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Probing the Function of Perilipin 5b

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ABSTRACT

Many modern health issues arise from aberrant lipid metabolism, among these are type II diabetes and non-alcoholic fatty liver disease. One commonality these diseases share is neutral lipid metabolism and storage. Regulation of neutral lipid metabolism is of vital importance in the body, and among the proteins responsible for such regulation are the perilipins. Perilipins are a family of five conserved proteins that are found on the surface of lipid storage droplets and play a central role in the regulation of cellular neutral lipid metabolism. Perilipin 5 specifically, is expressed in tissues with a high capacity for fatty acid oxidation such as cardiac muscle, oxidative muscle, and fasting liver. Using western blotting and reverse transcriptase PCR, we have identified a splice variant of perilipin 5 we have termed perilipin 5b. This shortened form of the protein retains the amino terminal 35 kDa of the protein but lacks the putative 4-helix bundle found in the C-terminus. Based on the reports in the literature, this protein would lack the carboxy terminal domains reported to be necessary for interactions with lipases and other proteins but would retain serine 155 that is requisite for PKA phosphorylation and translocation to the nucleus. We have constructed a carboxy terminal truncation of perilipin 5 and are expressing it in CHO cells to further define the function of this protein. Immunofluoresence microscopy of CHO cells expressing perilipin 5b shows localization on lipid storage droplets and on puncta in the cytosol. Stimulation of the PKA signaling pathway in these cells leads to the phosphorylation of perilipin 5 and a shift to the nucleus. Collectively these data indicate that perilipin 5b plays a role in lipid storage, but the function of this truncation is still unknown.

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INTRODUCTION

Constant developments in technology have led to Western civilizations leading a more sedentary lifestyle. As a result, lipid accumulation due to lack of exercise has led to metabolic diseases such as obesity, diabetes, insulin resistance, and nonalcoholic fatty liver disease gaining a greater prevalence throughout the Western population. Now more than ever it is crucial to gain an understanding into the pathologies that lead to such diseased states. The following introduction will explore the metabolic diseases associated with aberrant lipid metabolism, as well as many of the proteins crucial for lipid metabolism.

Metabolic Disease

The increasing prevalence of metabolic disease requires an understanding of the different associated diseases. The following explores several relevant metabolic diseases that are the result of abnormal lipid pathologies.

Obesity

Obesity is a worldwide epidemic responsible for increased metabolic diseases. Obese is defined as having a body mass index (BMI) $\geq 30 kg/m^2$. (1) If the current trends continue, it is estimated that by 2030, 38% of the world's adult population will be overweight, with another 20% considered obese (2). There are several diseases that are attributed to obesity, including cardiovascular disease, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD) (2).

Skeletal muscle is known to play a vital role in lipid metabolism. In a rested state, fatty acid (FA) oxidation occurring in the skeletal muscle contributes approximately 90% of the energy requirements for the muscle tissue(3). In those considered obese, high free fatty acid (FFA)

concentrations accompany an increased FA uptake, however the ability to oxidize the increased flux of lipid is not matched (2). Such a problem results in reduced enzymatic activity in crucial metabolic pathways such as beta oxidation, Krebs cycle, Electron Transport Chain (ETC), and FA transfer into mitochondria. The resulting diminished enzymatic activity may contribute to insulin resistance.

Insulin Resistance

Insulin is a hormone secreted by the pancreas in response to increased glucose levels in the blood. Insulin acts to increase the uptake of glucose by facilitating the conversion of glucose into glycogen for storage in the liver and into fat for storage in adipose tissue. The aforementioned metabolic alterations within muscle are believed to provide a link between obesity and insulin resistance (2).

Diabetes

Diabetes mellitus, commonly referred to as diabetes, is a group of metabolic diseases that are characterized by hyperglycemia as a result of aberrant insulin secretion, action, or both. There are several types of diabetes such as type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus. However, the majority of diabetes cases fall into the major categories of type 1 and type 2, both of which will be the primary focus moving forward (1, 4).

Type 1 diabetes mellitus is a disorder that is characterized by the destruction of pancreatic beta cells, resulting in absolute insulin deficiency. T1DM only accounts for approximately 5-10% of those affected with diabetes (4). The majority of the cases are due to an autoimmune-mediated destruction of beta cells (type 1a), whereas a small number of cases are the result of failure of

beta cells (type 1b) (4). In those considered obese, T1DM development could potentially be due to an imbalance between concentrations of adiponectin and leptin (5,6). Adiponectin and leptin are both hormones that are secreted by white adipose tissue (WAT). Adiponectin is an anti-inflammatory molecule that increases FA oxidation in the liver and skeletal muscle and decreases proportionally to fat mass. Leptin is a pro-inflammatory molecule that increases proportionally to fat mass. The proinflammatory activity of leptin increases the destruction of beta cells, accompanied by an increase in insulin resistance due to the decreased adiponectin concentration (5, 6).

Type 2 diabetes mellitus is a disorder that is characterized by insulin resistance and the inability of the body to produce enough insulin to overcome the resistant state (7). Approximately half of the individuals diagnosed with T2DM are obese. T2DM development in obese individuals is typically thought of as insulin resistance occurring first, followed by hyperinsulinemia, then hyperglycemia due to the failure of beta cells to produce enough insulin (8). With weight loss, insulin resistance may improve, however it is seldom restored to normal (4).

Nonalcoholic Fatty Liver Disease

The liver is a vital organ in the body that is responsible for metabolic processes such gluconeogenesis, glycogenolysis, and FA oxidation. As the obesity epidemic continues to grow, NAFLD grows in parallel as it is characterized by a $\geq$ 5% accumulation of fat in the liver. In 2016, NAFLD was estimated to have affected 30% of the US population and was projected to become the leading cause of end stage liver disease by 2020 (9). Insulin plays several roles in hepatocytes including increased lipogenesis. Elevated insulin levels and insulin resistance are then key players in the synthesis of NAFLD. High insulin levels will result in increased lipogenesis, and insulin resistance results in increased adipocyte lipolysis to release free fatty

acids. The fatty acids get esterified to triacylglycerol in the liver, thus resulting in the fat accumulation that characterizes NAFLD (9).

Lipid Overview

The proteins to be discussed later interact directly with lipids. As such, the following gives an overview of lipids and the pathologies related to lipids.

Lipids

There are several categories of lipids, the main ones being fatty acids, neutral lipids, phospholipids, steroids, and eicosanoids. While lipids are commonly thought of simply as storage molecules and energy sources, they can also act as signaling molecules and are integral parts of the cell membrane.

Free fatty acids are amphipathic carboxylic acids that get stored as neutral lipids, meaning they lack a charge due to the esterification of the carboxylic acid group to glycerol. A single FA esterified to glycerol forms monoacylglycerol (MAG), two FAs esterified to glycerol form diacylglycerol (DAG), and three FAs esterified to glycerol form triacylglycerol (TAG). TAGs act as the body's central energy source due to their reduced states and hydrophobic nature.

Lipid droplets

Lipid droplets (LD) are mostly found in adipose tissue but are present in nearly all tissue types. LDs consist of a hydrophobic core made up of triacylglycerols and sterol esters surrounded by a phospholipid monolayer. The monolayer is studded with several proteins including members of the perilipin family. In order to prevent lipotoxicity, free fatty acids are converted into neutral lipids and stored in lipid droplets [16].

Lipolysis

Lipolysis is the breakdown of triacylglycerols in the lipid droplet. The process of lipolysis consists of three steps. The first step is the breakdown of triacylglycerol to diacylglycerol via adipose triacylglycerol lipase (ATGL). ATGL binds to its cofactor, comparative gene identification-58 (CGI-58), in order to remove the fatty acid from TAG to result in DAG. Second, hormone sensitive lipase (HSL) removes the second fatty acid to convert diacylglycerol to monoacylglycerol. Lastly, monoacylgylcerol lipase (MGL) cleaves the remaining ester group, resulting in a free fatty acid and glycerol.

In order for lipolysis to begin, a hormone signal must come in the form of a catecholamine, such as epinephrine or norepinephrine, or glucagon. One of these hormones binds to a G-Protein Coupled Receptor (GPCR) which begins a cascade of events that ultimately activates protein kinase A (PKA). As a result of this activation, PKA is then able to activate HSL and perilipin 1 via phosphorylation. Once phosphorylated, HSL is translocated to the surface of the lipid droplet, and perilipin 1 undergoes structural changes that allow for the release of CGI-58 to then go and bind to its cofactor, ATGL, which is responsible for the conversion of TAG to DAG.

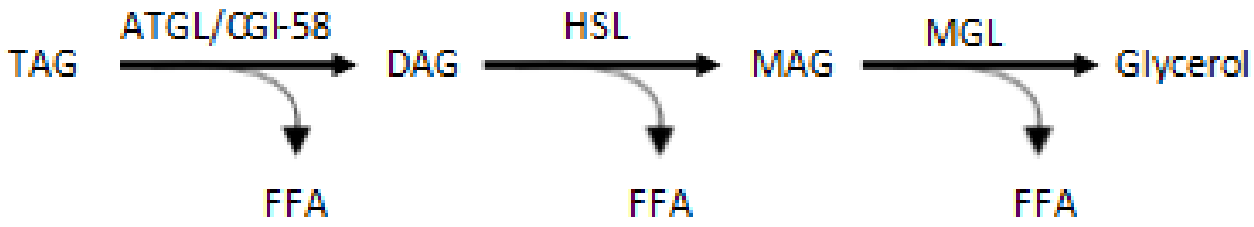

Figure 1: Mechanism of lipolysis. TAG is metabolized into FFAs and glycerol. Fatty acids are shuttled to muscle for beta-oxidation and liver for ketone body synthesis, while glycerol is shuttled to the liver for gluconeogenesis.

Lipotoxicity

Lipotoxicity is defined as the accumulation of lipids in tissues and organs where lipids are not normally found (10). It is estimated that approximately 25% of the adult US population is affected by lipotoxicity (10). When there are elevated FA levels in the body, insulin-secreting cells experience prolonged exposure to the high FA levels. As a result, insulin gene expression is reduced and apoptosis is induced. Exposure to lipids or FFAs have several negative effects, such as LD formation due to the cell's response to stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction. These negative effects lead to beta-cell damage and impaired insulin secretion (11).

PAT Family Proteins

The PAT family proteins are a family of lipid-droplet proteins that are related by their similarities in sequences and function within mammalian cells. The term, "PAT family," comes from the initial names of the first three identified members of the family: perilipin, adipocyte differentiation-related protein (ADRP), and tail-interacting protein of 47 kiloDaltons (TIP47). These members are now referred to as perilipin 1, perilipin 2, and perilipin 3, respectively (12).

Among the shared sequences within the family are the PAT domain and 11-mer repeat. The PAT domain is a highly conserved 100 amino acid domain located at the amino terminus found in each member of the family except perilipin 4 (12). The 11-mer repeat is shared throughout each member of the family and is proposed to be the site responsible for the ability of the proteins to bind lipid droplets.

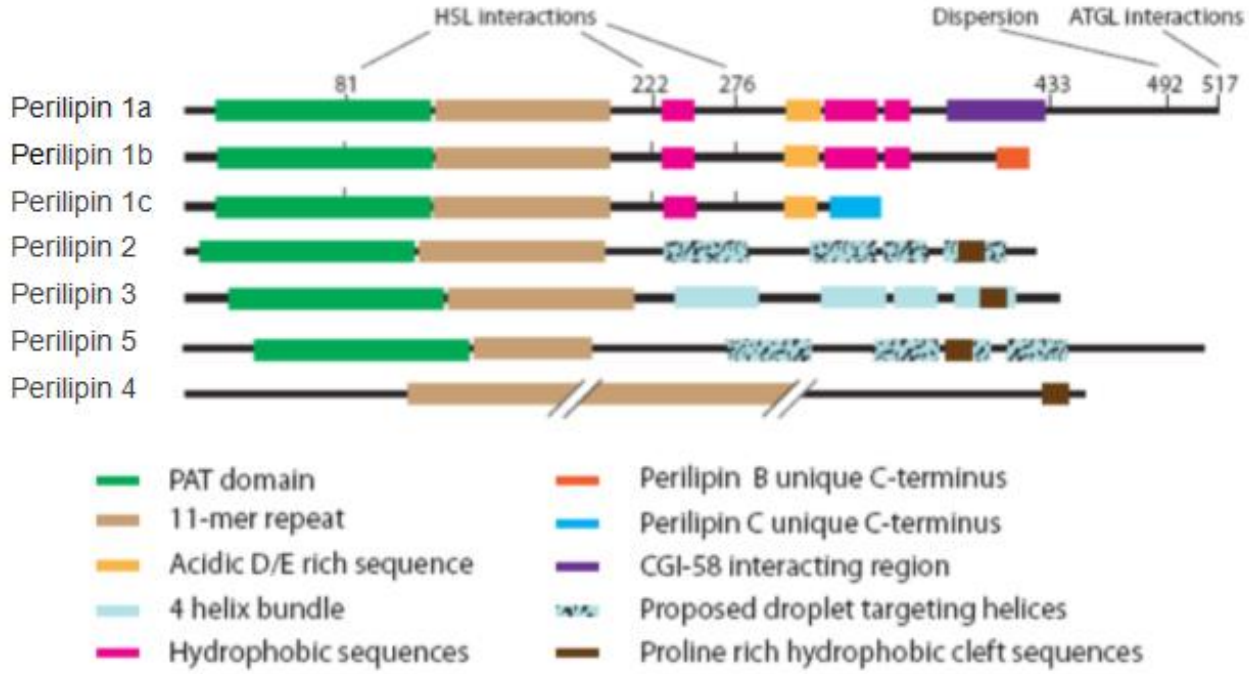

Figure 2: All members of the perilipin family, including known splice variants, adapted from Bickel et al 2009 (12).

Perilipin 1

Perilipin 1 was the first discovered and consequently most studied member of the PAT family. The highest concentrations of this protein are seen in white adipose tissue (WAT) and brown adipose tissue (BAT) (12).

Perilipin 1 is a major regulator of lipolysis in human adipocytes. On lipid droplets, perilipin 1 has a dual function of acting as a physical barrier to lipases under basal conditions and as a recruitment site for lipases during lipolysis (13). Two proteins that are known to interact with perilipin 1 are ATGL and CGI-58. ATGL is a TAG hydrolase that promotes the breakdown of stored fat and CGI-58 is its coactivator (14). Under basal conditions, CGI-58 is bound to perilipin 1 on the lipid droplet to prevent interaction with ATGL. PKA becomes activated once

cAMP levels are high, allowing for the phosphorylation of perilipin 1 and subsequent release of CGI-58. Newly released CGI-58 can then interact with ATGL, thus beginning the process of lipolysis (15).

Perilipin 2

Perilipin 2 is a 50 kDa protein that was first identified as a ribonucleic acid (RNA) transcript that was induced during differentiation of cultured adipocytes. Levels of perilipin 1 are highest when adipocytes are immature and lowest when adipocytes are mature. Immature adipocytes contain smaller lipid droplets coated by perilipin 2, whereas mature adipocytes contain larger lipid droplets coated by perilipin 1. If perilipin 2 is not bound to lipid droplet, it is quickly degraded (12).

Perilipins 1 and 2 have similar functions in the cell, however there are key differences between the two. Like perilipin 1, perilipin 2 limits the interactions of lipid droplets with lipases to promote neutral lipid accumulation. However, it does not regulate lipolysis to the same extent that perilipin 1 does. Also, perilipin 1 appears to outcompete perilipin 2 in the cell for binding of lipid droplets in adipocytes (12).

Perilipin 3

Perilipin 3 is a highly exchangeable protein that is involved in metabolic processes such as lipid storage, mobilization, and LD formation (13). Unlike perilipin 1, perilipin 3 is expressed in nearly all tissues (12), and unlike perilipin 2, perilipin 3 is stable and can freely exchange between the cytosol and lipid droplet based on the metabolic state of the cells (15). Perilipin 3 was first described as binding and mediating the transport of mannose-6-phosphate receptors throughout the cytosol, suggesting that the protein has other functions besides for mediating lipolysis of lipid droplets.

In order to gain more insight into how members of the PAT family may bind lipid droplets, a fragment of perilipin 3 was isolated and crystallized using X-ray crystallography. Results of the analysis revealed that the fragment has a four-helix bundle with a hydrophobic cleft, the overall structure resembling the shape of a boot. The four-helix bundle present is similar to the terminal half of apolipoprotein E (apoE) that is able to open and close for either the binding of lipoproteins or close to remain stable in solution. Such similarities between the two proteins suggest a mechanism for the reversible binding to lipoproteins and lipid droplets seen in the perilipins (12).

Perilipin 4

Perilipin 4 is expressed in white adipose tissue, skeletal muscle, and heart (12). Similar to perilipin 1, perilipin 4 was first identified as a protein induced during differentiation of adipocytes (16). It appears that the function of perilipin 4 is to associate with LDs in a hormone-substrate manner (16). In the absence of fatty acids, perilipin 4 is typically found in the cytosol. When fatty acids are present, similar to perilipin 3, perilipin 4 localizes to the lipid droplet to allow for storage of newly synthesized TAG (17, 18). This suggests that this protein is present in the formation of immature adipocytes.

Perilipin 5

Perilipin 5 is found within highly oxidative tissues such as brown adipose tissue, heart, skeletal muscle, and liver, and is a major regulator of lipolysis (12). It localizes to the surface of LDs and mitochondria, with pools also found in the cytosol and ER, although its functions at these sites are not yet known (19). Perilipin 5 has been shown to inhibit lipolysis through direct binding of ATGL and CGI-58 (20, 21) to protect the LD from lipases and subsequent TAG hydrolysis. It has also been observed that perilipin 5 has the ability to tether the mitochondria and cytosolic

lipid droplets (22), with the proposed function being the optimization of oxidative efficiency and regulation of excess FA (21).

Similar to perilipin 1, perilipin 5 is regulated by phosphorylation via PKA. Under basal conditions, perilipin 5 is bound to both ATGL and CGI-58 on the surface of the LD. Upon phosphorylation, perilipin 5 releases CGI-58, allowing it to interact with ATGL to begin the process of lipolysis.

Recently, fractions of perilipin 5 have been observed in an additional location in the cell: the nucleus. To better study these observations, a cell line that endogenously expresses perilipin 5, C2C12 skeletal muscle, was stimulated with isoproterenol (Iso) and 3-isobutyl-1-methylxanthine (IBMX) to activate the PKA pathway and to simulate prolonged fasting. Following stimulation, perilipin 5 protein expression not only increased, but nuclear localization of the protein was also induced by the 120-minute mark (23). After further study, it was determined that during catecholamine-stimulated lipolysis, perilipin 5 forms transcriptional complexes with PGC-1alpha and SIRT1 in the nucleus following phosphorylation by PKA. Such interactions identify perilipin 5 as a transcriptional co-regulator of the cellular lipid load and the oxidative capacity of the mitochondria (23). Additionally, it has been shown that nuclear translocation is specific to perilipin 5 and is not characteristic of the perilipin family as a whole.

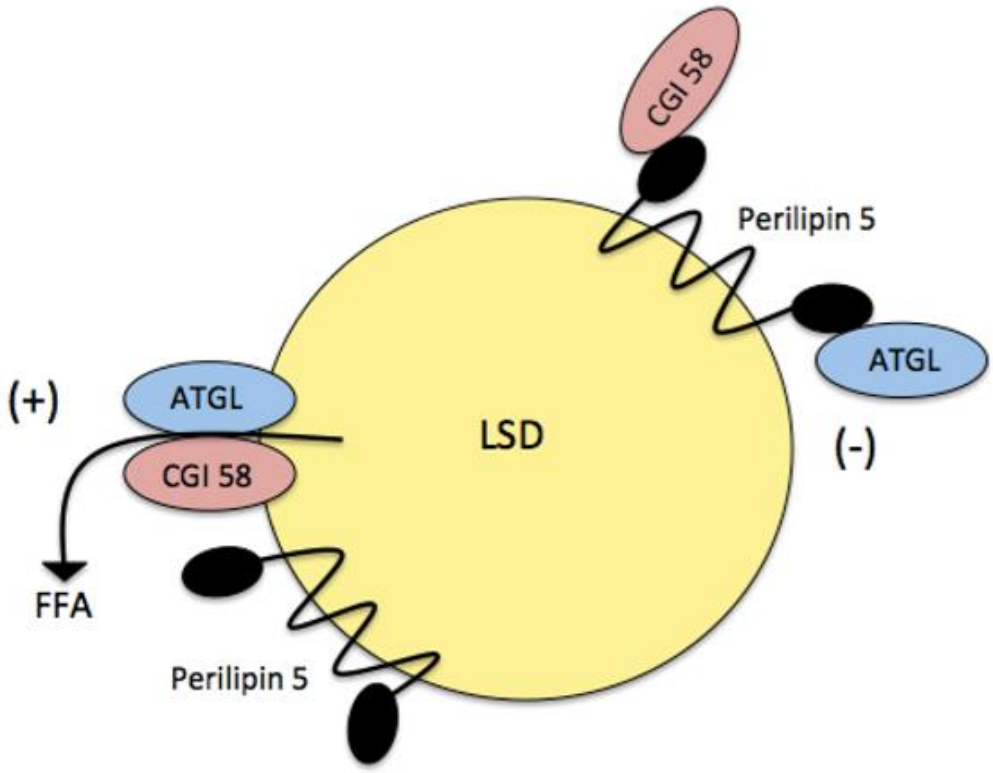

Figure 3: depiction of perilipin 5 under basal and stimulated conditions.

Relevant Biological Information

An important aspect of perilipin 5 is the intriguing ability to translocate to the nucleus. In order to discuss the possible functions of perilipin 5b, it is important to discuss all interactions with perilipin 5 requisite for its functions within the cell. The following will give an overview of the interaction partners of nuclear perilipin 5.

Protein kinase A

Protein kinase A PKA is a cyclic adenosine monophosphate dependent protein responsible for the activation of several pathways, including gluconeogenesis, lipolysis, and glycogenolysis. The structure of PKA consists of two regulatory subunits that allow for the binding of cAMP and two

catalytic subunits that dissociate following the binding of cAMP to go and phosphorylate the target protein(s). Activation of the PKA pathway typically begins with the binding of a ligand to a g-protein coupled receptor (GPCR).

Calmodulin-dependent protein kinase II

Calmodulin-dependent protein kinase II (CaMKII) is a crucial regulator of cardiac physiology and pathology. CaMKII is regulated by the Ca^{2+}/calmodulin complex. Upon elevated levels of intracellular Ca^{2+}, calmodulin is bound by Ca^{2+} to form a complex, of which binds the regulatory site of CaMKII. This binding of the complex to the regulatory site of CaMKII results in activation of the kinase. Along with PKA, CaMKII is one of the most abundant kinases in the heart (24). Due to the presence of perilipin 5 in heart tissue, CaMKII could also be an important regulator of perilipin 5.

Protein kinase C

Like PKA and CaMKII, protein kinase C (PKC) is a serine/threonine kinase responsible for several metabolic processes throughout the body. PKC is a several membered family containing 3 subfamilies that are categorized based on their dependence on cofactors. Conventional PKC isozymes are activated by DAG and Ca^{2+}, novel PKC isozymes are activated by DAG alone, and atypical PKC is regulated by protein: protein interactions (25). PKC is also commonly known as an oncoprotein due to it being a receptor for tumor promoting phorbol esters.

Nuclear pore complex

The nuclear pore complex (NPC) punctures the nuclear envelope and acts as a gate for molecular exchange between the cytoplasm and nucleus. In fact, molecular transport between the cytoplasm and nucleus occurs predominantly through the NPC. It is positioned in circular openings in the nuclear envelope where the inner and outer membranes of the envelope are fused. As visualized

by electron microscopy, the core structure of the NPC appears as three porous ring densities named according to their locations: cytoplasmic, inner, and nucleoplasmic. The nucleoplasmic side contains eight flexible rod-shaped extensions that connect at a distal ring to form the nuclear basket. There are also eight flexible extensions coming from the cytoplasmic ring. A small number of transmembrane nucleoporins allow for the anchoring of the complex to the nuclear envelope (26).

Nuclear transport mechanisms

Unlike many channels and transporters found in cells, the NPC does not have gates that open and close to allow for transport. Instead, it has a passive barrier, called the diffusion barrier, that allows select cargoes up to 40nm to move through. The diffusion barrier is made up of phenylalanine-glycine (FG) repeats in the central transport channel. While these FGs do not permit the passive diffusion of macromolecules greater than 40kDa, it allows the active transport or large cargoes.

To allow for the transport of large cargoes to the nucleus, there exist carrier proteins called karyopherins. These proteins bind to the FGs found in the diffusion barrier and recognize specific molecules, thus allowing for the shuttle of macromolecules into the nuclear envelope. Karyopherins specialize in either import of cargo or export of cargo, however some can perform both functions.

For karyopherin-mediated transport, the release of cargo on the appropriate side of the NPC is dependent on a gradient of proteins called Ran. Ran is a small GTPase protein that binds and hydrolyzes GTP to GDP. When a karyopherin shuttles cargo from cytoplasm to nucleus, it interacts with RanGTP to allow for the release of cargoes into the nucleus. Such a mechanism could be requisite for the transport of perilipin 5 into the nucleus (27, 28).

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear receptors and transcription factors (28). PPARs play a role in several metabolic function such as regulation of energy balance, glucose homeostasis, TAG and lipoprotein metabolism, FA synthesis, storage and export, and more. The family consists of 3 isotypes, alpha, beta/sigma, and gamma. The subtypes have conserved genes throughout, however the tissue distribution differs for each (28).

The peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) family includes multiple nuclear or non-nuclear receptors that control cell metabolism (29). One of those members is PGC-1α. PGC-1α was the first discovered member of the family and was identified in BAT in mouse studies of cold-induced thermogenesis (30). PGC-1α can be used in conjunction with different coactivators to induce changes in lipid oxidation, energy homeostasis, mitochondrial mass, and insulin sensitivity (29). Since it is a transcription factor, PGC-1α can bind to isotypes of PPARs to coordinate expression of mitochondrial genes to contribute to FA transport and utilization (31). PGC-1α also plays a role in oxidative phosphorylation by regulating the expression of nuclear and mitochondrial genes requisite for components of said phosphorylation (30).

The aim of this thesis is to uncover some of the functions and interactions of perilipin 5b, the splice variant of perilipin 5. Currently there is a lack of literature for the existence and/or function of the splice variant, and this thesis aims to fill the gaps in knowledge that the current lack of literature has provided. We hypothesize that, similar to the known splice variants of perilipin 1, perilipin 5b has altered functions within the cell.

METHODS

Sterile tissue culture

Sterile Tissue Culture CHO K1 cells were maintained in Ham's F-12 Nutrient Mix media (Invitrogen) containing 10% Cosmic Calf Serum (HyClone), 1% amphotericin B, 1% L-glutamine, and 1% penicillin streptomycin at 37ºC and 5% CO_2. Transfected CHO cells were maintained in the same media mix but supplemented with Geneticin®, G-418, for selection. Control cells were treated for 24 hours with fresh media.

Preparation of PLIN5b cDNA

Full length PLIN5 was obtained and cut with restriction enzymes XbaI and BglII and recircularized using DNA ligase. This was done to create a vector containing the shortened version of perilipin 5, perilipin 5b. Resulting DNA samples were electrophoresed along with a 1kB size marker through a 0.6% agarose gel to ensure the previous cutting and ligation were successful.

Transformation

5-alpha Competent *E. coli* cells were obtained from New England Biolab's High Efficiency Transformation Kit and thawed on ice for 10 minutes. 2μL of PLIN5b DNA was added to the cells and subsequently combined with SOC media. Two previously poured LB AMP plates were obtained and warmed to 37ºC. 10 μL of mixture was spread on plate 1 and 100 μL of mixture spread on plate 2. Plates were incubated overnight at 37ºC.

DNA isolation

Colonies were picked from transformation plates and grown in LB AMP media overnight. 1.5mL of the resulting culture was centrifuged at 6,500 rpm for 10 minutes to separate the DNA from the liquid culture. DNA was isolated from the pellet using Invitrogen's PureLink™ HiPure Plasmid Miniprep Kit. DNA was eluted with 50µL elution buffer and stored at -20 ºC until further use.

Transfection

To obtain CHO cells expressing the perilipin 5b vector, cells were transfected with the isolated DNA using the Lipofectamine® 2000 reagent and protocol (Invitrogen), using the 12µL concentration of lipofectamine for transfection. CHO K1 cells were split into a sterile six well plate and grown to approximately 70% confluency. Cells were transfected with the perilipin 5b DNA with the 3X-FLAG expression vector and selection was performed using 300µg/mL G418. Transfections were incubated for a 48-hour period at 37ºC and 5% CO_2 before using for experimentation.

Cellular lysate

Cellular lysate was prepared from transfected CHO cells grown on sterile plates to approximately 80% confluency. Media was removed, cells were washed with 1mL of 1x PBS, then the bottom of the plates were scraped with a spatula in order to remove the cells from the bottom of the plate and form a heterogeneous solution with the PBS. PBS/cell solution was transferred to an Eppendorf tube and centrifuged at 3000 rpm for 5 minutes. Supernatant was poured out following the centrifugation and 1mL of 10mM TRIS was added to lyse the cells. In order to ensure lysing occurred, the samples were sonicated 3x for 5 seconds each. Following

sonication, samples were centrifuged at 3000 rpm for 5 min and the resulting supernatant and pellet were separated into different Eppendorf tubes. The tube containing the pellet sample was brought back up in 800μL of 10mM TRIS to obtain approximately the same volume as the samples containing the supernatant. Samples were stored at -20 ºC until ready for use.

Immunoblots

Protein samples were prepared by adding Laemmli Sample Buffer and beta-mercaptoethanol and boiled to denature secondary structure before loading onto precast 10% Bis-Tris gels. The gel was electrophoresed for 1 hour at 160 V with 1X MOPS SDS-Page running buffer. 2μL of LI-COR fluorescent molecular marker 700 CW was loaded for size determination. Following electrophoresis, the separated protein was transferred to a nitrocellulose membrane for 16 hours at 12 V.

Following transfer, the membrane was removed and dried on the bench for at least an hour then rewet with 1X PBS to ensure maximal protein binding. Next, the membrane was blocked with 1% FAF BSA blocker (FAF BSA in PBS) for an hour with rocking at room temperature to bind any nonspecific recognition sites. The blot was incubated in primary antibody solution with block overnight at 4º C. The common primary antibody used was anti-FLAG (SIGMA) at a 1:5,000 concentration of antibody to blocker.

The next day, the membrane was washed three times in 1X PBS for five-minute intervals at room temperature with rocking. Following washing, the blot was incubated in secondary antibody for 55 minutes at room temperature while rocking. This step along with the following steps were light sensitive and thus performed in the dark. Secondary antibody was used at a 1:2000 concentration. For the FLAG primary antibody, a secondary of goat anti-mouse 800 CW

(LI-COR) was used. Three final washes of 1X PBS for 5 minutes at room temperature were done to remove nonspecific antibody following incubation. The blot was then detected at the 800, 600, and 700 nm channels for 10 minutes using a LI-COR Odyssey Fc imaging system.

Immunofluorescence

CHO cells expressing the perilipin 5b protein were plated on coverslips in a six-well tissue culture dish. Cells were washed with PBS three times then fixed in 3% paraformaldehyde for 50 minutes. Cells were blocked with 1x PBS, 5% Goat Serum, 0.1% Triton x-100 and glycine for 1 hour at room temperature. Coverslips were incubated with primary antibody 0.2% FLAG anti-mouse, in 1x PBS, 5% Goat Serum, 0.1% Triton X-100, overnight in a humidified chamber at 4ºC. The next day the coverslips were washed three times with 1 x PBS and Triton-X100 and incubated with secondary antibodies in antibody dilution buffer. Excess antibody was then removed by blotting on a Kimwipe, and coverslips were mounted with Prolong Gold anti-fade reagent with DAPI (Invitrogen). Coverslips were imaged immediately using Olympus B201 BX40 microscope.

RESULTS AND DISCUSSION

To ensure that the plin5b DNA was correctly synthesized, two samples of the DNA were cut with restriction enzyme PstI. Two samples were used to ensure that there was continuity throughout the synthesized DNA. If the two samples resulted in different band patterns, that would signify that the synthesizing and subsequent cloning was not successful. Based on the plasmid map of plin5b, cutting with PSTI should result in band sizes of approximately 4.5kb, 1.4kb, .9kb, and .4kb. Imaging following the electrophoresis of the digested samples showed the same banding pattern between both samples with band sizes of approximately 4.5kb, 1.4kb, .9kb, and .4kb. The resulting gel as seen in Figure 4 shows that the synthesis of the DNA was successful and ready for transfection into CHO cells.

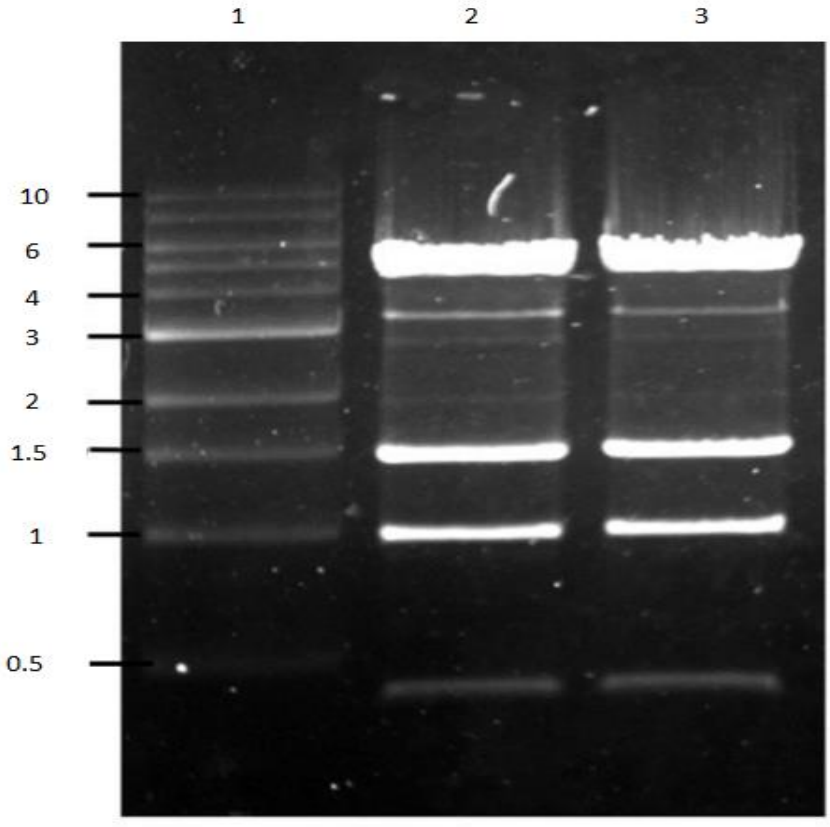

Figure 4. After the plin5b DNA was synthesized, two samples of the DNA were cut with restriction enzyme, PSTI. Lane 1 corresponds to the molecular weight marker (1kb), lanes 2 and 3 correspond to two different samples of prepared plin5b DNA.

bind to mitochondria, as well as a partial loss of ability to bind lipids, ATGL, and CGI-58. Such losses suggest that perilipin 5b may serve a different function in the cell than perilipin 5 does, specifically with regards to catecholamine stimulated lipolysis. However, with the existence of the 11-mer repeats, perilipin 5b appears to retain the ability to bind lipid droplets, as the 11-mer repeats are proposed to be responsible for the ability of perilipins to bind lipid droplets (12).

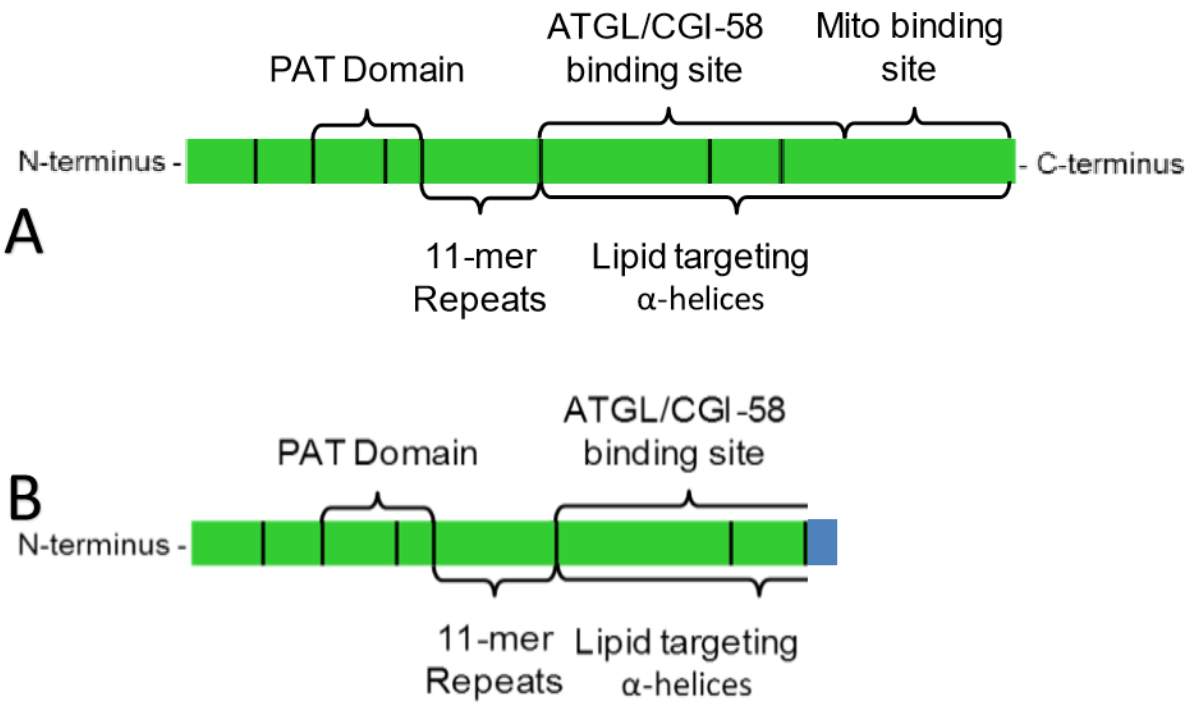

Figure 5. The proposed differences in exons and subsequent binding site availability of perilipin 5b as compared to perilipin 5. Panel A shows perilipin 5, panel B shows perilipin 5b.

Further analysis of the sequence of perilipin 5b as compared to perilipin 5 provided information that suggests perilipin 5b may still be phosphorylated by PKA like perilipin 5. A known

phosphorylation site in perilipin 5 is serine 155, shown in Figure 6. According to sequence analysis, perilipin 5b would retain this site. This suggests that, although previous analyzation shows that perilipin 5b may not bind ATGL and CGI-58 to regulate lipolysis, it still may be a key player in catacholamine stimulated lipolysis. Additionally, studies have shown that upon phosphorylation, perilipin 5 translocates to the nucleus to further regulate lipolysis. Retention of serine 155 in perilipin 5b provides the possibility that, upon stimulation, perilipin 5b could also be translocated to the nucleus. However, to deduce this, more research on the mechanism for how perilipin 5 is translocated to the nucleus would need to be done in order to infer that the same process could or could not occur with its splice variant, perilipin 5b.

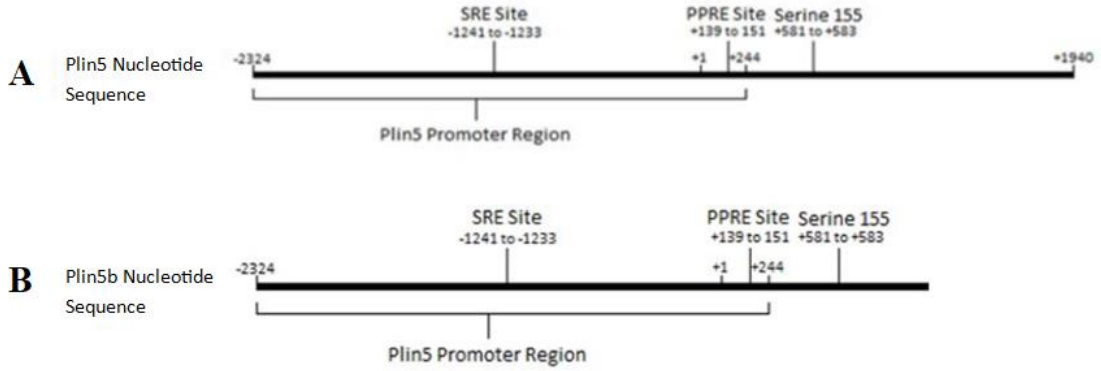

*Figure 6. Comparing the nucleotide sequences of perilipin 5 (**A**) and perilipin 5b (**B**). Perilipin 5b appears to have retained a known phosphorylation site in perilipin 5, serine 155.*

In order to determine localization of perilipin 5b, two samples of a CHO cell line expressing both perilipin 5 and perilipin 5b were separated into supernatant and pellet fractions. The prepared cellular fractions were separated into nuclear and supernatant fractions due to the cytosol and cellular organelles remaining in the supernatant following centrifugation and all nuclear contents forming a pellet. Under basal conditions, perilipin 5 can be found on the surface of LDs, mitochondria, as well as in pools within the cytosol and ER (19). Following catacholamine stimulation, perilipin 5 can also be found in the nucleus (23). The immunoblot, shown in Figure 7, showed presence of the protein in the supernatant fractions and not in the pellet fractions, suggesting that under basal conditions perilipin 5b is not found in the nucleus.

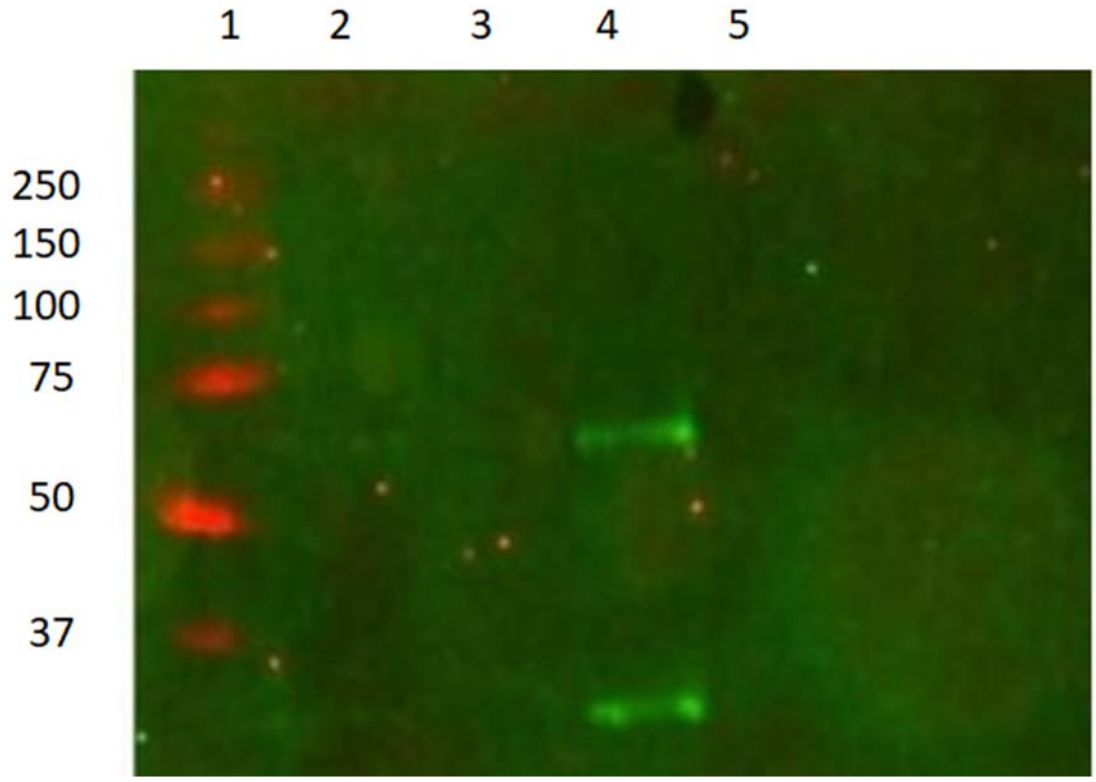

Figure 7. A cell line expressing both perilipin 5 and perilipin 5b was separated into pellet and supernatant fractions, electrophoresed, and transferred to an immunoblot to test for presence of perilipin 5b in the nucleus under basal conditions. Two samples of each fraction were electrophoresed. Lane 1 corresponds to the molecular weight marker in kDa, lanes 2 and 3 correspond to sample 1 supernatant and nuclear fractions respectively, and lanes 4 and 5 correspond to sample 2 supernatant and nuclear

In order to further elucidate the location of perilipin 5b, fluorescent microscopy was performed on perilipin 5b against a cell line of perilipin 5 acting as a control. As previously discussed, perilipin 5b is expected to be found in the cytosol under basal conditions like its parent protein, perilipin 5. Western blotting as shown in Figure 7 also suggested this hypothesis. However, the fluorescent microscopy shown in Figure 8 shows perilipin 5b all throughout the cell, not just in the cytosol. Although this finding was not seen in the previously shown western blot, it could be due to a low concentration of protein existing within the nuclear sample fraction, thus no banding was seen in the resulting blot. The microscopy also shows altered morphology of the nucleolus as compared to perilipin 5. Further investigation would be needed to determine the cause of the observed alteration to the morphology of the nucleolus.

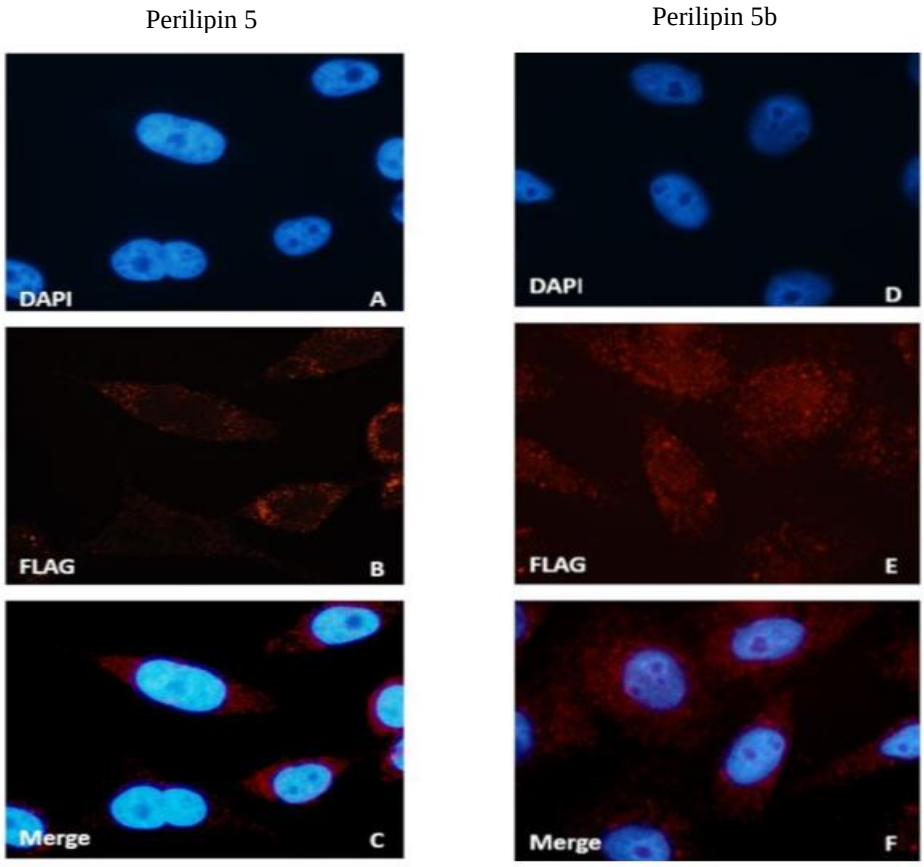

Figure 8. Two CHO cell lines separately expressing perilipin 5 and perilipin 5b were stained with fluorescent antibody FLAG while DAPI was used to visualize the nuclei. Panels A-C correspond to perilipin 5, panels D-F correspond to perilipin 5b.

CONCLUSION AND FUTURE WORK

There is still much to be discovered about the splice variant of perilipin 5, perilipin 5b. This thesis probes into only the beginning of what is to be discovered about the protein. However, the data of this thesis supports the hypothesis that perilipin 5b has altered functions within the cell as compared to perilipin 5. This can be seen in the sequencing data that shows the loss of binding sites in perlipin 5b vital for perilipin 5's function, as well as the fluroescent microscopy showing perilipin 5b localized throughout the cell as opposed to only the cytosol, the known localization of perilipin 5. Although the data suggests the altered functions within the cell, more work needs to be done in order to effectively conclude such an observation.

The mitochondria binding site has been proposed to have been lost completely along with partial loss of the ATGL/CGI-58 binding site in perilipin 5b. To test the proposed loss of the mitochondria binding site, fluroescent microscopy should be performed on a cell line expressing perilipin 5b with the use of an antibody that fluoresces green and mitotracker, which fluoresces red. Absence of green fluorescence within the mitochondria would suggest that perilipin 5b has indeed lost its binding ability. To test the partial loss of the ATGL/CGI-58 binding site, ATGL and CGI-58 antibodies should be used against a western blot. Loss of this site would signifiy that perilipin 5b does not aid in catecholamine stimulated lipolysis, again supporting the hypothesis of altered function within the cell. Such results could have great implications into the study of metabolic diseases and the function of lipid droplet proteins in relation to these diseases.

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APPENDIX

AMP	ampicillin
ATGL	adipose triglycerol lipase
apoE	apolipoprotein E
BAT	brown adipose tissue
BMI	body mass index
BSA	bovine serum albumin
CaMKII	calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CGI-58	comparative gene identification-58
CHO	Chinese hamster ovary
CW	center wavelength
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ETC	electron transport chain
FA	fatty acid
FFA	free fatty acid
FG	phenylalanine-glycine
GDM	gestational diabetes mellitus
GDP	guanine diphosphate
GPCR	g-protein coupled receptor
GTP	guanine triphosphate
IBMX	3-isobutyl-1-methylxanthine
kDa	kiloDaltons
LB	lysogeny broth
LSD	lipid storage droplet
MAGL	monoacylglycerol lipase
MGL	monoacylglycerol
MOPS	3-(N-morpholino) propanesulfonic acid
NAFLD	nonalcoholic fatty liver disease
NPC	nuclear pore complex
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC-1	peroxisome proliferator-activated gamma coactivator
PGC-1α	peroxisome proliferator-activated gamma coactivator alpha
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor

RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SIRT1	sirtuin 1
SOC	super optimal broth with catabolite repression
TAG	triacylglycerol
TG	triglycerol
TRIS	Tris Amino methane
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
WAT	white adipose tissue