THE PKA-MEDIATED PHOSPHORYLATION OF SERINE 492 OF PERILIPIN A PROMOTES LIPOLYSIS BY TRIGGERING LIPID DROPLET DISPERSION

by

XIAOFANG LIANG

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirement

for the degree of

Master of Science

Graduate Program in Nutritional Sciences

written under the direction of

Dr. Dawn L. Brasaemle

and approved by

_____

_____

_____

New Brunswick, New Jersey

October 2008

ABSTRACT OF THE THESIS

The PKA-mediated Phosphorylation of Serine 492 of Perilipin A Promotes Lipolysis by Triggering Lipid Droplet Dispersion

By XIAOFANG LIANG

Thesis Director:

Dr. Dawn L. Brasaemle

The lipid droplet protein perilipin A plays a key role in regulating triglyceride storage and hydrolysis. The phosphorylation of perilipin A by PKA facilitates lipolysis mediated by hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) in adipocytes. Previous studies show that the phosphorylation of serine 492 of perilipin A drives lipid droplet fragmentation and dispersion following PKA-activation. These studies address the hypothesis that lipid droplet dispersion triggered by the phosphorylation of serine 492 promotes PKA-stimulated lipolysis. Adenoviral constructs encoding ectopic perilipin A (PeriA) and three mutated forms were generated, including 1) Not5, with all PKA site serines mutated to alanine or glutamic acid except PKA site #5; 2) S492A, with a single mutation of the fifth PKA site serine to alanine; and 3) All5, with all PKA sites serines substituted with alanine or glutamic acid. All forms of perilipin A and β-galactosidase were expressed in NIH 3T3 CarΔ fibroblasts, which express ATGL but lack endogenous perilipin A and HSL. Cells expressing unmodified

perilipin A and mutated forms had similar levels of basal lipolysis. Six hours treatment with forskolin/IBMX in PeriA cells increases lipolysis by 2.2-fold, to levels similar to those of control cells expressing β-galactosidase. When compared to lipolysis in PeriA cells, phosphorylation of serine 492 alone (Not5) contributes to 77.5% of maximal lipolysis, whereas phosphorylation of all five other PKA sites (S492A) yields only 66.6% of maximal lipolysis. Stimulated lipolysis in cells expressing perilipin lacking all PKA sites (All5) increased only 16% over basal levels. Immunofluorescence microscopy shows that phosphorylation of serine 492 is necessary and sufficient to drive the full dispersion of lipid droplets following PKA-stimulation. In conclusion, PKA-mediated phosphorylation of serine 492 of perilipin A facilitates lipolysis at least partially through the stimulation of lipid droplet dispersion.

ACKNOWLEDGEMENTS

I am deeply and variously indebted to Dr. Dawn L. Brasaemle who not only served as my supervisor but also encouraged, challenged and nurtured me over this graduate program. She and my other committee members Dr. George Carman and Dr. Wendie Cohick provided me with valuable guidance and suggestions for this perilipin project. Moreover, their professionalism and great enthusiasm for science and research has influenced my way of thinking and acting as a scientist.

This thesis could not have been completed or even started without the help and contributions of my peers in Thompson Hall. Irina Aranovich, the former lab manager: I thank her for her patient training during my rotation in Dr. Brasaemle's lab. Her lovely personality and great interest in science fascinated me and convinced me to stay in this lab. Dr. Jorge Matias Caviglia is another extremely helpful person, who not only imparted in me solid lab techniques but also walked me through almost every part of the project. I thank him for sharing with me his insights and experience in research. My gratitude also goes to Mr. Sean Sullivan and Ms. Deanna Russell for assisting me with collecting data for my thesis; PhD candidate Derek McMahon for helping me with statistics, and making me laugh as often as he does; Dr. Natalia Scaglia, Dr. Yanxin Wang, and Yi-fang Huang for their consistent generosity and willingness to help. I appreciate you all and thank you for your support.

Over the past three years at Rutgers, I have been fortunate to have the company of many friends and great professors. They shared my laughter and tears; they helped me

to discover my own potential and encouraged me to pursue my goals; they, with their great passion in their own fields of study and charismatic personalities, deeply influenced me, giving me tremendous strength and energy to complete this Master's degree program. It is they who made my three years of study unforgettable; it is they who helped me to make my perspective of the world more integrative. I appreciate each of them for being around. For the future, I would like to integrate the uplifting values that I have assimilated from my friends and members of the faculty at Rutgers with my expertise and enthusiasm for food and nutritional sciences to build a career, in which I will serve human beings and the society.

Above all, I owe my profoundest thanks to my parents, Kangning and Huifang, for their patience, understanding, encouragement, inspiration, and unconditional love and support throughout my life. Because of them, I have enormous faith and the guts to fulfill my dreams. Thank you!

This thesis is for you, my dear parents!

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURE LEGENDS AND FIGURES

LIST OF ABBREVIATIONS

cAMP – Cyclic Adenosine Monophosphate

PKA – cAMP-dependent Protein Kinase A

HSL – Hormone Sensitive Lipase

ATGL – Adipose Triglyceride Lipase

POCS – Polycystic Ovary Syndrome

TGH – Triglyceride Hydrolase

ADRP – Adipocyte Differentiation-Related Protein

CGI-58 – Comparative Gene Identification-58

PCR – Polymerase Chain Reaction

CsCl – Cesium Chloride

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

EDTA – Ethylenediaminetetraacetic Acid

BSA – Bovine Serum Albumin

PBS – Phosphate Buffered Saline

IBMX – Isobutylmethylxanthine

IgG – Immunoglobulin G

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis

MEF – Murine Embryonic Fibroblasts

Introduction

Obesity is one of the biggest health concerns in the United States. According to data from the National Health and Nutrition Examination Survey, in 2005 and 2006, over 72 million people, that is, more than one-third of adults, were obese (http://www.cdc.gov/nchs/pressroom/07newsreleases/obesity.htm). Considering the high health risks of obesity-related diseases, identifying how stored lipids are mobilized and the underlying energy-regulatory system may help us to find an effective means to reduce the prevalence of obesity. Perilipin A, a lipid-droplet associated protein, due to its structural location on lipid droplets, has gained increasingly more attention in the past decade with regard to its inhibitory and facilitatory effects on lipid metabolism. The following paragraphs will give you a brief background about the major elements of triglyceride hydrolysis in adipose tissues, and how perilipin A is involved in it as well as the hypothesis of the present study.

Lipid droplets. Lipid droplets or adiposomes in adipocytes are the major and dynamic intracellular storage organelles for triglycerides in vertebrates. They are composed of a triglyceride core coated by a phospholipid monolayer with proteins attached to the surface (Londos *et al.* 1999). Adipocyte triglyceride-rich lipid droplets play an important role in maintaining whole body energy homeostasis and influencing metabolic health (Trayhurn & Beattie 2001). Small lipid droplets also exist in non-adipose tissues and cells such as liver, skeletal muscle, heart, kidney, intestine,

mammary gland, and macrophages (Schaffer 2003). Usually these tissues have a limited capacity for storage of lipids. However, continual consumption of excess calories not only causes the excessive accumulation of fat in adipose tissue, but also leads to increased import of free fatty acids into non-adipose tissues, resulting in cellular dysfunction or cell death, that is, lipotoxicity. It has been proposed that excess lipid accumulation in the heart, skeletal muscle, pancreas, liver, and kidney contribute to the genesis of heart failure, insulin resistance, type 2 diabetes, and pancreas dysfunction (Schaffer 2003). Since the overflow of free fatty acids from adipose tissue to non-adipose tissues originates from adipocyte lipolysis, it is critical to understand the mechanisms by which fat is stored in and mobilized from the adipocyte lipid droplets and how those processes are regulated.

Lipolysis and lipases. One of adipose tissue's functions is to serve as an energy reservoir for peripheral tissues. In the fasting state, free fatty acids and glycerol are released from the hydrolysis of triglycerides stored in fat cells, supplying important oxidative fuels for non-adipose tissues such as liver, skeletal muscle, kidney and the myocardium (Carmen & Victor 2006). The process is known as adipocyte lipolysis. Several pathways have been found to modulate this lipolysis, including endocrine and paracrine regulation, such as catecholamines, natriuretic peptides, as well as the regulation of some other antilipolytic agents like prostaglandins, adenosine, nicotinic acid, neuropeptide Y and peptide YY (Langin 2006). Catecholamines such as epinephrine and norepinephrine, are important lipolysis regulators, especially in

humans (Carmen & Victor 2006). The binding of catecholamines to β-adrenergic receptors on the surface of the adipocyte signals a receptor-associated stimulatory G-protein (Gαs), which leads to the activation of adenylate cyclase. The activated adenylate cyclase then catalyzes the conversion of AMP to cyclic AMP (cAMP), increasing intracellular cAMP levels, resulting in the activation of protein kinase A (PKA) and subsequent phosphorylation of downstream lipase(s) and lipid droplet-associated protein(s) such as hormone-sensitive lipase and perilipin A, respectively. Thus, the PKA stimulated lipolytic cascade occurs. (Tansey *et al.* 2004)

Hormone Sensitive Lipase (HSL). Hormone sensitive lipase is expressed in multiple tissues, organs and cells, including adipose tissue, steroidogenic tissues, heart, mammary gland, skeletal muscle and macrophages (Yeaman 2004). An 84 kDa polypeptide is the major isoform of HSL, which is composed of three domains: an N-terminal protein-protein and/or protein-lipid interaction domain, a C-terminal catalytic domain, and a C-terminal regulatory loop. HSL is active against a variety of lipid substrates, including triglycerides, diglycerides, monoglycerides, and cholesteryl esters (Yeaman 2004) with the highest specificity for diglyceride substrates (Fredrikson *et al.* 1981). HSL is phosphorylated by PKA. All of the known phosphorylation sites are found in the regulatory loop and are subject to reversible phosphorylation; phosphorylation modestly increases the catalytic activity of HSL following binding of lipolytic hormones such as catecholamines to their receptors (Khoo *et al.* 1973;Khoo *et al.* 1974;Belfrage *et al.* 1980;Nilsson *et al.* 1980;Kawamura *et al.* 1981;Belfrage *et al.*

1981;Fredrikson *et al.* 1981). In basal conditions, HSL is in the cytosol and cannot access perilipin-coated lipid droplets. Following the stimulation of lipolysis, both HSL and perilipin are phosphorylated by PKA. Phosphorylated HSL translocates from the cytosol and binds to lipid droplets via a protein-protein interaction with phosphorylated perilipin A (Sztalryd *et al.* 2003;Brasaemle 2007). Thus, the interaction between HSL and its triglyceride and diglyceride substrates is enhanced (Su et al. 2003;Sztalryd et al. 2003). In such conditions, lipolysis catalyzed by the enzyme can increase up to 100-fold (Yeaman 2004).

The thought that hormone sensitive lipase is the key lipolytic enzyme had existed for decades until it was challenged by findings from HSL-null mice. In comparison with wild-type mice, white adipose tissue in HSL knockout mice retained unchanged mass and had around 40% of triglyceride lipase activity. Accumulation of diglycerides was observed in adipocytes of HSL null adipocytes. (Osuga *et al.* 2000;Haemmerle *et al.* 2002). These results suggest that HSL is not the only lipase in white adipose tissue and has a preference for hydrolyzing diglycerides. Moreover, lipolytic activity in isolated HSL null adipocytes was not enhanced significantly by β_3-adrenergic stimulation, but was similar to that of normal control cells under basal unstimulated conditions. These findings indicate that HSL is required for catecholamine stimulated lipolysis and an HSL-independent lipolytic pathway exists in adipocytes, especially in basal conditions (Wang *et al.* 2001).

Adipose Triglyceride Lipase (ATGL). Recently, a novel non-HSL lipase named adipose triglyceride lipase (ATGL; also desnutrin) was identified by three laboratories (Jenkins *et al.* 2004;Villena *et al.* 2004;Zimmermann *et al.* 2004). Both the murine and human ATGL mRNAs are expressed in adipose tissue. Murine ATGL is highly expressed in white and brown adipose tissue, cardiac and skeletal muscles, and testis, to a lesser degree (Zimmermann *et al.* 2004). Murine ATGL protein is estimated to be 54 kDa and contains 486 amino acids, whereas the human ATGL protein has 504 amino acids with 86% homology to the mouse counterpart. The structural characteristics of known lipases, including the GXSXG motif for serine esterases and α/β hydrolase folds, are located in the N-terminal regions of both the murine and human ATGL (Zimmermann *et al.* 2004). Unlike the wide substrate specificity of HSL, ATGL exhibits a strong specificity for triglycerides, has very low diglyceride-hydrolase activity, and has no cholesteryl or retinyl esterases activities (Zimmermann *et al.* 2004).

The regulation of the enzymatic activity of ATGL is also different from that of HSL; it is not regulated via phosphorylation by PKA (Zimmermann *et al.* 2004). Instead, ATGL expression is up-regulated by fasting and down-regulated by insulin at the transcriptional level (Kershaw *et al.* 2006). Furthermore, human studies show that both mRNA and protein expression of ATGL and HSL are negatively correlated with the degree of insulin resistance in obese people (Jocken *et al.* 2007). In lean patients with polycystic ovary syndrome (POCS), a female disease with insulin resistance as one

of the symptoms, HSL protein expression was decreased significantly, whereas there was no change in ATGL protein levels (Ryden *et al.* 2007).

In contrast to HSL, which is involved in stimulated lipolysis, ATGL is more responsible for basal lipolysis than stimulated lipolysis in human adipocytes (Ryden *et al.* 2007;Langin *et al.* 2005). RNAi knockdown of ATGL in human adipocytes caused 40% reduction in basal lipolysis but no effect on stimulated lipolysis (Ryden *et al.* 2007). BAY, a potent HSL inhibitor, greatly blocked stimulated lipolysis in human adipocytes, but did not inhibit fatty acid release in unstimulated conditions, although glycerol release was inhibited (Langin *et al.* 2005). This means that ATGL acts upstream of HSL by hydrolyzing triglycerides, releasing diglycerides and free fatty acids. In white adipose tissue of ATGL-knockout mice, however, no significant difference was observed in the basal release of free fatty acids and glycerol when compared with control mice, whereas with PKA-stimulation, free fatty acid release was reduced by 74% and glycerol release was reduced by 78% (Haemmerle *et al.* 2006). Furthermore, the absence of ATGL in adipocytes and other peripheral tissues cannot be completely compensated for by HSL or triglyceride hydrolase (TGH). In addition, triglyceride hydrolase activity of ATGL can be increased by CGI-58 (comparative gene identification-58), a member of the esterase/thioesterase/lipase subfamily of proteins containing α/β hydrolase folds (Lass *et al.* 2006). The hydrolysis of triglycerides was enhanced by 20-fold in mixtures of COS-7 cell extracts expressing murine ATGL with the addition of CGI-58.

Lipid droplet-associated proteins. The perilipin family of proteins (PAT) includes adipophilin, perilipin, TIP47, OXPAT/MLDP and s3-12. One or more of these five proteins coats lipid droplets in the cells of vertebrates and they are the most abundant structural proteins on lipid droplets (Brasaemle 2007). Due to this important localization of PAT proteins, investigation of these proteins may shed some light on how lipases interact with these proteins and their lipid substrates in lipid droplets, and how these interactions are regulated. (Brasaemle *et al.* 2004;Fujimoto *et al.* 2004;Liu *et al.* 2004;Turro *et al.* 2006;Umlauf *et al.* 2004;Wu *et al.* 2000)

Adipocyte Differentiation-Related Protein (ADRP). Adipophilin or adipocyte differentiation-related protein (ADRP) is expressed widely in mammalian cells (Brasaemle *et al.* 1997b). In adipocytes, ADRP is expressed during early adipocyte differentiation and localizes on lipid droplet surfaces. It is displaced by perilipin, which is expressed 3 to 5 days after the initiation of adipose differentiation (Brasaemle *et al.* 1997b). Despite the observation that the N-terminal region of ADRP is homologous with that of perilipin A, ADRP cannot substitute functionally for perilipin A. In stimulated lipolysis, the translocation and docking of phosphorylated HSL on lipid droplets was prevented in differentiated perilipin-null embryonic fibroblasts that have ADRP-coated lipid droplets instead of perilipin A coated lipid droplets (Sztalryd *et al.* 2003). Furthermore, ADRP over-expression stimulates a parallel increase of intracellular triglyceride content (Imamura *et al.* 2002;Listenberger *et al.* 2007); the turnover of triglycerides from lipid droplets was reduced by ADRP over-expression

(Listenberger *et al.* 2007), most likely, because the association of ATGL with lipid droplets was also decreased.

Perilipin A. Among members of the PAT family of proteins, the most abundant proteins on the surfaces of adipocyte lipid droplets are perilipins (Brasaemle 2007). They are also found on lipid droplets in steroidogenic cells of adrenal cortex, testes and ovary (Servetnick *et al.* 1995). Perilipin A, B, and C are three protein isoforms encoded by a single copy gene and generated by alternative mRNA splicing (Greenberg *et al.* 1993;Lu *et al.* 2001). Perilipin A and B have the same N-terminal sequence (406 amino acids) with predicted molecular masses of 56 kDa and 46 kDa, respectively. The 42 kDa perilipin C has been found to be expressed in steroidogenic cells only (Servetnick *et al.* 1995).

Perilipin A is the major isoform that has been investigated extensively since the 1990s. It has been found that ectopic expression of perilipin A in fibroblastic 3T3-L1 pre-adipocytes lacking endogenous perilipin causes the clustering of lipid droplets rather than the dispersed patterns of lipid droplets observed in control cells without perilipin (Brasaemle *et al.* 2000). Compared with control cells, perilipin A-expressing cells have reduced triglyceride lipolysis and consequently store 6 to 30-fold more triglyceride under basal conditions. In contrast, perilipin null mice are leaner and have approximately 62%-70% smaller white adipocytes than wild type mice (Martinez-Botas *et al.* 2000;Tansey *et al.* 2001). The structure of the perilipin protein endows its role as a protector against the access of cytosolic lipases to stored neutral lipids in lipid droplets

under basal conditions. The central hydrophobic regions of the perilipin sequence facilitate the targeting and anchoring of perilipin A to lipid droplets (aa 242-260, aa 320-342, aa 349-364) (Garcia *et al.* 2004;Subramanian *et al.* 2004), whereas aa 122-222 and C-terminal aa 406-517 help to shield stored neutral lipid droplets from the attack of cytosolic lipases (Garcia *et al.* 2004;Zhang *et al.* 2003). Furthermore, perilipin A promotes lipolysis stimulated by PKA. In adipocytes from perilipin null mice, basal lipolysis is increased by a factor of 10, whereas stimulated lipolysis is attenuated dramatically in comparison with cells from wild type mice (Martinez-Botas *et al.* 2000;Tansey *et al.* 2001). The promotion of lipolysis by perilipin A under stimulated conditions is mainly contributed by the phosphorylation of its six PKA sites in response to PKA activation.

PKA sites of Perilipin. Perilipin A contains six PKA sites (aa #81, 222, 276, 433, 492, and 517 in the murine sequence), which have been thought to regulate the access of hormone sensitive lipase (HSL) and non-HSL lipases to stored neutral lipids (Zhang *et al.* 2003). Mutagenesis studies of the six PKA sites of perilipin A demonstrate that one or more of the three N-terminal PKA sites (serine 81, 222, 276) are critical for HSL catalyzed lipolysis, by facilitating the translocation of HSL from cytoplasm to the surfaces of lipid droplets when lipolysis is stimulated (Souza *et al.* 2002;Tansey *et al.* 2003;Zhang *et al.* 2003). Under these conditions, both perilipin A and HSL are phosphorylated in response to the inactivation of insulin signaling and binding of catecholamines to their receptors leading to elevation of intracellular cyclic AMP

(Miyoshi *et al.* 2006;Zhang *et al.* 2003). Phosphorylated perilipin A alters its conformation from basal conditions, provide a docking site for phosphorylated HSL on lipid droplets, and thereby enhances the access of HSL to triglyceride and diglyceride substrates in lipid droplets (Brasaemle 2007).

Furthermore, different from perilipin B and C, perilipin A contains three additional C-terminal PKA consensus sites (aa #433, 492, and 517) (Greenberg *et al.* 1993). In stimulated lipolysis, the phosphorylation of one or more of the C-terminal PKA sites is required to facilitate the access of non-HSL lipases to lipid droplets (Zhang *et al.* 2003). More specifically, the PKA site containing serine 517 has been suggested to be a global regulator of all PKA-stimulated lipolysis in adipocytes differentiated from perilipin null murine embryonic fibroblasts (MEF) expressing ectopic perilipin A and endogenous HSL and ATGL (Miyoshi *et al.* 2007;Greenberg *et al.* 1993). 30% of residual PKA-stimulated fatty acid release was observed when HSL was reduced by shRNA and endogenous ATGL expression was unmodified. However, this residual fatty acid release was abrogated in cells expressing mutated perilipin lacking serine 517. These findings suggest that the phosphorylation of PKA site serine 517 of perilipin A is required in ATGL-mediated lipolysis (Miyoshi *et al.* 2007;Greenberg *et al.* 1993). Moreover, the complete knockout of ATGL expression by shRNA in perilipin null MEF adipocytes containing endogenous HSL and ectopic perilipin A resulted in total abrogation of PKA-stimulated fatty acid and glycerol release, which suggests that stimulated lipolysis is initiated by ATGL, and that HSL acts downstream in the lipolytic

pathway by hydrolyzing mainly diglycerides. In addition, a single mutation of the PKA site at serine 492 of perilipin A caused ~30% inhibition of PKA-stimulated lipolysis, indicating that the phosphorylation of serine 492 plays a role in the PKA-stimulated lipolysis, together with serine 517.

The accessory role of serine 492 of perilipin A in PKA-stimulated lipolysis may be due to its contribution to lipid droplet fragmentation and dispersion (Marcinkiewicz *et al.* 2006). In fibroblasts expressing ectopic perilipin A, lipid droplets were arranged in a pattern of clusters in basal conditions (Brasaemle *et al.* 2000). However, the activation of PKA by forskolin and isobutylmethylxanthine (IBMX) caused clustered lipid droplets to disperse widely throughout the cytoplasm. The single mutation of serine 492 to alanine blocked this lipid droplet remodeling, whereas mutations of one or more of the other five PKA sites (#aa81, 222, 276, 433 and 517) did not (Marcinkiewicz *et al.* 2006). Moreover, perilipin A does not disperse into the cytosol or any other subcellular compartment during stimulated lipolysis, instead, it remains attached to the surfaces of fragmented microlipid droplets, and the total perilipin protein mass remains relatively unchanged (Marcinkiewicz *et al.* 2006). In addition, the mutation of all three C-terminal PKA site serines to glutamic acid failed to trigger lipid droplet dispersion under basal or stimulated conditions, indicating that a negative charge on amino acid residue 492 is not sufficient to mimic the behavior of phosphorylated serine.

Hypothesis. The discoveries that the phosphorylation of serine 492 of perilipin A enhances PKA-stimulated lipolysis and is necessary for lipid droplet remodeling and

dispersion lead to the questions, is the phosphorylation of serine 492 alone sufficient to drive the remodeling of lipid droplets, and does this remodeling contribute to PKA-stimulated lipolysis? Therefore, we hypothesize that the lipid droplet dispersion triggered by the phosphorylation of serine 492 alone promotes PKA-stimulated lipolysis. The goals of this thesis are:

1) To test the hypothesis that the lipid droplet remodeling stimulated by the phosphorylation of serine 492 of perilipin A facilitates PKA-stimulated lipolysis. To address the question fully, adenoviral constructs encoding ectopic perilipin A and three mutated forms are needed, including 1) Not5, with all PKA sites mutated except PKA site #5; 2) S492A, single mutation of PKA site #5; and 3) All5, with all PKA sites mutated. NIH 3T3 CarΔ fibroblasts expressing unmodified perilipin A and its mutated forms will be lipid loaded with tritium-labeled oleic acid for 12 hrs to stabilize nascent perilipin and displace endogenous ADRP on the surfaces of lipid droplets. After that, forskolin/IBMX stimulated lipolysis experiments will be conducted, and the amount of tritium-labeled fatty acid release into the medium will be measured under both basal and stimulated conditions.
2) To confirm that proteins synthesized from the generated adenoviral constructs of original perilipin and its mutants target to lipid droplets, and to further characterize the role of the phosphorylation of serine 492 of perilipin A during forskolin/IBMX stimulation in the fragmentation and dispersion of lipid droplets,

I will use immunofluorescence microscopy to observe the distribution of lipid droplets in the absence or presence of forskolin/IBMX in NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A and its mutated forms.

Materials and Methods

Materials. Fetal bovine serum, oleic acid, 3-isobutyl-1-methylxanthine (IBMX), forskolin, phosphatase inhibitor cocktail 1, and purified goat immunoglobulin G (IgG) were purchased from Sigma. Bovine serum and the Zero Blunt PCR Cloning Kit were purchased from Invitrogen (Carlsbad, CA). Triacsin C was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). *Pfu* DNA polymerase, AdEasy™ XL Adenoviral Vector System, and Quick Change II XL Site-Directed Mutagenesis Kit were purchased from Stratagene, Inc. (La Jolla, CA). Fatty acid-free bovine serum albumin was purchased from either Sigma or US Biological (Swampscott, MA). Coomassie Plus-The Better Bradford™ Assay Kit was purchased from Pierce. Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503 were purchased from Molecular Probes, Inc. (Eugene, OR). Phospho-(Ser/Thr) protein kinase A substrate antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Polyclonal antibodies raised against perilipin and ADRP have been described previously (Brasaemle *et al.* 1997b;Schultz *et al.* 2002). A polyclonal antibody raised against calnexin was purchased from StressGen Bioreagents Corp. (Victoria, BC, Canada).

Generation of adenoviral constructs. The cDNA of perilipin mutant S1234A6E (Not5), with alanine substituted for PKA sites #1, 2, 3, and 4 and glutamic acid substituted for PKA site #6, was created using perilipin S12346A as a template (provided by Amy Marcinkiewicz), whose PKA sites #1, 2, 3, 4, and 6 were mutated into alanine.

5’ (5’-GCATGGTACCATGTCAATAAACAAGGGC-3’) and 3’ (5’-CAAAAGCTTTCACTCCTTCTTGC-3’) oligonucleotide were used as primers (Table 1), which contain engineered *KpnI* and *HindIII* restriction sites, respectively. The DNA was amplified using Polymerase Chain Reaction (PCR) and P*fu* polymerase, and then ligated into pCR-Blunt vector, followed by restriction digestion and ligation into *KpnI* and *HindIII* restriction sites of the pShuttle-CMV vector. The linearized plasmid was then transformed into competent BJ5183-AD-1 *E. coli* (Stratagene). Positive recombinant plasmids of the adenoviral pShuttle-perilipin Not5 construct were identified by *Pac I* restriction digestion of isolated plasmids from single colonies. Additionally, recombinant plasmids of adenoviral constructs of pShuttle-perilipin S492A, which had the fifth PKA site mutated into alanine, and pShuttle-perilipin S12345A6E (All5), whose PKA sites #1, 2, 3, 4, and 5 were substituted by alanine and PKA site #6 was substituted by glutamic acid, were generated respectively by QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) (Table 1), and transformed into competent BJ5183-AD-1 *E. coli*. The pShuttle-unmodified perilipin A construct was used as a template to generate the S492A construct, and the newly generated pShuttle-Not5 construct was used as a template to make the All5 construct. The *Pac I* restriction digestion was also used to identify the positive recombinants of each of the two plasmids. All three of the recombinant plasmids were amplified either in JM109 *E. coli* or XL10-Gold *E. coli* (Stratagene). The recombined plasmids were purified using the endotoxin-free plasmid preparation kit (Qiagen, Santa Clarita, CA), linearized by *Pac I* restriction enzyme

digestion, and then transfected into AD293 cells following the MBS Mammalian Transfection Kit (Stratagene). The resultant viral stocks were harvested 7-10 days after transfection, and were amplified once in AD293 cells, yielding P2 viral stocks of perilipin Not5, perilipin S492A, and perilipin All5, respectively. Expression of perilipin driven by the crude mutated perilipin adenoviruses was confirmed in either 3T3-L1 CarΔ cells or NIH 3T3 CarΔ cells by western blots before making purified preparations of adenoviral constructs by cesium chloride (CsCl) ultracentrifugation. β-galactosidase and unmodified perilipin adenoviral constructs were amplified in AD293 cells from Dr. Vidya Subramanian's adenoviral stocks, and purified by CsCl ultracentrifugation too. Cesium chloride-purified preparations of all recombinant adenoviral constructs, including intact murine perilipin A, three perilipin mutants (Not5, S492A, and All5), and β-galactosidase were used in all experiments.

Cell Culture. AD 293 cells and 3T3-L1 CarΔ fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with a 5.5% CO_2 atmosphere. NIH 3T3 CarΔ fibroblasts were cultured in DMEM supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, 800μg/ml G418 antibiotic, and were grown in a 5.5% CO_2 atmosphere at 37°C.

Adenoviral Infection. NIH 3T3 CarΔ cells were transduced by incubation with purified adenoviral preparations for expression of perilipin, mutated forms of perilipin, or control β-galactosidase. The titers of each perilipin adenoviral constructs were

determined in three experiments to adjust the expression of perilipin and its mutants to a similar level.

Fatty acid release measurement. NIH 3T3 CarΔ cells were infected with adenoviruses for 28 hours at 37°C. The infected cells were then incubated in culture medium containing 600 μM tritium-labeled oleic acid (1 μCi/well in 6-well plates) for 12 hours. The oleic acid was complexed to fatty acid-free BSA at a 4:1 molar ratio. To stimulate lipolysis, 10 μM forskolin in 0.1% dimethyl sulfoxide (DMSO) and 0.5 mM IBMX were added to the cells in DMEM with 3% fatty-acid free BSA. To inhibit long-chain fatty acyl-CoA synthetase, 6 μM triacsin C was added to this medium. All cells were incubated in the same concentration of DMSO in each experiment. 200 μL of medium were collected after 0, 2, 4, and 6 hours of lipolytic stimulation to measure the release of fatty acids using scintillation counting (Beckman).

Immunofluorescence microscopy to observe lipid droplet distribution. NIH 3T3 CarΔ cells were infected with adenoviruses for 24 hours at 37°C, and then released with trypsin and EDTA and re-plated on glass coverslips for 10 hours (for fluorescence microscopy experiments), followed by 12 hrs lipid loading with 200 μM oleic acid. To stimulate lipolysis, 10 μM forskolin in 0.1% DMSO and 0.5 mM IBMX were added to the cells in DMEM containing 3% fatty-acid free BSA. After 6 hours of lipolytic stimulation, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature, followed by 1 hour incubation in blocking solution containing 0.5g/ml goat immunoglobulin G (IgG), 0.1 mg/ml saponin and 0.2 mM

glycine (Blanchette-Mackie et al. 1995). Blocked cells were probed with antibodies raised against perilipin for 1 hour at room temperate (1:5,000 dilution) or overnight at 4°C (1:10,000 dilution), and then co-stained with Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:1,000 dilution) and Bodipy 493/503 (0.01mg/ml) for 1 hour at room temperature to stain perilipin A and neutral lipids (Gocze & Freeman 1994). Stained cells were viewed with a Nikon Eclipse E800 fluorescence microscope armed with phase contrast lenses and a Power Macintosh G4-interfaced Hamamatsu Orca digital camera. Improvision Openlab softwere was used to capture and process images.

Immunoblotting. NIH 3T3 CarΔ fibroblasts were harvested and lysed in a hypotonic buffer containing 1 M Tris, pH 7.6, 0.5 M EDTA, 500 mM sodium fluoride, 10 μg/ml leupeptin, 500 μM benzamidine, 100 μM [4-(2-aminoethyl) benzenesulfonyl fluoride] hydrochloride, and protease inhibitor cocktail 1, followed by 9 seconds sonication to homogenize the samples (Branson Sonifier). Whole cell lysates containing 40 μg of protein determined by Bradford protein assay kit (Pierce) were resolved by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses (SDS-PAGE), and transferred electrophoretically to nitrocellulose membranes. The immunoblots were probed sequentially with rabbit polyclonal antibodies raised against 1) PKA (1:1000 dilution), 2) ADRP (1:50,000 dilution) and Calnexin (1:5,000), 3) perilipin (1: 25,000 dilution), and 4) β-galactosidase (1:50,000 dilution). The blots were then incubated with anti-rabbit IgG (1:5000 dilution). The protein bands were visualized using Amersham ECL Western blotting detection reagents (GE Healthcare).

Results

Generation of mutated forms of perilipin A. To study the role of the phosphorylation of serine 492 in lipolysis, we generated adenoviral constructs encoding three mutated forms of perilipin A. They are 1) Not5, with alanine substituted for PKA sites #1, 2, 3, and 4 and glutamic acid substituted for PKA site #6, leaving serine 492 in the fifth PKA site as the only substrate for PKA; 2) S492A, including intact PKA sites in positions 1, 2, 3, 4, and 6 with the fifth PKA site mutated to alanine to prevent phosphorylation; and 3) All5, all PKA sites were mutated to either alanine (PKA sites #1, 2, 3, 4, and 5) or glutamic acid (PKA site #6). The reason that we are using glutamic acid to substitute for the sixth PKA site rather than alanine is due to the difficulty in detecting expression of the mutated form of perilipin with alanine on the sixth PKA site in 3T3-L1 fibroblasts (Marcinkiewicz *et al.* 2006). This may due to the fact that the sixth PKA site (serine 517) is also the last amino acid residue on the protein sequence of perilipin A; hence, serine, or a polar residue may be needed for stable protein expression in the cells.

Testing perilipin A function in NIH 3T3 CarΔ fibroblasts. To identify the relationship between phosphorylation of serine 492 of perilipin A and lipolysis by non-HSL lipases, we chose NIH 3T3 CarΔ fibroblasts as our model, since this cell line does not express endogenous HSL or perilipin A, but does express ATGL. The expression of a modified coxsackie virus and adenovirus receptor gene lacking the

intracellular signaling domain (CAR Δ) in NIH 3T3 cells enhances the ability of the cells to take up adenoviruses (Ross *et al.* 2003), and therefore increases protein expression of perilipin A or β-galactosidase in the cells. However, newly formed perilipin A protein is easily degraded (Brasaemle *et al.* 1997a). Nascent perilipin must assemble onto lipid droplets to stabilize itself via hydrophobic interactions between its central domain and the surfaces of lipid droplets (Garcia *et al.* 2003). In our experiments, oleic acid was added to the cells to promote lipid droplet formation and stabilize the various forms of perilipin A. Endogenous ADRP is replaced on the surfaces of lipid droplets when ectopic perilipin A is expressed (Souza *et al.* 2002;Tansey *et al.* 2003), which gives us the ability to study the role of perilipin A, rather than ADRP, in the regulation of lipid metabolism. Figure 1 shows the titration of levels of adenovirus driving expression of perilipin A and its mutated forms All5, S492A and Not5 in NIH 3T3 CarΔ cells. The densitometric scan volumes for perilipin and ADRP bands were adjusted by the corresponding density of calnexin (loading control), and summarized in Table 2. The viral titers used in the experiments for all adenoviruses were perilipin A at 16.0μL/9.5cm^2 well, All5 at 22.0μL/9.5cm^2 well, S492A at 12.0μL/9.5cm^2 well, and Not5 at 6.5μL/9.5cm^2 well, respectively, which were determined based on similar perilipin protein levels and low ADRP signals in cells expressing ectopic unmodified or mutated forms of perilipin. The amount of β-galactosidase virus used was 6.7μL/9.5cm^2 well determined based on the detectable level of its protein expression in NIH 3T3 CarΔ cells.

Perilipin A plays dual roles in lipid metabolism. To identify and compare

contribution of perilipin mutants with that of ectopic perilipin A during basal and PKA-stimulated lipolysis with regard to their role in regulation of lipid metabolism, we conducted forskolin-stimulated lipolysis experiments. NIH 3T3 CarΔ cells expressing ectopic perilipin A (PeriA), its mutated forms (Not5, S492A, and All5) or β-galactosidase (control) were loaded with 600 μM ^{3}H-oleic acid for 12 hrs. Tritium, a radioactive isotope of hydrogen, can be easily incorporated into lipids. Therefore, we used tritium-labeled oleic acid as a 'visible' marker for synthesis and hydrolysis of lipids in the cells. The lipid-loading condition was optimized by Amy Marcinkiewicz, who found that lipid loading with 600 μM oleate for 12 hours was best to maximize the expression of perilipin, while displacing endogenous ADRP on the lipid droplets. To remove the unincorporated oleic acid, the cells were washed twice after the lipid-loading period with PBS containing 0.5% fatty acid-free bovine serum albumin (BSA), and then incubated for 6 h at 37°C in fresh medium containing 3% fatty-acid free BSA, 6 μM triacsin C, an acyl-CoA synthetase inhibitor used to prevent fatty acid re-esterification and re-incorporation into triglyceride, and in the absence or presence of 10 μM forskolin, which activates the enzyme adenylate cyclase, and 0.5 mM IBMX to increase and sustain intracellular levels of cyclic AMP (cAMP). The elevated cAMP then activates PKA, which phosphorylates the available serines on the PKA sites of ectopic perilipin or its mutated forms. The amounts of ^{3}H-fatty acid released into the medium over time were determined by scintillation counting of aliquots of culture medium. The 6 h forskolin-stimulation period was chosen based upon prior observations that 6 h

stimulation is long enough to observe lipid droplet fragmentation promoted by phosphorylation of serine 492 (Marcinkiewicz *et al.* 2006). Lipid droplet fragmentation was detectable by 2 h forskolin-stimulation, but became more obvious by 4 h, and was maximal by 8 h (Marcinkiewicz *et al.* 2006). Hence, collection of culture medium over 6 hrs should provide information about lipolysis that accompanies lipid droplet dispersion.

As shown in Fig. 2A, control cells expressing β-galactosidase, which have ADRP-coated lipid droplets, have similar maximal lipolytic activity under both basal and stimulated conditions. For example, the amounts of ^{3}H-fatty acids released in the absence and presence of forskolin and IBMX at the 6 h incubation were 204.6 ± 13.2 and 217.7 ± 6.9 dpm/μg of protein, respectively. In contrast, basal lipolysis in cells expressing ectopic perilipin A was significantly suppressed by 56% at the 6 h time point in comparison with that in the control cells. However, upon addition of forskolin and IBMX, lipolysis was enhanced by 2.2-fold in cells expressing ectopic perilipin A compared with unstimulated cells, and reached a level similar to that of control cells. These findings indicate that perilipin A functions normally in these fibroblasts by reducing lipolysis under unstimulated conditions and facilitating lipolysis upon its phosphorylation by PKA. The results also suggest that phosphorylated perilipin promotes lipolysis mediated by non-HSL lipase in forskolin-stimulated conditions.

Role of the phosphorylation of Serine 492 of perilipin A in lipolysis. The phosphorylation of serine 492 of perilipin A has been shown to stimulate lipid droplets

fragmentation (Marcinkiewicz *et al.* 2006). To examine if the phosphorylation of serine 492 plays a role in PKA-stimulated lipolysis, we conducted the forskolin-stimulated lipolysis experiment in NIH 3T3 CarΔ cells expressing ectopic perilipin A (PeriA), its mutated forms (Not5, S492A, and All5), or β-galactosidase (control), and measured ^{3}H-fatty acid release, following the same procedure described above. As shown in Fig. 2B, basal lipolysis in cells expressing each of the mutated forms of perilipin A was as low as that observed in cells expressing unmodified perilipin A. No significant differences in the amount of ^{3}H-fatty acid release were found either between perilipin mutants, or between each mutant and intact perilipin A ($P>0.05$) (Table 3), suggesting that each of the mutated forms of perilipin A protects stored neutral lipids from the attack of cytosolic lipases in unstimulated conditions. On the other hand, in the presence of forskolin and IBMX, cells expressing each of the perilipin A mutations released lower amounts of fatty acids, when compared with the PeriA cells. Unlike the 2.2-fold increase in lipolysis observed in the PeriA cells under stimulated conditions, the increase in lipolysis for the perilipin mutants Not5, S492A, and All5 was merely 1.70-fold ($P<0.001$), 1.60-fold ($P<0.001$), and 1.31-fold ($P<0.001$), respectively, above the level of basal lipolysis for each of the constructs at the 4 h time point. At the 6 h time point, a smaller relative increase in activation was observed compared with the data at 4 h stimulation. The perilipin mutants Not5, S492A and All5 showed increases in lipolysis of 1.58-fold ($P<0.001$), 1.45-fold ($P<0.001$), and 1.16-fold ($P<0.01$) above basal lipolysis, respectively, by 6 h PKA-stimulation. These data indicate that full

activation of lipolysis in these cells requires the phosphorylation of both serine 492 and one or more additional PKA sites of perilipin A. Compared with the lipolysis observed in cells expressing intact perilipin A, after 6 h treatment with forskolin and IBMX, the single PKA site mutation S492A (PKA site #5) showed 33.4% inhibition of maximal PKA-stimulated lipolysis, whereas the multiple mutations of perilipin at PKA sites #1, 2, 3, 4, and 6 (Not5) suppressed stimulated lipolysis by only 22.5% ($P<0.001$), suggesting that the fifth PKA site serine 492 is more important than the combination of all other PKA sites in promoting maximal stimulated lipolysis.

Our experiments allowed us to compare the relative contributions of phosphorylation of serine 492 and serine 517 to PKA-stimulated lipolysis. According to Fig. 2B, phosphorylation of perilipin mutant Not5 (containing a PKA site serine only in site #5) contributed to 77.5% of maximal stimulated lipolysis when compared with intact perilipin A at 6 h stimulation. In contrast, stimulated lipolysis in cells expressing All5, in which all PKA site serines have been mutated but amino acid 517 contains the phosphomimetic amino acid, glutamic acid, enhanced lipolysis by only 16% above its basal lipolysis. Hence, ^{3}H-fatty acid release was further attenuated by 26.1% in All5 cells compared with that in Not5 cells ($P<0.001$). These data indicate that the perilipin A Not5 mutant lacking serine 492 in the fifth PKA site cannot promote maximal lipolysis despite a phosphomimetic substitution of glutamic acid for serine 517. These observations suggest that phosphorylation of serine 492 is more critical than

phosphorylation of serine 517 or any of the other four PKA sites in non-HSL mediated lipolysis following the activation of PKA.

Confirmation of Lipolytic Stimulation and Perilipin Expression by Protein Immunoblots. The phosphorylation of the various mutated forms of perilipin A following the activation of PKA was visualized by probing immunoblots of cell lysates with phospho-(Ser/Thr) protein kinase A substrate antibody. As shown in Fig. 3, strong signals for phosphorylated serines on perilipin A were observed when perilipin A or the S492A perilipin A mutation were expressed, consistent with the phosphorylation of serine residues in 6 or 5 PKA sites, respectively. A much weaker signal was observed for the Not5 mutation of perilipin A, which is consistent with the single serine residue remaining in PKA site #5 for this perilipin variant. No signal was observed for the All5 mutation of perilipin A due to the fact that all serine residues in PKA sites were mutated. Furthermore, data from immunoblots probed with antibodies raised against perilipin show that the protein levels of ectopic perilipin A and its mutated forms are similar to each other. Under forskolin-stimulated conditions, reduced migration of perilipin A bands was observed, suggesting that perilipin A and mutated variants of perilipin were phosphorylated in response to PKA-stimulation. Although the expression of perilipin A or its mutants did not displace ADRP completely off of lipid droplets, the ADRP protein levels in cells expressing ectopic perilipin A or its mutated forms were significantly lower than those in control cells. According to densitometric scans of the immunoblots, the ADRP signals in PeriA cells were 66% and 52% lower

than those in control cells under basal and stimulated conditions, respectively, ($P<0.01$). Similarly, compared with their control counterparts, the ADRP signals in Not5, S492A, and All5 cells were 93%, 61% and 87% lower in basal conditions, and 58%, 48% and 76% lower in PKA-stimulated conditions, respectively. Thus, the lipolysis data address the specific contributions of perilipin A, rather than ADRP, to the regulation of lipid metabolism.

Activation of PKA in Fibroblasts Expressing Ectopic Perilipin A Promotes Lipid Droplet Dispersion. Under basal conditions, numerous small lipid droplets in NIH 3T3 CarΔ fibroblasts expressing ectopic Perilipin A aggregated together in the cytoplasm (Fig. 5, panel A), as reported previously (Brasaemle *et al.* 2000;Marcinkiewicz *et al.* 2006;Miyoshi *et al.* 2007). Incubation of cells with forskolin and IBMX, elevates cAMP and activates PKA. This treatment resulted in tremendous dispersion of perilipin A-coated lipid droplets widely across the cytoplasm (Fig. 5, panel B). To quantify the changing patterns of lipid droplet distribution in cells expressing perilipin A or mutated forms of perilipin A following treatment with forskolin and IBMX, I defined the following patterns of lipid droplet distribution: 1) densely clustered (Fig.4, panel A), 2) partially dispersed (Fig.4, panel B), and 3) fully dispersed (Fig.4, panel C). We scored cells expressing PeriA following treatment with forskolin and IBMX to quantify the distribution of lipid droplets: 99.5% of cells expressing PeriA show either fully (87.4%) or partially (12.1%) dispersed patterns of lipid droplets (Fig. 6; Table 4). These results confirm that phosphorylation of perilipin A drives lipid droplet fragmentation and

dispersion.

Phosphorylation of Serine 492 of Perilipin A is Essential to Drive the Full Dispersion of Lipid Droplets. Among the six PKA sites of perilipin A, it has been found that phosphorylation of the fifth PKA site serine 492 promotes the remodeling of lipid droplets (Marcinkiewicz *et al.* 2006). In the current study, we have observed that at the 6 h time point, the phosphorylation of serine 492 contributes to approximately 77.5% of lipolysis under stimulated conditions. This study includes the analysis of several new mutated forms of perilipin A. Hence, we asked if the promotion of lipolysis by phosphorylated serine 492 correlates to lipid droplet dispersion in response to PKA-stimulation, and whether the additional mutated forms of perilipin show dispersion patterns consistent with the original observation. We used immunofluorescence microscopy to examine the effect of PKA site mutations of perilipin A on lipid droplet distribution under both basal and stimulated conditions in NIH 3T3 CarΔ cells expressing either ectopic perilipin A or its mutants. For each condition, the lipid droplet distribution of 100-200 cells was graded according to the same categorization methods mentioned above (Fig. 4; Fig. 6; Table 4).

In basal conditions, all mutated forms of perilipin targeted to lipid droplets. Additionally, lipid droplets in the majority (73-86%) of cells expressing either intact perilipin A or mutations of perilipin A were clustered together (Fig. 5; Fig 6). This observation suggests that mutated forms of perilipin A maintained the protective role of perilipin A against the attack of cytosolic lipases to stored fat in lipid droplets under basal

conditions. In forskolin/IBMX-stimulated conditions, cells expressing perilipin A and Not5, respectively, showed significantly more fully dispersed lipid droplets than cells expressing S492A and All5 (Fig. 6; Table 4). 94% of the Not5 cells showed highly dispersed lipid droplets, with the remaining cells displaying a partially dispersed lipid droplet pattern. Since serine 492 can be phosphorylated in the Not5 mutant, this result is consistent with prior observations. In contrast, in response to PKA-stimulation, the lipid droplets in cells expressing perilipin A with the single mutation of serine to alanine on PKA site #5 (S492A) failed to disperse completely into micro-droplets. The S492A cells showed a significantly greater proportion of cells with clustered and partially dispersed lipid droplets when compared with the PeriA and Not5 cells in stimulated conditions (Fig.6; Table 4). The majority of lipid droplets in the S492A cells either aggregated loosely with each other, or dispersed into smaller bundles (Fig.5, panel F). Similarly, cells expressing perilipin A with substitutions of all six PKA sites with either alanine or glutamic acid (All5) showed reduced lipid droplet dispersion following activation of PKA. 48% of the cells expressing All5 manifested a clustered lipid droplet pattern, with the remaining cells showing either partially dispersed (33%) or fully dispersed (19%) patterns of lipid droplets. No significant differences were observed between the S492A and All5 cells when assessing lipid droplet distribution ($P>0.05$). These observations indicate that the phosphorylation of serine 492 is required for triggering complete lipid droplet fragmentation, and that the other five PKA sites are not as important as serine 492 in lipid droplet remodeling. In addition, based upon the

comparison of lipid droplet distribution patterns in Not5 and All5 cells, we may conclude that the phosphomimetic substitution of the sixth PKA site serine with glutamic acid in Not5 and All5 adenoviral constructs did not itself promote lipid droplet dispersion.

Discussion

The present study focuses on investigating the contribution of the phosphorylation of serine 492 (PKA site #5) by PKA to regulation of triglyceride hydrolysis. Three mutated forms of perilipin A were generated, including 1) Not5, having all PKA sites mutated into either alanine (PKA sites #1, 2, 3, 4) or glutamic acid (PKA site #6) except serine 492 (PKA site #5) as the only substrate for PKA; 2) S492A, maintaining intact PKA sites in positions 1, 2, 3, 4, and 6 with fifth PKA site mutated into alanine to prevent phosphorylation; and 3) All5, all PKA sites were mutated to either alanine (PKA sites #1, 2, 3, 4, and 5) or glutamic acid (PKA site #6). We conducted forskolin/IBMX-activated lipolysis experiments and immunofluorescence microscopy experiments, respectively, in NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (PeriA), its mutated forms (Not5, S492A, and All5) or β-galactosidase (control). The infected cells were lipid loaded with 600 μM ^{3}H-oleic acid during forskolin-stimulated lipolysis experiments, or 200 μM oleic acid during immunofluorescence microscopy experiments in order to stabilize the newly expressed perilipin protein coating around lipid droplets, and consequently to displace endogenous ADRP off of the lipid droplets.

In our study, in basal conditions, cells expressing ectopic perilipin A (PeriA), or its mutants (Not5, S492A, and All5) suppressed basal lipolysis by 53-56% over 6 hours, when compared with control cells expressing β-galactosidase, which have endogenous ADRP surrounding lipid droplets but no perilipin A. This indicates that both wild-type

and mutated forms of perilipin function normally as barriers against the access of lipases to the surfaces of lipid droplets in NIH 3T3 CarΔ fibroblasts under unstimulated conditions. The observed increases in basal lipolysis over 6 hours may have been due to the residual ADRP on the surfaces of lipid droplets, in other word, ectopic perilipin A or its mutated forms did not fully cover the surfaces of lipid droplets, providing binding space for lipase(s) to access stored triglyceride substrates. In PKA-stimulated lipolysis, cells expressing PeriA showed a 2.2-fold increase in the amount of fatty acid released at the 6 h time point, reaching similar levels as that observed in the β-galactosidase-expressing cells，indicating that perilipin A promotes lipolysis following its phosphorylation by PKA. Substitution of serine 492 with alanine (S492A) reduced PKA-stimulated lipolysis by 33.4% in NIH 3T3 CarΔ cells. Hence, phosphorylation of all five of the other PKA sites (PKA sites #1, 2, 3, 4, and 6) contributed to approximately 66.6% of maximal lipolysis observed in PeriA cells. Our observation is consistent with previous findings that a single mutation of serine 492 of perilipin A reduced PKA-stimulated lipolysis by ~30% in adipocytes differentiated from perilipin-null murine embryonic fibroblasts (MEF) expressing endogenous HSL and ATGL and ectopic mutated perilipin A (Miyoshi *et al.* 2007). Surprisingly, the major finding of our study is that at 6 h PKA-stimulation, the phosphorylation of serine 492 alone (Not5) is able to promote lipolysis at 77.5% of the level of lipolysis observed in cells expressing wild-type perilipin A. Our results suggest that the phosphorylation of serine 492 plays a more important role in promoting PKA-stimulated lipolysis than the combination of all other

five PKA sites does, however, additional PKA sites of perilipin A are required to drive full activation of lipolysis.

PKA-stimulated lipolysis in cells expressing perilipin mutant All5, which has the first five PKA sites mutated into alanine and the last amino acid, serine 517, mutated into glutamic acid, only increases to 16% above basal lipolysis at the 6 h time point. However, as mentioned above, the lipolytic activity in cells expressing Not5, which has just one amino acid different from the All5 construct (serine 492) was 77.5% of maximal levels of lipolysis observed in wild-type PeriA cells at 6 h PKA-stimulation. These findings indicate that without phosphorylation of serine 492, a phosphomimetic amino acid at the 517th residue of perilipin A is not potent enough to drive PKA-stimulated lipolysis to a maximal level. Our observations contrast with those of the Greenberg group (Miyoshi *et al.* 2007), who suggested that the sixth PKA site, serine 517, of perilipin A is a global regulator of PKA-stimulated lipolysis. They found that mutation of serine 517 (PKA site #6) to alanine totally blocked stimulated lipolysis in perilipin-null MEF adipocytes, and that the single mutation of serine 517 to a phosphomimetic aspartic acid residue enhanced stimulated lipolysis by 35% above that observed for ectopic perilipin A.

One explanation for the differences between our observations and those of the Greenberg group is that all of the adenoviral constructs for perilipin A and its mutants generated by Greenberg's lab contain a Flag epitope tag on the carboxyl terminus of each construct (Miyoshi *et al.* 2007;Zhang *et al.* 2003). This epitope tag adds five

aspartic acid residues adjacent to serine 517, at the C-terminus. This may have lead to an exaggerated phosphomimetic effect for the sixth PKA site in response to PKA-stimulation, which complicates study of the contribution of serine 517 to lipolytic regulation. In summary, our findings suggest that serine 492 plays a more essential role in regulating lipolysis than either serine 517 or the combination of all five of the other PKA sites following phosphorylation by PKA.

Previously, it was found that phosphorylation of one or more of the three N-terminal PKA sites of perilipin A is required to assist the translocation of phosphorylated HSL to the surfaces of lipid droplets in response to PKA-stimulation, and therefore, to facilitate HSL-mediated lipolysis (Souza et al. 2002;Zhang et al. 2003). Mutation of the three N-terminal PKA sites of perilipin A in combination attenuated PKA-stimulated lipolysis by 40% in perilipin-null MEF adipocytes expressing both HSL and ATGL (Miyoshi *et al.* 2007). In our study, mutation of PKA sites #1, 2, 3, and 4, partially attenuated forskolin-stimulated lipolysis in NIH 3T3 CarΔ fibroblasts lacking HSL but not ATGL. Provided that the glutamic acid substitution of site 6 acts as a true phosphomimetic amino acid, these observations suggest that in addition to their role in HSL-mediated lipolysis, one or more of PKA sites #1, 2, 3, and 4, of perilipin A may promote lipolysis mediated by ATGL or additional lipases other than HSL.

In this study, we made a series of novel mutations of perilipin A to isolate the effects of phosphorylation of serine 492 from the effects of phosphorylation of the other residues. Since these novel mutated forms of perilipin A had not been previously characterized, we

used immunofluorescence microscopy to test whether they target to lipid droplets and how fragmentation and dispersion of lipid droplets proceeds in NIH 3T3 CarΔ fibroblasts expressing perilipin mutants. Patterns of lipid droplet distribution were compared in NIH 3T3 CarΔ cells expressing ectopic perilipin A and the mutated forms, Not5, S492A, and All5, in the absence or presence of forskolin and IBMX. We found that the phosphorylation of serine 492 alone (Not5) was sufficient to trigger the conversion of clustered lipid droplets into tiny micro-droplets scattered throughout the cytoplasm in NIH 3T3 CarΔ fibroblasts following activation of PKA. Addition of the phosphomimetic residue, glutamic acid, to PKA site #6 did not interfere with the contribution of serine 492 in this aspect; lipid droplets in most cells expressing All5 displayed clustered patterns in both basal and stimulated conditions. As observed previously (Marcinkiewicz *et al.* 2006), mutation of serine 492 to alanine eliminated lipid droplet dispersion. These findings suggest that complete lipid droplet fragmentation stimulated by phosphorylation of serine 492 correlates to a role for phosphorylated serine 492 in the promotion of PKA-stimulated lipolysis. In basal conditions, lipid droplets in cells expressing ectopic perilipin A (PeriA), or its mutated forms (Not5, S492A, and All5) were predominantly present in densely clustered patterns, suggesting that mutation of PKA sites of perilipin A did not affect the mechanism by which perilipin A organizes lipid droplets into tight aggregates in the cytoplasm.

In contrast to the previous findings (Marcinkiewicz *et al.* 2006), following stimulation of PKA, lipid-droplets in most cells expressing S492A distributed into an

intermediate dispersion pattern, by breaking into smaller groups of lipid droplets that aggregated loosely with each other, rather than in dense clusters. Since the lipid droplet distribution data were generated by having 2 or 3 individuals score 100-200 cells according to the categorization scheme described in Fig.4, the scoring results were subjective due to differences in the interpretation of scatter patterns by each individual. Hence, results of this study may be somewhat inconsistent with the previous findings. However, the identical appearance of Not5 cells relative to PeriA cells in stimulated conditions suggests that serine 492 is the major determinant of lipid droplet remodeling.

Adipose triglyceride lipase (ATGL) is considered to be the rate-limiting lipase for triglyceride hydrolysis in adipose tissue (Haemmerle *et al.* 2006). It acts upstream of hormone-sensitive lipase (HSL) by hydrolyzing triglyceride; HSL then hydrolyzes diglyceride (Zimmermann *et al.* 2004). Our cell model of NIH 3T3 CarΔ fibroblasts lacks endogenous HSL (Souza *et al.* 2002), and allows us to focus on non-HSL-mediated lipolysis; this lipolysis is most likely catalyzed by ATGL (Zimmermann *et al.* 2004). Hence, our results suggest that the phosphorylation of serine 492 is a crucial regulator of ATGL-mediated lipolysis, and serine 517 plays only a complementary role. Furthermore, one or more of PKA sites #1, 2, 3 and 4 may also be involved in lipolysis mediated by ATGL or lipases other than HSL.

Then we may ask how serine 492 phosphorylation is involved in ATGL-mediated lipolysis? Is it simply through fragmentation of lipid droplets stimulated by the phosphorylation of serine 492, or through other more complex mechanisms? The

mass of perilipin A did not increase significantly during lipid droplet dispersion in 3T3-L1 fibroblasts expressing ectopic perilipin A or in 3T3-L1 adipocytes expressing endogenous perilipin A (Marcinkiewicz *et al.* 2006). Since the sum of the surface area of tiny micro-droplets after the dispersion process is greater than that of larger or clustered lipid droplets before the process, the barrier function of perilipin A on the surface of lipid droplets may become weaker. ATGL may bind to the surface of lipid droplets due to this thinning of the perilipin coating as it provides additional surface availability to cytosolic proteins, resulting in enhanced triglyceride hydrolysis. Since fatty acid release can be detected within 30 min after the cAMP cascade is triggered, whereas the serine 492-mediated lipid droplets remodeling is detectable much later, I speculate that phosphorylation of perilipin A facilitates ATGL-mediated lipolysis by changing its conformation, revealing the lipid droplet surface, in the early phase of PKA-stimulation, and promoting fragmentation and dispersion of lipid droplets in the late phase. Additionally, a proteomic study of lipid droplets isolated from 3T3-L1 adipocytes found that ADRP, s3-12, and many additional proteins became localized on the surfaces of lipid droplets in lipolyticaly stimulated 3T3-L1 adipocytes (Brasaemle *et al.* 2004). This supports the idea that phosphorylation of perilipin A promotes a conformational change in the protein that uncovers the lipid droplet, revealing binding sites for additional proteins that may include ATGL.

In conclusion, we have demonstrated that the PKA-mediated phosphorylation of serine 492 of perilipin A plays a critical role in regulating and facilitating

non-HSL-mediated lipolysis, most likely catalyzed by ATGL. Lipolysis is promoted partially following the fragmentation of lipid droplets into tiny lipid micro-droplets, thus expanding the surface area of the droplets. The remodeling process enhances the interaction of lipolytic enzymes with stored triglycerides in lipid droplets. Without phosphorylation of serine 492, stimulated lipolysis is attenuated by 33.4%, and the complete dispersion of lipid droplets is blocked.

Table 1. Subcloning and mutagenic primers used to generate adenoviral constructs of mutated forms of perilipin A. Forward (F) and reverse (R) subcloning primers contain *KpnI* and *HindIII* restriction sites, respectively, which were used to subclone the desired perilipin cDNA sequence into either the pCR-Blunt vector or pShuttle-CMV vector. Mutagenic oligonucleotide primers replace a single nucleotide to alter a single amino acid from alanine to glutamic acid (Not5), or from serine to alanine (S492A, and All5).

Adenoviral Constructs	**Oligonucleotide Sequence 5' to 3'**	
Not5	Subcloning (F)	GCATGGTACCATGTCAATAAACAAGGGC
	Subcloning & Mutagenic (R)	CAAAAGCTTTCACTCCTTCTTGC
S492A	Subcloning (F)	GCATGGTACCATGTCAATAAACAAGGGC
	Subcloning (R)	ATGCAAGCTTTCAGCTCTTCTTGCGC
	Mutagenic (F)	CAGAGTCGCCGACAGCTTCTTCC
	Mutagenic (R)	GGAAGAAGCTGTCGGCGACTCTG
All5	Subcloning (F)	GCATGGTACCATGTCAATAAACAAGGGC
	Subcloning (R)	CCAAAAGCTTTCACTCCTTCTTGCG
	Mutagenic (F)	CAGAGTCGCCGACAGCTTCTTCC
	Mutagenic (R)	GGAAGAAGCTGTCGGCGACTCTG

Table 2. Densitometric scan volumes of bands of perilipin A and ADRP. Films from immunoblots probed for perilipin A, ADRP and calnexin shown in Figure 1 were scanned by a laser-scanning densitometer. Densities of perilipin A and ADRP signals were quantified by volume, and adjusted by the corresponding volume of calnexin.

Adenoviral Construct (µl/9.5cm^2 well)	Adjusted Perilipin A	Adjusted ADRP
Uninfected	-	1908.39
β-galactosidase 6.7	-	1980.61
PeriA 14.0	912.98	1250.25
PeriA 15.0	2516.86	859.95
All5 20.0	2608.99	402.88
All5 22.5	2913.44	325.75
S492A 12.0	3651.71	663.07
S492A 14.0	5169.01	433.11
S492A 15.0	4334.95	624.88
Not5 3.0	449.21	1156.25
Not5 4.0	1069.04	752.93
Not5 5.0	2143.26	681.55
Not5 7.0	2985.45	450.34

Table 3. Summary of statistical analyses of data collected for ^{3}H-fatty acid release under basal and forskolin-stimulated conditions. Two-way analysis of variance was used to compare data depicted in Figures 2A and 2B.

	β-gal stm	**PeriA basal**	**PeriA stm**	**Not5 basal**	**Not5 stm**	**S492A basal**	**S492A stm**	**All5 basal**	**All5 stm**
β-gal basal	ns, except at 6hr (P<0.05)	**P<0.001**	ns	**P<0.001**	P<0.001	**P<0.001**	P<0.001	**P<0.001**	P<0.001
β-gal stm		P<0.001	ns, except at 6hr (P<0.001)	P<0.001	4hr, P<0.05; 6hr, P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
PeriA basal			**P<0.001**	ns	P<0.001	ns	P<0.001	ns	P<0.001
PeriA stm				P<0.001	**P<0.001**	P<0.001	**P<0.001**	P<0.001	**P<0.001**
Not5 basal					**P<0.001**	ns	P<0.001	ns	4hr and 6hr, P<0.01
Not5 stm						P<0.001	**P<0.001**	P<0.001	**P<0.001**
S492A basal							**P<0.001**	ns	P<0.001
S492A stm								P<0.001	ns, except at 6 hr (P<0.001)
All5 basal									**4hr, P<0.001; 6hr, P<0.01**

Table 4. Mutation of serine 492 to alanine interferes with complete lipid droplet dispersion. NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (PeriA) or its mutated forms (Not5, S492A, and All5) were incubated for 6 h at 37°C in medium containing 3% fatty-acid free bovine serum albumin, and 10 μM forskolin in 0.1% dimethyl sulfoxide (DMSO) and 0.5 mM IBMX (STM), or an equivalent amount of DMSO (Basal). Cells were fixed with 4% paraformaldehyde in PBS, and incubated with anti-perilipin antisera, and then co-stained with Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503. Cells were then scored for lipid droplet distribution according to the three patterns described in Figure 4: clustered, partially dispersed (intermediate), and fully dispersed. Data were collected from one experiment and included cell counts by 2 or 3 individuals. Data are the means of the percent of each pattern from 200-300 cell counts total and were analyzed by two-way ANOVA.

Perilipin	**PeriA**		**Not5**		**S492A**		**All5**	
	Basal	**STM**	**Basal**	**STM**	**Basal**	**STM**	**Basal**	**STM**
Clustered LDs (% Cells)	74.3	0.4	77.6 ns[1]	0.0 ns[2]	85.9 ns[1]	36.1**[a, b]	72.6 ns[1]	47.9***[b]
Intermediate LDs (% Cells)	22.1	12.1	18.7 ns[1]	5.7 ns[2]	11.4 ns[1]	43.0**[b, *]	21.0 ns[1]	32.9 ns[2]
Dispersed LDs (% Cells)	3.6	87.4	3.7 ns[1]	94.3 ns[2]	2.8 ns[1]	20.8***[a]	6.4 ns[1]	19.2***[a]
Number of cells scored	280	218	277	208	360	222	324	239
N	2	2	2	2	3	2	3	2

ns[1], not significantly different from PeriA Basal, P>0.05
ns[2], not significantly different from PeriA STM, P>0.05
***a, significantly lower than PeriA STM and Not5 STM, P<0.001
***b, significantly greater than PeriA STM, P<0.001
**a, significantly greater than PeriA STM, P<0.01
**b, significantly greater than Not5 STM, P<0.01
*, significantly greater than PeriA STM, P<0.05

FIGURE LEGENDS

Figure 1. Titration of adenovirus levels for expression of perilipin A and its mutants. NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (PeriA), its mutants (Not5, S492A, and All5), or β-galactosidase (control) were incubated in medium containing 600 μM oleic acid for 6 h at 37°C, and then harvested in hypotonic lysis buffer. Proteins were separated on SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were probed with antibodies raised against perilipin A, ADRP, β-galactosidase, and calnexin as a loading control. Data shown here are representative of three experiments.

Figure 2. Time course of ^{3}H-fatty acid release under basal and forskolin-stimulated conditions. A) Confluent NIH 3T3 L1 CarΔ fibroblasts expressing ectopic perilipin A (PeriA), or β-galactosidase (control), or B) perilipin A (PeriA), its mutated forms (Not5, S492A, and All5), or β-galactosidase (control), were lipid loaded with 600 μM ^{3}H-oleic acid for 12 hrs. Cells were washed twice after fatty acids were removed, and then incubated for 6 hrs at 37°C in medium containing 6 μM triacsin C, 3% fatty-acid free bovine serum albumin, and either 10 μM forskolin in 0.1% dimethyl sulfoxide (DMSO) and 0.5 mM IBMX, or 0.1% DMSO. The efflux of radioactivity into the medium was measured by scintillation counting of 200 μl aliquots of culture medium at 0, 2, 4, and 6 h time points, and was expressed relative to total

cellular protein. Measurements for each data point were obtained from triplicate dishes of cells. Data of one representative experiment out of four are shown as mean ± S.D. for triplicate samples, analyzed by two-way analysis of variance. Some error bars are contained within the symbols.

Figure 3. The expression of cellular proteins under basal and forskolin-stimulated conditions. NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (PeriA), its mutants (Not5, S492A, and All5), or β-galactosidase (control) were incubated for 2 hrs at 37°C in medium containing 3% fatty-acid free bovine serum albumin, and in the absence or presence of 10 μM forskolin and 0.5 mM IBMX. Proteins were harvested in hypotonic lysis buffer, separated on SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were probed with antibodies raised against phospho-(Ser/Thr) protein kinase A substrate, perilipin A, ADRP, β-galactosidase, and calnexin as a loading control. Data shown here are representative of four experiments.

Figure 4. Patterns of lipid droplet distribution in NIH 3T3 CarΔ fibroblasts. Three patterns of lipid droplet distribution were observed and defined in NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (A), and its mutated form Not5 (B, and C). These three patterns include A) densely clustered (image selected from PeriA cells under basal conditions), B) partially dispersed, with lipid droplets aggregated loosely together (image selected from Not5 cells under stimulated conditions), and C) fully dispersed,

with lipid droplets dispersed into tiny micro-lipid droplets with no detectable clusters (image selected from Not5 cells under stimulated conditions). This categorization of lipid droplet distribution patterns was used for scoring the cells shown in Figure 6 and summarized in Table 4.

Figure 5. Mutation of serine 492 to alanine prevents full lipid droplet dispersion. Confluent NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A or its mutated forms (Not5, S492A, and All5) were lipid loaded with 200 μM oleic acid for 12 hrs. Cells were washed once after fatty acids were removed, and then incubated for 6 hrs at 37°C in medium containing 3% fatty-acid free bovine serum albumin, and either 10 μM forskolin in 0.1% dimethyl sulfoxide (DMSO) and 0.5 mM IBMX, or 0.1% DMSO. Cells were fixed with 4% paraformaldehyde in PBS, incubated with anti-perilipin antisera, and then co-stained with Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503. Images displayed are representative of the major pattern of lipid droplet distribution for each condition.

Figure 6. Mutation of serine 492 to alanine prevents complete lipid droplet dispersion. NIH 3T3 CarΔ fibroblasts expressing ectopic perilipin A (PeriA) or its mutated forms (Not5, S492A, and All5) were incubated for 6 hrs in at 37°C with (B) or without (A) forskolin and IBMX. Cells were fixed with 4% paraformaldehyde in PBS, incubated with anti-perilipin antisera, and then co-stained with Alexa Fluor

546-conjugated goat anti-rabbit IgG and Bodipy 493/503. 100 to 200 cells were then scored for lipid droplet distribution according to the three patterns described in Figure 4: clustered, partially dispersed (intermediate), and fully dispersed (dispersed). Data were collected from one experiment and included cell counts by 2 or 3 individuals. Data are the means of the percent of cells showing each pattern from 200-300 cell counts total and were analyzed by two-way ANOVA. Some error bars are contained within the symbols.

Fig. 1

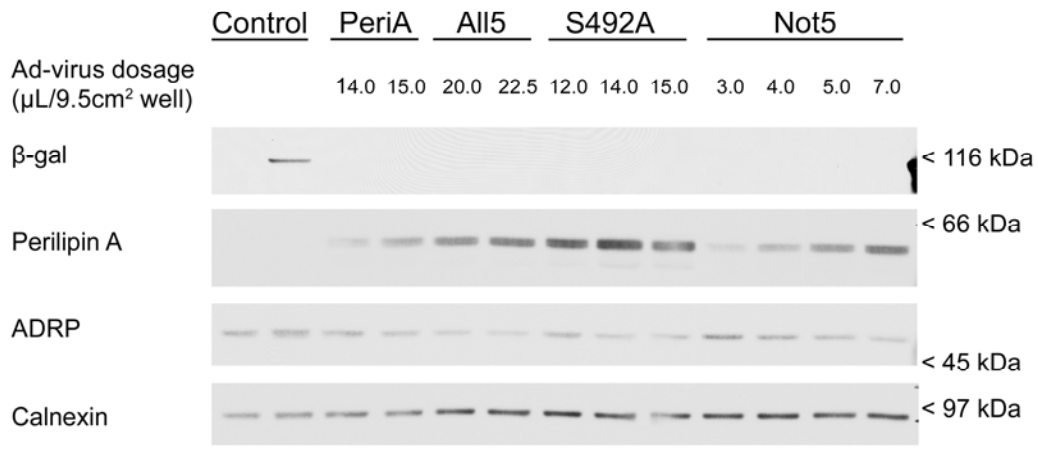
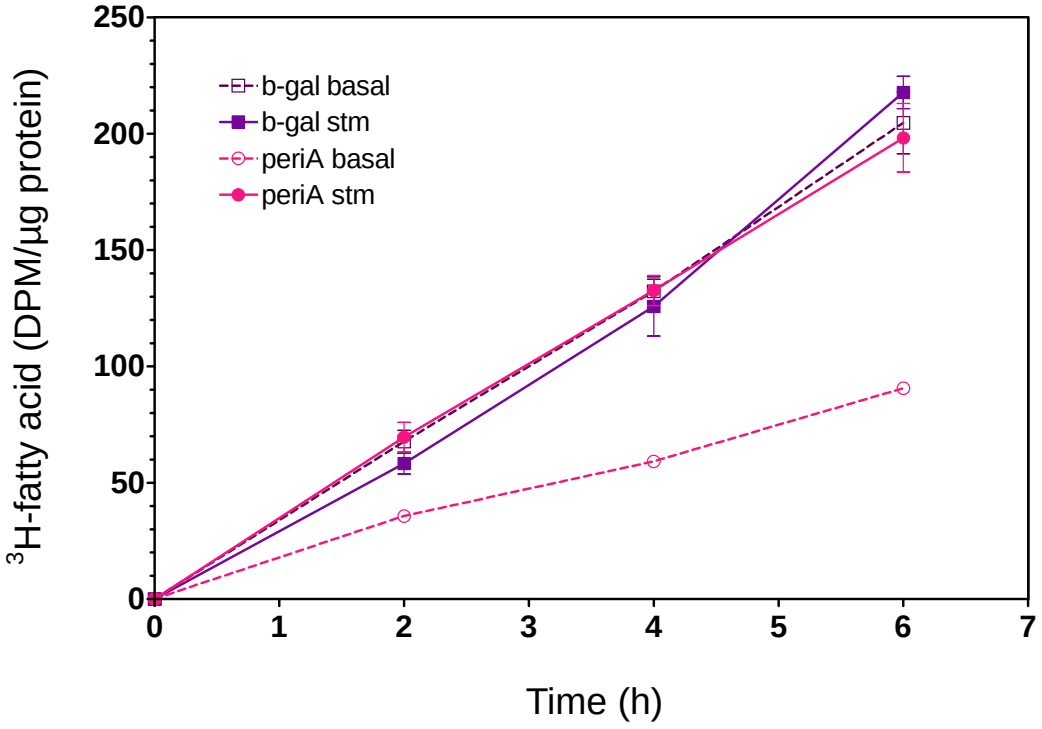

Control
PeriA
All5
S492A
Not5
Ad-virus dosage
(μL/9.5cm² well)
14.0 15.0 20.0 22.5 12.0 14.0 15.0 3.0 4.0 5.0 7.0
β-gal
< 116 kDa
Perilipin A
< 66 kDa
ADRP
< 45 kDa
Calnexin
< 97 kDa

Fig. 2

A


b-gal basal
b-gal stm
periA basal
periA stm
^{3}H-fatty acid (DPM/µg protein)
Time (h)
0
50
100
150
200
250
0
1
2
3
4
5
6
7

B

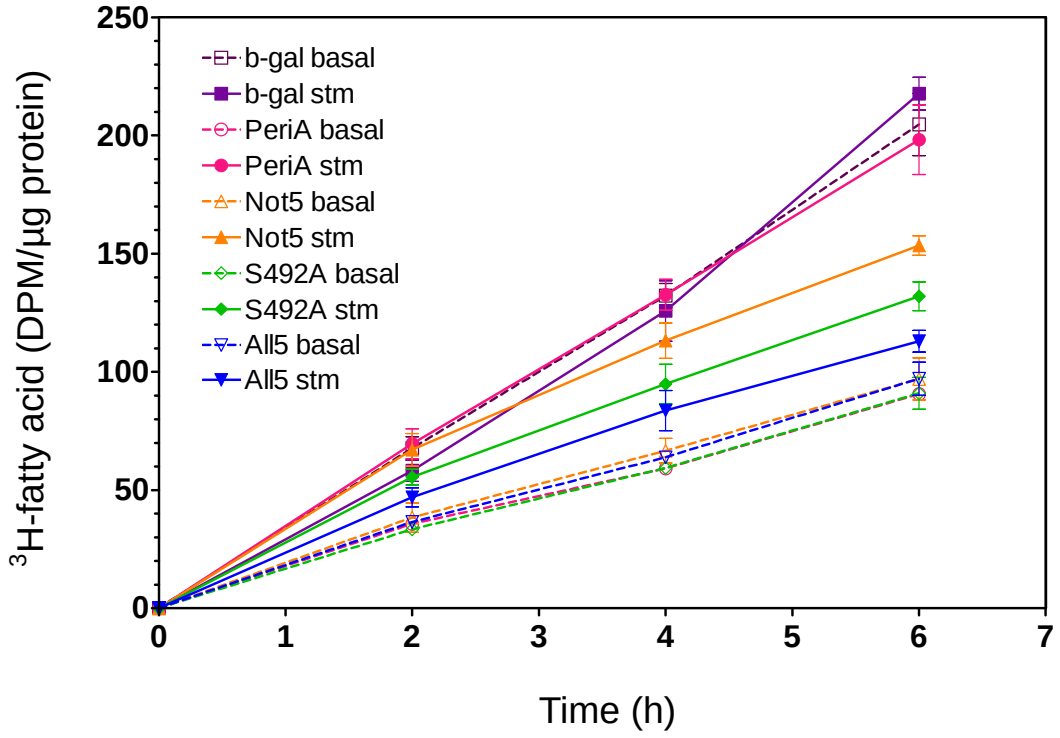

b-gal basal
b-gal stm
PeriA basal
PeriA stm
Not5 basal
Not5 stm
S492A basal
S492A stm
All5 basal
All5 stm
^{3}H-fatty acid (DPM/µg protein)
Time (h)
0
50
100
150
200
250
0
1
2
3
4
5
6
7

Fig. 3

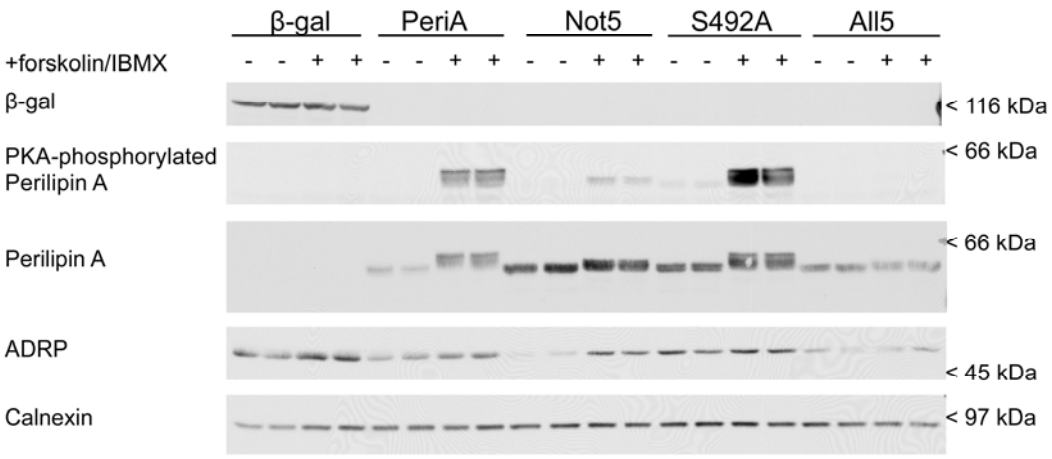

β-gal
PeriA
Not5
S492A
All5
+forskolin/IBMX
- - + + - - + + - - + + - - + + - - + +
β-gal
< 116 kDa
PKA-phosphorylated Perilipin A
< 66 kDa
Perilipin A
< 66 kDa
ADRP
< 45 kDa
Calnexin
< 97 kDa

Fig. 4

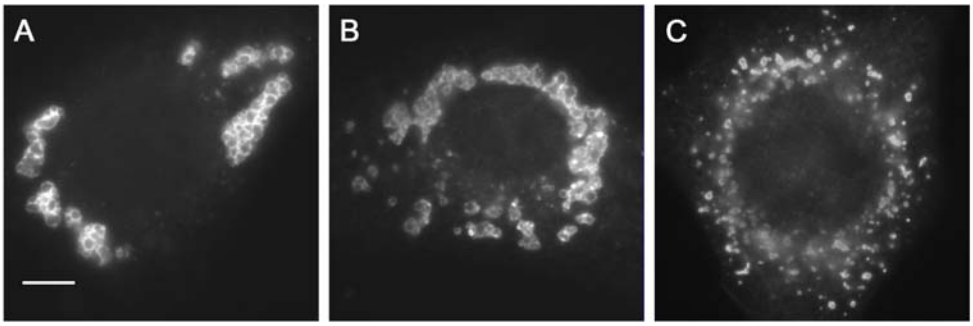


Fig. 5

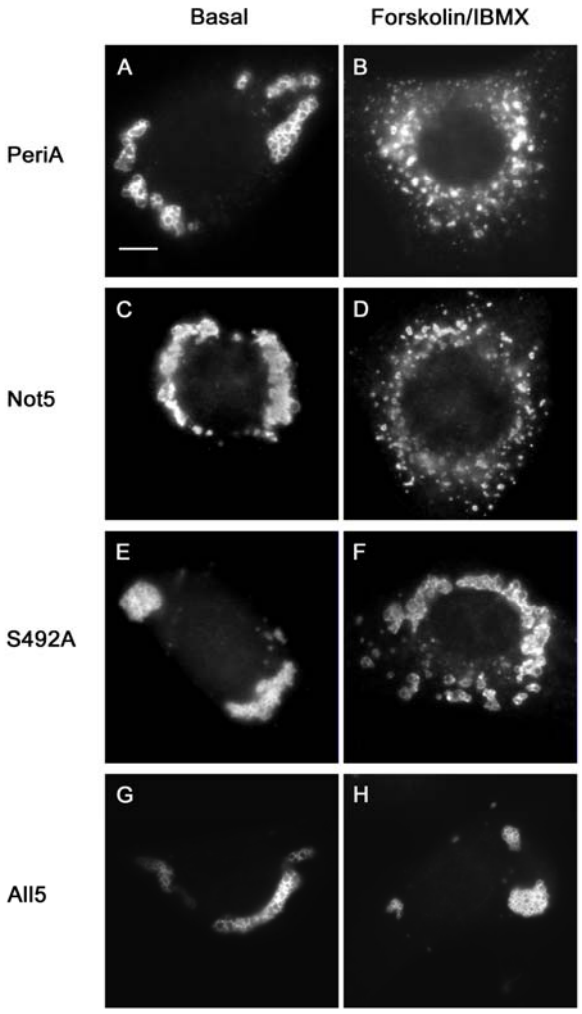
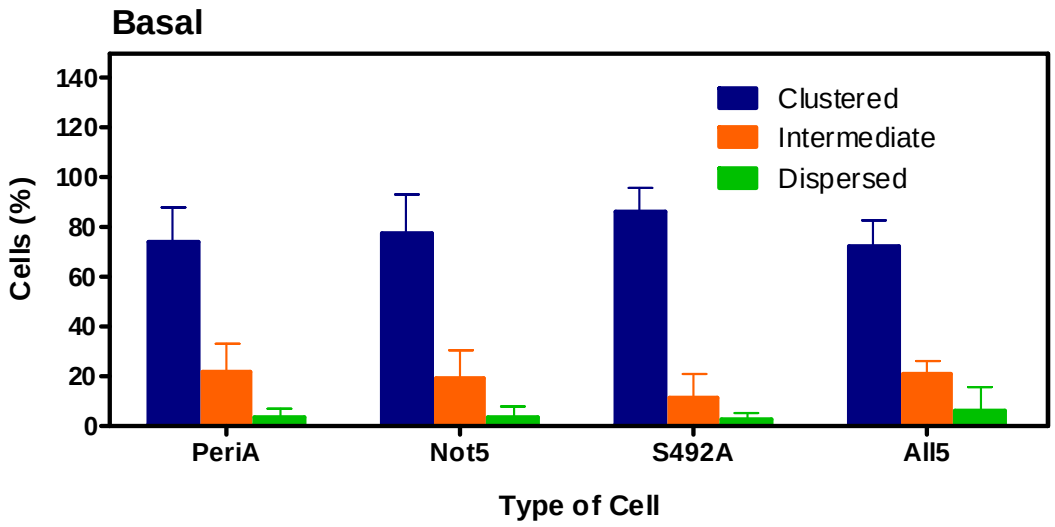
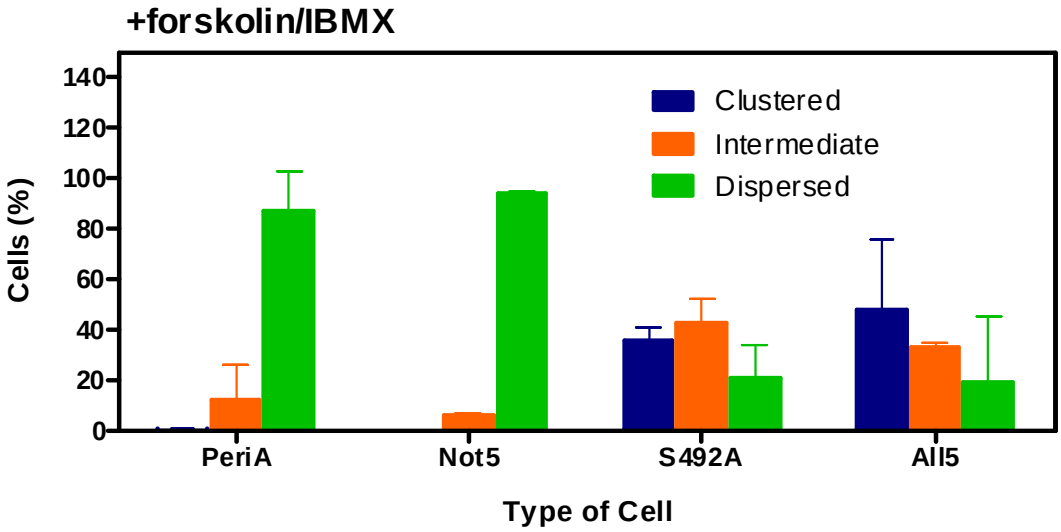


Fig. 6

A


Basal
140
120
100
80
60
40
20
0
Cells (%)
Clustered
Intermediate
Dispersed
PeriA
Not5
S492A
All5
Type of Cell

B


+forskolin/IBMX
140
120
100
80
60
40
20
0
Cells (%)
Clustered
Intermediate
Dispersed
PeriA
Not5
S492A
All5
Type of Cell

References

Belfrage P., Fredrikson G., Nilsson N. O., & Stralfors P. (1980) Regulation of adipose tissue lipolysis: phosphorylation of hormones sensitive lipase in intact rat adipocytes. *FEBS Lett.* 111: 120-124.

Belfrage P., Fredrikson G., Nilsson N. O., & Stralfors P. (1981) Regulation of adipose-tissue lipolysis by phosphorylation of hormone-sensitive lipase. *Int.J.Obes.* 5: 635-641.

Blanchette-Mackie E. J., Dwyer N. K., Barber T., Coxey R. A., Takeda T., Rondinone C. M., Theodorakis J. L., Greenberg A. S., & Londos C. (1995) Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J.Lipid Res.* 36: 1211-1226.

Brasaemle D. L. (2007) Thematic review series: Adipocyte Biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J.Lipid Res.* 48: 2547-2559.

Brasaemle D. L., Barber T., Kimmel A. R., & Londos C. (1997a) Post-translational regulation of perilipin expression. Stabilization by stored intracellular neutral lipids. *J.Biol.Chem.* 272: 9378-9387.

Brasaemle D. L., Barber T., Wolins N. E., Serrero G., Blanchette-Mackie E. J., & Londos C. (1997b) Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J.Lipid Res.* 38: 2249-2263.

Brasaemle D. L., Dolios G., Shapiro L., & Wang R. (2004) Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J.Biol.Chem.* 279: 46835-46842.

Brasaemle D. L., Rubin B., Harten I. A., Gruia-Gray J., Kimmel A. R., & Londos C. (2000) Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J.Biol.Chem.* 275: 38486-38493.

Carmen G. Y. & Victor S. M. (2006) Signalling mechanisms regulating lipolysis. *Cell Signal.* 18: 401-408.

Fredrikson G., Stralfors P., Nilsson N. O., & Belfrage P. (1981) Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. *J.Biol.Chem.* 256: 6311-6320.

Fujimoto Y., Itabe H., Sakai J., Makita M., Noda J., Mori M., Higashi Y., Kojima S., & Takano T. (2004) Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7. *Biochim.Biophys.Acta* 1644: 47-59.

Garcia A., Sekowski A., Subramanian V., & Brasaemle D. L. (2003) The central domain is required to target and anchor perilipin A to lipid droplets. *J.Biol.Chem.* 278: 625-635.

Garcia A., Subramanian V., Sekowski A., Bhattacharyya S., Love M. W., & Brasaemle D. L. (2004) The amino and carboxyl termini of perilipin a facilitate the storage of triacylglycerols. *J.Biol.Chem.* 279: 8409-8416.

Gocze P. M. & Freeman D. A. (1994) Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. *Cytometry* 17: 151-158.

Greenberg A. S., Egan J. J., Wek S. A., Moos M. C., Jr., Londos C., & Kimmel A. R. (1993) Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proc.Natl.Acad.Sci.U.S.A* 90: 12035-12039.

Haemmerle G., Lass A., Zimmermann R., Gorkiewicz G., Meyer C., Rozman J., Heldmaier G., Maier R., Theussl C., Eder S., Kratky D., Wagner E. F., Klingenspor M., Hoefler G., & Zechner R. (2006) Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. *Science* 312: 734-737.

Haemmerle G., Zimmermann R., Strauss J. G., Kratky D., Riederer M., Knipping G., & Zechner R. (2002) Hormone-sensitive lipase deficiency in mice changes the plasma lipid profile by affecting the tissue-specific expression pattern of lipoprotein lipase in adipose tissue and muscle. *J.Biol.Chem.* 277: 12946-12952.

Imamura M., Inoguchi T., Ikuyama S., Taniguchi S., Kobayashi K., Nakashima N., & Nawata H. (2002) ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am.J.Physiol Endocrinol.Metab* 283: E775-E783.

Jenkins C. M., Mancuso D. J., Yan W., Sims H. F., Gibson B., & Gross R. W. (2004) Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J.Biol.Chem.* 279: 48968-48975.

Jocken J. W., Langin D., Smit E., Saris W. H., Valle C., Hul G. B., Holm C., Arner P., & Blaak E. E. (2007) Adipose triglyceride lipase and hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. *J.Clin.Endocrinol.Metab* 92: 2292-2299.

Kawamura M., Jensen D. F., Wancewicz E. V., Joy L. L., Khoo J. C., & Steinberg D. (1981) Hormone-sensitive lipase in differentiated 3T3-L1 cells and its activation by cyclic AMP-dependent protein kinase. *Proc.Natl.Acad.Sci.U.S.A* 78: 732-736.

Kershaw E. E., Hamm J. K., Verhagen L. A., Peroni O., Katic M., & Flier J. S. (2006)

Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin. *Diabetes* 55: 148-157.

Khoo J. C., Aquino A. A., & Steinberg D. (1974) The mechanism of activation of hormone-sensitive lipase in human adipose tissue. *J.Clin.Invest* 53: 1124-1131.

Khoo J. C., Steinberg D., Thompson B., & Mayer S. E. (1973) Hormonal regulation of adipocyte enzymes. The effects of epinephrine and insulin on the control of lipase, phosphorylase kinase, phosphorylase, and glycogen synthase. *J.Biol.Chem.* 248: 3823-3830.

Langin D. (2006) Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome. *Pharmacol.Res.* 53: 482-491.

Langin D., Dicker A., Tavernier G., Hoffstedt J., Mairal A., Ryden M., Arner E., Sicard A., Jenkins C. M., Viguerie N., van H., V, Gross R. W., Holm C., & Arner P. (2005) Adipocyte lipases and defect of lipolysis in human obesity. *Diabetes* 54: 3190-3197.

Lass A., Zimmermann R., Haemmerle G., Riederer M., Schoiswohl G., Schweiger M., Kienesberger P., Strauss J. G., Gorkiewicz G., & Zechner R. (2006) Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metab* 3: 309-319.

Listenberger L. L., Ostermeyer-Fay A. G., Goldberg E. B., Brown W. J., & Brown D. A. (2007) Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J.Lipid Res.* 48: 2751-2761.

Liu P., Ying Y., Zhao Y., Mundy D. I., Zhu M., & Anderson R. G. (2004) Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J.Biol.Chem.* 279: 3787-3792.

Londos C., Brasaemle D. L., Schultz C. J., Segrest J. P., & Kimmel A. R. (1999) Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin.Cell Dev.Biol.* 10: 51-58.

Lu X., Gruia-Gray J., Copeland N. G., Gilbert D. J., Jenkins N. A., Londos C., & Kimmel A. R. (2001) The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. *Mamm.Genome* 12: 741-749.

Marcinkiewicz A., Gauthier D., Garcia A., & Brasaemle D. L. (2006) The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and

dispersion. *J.Biol.Chem.* 281: 11901-11909.

Martinez-Botas J., Anderson J. B., Tessier D., Lapillonne A., Chang B. H., Quast M. J., Gorenstein D., Chen K. H., & Chan L. (2000) Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nat.Genet.* 26: 474-479.

Miyoshi H., Perfield J. W., Souza S. C., Shen W. J., Zhang H. H., Stancheva Z. S., Kraemer F. B., Obin M. S., & Greenberg A. S. (2007) Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis in adipocytes. *J.Biol.Chem.* 282: 996-1002.

Miyoshi H., Souza S. C., Zhang H. H., Strissel K. J., Christoffolete M. A., Kovsan J., Rudich A., Kraemer F. B., Bianco A. C., Obin M. S., & Greenberg A. S. (2006) Perilipin promotes hormone-sensitive lipase-mediated adipocyte lipolysis via phosphorylation-dependent and -independent mechanisms. *J.Biol.Chem.* 281: 15837-15844.

Nilsson N. O., Stralfors P., Fredrikson G., & Belfrage P. (1980) Regulation of adipose tissue lipolysis: effects of noradrenaline and insulin on phosphorylation of hormone-sensitive lipase and on lipolysis in intact rat adipocytes. *FEBS Lett.* 111: 125-130.

Osuga J., Ishibashi S., Oka T., Yagyu H., Tozawa R., Fujimoto A., Shionoiri F., Yahagi N., Kraemer F. B., Tsutsumi O., & Yamada N. (2000) Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. *Proc.Natl.Acad.Sci.U.S.A* 97: 787-792.

Ross S. A., Song X., Burney M. W., Kasai Y., & Orlicky D. J. (2003) Efficient adenovirus transduction of 3T3-L1 adipocytes stably expressing coxsackie-adenovirus receptor. *Biochem.Biophys.Res.Commun.* 302: 354-358.

Ryden M., Jocken J., van H., V, Dicker A., Hoffstedt J., Wiren M., Blomqvist L., Mairal A., Langin D., Blaak E., & Arner P. (2007) Comparative studies of the role of hormone-sensitive lipase and adipose triglyceride lipase in human fat cell lipolysis. *Am.J.Physiol Endocrinol.Metab* 292: E1847-E1855.

Schaffer J. E. (2003) Lipotoxicity: when tissues overeat. *Curr.Opin.Lipidol.* 14: 281-287.

Schultz C. J., Torres E., Londos C., & Torday J. S. (2002) Role of adipocyte differentiation-related protein in surfactant phospholipid synthesis by type II cells. *Am.J.Physiol Lung Cell Mol.Physiol* 283: L288-L296.

Servetnick D. A., Brasaemle D. L., Gruia-Gray J., Kimmel A. R., Wolff J., & Londos C.

(1995) Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. *J.Biol.Chem.* 270: 16970-16973.

Souza S. C., Muliro K. V., Liscum L., Lien P., Yamamoto M. T., Schaffer J. E., Dallal G. E., Wang X., Kraemer F. B., Obin M., & Greenberg A. S. (2002) Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. *J.Biol.Chem.* 277: 8267-8272.

Su C. L., Sztalryd C., Contreras J. A., Holm C., Kimmel A. R., & Londos C. (2003) Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. *J.Biol.Chem.* 278: 43615-43619.

Subramanian V., Garcia A., Sekowski A., & Brasaemle D. L. (2004) Hydrophobic sequences target and anchor perilipin A to lipid droplets. *J.Lipid Res.* 45: 1983-1991.

Sztalryd C., Xu G., Dorward H., Tansey J. T., Contreras J. A., Kimmel A. R., & Londos C. (2003) Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J.Cell Biol.* 161: 1093-1103.

Tansey J. T., Huml A. M., Vogt R., Davis K. E., Jones J. M., Fraser K. A., Brasaemle D. L., Kimmel A. R., & Londos C. (2003) Functional studies on native and mutated forms of perilipins. A role in protein kinase A-mediated lipolysis of triacylglycerols. *J.Biol.Chem.* 278: 8401-8406.

Tansey J. T., Sztalryd C., Gruia-Gray J., Roush D. L., Zee J. V., Gavrilova O., Reitman M. L., Deng C. X., Li C., Kimmel A. R., & Londos C. (2001) Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc.Natl.Acad.Sci.U.S.A* 98: 6494-6499.

Tansey J. T., Sztalryd C., Hlavin E. M., Kimmel A. R., & Londos C. (2004) The central role of perilipin a in lipid metabolism and adipocyte lipolysis. *IUBMB.Life* 56: 379-385.

Trayhurn P. & Beattie J. H. (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc.Nutr.Soc.* 60: 329-339.

Turro S., Ingelmo-Torres M., Estanyol J. M., Tebar F., Fernandez M. A., Albor C. V., Gaus K., Grewal T., Enrich C., & Pol A. (2006) Identification and characterization of associated with lipid droplet protein 1: A novel membrane-associated protein that resides on hepatic lipid droplets. *Traffic.* 7: 1254-1269.

Umlauf E., Csaszar E., Moertelmaier M., Schuetz G. J., Parton R. G., & Prohaska R. (2004) Association of stomatin with lipid bodies. *J.Biol.Chem.* 279: 23699-23709.

Villena J. A., Roy S., Sarkadi-Nagy E., Kim K. H., & Sul H. S. (2004) Desnutrin, an

adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J.Biol.Chem.* 279: 47066-47075.

Wang S. P., Laurin N., Himms-Hagen J., Rudnicki M. A., Levy E., Robert M. F., Pan L., Oligny L., & Mitchell G. A. (2001) The adipose tissue phenotype of hormone-sensitive lipase deficiency in mice. *Obes.Res.* 9: 119-128.

Wu C. C., Howell K. E., Neville M. C., Yates J. R., III, & McManaman J. L. (2000) Proteomics reveal a link between the endoplasmic reticulum and lipid secretory mechanisms in mammary epithelial cells. *Electrophoresis* 21: 3470-3482.

Yeaman S. J. (2004) Hormone-sensitive lipase--new roles for an old enzyme. *Biochem.J.* 379: 11-22.

Zhang H. H., Souza S. C., Muliro K. V., Kraemer F. B., Obin M. S., & Greenberg A. S. (2003) Lipase-selective functional domains of perilipin A differentially regulate constitutive and protein kinase A-stimulated lipolysis. *J.Biol.Chem.* 278: 51535-51542.

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