMP signaling (Kirschner, Yin et al. 2009). During fasting, catecholamine hormones are the most important lipolytic stimuli and they mediate their effects by activating beta-adrenergic receptors (GPCR) which activates the enzyme adenylate cyclase and raises cAMP levels, consequently causing activation of PKA. This activated PKA is capable of phosphorylating rat HSL at residues Ser563, Ser659, and Ser660 (corresponding to murine Ser557, Ser650, Ser651), resulting in significant increases in HSL activity (Anthonsen, Ronnstrand et al. 1998) and translocation of HSL from the cytosol to the LD (Brasaemle, Levin et al. 2000). At the LD, phosphorylated HSL interacts with perilipin1. Perilipin 1 is another major component of β -adrenergic signaling which is present on the lipid droplet surface and contains six consensus sites for serine phosphorylation by PKA. Although phosphorylation at all sites has not been well characterized, it was shown that phosphorylation in at least three of these sites (Ser-81, Ser-222, Ser-276 of murine PLIN1) is required to facilitate HSL access to lipid substrates on the lipid droplet surface (Brasaemle, Subramanian et al. 2009). Phosphorylation of serine 492 and serine 517 of murine perilipin 1 (PLIN1) is additionally required for the release of CGI-58 from PLIN1, allowing CGI-58 to directly interact with ATGL and initiating hydrolysis of TAG (Granneman, Moore et al. 2009).

Apart from above mentioned phosphorylation sites additional sites were identified in a quantitative phosphoproteomic study of beta-adrenergic stimulated murine white adipose tissue, one on them is on HSL serine 559 and two are on PLIN1 at

serine 410 and 460 (Kanshin, Wang et al. 2009). Additionally, murine HSL serine 559 has also been described to be a possible substrate for AMP-activated protein kinase (AMPK) (Daval, Diot-Dupuy et al. 2005). Moreover, serine 196 of isoform1 of human HSL was identified in a large scale phosphoproteomic screen. Additionally, ATGL also has been proposed to become phosphorylated by AMPK at serine 406 thereby increasing its TAG hydrolase activity (Ahmadian, Abbott et al. 2011). These continued identifications of novel phosphorylation sites on important players of β -adrenergic signaling in intracellular lipid metabolism indicate that more phosphorylation sites remain to be discovered and even more to be functionally characterized. Whether regulatory proteins of ATGL (CGI-58 and G0S2) are phosphorylated was unknown. Thus an important aim is to identify novel target phosphorylation sites on CGI-58 and G0S2 by PKA and other kinases involved in lipid metabolism followed by functionally studying those newly identified sites. Part 1 of my thesis focuses on increasing our understanding of whether and if yes how CGI-58 is regulated by phosphorylation.

5. HYPOTHESIS AND AIM

Lipolysis is regulated by phosphorylation. The aim of a major part of this thesis is the identification and functional elucidation of novel phosphorylation sites of protein factors controlling intracellular lipid storage and mobilization (Fig. 2).

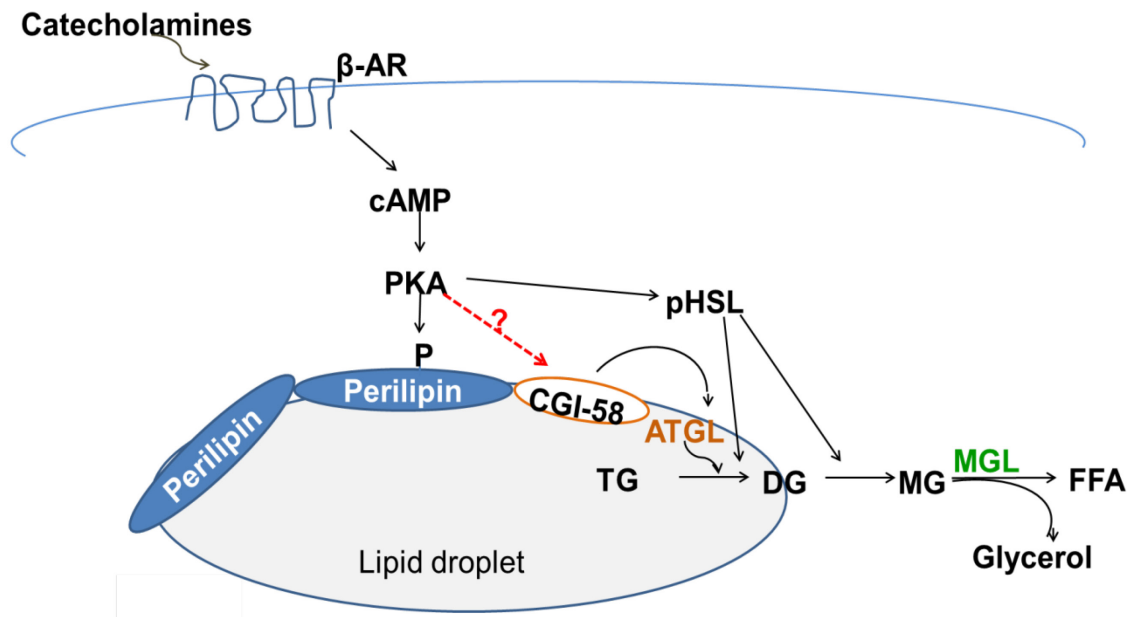


Figure 2. Catecholamine-stimulated lipolysis: occurs by activating adenylate cyclase and raising cAMP levels, thereby increasing protein kinase A (PKA) activity. This results in phosphorylation and modulated activity of several key lipolytic proteins. Adipose triglyceride lipase (ATGL) is the primary lipase for the initial step in TAG hydrolysis, and ATGL activity is increased during stimulated lipolysis. CGI-58 is a positive modulator of ATGL but the mechanism by which it regulates ATGL is not well defined.

Hence, we hypothesized that:

- 1) CGI-58 is a PKA target.
- 2) Phosphorylation of CGI-58 affects lipid mobilization.

6. MATERIALS AND METHODS:

(Methods are mainly reused from Sahu-Osen, Montero-Moran et al. (Sahu-Osen, Montero-Moran et al. 2015) with some modifications. JLR permits authors to reuse the content of their paper for the authors' PhD theses.)

6.1 Expression and purification of recombinant mouse CGI-58, S239A, S240A, and S239A/S240A mutants in pET28a (+) Vector:

Preparation of mouse CGI-58 and its mutant cDNA subcloned into the pET-28A(+) bacterial expression vector and purifications were done in Prof. Dawn L. Braasemle's laboratory (Rutgers, USA) and have been described previously (Montero-Moran, Caviglia et al. 2010); recombinant CGI-58 included 2 tandem amino-terminal 6-histidine fusion sequences. Briefly, a mouse CGI-58 cDNA was amplified using two step PCR with PfuUltra polymerase, followed by insertion into pET28a(+) bacterial expression vector (Novagen, EMD Chemicals, Inc., Gibbstown, NJ) into the NdeI and SalI restriction sites. Mutant variants of CGI-58 cDNA (S239A, S240A and S239A/S240A) were prepared in a similar way by using the template of 12-His-CGI-58 cDNA and following set of primers: For the S239A variant of CGI-58 forward (5' AAGTACGCGTCTATGTTTGAGATGACAC-3'), and reverse (5'-ACATAGACGCGTACTTCCGCT-TGAAATCAGG-3') primers were used. Mutagenic oligonucleotide primers for the S240A mutated variant of CGI-58 were: forward (5'-AAGTACTCCGCAATGTTTGAAGATGACACGG-3') and reverse (5'-AAACATGCGGAGTACTTCCG-CTTGAATCAGG-3') primers. The cDNA for the S239A/SS240A double mutated variant of CGI-58 was similarly prepared and cloned in to pET28a (+) vector. All mutations were confirmed by DNA sequencing before expressing them into BL21 (DE3) cells. Recombinant 12-His-tagged wild type (WT) and mutated (S239A, S240A, and S239A/S240A) variants of CGI-58 were expressed and purified from *E. coli* cell extracts with nickel-nitrilotriacetic acid agarose, as described (Montero-Moran, Caviglia et al. 2010). In short, BL21 cells expressing WT or mutant variants of CGI-58 were induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside. After 5 hours' induction at 37°C bacterial cells were

lysed in lysis buffer containing 300 mM sodium chloride, 50 mM Tris-HCL, pH 8.0, 20 mM imidazole, with or without protease inhibitors including 10 mg/l leupeptin, 500 μ M benzamidine, and 100 μ M 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride. Cleared supernatants were achieved firstly by centrifugation for cell debris removal, and secondly by 0.2% polyethyleneimine treatment to precipitate nucleic acid. The resulting supernatant was loaded onto a Ni²⁺-NTA-agarose column and eluted with imidazole. All the steps of protein purifications were performed at 4°C; final preparations were stored at -20°C.

6.2 Expression and purification of recombinant mouse CGI-58, S239D/E mutant and mouse ATGL in His-pSumo Vector:

Mouse CGI-58 and ATGL were cloned into His-pSumo vector (kindly provided by Dr. Christopher D. Lima, Sloan Kettering Institute), expressed and purified in Prof. Monika Oberer's laboratory (Karl-Franzens University, Graz, Austria). Sequences containing complete coding region of mCGI-58 and ATGL cDNAs were amplified as described earlier (Gruber, Cornaciu et al. 2010). For mouse CGI-58, it was first cloned into the pProEX HTb vector (Addgene, Cambridge, MA), from which it was further subcloned into the His-pSumo vector. To do so cDNAs were amplified with PCR using either the Phusion Polymerase kit (New England Biolabs) or the Fail-Safe™ PCR system (Epicenter Biotechnologies, Madison, WI) with following primers, forward (5'-CGAAG-CAGAGAGCTCGAAAACCTGTATTTTCAGG-3') and reverse (5'-GGAACCTC-GAGTCATC-AGTCTACTGTGTGGC-3'), which included endonuclease cleavage sites for subcloning and a 5' tobacco etch virus cleavage site from the pProEX HTb vector. The S239D and S239E variants of CGI-58 were produced by QuickChange® site-directed mutagenesis kit (Agilent technologies, CA, USA) as per manufacturer's protocol with forward (5'-CCTGATTTCAAGCGGAAGTACGACTCTATGTTTGAAGATGACACG-3') and reverse (5'-CGTGTCATCTTCAAACATAGAGTCGTA CTTC-CGCTTGAAATCAGG-3') mutagenic primers for S239D CGI-58 and forward (5'-CCTGATTTCAAGCGGAAGTACGAGTCTATGTTTGAAGATGACACG-3') and reverse (5'-CGTGTCATCTTCAAACATAGACTCGTA CTTC CGCTTGAAATCAGG-3') mutagenic primers for S239E. In short, 125 ng of each primer, 1x reaction buffer,

0.2 mM dNTPs, 3 μ L of quicksolution and 2.5 units of *pfu* ultra DNA polymerase containing site directed mutagenesis PCR reactions were set. The amplified products were treated with DpnI enzyme to digest the original methylated template DNA. Then the remaining undigested product was transformed into XL-10 gold *E. Coli* cells.

The mouse ATGL cDNA encoding amino acid 1-288 subcloning into His-pSumo included forward (5'-GCTATGGATCCATGTTCCCGAGGG-3') and the reverse (5'-GGCGCTCGATCATTTCGAACTGCGGGTGGCTCCAATCCTCCTCTCCAGC-3') oligonucleotide primers containing endonuclease cleavage sites and a stop codon following the nucleotide sequence after D288, followed by the sequence encoding a Strep-tag® (IBA, Göttingen, Germany). All the sequences, of wild type and mutants, were confirmed by DNA sequencing (Integrated DNA Technologies, Coralville, IA and Agowa, Berlin, Germany).

BL21 (DE3) cells containing His-pSumo-CGI-58 and its variants, His-pSumo-ATGL 1-288 and the His-pSumo vector encoding 6-His-smt proteins were induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Induced His-pSumo-CGI-58 cells were grown for 9-12 hours, while His-pSumo-ATGL 1-288 and His-pSumo cells were grown for 4 hours at 30°C. Expression of the proteins was confirmed with SDS-PAGE.

Recombinant His-sumo tagged CGI-58 was purified by lysing *E. coli* in to buffer containing 20 mM Tris-HCl, 500 mM NaCl, 30 mM imidazole, 0.1% NP-40, 3.5 mM β -mercaptoethanol, pH 7.8, supplemented with protease inhibitor cocktail (Complete, EDTA-free Tabs, Roche Diagnostics, IN, USA), 750 U benzonase nuclease HC (Novagen/EMD Millipore, San Diego, CA, USA) and 1 mg/ml lysozyme with probe sonication. The lysed cells were centrifuged at 30,000 x g for 40 min. The clarified supernatant was further purified with affinity chromatography by using a 5 ml His-Trap FF column (GE Healthcare) to obtain recombinant CGI-58. The column was extensively washed with lysis buffer. Then the elution was done in buffer containing 20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, 10% glycerol, 3.5 mM β -mercaptoethanol, pH 7.8 with a linear gradient. Tobacco etch virus protease cleavage was performed at room temperature for 4 hours. The resulting cleaved CGI-58 was further purified on a Superdex 200 (Sigma-Aldrich) column equilibrated in buffer composed of 20 mM Tris-HCl, 300 mM NaCl, 1 mM dithio-

threitol (DTT), 1 mM EDTA pH 7.8. Remaining 6-His-smt was removed by reverse affinity chromatography with a 5 ml His-Trap FF column. Final purified recombinant CGI-58 was then dialyzed into the PKA assay buffer.

Similar to CGI-58 recombinant ATGL-1-288 and His-smt protein were purified. *E. coli* cells expressing recombinant constructs were lysed with sonication in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM benzamidine, 1 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.8) supplemented with 0.1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail. All soluble proteins in the lysates after clearing them by centrifugation, were loaded onto 1 ml His-Trap FF columns, which were washed with the lysis buffer. Elutions of recombinant proteins were done with 12 column volumes (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 500 mM imidazole, 1 mM benzamidine, 1 mM TCEP, pH 7.8). Afterwards, ATGL 1-288 was dialyzed into buffer-6 (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM TCEP, pH 7.8) and used directly in the TAG hydrolase activity assay.

6.3 Phosphorylation of recombinant mouse CGI-58 and synthetic peptides with PKA:

CGI-58 peptides of 10 amino acids including serine 239 RKYSS and its mutant variants RKYSA, RKYAS, and RKYAA and recombinant CGI-58 and its mutant proteins were tested for phosphorylation by *in-vitro* PKA kinase assay, which is based on the transfer of radioactive phosphate group from ATP to a substrate by utilizing a kinase.. For kinase assays, either purified peptide or recombinant protein was incubated with kinase assay buffer containing 10 mM MgCl₂, 60 mM DTT, 50 mM Tris-HCl, pH 8.0, 50 μM [³²P]ATP (~230,000-470,000 cpm/nmol) with varying concentration of PKA catalytic subunit (Promega GmbH, Mannheim, Germany) for 10 min at 30°C. Kinase reactions were stopped by adding Laemmli's sample buffer followed by SDS-PAGE to separate radiolabeled protein from radioactive ATP, and autoradiography was performed to detect the radioactive phosphate incorporation into its substrate. For some experiments kinase assay reactions were spotted on Whatman P81 phosphocellulose filter papers (Upstate cell signaling solution, NY,USA), which were extensively washed with 75mM phos-

phoric acid and dried for liquid scintillation counting. Additionally, for mass spectrometry analysis, the kinase assay reactions were performed with nonradioactive ATP. All phosphorylation reactions were performed in triplicate. A unit of PKA was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min.

6.4 Phosphoamino acid analysis:

A PVDF membrane containing ^{32}P labelled recombinant CGI-58 product of kinase reaction was subjected to acid hydrolysis with 6 N HCl at 100°C for 4 hours (Yang and Carman 1996). The hydrolysates were dried under vacuum and applied to cellulose TLC plates (EM science) with standard phosphoamino acids, phosphoserine, phosphothreonine, and phosphotyrosine. Amino acids were separated by 2-dimensional TLC using formic acid: acetic acid: water (50:156:1794, v/v) in the first dimension and acetic acid: pyridine: water (100:10:1890, v/v) in the second dimension (Boyle, van der Geer et al. 1991). Following the separation of molecules, the TLC plates were dried and subjected to phosphorimaging analysis with a Molecular Dynamics Storm Phosphorimager. Standard phosphoamino acids were visualized by spraying the plate with 0.25% ninhydrin in acetone.

6.5 Mass Spectrometry:

Identification of CGI-58 serine 239 was received from liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Purified recombinant CGI-58 protein was phosphorylated *in vitro* by incubation with PKA and non-radioactive ATP as explained above in 6.3. First protein was subjected to reduction and alkylation with 5 mM DTT and 20 mM iodoacetamide (IAA) solution respectively. Secondly, enzymatic digestion with AspN (Sigma-Aldrich, Saint Louis, MO, USA), modified trypsin (Promega GmbH, Mannheim, Germany) or chymotrypsin (Roche, Indianapolis, IN, USA) were carried out followed by acidifying the digests with 0.5% trifluoroacetic acid. Separation of 0.5 µg CGI-58 digest was performed on a nano-HPLC (Dionex Ultimate 3000) equipped with a µ-precolumn (C18, 5 µm, 100 Å, 5 x 0.3 mm) and an Acclaim PepMap RSLC nanocolumn (C18, 2 µm, 100 Å,

150 x 0.075 mm) (all Thermo Fisher Scientific, Vienna, Austria). Samples were concentrated on the enrichment column for 2 min at a flow rate of 20 μ l/min, with 0.5 % trifluoroacetic acid as an isocratic solvent. Separation was carried out on the nanocolumn at a flow rate of 300 nl/min using the following gradients, where solvent A was 0.3% formic acid in water and solvent B was a mixture of 80% acetonitrile in water containing 0.3% formic acid: 0-2 min 4% B, 2-35 min 4-28% B, 35-47 min 28-50% B, 47-48 min 50-95% B, 48-58 min 95% B, 58-58,1 min 95-4% B, 58,1-70 min 4% B; or 0-2 min 4% B, 2-180 min 4-28% B, 180-255 min 28-50% B, 255-260 min 50-95% B, 260-279 min 95% B, 279-280 min 95-4% B, 280-300 min 4% B. Ionization of the sample was done in the nanospray source (NSI-1 Dynamic nanospray probe with the NSI-1 base) equipped with stainless steel emitters (ES528, Thermo Fisher Scientific, Vienna, Austria) and analyzed in a Thermo Orbitrap Velos Pro™ mass spectrometer in positive ion mode by altering full scan MS (m/z 400 to 2000) in the ion cyclotron and MS/MS by high energy collision induced dissociation (HCD) of the 10 most intense peaks (MS^1 , $R=60,000$ and MS^2 , $R=7500$).

The obtained LC-MS/MS data were evaluated by conducting a search using mammalian SwissProt public database (downloaded on July 13, 2012) by using Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) and Mascot 2.4 (MatrixScience, London, UK) search softwares. During the search carbamidomethylation on cysteines was kept as a fixed modification due to alkylation of protein in the first few steps of sample preparation. Oxidation on methionine and phosphorylation on serine or threonine was entered as a dynamic modification to identify possible phosphorylation sites on CGI-58. A maximum false discovery rate of 5% using decoy database search, a Mascot ion score cut off of 30 and a minimum of 2 identified peptides were chosen as identification criteria. For identified peptides, search criteria also included a precursor mass tolerance of 10 ppm and a precursor mass error tolerance of 0.7 Da.

For detection of *in vivo* phosphorylation of CGI-58, white adipose tissue (WAT) was treated with IBMX, 500 μ M and isoproterenol, 10 μ M in DMEM medium with 2% BSA at 37°C for 30 min. Tissues were homogenized and the proteins from lysates were precipitated by using 50% TCA. Precipitated proteins were solubilized, alkylated, reduced and digested with AspN enzyme. Resultant AspN peptides were used in a targeted approach to detect the defined m/z and retention

time. These defined m/z and retention time values were obtained from a custom made synthetic phosphopeptide (DFKRKYSpSMFE) as standard. For detection of correct retention time of the above mentioned peptide, catecholamine treated WAT sample was spiked with custom made synthetic CGI-58 phosphopeptide (DFKRKYSpSMFE) which is the predicted product of CGI-58 AspN digestion. Following m/z were targeted: 1517.63 $[M+H]^+$, 759.32 $[M+H]^{2+}$ and 506.55 $[M+H]^{3+}$ for phosphorylated AspN peptide. For unphosphorylated AspN putative peptide 1437.67 $[M+H]^+$, 719.33 $[M+H]^{2+}$ and 497.89 $[M+H]^{3+}$ were targeted. The normalized collision energy (NCE) was set to 30 for top10 HCD.

6.6 Measurement of CGI-58 co-activation of ATGL in vitro:

To determine the effect of CGI-58 phosphorylation on ATGL lipase activity, non-radioactively phosphorylated and unphosphorylated recombinant CGI-58 proteins were incubated with Sf9 cell lysate expressing recombinant mouse ATGL (McMahon, Dinh et al. 2014). 40 μ g protein of Sf9 cell lysate in a volume of 100 μ L were mixed with 1 μ g of phosphorylated and unphosphorylated recombinant CGI-58, respectively. This reaction was further mixed with 100 μ L of a radioactive triacylglycerol substrate having 330 μ M [9, 10- ^{3} H] triolein emulsified with phospholipids, 145 μ M PC-PI (3:1); triolein is a commonly used substrate to determine TAG hydrolase activity. Reactions were then incubated in a 37°C water bath for 60 min and stopped by adding methanol-chloroform-heptane [10:9:7 (v/v/v)] and 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After centrifugation at 800 x g for 20 min free fatty acids were quantified by scintillation counting in the extracted upper phase (Lass, Zimmermann et al. 2006).

In additional experiments, recombinant CGI-58, CGI-58-S239D and CGI-58-S239E were expressed in *E. coli*, followed by the disruption of the cells by sonication and the removal of cell debris by centrifugation for 10 min at 2700 x g. Protein concentration of the extracts were determined by Bradford assay. 26 μ g of the supernatants with variants of recombinant CGI-58 were used in TAG hydrolase assays with 8 μ g of partially purified recombinant ATGL 1-288 as described (Schweiger, Eichmann et al. 2014).

6.7 Generation of Recombinant Adenovirus:

Adenovirus provides a versatile tool for a very high expression of a gene; most of the adenoviruses used in this thesis were generated as per the manufacturer's protocol supplied with AdEasy XL Adenoviral vector systems (Agilent technologies). Adenoviral expression vectors driving the expression of mouse CGI-58 and β -galactosidase were described previously (Montero-Moran, Caviglia et al. 2010). For CGI-58-S239A/S240A, mutant variant of CGI-58 and perilipin 1A or a mutated perilipin 1A, lacking 6 serine residues (five mutated to alanine, one to glutamate) which are putative PKA target sites, adenovirus generation was done by inserting respective cDNAs in to the pShuttle vector. The mutated perilipin 1A cDNA encoded alanine substitutions for serine residues at positions 81, 222, 276, 433, and 492 to prevent phosphorylation by PKA, and a glutamate substitution for serine 517 to permit targeting of the mutated perilipin 1A to lipid droplets (Marcinkiewicz, Gauthier et al. 2006). Resulting constructs were linearized with PmeI and transformed into BJ5183 *E.coli* cells for recombination with adenoviral elements. Screening of clones was done after digesting multiple clones with PacI enzyme then the positive clones were transformed in to XL-10-Gold cells. Assembly of virions was done in cultured AD-239 human embryonic kidney cells which were further passaged to 3rd-4th generation to increase the multiplicity of infection (MOI). At every passage titration of multiplicity of infection (MOI) was done by infecting AD293 cells for 4-5 days. Finally crude adenovirus preparations were purified over cesium chloride gradients.

6.8 Cell Culture and Adenoviral Transduction:

Normal human skin fibroblasts (WS1) were obtained from American Type Culture Collection (Manassas, VA); human skin fibroblasts from an individual with neutral lipid storage disorder (NLSD) were generously provided by Dr. Rosalind A. Coleman (University of North Carolina, Chapel Hill, NC). WS1 and NLSD fibroblasts were cultured in MEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 10 mM sodium pyruvate,

and 10 units/mL penicillin and 100 µg streptomycin at 37°C in 5% CO₂. NLSF fibroblasts were transduced with purified adenoviral preparations for expression of mouse CGI-58 (WT, or S239A/S240A mutated CGI-58) or β-galactosidase; cells were collected at various times following transduction for the determination of TAG levels. For collection cells were washed with PBS and collected by trypsinization followed by centrifugation at 800 x g for 10 min.

Resulting cell pellets were suspended in 300 µL hypotonic lysis buffer (20 mM Tris pH7.4, 1 mM EDTA, 10 mM sodium fluoride and 1x protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) after incubation on ice for 10 min lysis was completed with sonication. Expression of CGI-58 was normalized between samples using densitometry of immunoblots which were probed with CGI-58 antibody.

For immunofluorescence microscopy, Cos-7 cells or NIH3T3-CARΔ fibroblasts cells were used. NIH3T3CARΔ fibroblasts are NIH3T3 fibroblasts stably expressing a truncated version of the Coxsackie and adenovirus receptor (CARΔ) lacking the cytoplasmic signaling domain (Orlicky, DeGregori et al. 2001) to increase the uptake of adenoviral vectors. Cells were transduced with purified adenoviruses for the expression of either WT mouse perilipin 1A or the mutated variant of mouse perilipin 1A (Plin1Aall6) that lacks 6 serine residues in PKA consensus sequences. At the same time, the cells were also transduced with either WT CGI-58 or S239/S240A CGI-58 and incubated for 48 hours. Cos-7 cells were cultured in DMEM supplemented with 10% FBS, 10 units/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. For the NIH3T3CARΔ fibroblasts 800 µg/ml G418 was supplemented to the same growth medium to maintain selection of cells expressing CARΔ.

6.9 Immunofluorescence Microscopy:

After 24 hours of adenoviral transduction cells expressing perilipin 1A or perilipin 1A-all6 and CGI-58-WT or CGI-58-S239A/S240A were transferred to a glass surface. 36 hours after transduction, cell were lipid loaded with 200 µM oleic acid complexed to fatty acid-free BSA at a 4:1 molar ratio to increase the synthesis and storage of TAG in lipid droplets. After 48 hours of transduction cells were treated in

DMEM complemented with 1% fatty acid-free BSA for 30 minutes with 10 μ M forskolin and 0.5 mM IBMX to activate β -adrenergic signaling for stimulated conditions or with mock (DMSO) for basal conditions. Cells were washed with 37°C warmed PBS followed by incubating with 4% formaldehyde in PBS at room temperature for 10-15 minutes. Further, multiple steps of washing with 500 μ L PBS for 10 min at room temperature were performed in order to remove the traces of formaldehyde. Cells were probed overnight at 4°C with goat polyclonal antisera raised against an amino-terminal peptide of perilipin 1A (kindly donated by Dr. Constantine Londos, formerly of National Institutes of Health, Bethesda, MD, deceased) and rabbit polyclonal antisera raised against recombinant mouse CGI-58 (Subramanian, Rothenberg et al. 2004) in PBS containing 0.1 mg/mL saponin, 0.2 mg/mL rat IgG. Secondary antibody staining was carried out with anti-goat Alexa Fluor 555 and anti-rabbit Alexa Fluor 647 for 2 hours at room temperature. Nuclei were stained with Hoechst 33422 and lipid droplets were stained with BODIPY 493/503 (Life Technologies), both were used in 0.1 μ g/ml concentration in PBS. Images were taken either by a Nikon Eclipse E800 fluorescence microscope equipped with a Photometrics CoolSNAP EZ digital camera or an LSM510 Meta Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany) using a 63X oil immersion lens (NA 1.4). Images were processed using Zen 2008 and ImageJ software. Cells were manually scored for protein localization patterns by at least two observers blinded to sample identity and automatically scored using the ImageJ JACoP plugin (Bolte and Cordelieres 2006) to determine Manders coefficients; more than 50 cells were counted for each determination.

6.10 Measurement of Cellular Triacylglycerol:

Human NLSD fibroblasts were transduced with adenovirus to drive the expression of WT CGI-58, or S239A/S240A mutated CGI-58; cells were harvested at various time points post transduction and cell lysates were prepared as mentioned above in 6.8. Lipids were extracted in glass tubes from cell lysates by using isopropanol:hexane:water (80:20:2, v/v/v) followed by lipid phase collection. Equal volumes of all the samples were taken and organic phase was evaporated in

speed vacuum concentrator equipped with a cold trap (ThermoSavant, Farmingdale, NY). Lipid extracts of corn oil were used as TAG standards. The cellular TAG content was determined using an enzymatic assay Infinity Triglyceride stable reagent from Thermo Scientific. Samples were transferred to 96 well plate and absorbance was determined at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The triacylglycerol measurements were expressed relative to cellular protein content measured by the Bio-Rad Laboratories, Hercules, CA, USA DCTM Protein Assay.

In case of the PKA stimulated experiment, NLSD fibroblasts were transduced with CGI-58-WT and S239A/S2240A for 6 hours followed by treatment with 10 μ M forskolin and 0.5 mM IBMX to increase adenylyl cyclase activity, in turn, activating PKA. Cells were collected at 0, 0.5, 1, and 2 hours and lysates were subjected to determination of TAG levels as mentioned above.

6.11 Immunoblotting:

Equal amount of proteins from whole cell homogenates were solubilized and denatured in SDS-PAGE buffer, followed by separation on SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis. Blots were probed with either antiserum raised against mouse CGI-58 or mouse perilipin A in 1:50,000 and 1:10,000 dilution respectively. As loading control β -actin probed in 1:7,000 dilution was used. All dilutions were done in 5% BSA in Tris buffered saline-Tween 20 (TBS-Tween: 50 mM Tris, 150 mM NaCl, pH 7.4, containing 0.1% Tween 20). To detect antigen-antibody complex formed between separated protein and primary antibody, the membrane was probed with peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse IgG. Chemiluminescent detection was performed with chemiluminescence reagent (Amersham Biosciences, Vienna, Austria) and quantification of relative protein expression levels were done by densitometry analysis using a BioRad (Hercules, CA, USA) densitometer.

6.12 Adipose tissue collection:

Male C57Bl/6 mice (age 8-12 weeks, body weight 20-30 g) were kindly provided by Prof. Dagmar Kratky (Medical University Graz, Graz, Austria). They were cared for according to the standards established by the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments (Vienna, Austria) and protocols were approved by an institutional review board. Mice had free access to food (chow diet, Ssniff®, Soest, Germany) and water and they were housed under a 12 h light/12 h dark cycle in a temperature-controlled environment. On the day of the experiment the animals were fasted for 6 hours of day time prior to sacrifice; gonadal white adipose tissues were collected and cut into small pieces (~1 mm³). Tissues were briefly washed with 37°C warmed PBS to remove any blood or hair contamination before cutting into small pieces. Pieces of white adipose tissues were treated either with DMSO for basal or 10 µM isoproterenol and 0.5 mM IBMX for stimulated experimental conditions in DMEM containing 2% fatty acid-free BSA for 20 min in a 37°C water bath with continuous gentle shaking. Tissues were then washed with ice cold PBS and snap frozen in liquid nitrogen, followed by lysis by sonication either in PBS pH 7.4 with protease and phosphatase inhibitor or in immunoprecipitation buffer described below. Lysates were cleared by centrifugation at 10,000 x g for 10 min at 4°C. Protein content of supernatants was determined by Bradford Assay (Bradford 1976).

6.13 Immunoprecipitation (IP):

CGI-58 antibody (CGI-58 antiserum) was cross-linked to protein A-sepharose beads (GE healthcare, Buckinghamshire, UK) by incubation with 50 mM dimethyl pimelimidate (DMP) for 1 hour at room temperature. As a negative control, non-specific control IgG were crossed linked. Unbound CGI-58, IgG antibody and excess DMP was removed by washing the cross-linked antibodies with 0.1 M triethanolamine in PBS. Further, prior to immunoprecipitation of CGI-58, lysates of treated and untreated white adipose tissue expressing endogenously CGI-58 and lysates from NIH3T3CARΔ fibroblasts ectopically expressing CGI-58-WT or S239A/S240A mutant were first precleaned with protein A-sepharose beads (GE

healthcare, Buckinghamshire, UK). Precleaned lysates were then incubated with cross-linked antibodies for 16 hours at 4°C with continuous mixing, in total 1 mg of protein was used for each IP. Unbound antigen was removed by washing the beads with immunoprecipitation buffer containing 50 mM Tris-HCl, 0.1% tween 20, 300 mM NaCl, 5 mM EDTA, 50 mM NaF and 0.02% NaN₃ with protease and phosphatase inhibitors. Immunoprecipitates were collected by centrifugation at 1000 x g for 1 min at 4°C. Immunoprecipitated proteins were eluted from the beads by heating the sample with SDS-PAGE XT sample buffer (Bio-Rad Laboratories GmbH, Vienna, Austria) at 90°C for 10 minutes followed by separating them on 4-20% gradient gels for SDS-PAGE (Bio-Rad Laboratories GmbH, Vienna, Austria) and transferred to nitrocellulose membranes. Membranes were incubated with anti-CGI-58 (1:50,000) or phospho-S/T-PKA substrate antibody (1:1000) (Cell Signaling Technology #9621; Danvers, MA) in 5% BSA in TBS with 0.1% tween 20 overnight at 4°C. Blots were incubated with Clean-Blot IP Detection Reagents (Thermo Scientific, Ottawa, Ontario, Canada) (1:2500) in 5% non-fat milk in TBS with 0.02% Tween-20 for 2 h at 25°C, followed by detection with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Ottawa, Ontario, Canada).

6.14 SDS-PAGE, two-dimensional electrophoresis and immunoblot analysis:

Two-dimensional gel electrophoresis of DMSO and catecholamine treated white adipose tissue lysates was done to obtain higher resolution of protein separation and separate protein isoforms. Standard protocols for 2D-gel electrophoresis and immunoblotting of proteins were used. Immunoblotting is described in detail in 6.11. For 2D-gel electrophoresis proteins were precipitated with pre-chilled (-20°C) acetone, resulting pellets were then suspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.0025% bromophenol blue, 0.5% pharmalyte (pH 3-10), 50 mM DTT). Proteins were separated in the first dimension on immobilized non-linear pH gradient strips (pH 3-10) (Bio-Rad Laboratories GmbH, Vienna, Austria) by isoelectric focusing by gradual increase in voltage from 200 V to 8000 V at the

rate of 4 V/min (28 kVh in total). Rehydrated strips were reduced and alkylated by rinsing it with 1% (w/v) DTT and 2.5% (w/v) IAA respectively. The second dimensional separation was performed using 4-12% gradient SDS-PAGE gels (Bio-Rad Laboratories GmbH, Vienna, Austria). Antibodies used for immunoblotting were rabbit polyclonal anti-mouse CGI-58 antiserum and phospho-S/T-PKA substrate antibody (Cell Signaling Technology #9621; Danvers, MA) as described above. Proteins were detected using peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) and enhanced chemiluminescence reagents (Amersham Biosciences, Vienna, Austria or Thermo Scientific, Ottawa, Ontario, Canada).

6.15 Data and statistical analyses:

Kinetics data were analyzed according to the Michaelis-Menten equation using the GraphPad Prism kinetic model-fitting program. Data are presented as mean $\pm$ standard deviation (SD) or standard error and were analyzed using two-way ANOVA and Tukey's post-hoc test with SPSS software (IBM, version 20). Differences between samples were considered significant at $p < 0.05$.

7. RESULTS

7.1 PKA, PKC and AMPK kinase assays on recombinant CGI-58 and G0S2:

Many kinases share similar consensus sequences (RXXS/T) which are substrate serine/threonine residues in close vicinity to an essential arginine (R) residue. Therefore we screened the amino acid sequence of CGI-58 and G0S2, two important regulators of ATGL, *in-silico* with NetPhos 2.0 software (University of Denmark). This exposed Ser239 and Ser240 in CGI-58 in the consensus sequence RKYSS as potential targets for phosphorylation by PKA with high scores 0.984 and 0.996 respectively (threshold=0.500). In G0S2 we identified Ser97 as a potential target of PKC and PKA with score of 0.75 and 0.59 respectively. Apart from PKA and PKC, AMPK is another important kinase in energy homeostasis which shares a similar consensus sequence (XRXS/T) and could therefore also play a role in CGI-58 and/or G0S2 phosphorylation. Based on the above information we screened CGI-58 and G0S2 for their phosphorylation by PKA, PKC and AMPK with a radioactive kinase assay. Recombinant CGI-58 and G0S2 were incubated with the respective kinases and incorporation of radioactive ^{32}P was detected by autoradiography. Kinase assay results revealed CGI-58 and G0S2 as PKA, PKC and AMPK substrates *in vitro* (Fig 3A). Further, we analyzed whether the consensus sequence RKYSS was conserved in CGI-58. In vertebrate species it is well conserved, but not in CGI-58 orthologs like *Caenorhabditis elegans* or *Drosophila melanogaster* (Fig. 3B). Phosphoamino acid analysis of the ^{32}P -labeled protein showed that PKA phosphorylates CGI-58 at a serine residue (Fig. 3C).

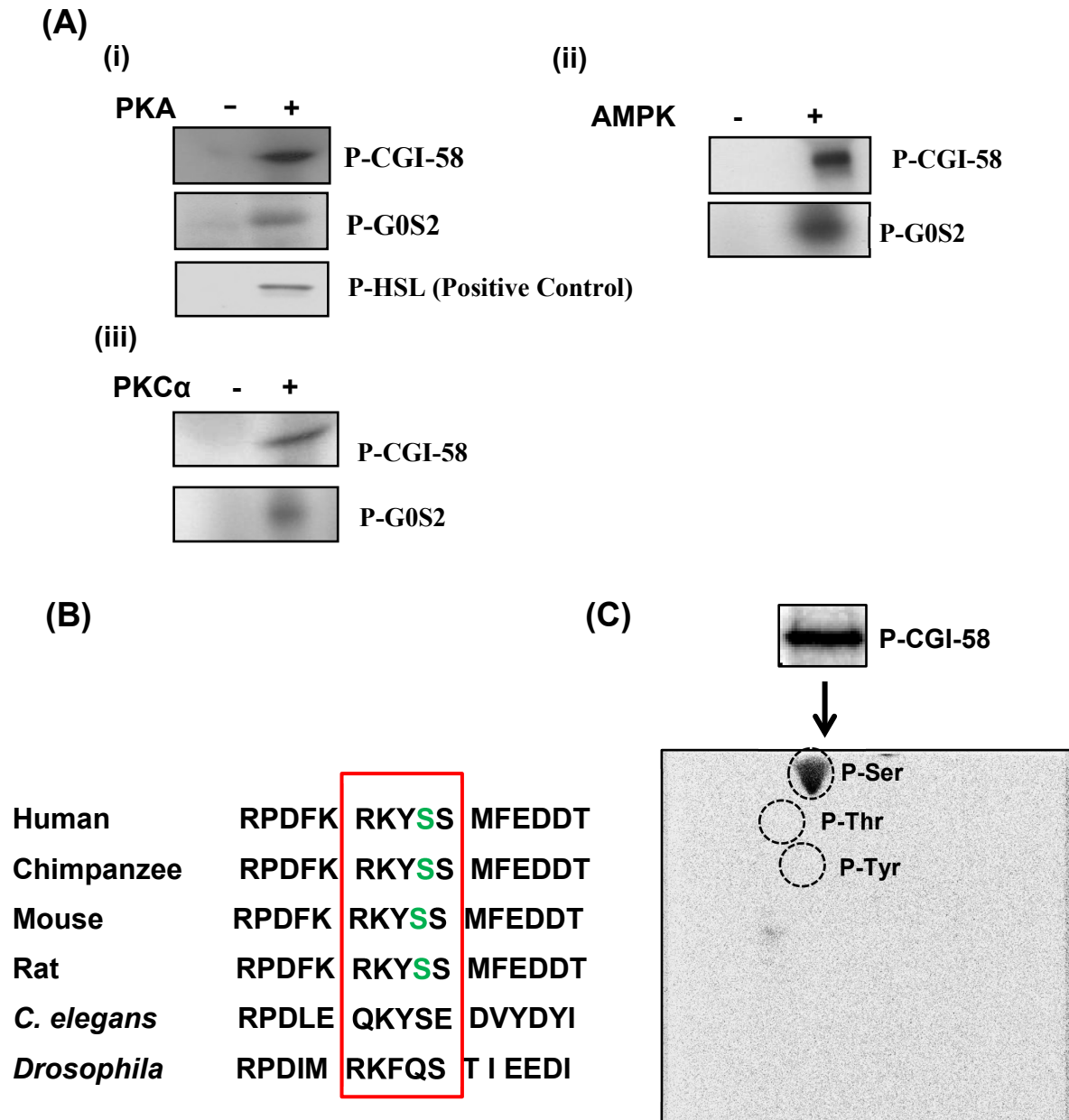


Figure 3. CGI-58 and G0S2 are phosphorylated by PKA, AMPK and PKC *in-vitro*: (A) Autoradiography of recombinant CGI-58 and G0S2 subjected to [γ - ^{32} P] ATP (i) PKA, (ii) AMPK and (iii) PKC α kinase assays. Negative control reactions were performed without kinases. (B) Sequence of putative phosphorylation site (S239) of CGI-58 by PKA is conserved between different mammalian species but does not exist in non-mammalian species. Amino acid sequences of CGI-58 are aligned with human (NP_057090), chimpanzee (XP_516397), mouse (NP_080455), rat (NP_997689), *C. elegans* (hypothetical protein C37H5.2, NP_504299.2), and *D. melanogaster* (CG1882, isoform B, NP_724609.1). (C)

Phosphoamino acid analysis of purified recombinant mouse CGI-58 was performed after phosphorylating it in a kinase assay by PKA followed by SDS-PAGE and transfer to PVDF membrane. The CGI-58 corresponding band from the PVDF membrane was subjected to acid hydrolysis by HCl then applied to 2D TLC analysis. The positions of the standard phosphoamino acids, phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr), are indicated. The shown data are representatives of two independent experiments.

7.2 Kinetics of recombinant CGI-58 phosphorylation by PKA:

Detailed kinetics of *in vitro* phosphorylation was done to study the effect of various factors involved in the assay. Results showed that phosphorylation of CGI-58 by PKA displayed saturation kinetics and was dependent on factors like protein concentrations of CGI-58 (Fig. 4A, 4E), PKA (4B), and ATP (Fig. 4C), and on time of reaction (Fig. 4D). Analysis of the data using the Michaelis-Menten equation yielded apparent K_m values for ATP and recombinant CGI-58 of $14.0 \pm 2.8 \mu\text{M}$ and $71.5 \pm 13.9 \mu\text{g/ml}$, respectively. This experiment was performed in Prof. Dawn L. Braasemle's laboratory at Rutgers University, New Jersey, USA.

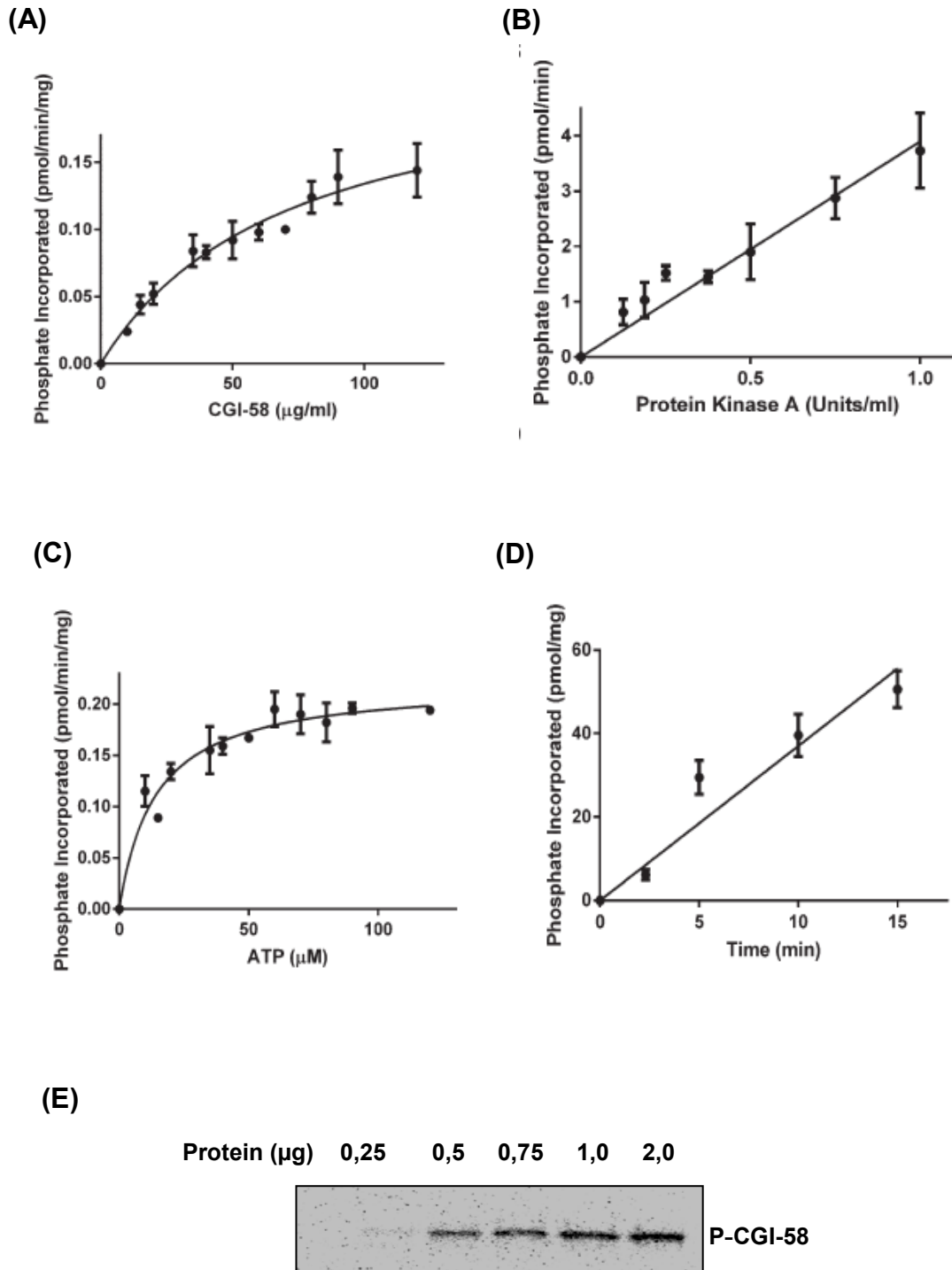


Figure 4. Phosphorylation Kinetics by PKA on recombinant CGI-58: In panel A-D varying setups of the reaction were tested: **(A) Concentration of CGI-58:** Varying protein concentrations of mouse recombinant CGI-58 were incubated with mammalian PKA (0.32 units/ml) and 50 μM [γ - ^{32}P] ATP for 10 minutes. After the phosphorylation reactions, the samples were spotted onto Whatman P81 paper,

washed with 75 mM phosphoric acid, and incorporation of radioactivity was determined by scintillation counting. **(B) Concentration of PKA:** Recombinant CGI-58 (1 μ g) was incubated with the specified concentrations of PKA and 50 μ M [γ - ^{32} P] ATP for 10 minutes. The data show one representative of two independent experiments. **(C) Concentration of ATP:** Recombinant CGI-58 (1 μ g) was incubated with PKA (0.32 units/ml) and the designated concentrations of [γ - ^{32} P] ATP for 10 minutes. **(D) Time of reaction:** Kinase assay with varying reaction time as shown in the figure depicted the effect of time on CGI-58 phosphorylation. Each data point represents the average of triplicate determinations. Bars represent $\pm$ standard deviation (SD). **(E) PKA kinase assay reactions to phosphorylate different amounts of recombinant CGI-58 protein** were performed. Then proteins were separated on SDS-PAGE followed by autoradiography for detection of signal.

7.3 Serine 239 is the major phosphorylation site by PKA in vitro:

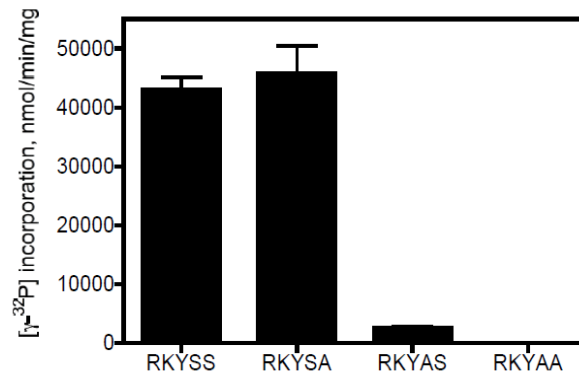
To determine the exact phosphorylation site of CGI-58 by PKA, a peptide of 10 amino acids containing the predicted consensus sequence of mouse CGI-58 was synthesized. Different mutant variants of the peptide replacing serine 239 and serine 240 were also synthesized, which included S239A, S240A and S239A/S240A. Results clearly showed that a peptide with intact S239 was a major substrate for phosphorylation by PKA *in vitro*. On the other hand, peptides which contained serine to alanine single or double mutations served as poor substrates or no substrates of PKA in *in vitro* kinase assays. PKA with S239A single mutant peptide showed only 6% of the activity when compared to intact RKYSS peptide (Fig. 5A). Peptides possessing a double mutation of both the serine residues S239A/S240A did not show any sign of phosphorylation.

After confirming serine 239 as the main residue being phosphorylated by PKA with CGI-58 derived synthetic peptides, we moved on to confirm the same phosphorylation site on intact CGI-58 recombinant protein (Fig. 5B). The wild type form and mutants of CGI-58 were expressed and purified from *E.coli*. Then they were subjected to a kinase assay to determine the residue targeted by PKA for

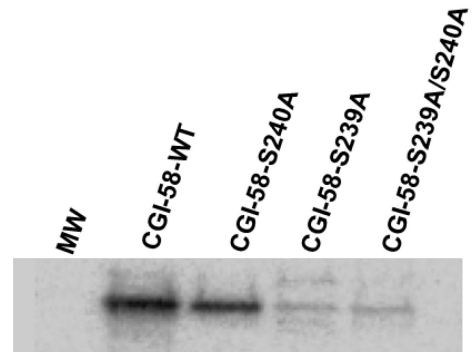
phosphorylation in intact full length CGI-58. Incorporated amount of radioactive ATP into recombinant CGI-58 wild type and S239A mutant confirmed the above mentioned results of the peptide kinase assay, where S239A (RKYAS) mutant was a weak substrate of PKA but wild type (RKYSS) and S240A single mutant (RKYSA) were excellent substrates of PKA. Additionally, we used mass spectrometry to further confirm the site of phosphorylation of CGI-58 by PKA (Fig. 5C, 5D). Full length phosphorylated recombinant CGI-58 (WT) protein by PKA was subjected to reduction, alkylation and enzymatic digest with trypsin, chymotrypsin or endoprotease (flavastacin/AspN). The result of LC-MS/MS and proteome discoverer search using SwissProt database clearly identified serine 239 of CGI-58 as phosphorylation site using the three different digests of CGI-58 after *in-vitro* PKA kinase reaction. We identified three different peptides of CGI-58 containing serine 239 in phosphorylated states (Table 1).

In summary, from peptide based and full length CGI-58 kinase assays of various mutants and mass spectrometry analysis, serine 239 was discovered as the main residue for PKA phosphorylation *in vitro*.

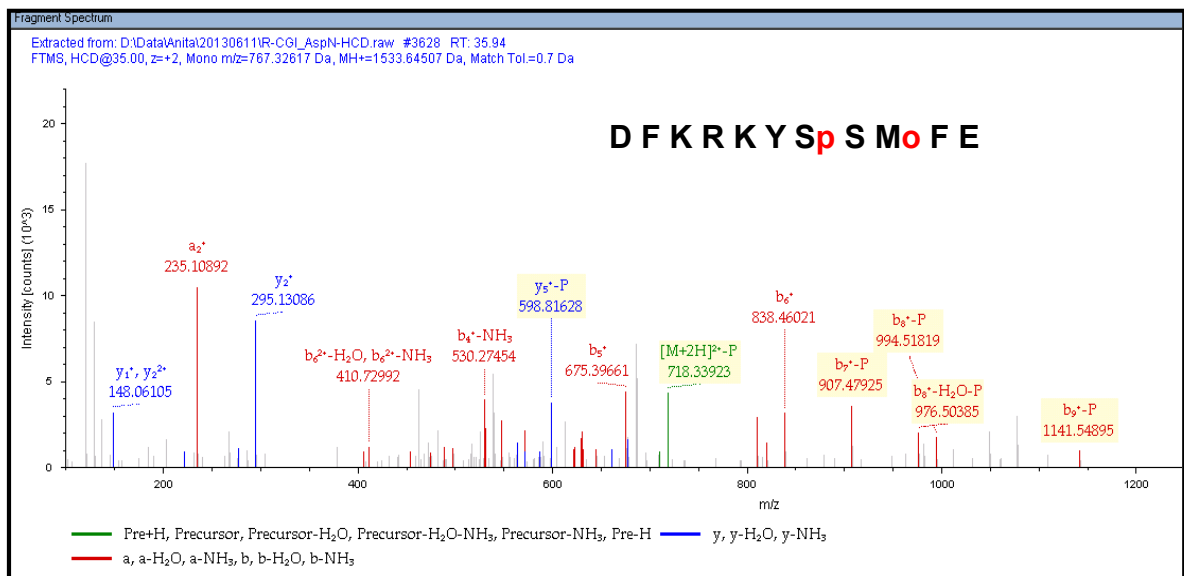
(A)



(B)



(C)



(D)

Ion Series	Phosphorylation Losses	Neutral Losses	Multiple Neutral Losses	Precursor Ions				
#1	a ⁺	a ²⁺	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	88.03931	44.52329	116.03423	58.52075	D			11
2	235.10773	118.05750	263.10265	132.05496	F	1418.61747	709.81237	10
3	363.20270	182.10499	391.19762	196.10245	K	1271.54905	636.27816	9
4	519.30382	260.15555	547.29874	274.15301	R	1143.45408	572.23068	8
5	647.39879	324.20303	675.39371	338.20049	K	987.35296	494.18012	7
6	810.46211	405.73469	838.45703	419.73215	Y	859.25799	430.13263	6
7	977.46047	489.23387	1005.45539	503.23133	S-Phospho	696.19467	348.60097	5
8	1064.49250	532.74989	1092.48742	546.74735	S	529.19631	265.10179	4
9	1211.52792	606.26760	1239.52283	620.26505	M-Oxidation	442.16428	221.58578	3
10	1358.59634	679.80181	1386.59125	693.79926	F	295.12886	148.06807	2
11					E	148.06044	74.53386	1

Figure 5. CGI-58 is phosphorylated in vitro by PKA: To identify the amino acid position at which CGI-58 is getting phosphorylated by PKA, we used either 10 amino acid synthetic peptide (0.5 mM) or full length recombinant CGI-58 (1 µg) containing either WT, S240A, S239A, or S239A/S240A sequences. These peptides and full length recombinant CGI-58 containing various sequences were incubated with PKA (0.32 units/ml) and 50 µM [γ - ^{32} P] ATP/ATP for 10 min in kinase assay buffer to complete kinase reaction and phosphorylate the substrate. Resultant radioactive reaction products were transferred to either P81 Whatman paper (peptides) or run on SDS-PAGE (recombinant CGI-58) for separating it based on molecular weight prior to transfer onto PVDF membrane. Reaction products from non-radioactive kinase assays were subjected to mass spectrometry. **(A)** Synthetic peptides were measured by scintillation counting. Data are mean $\pm$ standard deviation of triplicate reactions. **(B)** Phosphoimaging analysis of recombinant full length CGI-58 kinase assay products. Different lanes represent kinase reaction products of recombinant CGI-58 containing different sequence at putative phosphorylation site by PKA: Lane 1 - MW molecular weight markers, Lane 2 - WT CGI-58, Lane 3 - S240A mutated CGI-58, Lane 4 - S239A mutated CGI-58, Lane 5 - S239A/S240A mutated CGI-58. **(C)** After kinase assay reaction full length recombinant CGI-58 WT was reduced and alkylated then enzymatically digested for LC-MS/MS analysis. Spectrum of AspN digested CGI-58 fragmentation of parent ion is shown where neutral loss of phosphate group is detected at serine239 residue. **(D)** “b” and “y” ion series of the peptide detected in mass spectrometry analysis corresponding to the spectrum shown in (C) which is a result of cleavage of CO-NH bond in a peptide. The mass difference between two neighboring amine acids “b” or “y” ions is indicative of particular amino acid residue. Phosphorylated serine 239 is circled in green.

Table1. Phosphorylated peptides of CGI-58 identified by mass spectrometry:

Purified recombinant CGI-58 was incubated with PKA *in vitro*, digested with either trypsin, AspN, or chymotrypsin and analyzed by LC-MS/MS. Peptides were identified by searching the mammalian SwissProt database with Proteome Discoverer and Mascot software. Modified amino acids are shown in small letters (*s* designates phosphorylated serine, *m* designates oxidized methionine). Mass/charge ratio (*m/z*) and mass error (Δm) of measured peptides, as well as Mascot ion scores and expect values are listed. AspN peptide spectrum and *b* and *y* ion series is shown in detail in Fig. 5C and 5D above respectively.

Enzyme	Sequence	<i>m/z</i>	Δm (ppm)	Ion score	Expect value
Trypsin	KYsSMFEDDTVTEY- IYHCNVQTPSGETAfK	1209.85059	1.09	104	2.9E-009
AspN	DFKRKYsSmFE	767.32617	0.42	32	7.5E-003
Chymotrypsin	KRKYsSMF	571.75867	3.16	30	6.9E-003

7.4 CGI-58 is a protein kinase A target in cells and adipose tissue:

After successful validation of serine 239 as main PKA target site *in vitro*, we investigated whether CGI-58 is a PKA target also *in vivo*. To this end we treated murine gonadal white adipose tissue *ex vivo* with isoproterenol and IBMX to activate catecholamine stimulated G-protein coupled receptor (GPCR) signaling to activate PKA by elevating cAMP levels. To detect the effect of PKA stimulation on CGI-58 phosphorylation we performed 2D western blotting from treated and control (DMSO) tissue lysates. We probed the blots with CGI-58 antibody and based on molecular weight and isoelectric point (pI) detected diverse spots of CGI-58, showing a shift towards negative charge in the treated sample as compared to the control, which is most likely due to the addition of a negatively charged phosphate group onto the protein (circle marked in blue, Fig. 6A). In addition, we immunoprecipitated CGI-58 from treated and control white adipose tissue lysates and then blotted it with phospho-S/T PKA substrate antibody or CGI-58 antibody. Results showed a clear signal of phosphorylation in PKA stimulated tissue while no signal was detected in the unstimulated sample (Fig. 6B). Further, we ectopically expressed CGI-58 in NIH3T3CARAΔ cells, consisting CGI-WT or its mutant, S239A/S240A. Next cells were treated with both IBMX and forskolin to activate PKA or DMSO as control. After treatment cells were lysed and lysates were processed for immunoprecipitation (IP) of CGI-58. Immunoprecipitated CGI-58 containing various WT and mutated sequences at the putative PKA site were then run on SDS-PAGE and blotted with pS/T PKA substrate antibody to prove S239 as major PKA site *in vivo*. The results show phosphorylated CGI-58 in stimulated cells, where ectopically expressed CGI-58 has intact S239 (Fig. 6C). Finally, the above results were confirmed by LC-MS/MS analysis using targeted higher-energy collisional dissociation (HCD) of white adipose tissue with or without spiking with a 10 amino acid peptide carrying the S239 phosphorylation. In both treated spiked and unspiked samples of white adipose tissue, the peptide carrying serine 239 phosphorylation could be detected at the same retention time (Fig. 7i, ii)

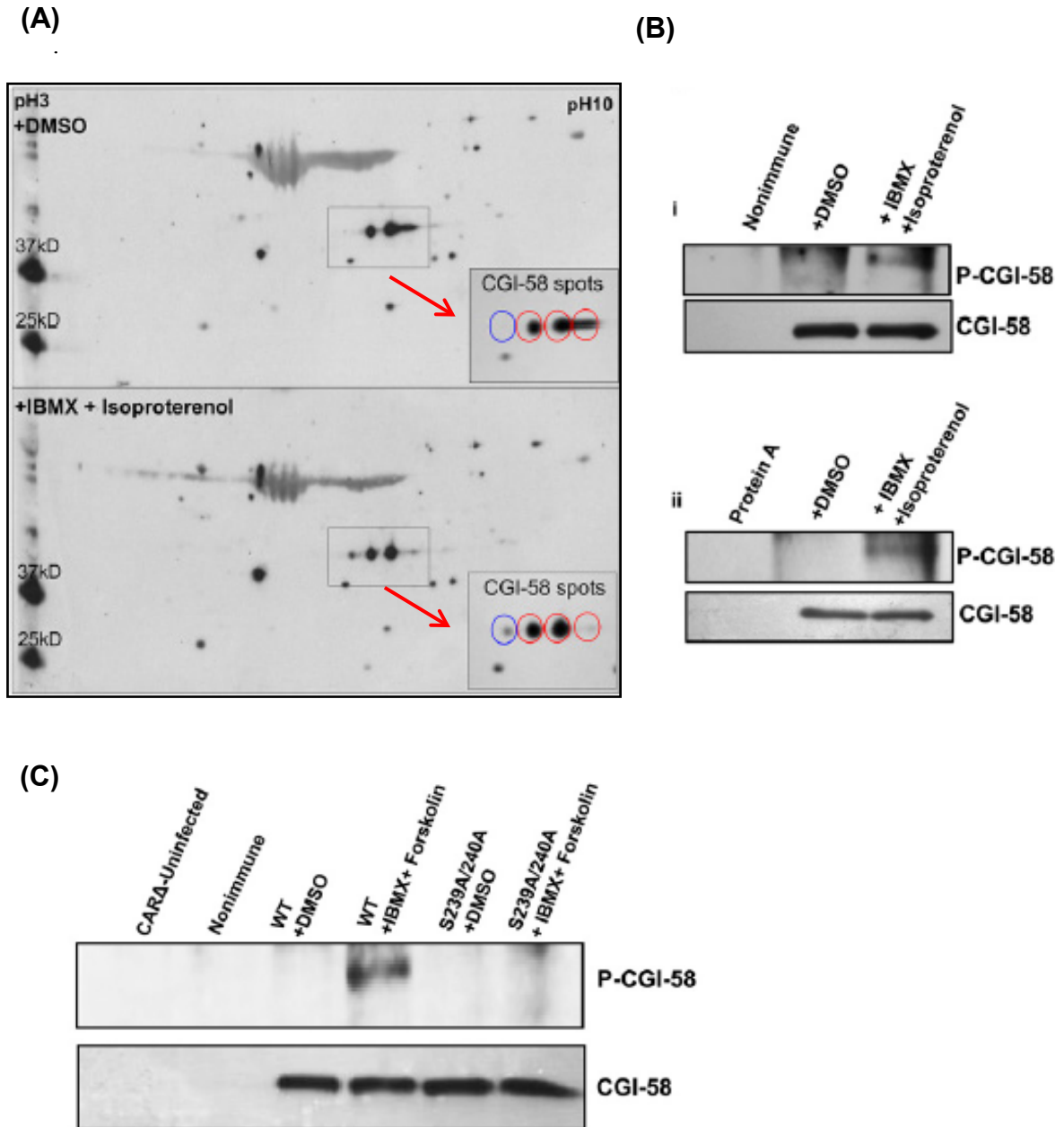
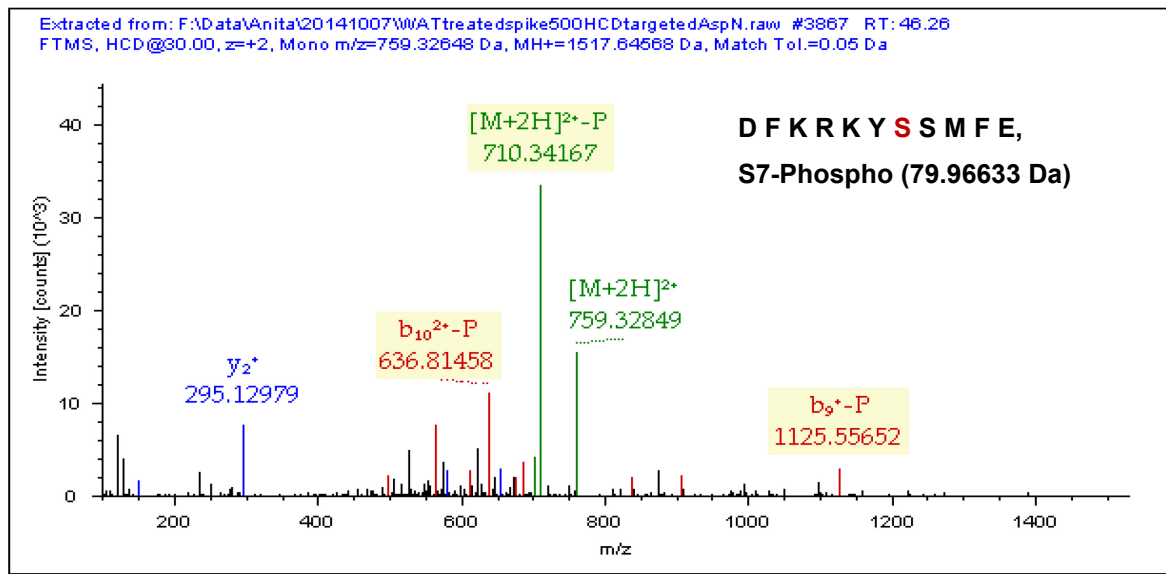


Figure 6. CGI-58 is phosphorylated in vivo in cells and by PKA: **A)** 2D immunoblot of CGI-58 from murine white adipose tissue (C57Bl/6) incubated either with DMSO (control) or 0.5 mM IBMX and 10 μ M isoproterenol (treated). **B)** Immunoprecipitated CGI-58 from white adipose tissue blotted with either pS/T PKA substrate antibody or CGI-58 antibody (loading control). Non-immune IgG (i) and protein A beads without CGI-58 antibody crosslinking (ii) were included as controls. **C)** NIH3T3CARA Δ cells expressing different variants of CGI-58 as shown in the figure were treated with either IBMX and forskolin or DMSO. After the PKA stimulation cells were lysed and CGI-58 containing WT or S239A/S240A sequenc-

es were immunoprecipitated. Immunoprecipitated CGI-58 variants were then separated on SDS-PAGE, blotted onto nitrocellulose membrane and blots were probed with either pS/T PKA substrate antibody or CGI-58 antibody.

(i)



(ii)

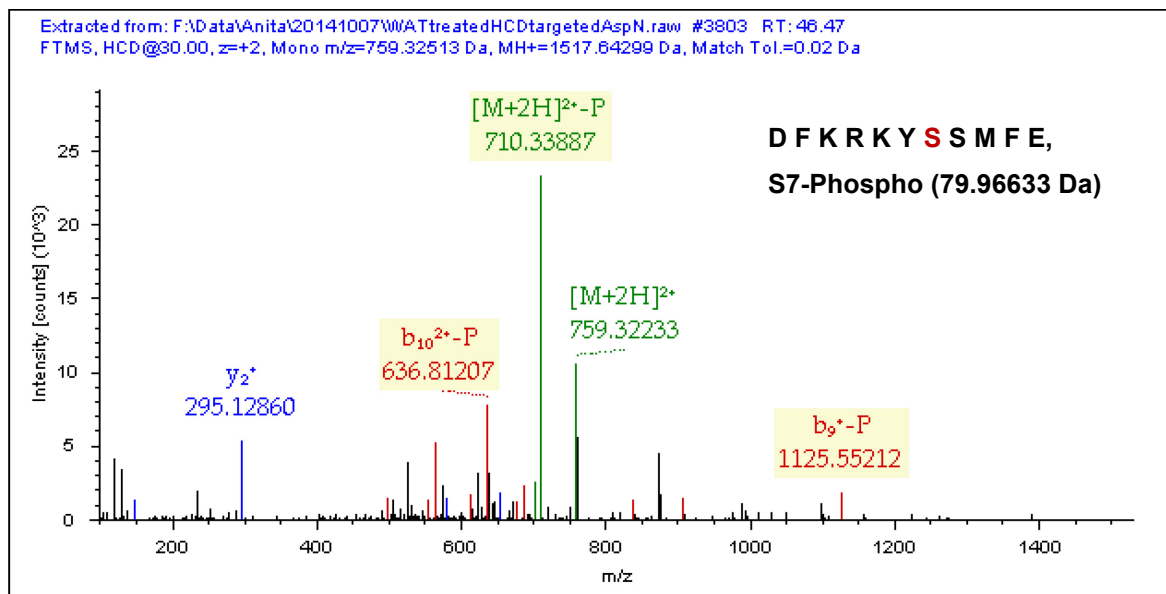


Figure 7. Confirmation that CGI-58 is phosphorylated in adipose tissue in vivo by mass spectrometry: Catecholamine treated white adipose tissue lysate was precipitated with TCA then reduced and alkylated prior to AspN enzyme di-

gestion. Half of the sample was (i) spiked with phosphorylated synthetic peptide (D F K R K Y Sp S M F E) (500 attomole), the other half was left (ii) unspiked. The MS2 spectrum showed a similar neutral loss of the phosphate group as well as an identical fragmentation pattern, and the parent peak of the AspN digested peptide containing S239 had the same m/z and retention time as the internal standard peptide.

7.5 Phosphorylated CGI-58 at serine 239 by PKA has no effect on TAG hydrolase activity of ATGL *in vitro*:

As serine 239 of CGI-58 was confirmed as a key PKA phosphorylation site *in vitro* and *in vivo*, we aimed to delineate the effect of the newly discovered phosphorylation on ATGL activation, which is a well-defined target of CGI-58 in lipid metabolism in literature to date. For this purpose recombinant CGI-58 was phosphorylated in a non-radioactive *in vitro* PKA kinase assay which was then incubated with a Sf9 insect cell lysate containing recombinant ATGL and an emulsified radioactive triacylglycerol substrate. Results showed no difference in fatty acid release irrespective of CGI-58 phosphorylation status. The phosphorylated and non-phosphorylated form of CGI-58 showed the same effect on ATGL activation to hydrolyze TAG (Fig. 8A). Further, different versions of recombinant ATGL (truncated, 1-288 amino acid) were tested for its hydrolase activity together with different recombinant variants of CGI-58 (WT, S239D and S239D), which mimic the negative charge status of phosphorylated CGI-58. The data suggest that the phosphorylation of CGI-58 neither facilitates nor obstructs the activation of ATGL (Fig. 8B). Furthermore, *in vitro* data indicate that neither the serine 239 (S) to alanine (A) mutation, which is disruption of phosphorylation site, nor serine 239 to aspartic acid (D) or glutamic acid (E) exchange, which leads to a consecutive phosphorylation like negatively charged status, has any effect on ATGL TAG-hydrolase activity.

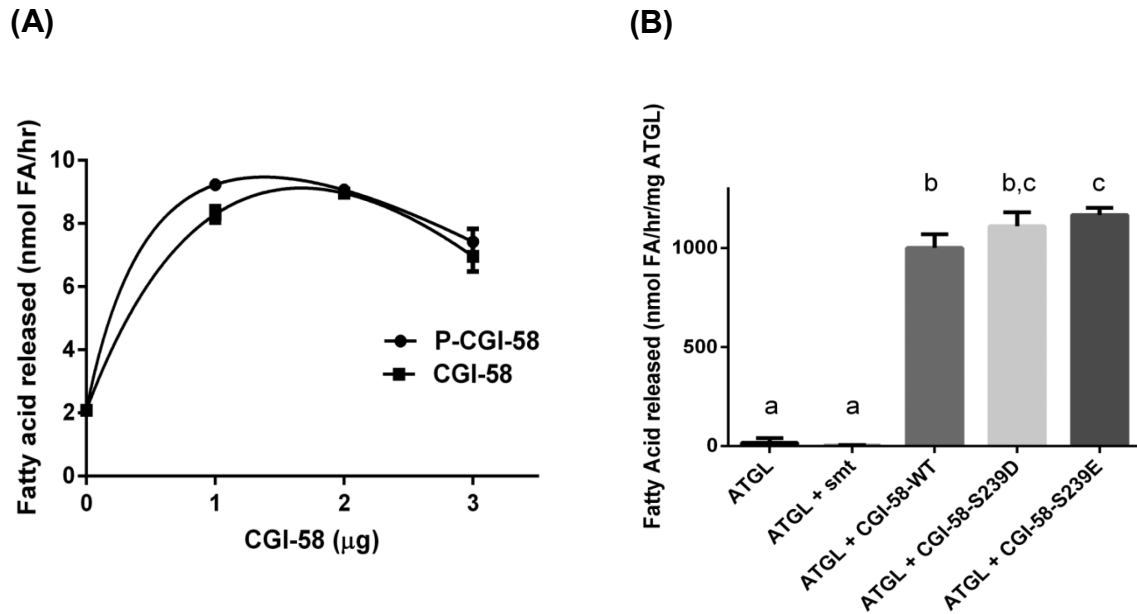


Figure 8. Effect of various mutants of CGI-58 on in vitro TAG hydrolysis by ATGL: (A) Sf9 insect cell extracts containing recombinant ATGL were incubated with in vitro PKA phosphorylated and non-phosphorylated CGI-58. This reaction contained radioactive triolein emulsified with 3:1 phosphatidylcholine: phosphatidylinositol, as an ATGL substrate. Reactions were terminated and lipids were extracted by adding methanol-chloroform-heptane [10:9:7 (v/v/v)] and 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5, which separated radioactive labeled lipid products into a chloroform phase. Released free fatty acids in the aqueous phase were quantified by scintillation counting. Data are depicted as mean $\pm$ standard deviation of triplicate reactions. One representative experiment out of three is shown. (B) Effect of phosphorylation mimicking (S329D) recombinant CGI-58 on TAG hydrolase activity of recombinant truncated ATGL was assessed in vitro. E.coli extracts expressing recombinant CGI-58 WT, S239D, S239E or control vector driving the production of a 6-His-Sumo protein were screened for their effect on truncated ATGL activity by co-incubating them with the radioactive triolein substrate (as described above). Data are reported as mean $\pm$ standard deviation of triplicate samples from one representative experiment out of two. Data were analyzed by ANOVA; data with the same alphabetic letter subscript showed no significant difference; data with different letters are changed significantly ($p < 0.05$).

7.6 Phosphorylated CGI-58 at serine 239 by PKA has no effect on TAG hydrolase activity of ATGL in NLSD cells:

Next, we investigated the functional impact of S239 phosphorylated CGI-58 in intact cells. We used an adenoviral expression system in human NLSD fibroblast to drive the expression of CGI-58 WT or CGI-58 S239A/S240A mutant. The NLSD cells lack functional CGI-58 and have perilipin 2 coated lipid droplets, however they express endogenous ATGL (Fig. 9A), but are unable to hydrolyze TAG in the absence of CGI-58. Whereas NLSD cells expressing β -galactosidase have 15-fold increased TAG levels, addition of either WT or S239A/S240A variant of CGI-58 by adenovirus led to a significant decrease in intracellular TAG compared to non-transduced cells. This decrease in TAG level due to addition of CGI-58 variants continued up to 48 hours and was similar in cells expressing either WT or S239A/S240A CGI-58 (Fig. 9B, 9C). Effect of CGI-58 and its mutants in assisting the turnover of intracellular TAG in NLSD cells were compared in both basal and stimulated (forskolin and IBMX treated) conditions. Under basal condition, intracellular TAG measurements were performed in cells collected at various time points between t=0 to t=48 hours post adenoviral transduction of CGI-58 variants. For the stimulated condition, NLSD cells were transduced with adenovirus of CGI-58 variants for 6 hours then stimulated (forskolin and IBMX) for different time points between t=0 to t=2 hours Prior to TAG measurement in cell lysates. Data suggested that CGI-58 S239A/S240A mutant is equally effective as the WT variant in stimulating TAG hydrolysis in NLSD cells in basal (Fig. 9C) and stimulated (Fig. 9D) conditions. Further, the protein levels of WT and mutated CGI-58 were shown to be similar in basal and stimulated condition (Fig. 9E, 9F). Therefore PKA is not a major modulator of lipolysis, and phosphorylation of Ser239 (or Ser240) is not required for the function of CGI-58 in facilitating TAG hydrolysis in human NLSD fibroblasts. It is important to note that NLSD fibroblasts lack two major components of PKA-mediated lipolysis, perilipin 1 and hormone-sensitive lipase (HSL).

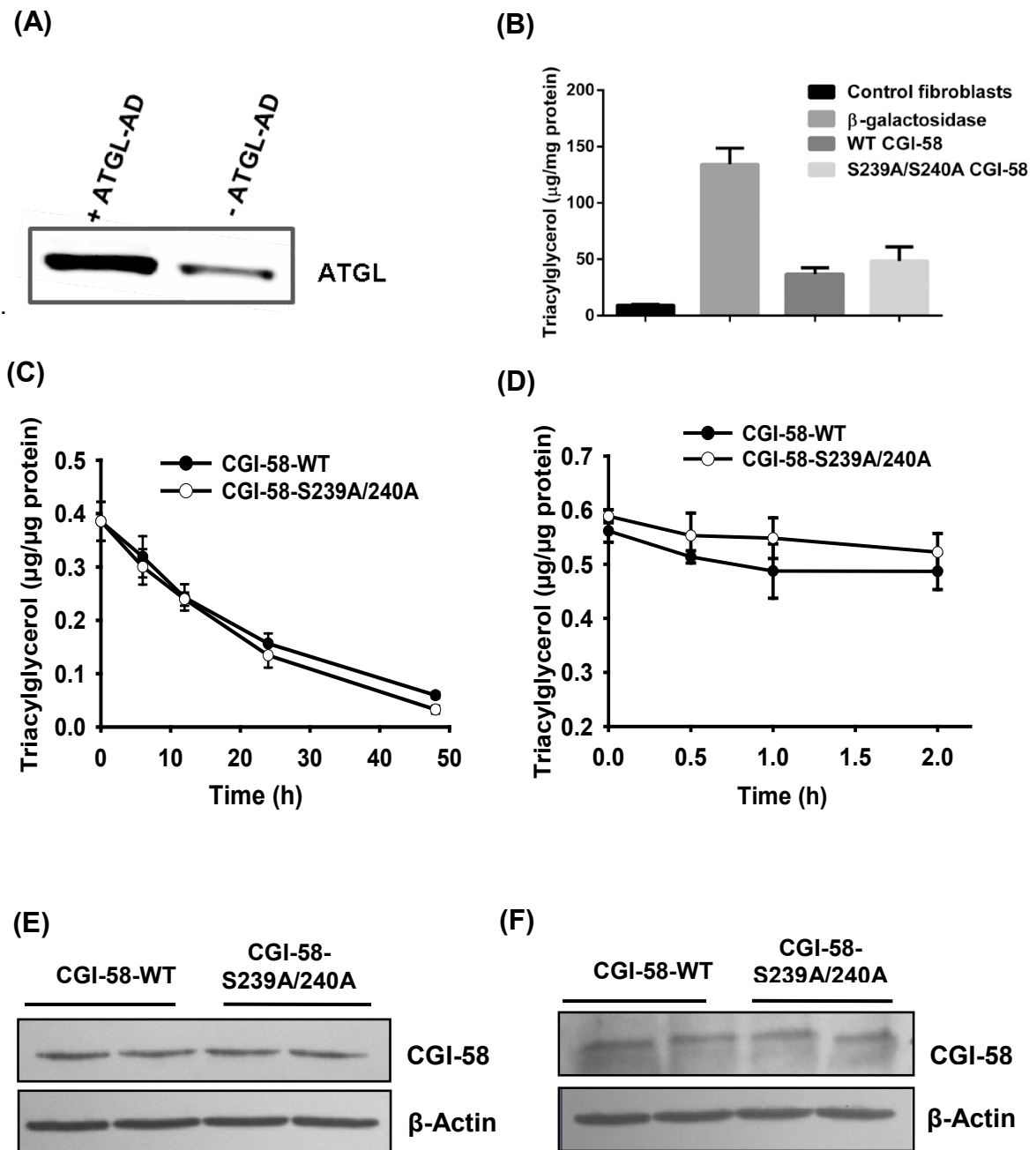


Figure 9. Effect of serine 239 mutant of CGI-58 on TAG hydrolysis in NLSD cells: (A) Immunoprecipitation blot of ATGL with and without transduction with ATGL adenovirus to determine the presence of endogenous ATGL in human NLSD fibroblast. (B) TAG levels were measured in control fibroblasts (WS1) expressing active endogenous CGI-58 and in NLSD cells post adenoviral transduction with CGI-WT, S239A/S240A and β-galactosidase. Human NLSD fibroblasts lack functional CGI-58 and store excess TAG which can be rescued by ectopic

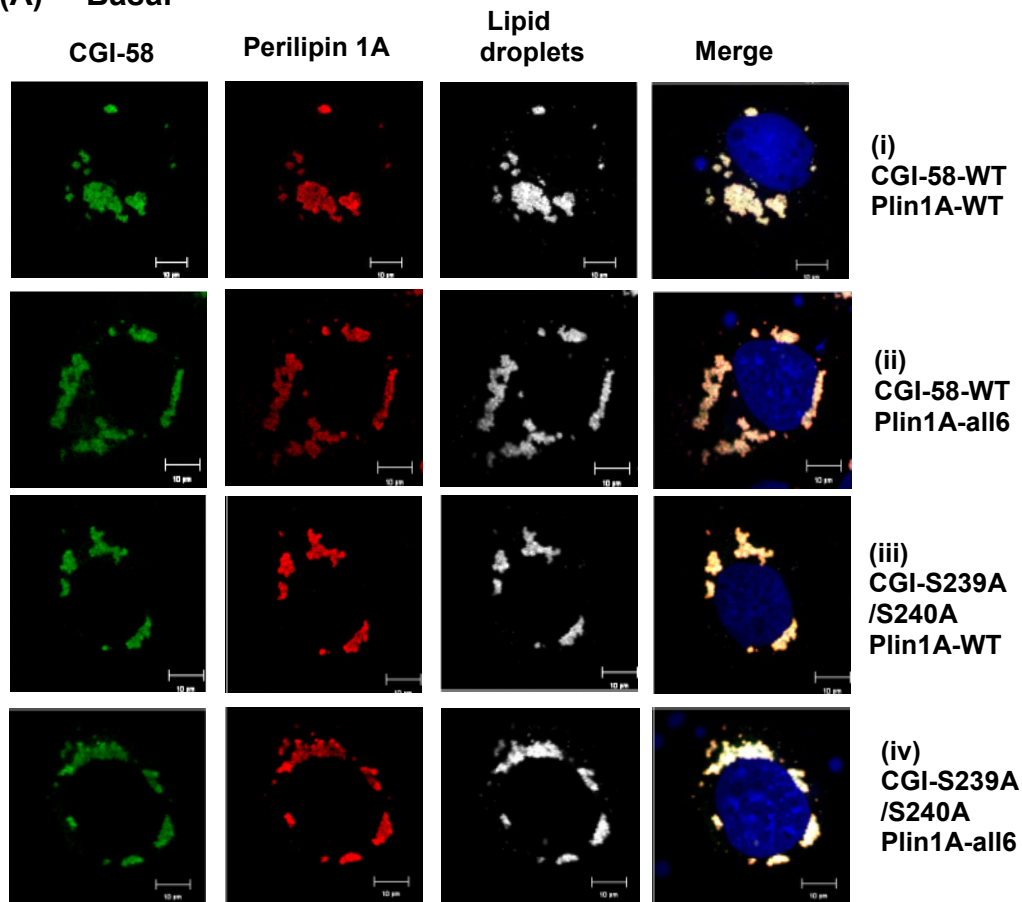
expression of CGI-58. **(C)** For basal TAG determination adenovirally transduced NLSD cells were collected at different time points as mentioned in the figure. Lipids were solvent extracted and TAG was measured by an enzymatic assay. **(D)** Similar to Fig. 8C, TAG was extracted and measured in stimulated (forskolin and IBMX) NLSD cells at different time points, which were transduced with CGI-58 WT and S239A/S240A for 6 hours prior to the treatment. Data in 7C and 7D are depicted as mean $\pm$ standard error of triplicate samples from a representative experiment out of two. Data were analyzed by ANOVA, no significant differences were observed for CGI-58 WT or S239A/S240A neither in basal nor in stimulated condition. **(E)** Immunoblot from basal (7C) and **(F)** from stimulated (7D) NLSD fibroblast cell lysates expressing CGI-58 WT or S239A/S240A.

7.7 CGI-58 serine 239 phosphorylation alters the subcellular localization of CGI-58 upon PKA stimulation:

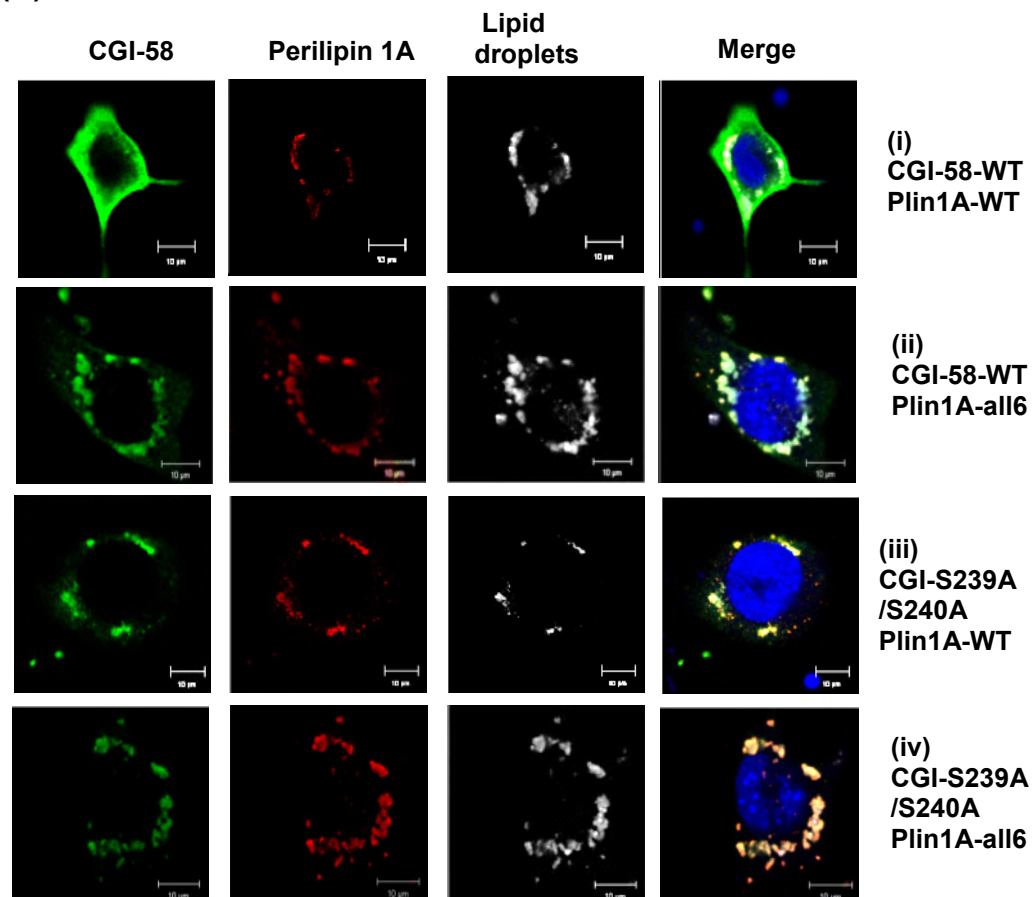
After analyzing the effect of PKA mediated serine 239 CGI-58 phosphorylation on TAG hydrolysis, we next examined the effect of the phosphorylation on CGI-58 subcellular localization. PKA stimulated intracellular lipolysis is a well-defined stepwise process where the major players get either localized to lipid droplets or to the cytosol upon phosphorylation. In adipocytes perilipin 1A coats lipid droplets and sequesters CGI-58 to LDs by binding to it. PKA-phosphorylated perilipin 1A no longer anchors CGI-58 to the LD hence it moves to the cytosol (Granneman, Moore et al. 2009). As perilipin 1A phosphorylation induces the relocation of CGI-58 from LDs to the cytosol, we hypothesized that the PKA-mediated phosphorylation of CGI-58 might also contribute to the translocation of CGI-58 into the cytoplasm in beta-adrenergic stimulated cells. To validate this we used two different cell types, namely Cos7 and NIH3T3CAR Δ fibroblasts, both lacking endogenous CGI-58. Different variants of CGI-58, either WT or S239A/S240A mutated CGI-58, were co-expressed with perilipin 1A WT (Plin1A-WT) or Plin1A-all6 (perilipin 1A lacking serine residues in all 6 known consensus PKA sites) were transduced into both cell types by adenovirus transduction. Adenoviral titers were adjusted to drive equivalent levels of protein expression for both variants of perilipin 1A and checked by immunoblotting (Fig. 10E i-ii). Cells were lipid loaded for 12 hours to

increase LD formation, which is required for perilipin 1A protein stabilization. This was followed by treatment with either DMSO (basal condition) or forskolin and IBMX (stimulated condition). The cells were then fixed with formaldehyde and immunostained for perilipin 1 and CGI-58. Microscopy results show that under basal conditions CGI-58 WT and S239A/S240A are localized to LDs, where the docking protein perilipin1A is present in either WT or mutant form (Fig. 10A i-iv). Under PKA stimulated conditions in combination with perilipin 1A CGI-58 WT is localized to the cytosol (Fig. 10B i). On the other hand, S239A/S240A mutated CGI-58 either in combination with Plin1A-WT or Plin1A-all6 mutant did not translocate to the cytosol under stimulated conditions (Fig. 10B ii-iv). The effect was greater when both proteins (CGI-58 and perilipin 1A) were mutated (Fig. 10D). This proves that PKA mediated CGI-58 phosphorylation on serine 239 contributes to its dispersion from LDs where perilipin1A is expressed.

(A) Basal



(B) Stimulated



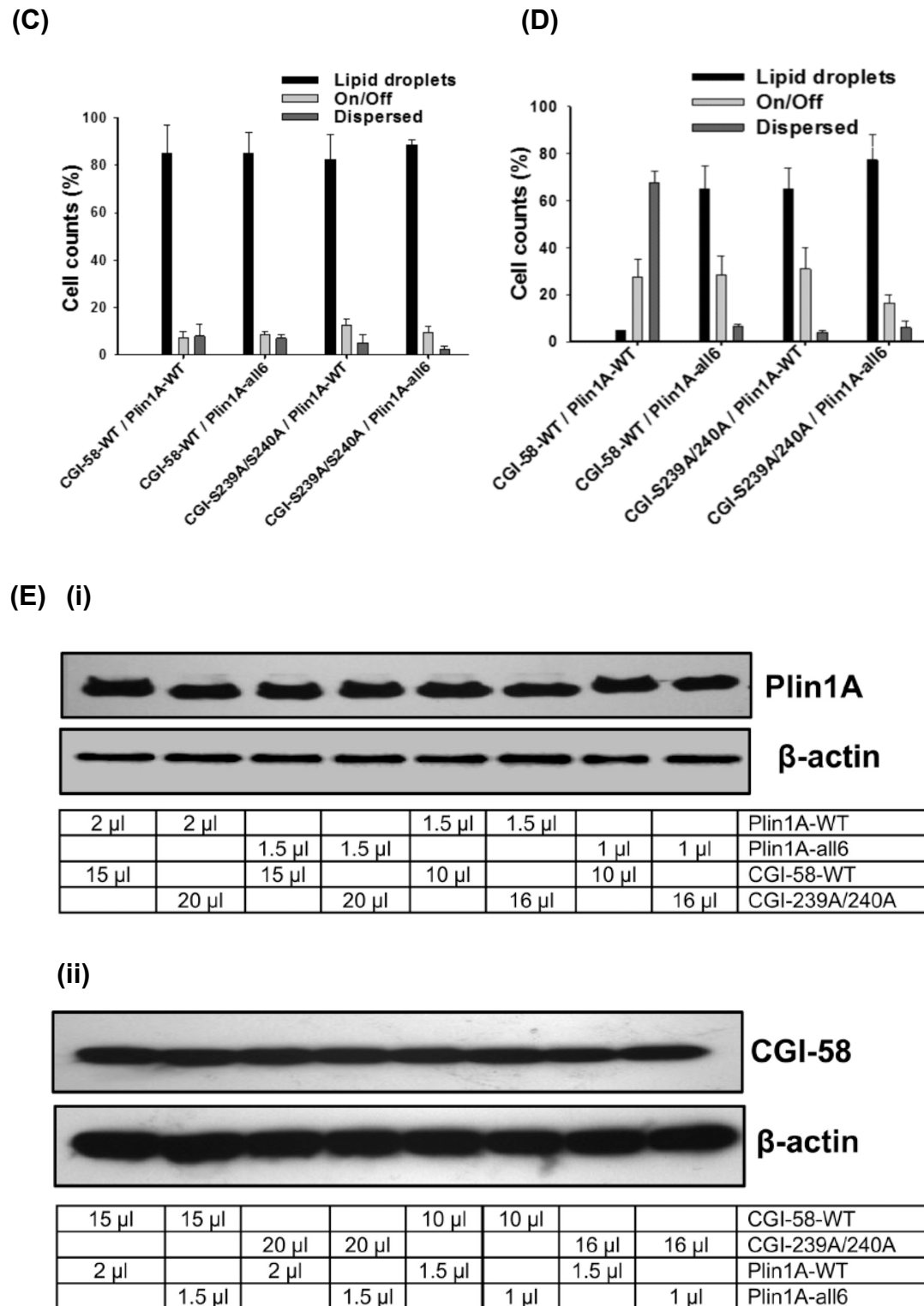


Figure 10.: CGI-58 phosphorylation interrupts its localization to the cytosol upon PKA stimulation: (A) Confocal microscopy images of Cos7 cells under basal condition (DMSO), showing the localization of WT or S239A/S240A CGI-58 to lipid droplets together with perilipin 1A and perilipin 1A-all6 PKA sites mutant (A i-iv). (B) Micrographs of Cos7 showing the localization of CGI-58 WT and

S239A/S240A in combination with perilipin1A and perilipin 1A-all6 PKA sites mutant under PKA stimulated condition (B i-iv). To stimulate PKA cells were treated with forskolin + IBMX subsequently fixed and immunostained with CGI-58 and perilipin antisera. Cells were additionally stained with Hoechst 33422 for the detection of nuclei, and BODIPY 493/503 for the detection of lipid droplets. **(C)** Average cell counts were determined by counting more than 50 cells per condition for both basal and **(D)** stimulated cells. The figure legends “Lipid Droplets” describes signal of CGI-58 mainly observed on lipid droplets. “On/Off” defines the signal for CGI-58 found both on lipid droplets and partially diffuse in the cytoplasm. “Dispersed” describes the signal for CGI-58 predominantly found in the cytoplasm. Images are representative of one experiment out of 5. **(E)** Immunoblot after adjusting the adenoviral titer of different mutants to drive equivalent protein expression. Cos7 cells were transduced with different amounts of CGI WT, CGI-58 S239A/S240A, perilipin1A WT and Perilipin1A-all6 adenovirus in combinations for 48 hours as shown in Fig. 10E, followed by immunoblotting for either CGI-58 (i) or perilipin 1A (ii). Endogenous actin was used as protein loading control (lower panels of Fig. 10E).

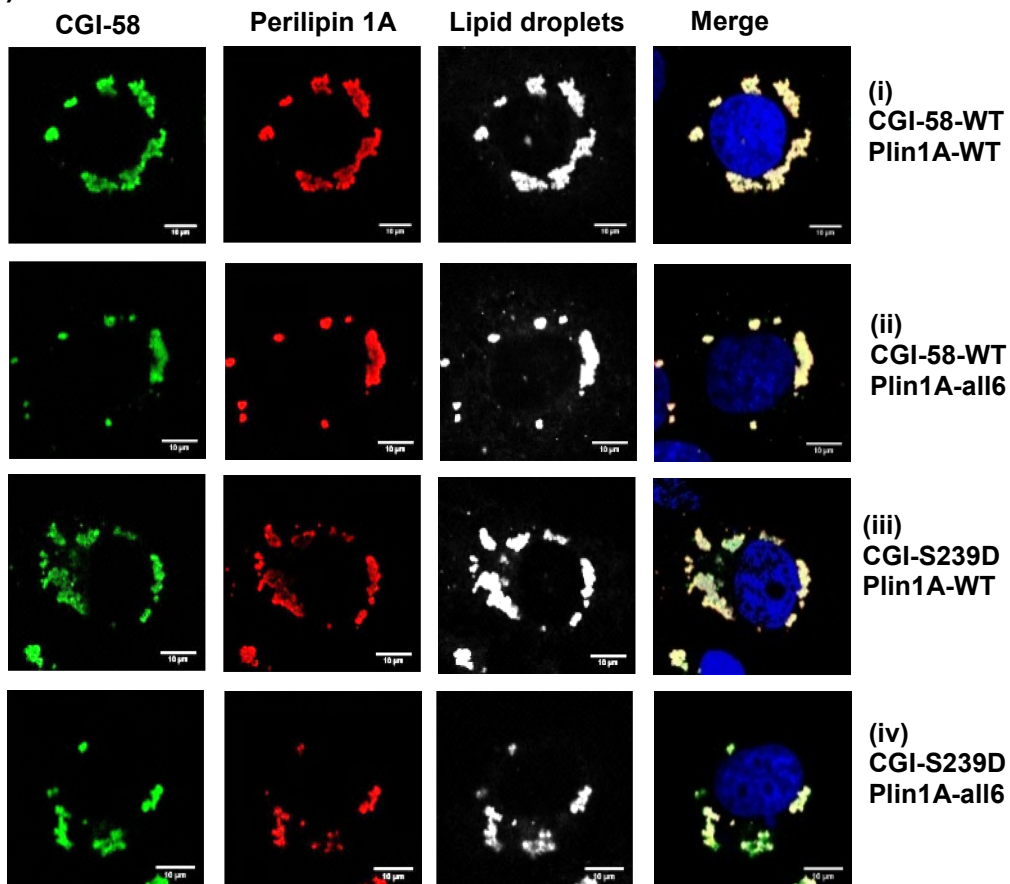
Table 2. Protein colocalization analysis by determination of Manders coefficients using ImageJ: Micrographs from Fig. 9 (basal and stimulated condition) were subjected to automatic analysis using ImageJ JACoP plugin for colocalization of CGI-58 with either perilipin 1A or lipid droplets. Manders coefficients 2 (M2) define the overlap between the fluorescent signals with different wavelengths; an M2 of 0.00 indicates no colocalization, whereas 1.00 indicates complete colocalization.

Expressed proteins		Colocalization of CGI-58 with perilipin 1A (M2)		Colocalization of CGI-58 with lipid droplets (M2)	
CGI-58	Perilipin 1A	basal	stimulated	basal	stimulated
WT	WT	0.86 ± 0.03	0.46 ± 0.03	0.86 ± 0.02	0.46 ± 0.03
WT	All6	0.98 ± 0.01	0.65 ± 0.03	0.96 ± 0.01	0.63 ± 0.03
239A/240A	WT	0.88 ± 0.02	0.65 ± 0.03	0.79 ± 0.03	0.62 ± 0.03
239A/240A	All6	0.83 ± 0.03	0.84 ± 0.04	0.80 ± 0.03	0.88 ± 0.03

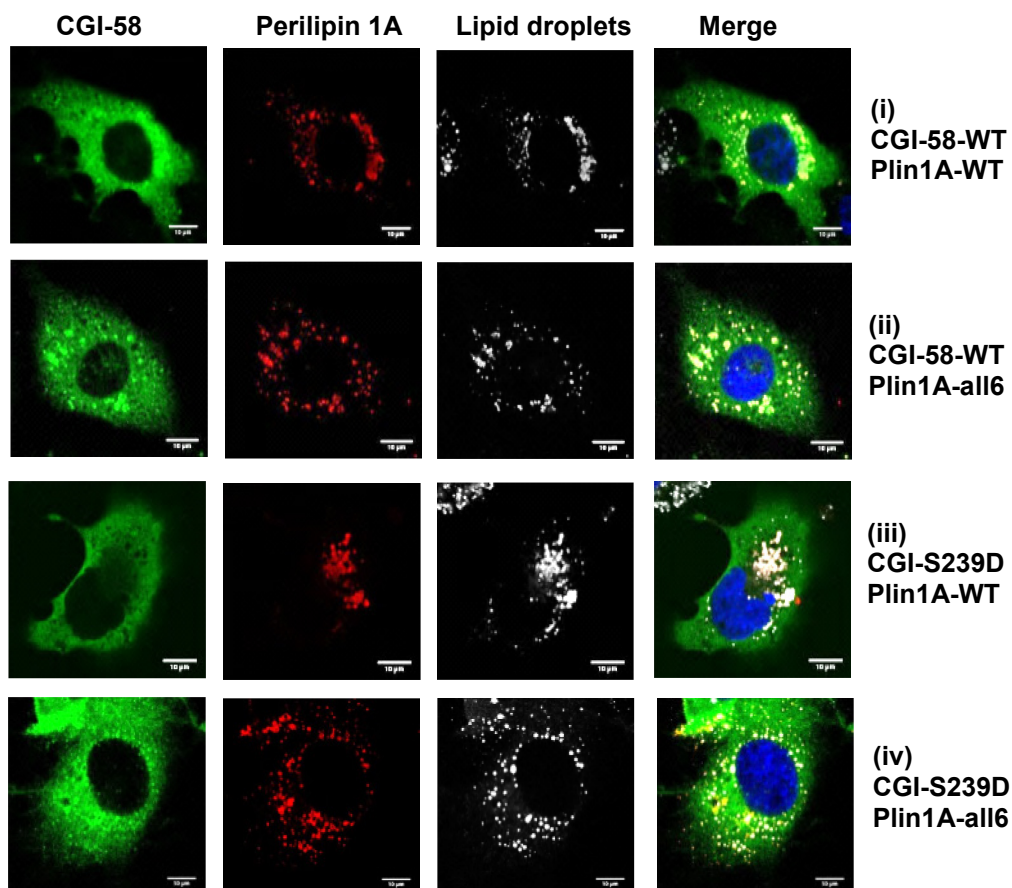
7.8 In contrast to CGI-58 S239A, CGI-58 S239D mutant effectively disperses into the cytosol upon PKA stimulation:

Next we examined the effect of constitutively phosphorylated serine 239 on the translocation of CGI-58. This was accomplished by mutating serine 239 to aspartic acid (D) which mimics the negative charge of a phosphate group. The effect of S239D mutation was tested for colocalization in basal and PKA stimulated conditions similar to S239A in section 7.7. Results show that the addition of a negative charge to amino acid 239 of CGI-58 is insufficient to cause a dispersion of CGI-58 from perilipin 1A-coated lipid droplets under basal conditions. However, PKA stimulation leads to efficient dispersion of S239D CGI-58 into the cytoplasm (Fig. 11). These data suggest that the negative charge at position 239 is important for CGI-58 dispersion into the cytosol after phosphorylation by PKA.

(A) Basal
CGI-58



(B) Stimulated



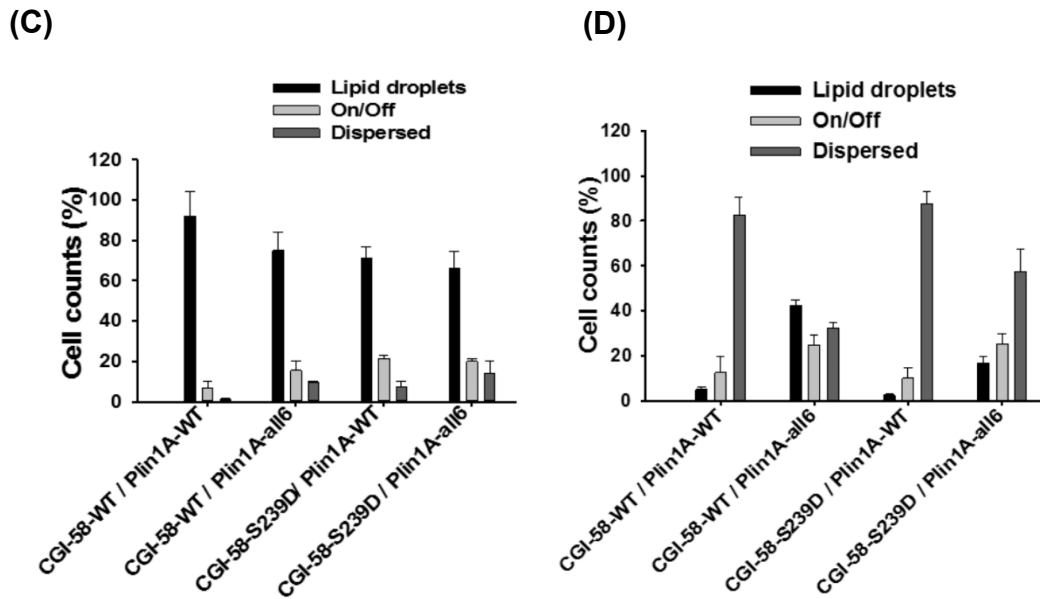


Figure 11. S239D mutated CGI-58 localizes to lipid droplets coated with perilipin 1A under basal conditions and disperses into the cytosol under PKA stimulated conditions: Cos7 cells were transduced with adenovirus for the expression of WT perilipin 1A (Plin1A-WT; i, iii) or mutated perilipin 1A (Plin1A-all6; ii, iv) for 12 hours. CGI-58-WT (i, ii) or CGI-58 S239D (iii, iv) in the pcDNATM4/HisMax vector (Life Technologies) were transfected by using Lipofectamine[®] 3000 (Life Technologies) for 12 hours. Afterward, cells were lipid loaded with oleic acid (200 μ M) for 12-16 hours. Cells were then incubated with either DMSO (Basal) or PKA activator (10 μ M forskolin, 0.5 mM IBMX) (stimulated). in 2% fatty acid-free BSA in DMEM for 30 min at 37°C and 5% CO₂. Cells were processed for immunostaining as explained earlier in this thesis, with CGI-58 and perilipin 1A antisera. Lipid droplets were stained with Bodipy 493/503 (white) and nuclei were also counter stained with Hoechst 33422 (blue). (A) Micrographs showing staining and localization under basal conditions. (B) Micrographs of stimulated cells. Blind cell count averages of 50 cells per condition counted by 2 individuals in both (C) basal and (D) stimulated conditions were plotted to check average colocalization. “Lipid Droplets” designates the signal for CGI-58 primarily on lipid droplets. “On/Off” designates the signal for CGI-58 both on lipid droplets and diffuse throughout the cytoplasm. “Dispersed” designates the signal for CGI-58 primarily diffused throughout the cytoplasm.

8. DISCUSSION:

In this part of the thesis the role of the newly identified CGI-58 serine 239 phosphorylation site was established. The central aim of this part of the thesis was to determine if new unknown phosphorylation events by PKA could contribute to the regulation of intracellular TAG hydrolysis, as little is known about the mechanism by which CGI-58 could regulate ATGL. We have used a variety of approaches to identify a unique site at CGI-58 which is phosphorylated by PKA *in vitro* and *in vivo*. We also put in substantial effort to identify the functional importance of this newly discovered phosphorylation site on CGI-58. A number of experimental approaches were used to provide enough evidences to prove that CGI-58 is a PKA substrate. Firstly, CGI-58 recombinant proteins carrying different mutations were used to identify *in-vitro* phosphorylation of CGI-58 by PKA in a kinase reaction. Secondly, *in-vivo* occurrence of the CGI-58 phosphorylation was confirmed by immunoprecipitating CGI-58 from fibroblast cells and white adipose tissue in basal and PKA stimulated states. Immunoprecipitated CGI-58 was blotted and probed with pS/T PKA substrate antibody. Additionally, we also confirmed the site of phosphorylation at serine 239 by different mutational studies and mass spectrometry. Most of our studies were conducted using murine CGI-58; human CGI-58 is likely to be an equal substrate for PKA, given the complete conservation of the PKA consensus sequence in chordates.

PKA mediated phosphorylation of multiple substrates including perilipin 1A are critically important for hormone stimulated intracellular lipolysis in adipocytes. Perilipin 1A on the lipid droplet surface regulates lipolysis in adipose tissue. On the one hand it acts as barrier for lipases under basal condition; on the other hand upon multiple phosphorylation it allows access of lipases to lipids to hydrolyze them in order to fulfill the energy demands in stimulated condition. Apart from perilipin 1A PKA has another important substrate HSL, which plays an important role in intracellular lipolysis. PKA mediated phosphorylation of HSL is required to trigger the movement of the lipase to lipid droplets from the cytosol (Egan, Greenberg et al. 1992, Brasaemle, Levin et al. 2000, Su, Sztalryd et al. 2003, Sztalryd, Xu et al. 2003), and to activate lipase activity (~100 fold), yet by a poorly understood mechanism. Another two essential components involved in intracellular lipolysis are CGI-58 and ATGL. CGI-58 binds to unphosphorylated perilipin 1A. PKA stimulated phosphorylation of perilipin1A releases CGI-58 from LDs to the cytosol where it

can interact with ATGL (Granneman, Moore et al. 2009). Fluorescence studies in stimulated lipolysis have shown that ATGL and CGI-58 colocalize in close vicinity on lipid droplets to activate TAG hydrolysis (Granneman, Moore et al. 2009). This ATGL/CGI-58 duo efficiently degrades TAG to DAG and FFA. In this thesis we establish that PKA mediated phosphorylation of CGI-58 also plays a role in the dispersion of CGI-58 from the perilipin scaffold, to enable CGI-58 interaction with ATGL. A previous study has demonstrated that human truncation mutations in perilipin 1A that disrupt sequestration of CGI-58 to lipid droplet under basal condition lead to elevated lipolysis (Gandotra, Lim et al. 2011). Hence, the contribution of subcellular localization of CGI-58 in regulating lipolysis is an important fact.

In this study we present evidence that PKA mediated phosphorylation of CGI-58 is an important step in the activation of lipolysis where perilipin 1A is the major LD associated protein. However in the cells where other members of the perilipin family are expressed, this phosphorylation might not be an equally significant event in lipolysis. Five members of the perilipin family are the main constituents of lipid droplet associated proteins to date (perilipin 1-5). Perilipin 1 expression is robust and mainly limited to adipocytes from white and brown adipose tissue whereas in non-adipose tissue cell types like adrenal cortex, testes and ovaries it is basically missing (Greenberg, Egan et al. 1991). Nevertheless, CGI-58 is expressed in these above mentioned steroid producing cells yet not much is known about its role in lipolysis. Perilipin 2 is present in the cells where smaller lipid droplets are present like fibroblasts; it has been shown to play a role in the control of lipolysis in non-adipose tissue but not to be phosphorylated by PKA. However, protein-protein interaction between perilipin 2 and CGI-58 has been reported (Yamaguchi, Omatsu et al. 2006). In human skin fibroblast cells, where lipid droplets are smaller in size and are coated with perilipin 2 it is unlikely that phosphorylation of CGI-58 plays a role in facilitating lipolysis since our results show that stimulated NLSD fibroblasts did not exhibit any acceleration in lipolysis despite heterologous CGI-58 expression. Moreover, CGI-58 shows a diffuse pattern in cytosol when perilipin 2 is the predominant lipid droplet protein (Subramanian, Rothenberg et al. 2004). Further experiments should be carried out in the system containing perilipin 1 to study the effect of CGI-58 phosphorylation on serine 239 on TAG hydrolysis. Perilipin 5 is another important perilipin family member coating

lipid droplets which has been recently shown to play a role in lipolysis in oxidative tissues like skeletal muscle, heart and liver (Wolins, Quaynor et al. 2006, Yamaguchi, Matsushita et al. 2006, Dalen, Dahl et al. 2007). Perilipin 5 interacts with CGI-58 and localizes on LDs in basal condition, and the release of CGI-58 from perilipin 5 is required for PKA stimulated lipolysis. Interestingly CGI-58 is not required for ATGL localization to lipid droplets (Wang, Bell et al. 2011). It has been shown before that ATGL is not a PKA substrate but perilipin 5 is a possible substrate of PKA, as cells expressing perilipin 5 have been shown to exhibit increased lipolysis under PKA stimulation (Wang, Bell et al. 2011). Additional studies are required to determine if PKA mediated CGI-58 phosphorylation has an impact on lipolysis in cells expressing perilipin 5 through a dispersion mechanism similar to that observed in perilipin 1A expressing cells.

Even though phosphorylated CGI-58 switches its location to cytosol which in turn increases its interaction chances with ATGL in adipocyte, phosphorylation of CGI-58 does not affect the activation of ATGL itself. Studies in the recent years suggest that lipolysis is a complex process which is controlled by multiple phosphorylation steps and protein-interaction between perilipin, lipases and factor(s) which can modulate lipase activity like CGI-58 and G0S2. The mechanism by which CGI-58 activates ATGL to hydrolyze TAG and G0S2 inhibits ATGL to halt lipolysis is yet to be discovered. It has been shown that the N-terminal region in ATGL is the place where CGI-58 and G0S2 bind. (Schweiger, Schoiswohl et al. 2008, Yang, Lu et al. 2010). However, there is no clear evidence that the regulation of ATGL by CGI-58 and G0S2 is not directly competitive for binding sites. We also identified an *in vitro* phosphorylation site of G0S2 at serine 97 which might shed some light on its regulatory mechanism how G0S2 mediates its inhibitory effects on ATGL. However further studies are required to determine its physiological relevance, function and mechanism, as well as the upstream kinase. G0S2 phosphorylation might affect protein stability, protein localization and/or protein-protein interaction by inducing reversible conformational changes.

Here we show that Ser239 of CGI-58 is phosphorylated by PKA *in vitro*, in tissue samples and in cultured cells. Interestingly, 2D electrophoresis revealed 3-4 isoforms of CGI-58 upon PKA stimulation suggesting additional post-translational

modifications. Further studies are needed to address this hypothesis. Another important possibility is that this newly discovered phosphorylation site of CGI-58 could have an ATGL independent function in another PKA mediated mechanism. Besides PKA we have also identified that PKC and AMPK could phosphorylate G0S2 and CGI-58 *in vitro*, but then again these findings are very preliminary and further experiments need to be done to identify the phosphorylation sites and their *in vivo* impact on lipolysis and upstream kinases. It will be fascinating to understand the signaling network between these kinases and their substrates to obtain a bigger picture of how these phosphorylations may act together to maintain energy homeostasis within the cell and tissue.

PART 2

Identification of cyclophilin 1 as a potential novel regulator of lipid droplet size by quantitative proteomics of larval fat body lipid droplets of perilipin deficient flies

9 INTRODUCTION

As mentioned in the first part of the thesis, lipid related diseases, such as obesity, metabolic syndrome, fatty liver disease, diabetes, cardiovascular disorders and cancer are a major public health concern. All of these diseases are associated with a disturbed energy homeostasis leading to abnormal fat accumulation. A better understanding of the underlying mechanism(s) involved in the homeostasis of energy flux would therefore be a promising way to develop alternative therapies. Controlled energy homeostasis is vital for all organisms including humans and insects to ensure survival under diverse conditions of low and high availability of nutrients. Lipids are one of the major sources to provide energy in many organisms during various metabolic stages. Intracellularly they are efficiently packed into lipid droplets (LDs), which serve as specialized organelles to store excess energy in the form of triacylglycerol and sterol esters. Despite the existing knowledge about the role of LDs in energy storage, their function in (patho)physiological processes are poorly understood. Therefore, this part of the thesis focuses on investigation of the lipid droplet proteome which could contribute in regulating the size and number of lipid droplets and consequently to altered energy homeostasis.

9.1 *Drosophila melanogaster* as model:

The fruit fly *Drosophila melanogaster* has been used to study various signaling pathways and genes associated with human diseases due to the availability of the whole genome and having clear orthologs of a large number of disease-associated genes (Reiter, Potocki et al. 2001). Moreover, the fly model is cost effective and due to its short life span and the presence of only four chromosomes makes it attractive for easy generation of transgenic flies. Similarly, availability of large-scale RNAi (RNA interference) libraries allows *Drosophila melanogaster* to be used as model for studying function of a specific gene (loss-of function phenotype) by knock down (KD). Additionally, the use of the GAL4/UAS system allows the expression of specific gene or RNAi in tissue specific manner (Brand and Perrimon 1993, Dietzl, Chen et al. 2007) (Fig. 12).

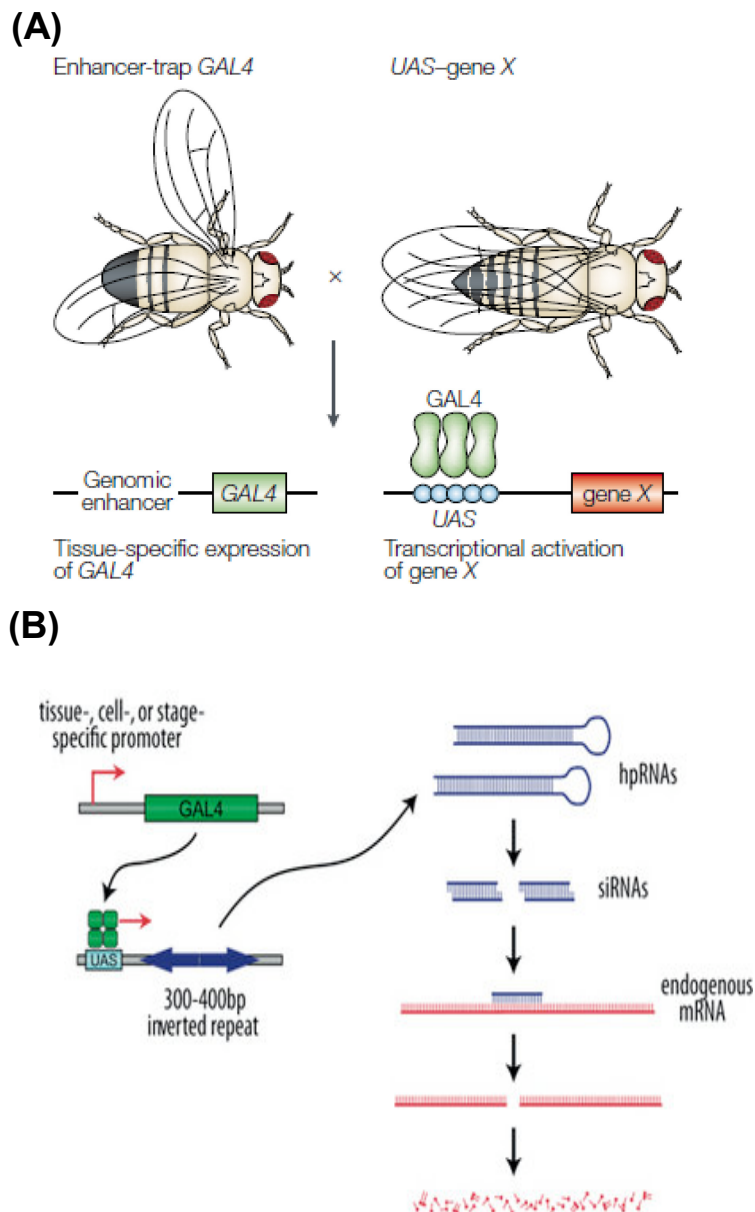


Figure 12. (A) The GAL4/UAS targeted gene expression system (St Johnston 2002): In *D. melanogaster* Gal4 is used for targeted gene expression by inserting Gal4 sequence under a tissue specific promoter. When Gal4 is expressed it can bind to the UAS (Upstream Activating Sequences) element and activate the expression of a gene which is under control of its promoter UAS-element. In the absence of Gal4 the target gene is silent while binding of Gal4 to the UAS-element causes activation of the target gene (gene x) expression. To drive the expression of specific gene flies carrying the target, UAS-gene X are crossed with the flies expressing enhancer-Gal4, termed as drivers. Therefore the expression of the gene X is driven in offspring where Gal4 is expressed. . **(B) The GAL4/UAS transgenic RNAi system in *D. melanogaster*** (modified from

<http://stockcenter.vdrc.at/>): Similar to targeted gene expression Gal4/UAS system is also used for specific gene inactivation in vivo in combination with RNAi. UAS-driven RNAi can be triggered by expression of double stranded RNAs like hairpins RNAs, which is processed into small fragments, that is also known as small interfering RNAs (siRNA) by Dicer enzymes. These siRNAs are capable of binding to the endogenous complementary mRNAs in combination with Argonaut protein leading to sequence specific degradation of target mRNA and consequently to target gene inactivation.

Considering the above facts *Drosophila* provides a convenient model for studying the function of a gene/protein involved in various cellular processes.

9.2 Intracellular lipid metabolism in fat body of *D. melanogaster*:

Like in mammals, intracellular lipid storage and mobilization is highly regulated in insects. Triacylglycerols (TAGs) in LDs are the main form of stored lipid and energy in *D. melanogaster* and LDs are present at all developmental stages. LD have a neutral lipid core which is surrounded by a phospholipid monolayer where LD associated proteins are present on the monolayer playing important roles in the regulation of TAG hydrolysis in coordination with energy requirements of the organism as well as many other processes. Recently *D. melanogaster* has appeared as a highly relevant model for LD related metabolism studies due to the conservation of many metabolic pathways in humans and flies and the presence of LD associated proteins in flies that have been shown to have clear orthologs in humans. Perilipins (lipid droplet storage 1 and 2, LSD1 and 2) (Greenberg, Egan et al. 1991, Beller, Bulankina et al. 2010) and ATGL (Brummer) (Gronke, Mildner et al. 2005, Lass, Zimmermann et al. 2006) are the key regulators of TAG lipolysis in mammals as well as in *D. melanogaster* respectively. Not only the above-mentioned proteins but also the overall emerging model of hormone-regulated lipolysis seems evolutionarily preserved between mammals and *D. melanogaster*. In this model perilipin 1 (LSD1) can be phosphorylated upon release of circulating adipokinetic hormone (Akh) which mediates activation of Protein kinase A. PKA activation leads to an increase lipolytic activity by scaffolding different lipases like brummer

and its activators onto the surface of lipid droplets in the fat body (Gronke, Mildner et al. 2005, Patel, Soulages et al. 2005). Similarly, in mammals catecholamine binds to G-protein coupled receptor leading to PKA activation which phosphorylates multiple substrates including mPLIN1 (mammalian perilipin 1) followed by recruitment of mATGL (mammalian Adipose triglyceride lipase) and mHSL (mammalian hormone sensitive lipase) on lipid droplets causing an increase in TAG hydrolysis in adipose tissue (Lass, Zimmermann et al. 2011).

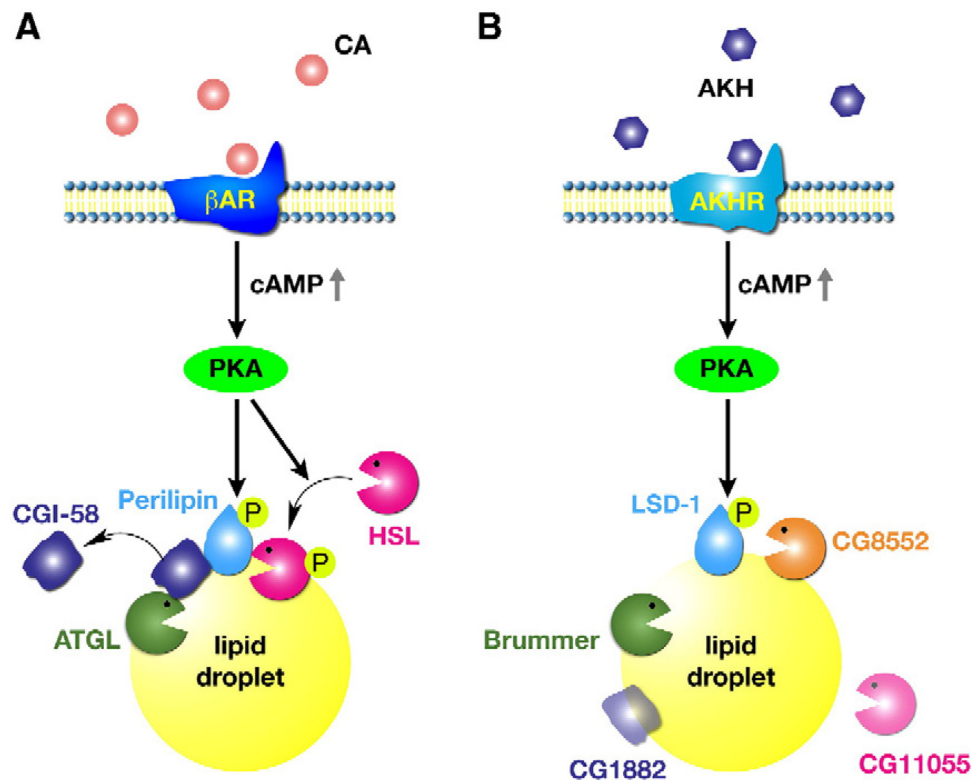


Figure 13. Hormone-stimulated lipolysis is preserved between mammals and *D. melanogaster* (Bickel, Tansey et al. 2009): Scheme depicting resemblances between A) mammalian and B) insect mechanism of hormone regulated lipolysis. In insects lipolysis is stimulated by adipokinetic hormone (AKH) binding to G protein coupled receptor, adipokinetic hormone receptor (AKHR) whereas in mammals this is accomplished by catecholamine binding to β -adrenergic receptor. In both cases this leads to increase cAMP concentration which activates protein kinase A (PKA). Activated PKA phosphorylates mPLIN1 and dPLIN1 (drosophila

perilipin1/LSD1) which recruits lipases to lipid droplet surface providing the access to stored TAGs. In Fly Bummer (bmm/dATGL) an ATGL ortholog and CG11055 an HSL ortholog seems to be major lipases known so far playing main role in TAG hydrolysis. In mammals PKA stimulation phosphorylate multiple substrates including mPLIN1, mHSL and mATGL leading to change in the localization and interaction behavior of each of them. Phosphorylated perilipin in mammals leads to release of CGI-58 to cytosol where mATGL and mCGI-58 interacts followed by recruiting mATGL to LD surface to access the stored TAG. In fly ortholog of CGI-58 (CG1882) has been found to be associated with lipid droplets though functional characterization of it is yet to be elaborated.

9.3 PERILIPINS - PAT family LD proteins and fat storage in *D. melanogaster*:

The fly fat body corresponds to mammalian adipose tissue, which not only plays a role in energy storage and utilization but also as an endocrine organ. The fat body is a loose tissue surrounded by hemolymph to provide quick response to changes in energy state (Arrese and Soulages 2010). Fat body cells are filled with different sizes of lipid droplets providing the major energy storage site in insects (Beller, Thiel et al. 2010). The morphology of the fat body changes during developmental stages of the fly: for example, during larval stage it occupies the majority of the space of the body cavity since the fly needs to accumulate substantial amount of energy reserves in the fat body to go through metamorphosis (Arrese and Soulages 2010). Intracellular energy is stored in the form of triacylglycerides in LDs; its expenditure requires mobilization of triacylglycerides by hydrolysis, which is highly regulated by LD surface proteins like perilipins (Zechner, Zimmermann et al. 2012) as described above.

Perilipin was first discovered in laboratory of Prof. Constantine Londos in 1990s as lipid droplet associated PKA target protein serving as a barrier between lipases and stored TAG in adipocytes (Egan, Greenberg et al. 1990, Greenberg, Egan et al. 1991). Eventually, more lipid droplet proteins having sequence similarity with perilipin were discovered; all together these proteins are known as the PAT family proteins. Mammalian PAT family proteins include (old nomenclature in brackets)

perilipin 1 (perilipin), perilipin 2 (adipocyte differentiation-related protein (ADRP), and perilipin 3 (tail-interacting protein of 47 kDa (TIP47)), perilipin 4 (S3-12) and perilipin 5 (OXPAT). Non-mammalian members include perilipin 1 (lipid storage droplet 1 (LSD1), perilipin 2 (lipid storage droplet 2 (LSD2) in flies and MPL1 in fungi (Gronke, Beller et al. 2003, Wang and St Leger 2007). This evolutionary conservation between species from fungi to human indicates towards the significance of the PAT family proteins in regulation of intracellular lipid metabolism. Expression of these different mammalian perilipin (1-5) members varies in different tissues as well as at different stages of lipid droplet biogenesis. Perilipin 1 is exclusively expressed in adipose tissue while perilipin 2 (ADRP) and perilipin 3 (TIP47) are ubiquitously expressed (Brasaemle 2007). Perilipin 1 and 2 polymorphisms in mammals have been associated with metabolic diseases. Perilipin 1 single nucleotide polymorphism (PLIN 14995A>T) is proposed as an obesity risk marker (Qi, Tai et al. 2005). While perilipin 1 is intensively studied, very little is known about perilipin 4 (S3-12) whose expression has been found in limited amounts but in multiple mammalian tissues with the highest in white adipose tissue and little in heart and skeletal muscle. Perilipin 4 mainly coats nascent lipid droplets and has thus been hypothesized to be involved in LD biogenesis (Wolins, Skinner et al. 2003). Not only perilipin 4 but also perilipin 2 and 3 have also been documented in playing a role in LD biogenesis (Bulankina, Deggerich et al. 2009). On the other hand perilipin 5 (OXPAT) has been more recently found to be expressed in fatty acid oxidizing tissues like heart, skeletal muscle, brown adipose tissue (BAT) and liver (Dalen, Dahl et al. 2007). Existence of multiple perilipins and their differential expression pattern within an organism and their functional redundancy makes it difficult to understand the specific role of a particular perilipin as it would be required to have an organism model which could express only one perilipin at a time. Therefore, drosophila could serve as an important model to study the role of perilipins, as the complexity of the perilipin system is relatively simple due to the presence of only two perilipins.

The N-terminal ~100 amino acid sequence (PAT region) of fly perilipin 1 (LSD1) and perilipin 2 (LSD2) is conserved which is important for lipid droplet localization (Brasaemle 2007). Not only sequence similarity to mammalian perilipins, but also their similar regulation by PKA and functional similarity in regulation of lipid metab-

olism makes them interesting targets for functional investigations. Both insect PAT proteins have been shown to be present on the lipid droplet surface in different developmental stages, such as embryonic, larval and adult stages. Fly perilipin 2 (LSD2) mutation leads to 50% reduced TAG levels verifying its role in lipid storage (Teixeira, Rabouille et al. 2003) and overexpression leads to increase fat accumulation in fly fat bodies. Similarly, perilipin 2 knock out mice on long term high fat diet show decreased serum TAG levels and resistance to diet induced obesity (McManaman, Bales et al. 2013). In contrast, drosophila perilipin 1 (LSD1) mutation leads to excess fat accumulation leading to onset of obesity and hyperphagia (Beller, Bulankina et al. 2010). However, perilipin1 knock out mice were not only lean with substantial reduction in fat depot, but also showed resistance towards diet induced obesity. Moreover, they showed a higher abundance of perilipin 2, (Tansey, Sztalryd et al. 2001) which may indicate functional redundancy of perilipin 1 and 2 by partly compensating perilipin 1 absence.

Different perilipins not only contribute in fat storage and mobilization, but they have also emerged as important structural proteins for lipid droplets. Prof. Ronald P. Kuehnlein's laboratory has recently shown the structural impact of perilipins on LD size in *Drosophila melanogaster*. Perilipin1 (LSD1) deficient flies are obese and their fat bodies depict a giant lipid droplet phenotype (Beller, Bulankina et al. 2010). However, the underlying mechanism remains to be studied. This giant LD phenotype appears during larval stage and becomes predominant in immature adults by acquiring uniocular fat body cell appearance. Fascinatingly, this giant LD phenotype is independent of excess fat accumulation in *plin1* mutant flies, which they have confirmed by looking at the size of different *mdy plin1* and *mdy bmm* double mutant flies, who have similar body fat content and are similarly lean animals. However, lipid droplets of *mdy plin1* are bigger in size than *mdy bmm* double mutants (Beller, Bulankina et al. 2010). Here, *mdy* represents a mutant of drosophila diglyceride acyltransferase (dDGAT1), which catalyzes TAG synthesis from DAG, and *bmm* represents brummer (dATGL). Ronald Kuehnlein's laboratory has shown multiple functions of drosophila perilipin 1 including the involvement in fat storage control and mobilization by being a component of the AKH/AKHR signaling pathway, modulating brummer lipase localization and determining size of lipid droplets in fat body. However, mechanism and factors involved in lipid droplet

size regulation are yet unclear. Therefore, to increase our understanding of factors involved in LD size regulation, we have used different mutants of perilipins from *D. melanogaster*, hypothesizing that the protein coat of LDs is involved in the regulation of LD size.

9.4 Regulation of lipid droplets size:

Lipid droplets are not only a way for cells to cope with excess lipids or provide energy in crisis but have been discovered to play many other roles which includes storing proteins like histones, being an active member of the cellular membrane transport system etc (Zehmer, Huang et al. 2009, Li, Thiel et al. 2012). Lipid droplets exist in various sizes depending upon many factors like cell type, organism and the metabolic status of the organism (Suzuki, Shinohara et al. 2011). Their size can vary from few hundred μm in mammalian adipocytes to less than 1 μm in diameter in insect fat body and can change upon pathological conditions like liver steatosis (Brasaemle, Dolios et al. 2004, Beller, Riedel et al. 2006, Matsusue, Kusakabe et al. 2008). Generally, adipocytes are known to have larger LDs than non-adipocyte cells. Proteins like PAT family members not only play important roles in LD size regulation in adipocytes, but absence of them in mouse hepatocytes has been shown to lead to a strong increase in LD size and decrease in the number of LDs (Bell, Wang et al. 2008). Giant lipid droplets can efficiently store excess lipids to prevent immediate lipotoxic stress but the increase in volume causes a decrease in available surface area for accommodating proteins required for lipolysis on the LD surface. LDs are usually found in close proximity to the endoplasmic reticulum (ER), mitochondria and peroxisomes irrespective of cell types (Novikoff, Novikoff et al. 1980, Stemberger, Walsh et al. 1984). This physical close contiguity indicates active exchange between LDs and these organelles. Core neutral lipids and surface phospholipids, required for either LD formation or growth, are largely synthesized in the ER, which harbors diacylglycerol acyl-CoA acyltransferases (DGAT) and cholesterol acyl-CoA acyltransferases (ACAT), two important enzymes catalyzing the last steps of TAG and sterol ester synthesis. This flow of lipids between LDs and ER is thought to be bidirectional and also applies for trafficking of protein between them (Suzuki, Shinohara et al. 2011). Simi-

larly, mitochondrial and peroxisome interaction with LDs are required for the availability of fatty acids for β -oxidation. However, in all these exchanges and trafficking physical contact alone is not sufficient and requires active involvement of proteins like lipid-transfer proteins (LTPs) (Lev 2010).

Biogenesis and growth of LDs remains to be fully explored, however, at least two models for LD growth have been proposed (Figure 14). According to these models, LD expand firstly by on site synthesis of TAG and phospholipids and secondly by coalescence or fusion with each other. For fusion of two LDs they need to come very close to each other, which can involve multiple different types of physical forces. The close proximity leads to the complete fusion of two LDs by a pore formation, which is favored by high surface tension and low line tension. Additionally, maturation of LDs can occur while transfer of molecules by small droplets continuously happens towards bigger droplets. The direction of molecules is decided by Laplace pressure, which is defined as the pressure difference between the inside and outside of a curved surface. (Thiam, Farese et al. 2013). This remodeling of LDs could occur due to a change in metabolic state of the cell or a change in composition of certain lipids like phosphatidylcholine (PC) and phosphatidic acid (PA). Multiple studies have shown that reduced PC/PA ratio correlates with larger lipid droplets (Yang, Galea et al. 2012). Fusion of lipid droplets often requires additional protein factors apart from the physical pressure. Protein like fat-specific protein-27 (FSP-27) or cell death-inducing DFF-54 (CIDE) proteins have been shown to play a role in LD size regulation. Ectopic expression of FSP-27 in adipocytes leads to increase size and decrease LD numbers (Nishino, Tamori et al. 2008). FSP-27/CIDE are LD associated proteins believed to be involved in the pore formation between the droplets and thus facilitating TAG transfer from one droplet to another (Gong, Sun et al. 2011). Another mechanism, which can have a drastic impact on LD size, is expansion by onsite TAG synthesis. Additional proteins, namely seipin and fat storage-induced transmembrane proteins (FITMs), are ER resident proteins capable of altering LD size by so far unknown mechanisms but deficiency of both has led to altered TAG storage and reduced LD size. However, FITMs do not contribute to TAG synthesis but only promote TAG partitioning to LDs (Kadereit, Kumar et al. 2008). In yeast the deletion of seipin leads to formation of LDs which are 20 times larger in volume; similarly in seipin deficient drosophila salivary gland

giant LDs have been observed; however in mammalian cultured cells depletion of seipin is associated with small LD formation (Yang, Galea et al. 2012)

In addition, LD size expansion could be due to relocation to LDs from ER of certain important enzymes involved in TAG and PC synthesis like DGAT2 (diacylglycerol O-acyltransferase 2) and CCT, CTP:phosphocholine cytidyltransferase (Yang, Galea et al. 2012, Wilfling, Wang et al. 2013). LD size also appears to be related to the ratio of TAG/PC which is regulated by the rates of local TAG and PC synthesis and thus to the relative activity of above mentioned enzymes. Larger LDs have a higher TAG/PC ratio as compared to smaller LDs (Krahmer, Guo et al. 2011).

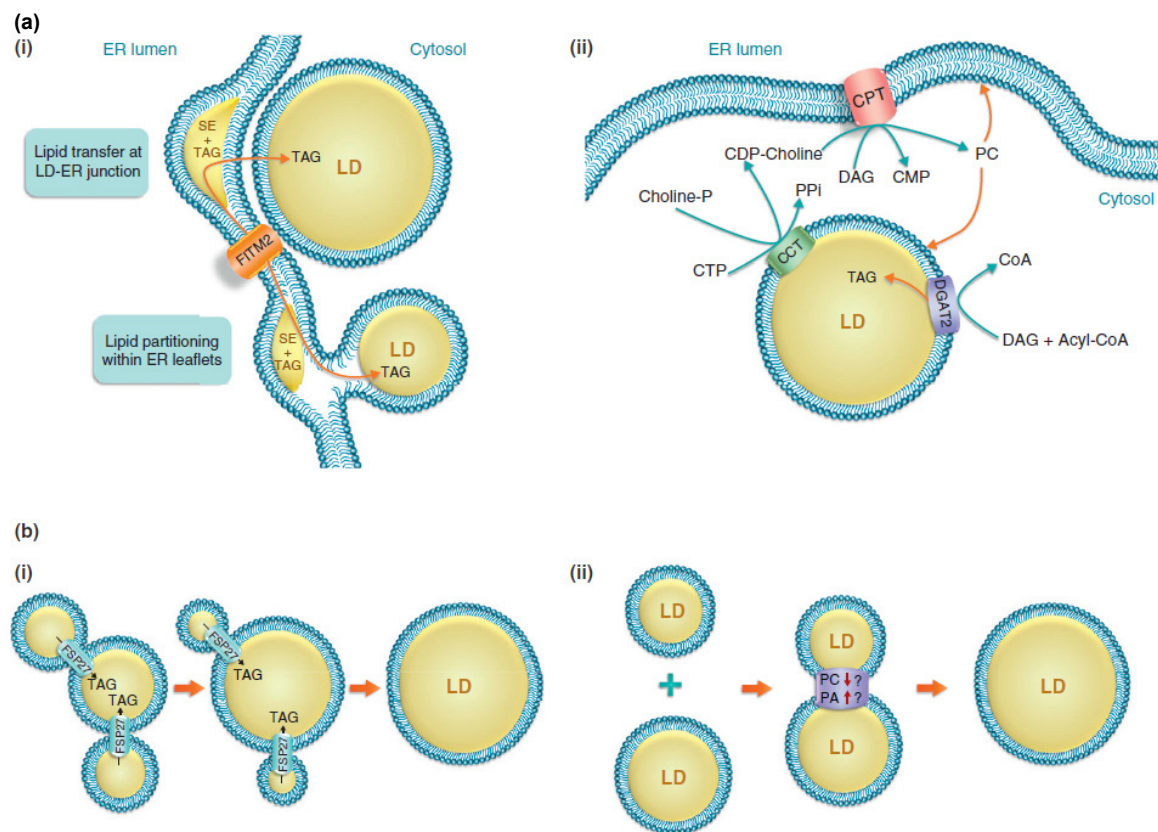


Figure 14. Regulation of LD size (Yang, Galea et al. 2012): Lipid droplet formation may occur within the ER by budding and forming a round structure covered with phospholipid monolayer containing newly synthesized lipid esters. LDs can grow by targeted delivery of TAG into LDs which involves ER resident six trans-membrane domain containing proteins FITM1/2 (ai). LDs as well can grow by locally synthesizing lipids (TAG) and CDP-choline, the precursor of phosphatidylcho-

line (PC). Additional PC synthesis is required to fulfil the increased demand to coat the enlarged surface of growing LDs. This involves CCT, CTP:phosphocholine cytidylyltransferase, which belongs to the family of transferases and catalyzes the rate limiting step in the Kennedy pathway of PC synthesis. Local TAG synthesis required for LD growth is accomplished by DGAT2 (diacylglycerol O-acyltransferase 2). DGAT2 uses diacylglycerol (DAG) and fatty acyl CoA as substrates (aii). In addition, LD can grow through fusion, which can firstly be mediated by LD associated protein fat specific protein-27kDa (FSP-27). FSP-27 might form junctions between droplets to facilitate the exchange of TAG between droplets. However fusion of small LDs to larger LDs could be due to higher internal pressure in the smaller LDs in FSP-27 coated LDs (bi). Secondly, concentration changes in two important phospholipids, phosphatidic acid (PA) and phosphatidylcholine (PC) could result in LD fusion. Detailed mechanism and factors involved remain to be investigated.

Perilipins are additional important protein factors, which have been repeatedly shown to be associated with altered or differential size of LDs. Perilipins have been shown to bind to the ER during LD formation (Wolins, Brasaemle et al. 2006). Perilipin 1 and 2 are only stable when associated with the LD surface however perilipin 3, 4 and 5 can also be stable in the cytosol. Perilipin 1 has been shown to replace perilipin 2 on larger LD surface, whereas perilipin 1 knock out mice have shown reduced LD size, *D. melanogaster* shows inverse results upon perilipin 1 (LSD1) deficiency. Moreover, in mammalian adipocytes perilipin 1 has been seen to activate FSP-27, when co-expressed together lead to larger LDs (Sun, Gong et al. 2013). Above-mentioned facts indicate that the LD proteome plays a critical role in regulating LD size.

Although LDs are getting much attention for their role in lipid storage, lipid mobilization and their association with multiple diseases, the functional association of different sized LDs with these processes and the factors which can influence the size of LDs are not clearly understood. Therefore in collaboration with Prof. Ronald P. Kühnlein, we studied the lipid droplet proteome in dependence of the presence of both, only one or no perilipin in *D. melanogaster*, in order to identify protein fac-

tors regulated by the presence of perilipins and possibly involved in LD size regulation.

9.5 Quantitative proteomics and different isotopic labeling strategies:

Proteomics is a powerful tool to perform large-scale studies to identify novel protein factors and analyze their function in order to answer complex biological questions. The proteome is defined as the entire set of proteins including modifications expressed in a certain cell, tissue or organism at a certain time (<http://www.uts.edu.au/about/faculty-science>). This set of proteins is highly dynamic and dependent on the state and environment of an organism and thus affected by starvation, stress, disease etc. Proteomics thus provides deeper insight into the variation of the proteome, post translation modifications and altered networks. Likewise studying the proteome of certain mutant as compared to its wild type control helps to understand the function of that particular protein, as well as its interaction partners and recruitment of novel proteins in the absence of that particular protein. Proteomics does not only allow identification of proteomes but it allows to measure abundance of proteins with the help of different labeling techniques permitting absolute and relative quantification of proteins in two different samples. In more precise relative quantification approaches peptides are labeled with stable isotopes leading to the addition of a predictable mass difference between the peptides from two different samples. Incorporation of stable isotopes into peptides is possible at different points during sample preparation: 1) by chemical labeling of intact protein, or 2) enzymatically digested peptide or 3) by metabolic incorporation of stable isotope labeled amino acids in cell culture (SILAC) or into the food of a whole organism (Fig. 15).

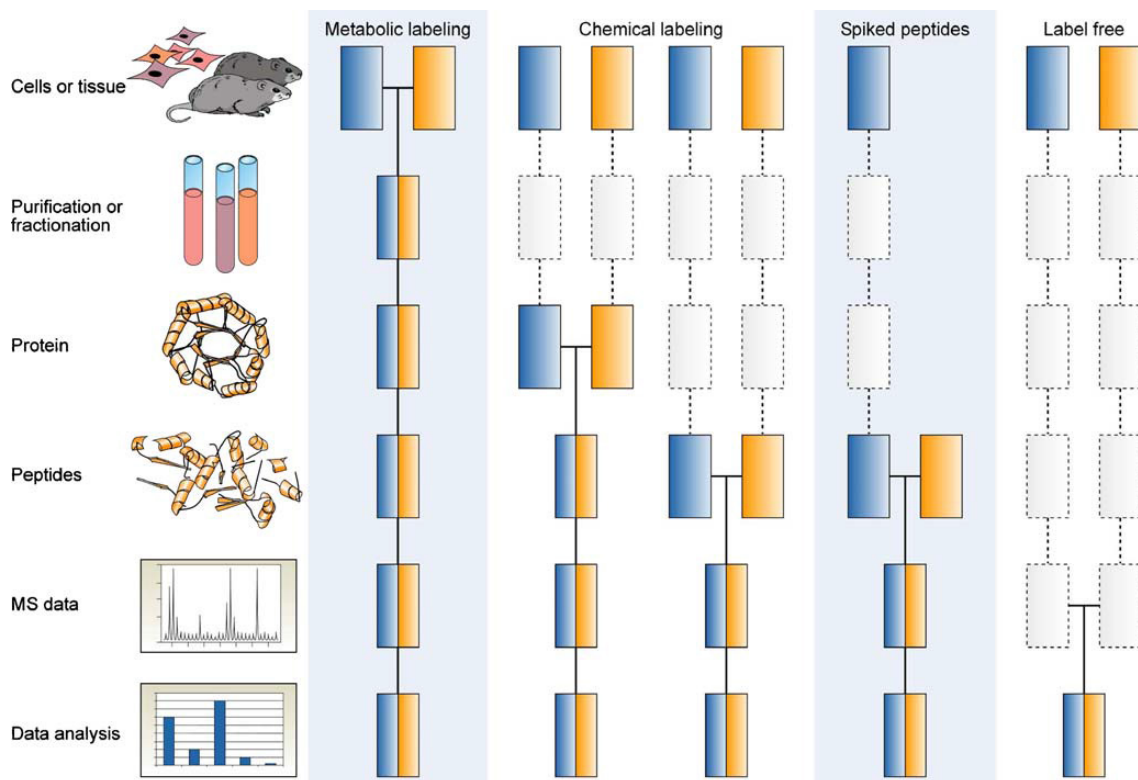


Figure 15. Different quantitative proteomics workflows (Bantscheff, Schirle et al. 2007): Scheme depicting the different labeling strategies to compare two different samples (blue and yellow) by quantitative proteomics. Solid horizontal lines indicate the point when samples are combined and dotted line and boxes indicates where samples are handled separately. As the metabolic labeling happens at the earliest point possible it is considered to be most accurate due to less room for errors during sample preparation. Similarly, the more steps it takes or the later samples are combined increases the possibility of errors. Thus label free quantification is considered to be the least accurate method of the depicted workflows. .

9.6 Stable isotope labeling with amino acids in cell culture (SILAC, metabolic labeling):

As described above (Fig. 15) the earliest point during the sample preparation where samples can be labeled with different stable isotope is metabolic labeling. The SILAC method uses *in vivo* metabolic incorporation of either ^{13}C or ^{15}N heavy labeled amino acids (usually arginine and lysine) into proteins (Oda, Huang et al. 1999, Ong, Blagoev et al. 2002) by growing cells or organisms in medium/food

containing either light (naturally existing) or heavy isotope labeled amino acids. This will produce two almost identical proteomes only their enzymatically-digested peptides from heavy labeled samples will have a constant mass increment compared to the non-labeled/light sample. Mass spectrometry analysis of m/z (m =mass, z =charge) shift is then observed and interpreted with the help of analyzing software to calculate heavy to light ratio, therefore allowing the simultaneous quantification of proteins in two different samples with a minimal introduction of error. Metabolic labeling has many advantages over chemical labeling like its high reproducibility, high labeling efficiency and reduced sample variation or error. However all these advantages come with higher cost of special media/food and the necessity to obtain an almost complete labeling making SILAC less attractive for many experiments. Additionally not every cell line (especially primary cells) or organism grows efficiently in restricted SILAC medium/food and in human tissues metabolic labeling will be practically impossible. Thus chemical labeling is often the preferred, cheaper and more straightforward method used in quantitative proteomics.

9.7 Dimethyl labeling (chemical labeling):

Incorporation of isotopic tags into peptides via chemical labeling is another popular approach for quantitative proteomics which is less expensive compared to SILAC and can be more broadly applied (Boersema, Raijmakers et al. 2009). However, stable isotope incorporation happens at a later stage as compared to SILAC. One of the commonly used chemical labeling strategies is dimethylation of primary amines of enzymatically-digested peptides. Dimethylation is performed to label the N-terminus of the peptides and the ϵ -amino group of lysine residues by using either regular (light) or deuterated (heavy) forms of formaldehyde allowing forming of a Schiff base that is reduced by addition of sodium cyanoborohydride to the reaction to form secondary amines. With dimethyl labeling quantitative analysis of three samples in one run is possible due to the opportunity to generate three different, light (2 CH_3), medium/intermediate ($2 \text{ CD}_2\text{H}$), and heavy ($2 \text{ }^{13}\text{CD}_3$) dimethyl groups on primary amines (Fig. 16). Light labeling is achieved by a combination of regular formaldehyde and cyanoborohydride giving rise to a mass difference of

+28 Da as compared to the unlabeled amine group. A mass difference of +32 Da is possible by using a combination of deuterated formaldehyde and cyanoborohydride, which is termed as intermediate or medium label. Heavy dimethyl label which adds a mass of +36 Da to the amine group, is a product of its reaction with ^{13}C -labeled formaldehyde and cyanoborodeuteride.

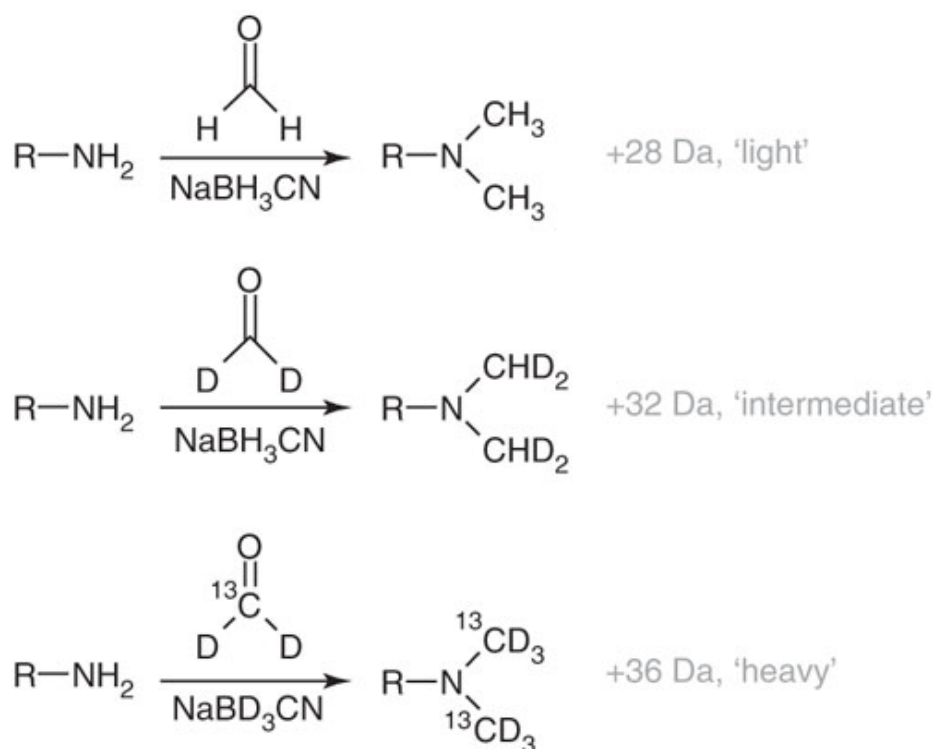


Figure 16. Scheme representing triplex stable isotope dimethyl labeling on N-term of the peptide (adapted from (Boersema, Raijmakers et al. 2009)): The resultant mass differences are due to use of different formaldehyde molecules (regular, deuterated, and deuterated in addition to ^{13}C -labeled formaldehyde) in combination with either cyanoborohydride or cyanoborodeuteride in the dimethylation reaction. .

10 AIM OF STUDY

We hypothesized that perilipins affect recruitment of other proteins to lipid droplets and that the absence of perilipins may thus alter the protein coat of lipid droplets. Moreover, proteome changes may be causative or a consequence of the observed increase in lipid droplet size of perilipin 1 mutants as compared to perilipin 2 mutants, perilipin 1 and 2 double mutants or wildtype. The aim of this second part of my thesis was thus to elucidate how the lipid droplet proteome is altered in the absence of PAT family proteins using *Drosophila melanogaster* (fruit fly) as a model and whether proteome changes can be correlated and maybe even functionally linked to changes in lipid droplet size. In order to address these questions the following approach was taken:

1. Quantitative and qualitative changes of the lipid droplet proteomes of *plin1* (*LSD1*) mutant, *plin2* (*LSD2*) mutant and *plin1/plin2* (*LSD1/LSD2*) double mutant *D. melanogaster* as compared to control flies were analyzed by metabolic and dimethyl labeling to identify LD associated proteins which may be involved in LD size regulation in combination with PAT family proteins.
2. The impact of one selected newly identified protein candidate, namely cyclophilin 1, on lipid droplet size in *D. melanogaster* fat body was investigated.

11 MATERIAL AND METHODS

11.1 Fly maintenance:

If not mentioned otherwise all the flies were maintained and propagated on standard Goettingen food at 25°C and ~60% humidity with a 12h light-dark cycle (standard conditions). Standard Goettingen food typically comprises of 20 L H₂O, 125 g agar, 360 g dry yeast, 200 g soy flour, 440 g treacle, 1.6 kg cornmeal, 1.6 kg malt, 125 ml propionic acid and 30 g nipagine supplemented with few particles of dry yeast in the vial. Young virgin females and males were collected within 4-6 hours of hatching for setting up different crosses, and were kept separated in standard conditions (25°C and 60% humidity, 12h light-dark cycle) until used for a cross. Crawling third instar larvae were collected for fat body isolation to perform different experiments.

11.2 Fly stocks:

Following fly strains used in this study were either received from Prof. Ronald Kuehnlein's laboratory at MPI-Göttingen, Germany, or from the BDSC (Harvard, Boston, MA, USA) TRiP library (www.flyrnai.org) (Table 3).

Table 3. Fly strains used in this thesis: The table indicates the names of the fly strains used in the thesis, internal stock numbers at Prof. Ronald Kuehnlein's laboratory, MPI-Göttingen, Germany and their sources.

No	Name	Stock number	Genotype	Reference/ source
1	white-control	RKF1084	<i>w[1118]</i>	VDRC60000
2	Fat body driver	RKF1421	<i>w*</i> ; <i>+/+</i> ; <i>P{Lpp-GAL4.B}c4/TM3</i> , <i>Sb* float.</i>	Kuehnlein
3	Fat body driver With PLIN1 KO	RKF910	<i>w*</i> ; <i>P{w[+mW.hs]=GawB}FB+</i> <i>SNS / CyO float. ; plin1 [1] /</i> <i>TM6C, Sb [1] Tb [1] float.</i>	Kuehnlein

4	Control	RKF676	w*; plin1[rev1]	Kuehnlein
5	PLIN1 KO	RKF649	w*; plin1[1] / TM3 Sb e float	Kuehnlein
6	PLIN2 KO	RKF610	y* plin2[51] / FM7i; P{w[+mC]= ActGFP} JMR3 or hom or Dp(1;Y) y[+]	Kuehnlein
7	PLIN1/PLIN2 KO	RKF989	y* plin2[51] / FM7i, P{w[+mC] =ActGFP} JMR3 float ; plin1[1]	Kuehnlein
8	Cyp1 RNAi	BDSC330 01	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] =TRiP.HMS00801} attP2	(www.flyrnai.org).
9	Cyp1 RNAi	BDSC339 50	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] =TRiP.HMS00902} attP2	(www.flyrnai.org).
10	Cyp1RNAi/PLIN1 KO	ASF1562	y[1], v[1]; +/+; UAS-Cyp1-RNAi (BDSC33001), Plin1 / TM3, Sb[1] (floating)	Kuehnlein
11	Cyp1RNAi/PLIN1 KO	ASF1563	y[1], v[1]; +/+; UAS-Cyp1-RNAi (BDSC33950), Plin1 / TM3, Sb[1] (floating)	Kuehnlein
12	Cyp1-eGFP	RKF1583	w*; P{UAS- Cyp1::eGFP} attP40 #2M	Kuehnlein

11.3 SILAC labelling of *D. melanogaster*:

The lysine auxotrophic *S. cerevisiae* strain BY4741-lys2 (MATa; his3 Δ 1; leu2 Δ 0; lys2 Δ 0; ura3 Δ 0) was cultured in conditional labelling medium which contained 1.7 g/L yeast nitrogen base (without amino acids and without ammonium sulfate), 20 g/L D-glucose, 5 g/L ammonium sulfate, 200 mg/L adenine hemisulfate, 20 mg/L uracil, 100 mg/L Tyr, 10 mg/L His, 60 mg/L Leu, 10 mg/L Met, 60 mg/L Phe, 40 mg/L Trp, 100 mg/L Arg, and 30 mg/L [$^{12}\text{C}_6$, $^{14}\text{N}_2$]Lys (Lys0) or 30 mg/L [$^{13}\text{C}_6$, $^{15}\text{N}_2$]Lys (Lys8) (all from Sigma-Aldrich) (Sury, Chen et al. 2010). First a pre-culture was grown overnight, which was diluted 1:1000 to expand it into a large scale culture, grown for 16 h at 30°C and harvested in its exponential growth

phase. Cell pellets were collected by centrifugation of the culture at 3,000 g for 10 min at 4°C, lyophilized and stored at -20°C. The extent of labelling of the yeast with either Lys0 (Lysine 0) or Lys8 (Lysine8) was determined by LC-MS/MS respectively. To this end yeast cell walls were first digested with zymolyase (2mg/g wet weight of yeast) in 20 mM phosphate buffer, pH 7.4, at 30°C for 60 minutes, followed by sonication. Protein estimations were done with Bradford assay (Bradford 1976). Then proteins were precipitated with 50% trichloroacetic acid (TCA) (Sigma-Aldrich, Vienna, Austria) for 2h at 4°C and resolubilized in 6 M urea. Protein samples were reduced and alkylated by using 5mM Dithiothreitol (DTT) and 20mM, iodoacetamide (IAA) respectively. Resulted protein samples were subjected to enzymatic digestion with trypsin (1 µg trypsin/50 µg proteins) (Promega GmbH, Mannheim, Germany). Peptides (~1 µg) were run on a nanoLC coupled LTQ-FT (Thermo Fischer Scientific, Waltham, MA, USA) for checking the labelling efficiency using a 120 minutes multistep - gradient changing from aqueous to organic solvent. Peptide separation was done by RSLC nanocolumn (C18, 2 µm, 100 Å, 150 x 0.075 mm) (Thermo Fisher Scientific, Vienna, Austria). Samples were concentrated on the enrichment column for 2 min at a flow rate of 20 µl/min, with 0.5 % trifluoroacetic acid as an isocratic solvent. Separation was carried out on the nanocolumn at a flow rate of 300 nl/min using the following gradients, where solvent A was 0.05% TFA (trifluoroacetic acid) (Sigma-Aldrich, Vienna, Austria) in H₂O and solvent B 0.05% TFA in 80% acetonitrile (ACN) Sigma-Aldrich, Vienna, Austria) : 0-4 min 4% B, 4- 60 min 4-40% B, 60-95 min 40-95% B, 95-105 min 95-95% B, 105-120 min 4% B. Data were analyzed by Proteome Discoverer software for differentially labelled peptides. The obtained mass spectra was search against *S. cerevisiae*, NCBI public data base and heavy and light peptides were quantified by their intensities. For quantification of peptides following setting were made in the program: up to 2 missed cleavage were allowed, precursor ion mass tolerance: 10 ppm; fragment mass tolerance: 0.7 Da; dynamic modification were chosen heavy lysine (¹³C₆, ¹⁵N₂), light lysine (¹²C₆, ¹⁴N₂), oxidation on methionine, and cysteine carbamidomethylation was chosen as static modification. After confirming the yeast labelling efficiency to above 99%, it was used to label *D. melanogaster*. *Drosophila* embryos were collected on apple juice agar plates and 150 embryos were further transferred to a vial containing SILAC medium, which comprised 9 g of dry weight of *S. cerevisiae* yeast either labelled with Lys8 or Lys0,

320 mM sucrose, 0.5% agarose, 0.05% propionic acid, 0.25% phosphoric acid, 6 mM methylparaben and 0.3 mM ampicillin (all from Sigma-Aldrich, Vienna, Austria). Embryos were incubated at 30°C until they became third instar larvae (L3). Fat bodies from L3 larvae were isolated under the microscope from RKF676 (control) labeled with Lys0 and RKF 649 (PLIN1 KO) labeled with Lys8, equal numbers of fat bodies were combined for LD isolation.

11.4 Lipid droplet isolation:

Fat bodies from approximately 150 larvae were subjected to lysis by nitrogen gas cavitation using a cell disruption bomb (Parr instrument GmbH, Frankfurt, Germany) in ice cold disruption buffer (20 mM potassium phosphate pH 7.4, 250 mM sucrose, 1 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich, Vienna, Austria). Disruptions were carried out at 800 psi for 5 min two times then sample was collected by slowly opening the output valve. Cell debris was removed by centrifuging the homogenate at 1000 g for 5 min at 4°C. Cleared supernatants were transferred to ultracentrifuge tubes (SW41) and overlaid with ice cold overlay buffer (50 mM potassium phosphate pH 7.4, 100 mM KCl, 1 mM EDTA and protease inhibitor) up to ~3 mm below the top of the tube. Ultracentrifugation was performed using SW41 swing bucket rotter (Beckman Coulter GmbH, Krefeld, Germany) at 40,000 rpm for 1 hour at 4°C. During centrifugation LDs float up due to their lower density. This upper layer LD fraction was isolated with the help of a tube cutter. The LD fraction was washed two times with overlay buffer by centrifugation in Eppendorf tubes at 20,000 g for 10 min at 4°C and removal of as much buffer as possible after each centrifugation (Ding, Zhang et al. 2013). Small aliquots were taken out for protein estimation by BCA protein (Thermo Scientific, Rockford, IL, USA) and the rest was stored at -20°C for mass spectrometry analysis.

11.5 In solution stable isotope dimethyl labeling:

For dimethyl labelling of lipid droplet proteome peptides, lipids were removed by ether extraction. In order to perform ether extraction 3 times (v/v) diethyl ether

(Sigma-Aldrich, Vienna, Austria) were added to lipid droplets and incubated for 5 minutes with continuous shaking. Upper layer containing lipids was carefully removed without disturbing the interphase which contains proteins. This extraction was repeated 3-4 times. Finally, residual ether was removed with nitrogen gas. Finally, protein fractions were concentrated using 3 kDa cut off Amicon ultra filters following the manufacturer's instructions (Millipore, Billerica, MA, USA). Concentrated and extracted protein samples of RKF 676 (control), RKF989 (PLIN1/PLIN2 double KO) and RKF610 (PLIN2 KO) were reduced, alkylated and digested with trypsin (Promega, Madison, WI, USA) at 37°C for 16 h with continuous shaking. Resultant peptides were subjected to dimethyl labelling. Sample volumes were made up to 50 µL with 100 mM tetraethylammonium bromide (TEAB, pH 8.0) followed by addition of different formaldehyde isotopes: CH₂O (2 µL of 4% (v/v)) for light and CD₂O (2 µL of 4% (v/v)) for intermediate labeling. Samples were briefly mixed and spun down followed by addition of 2 µL of NaBH₃CN. The labeling reactions were carried out at 15-22°C for 1 hour with continuous mixing. To quench the labeling reactions 8 µL of 1% (v/v) ammonia solution was added followed by brief mixing. Additionally, 4 µL of 5% (v/v) formic acid was added to neutralize the reactions. Labeled peptides were dried in a vacuum concentrator and redissolved in 10 µL of 0.5% TFA. Labeling efficiencies were checked using LTQ-Orbitrap velos pro. To do so samples were separated by the Acclaim® PepMap RSLC analytical column (75 µm x 15 cm). Solution A consisted of Milli-Q water and 0.05% TFA, solution B of 80% ACN and 0.05% TFA in Milli-Q water. For loading the sample solution A was used and separation was done using following gradient in a 70 min run; 0-4 min 4% B, 4-30 min 4-30% B, 30-48 min 30-95% B, 48-58 min 95% B, 58-70 min 4% B. Mass spectrometry detections were done using collision induced dissociation (CID) fragmentation of top 10 peaks. The LC-MS/MS data were analyzed by searching the SwissProt *Drosophila* fruit fly public database (541,762 entries) with Proteome Discoverer 1.4 (Thermo Fisher Waltham, MA, USA). Finally samples were mixed for quantitative proteomics analysis based on the total area identified in desired samples.

11.6 Mass spectrometry:

SILAC labeled or stable isotopic dimethyl labeled samples (~1 µg) were run on to either on LTQ-FT (for SILAC samples) or LTQ orbitrap (for dimethyl labeled sample) using collision induced dissociation (CID) and a 5 hour gradient. Samples were first separated by the Acclaim® PepMap RSLC analytical column (75 µm x 15 cm) at 200 nL/min flow rate. Solution A consisted of Milli-Q water and 0.05% TFA, solution B of 80% ACN and 0.05% TFA in Milli-Q water. For loading the sample solution A was used and separation was done using the following gradient in a 300 min run; 0-2 min 4%B, 2-180 min 4-28% B, 180-255 min 28-50% B, 255-260 min 50-95% B, 260-279 min 95% B, 280-300 min 4%B. The sample was ionized in the nanospray source equipped with stainless steel emitters (ES528, Thermo Fisher Scientific, Vienna, Austria). It was analyzed in a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) or LTQ-orbitrap velos pro operated in positive ion mode, applying alternating full scan MS (m/z 300 to 2000) in the ion cyclotron and MS/MS by collision induced dissociation 5 most intense peaks. The LC-MS/MS data were analyzed by searching the *Drosophila* fruit fly SwissProt whole public database (541,762 entries) with Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) using carbamidomethylation on Cys as fixed modification and oxidation on methionine as variable modification. For quantification lysine (K) side chain modification were specified as dimethyl light (K) (+28.031 Da), dimethyl medium (K) (+32.056 Da) or SILAC heavy Lys8 (K) ($^{13}\text{C}_6$ $^{15}\text{N}_2$ /+8.014 Da) respectively (all as variable modification). A maximum false discovery rate of 5% using decoy database search, a Mascot ion score cut off of 20 and a minimum of 2 identified unique peptides were chosen as identification criteria. Ratios were reported as heavy (intermediate)/light with 100 as maximum fold change. WT (controls) were always labeled with light and KO (mutant) were always labeled with heavy/intermediate label.

11.7 Immunohistochemical Staining:

Larval fat bodies from RKF 676 (control) and different perilipin knock outs were isolated in phosphate buffer saline (PBS) to detect the localization of hit candidates. Unfortunately antibodies for all identifies candidates were not available. Isolated fat bodies were carefully washed with PBS and fixed in 4 % formaldehyde for 15-20 minutes at room temperature. Fixed fat bodies were intensively washed with BBT solution (10 mM Tris, 55 mM NaCl, 40 mM KCl, 7 mM MgCl₂, 20 mM glucose, 50 mM sucrose, pH 7.0 and 0.1% tween 20) and 0.2% BSA to completely remove traces of formaldehyde and blocked in 3% (v/v) horse serum (diluted in BBT) for 2 hours at room temperature. Further, blocked fat bodies were incubated with ApoL1, ApoL2 (1:500) (Brankatschk and Eaton 2010), ADH (1:200), FBP1 (p49.8) (1:200), FBP1 (p19) (1:200) and, FBP1 (p48.8) (1:200) (Burmester, Antoniewski et al. 1999) antibodies (all diluted in blocking solution overnight) at 4°C. After washing with 0.2% BSA in BBT solution, fat bodies were incubated with pre-absorbed fluorescently labelled secondary antibodies (Alexa Fluor 568) reacting to different host IgG, in accordance with the primary antibody (rabbit, rat, mouse and goat), in blocking solution in 1:500 dilution for 2 hours at room temperature. Fat bodies were then incubated with BODIPY for lipid droplets, Cell mask for membranes, and Hoechst 33342/DAPI for nucleus (all from Life Technologies, Carlsbad, CA, USA) in 1:1000 dilutions in BBT. After fat bodies were intensively washed and mounted in mowiol 4-88 (Sigma-Aldrich, Hamburg, Germany) mounting medium (4.8 g mowiol 4-88, 12 g glycerol in 24 ml 0.2 M Tris HCl, pH 8.5) images of fat bodies were taken with Zeiss LSM-780 confocal microscope by using 40x water object and ZEN 2010 LSM software. Some fat bodies were intentionally broken to release lipid droplets for identifying localization of protein. Fat body protein 1 (FBP1) and apolipoprotein (ApoL) antibodies were generous gifts from Prof. Jean-Antoine Lepasant (Institut Jacques Monod, CNRS-Universite Paris Diderot, France) and Prof. Suzanne Eaton (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), respectively.

11.8 Live tissue imaging:

To analyze the effect of Cyp1 on lipid droplet size, flies carrying the FB-Gal4 (RKF 1421, fat body driver) were crossed with either control (RKF1084) or with Cyp1 RNAi lines (BDSC 33001 and BDSC 33950). Fat bodies attached to salivary glands were isolated by dissecting crawling L3 larvae in artificial hemolymph 3.1 (HL3.1) (70 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂, 10 mM NaHCO₃, 10 mM glucose, 115 mM sucrose 5 mM HEPES, pH 7.0) (Feng, Ueda et al. 2004). Subsequently, fat bodies were incubated in PBS containing BODIPY 493/503 or LD540 (for lipid droplets), Hoechst33342 or DAPI (for nuclei) and CellMask (for membranes) stains (all from Life Technologies, Carlsbad, CA, USA). Images were acquired using Zeiss LSM-780 with 40 x magnification water objective in a fix gain mode. Images were acquired in such a way that most of the cells had the highest diameter of nucleus as fixed parameter. Lipid droplet size was determined with FIJI software (just another imageJ) using partial size analysis of XY panel of binary images (Deutsch, Schriever et al. 2014). Results are presented as mean $\pm$ standard error from one experiment of at least two images from independent fat bodies. Approximately 500-600 LDs were quantified in each image.

11.9 RNA isolation and cDNA synthesis:

Five to ten L3 larval fat bodies were snap frozen in liquid nitrogen. RNA was extracted using Direct-zol RNA MiniPrep-kit (Zymo Research, Freiburg, Germany) according to the instructions of the manufacturer. Briefly 5-10 larval fat bodies were homogenized in 700 μ L TRI reagent by using ceramic-cylinder beads in 2 mL screw cap tubes with Precellys 24 homogenizer (Precellys, MD, USA) at 5000 rpm for 10 seconds. Samples were centrifuged for 1 min at 5000 rpm. An equal volume (1:1) of ethanol (95-100%) was added to the resultant supernatant; then the mixture was loaded onto Zymo-spin IIC columns. After the Brief centrifugation columns were prewashed twice with Direct-zol RNA preWash buffer. After the pre-wash columns were additionally washed 1 time with RNA wash buffer. Finally, RNA was eluted in DNase/RNase free water. Eluted RNA was directly used for

complementary DNA (cDNA) synthesis and leftover RNA was stored at -80°C. The cDNA synthesis from total RNA (1 µg) was done using QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions.

11.10 Generating of Cyp1-eGFP transgenic fly:

Transgenic fly lines expressing Cyp1-eGFP fusion protein were generated using a PhiC31-mediated transgene insertion into the strain attP40 on the 2nd chromosome at the 25C6 cytosite. Constructs were cloned into pUASTattb vector using Gibson assembly cloning kit (New England BioLabs, Ipswich, MA, USA) using manufacturer's instructions. Following primers were generated by using NEBuilder for Gibson assembly tool to generate Cyp1 and eGFP overlapping fragments with pUAST vector:

Cyp1 forward (blue)	–	5'-attgggaattcgtaaacaATGGTCTCTTTTGTGCAAC-3'
Cyp1 reverse (green)		5'-ctcaccatAAGAGAACCAGAGTTAGC-3'
eGFP forward (green)		5'-ggttctcttATGGTGAGCAAGGGCGAG-3'
eGFP reverse (red)		5'-gatacctctagaggtacTTACTTGTACAGCTCGTCCATG-3'

(Color code next to primers refers to colors used in Fig. 17 for binding site.). Uppercase in primers indicate gene specific primer and lowercase indicates overlapping part of the primer with other fragments. Overlapping fragments were then generated by PCR amplification (Barnes 1994). The vector, pUAST was linearized for assembly by digesting it with KpnI and BglII restriction enzymes. Vector and fragments to be cloned (Cyp1 and eGFP) were gel purified and assembled in a thermocycler at 50°C following instructions from the Gibson assembly cloning kit (Fig. 17). Assembled product was then transformed into chemically competent cells and clones were screened by restriction digestion and plasmid DNA sequencing. Afterwards plasmid DNA was injected into y1 w67c23; P{CaryP}attP40 strain embryos by using 25C6 CytoSite. Injection, generation and screening of positive transformants based on red eye color were done at BestGen Inc. (www.thebestgene.com). Positive transformants were made homozygous by crossing male and virgin female sibling positive transformants which were received.

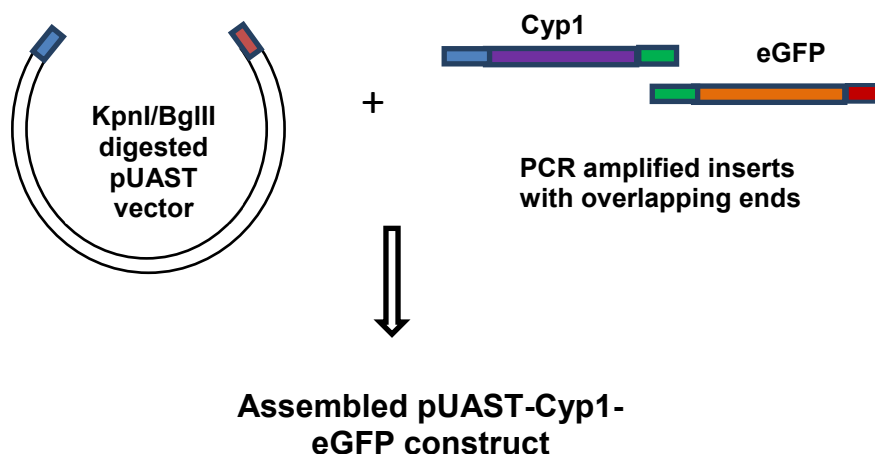


Figure 17. Gibson Assembly workflow: Gibson Assembly cloning strategy is an exonuclease-based method to assemble multiple DNA fragments in the correct order. First primers for desired fragments (eGFP and Cyp1) were designed and DNA fragment containing overlapping part as shown in the figure were generated by PCR amplification. In parallel pUAST vector was digested with KpnI and BglII restriction enzymes (RE). Finally RE digested vector and PCR amplified fragments with overlapping ends were incubated with Gibson Assembly master mix at 50°C for 60 min. Gibson Assembly contains three enzymatic activities in order to generate a complete construct: first a 5' exonuclease generates long overhangs, a polymerase fills in the gaps of the annealed single strand regions, and a DNA ligase seals the nicks. Figure has been adapted from www.neb.com.

11.11 Image analysis:

FIJI software was used to perform lipid droplet size analysis using ImageJ. Images were acquired keeping sure that most of the fat body cells contained the largest diameter of the nucleus in order to have consistency in focal planes. Detailed procedure of live tissue imaging is explained in section 11.8 of this thesis. Analysis was performed using binary images, which consist only of the pixels representing the lipid droplets. Therefore, Images were first converted to gray scale; then the threshold was adjusted for the color saturation of the lipid droplets, followed by

binarization of images. Subsequently watershed object separation was performed, which separated borders of droplets in case they were very close and fused with each other. Manual comparisons of binary images with the original image were performed to control correct binary conversion. To measure the size of lipid droplets the partial analysis tool of FIJI was used where the size of lipid droplets in the image were measured as surface area in square micrometers (μm^2) (Deutsch, Schriever et al. 2014). Edges of images were not included to exclude LDs which were not fully sphere shaped.

12. RESULTS

The results presented here are still unpublished preliminary data and currently followed up in the laboratories of Prof. Ruth Birner-Grünberger (Medical University of Graz, Austria) and Prof. Ronald P. Kühnlein (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany).

12.1 LD proteomes of *plin1*, *plin2* single and *plin1/plin2* double mutants:

Perilipins may affect recruitment of other proteins to LDs and thus the absence of perilipins may alter the protein coat of LDs. Proteome changes may be causative or a consequence of the observed increase in lipid droplet size of perilipin 1 mutants as compared to perilipin 2 mutants, perilipin 1 and 2 double mutants or control/WT (Beller, Bulankina et al. 2010). To this end we performed quantitative proteomics analysis using SILAC metabolic labeling and dimethyl chemical labeling methods. Data revealed a unique set of proteins present on the LD surface of perilipin mutants which are regulated differentially. In perilipin1 mutant larval fat body LDs in total 71 proteins were quantified, out of which 3 were upregulated and 11 were down regulated while the rest remained unchanged (Fig. 18). Peptidyl-prolyl cis-trans isomerase (P25007) also known as cyclophilin 1 was highly upregulated (20 fold) in perilipin 1 knock out (*plin1* KO) compared to control fly LDs which made it our prime candidate. Additionally, cyclophilin 1 has been indicated to have an impact on lipid droplet size in HCV (hepatitis C virus) replicon cells. Anderson et al have shown that upon treatment with cyclophilin inhibitor (NIM81) in sg-1b replicon cells LD size doubled. (Anderson, Lin et al. 2011). However they emphasized that this effect in change of LD size requires presence of HCV protein because the effect was seen only in sg-1b replicon cells but not in naïve Huh7 or sg-1b NIMr NIM81 resistant cells. In perilipin 2 knock out (*plin2* KO) larval fat bodies in total 134 LD proteins were identified and 106 were quantified out of which 49 were down-regulated and 25 were upregulated. Providentially, cyclophilin 1 is unchanged in perilipin 2 mutant LDs. We as well performed qproteomics of perilipin 1 and perilipin 2 (*plin1/plin2* dKO) double knock out larval fat body LDs in which 149 proteins were quantified, of which 23 were up regulated and 16 down regulated.

Interestingly, cyclophilin 1 remained unchanged in larval LDs of *plin1/plin2* dKO. This quantitative difference is expected if cyclophilin 1 amounts on LDs are related to LD size as LD size of *plin1/plin2* dKO is unchanged or similar to control flies. Based on qproteomics data protein candidates which are differential regulated in *plin1* KO and *plin1/plin2* dKO larval LDs were selected as candidates which could be of interest in LD size and lipid metabolism regulation (Table 4). A compilation of the qproteomics data can be found in the appendix of this thesis.

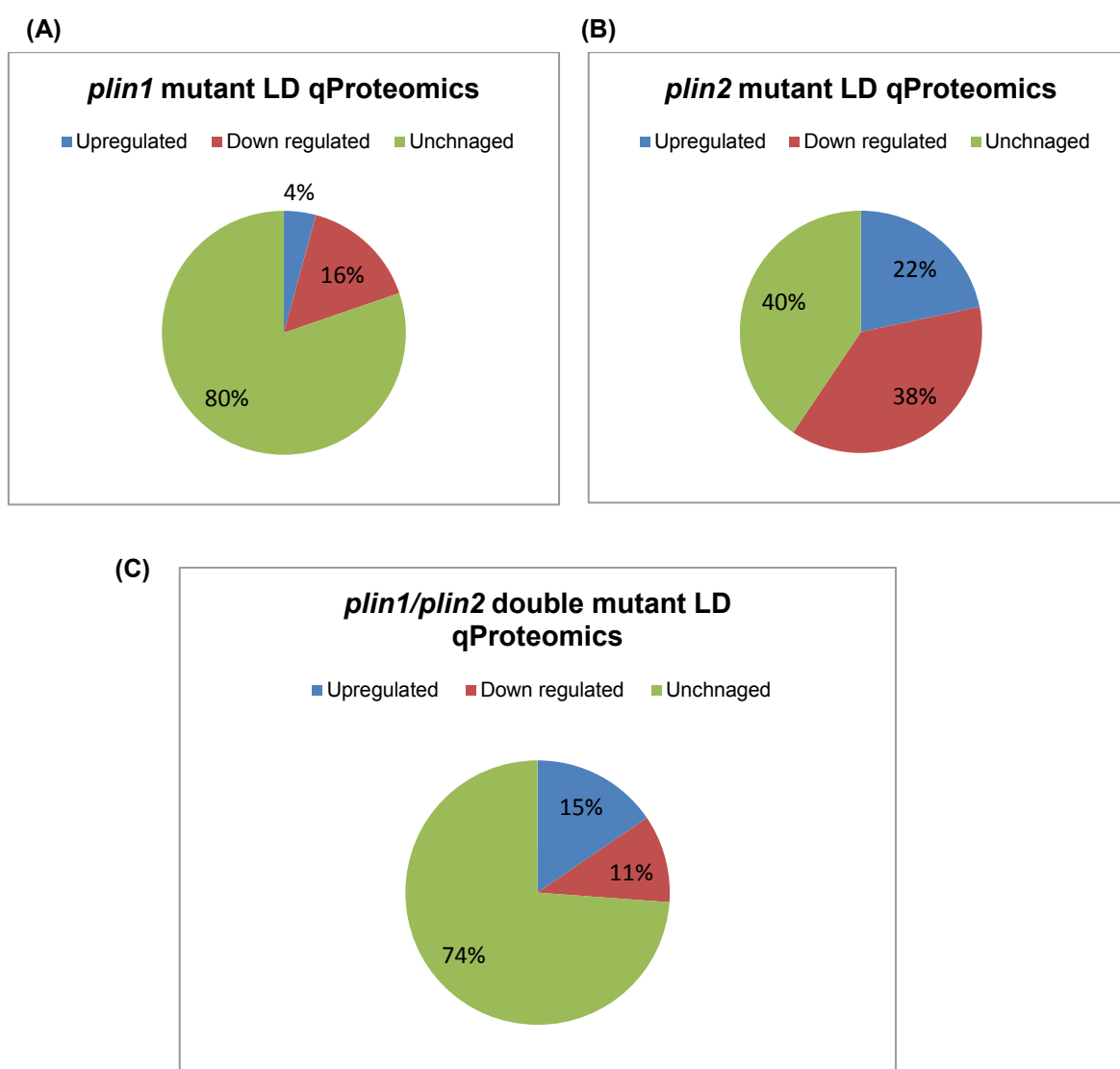


Figure 18. Quantitative proteomics data representing up and down regulated proteins in LDs of fat bodies of larvae of different perilipin mutants: (A) Perilipin 1 knock out, (B) perilipin 2 knock out, (C) perilipin 1 and 2 double knock out larval lipid droplets. Protein which shows a heavy/light ratio above 2.0 was consid-

ered upregulated and below 0.5 was considered down regulated, while the rest were considered unchanged.

Table 4: Candidates of interest: Proteins were selected based on their differential abundance on lipid droplets in different perilipin mutants. Cyclophilin 1 is most prominently upregulated in perilipin1 knock out, whereas unchanged in perilipin 2 and double knock out making it our prime candidate whose abundance might be related to LD size. Ratios of protein abundance of perilipin mutants versus their controls are shown.

No	Uniprot Accession no	Description	Ratio (H/L or M/L)		
			PLIN1 KO / WT	PLIN2 KO / WT	PLIN1/PLIN2 dKO / WT
1	P25007	Cyclophilin 1 (Cyp1)	20.1	1.4	1.0
2	P61858	tubulin beta 2	0.02	Not identified (Tubulin beta 1 M/L=0.3)	Not identified (Tubulin alpha 1 M/L=1.1)
3	P54398	Fat body protein 2	0.06	Not identified	3.4
4	Q9V496	apolipoprotein (ApoL)	0.6	1.2	3.3
5	Q9VU58	neuropeptide like 2	0.3	1	1.3
6	P62152	calmodulin	1.3	Identified but not quantified	0.5
7	Q04691	fat body protein 1	Not identified	0.14	0.7
8	P00334	alcohol dehydrogenase (ADH)	0.9	1.1	1.6

12.2 PAT family proteins show partial redundancy:

It has been shown that different perilipins of *Drosophila* show partial redundancy by a previous report (Bi, Xiang et al. 2012); our data set confirms this partial redundancy. As shown in Table 5 LDs of perilipin 1 (*lsc1*) mutant carry more perilipin 2 (*lsc2*) as compared to the control and *vice versa*. As expected perilipin 1 is highly down regulated in *plin1* 1 KO and *plin1/plin2* dKO, while perilipin 2 is highly down regulated in *plin2* KO and *plin1/plin2* dKO.

Table 5. Abundance of PAT family proteins: Ratios of protein abundance of perilipin mutants versus their controls are shown.

No	Accession number	Description	Ratio (H/L or M/L)		
			<i>plin1</i> KO / WT	<i>plin2</i> KO / WT	<i>plin1/plin2</i> dKO / WT
1	Q9VCI3	Lipid storage droplets surface-binding protein 1 (LSD1)	0.06	100	0.04
2	Q9VXY7	Lipid storage droplets surface-binding protein 2 (LSD2)	3.5	0.01	0.1

12.3 Identified protein candidates are not mainly and not exclusively localized to LDs:

To validate the LD proteome data set we used immunohistochemistry (IHC) to confirm the localization of some of the candidates limited by the availability of specific antibodies. We identified alcohol dehydrogenase (ADH), apolipoprotein (ApoL) and the fat body protein 1 (FBP1) as lipid droplet proteins in the fly fat body using this technique (Fig. 18). However, they are not solely located on lipid droplets but appear to localize as well to other compartments in the fat body confounding their clear localization to LDs. To overcome the difficulty of multiple signals and to obtain clear localization of proteins we broke the fat body cells by mechanical means and used free-floating LDs for imaging. Confocal images of fat bodies from RKF 676 (control), which were subjected to immunohistochemistry and counter stained with BODIPY for lipid droplets, were taken. Images from intact fat body cells and free-floating LDs revealed localization of FBP1, ApoL and ADH on lipid droplets as well as in other compartments. However signals from the lipid droplets were much weaker than from other compartments. Therefore free-floating LDs provided much clearer evidences due to reduced background coming from other compartments of the cells.

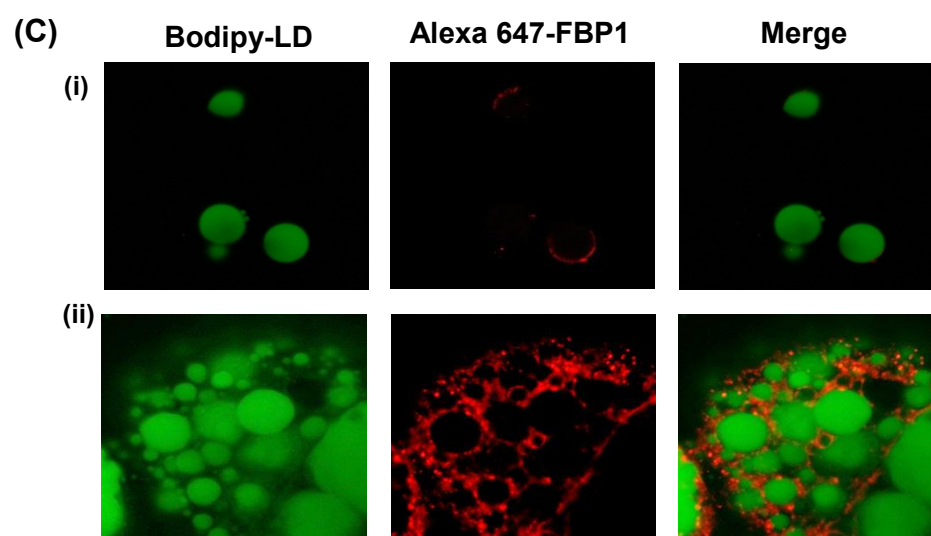
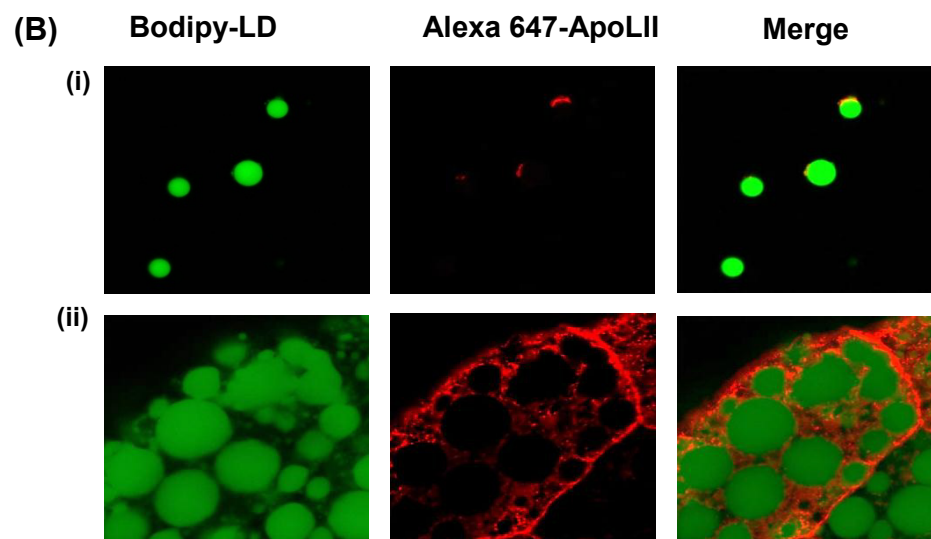
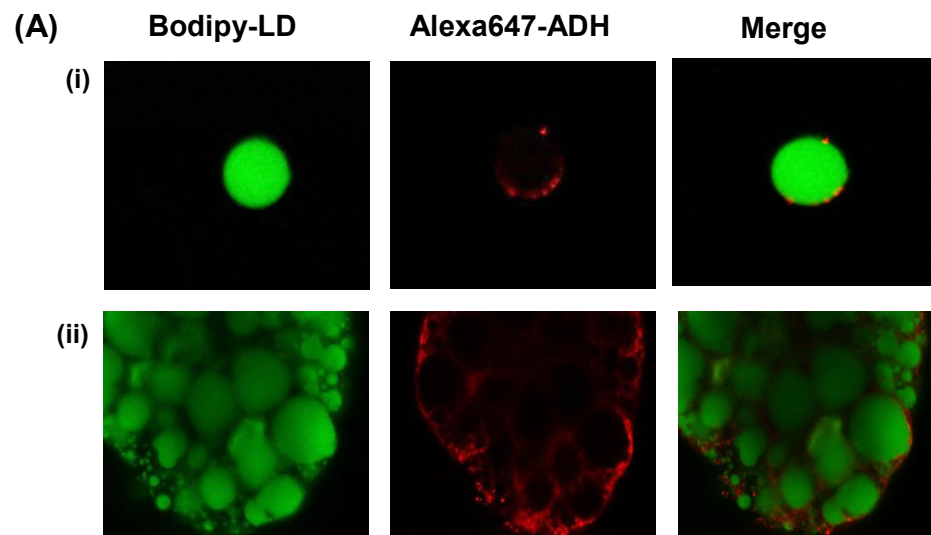


Figure 19. Localization of the protein candidates identified on lipid droplets by immunohistochemistry: (A) ADH (B) ApoLII and (C) FBP1 antibodies were used to identify the localization of protein on (i) free-floating lipid droplets or in (ii) intact fat body tissue of *D. melanogaster* RKF676 (Control). Control genotype (RKF676) was selected for this experiment due to the fact that when above antibodies were tested prior to immunohistochemistry by western blot RKF676 showed the best signal. Counter staining for neutral lipids was done with BODIPY 493/503.

As described above cyclophilin 1 emerged as our top candidate whose abundance on LDs may be related to LD size in the quantitative proteome study of perilipin knock out *D. melanogaster* fat body lipid droplets. Thus we wanted to confirm the localization of Cyp1 to lipid droplets in the fly fat body. To this end we generated flies expressing cyclophilin 1- eGFP fusion protein under UAS control and used L3 larval fat bodies for live imaging. Additionally fat bodies were counter stained for nucleus and lipid droplets with DAPI and LD-540, respectively. Resultant images revealed that Cyp1-eGFP may be localized to lipid droplets but in much lesser quantity as compared to other cellular locations (Fig. 19). Below pictures show a very weak signal on lipid droplets due to confounding stronger signals coming from other parts of the cell. Absence of availability of *Drosophila melanogaster* Cyp1 specific antibody makes it difficult to provide clear evidence of localization of Cyp1 to lipid droplets since we cannot exclude a bias caused by fusion to eGFP. Localization of Cyp1 to the nucleus and cytoplasm was shown in drosophila BG2 neuroblast-derived tissue culture cells in a punctuated manner (Lee, Ramirez et al. 2014). In addition, cyclophilin 1 as well has been identified as a microtubule associated protein in a co-sedimentation assay (Hughes, Meireles et al. 2008).

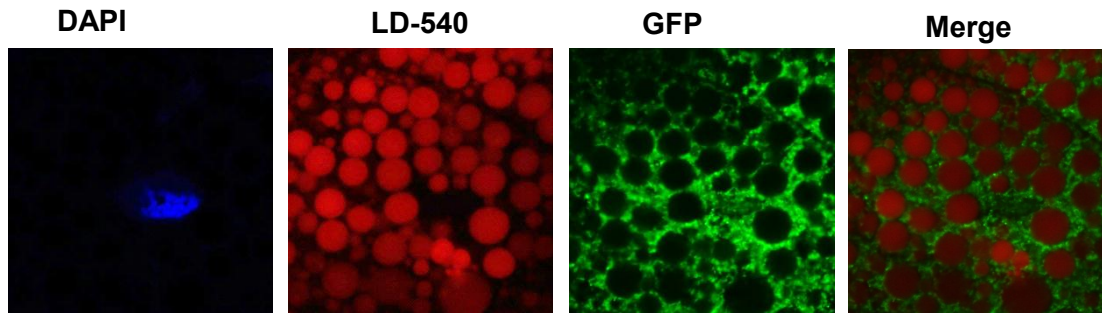
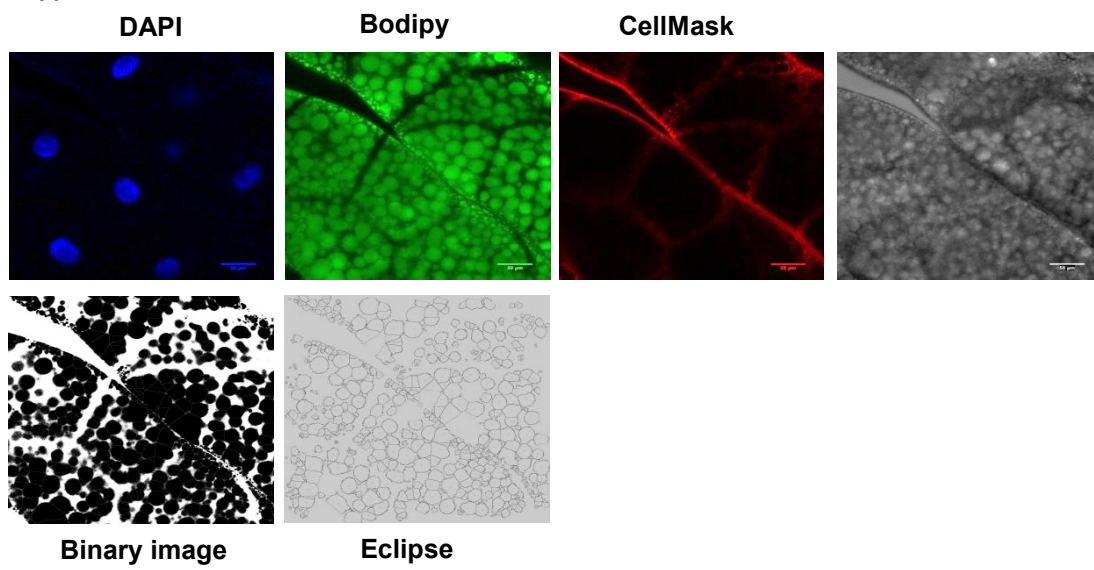


Figure 20. Localization of cyclophilin 1 in *D. melanogaster* fat body showing cyclophilin 1-eGFP fusion protein present on and around lipid droplets in a punctuated manner: Blue (nucleus), red (lipid droplets) and green (Cyp1-eGFP fusion protein).

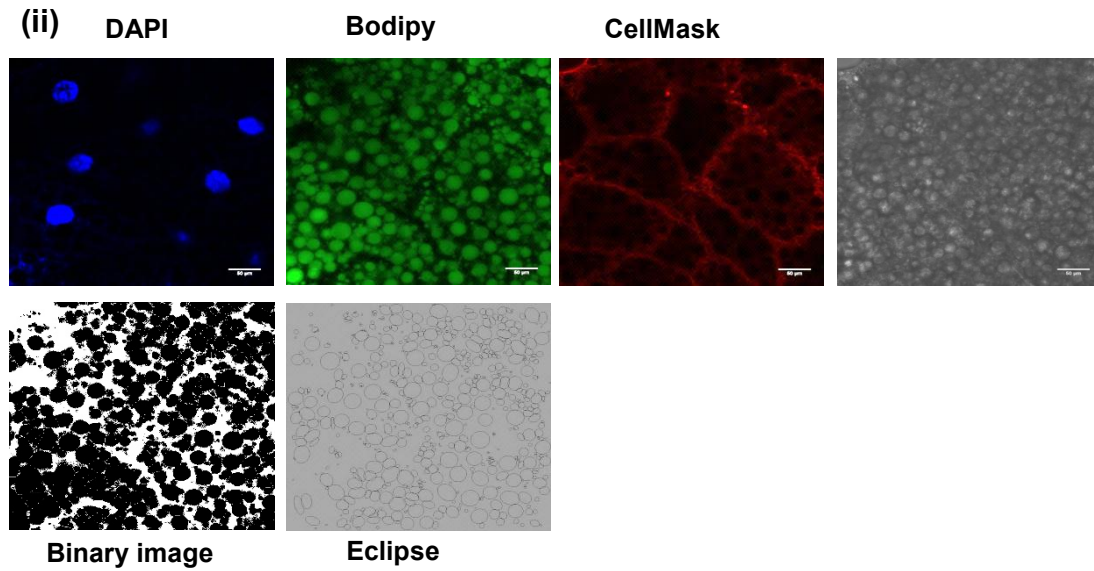
12.4 Cyclophilin 1 expression affects LD size in *plin1 (lsd1)* knock out *Drosophila melanogaster* fat body:

To understand the functional role of cyclophilin 1 in regulation of LD size we used BDSC Cyp1 RNAi lines under UAS control and crossed them with conditional fat body tissue specific FB-Gal4 driver, therefore taking advantage of UAS-GAL4 system. L3 progeny from the crosses were used to isolate the salivary gland attached fat body for live imaging of LDs. Since LD size might not be constant over different parts of the fat body we used the same part of the fat body attached to the salivary gland for all of our imaging experiments (Fig. 20A). The larval fat bodies were isolated and incubated in hemolymph 3.1 containing BODIPY493/503 for neutral lipid droplets, DAPI for nucleus and CellMask for cell membrane. Images of the XY panel where most of the cells contained the highest diameter of the nuclei were acquired by using 40x water objective on LSM780 confocal microscope and images were analyzed by using FIJI (just another imageJ software) following recently published procedures (Deutsch, Schriever et al. 2014). Our results show that Cyp1 contributes in to regulation of lipid droplet size. For the analysis the BODIPY channel which stained neutral lipids and hence LDs were subjected to binary conversion followed by particle size analysis. Image analysis revealed that inactivation of Cyp1 by RNAi leads to a significant decrease in size of LDs in the fat body of *D. melanogaster* (Fig. 20B, 20Ci, ii, iii).

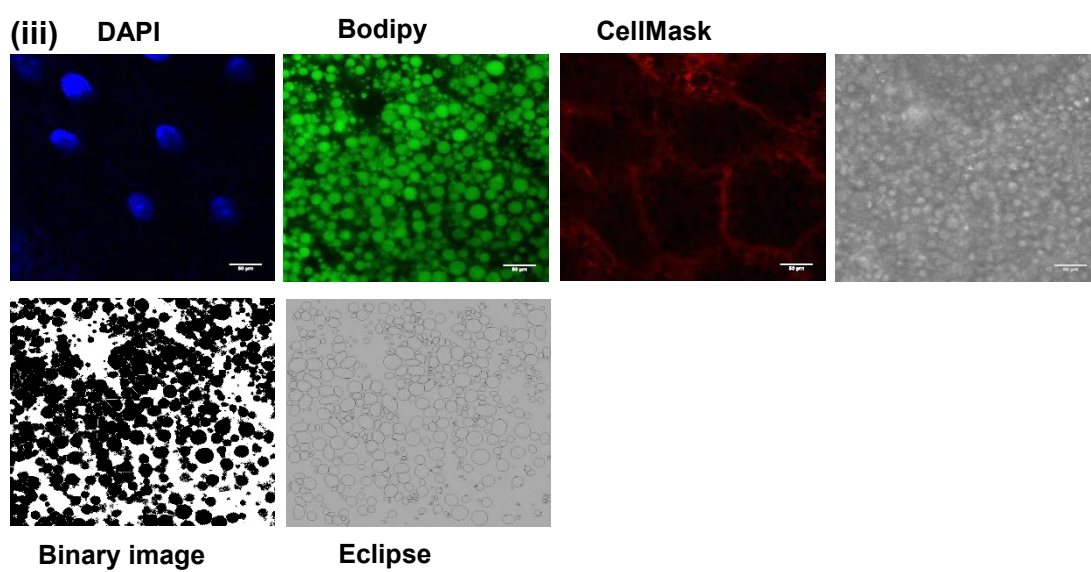
(A)
(i)



(ii)



(iii)



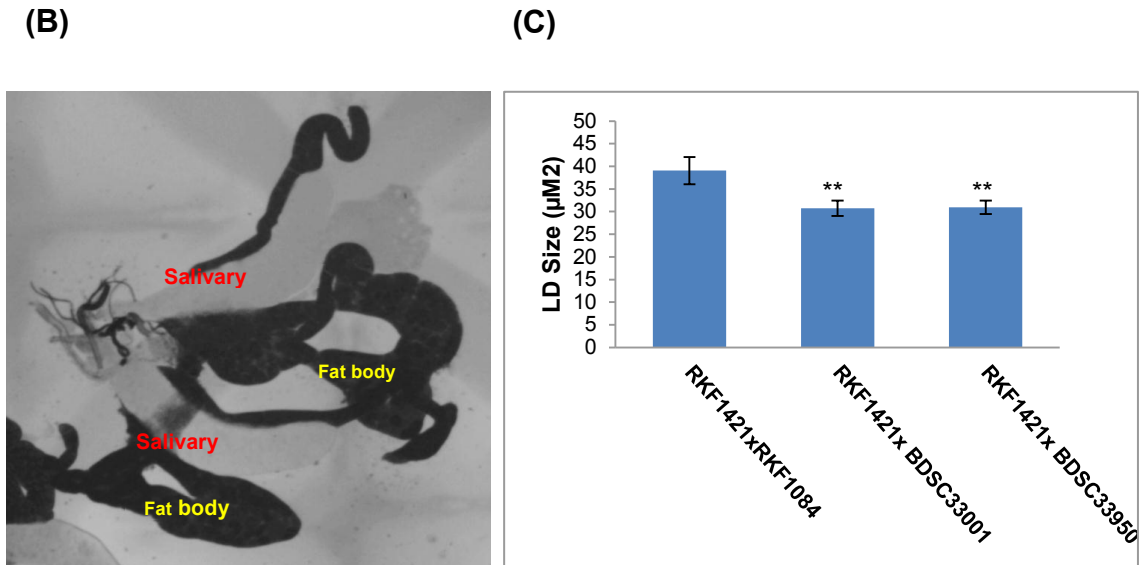
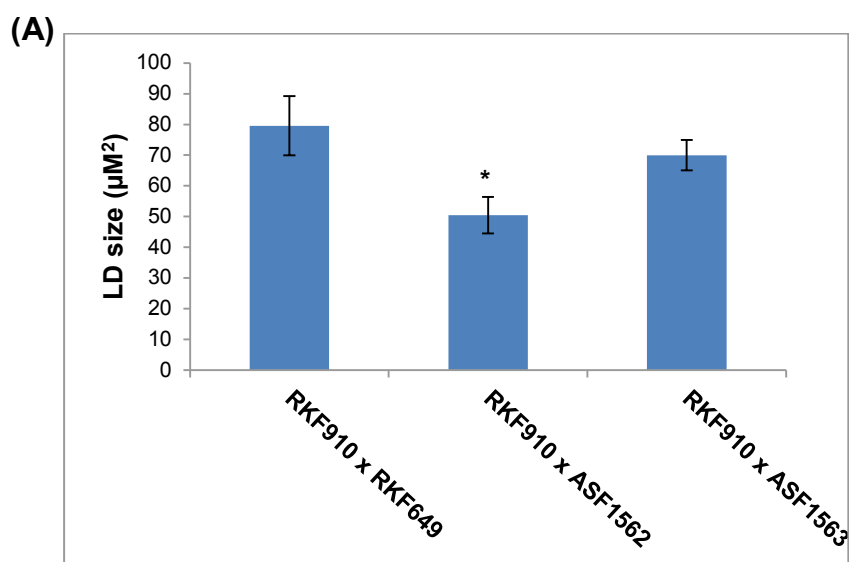


Figure 21. Effect of cyclophilin 1 RNAi on fat body lipid droplet size of *D. melanogaster*: Salivary gland attached fat body parts were used for imaging (B). Image J was used to identify lipid droplet size by particle size analysis (C) by converting image to binary then applying watershed tool for the purpose of separating particles which show fused patterns. More than 500 lipid droplets were analyzed per sample. Two separate lines from BDSC for *cyp1* RNAi BDSC 33001(Aii), BDSC 33950 (Aiii) and white eyed control (Ai) were crossed with fat body driver (RKF 1421) to drive the RNAi to fat body. Selected L3 larval progeny were dissected for isolating salivary gland attached fat body followed by live staining for nucleus (blue), cell border (red) and lipid droplets (green). Images were acquired using 40x water objective of the confocal microscope. Bars represent mean $\pm$ SEM. *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$, versus RNAi control group.

12.5 Effect of Cyclophilin1 down regulation on lipid droplet size in perilipin 1 knock out genetic background:

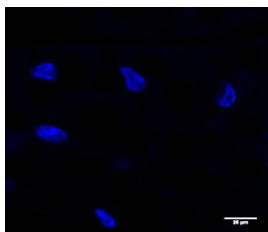
As described above the quantitative larval fat body LD proteome analysis of the various drosophila perilipin mutants identified Cyp1 as highly upregulated (20 fold) protein in perilipin 1 knock out LDs, however not changed in perilipin 1 and 2 double knock out. In addition, *plin1* knock out fly fat body has been shown to possess a larger LD phenotype (Beller, Bulankina et al. 2010). Besides, as shown above 10.4 *cyp1* RNAi leads to a decrease in lipid droplet size in L3 larval fat body. Therefore, we wanted to analyze the impact of *cyp1* knock down in absence of perilipin 1 on size of LDs. To this end, we produced a fat body specific knock down of Cyp1 by crossing two independent RNAi effector lines (BDSC 33001 and BDSC 33950) into the perilipin 1 knock out genetic background which contained a fat body specific driver. Consistent with the effect in the wildtype background (Fig. 20) size analysis of more than 500 lipid droplets from larval fat body images showed that *cyp1* knock down leads also to a significant decrease in LD size in perilipin 1 knock out (Fig. 21A). Hence, Cyp1 appears to be an important lipid droplet associated protein playing a role in regulation of size of the larval fat body LDs in *D. melanogaster*.



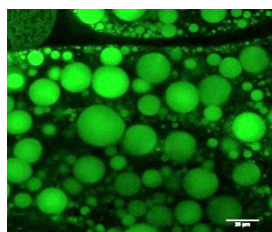
(B)

(i)

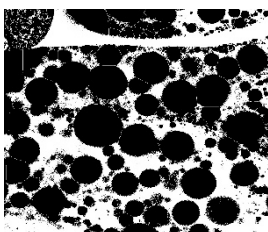
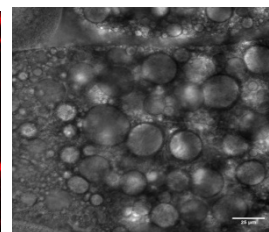
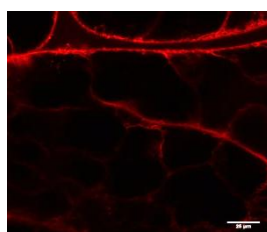
DAPI



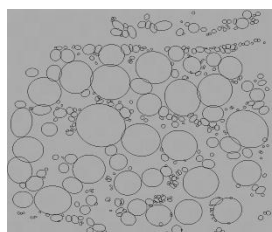
Bodipy



CellMask



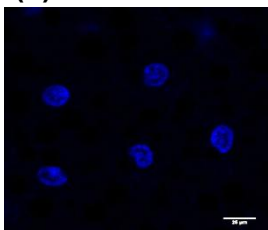
Binary image



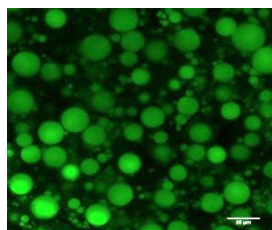
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(ii)

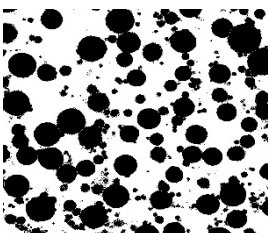
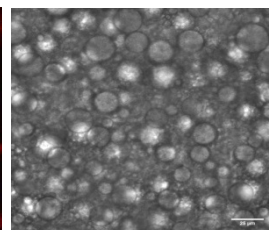
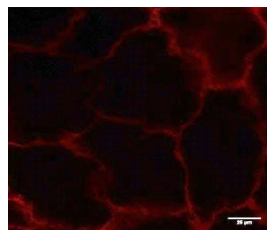
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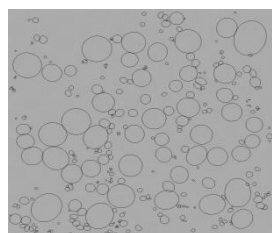
Bodipy



CellMask



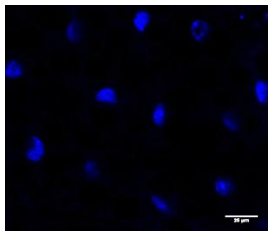
Binary image



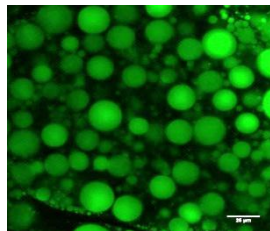
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(iii)

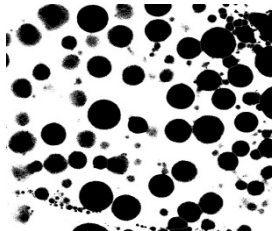
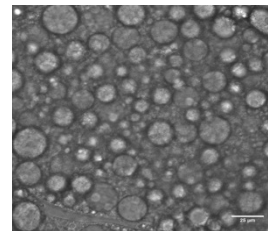
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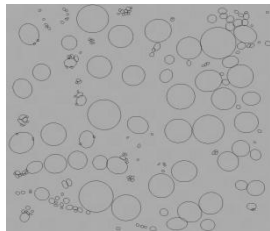
Bodipy



CellMask



Binary image



Eclipse

Figure 22. Cyclophilin 1 RNAi in perilipin1 background reduces lipid droplets size: (A) ImageJ analysis of L3 larval fat bodies in *plin1* knock out background showed down regulation of lipid droplet size by *cyp1* knock down. (B) Larval fat body images from progeny of *ts-fat* body driver in *plin1* KO genetic background crossed with (i) *plin1* KO out (RKF 649), (ii) *cyp1* RNAi line (BDSC33001) in *plin1* KO genetic background (ASF1562) and (iii) *cyp1* RNAi line (BDSC33950) in *plin1* KO genetic background (ASF1563). More than 500 LDs were analyzed with the particle size tool of imageJ. Images were acquired using 40x water objective of a confocal microscope. Blue: DAPI stain for nucleus; green: Bodipy stain for LDs; red: CellMask stain for cell membrane; black-white-binary image of LD channel and eclipse-particles considered for analysis by imageJ. Bars represent mean $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, versus RNAi control group.

13. DISCUSSION:

Lipid droplets are evolutionarily conserved from bacteria to mammals and their differential number and size has not only been associated with different cell types but also with different disease states (Fujimoto and Parton 2011, Greenberg, Coleman et al. 2011, Krahmer, Farese et al. 2013). As well, it is widely accepted that a differential LD proteome determines the structural and biochemical properties of LDs (McDonough, Agustin et al. 2009, Suzuki, Shinohara et al. 2011). Both structural and biochemical characteristics of LDs in *D. melanogaster* fat body cells are strongly influenced in different developmental stages from larva to adult by the presence and absence of the PAT family proteins Plin1 (perilipin 1) and Plin2 (perilipin 2). Absence of perilipin 1 has been well documented for causing a giant lipid droplet phenotype (Beller, Bulankina et al. 2010). Therefore, we focused on the question if the absence of perilipin 1 will change the LD proteome, which in turn may control the structural and functional properties of the LDs.

The present study shows the identification of novel proteins on lipid droplets of the fat body of different perilipin knock out mutants of *D. melanogaster* by quantitative proteomics.

In this work, cyclophilin1 has appeared as a potential player in regulation of lipid droplet size since it was found to be highly enriched on LDs of larval fat bodies of perilipin1 knock out *Drosophila melanogaster*. Functional analysis of the role of cyclophilin 1 in lipid droplet size regulation was studied by fat body specific RNAi knockdown. This study could prove to be important in identifying mechanism of perilipins in regulating LD size.

LD quantitative proteome analysis of perilipin mutants are an ideal system to study the impact of PAT family proteins in regulation of lipid droplet associated metabolism, as newly identified candidates could play an important role in this process. Our current study confirms some of the important properties of the lipid droplet proteome which have been published. One example are the redundant functions of drosophila perilipins and their influence on lipid droplet size which have been shown recently (Bi, Xiang et al. 2012). Our results confirmed this redundancy of PAT family proteins. We show here that in LDs from perilipin 1 knock out, perilipin 2 was found upregulated (3.5 fold). Vice versa, in qproteome analysis of perilipin 2 knock out LDs perilipin 1 was greatly upregulated (100 fold) (Table 5). Apart from

this we identified a proteome set which has been shown previously to be associated to lipid droplets such as various histone proteins which appear to be stored on LDs until needed (Cermelli, Guo et al. 2006). Other proteins which were identified by above study such as ATP synthase, heat shock proteins and actin were as well identified in our qproteomics analysis of different perilipin mutant lipid droplets of *D. melanogaster* (Appendix supplementary tables 1, 2, 3). Apart from these we have identified eight candidate proteins (Table 4) which show interesting differential abundance patterns on lipid droplets of fat bodies of different perilipin deficient fly mutants. Functional analysis of these identified candidates either with RNAi/deletion or overexpression may reveal whether they play a role in lipid droplet size regulation. We so far selected only Cyp1 for functional analysis of its impact on LD size regulation by RNAi. It would be interesting to look at the other candidate proteins as well, especially tubulin, which is a major cytoskeletal protein. Interestingly, it has been shown in perilipin 2 carrying LDs of mammalian fibroblast cells that the cytoskeleton plays a major role in regulation of cytosolic LD size, mechanistically involving microtubule-dependent formation of lipid droplet complexes which are required for LD growth (Bostrom, Rutberg et al. 2005). Additionally, perilipin 2 genetic disruption in *D. melanogaster* embryos has been shown to play a role in regulation of microtubule motor protein as well as lipid accumulation in *D. melanogaster* embryos, indicating that droplet motion could play role in control of lipid metabolism (Welte, Cermelli et al. 2005). A high throughput RNAi screen in *D. melanogaster* fat body cells in Ronald Kuehnlein's lab indicated increased LD size upon knock down of Tub85D (unpublished data, appendix supplementary figure1). Recently another interesting ER-LD shuttle protein spastin has emerged as being involved in regulation of LD size and number. In *Drosophila* overexpression of spastin leads to increase in size and decrease in numbers of LDs in the fat body. On the other hand RNAi mediated down-regulation results in decreased LD size in larval fat body (Papadopoulos, Orso et al. 2015). Taken together some of the above points, these proteome data sets could also give us an indication towards modified interaction of LDs with proximal organelles.

Cell fractionation is a powerful method to provide organelle fractions, however drawbacks of the method are that identified proteins may be true residents or contaminants coming from other compartments which exist in very close proximity, for

instance mitochondria and endoplasmic reticulum are consistently found in close contact with LDs. For that reason we checked LD localization of some of the more interesting candidates' by an additional approach like immunohistochemistry or expression of a GFP fusion protein for confocal microscopy. Our immunohistochemistry results from fat body protein 1 (FBP1), apolipoprotein (ApoL) and alcohol dehydrogenase (ADH) confirmed their partial localization on LDs. However this could be concluded only be from free-floating lipid droplets, which were purposely let out by mechanical force during the sample preparation. Difficulties in confirmation of LD localization occurred due to the presence of the proteins on multiple locations in the intact fat body. Moreover weaker signal on lipid droplet compared to other compartments did not allow for a clear proof of LD localization of the protein in the intact fat body tissue. Apart from immunohistochemistry for identifying localization of our candidate proteins we expressed cyclophilin1 as a Cyp1-eGFP fusion protein due to unavailability of an antibody. We also observed multiple localization and weak presence on LDs for the fusion protein. For clearer localization images of free-floating LDs would be beneficial.

Perilipin 1 is the most extensively studied and characterized lipid droplet surface protein present on giant lipid droplets (Brasaemle and Wolins 2012). Deficiency of perilipin 1 in mice leads to radically reduced LD size whereas in *D. melanogaster* it leads to adversely giant lipid droplet phenotype in fat body tissue (Tansey, Sztalryd et al. 2001, Beller, Bulankina et al. 2010). This indicates an additional role of perilipin 1 in regulation of LD size apart from regulating TAG metabolism. Our results suggest that cyclophilin 1 is a newly identified protein factor involved in the regulation of LD size in fat body tissue of *D. melanogaster*.

Cyclophilins are highly conserved from drosophila to human suggesting its important role. 7 human (hCypA-E, hCyp40 and hCypNK), 8 yeast (Cpr1-8) and 9 drosophila cyclophilins have been identified (Wang and Heitman 2005). Human cyclophilins were discovered as binding proteins for the immunosuppressive drug cyclosporin A (CsA) (Handschumacher, Harding et al. 1984). *Drosophila melanogaster* cyclophilin 1 has ~73% amino acid sequence similarity to human cyclophilin A (CypA) whereas other human cyclophilins (C, D, E) show less than 50% amino acid sequence similarity to *D. melanogaster* Cyp1. Cyclophilins possess peptidyl-propyl isomerase (PPIase) activity that is required for accurate protein folding and

repairing damaged proteins accordingly acting as protein chaperons as well (Yao, Li et al. 2005). A retina specific integral membrane protein *Drosophila melanogaster* cyclophilin, ninaA, an ortholog of mammalian CypC is required for critical folding of rhodopsins (Colley, Baker et al. 1991, Stamnes, Shieh et al. 1991). However cyclophilins are not functionally conserved as a yeast mutant lacking all eight yeast cyclophilins was viable (Dolinski, Muir et al. 1997). Cyclophilins are either intracellular or secreted proteins found in multiple tissues such as: CypA, Cyp40, CypNK are mainly cytosolic; CypB and CypC are mainly present in endoplasmic reticulum (ER); whereas CypD is mainly localized to mitochondria and CypE to the nucleus (Wang and Heitman 2005, Yao, Li et al. 2005). Furthermore, Cyclophilin inhibitor (NIM811) has been shown to have an increasing effect on the size of lipid droplets in presence of Hepatitis C virus (HCV) (Anderson, Lin et al. 2011). However, the mechanism by which cyclophilin can regulate LD size is not clear. Our identification of cyclophilin1 in upregulated manner in Plin1 knock out LD proteome of *D. melanogaster*, which has a giant lipid droplet phenotype, indicates towards a possible role in LD size regulation. Our RNAi studies showed that upon knock down of cyclophilin1 LD size is significantly reduced in *D. melanogaster* fat body in control as well as in Plin1 knock out genetic background. Certainly, it would be interesting to look at the effect of cyclophilin1 overexpression on lipid droplet size. Taken together, cyclophilins proteins are functionally versatile proteins present in multiple locations of cell with a novel role in LD homeostasis.

In conclusion, the presented work from part two of this thesis provides compelling evidence that cyclophilin1 plays an important role in regulation of lipid droplet size in *Drosophila melanogaster*. Apart from identifying the influence of cyclophilin1 on lipid droplet size this work revealed the lipid droplet proteome in absence of different perilipins as well as in a perilipin free system. Future studies will have to address whether other identified proteins of the current study play a role in regulation of lipid droplet size. It will be also interesting to look at the functional impact of these identified candidates on the regulation of lipid homeostasis and their interplay.

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Signature

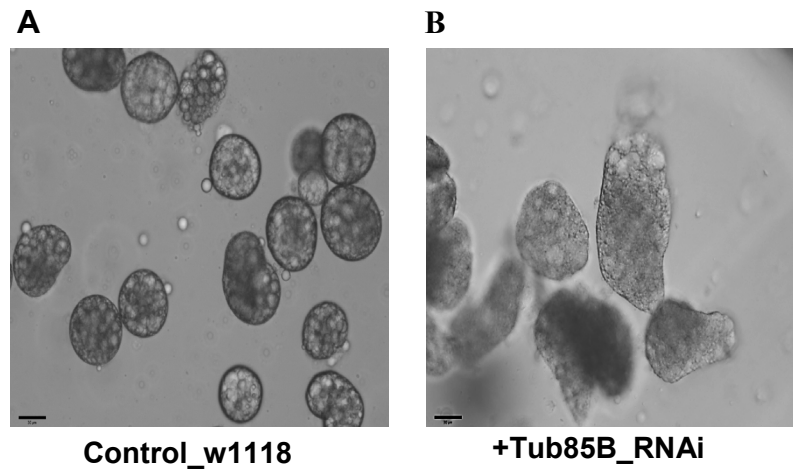
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APPENDIX:

Published manuscript:

Sahu-Osen, A., G. Montero-Moran (co-first author), M. Schittmayer, K. Fritz, A. Dinh, Y. F. Chang, D. McMahon, A. Boeszoermenyi, I. Cornaciu, D. Russell, M. Oberer, G. M. Carman, R. Birner-Gruenberger (co-corresponding author) and D. L. Brasaemle (2015). **"CGI-58/ABHD5 is phosphorylated on Ser239 by protein kinase A: control of subcellular localization."** J Lipid Res **56**(1): 109-121.

Supplementary figures and tables:



Supplementary Figure 1. Tubulin 85B dsRNA-mediated RNAi knock down in *D. melanogaster* fat body: In-vivo tubulin 85B RNAi (B) appeared to decrease fat body LD size compared to control (A). Transmission microscopy images after staining lipid droplets with BODIPY stain. These pictures were obtained in a large scale RNAi screening in Prof. Ronald P. Kühnlein's lab at MPI-Göttingen, Germany.

Supplementary Table1. Quantitative proteomics data of perilipin 1 knock out lipid droplets from larval *D. melanogaster* fat body: Heavy (H) to light (L) ratio indicates up (H/L>1) or down regulation (H/L<1) of a certain protein in perilipin 1 knock out LDs whereas H/L=1 indicates that the protein is equally expressed in knock out and control LDs. List is sorted based on the number of peptide spectral matches (PSMs), which is a measure for protein abundance. Yellow = candidates used for further analysis, green = additional interesting candidates.

Accession	Description	Heavy/Light	PSMs	Peptides	Heavy/Light Count	Heavy/Light Variability [%]	Reference
P35381	ATP synthase subunit alpha, mitochondrial OS=Drosophila melanogaster GN=blw PE=1 SV=2 - [ATPA_DROME]	1.0	75	12	6	22	
Q94920	Voltage-dependent anion-selective channel OS=Drosophila melanogaster GN=porin PE=1 SV=3 - [VDAC_DROME]	0.7	58	9	12	32	
Q26365	ADP,ATP carrier protein OS=Drosophila melanogaster GN=sesB PE=2 SV=4 - [ADT_DROME]	1.0	54	6	7	9	
P10987	Actin-5C OS=Drosophila melanogaster GN=Act5C PE=1 SV=4 - [ACT1_DROME]	1.5	41	7	3	25	(Beller, Riedel et al. 2006)
P53501	Actin-57B OS=Drosophila melanogaster GN=Act57B PE=3 SV=1 - [ACT3_DROME]	1.4	37	6	1		
P54399	Protein disulfide-isomerase OS=Drosophila melanogaster GN=Pdi PE=2 SV=1 - [PDI_DROME]	0.9	30	9	9	12	(Beller, Riedel et al. 2006)
P05661	Myosin heavy chain, muscle OS=Drosophila melanogaster GN=Mhc PE=1 SV=4 - [MYSA_DROME]	0.9	28	5	3	0	
P07764	Fructose-bisphosphate aldolase OS=Drosophila melanogaster GN=Ald PE=1 SV=5 - [ALF_DROME]	0.8	24	4	4	16	
P05303	Elongation factor 1-alpha 2 OS=Drosophila melanogaster GN=Ef1alpha100E PE=1 SV=2 - [EF1A2_DROME]	1.0	20	5	5	41	(Beller, Riedel et al. 2006)
P00334	Alcohol dehydrogenase OS=Drosophila melanogaster GN=Adh PE=1 SV=2 - [ADH_DROME]	0.9	20	4	3	43	(Beller, Riedel et al. 2006)
P25007	Peptidyl-prolyl cis-trans isomerase OS=Drosophila melanogaster GN=Cyp1 PE=1 SV=2 - [PPIA_DROME]	20.5	18	2	3	51	
P13060	Elongation factor 2 OS=Drosophila melanogaster GN=Ef2b PE=1 SV=4 - [EF2_DROME]	1.2	18	6	2	44	(Beller, Riedel et al. 2006)
Q05825	ATP synthase subunit beta, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-beta PE=1 SV=3 - [ATPB_DROME]	0.9	15	4	3	4	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
O18404	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Drosophila melanogaster GN=scu PE=1 SV=1 - [HCD2_DROME]	0.4	14	3	2	88	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P06603	Tubulin alpha-1 chain OS=Drosophila melanogaster GN=alphaTub84B PE=2 SV=1 - [TBA1_DROME]	1.0	12	3	1		
P11147	Heat shock 70 kDa protein cognate 4	0.9	12	3	2	12	

	OS=Drosophila melanogaster GN=Hsc70-4 PE=1 SV=3 - [HSP7D_DROME]						
P29844	Heat shock 70 kDa protein cognate 3 OS=Drosophila melanogaster GN=Hsc70-3 PE=2 SV=2 - [HSP7C_DROME]	1.3	11	2	2	1	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P84236	Histone H3 OS=Drosophila hydei GN=His3 PE=3 SV=2 - [H3_DROHY]	0.8	11	1	2	6	
Q24439	ATP synthase subunit O, mitochondrial OS=Drosophila melanogaster GN=Oscp PE=2 SV=2 - [ATPO_DROME]	0.8	11	5	5	14	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q9V496	Apolipoproteins OS=Drosophila melano- gaster GN=Rfabg PE=1 SV=2 - [APLP_DROME]	0.6	11	9	5	63	
Q9W237	40S ribosomal protein S16 OS=Drosophila melanogaster GN=RpS16 PE=2 SV=1 - [RS16_DROME]	0.4	11	3	2	93	
P11997	Larval serum protein 1 gamma chain OS=Drosophila melanogaster GN=Lsp1gamma PE=2 SV=2 - [LSP1G_DROME]	0.2	11	4	4	68	(Beller, Riedel et al. 2006)
Q9VAI6	General odorant-binding protein 99b OS=Drosophila melanogaster GN=Obp99b PE=2 SV=1 - [OB99B_DROME]	0.2	11	2	3	100	
P48610	Arginine kinase OS=Drosophila melanogas- ter GN=Argk PE=1 SV=2 - [KARG_DROME]	1.6	10	3	2	37	
Q9VGS2	Translationally-controlled tumor protein homolog OS=Drosophila melanogaster GN=Tctp PE=1 SV=1 - [TCTP_DROME]	0.8	10	1	1		
O61510	Fat body protein 2 OS=Drosophila similans GN=Fbp2 PE=3 SV=1 - [FBP2_DROSI]	0.1	10	2	4	113	
P09180	60S ribosomal protein L4 OS=Drosophila melanogaster GN=RpL4 PE=2 SV=2 - [RL4_DROME]	1.0	9	3	3	2	
P41092	60S ribosomal protein L27a OS=Drosophila melanogaster GN=RpL27A PE=2 SV=2 - [RL27A_DROME]	1.1	7	3	1		
P48588	40S ribosomal protein S25 OS=Drosophila melanogaster GN=RpS25 PE=1 SV=3 - [RS25_DROME]	0.7	7	1	1		
P30104	Glutathione S-transferase 1-1 (Fragment) OS=Drosophila erecta GN=GstD1 PE=3 SV=1 - [GSTT1_DROER]	0.1	7	2	2	11924	
Q9VXY7	Lipid storage droplets surface-binding protein 2 OS=Drosophila melanogaster GN=Lsd-2 PE=1 SV=1 - [LSD2_DROME]	3.0	6	2	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P41126	60S ribosomal protein L13 OS=Drosophila melanogaster GN=RpL13 PE=1 SV=1 - [RL13_DROME]	1.5	6	2	2	70	
P62152	Calmodulin OS=Drosophila melanogaster GN=Cam PE=1 SV=2 - [CALM_DROME]	1.3	6	1	1		
P08570	60S acidic ribosomal protein P1 OS=Drosophila melanogaster GN=RpLP1 PE=1 SV=2 - [RLA1_DROME]	1.1	6	2	1		
P42281	Acyl-CoA-binding protein homolog OS=Drosophila melanogaster GN=Dbi PE=2 SV=1 - [ACBP_DROME]	0.9	6	1	1		
P80455	40S ribosomal protein S12 OS=Drosophila melanogaster GN=RpS12 PE=2 SV=2 - [RS12_DROME]	0.8	6	2	2	12	
Q24407	ATP synthase-coupling factor 6, mitochon- drial OS=Drosophila melanogaster GN=ATPsyn-Cf6 PE=1 SV=1 - [ATP5J_DROME]	0.6	6	2	1		

P84041	Histone H4 OS=Drosophila erecta GN=His4 PE=3 SV=2 - [H4_DROER]	0.6	6	1	2	0	
Q9VJY6	60S ribosomal protein L24 OS=Drosophila melanogaster GN=RpL24 PE=1 SV=1 - [RL24_DROME]	1.2	5	1	1		
P54611	V-type proton ATPase subunit E OS=Drosophila melanogaster GN=Vha26 PE=1 SV=1 - [VATE_DROME]	1.2	5	1	1		
P18432	Myosin regulatory light chain 2 OS=Drosophila melanogaster GN=Mlc2 PE=1 SV=2 - [MLR_DROME]	1.1	5	2	1		
Q9VXK0	Protein NipSnap OS=Drosophila melanogaster GN=Nipsnap PE=1 SV=2 - [NIPSN_DROME]	0.8	5	1	1		
P17271	Histone H2B OS=Drosophila hydei GN=His2B PE=3 SV=2 - [H2B_DROHY]	0.8	5	1	1		
P19889	60S acidic ribosomal protein P0 OS=Drosophila melanogaster GN=RpLP0 PE=1 SV=1 - [RLA0_DROME]	0.6	5	1	1		
O01666	ATP synthase subunit gamma, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-gamma PE=2 SV=2 - [ATPG_DROME]	1.3	4	1	1		(Beller, Riedel et al. 2006)
P29413	Calreticulin OS=Drosophila melanogaster GN=Crc PE=1 SV=2 - [CALR_DROME]	1.1	4	1	1		(Beller, Riedel et al. 2006)
Q8MLY8	40S ribosomal protein S8 OS=Drosophila melanogaster GN=RpS8 PE=1 SV=1 - [RS8_DROME]	1.0	4	1	1		
O18640	Guanine nucleotide-binding protein subunit beta-like protein OS=Drosophila melanogaster GN=Rack1 PE=2 SV=2 - [GBLP_DROME]	0.7	4	1	1		
Q9W141	Putative ATP synthase subunit f, mitochondrial OS=Drosophila melanogaster GN=CG4692 PE=1 SV=1 - [ATPK_DROME]	0.6	4	1	1		(Beller, Riedel et al. 2006)
Q9VU58	Neuropeptide-like 2 OS=Drosophila melanogaster GN=Nplp2 PE=1 SV=1 - [TNLP2_DROME]	0.3	4	3	2	122	
P61858	Tubulin beta-2 chain OS=Drosophila hydei GN=betaTub85D PE=2 SV=1 - [TBB2_DROHY]	0.0	4	1	1		
P61853	Superoxide dismutase [Cu-Zn] OS=Drosophila mauritiana GN=Sod PE=3 SV=2 - [SODC_DROMA]	1.3	3	1	1		
P48159	60S ribosomal protein L23 OS=Drosophila melanogaster GN=RpL23 PE=1 SV=2 - [RL23_DROME]	1.1	3	1	1		
Q24251	ATP synthase subunit d, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-d PE=1 SV=2 - [ATP5H_DROME]	1.0	3	1	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P32100	60S ribosomal protein L7 OS=Drosophila melanogaster GN=RpL7 PE=2 SV=2 - [RL7_DROME]	0.9	3	2	1		
P07486	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Drosophila melanogaster GN=Gapdh1 PE=2 SV=2 - [G3P1_DROME]	0.6	3	2	1		(Cermelli, Guo et al. 2006)
P39018	40S ribosomal protein S19a OS=Drosophila melanogaster GN=RpS19a PE=2 SV=3 - [RS19A_DROME]	0.6	3	1	1		
P05389	60S acidic ribosomal protein P2 OS=Drosophila melanogaster GN=RpLP2 PE=1 SV=1 - [RLA2_DROME]	0.5	3	1	1		
Q29K06	GTP-binding protein 10 homolog OS=Drosophila pseudoobscura pseudoobscura GN=GA10450 PE=3 SV=1 - [GTPBA_DROPS]	3.5	2	1	1		
P46223	60S ribosomal protein L7a OS=Drosophila	1.1	2	2	1		

	melanogaster GN=RpL7A PE=2 SV=2 - [RL7A_DROME]						
B3N1G9	40S ribosomal protein S3a OS=Drosophila ananassae GN=RpS3A PE=3 SV=1 - [RS3A_DROAN]	1.0	2	2	2	1	
P54385	Glutamate dehydrogenase, mitochondrial OS=Drosophila melanogaster GN=Gdh PE=1 SV=2 - [DHE3_DROME]	1.0	2	1	1		
P41093	60S ribosomal protein L18a OS=Drosophila melanogaster GN=RpL18A PE=1 SV=1 - [RL18A_DROME]	1.0	2	1	1		
O16797	60S ribosomal protein L3 OS=Drosophila melanogaster GN=RpL3 PE=1 SV=3 - [RL3_DROME]	0.9	2	2	1		
Q24008	Inactivation-no-after-potential D protein OS=Drosophila melanogaster GN=inaD PE=1 SV=2 - [INAD_DROME]	0.9	2	1	1		
Q29HB2	Probable methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial OS=Drosophila pseudoobscura pseudoobscura GN=GA14712 PE=3 SV=1 - [MMSA_DROPS]	0.7	2	1	1		
Q9V429	Thioredoxin-2 OS=Drosophila melanogaster GN=Trx-2 PE=1 SV=2 - [THIO2_DROME]	0.7	2	1	1		
Q94516	ATP synthase subunit b, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-b PE=1 SV=2 - [AT5F1_DROME]	0.7	2	1	1		(Beller, Riedel et al. 2006)
Q9VWH4	Probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial OS=Drosophila melanogaster GN=I(1)G0156 PE=2 SV=1 - [IDH3A_DROME]	0.4	2	1	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q9W5R8	60S ribosomal protein L5 OS=Drosophila melanogaster GN=RpL5 PE=1 SV=2 - [RL5_DROME]	0.3	2	1	1		
Q94514	Cytochrome c oxidase subunit 5A, mitochondrial OS=Drosophila melanogaster GN=CoVa PE=1 SV=2 - [COX5A_DROME]	1.9	1	1	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q9NJH0	Elongation factor 1-gamma OS=Drosophila melanogaster GN=Ef1gamma PE=1 SV=2 - [EF1G_DROME]	1.0	1	1	1		(Beller, Riedel et al. 2006)
O96827	Probable elongation factor 1-beta OS=Drosophila melanogaster GN=Ef1beta PE=1 SV=3 - [EF1B_DROME]	0.8	1	1	1		(Beller, Riedel et al. 2006)
P0CG69	Polyubiquitin OS=Drosophila melanogaster GN=Ubi-p63E PE=3 SV=1 - [UBIQP_DROME]	0.2	1	1	1		
Q9VCI3	Lipid storage droplets surface-binding protein 1 OS=Drosophila melanogaster GN=Lsd-1 PE=1 SV=2 - [LSD1_DROME]	0.1	1	1	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q00725	Salivary glue protein Sgs-4 OS=Drosophila melanogaster GN=Sgs4 PE=2 SV=1 - [SGS4_DROME]	0.0	1	1	1		

Supplementary Table2. Quantitative proteomics data of perilipin 2 knock out lipid droplets from larval *D. melanogaster* fat body: Medium (M) to light (L) ratio indicates up (M/L>1) or down (M/L<1) regulation of a certain protein in perilipin 2 knock out LDs whereas M/L=1 indicates that the protein is equally expressed in knock out and control LDs. List is sorted based on the number of peptide spectral matches (PSMs). Yellow = candidates used for further analysis, green = additional interesting candidates.

Accession	Description	Medium /Light ratio	PSMs	Peptides	Medium/Light Count	Medium/Light Variability [%]	Reference
P82147	Protein lethal(2)essential for life OS=Drosophila melanogaster GN=l(2)efl PE=1 SV=1 - [L2EFL_DROME]	2.6	31	13	10	6705	
P53501	Actin-57B OS=Drosophila melanogaster GN=Act57B PE=1 SV=1 - [ACT3_DROME]	0.1	24	9	1		
P10987	Actin-5C OS=Drosophila melanogaster GN=Act5C PE=1 SV=4 - [ACT1_DROME]		23	8			
P05661	Myosin heavy chain, muscle OS=Drosophila melanogaster GN=Mhc PE=1 SV=4 - [MYSA_DROME]	0.1	22	14	7	308	(Beller, Riedel et al. 2006)
Q9V496	Apolipoporphins OS=Drosophila melanogaster GN=Rfabg PE=1 SV=2 - [APLP_DROME]	1.2	21	12	8	44	
P48610	Arginine kinase OS=Drosophila melanogaster GN=Argk PE=2 SV=2 - [KARG_DROME]	0.7	15	11	6	479	
P07764	Fructose-bisphosphate aldolase OS=Drosophila melanogaster GN=Ald PE=1 SV=5 - [ALF_DROME]	3.6	13	9	4	138	
Q9VAI6	General odorant-binding protein 99b OS=Drosophila melanogaster GN=Obp99b PE=2 SV=1 - [OB99B_DROME]	7.2	13	5	6	12	
Q9VU58	Neuropeptide-like 2 OS=Drosophila melanogaster GN=Nplp2 PE=1 SV=1 - [NPLP2_DROME]	1.0	11	5	2	132628633 8257	
P11147	Heat shock 70 kDa protein cognate 4 OS=Drosophila melanogaster GN=Hsc70-4 PE=1 SV=3 - [HSP7D_DROME]	0.1	11	6	1		
Q9V521	Phenoloxidase subunit A3 OS=Drosophila melanogaster GN=proPo-A3 PE=1 SV=1 - [PRPA3_DROME]	4.0	10	6	2	445	
P15007	Enolase OS=Drosophila melanogaster GN=Eno PE=1 SV=2 - [ENO_DROME]	1.1	10	4	4	45364	(Beller, Riedel et al. 2006)
Q04691	Fat-body protein 1 OS=Drosophila melanogaster GN=Fbp1 PE=2 SV=2 - [FBP1_DROME]	0.1	10	7	4	195	(Beller, Riedel et al. 2006)
P00334	Alcohol dehydrogenase OS=Drosophila melanogaster GN=Adh PE=1 SV=2 - [ADH_DROME]	1.1	9	8	2	111	(Beller, Riedel et al. 2006)

P11996	Larval serum protein 1 beta chain OS=Drosophila melanogaster GN=Lsp1beta PE=2 SV=3 - [LSP1B_DROME]	0.9	8	6	3	45	(Beller, Riedel et al. 2006)
P29413	Calreticulin OS=Drosophila melanogaster GN=Crc PE=1 SV=2 - [CALR_DROME]	0.8	8	7	4	8	(Beller, Riedel et al. 2006)
Q24388	Larval serum protein 2 OS=Drosophila melanogaster GN=Lsp2 PE=1 SV=2 - [LSP2_DROME]	1.1	8	6	3	5	Beller et al., 2006
P07159	Alcohol dehydrogenase OS=Drosophila orena GN=Adh PE=3 SV=2 - [ADH_DROOR]	6.6	7	6	1		
Q9VCI3	Lipid storage droplets surface-binding protein 1 OS=Drosophila melanogaster GN=Lsd-1 PE=1 SV=2 - [LSD1_DROME]	100.0	7	4	3	0	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P35381	ATP synthase subunit alpha, mitochondrial OS=Drosophila melanogaster GN=blw PE=1 SV=2 - [ATPA_DROME]	0.5	7	6	4	38	
Q6XI71	Nucleoside diphosphate kinase (Fragment) OS=Drosophila yakuba GN=awd PE=2 SV=1 - [NDKA_DROYA]	100.0	7	4	1		
P29844	Heat shock 70 kDa protein cognate 3 OS=Drosophila melanogaster GN=Hsc70-3 PE=2 SV=2 - [HSP7C_DROME]	0.2	7	5	2	14	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
O44104	Glyceraldehyde-3-phosphate dehydrogenase 2 OS=Drosophila pseudoobscura pseudoobscura GN=Gapdh2 PE=2 SV=2 - [G3P2_DROPS]	0.3	7	6	1		
P08736	Elongation factor 1-alpha 1 OS=Drosophila melanogaster GN=Ef1alpha48D PE=1 SV=2 - [EF1A1_DROME]	0.8	6	3	2	54	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
O62619	Pyruvate kinase OS=Drosophila melanogaster GN=PyK PE=2 SV=2 - [KPYK_DROME]	0.2	6	5	5	272	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P14318	Muscle-specific protein 20 OS=Drosophila melanogaster GN=Mp20 PE=2 SV=2 - [MP20_DROME]	0.8	6	5	4	36	
P06754	Tropomyosin-1, isoforms 9A/A/B OS=Drosophila melanogaster GN=Tm1 PE=1 SV=2 - [TPM1_DROME]	0.3	6	6	3	15116757	
P11997	Larval serum protein 1 gamma chain OS=Drosophila melanogaster GN=Lsp1gamma PE=2 SV=2 - [LSP1G_DROME]	0.0	6	4	3	231	(Beller, Riedel et al. 2006)
P19351	Troponin T, skeletal muscle OS=Drosophila melanogaster GN=up PE=1 SV=3 - [TNNT_DROME]	0.0	6	3	2	151	
P07188	Larval cuticle protein 3 OS=Drosophila melanogaster GN=Lcp3 PE=1 SV=1 - [LCP3_DROME]	95.0	5	1	2	8	
Q06559	40S ribosomal protein S3	0.2	5	3	2	45	

	OS=Drosophila melanogaster GN=RpS3 PE=1 SV=1 - [RS3_DROME]						
P92177	14-3-3 protein epsilon OS=Drosophila melanogaster GN=14-3-3epsilon PE=1 SV=2 - [1433E_DROME]	2.7	5	4	2	265	
Q9V3S9	Very long-chain-fatty-acid-- CoA ligase bubblegum OS=Drosophila melanogaster GN=bgm PE=2 SV=1 - [BGM_DROME]	0.2	5	3	2	81	
P53777	Muscle LIM protein 1 OS=Drosophila melanogaster GN=Mlp60A PE=2 SV=1 - [MLP1_DROME]	0.8	5	2	2	13	
Q24560	Tubulin beta-1 chain OS=Drosophila melanogaster GN=betaTub56D PE=1 SV=2 - [TBB1_DROME]	1.9	4	4	2	32	
P82384	Larval cuticle protein 9 OS=Drosophila melanogaster GN=Lcp9 PE=1 SV=2 - [LCP9_DROME]		4	1			
P11995	Larval serum protein 1 alpha chain OS=Drosophila melano- gaster GN=Lsp1alpha PE=2 SV=3 - [LSP1A_DROME]	1.2	4	3	1		(Beller, Riedel et al. 2006)
Q9W303	Chitinase-like protein Idgf4 OS=Drosophila melanogaster GN=Idgf4 PE=2 SV=1 - [IDGF4_DROME]	4.9	4	2	2	584	
P54399	Protein disulfide-isomerase OS=Drosophila melanogaster GN=Pdi PE=2 SV=1 - [PDI_DROME]	1.1	4	4	3	73	(Beller, Riedel et al. 2006)
P17271	Histone H2B OS=Drosophila hydei GN=His2B PE=3 SV=2 - [H2B_DROHY]	0.2	4	2	2	7	
Q23997	Chitinase-like protein CG5210 OS=Drosophila melanogaster GN=CG5210 PE=1 SV=2 - [C5210_DROME]	5.8	4	2	1		
Q94920	Voltage-dependent anion- selective channel OS=Drosophila melanogaster GN=porin PE=1 SV=3 - [VDAC_DROME]		4	2			
Q9VMD9	Tiggrin OS=Drosophila mela- nogaster GN=Tig PE=1 SV=2 - [TIG_DROME]	0.4	4	2	2	87	
Q6XI08	40S ribosomal protein S14a OS=Drosophila yakuba GN=RpS14a PE=2 SV=1 - [RS14A_DROYA]	0.3	3	2	1		
P06603	Tubulin alpha-1 chain OS=Drosophila melanogaster GN=alphaTub84B PE=2 SV=1 - [TBA1_DROME]	0.3	3	3	2	98	
Q24186	40S ribosomal protein S5a OS=Drosophila melanogaster GN=RpS5a PE=2 SV=1 - [RS5A_DROME]	0.4	3	2	2	19	
Q05825	ATP synthase subunit beta, mitochondrial OS=Drosophila melanogaster GN=ATPsyn- beta PE=2 SV=3 - [ATPB_DROME]	3.1	3	3	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P29310	14-3-3 protein zeta OS=Drosophila melanogaster	1.2	3	3	3	34	

	GN=14-3-3zeta PE=1 SV=1 - [1433Z_DROME]						
Q9VLU0	Barrier-to-autointegration factor OS=Drosophila melanogaster GN=baf PE=3 SV=1 - [BAF_DROME]	0.6	3	2	2	23	
Q27415	Nucleoplasmin-like protein OS=Drosophila melanogaster GN=Nlp PE=1 SV=1 - [NLP_DROME]	100.0	3	1	1		
P02840	Salivary glue protein Sgs-3 OS=Drosophila melanogaster GN=Sgs3 PE=2 SV=1 - [SGS3_DROME]	1.1	3	1	1		
P07189	Larval cuticle protein 4 OS=Drosophila melanogaster GN=Lcp4 PE=1 SV=2 - [LCP4_DROME]	100.0	3	2	1		
Q9VMP9	Glucosamine-6-phosphate isomerase OS=Drosophila melanogaster GN=Gnpda1 PE=2 SV=1 - [GNPI_DROME]	2.1	3	1	1		
P39018	40S ribosomal protein S19a OS=Drosophila melanogaster GN=RpS19a PE=2 SV=3 - [RS19A_DROME]	0.0	3	1	1		
P13060	Elongation factor 2 OS=Drosophila melanogaster GN=Ef2b PE=2 SV=4 - [EF2_DROME]		3	2			(Beller, Riedel et al. 2006)
P84052	Histone H2A OS=Drosophila erecta GN=His2A PE=3 SV=2 - [H2A_DROER]		3	2			
Q26365	ADP,ATP carrier protein OS=Drosophila melanogaster GN=sesB PE=2 SV=4 - [ADT_DROME]		3	2			
Q24251	ATP synthase subunit d, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-d PE=2 SV=2 - [ATP5H_DROME]	0.4	3	2	1		(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
B4PPT4	Adenylosuccinate synthetase OS=Drosophila yakuba GN=GE25034 PE=3 SV=1 - [PURA_DROYA]		3	2			
P05389	60S acidic ribosomal protein P2 OS=Drosophila melanogaster GN=RpLP2 PE=1 SV=1 - [RLA2_DROME]		3	1			
O61493	Protein hook OS=Drosophila virilis GN=hk PE=3 SV=1 - [HOOK_DROVI]		2	1			
O77460	Inorganic pyrophosphatase OS=Drosophila melanogaster GN=Nurf-38 PE=1 SV=3 - [IPYR_DROME]	0.5	2	1	1		
Q9V3P0	Peroxiredoxin 1 OS=Drosophila melanogaster GN=Jafrac1 PE=1 SV=1 - [PRDX1_DROME]	1.9	2	2	1		
P20478	Glutamine synthetase 2 cytoplasmic OS=Drosophila melanogaster GN=Gsl2 PE=2 SV=3 - [GLNA2_DROME]	5.4	2	2	2	1173252	
P02842	Salivary glue protein Sgs-8 OS=Drosophila melanogaster GN=Sgs8 PE=2 SV=1 - [SGS8_DROME]	0.6	2	2	1		
O02192	Heat shock protein 83	0.7	2	2	2	22	

	OS=Drosophila auraria GN=Hsp83 PE=3 SV=1 - [HSP83_DROAV]						
Q95028	L-lactate dehydrogenase OS=Drosophila melanogaster GN=ImpL3 PE=2 SV=1 - [LDH_DROME]		2	2			
P54366	Homeobox protein goosecoid OS=Drosophila melanogaster GN=Gsc PE=2 SV=1 - [GSC_DROME]		2	1			
P16911	Protein kinase DC1 OS=Drosophila melanogaster GN=Pka-C2 PE=2 SV=1 - [KDC1_DROME]	0.0	2	1	1		
P84029	Cytochrome c-2 OS=Drosophila melanogaster GN=Cyt-c-p PE=1 SV=2 - [CYC2_DROME]	1.0	2	2	1		
P29613	Triosephosphate isomerase OS=Drosophila melanogaster GN=Tpi PE=1 SV=3 - [TPIS_DROME]	1.0	2	2	1		
Q6AWN0	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase OS=Drosophila melanogaster GN=CG32068 PE=2 SV=2 - [MTND_DROME]	1.8	2	2	1		
Q9VXY7	Lipid storage droplets surface-binding protein 2 OS=Drosophila melanogaster GN=Lsd-2 PE=1 SV=1 - [LSD2_DROME]	0.0	2	2	2	4	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006))
P47948	Troponin C, isoform 2 OS=Drosophila melanogaster GN=TpnC47D PE=2 SV=2 - [TNNC2_DROME]	0.9	2	2	1		
Q8T3U2	40S ribosomal protein S23 OS=Drosophila melanogaster GN=RpS23 PE=2 SV=1 - [RS23_DROME]	0.4	2	1	1		
P30107	Glutathione S-transferase 1-1 (Fragment) OS=Drosophila teissieri GN=GstD1 PE=3 SV=1 - [GSTT1_DROTE]	4.0	2	2	1		
P22464	Annexin-B9 OS=Drosophila melanogaster GN=AnnIX PE=2 SV=2 - [ANXB9_DROME]	3.9	2	1	1		
Q27556	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Drosophila americana GN=Gpdh PE=3 SV=4 - [GPDA_DROAE]	1.0	2	1	1		
P09491	Tropomyosin-2 OS=Drosophila melanogaster GN=Tm2 PE=2 SV=1 - [TPM2_DROME]	0.2	2	2	1		
Q27331	V-type proton ATPase catalytic subunit A isoform 2 OS=Drosophila melanogaster GN=Vha68-2 PE=1 SV=2 - [VATA2_DROME]	0.2	2	1	1		
P50887	60S ribosomal protein L22 OS=Drosophila melanogaster GN=RpL22 PE=1 SV=2 - [RL22_DROME]	0.3	2	1	1		
P13008	40S ribosomal protein S26 OS=Drosophila melanogaster GN=RpS26 PE=1 SV=1 - [RS26_DROME]	0.2	2	2	1		
P48375	12 kDa FK506-binding protein		1	1			

	OS=Drosophila melanogaster GN=FK506-bp2 PE=3 SV=2 - [FKB12_DROME]						
Q7K0F0	Coiled-coil domain-containing protein 130 homolog OS=Drosophila melanogaster GN=CG15084 PE=2 SV=1 - [CC130_DROME]	0.0	1	1	1		
O96827	Probable elongation factor 1- beta OS=Drosophila melano- gaster GN=Ef1beta PE=1 SV=3 - [EF1B_DROME]	1.1	1	1	1		(Beller, Riedel et al. 2006)
P0CG69	Polyubiquitin OS=Drosophila melanogaster GN=Ubi-p63E PE=3 SV=1 - [UBIQP_DROME]	0.8	1	1	1		
Q9V813	S-methyl-5'-thioadenosine phosphorylase OS=Drosophila melanogaster GN=CG4802 PE=1 SV=1 - [MTAP_DROME]	4.1	1	1	1		
Q24407	ATP synthase-coupling factor 6, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-Cf6 PE=3 SV=1 - [ATP5J_DROME]	2.7	1	1	1		
P06742	Myosin light chain alkali OS=Drosophila melanogaster GN=Mlc1 PE=2 SV=4 - [MLC1_DROME]	0.1	1	1	1		
Q9W401	Probable citrate synthase, mitochondrial OS=Drosophila melanogaster GN=kdn PE=2 SV=1 - [CISY_DROME]	0.2	1	1	1		
Q94518	Nascent polypeptide- associated complex subunit alpha OS=Drosophila melano- gaster GN=Nacalpha PE=1 SV=2 - [NACA_DROME]	0.8	1	1	1		
P07187	Larval cuticle protein 2 OS=Drosophila melanogaster GN=Lcp2 PE=1 SV=1 - [LCP2_DROME]		1	1			
P17336	Catalase OS=Drosophila mela- nogaster GN=Cat PE=1 SV=2 - [CATA_DROME]	2.4	1	1	1		
P18091	Alpha-actinin, sarcomeric OS=Drosophila melanogaster GN=Actn PE=2 SV=2 - [ACTN_DROME]		1	1			
Q9XTL9	Glycogen phosphorylase OS=Drosophila melanogaster GN=GlyP PE=2 SV=2 - [PYG_DROME]	100.0	1	1	1		
P12613	T-complex protein 1 subunit alpha OS=Drosophila melano- gaster GN=T-cp1 PE=2 SV=2 - [TCPA_DROME]	3.6	1	1	1		
P23226	205 kDa microtubule- associated protein OS=Drosophila melanogaster GN=Map205 PE=1 SV=2 - [MA205_DROME]	2.7	1	1	1		
P48598	Eukaryotic translation initiation factor 4E OS=Drosophila melanogaster GN=eIF-4E PE=1 SV=1 - [IF4E_DROME]	1.4	1	1	1		
P92192	Larval cuticle protein 5 OS=Drosophila melanogaster GN=Lcp65Ab1 PE=1 SV=1 - [LCP5_DROME]		1	1			
P84041	Histone H4 OS=Drosophila erecta GN=His4 PE=3 SV=2 -	0.5	1	1	1		

	[H4_DROER]						
P62085	40S ribosomal protein S7 OS=Drosophila yakuba GN=RpS7 PE=2 SV=1 - [RS7_DROYA]	0.0	1	1	1		
P25007	Peptidyl-prolyl cis-trans iso- merase OS=Drosophila mela- nogaster GN=Cyp1 PE=1 SV=2 - [PPIA_DROME]	1.4	1	1	1		
Q9VJ33	NEDD8 OS=Drosophila mela- nogaster GN=Nedd8 PE=1 SV=1 - [NEDD8_DROME]	1.0	1	1	1		
Q02748	Eukaryotic initiation factor 4A OS=Drosophila melanogaster GN=eIF-4a PE=2 SV=3 - [IF4A_DROME]	0.5	1	1	1		
P18432	Myosin regulatory light chain 2 OS=Drosophila melanogaster GN=Mlc2 PE=1 SV=2 - [MLR_DROME]		1	1			
O16797	60S ribosomal protein L3 OS=Drosophila melanogaster GN=RpL3 PE=2 SV=3 - [RL3_DROME]	0.2	1	1	1		
Q7JZV0	Cuticular protein 47Eg OS=Drosophila melanogaster GN=Cpr47Eg PE=1 SV=1 - [LCP2A_DROME]		1	1			
P61853	Superoxide dismutase [Cu-Zn] OS=Drosophila mauritiana GN=Sod PE=3 SV=2 - [SODC_DROMA]		1	1			
Q9VXX8	Probable 60S ribosomal pro- tein L37-A OS=Drosophila melanogaster GN=RpL37a PE=3 SV=1 - [RL371_DROME]	0.3	1	1	1		
B4K934	Werner Syndrome-like exonu- clease OS=Drosophila moja- vensis GN=WRNexo PE=3 SV=1 - [WRNXO_DROMO]		1	1			
Q9VH69	40S ribosomal protein S29 OS=Drosophila melanogaster GN=RpS29 PE=1 SV=1 - [RS29_DROME]	0.0	1	1	1		
Q23979	Myosin-IB OS=Drosophila melanogaster GN=Myo61F PE=1 SV=3 - [MY61F_DROME]		1	1			
P31409	V-type proton ATPase subunit B OS=Drosophila melano- gaster GN=Vha55 PE=2 SV=1 - [VATB_DROME]	0.4	1	1	1		
P19889	60S acidic ribosomal protein P0 OS=Drosophila melano- gaster GN=RpLP0 PE=1 SV=1 - [RLA0_DROME]	0.2	1	1	1		
P41042	40S ribosomal protein S4 OS=Drosophila melanogaster GN=RpS4 PE=2 SV=2 - [RS4_DROME]	0.4	1	1	1		
P40793	Cdc42 homolog OS=Drosophila melanogaster GN=Cdc42 PE=1 SV=1 - [CDC42_DROME]	0.0	1	1	1		
B3MIW0	Protein clueless OS=Drosophila ananassae GN=clu PE=3 SV=1 - [CLU_DROAN]		1	1			
P54611	V-type proton ATPase subunit E OS=Drosophila melanogas- ter GN=Vha26 PE=2 SV=1 -	0.0	1	1	1		

	[VATE_DROME]						
P50882	60S ribosomal protein L9 OS=Drosophila melanogaster GN=RpL9 PE=2 SV=2 - [RL9_DROME]	0.1	1	1	1		
P41094	40S ribosomal protein S18 OS=Drosophila melanogaster GN=RpS18 PE=1 SV=1 - [RS18_DROME]	0.3	1	1	1		
Q04688	DNA-binding protein Ets97D OS=Drosophila melanogaster GN=Ets97D PE=1 SV=2 - [ELG_DROME]		1	1			
B3MRX2	40S ribosomal protein SA OS=Drosophila ananassae GN=sta PE=3 SV=1 - [RSSA_DROAN]		1	1			
Q9V3Y3	Defense protein I(2)34Fc OS=Drosophila melanogaster GN=I(2)34Fc PE=2 SV=1 - [DFP_DROME]		1	1			
Q9W334	40S ribosomal protein S28 OS=Drosophila melanogaster GN=RpS28b PE=3 SV=2 - [RS28_DROME]	0.1	1	1	1		
Q03334	40S ribosomal protein S13 OS=Drosophila melanogaster GN=RpS13 PE=2 SV=3 - [RS13_DROME]	0.0	1	1	1		
Q9VQ36	WD repeat-containing and planar cell polarity effector protein fritz OS=Drosophila melanogaster GN=fritz PE=2 SV=1 - [FRITZ_DROME]	0.3	1	1	1		
P41964	Drosomycin OS=Drosophila melanogaster GN=Drs PE=1 SV=1 - [DMYC_DROME]	0.4	1	1	1		
Q07171	Gelsolin OS=Drosophila mela- nogaster GN=Gel PE=1 SV=2 - [GELS_DROME]	0.3	1	1	1		
P26270	26S proteasome non-ATPase regulatory subunit 7 OS=Drosophila melanogaster GN=Mov34 PE=1 SV=6 - [PSMD7_DROME]		1	1			
P29746	Protein bangles and beads OS=Drosophila melanogaster GN=bnb PE=1 SV=1 - [BNB_DROME]	0.8	1	1	1		
Q9VZ49	Poly(U)-specific endoribonu- clease homolog OS=Drosophila melanogaster GN=CG2145 PE=1 SV=1 - [ENDOU_DROME]		1	1			
Q9W4E2	Neurobeachin OS=Drosophila melanogaster GN=rg PE=1 SV=3 - [NBEA_DROME]		1	1			
P62152	Calmodulin OS=Drosophila melanogaster GN=Cam PE=1 SV=2 - [CALM_DROME]		1	1			

Supplementary Table 3. Quantitative proteomics data of perilipin 1 and perilipin 2 double knock out lipid droplets from larval *D. melanogaster* fat body: Medium (M) to light (L) ratio indicates up (M/L>1) or down (M/L<1) regulation of a certain protein in double knock out LDs whereas M/L=1 indicates that the protein is equally expressed in knock out and control LDs. List is sorted based on the number of peptide spectral matches (PSMs). Yellow = candidates used for further analysis, green = additional interesting candidates.

Accession	Description	Medium/Light	PSMs	Peptides	Medium/Light Count	Medium/Light Variability [%]	Reference
P54399	Protein disulfide-isomerase OS=Drosophila melanogaster GN=Pdi PE=2 SV=1 - [PDI_DROME]	0.4	92	29	32	34	(Beller, Riedel et al. 2006)
P11997	Larval serum protein 1 gamma chain OS=Drosophila melanogaster GN=Lsp1gamma PE=2 SV=2 - [LSP1G_DROME]	3.7	84	28	30	48	(Beller, Riedel et al. 2006)
Q9V496	Apolipoporphins OS=Drosophila melanogaster GN=Rfabg PE=1 SV=2 - [APLP_DROME]	3.3	70	39	36	27	
Q24388	Larval serum protein 2 OS=Drosophila melanogaster GN=Lsp2 PE=1 SV=2 - [LSP2_DROME]	2.5	70	19	25	35	(Beller, Riedel et al. 2006)
P11996	Larval serum protein 1 beta chain OS=Drosophila melanogaster GN=Lsp1beta PE=2 SV=3 - [LSP1B_DROME]	1.9	70	24	20	22	(Beller, Riedel et al. 2006)
P11995	Larval serum protein 1 alpha chain OS=Drosophila melanogaster GN=Lsp1alpha PE=2 SV=3 - [LSP1A_DROME]	1.1	60	23	19	66	(Beller, Riedel et al. 2006)
Q04691	Fat-body protein 1 OS=Drosophila melanogaster GN=Fbp1 PE=2 SV=2 - [FBP1_DROME]	0.7	60	26	26	23	(Beller, Riedel et al. 2006)
P53501	Actin-57B OS=Drosophila melanogaster GN=Act57B PE=1 SV=1 - [ACT3_DROME]	2.7	56	11	2	37	
P10987	Actin-5C OS=Drosophila melanogaster GN=Act5C PE=1 SV=4 - [ACT1_DROME]	2.1	56	13	4	15	
P29844	Heat shock 70 kDa protein cognate 3 OS=Drosophila melanogaster GN=Hsc70-3 PE=2 SV=2 - [HSP7C_DROME]	0.6	48	20	18	29	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P11147	Heat shock 70 kDa protein cognate 4 OS=Drosophila melanogaster GN=Hsc70-4 PE=1 SV=3 - [HSP7D_DROME]	1.2	44	14	12	36	
P07764	Fructose-bisphosphate aldolase OS=Drosophila melanogaster GN=Ald PE=1 SV=5 - [ALF_DROME]	1.7	39	15	16	23	

Q05825	ATP synthase subunit beta, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-beta PE=2 SV=3 - [ATPB_DROME]	0.6	39	16	14	21	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P82147	Protein lethal(2)essential for life OS=Drosophila melanogaster GN=l(2)efl PE=1 SV=1 - [L2EFL_DROME]	0.6	38	12	14	13	
P29413	Calreticulin OS=Drosophila melanogaster GN=Crc PE=1 SV=2 - [CALR_DROME]	0.4	37	14	15	34	(Beller, Riedel et al. 2006)
P08736	Elongation factor 1-alpha 1 OS=Drosophila melanogaster GN=Ef1alpha48D PE=1 SV=2 - [EF1A1_DROME]	1.4	36	10	13	101	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q9VXY7	Lipid storage droplets surface-binding protein 2 OS=Drosophila melanogaster GN=Lsd-2 PE=1 SV=1 - [LSD2_DROME]	0.1	35	10	9	172	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
O44104	Glyceraldehyde-3-phosphate dehydrogenase 2 OS=Drosophila pseudoobscura pseudoobscura GN=Gapdh2 PE=2 SV=2 - [G3P2_DROPS]	0.8	32	8	2	8	
P00334	Alcohol dehydrogenase OS=Drosophila melanogaster GN=Adh PE=1 SV=2 - [ADH_DROME]	1.6	28	9	2	118	(Beller, Riedel et al. 2006)
Q6XI71	Nucleoside diphosphate kinase (Fragment) OS=Drosophila yakuba GN=awd PE=2 SV=1 - [NDKA_DROYA]	1.8	27	7	8	24	
Q9VAI6	General odorant-binding protein 99b OS=Drosophila melanogaster GN=Obp99b PE=2 SV=1 - [OB99B_DROME]	1.7	27	5	7	11	
P06603	Tubulin alpha-1 chain OS=Drosophila melanogaster GN=alphaTub84B PE=2 SV=1 - [TBA1_DROME]	1.2	27	9	11	8	
P35381	ATP synthase subunit alpha, mitochondrial OS=Drosophila melanogaster GN=blw PE=1 SV=2 - [ATPA_DROME]	0.9	27	11	11	16	
Q24560	Tubulin beta-1 chain OS=Drosophila melanogaster GN=betaTub56D PE=1 SV=2 - [TBB1_DROME]	1.1	24	12	11	28	
O62619	Pyruvate kinase OS=Drosophila melanogaster GN=PyK PE=2 SV=2 - [KPYK_DROME]	0.4	24	12	8	49	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P14318	Muscle-specific protein 20 OS=Drosophila melanogaster GN=Mp20 PE=2 SV=2 - [MP20_DROME]	2.1	22	5	5	31	
Q9VU58	Neuropeptide-like 2 OS=Drosophila melanogaster GN=Nplp2 PE=1 SV=1 - [NPLP2_DROME]	1.3	22	3	3	0	
Q9VCI3	Lipid storage droplets surface-binding protein 1 OS=Drosophila melanogaster	0.0	21	8	9	184	(Beller, Riedel et al. 2006,

	GN=Lsd-1 PE=1 SV=2 - [LSD1_DROME]						Cermelli, Guo et al. 2006)
P15007	Enolase OS=Drosophila melanogaster GN=Eno PE=1 SV=2 - [ENO_DROME]	1.8	19	8	9	49	(Beller, Riedel et al. 2006)
P48610	Arginine kinase OS=Drosophila melanogaster GN=Argk PE=2 SV=2 - [KARG_DROME]	5.1	17	9	8	34	
P07189	Larval cuticle protein 4 OS=Drosophila melanogaster GN=Lcp4 PE=1 SV=2 - [LCP4_DROME]	2.1	17	3	6	18	
Q06559	40S ribosomal protein S3 OS=Drosophila melanogaster GN=RpS3 PE=1 SV=1 - [RS3_DROME]	0.7	14	7	4	22	
Q6XI08	40S ribosomal protein S14a OS=Drosophila yakuba GN=RpS14a PE=2 SV=1 - [RS14A_DROYA]	0.5	14	4	4	55	
O02192	Heat shock protein 83 OS=Drosophila auraria GN=Hsp83 PE=3 SV=1 - [HSP83_DROAV]	3.6	13	8	5	19	
P07188	Larval cuticle protein 3 OS=Drosophila melanogaster GN=Lcp3 PE=1 SV=1 - [LCP3_DROME]	1.3	13	4	3	5	
P31409	V-type proton ATPase subunit B OS=Drosophila melanogaster GN=Vha55 PE=2 SV=1 - [VATB_DROME]	1.2	13	7	5	38	
P84029	Cytochrome c-2 OS=Drosophila melanogaster GN=Cyt-c-p PE=1 SV=2 - [CYC2_DROME]	0.4	13	6	5	22	
P17336	Catalase OS=Drosophila melanogaster GN=Cat PE=1 SV=2 - [CATA_DROME]	1.1	12	7	5	27	
Q94920	Voltage-dependent anion-selective channel OS=Drosophila melanogaster GN=porin PE=1 SV=3 - [VDAC_DROME]	0.9	12	6	6	27	
P50887	60S ribosomal protein L22 OS=Drosophila melanogaster GN=Rpl22 PE=1 SV=2 - [RL22_DROME]	0.1	12	3	5	17	
P42281	Acyl-CoA-binding protein homolog OS=Drosophila melanogaster GN=Dbi PE=2 SV=1 - [ACBP_DROME]	1.2	11	4	6	23	
P09180	60S ribosomal protein L4 OS=Drosophila melanogaster GN=Rpl4 PE=2 SV=2 - [RL4_DROME]	0.6	11	7	5	4	
P92192	Larval cuticle protein 5 OS=Drosophila melanogaster GN=Lcp65Ab1 PE=1 SV=1 - [LCP5_DROME]	2.7	10	3	3	94	
Q8IN44	Protein Turandot A OS=Drosophila melanogaster GN=TotA PE=1 SV=1 - [TO-TA_DROME]	1.0	10	6	6	15	
Q8MLY8	40S ribosomal protein S8 OS=Drosophila melanogaster GN=RpS8 PE=1 SV=1 - [RS8_DROME]	0.7	10	5	4	14	
Q02748	Eukaryotic initiation factor 4A OS=Drosophila melanogaster	2.5	9	4	3	7	

	GN=eIF-4a PE=2 SV=3 - [IF4A_DROME]						
Q7JNS1	Triosephosphate isomerase OS=Drosophila simulans GN=Tpi PE=3 SV=1 - [TPIS_DROSI]	1.9	9	6	6	34	
Q24251	ATP synthase subunit d, mitochondrial OS=Drosophila melanogaster GN=ATPSyn-d PE=2 SV=2 - [ATP5H_DROME]	0.4	9	4	3	4	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
P29310	14-3-3 protein zeta OS=Drosophila melanogaster GN=14-3-3zeta PE=1 SV=1 - [1433Z_DROME]	1.9	8	4	3	41	
P09491	Tropomyosin-2 OS=Drosophila melanogaster GN=Tm2 PE=2 SV=1 - [TPM2_DROME]	1.0	8	3	4	16	
P07187	Larval cuticle protein 2 OS=Drosophila melanogaster GN=Lcp2 PE=1 SV=1 - [LCP2_DROME]	0.8	8	2	4	13	
P41126	60S ribosomal protein L13 OS=Drosophila melanogaster GN=Rpl13 PE=2 SV=1 - [RL13_DROME]	0.6	8	4	2	66	
P54398	Fat body protein 2 OS=Drosophila melanogaster GN=Fbp2 PE=2 SV=2 - [FBP2_DROME]	3.5	7	3	2	0	(Beller, Riedel et al. 2006)
Q27331	V-type proton ATPase catalytic subunit A isoform 2 OS=Drosophila melanogaster GN=Vha68-2 PE=1 SV=2 - [VATA2_DROME]	2.0	7	5	5	3	
P48375	12 kDa FK506-binding protein OS=Drosophila melanogaster GN=FK506-bp2 PE=3 SV=2 - [FKB12_DROME]	1.1	7	3	3	64	
P61853	Superoxide dismutase [Cu-Zn] OS=Drosophila mauritiana GN=Sod PE=3 SV=2 - [SODC_DROMA]	1.1	7	3	2	4	
P19889	60S acidic ribosomal protein P0 OS=Drosophila melanogaster GN=RplP0 PE=1 SV=1 - [RLA0_DROME]	0.9	7	4	3	42	
P02518	Heat shock protein 27 OS=Drosophila melanogaster GN=Hsp27 PE=1 SV=2 - [HSP27_DROME]	0.8	7	4	3	6	
P22700	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type OS=Drosophila melanogaster GN=Ca-P60A PE=1 SV=2 - [ATC1_DROME]	0.8	7	4	4	37	(Cermelli, Guo et al. 2006)
Q26365	ADP,ATP carrier protein OS=Drosophila melanogaster GN=sesB PE=2 SV=4 - [ADT_DROME]	0.7	7	4	5	32	
Q24407	ATP synthase-coupling factor 6, mitochondrial OS=Drosophila melanogaster GN=ATPSyn-Cf6 PE=3 SV=1 - [ATP5J_DROME]	0.3	7	3	3	24	
Q94514	Cytochrome c oxidase subunit 5A, mitochondrial OS=Drosophila melanogaster GN=CoVa PE=1 SV=2 - [COX5A_DROME]	0.3	7	4	3	9	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)

P29681	20-hydroxyecdysone protein OS=Drosophila melanogaster GN=ImpE2 PE=1 SV=1 - [IMPE2_DROME]	0.1	7	4	3	21	
Q9XTL9	Glycogen phosphorylase OS=Drosophila melanogaster GN=GlyP PE=2 SV=2 - [PYG_DROME]	2.4	6	5	3	49	
O96827	Probable elongation factor 1- beta OS=Drosophila melano- gaster GN=Ef1beta PE=1 SV=3 - [EF1B_DROME]	2.1	6	4	4	14	(Beller, Riedel et al. 2006)
Q9V521	Phenoloxidase subunit A3 OS=Drosophila melanogaster GN=proPo-A3 PE=1 SV=1 - [PRPA3_DROME]	2.1	6	5	5	24	
Q95028	L-lactate dehydrogenase OS=Drosophila melanogaster GN=ImpL3 PE=2 SV=1 - [LDH_DROME]	1.2	6	3	2	22	
P05661	Myosin heavy chain, muscle OS=Drosophila melanogaster GN=Mhc PE=1 SV=4 - [MYSA_DROME]	1.0	6	4	4	30	(Beller, Riedel et al. 2006)
P25007	Peptidyl-prolyl cis-trans iso- merase OS=Drosophila mela- nogaster GN=Cyp1 PE=1 SV=2 - [PPIA_DROME]	1.0	6	3	4	356	
P37159	Lysozyme E OS=Drosophila melanogaster GN=LysE PE=2 SV=2 - [LYSE_DROME]	0.2	6	2	2	103	
P08570	60S acidic ribosomal protein P1 OS=Drosophila melano- gaster GN=RpLP1 PE=1 SV=2 - [RLA1_DROME]	0.4	6	2	3	24	
P13060	Elongation factor 2 OS=Drosophila melanogaster GN=Ef2b PE=2 SV=4 - [EF2_DROME]	11.9	5	5	4	544	(Beller, Riedel et al. 2006)
P54611	V-type proton ATPase subunit E OS=Drosophila melanog- aster GN=Vha26 PE=2 SV=1 - [VATE_DROME]	1.4	5	4	5	18	
Q95ZE8	60S ribosomal protein L14 OS=Drosophila virilis GN=RpL14 PE=3 SV=1 - [RL14_DROVI]	0.9	5	2	2	17	
O02649	60 kDa heat shock protein, mitochondrial OS=Drosophila melanogaster GN=Hsp60 PE=1 SV=3 - [CH60_DROME]	0.6	5	5	5	5	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q9VWG3	40S ribosomal protein S10b OS=Drosophila melanogaster GN=RpS10b PE=1 SV=2 - [RS10B_DROME]	0.5	5	2	3	16	
P12881	Proteasome subunit alpha type-1 OS=Drosophila mela- nogaster GN=Pros35 PE=1 SV=1 - [PSA1_DROME]	0.2	5	4	2	331	
P05389	60S acidic ribosomal protein P2 OS=Drosophila melano- gaster GN=RpLP2 PE=1 SV=1 - [RLA2_DROME]	0.2	5	3	2	150	
P07701	Salivary glue protein Sgs-5 OS=Drosophila melanogaster GN=Sgs5 PE=2 SV=1 - [SGS5_DROME]	1.2	4	2	2	3	
B3N1G9	40S ribosomal protein S3a OS=Drosophila ananassae	1.1	4	2	2	43	

	GN=RpS3A PE=3 SV=1 - [RS3A_DROAN]						
P0CG69	Polyubiquitin OS=Drosophila melanogaster GN=Ubi-p63E PE=3 SV=1 - [UBIQP_DROME]	0.9	4	3	2	4	
Q9W237	40S ribosomal protein S16 OS=Drosophila melanogaster GN=RpS16 PE=2 SV=1 - [RS16_DROME]	0.8	4	3	3	4	
P55828	40S ribosomal protein S20 OS=Drosophila melanogaster GN=RpS20 PE=1 SV=1 - [RS20_DROME]	0.8	4	2	2	7	
P62085	40S ribosomal protein S7 OS=Drosophila yakuba GN=RpS7 PE=2 SV=1 - [RS7_DROYA]	0.7	4	4	3	40	
P41042	40S ribosomal protein S4 OS=Drosophila melanogaster GN=RpS4 PE=2 SV=2 - [RS4_DROME]	0.7	4	2	2	38	
P39018	40S ribosomal protein S19a OS=Drosophila melanogaster GN=RpS19a PE=2 SV=3 - [RS19A_DROME]	0.2	4	3	2	170	
Q9I7U4	Titin OS=Drosophila melanogaster GN=sls PE=1 SV=3 - [TITIN_DROME]	1.7	3	1	2	12	
P17271	Histone H2B OS=Drosophila hydei GN=His2B PE=3 SV=2 - [H2B_DROHY]	1.6	3	2	2	13	
Q94518	Nascent polypeptide-associated complex subunit alpha OS=Drosophila melanogaster GN=Nacalpha PE=1 SV=2 - [NACA_DROME]	1.2	3	2	2	20	
Q8T3U2	40S ribosomal protein S23 OS=Drosophila melanogaster GN=RpS23 PE=2 SV=1 - [RS23_DROME]	0.7	3	2	2	39	
P22769	Proteasome subunit alpha type-7-1 OS=Drosophila melanogaster GN=Pros28.1 PE=1 SV=2 - [PSA71_DROME]	0.5	3	2	2	63	
P62152	Calmodulin OS=Drosophila melanogaster GN=Cam PE=1 SV=2 - [CALM_DROME]	0.5	3	3	3	36	
P40304	Proteasome subunit beta type-1 OS=Drosophila melanogaster GN=Pros26 PE=2 SV=2 - [PSB1_DROME]	0.2	3	2	2	628	
P53777	Muscle LIM protein 1 OS=Drosophila melanogaster GN=Mlp60A PE=2 SV=1 - [MLP1_DROME]	0.2	3	2	2	16	
P48588	40S ribosomal protein S25 OS=Drosophila melanogaster GN=RpS25 PE=1 SV=3 - [RS25_DROME]	0.1	3	2	2	1559	
O01666	ATP synthase subunit gamma, mitochondrial OS=Drosophila melanogaster GN=ATPsyn-gamma PE=2 SV=2 - [ATPG_DROME]	1.4	2	2	2	37	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)
Q24439	ATP synthase subunit O, mitochondrial OS=Drosophila melanogaster GN=Oscp PE=2 SV=2 - [ATPO_DROME]	0.8	2	1	2	5529362 2	(Beller, Riedel et al. 2006, Cermelli, Guo et al. 2006)

Q9VNA5	Proteasome subunit beta type-4 OS=Drosophila melanogaster GN=Prosbeta7 PE=2 SV=1 - [PSB4_DROME]	0.7	2	2	2	34	
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