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# Insights into the cellular trafficking of perilipin 5

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# ABSTRACT

Perilipins are a family of five proteins found on the surface of lipid storage droplets in nearly all tissues. These proteins act as cofactors for lipases and scaffolding for other proteins involved in lipid metabolism. In addition to the lipid droplet surface, members of the perilipin family have been found in the cytosol, endoplasmic reticulum, plasma membrane and mitochondria. The localization of these proteins is in part due to the phosphorylation state of the perilipin in question. Many other biological processes occur through kinase pathways, which have numerous cellular outcomes. Recently, perilipin 5 has been shown to localize to the nucleus when phosphorylated by PKA on serine 155. In the nucleus, it interacts with the peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1α) to regulate gene expression. In silico analysis of a phosphomimetic mutant (Ser155Asp) indicates that this site is part of a potential nuclear localization sequence. Using a CHO model cell line, we observed that translocation to the nucleus could be stimulated by treatment with forskolin and isobutylmethylxanthine (IBMX) and occurred independently of oleic acid treatment to promote triacylglycerol deposition. This thesis aims to explore the nuclear transport mechanism of perilipin 5 through investigating other phosphorylation pathways and nuclear recognition motifs that affects the subcellular localization. These data have the potential to gain insights to the regulation of perilipin 5 trafficking into the nucleus, interactions with other proteins such as PGC-1α and regulation of processes such as mitochondrial biogenesis.

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# INTRODUCTION

Lipid related pathologies have become increasingly relevant in modern civilization as technological advances have led to more sedentary lifestyles and western diets. The alarming rate at which these metabolic diseases such as obesity, diabetes, and non-alcoholic fatty liver disease, are gaining prevalence leads to a need to learn more about the underlying mechanisms that lead to disease states. A greater understanding of the underlying mechanisms can lead to effective treatment options and prevention methods to improve quality of life. This introduction starts with an overview of metabolic diseases, moves into a description of some of the lipids involved in this process, and is followed by an overview of the perilipins specifically focusing on perilipin 5 and ends with the pathways and cellular processes that are relevant to perilipin 5 function.

## Section 1 Metabolic Disease

With metabolic disease on the rise, it is important to have a greater understanding of the different diseases as a whole. This section focuses on lipid related pathologies and diseases that alter metabolic states.

## Section 1.1 Obesity

Obesity is a rising epidemic in modern cultures. In 2015, around 70% of the United States (US) population was reported to be obese or overweight, it is projected that in the year 2030 an even higher percentage of the US population will fall into this category (1). Obesity is a known risk factor for cardiovascular disease morbidity and mortality, along with type 2 diabetes, nonalcoholic fatty liver disease and other metabolic disorders.

Leptin and insulin are two key hormones that play important roles in the regulation of body weight. Leptin is an anorexigenic hormone synthesized in adipose tissue that regulates caloric intake. The

hormone is capable of crossing the blood-brain barrier to down-regulate appetite stimulating neuropeptides (1). In obesity, the blood concentration of leptin is elevated which promotes leptin resistance, a decreased response of tissues to leptin (1), which leads to continued appetite even after a high caloric intake.

Insulin is a pancreatic hormone which is critical for glucose homeostasis in the body. After a meal, in the fed state, insulin levels rise to keep blood glucose in check. Excess glucose is converted to glycogen in the liver and fat in adipose tissue. Glucose is imported in cells for conversion to glycogen through the major glucose transporter GLUT4, which facilitates diffusion of glucose through the plasma membrane. Metabolic alterations within muscle, such as cellular location changes in fatty acid transport proteins, defects in mitochondrial morphology, and enzyme activity are believed to play a role in linking obesity with insulin resistance (2).

Resistance of these two hormones leads to elevated circulating free fatty acid levels (FFA) in obese individuals in comparison to healthy people. There is also evidence of accumulation of intramuscular lipid which increases the mitochondrial burden of lipid and possibly leads to disconnection between standard metabolic pathways (3).

## Section 1.2 Diabetes

Diabetes mellitus, more commonly referred to as diabetes, is a condition tied to elevated levels of glucose in the bloodstream due to defective insulin signaling pathways. Type 1 and type 2 diabetes are the most commonly discussed forms of the disease, but less common types do occur. Monogenic diabetes is due to genetic mutation rather than environmental factors, and secondary diabetes is due to other health problems. Causes of secondary diabetes can range from hormonal disturbances, pancreas diseases, or a side effect from pharmaceuticals such as corticosteroids. Gestational diabetes, elevated blood glucose levels during pregnancy is also gaining prevalence. In 2017 it was reported that 46 million people in the North American continent and Caribbean have diabetes (4). This number is estimated to increase by 35%

by 2045 to 62 million individuals (4). The largest predicted increase is in Africa, which is looking at a 156% rise in the number of people effected by diabetes (4).

Type 1 diabetes is an autoimmune disease that leads to the destruction of pancreatic islet β-cells which produce insulin. The destruction of these cells leads to extremely low or non-existent insulin levels in the body. The hormone insulin regulates metabolism of carbohydrates, fats, and proteins by promoting glucose absorption into tissue from the bloodstream. Without insulin, glucose is trapped in the bloodstream and not absorbed into cells, leading to hyperglycemia and a lack of energy available to cells which can cause tissue damage if left untreated. Daily insulin treatments can allow for individuals with type 1 diabetes to live a relatively healthy life. Maintaining a healthy diet and proper glucose monitoring can assist in avoiding complications associated with the disease such as glaucoma, hypertension, heart, kidney and cardiovascular disease, and limb amputation.

Type 2 diabetes mellitus makes up approximately 90% of all diabetes cases, making it the most common form of diabetes (4). This high prevalence of the disease is likely linked to rising obesity levels, increased sedentary lifestyle and poor diet, which has led to the disease being increasingly seen in children, adolescents and young adults instead of being isolated to adult populations. The disease is characterized by inadequate insulin production and insulin resistance. Insulin resistance is a state in which cells do not respond fully to insulin even though pancreatic islet β-cells are still functional and producing the hormone. This resistance takes time to build until it leads to noticeable side effects in some individuals, prolonging time to diagnosis and treatment. Therefore, monitoring of risk factors in individuals, especially those with a familial history, is valuable in prevention of the disease. Body weight, physical activity, and proper nutrition are the most easily managed and controlled for in at risk populations and can lead to prevention of type 2 diabetes. Along with environmental factors, genetic factors can lead to onset and progression of the disease.

Insulin resistance can arise due to excess of circulating lipid and fatty acids, which can cause lipotoxicity. Excess lipid accumulation into visceral adipose tissue around vital organs, such as heart,

liver, pancreas, or skeletal muscle, leads to tissue damage and islet β-cell function decreasing (5). Reduced glucose transport is believed to be due to the alterations fatty acids can have on GLUT4 trafficking, budding, fusion, and activity (6). GLUT4 is an insulin regulated glucose transporter which allows the diffusion of glucose from the bloodstream into cells. Interference with GLUT4 makes decreases insulin's ability to stimulate glucose uptake or inhibit lipolysis. The overall effect is prolonged hyperglycemia and continual release of FFA in the fed state.

## Section 1.3 Nonalcoholic Fatty Liver Disease

The liver is a highly important organ in the regulation of energy homeostasis, playing a critical role in management of glycolysis, gluconeogenesis and lipid transport (7) Due to the obesity epidemic, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in developed countries (7). The prevalence of NAFLD is greater than expected and approaches 33% in some countries. Epidemiological data shows that most societies have approximately 30% of their population affected by the disease (8). NAFLD is a metabolic disorder that is predominately asymptomatic until further progression of the disease, and the gold standard for diagnosis is liver biopsy. The diagnosis of the disease is based on ≧5% of hepatic fat accumulation. It is assumed that obesity and insulin resistance play strong roles in NAFLD since the disease is an accumulation of fat in liver cells. Prevalence of NAFLD among obese populations ranges from 30-37% and more than three-quarters of diabetic patients reportedly have the disease (8). Metabolic diseases such as obesity and diabetes alter lipolysis leading to chronic accumulation of fat. Instead of fat being broken down and used for other cellular pathways, the FFA is re-esterified into TAG leading to an increase in hepatic TAG. This increase in hepatic TAG leads to the characterization of NAFLD. A portion of individuals affected by NAFLD will have further disease progression leading to nonalcoholic steatohepatitis (NASH) and even liver failure if left untreated.

## Section 1.4 Metabolic Syndrome

Metabolic syndrome, or Syndrome X, is a group of interrelated metabolic traits that are linked to individuals developing diseases such as cardiovascular disease and type 2 diabetes. Metabolic traits such as truncal obesity, diabetes, hypertension, hypertriglyceridemia, and hyperinsulinemia are all linked to the syndrome. To meet the criteria, three of five factors are required: abdominal obesity, elevated triglycerides, reduced HDL cholesterol, elevated blood pressure, and impaired fasting glucose (9). The prevalence of metabolic syndrome has increased with the growing obesity epidemic.

## Section 2.0 An Overview of Lipids

The pathologies and proteins that are going to be discussed deal with regulation of intracellular lipid levels and lipid metabolism. This section gives a brief description overview of lipid information and a brief overview of lipid metabolism.

## Section 2.1 Lipids

The main categories of lipids are fatty acids, neutral lipids, steroids, bile salts, and eicosanoids. The different types of lipids are related to one another by their hydrophobic properties, and are essential for numerous aspects of life. While fatty acids and neutral lipids are known for being forms of energy storage, steroids act as signaling molecules, and various other lipids are vital components of cellular membranes.

Free fatty acids (FFA) contain carboxylic acids which can be harmful to cells because the charged group can act as a detergent and affect intracellular pH, which is vital to maintain. To prevent breakdown due to the negative effect of the carboxylic acid, the group is esterified to glycerol or cholesterol to neutralize charge and store the FFA as neutral lipid. When a single FFA is tied to glycerol, it is termed monoacylglycerol (MAG), two FFA is diacylglycerol (DAG), and three fatty acids is triacylglycerol (TAG). In the cell most FFAs are typically stored as the neutral lipid TAG. Large accumulations of TAG

are readily found in adipose tissue, but all tissue types do contain the neutral lipid in some capacity in lipid droplets.

## Section 2.2 Lipid Droplets

Lipid droplets (LDs) are dynamic cytoplasmic organelles that are found predominately in adipose tissue but are also present in almost all tissue types. Size and number of LDs vary between cell types or even individual cells (10). The overall physical structure of LDs is a protein-studded phospholipid monolayer that surrounds the hydrophobic core of neutral lipids and triacylglycerols and sterol esters, (11). Cellular functions of LDs are storage of neutral lipids, membrane synthesis, viral replication, and protein degradation (10). Originally thought to be inert lipid globules and irrelevant to cell function, the organelle did not gain interest until the characterization of perilipin 1 (previously referred to as perilipin A), the first member of the PAT family of proteins (12).

## Section 2.3 Lipolysis

Lipolysis is the hydrolysis of triacylglycerols (TAG) which are stored within the cell. Released fatty acids, monoacylglycerols (MAG) and diacylglycerols (DAG) serve as substrates for other cellular pathways and processes. The entirety of lipolysis is a three-step process in which hydrolytic cleavage of TAG produces non-esterified fatty acids or free fatty acids (FFA). The release of the FFA into circulation controls the plasma level of FFA in the body. Lipolysis is dependent upon lipases being able to access the three enzymes that have been found to be vital in completing TAG hydrolysis. Adipose triglyceride lipase (ATGL) performed the first step in hydrolyzing TAG to DAG, releasing a FFA in the rate limiting step in the reaction. Hormone sensitive lipase (HSL) hydrolyzes DAG to produce MAG and a FFA, and finally monoglyceride lipase (MGL) cleaves MAG into glycerol and a FFA. This process is depicted in Figure 1. Lipolysis is regulated by varying pathways and interaction partners to regulate fat deposition and downstream metabolic pathways (13).

The most studied and crucial activator of ATGL is Comparative Gene Identification 58 (CGI-58) also known as ABDH5 (alpha/beta-hydrolase domain-containing protein 5). CGI-58 is unique due to the fact that it lacks lipase activity itself but does regulate ATGL activity. In unstimulated conditions, basal cAMP levels, CGI-58 is tightly bound to perilipin 1 and is unable to activate ATGL promoting lipid storage. Following hormonal stimulation, cAMP levels lead to stimulation of the PKA pathway phosphorylating perilipin 1 to allow for CGI-58 dissociation and TAG hydrolysis (14, 15).

Fatty acid binding protein-4 (FABP4) and lipotransin have been found to interact with HSL to regulate the second step of lipolysis. FABP4 is specifically a molecular chaperone which allows for FFA to be shuttled into and out of the cell for metabolism (14). The second interaction partner of HSL, lipotransin, is proposed to dock at the surface of HSL and therefore affect lipolysis after translocating (14). Lipotransin is a known member of the kantin family, which are enzymes that are critical for the remodeling of microtubule structures in cell division, motility, morphogenesis and signaling. While the family, lipotransin does not play an obvious role in lipid metabolism. Yet the ATPase activity of lipotransin appears to be critical in regulating HSL (14).

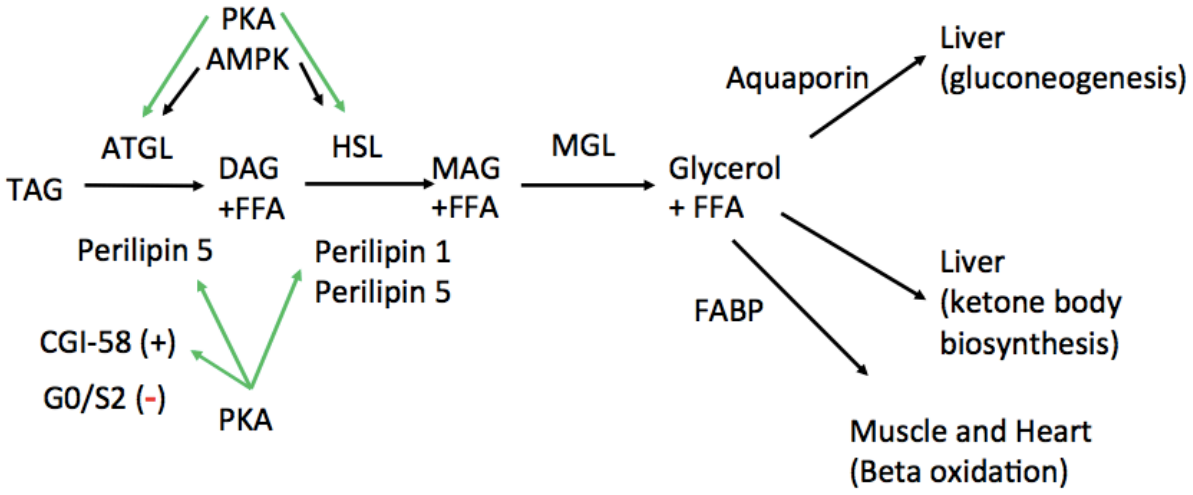



*Figure 1. Overview of Lipolysis. TAG is broken down in three steps to produce free fatty acids and a free glycerol. Fatty acids can be further processed into ketone body biosynthesis in the liver of beta oxidation in the muscle/heart. The glycerol is transported to the liver for gluconeogenesis.*

A major regulator of lipolysis is hormonal response, the best described hormone regulators being insulin and catecholamines. The release of lipolytic hormones is triggered due to altered levels of cyclic-adenosine monophosphate (cAMP) which acts as a second messenger to stimulate pathways that directly regulate lipid metabolism. Catecholamines bind to β-adrenergic receptors located on the cellular surface, most commonly discussed in adipocytes (15). Binding of the small molecule initiates G-protein-mediated signaling cascades which activate adenylate cyclase, raising the intracellular levels of cAMP. cAMP then further affects signaling pathways, such as PKA, which is activated and phosphorylates proteins involved in TAG hydrolysis and mobilization. Different catecholamines have different binding affinities and downstream effects to control lipolysis with different metabolic conditions and cellular responses. Dopamine, epinephrine, and norepinephrine are common catecholamines that are known for reward-motivated behavior and the fight-or-flight response.

Insulin is produced and secreted by pancreatic β-cells and stimulates the major antilipolytic pathway. Due to the implications of diabetes, insulin is widely recognized for signaling the increase of cellular glucose uptake from the bloodstream. After consumption blood-glucose levels are elevated, stimulating secretion of the hormone from pancreatic β-cells. The insulin signaling pathway is stimulated

and GLUT4 vesicles, an insulin-related glucose transporter, are synthesized and facilitate glucose transport to lower blood levels of glucose and raise intracellular glucose levels. In metabolism, insulin activates the phosphoinositide cascade via phosphatidylinositol 3-kinase (PI3K). This cascade activates Akt (Protein Kinase B) which has the downstream effect of phosphorylating and therefore inhibiting glycogen synthase kinase. Insulin is proposed to have antilipolytic action due to phosphodiesterase 3B (PDE3B). PDE3B is phosphorylated by Akt and leads to the accelerated conversion and degradation of cAMP to AMP (16). Even though this is a proposed pathway, it is not required for insulin to suppress lipolysis in adipocytes (16).

## Section 2.4 Lipotoxicity

Lipotoxicity is dysregulation of the lipid environment or intracellular lipid composition, which leads to accumulation of harmful lipids and further issues such as organelle dysfunction, cell injury, or death. Toxic lipid species include saturated free fatty acids, ceramides, and free cholesterol. Several mechanisms lead to toxic lipids causing cellular damage. Harmful lipids can lead to intracellular organelle modifications in the ER and mitochondria, and can also modify intracellular signaling pathways that relate to metabolism and inflammation (17). Signaling pathway modifications can arise through direct interactions between lipids and surface or cytoplasmic kinases.

## Section 3 PAT family proteins

The PAT family of proteins, most commonly referred to today as the perilipin proteins, is a group of lipid-droplet proteins related by their sequence homology and function within mammalian cells. The five family members are typically studied in mammalian models but can also be found in evolutionarily distant organisms (18).

With the exception of perilipin 4, the proteins share a 100-amino acid region near the amino terminus deemed the PAT domain, and perilipins 1-5 contain an 11-mer repeat motif (Figure 2). The PAT

domain is believed to interact with hormone sensitive lipase (HSL) (19), and an amino acid region of 141-200 is proposed to directly interact with HSL in perilipin 1 (20). The 11-mer domain is proposed to target lipid droplets by folding into amphipathic helices on the surface of lipid droplets to mediate the hydrophobic lipid core interface with its hydrophilic surroundings (19). This function potentially contributes to the localization of the proteins on the surface of lipid droplets (18).

While the perilipins share the same general function of regulating internal lipid stores, they otherwise differ in size, how they are regulated through transcription, tissue expression, and distinct cellular function (18). This is due to the variance in the carboxy terminus composition.

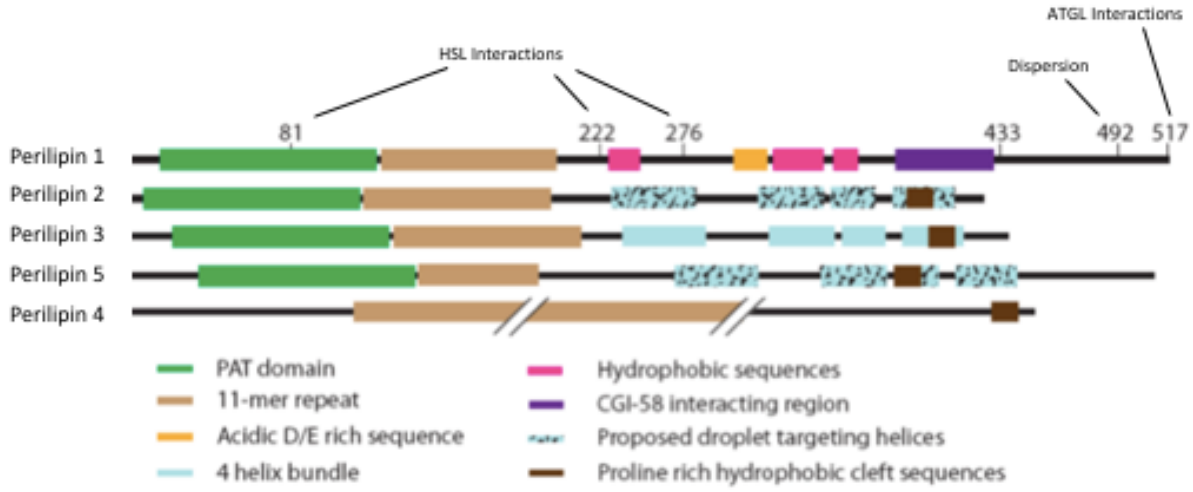



*Figure 2. Sequence alignment of the perilipin family of proteins adapted from Bickel, et al 2009*

## Section 3.1 Perilipin 1

First described by Londos and associates in 1991, perilipin 1, also known as perilipin/ perilipin A/perilipin 1A, is a 62 kDa protein specific to adipocytes (12). Perilipin 1 is the most studied family member of the protein and is found in highest concentrations in brown adipose tissue (BAT) and white adipose tissue (WAT) associated to lipid droplets (18). Perilipin 1 is considered a major regulator of lipolysis in adipose tissue for this reason. Under basal conditions, perilipin 1 acts as a barrier to lipolysis,

inhibiting activation of ATGL by CGI-58. This activity is due to perilipin 1 binding to CGI-58, preventing it from activating ATGL for breakdown of TAG (21). Under stimulated conditions, activation by PKA due to increased cellular levels of cAMP, perilipin 1 is phosphorylated and releases CGI-58. CGI-58 then complexes and activates ATGL allowing for TAG to be broken down into DAG (21). This phosphorylation specific control of lipid metabolism allows for tight regulation of cellular lipid levels.

PKA phosphorylation of perilipin 1 also leads to lipid droplet fragmentation when continuously stimulated (15). Serine-492 was found to be the specific site which lead to the effect of LD dispersion (15). Large peri-nuclear LDs were shown to fragment into microlipid droplets and disperse throughout the cytoplasm allowing for increased lipid metabolism (15).

## Section 3.2 Perilipin 2

The second family member, perilipin 2, is found on the lipid droplet but protein concentrations decrease as adipocytes continue to differentiate (18). Perilipin 2 is found throughout the body and is the major LD perilipin when perilipin 1 or 5 are not expressed (22). The protein was found to interact with CGI-58 but does not facilitate stimulated lipolysis in the same way or capacity of that of perilipin 1 and is characterized as being a less robust lipase barrier (18). Potential functions of the protein are to slow TAG hydrolysis and alter lipid droplet synthesis by crowding phospholipids (23). This crowding leads to exclusion of other proteins that rely on hydrophobic interactions of the neutral lipid core to associate to the LD (23).

When released from the LD the protein is quickly degraded, and in adipocytes perilipin 2 is outcompeted by perilipin 1 (18). Overexpression of perilipin 2 reduces access of ATGL to LDs preventing lipolysis, but this is a weak barrier (22). During maturation of lipid droplets perilipin 1 replaces perilipin 2 due to the fact that it is a stronger barrier to lipolysis and allows for LD growth (18).

Perilipin 2 has been reported to play a role in lipid accumulation in liver and wall of blood vessels, making it a protein of interest in diseases such as NAFLD and cardiovascular disease (18). This makes perilipin 2 of interest for therapeutic treatment to sequester lipid accumulation in undesired tissues.

## Section 3.3 Perilipin 3

The third family member can be found bound to lipid or free in the cytosol. The function of perilipin 3 is proposed to act as a major regulator of lipid droplet formation (24). When cultured in cells with high lipid levels, perilipin 3 was found to localize to small lipid droplets but over time is replaced by perilipin 2 as maturation continues (22). The protein does have high sequence homology with the other family members but is not regulated in the same way or appear to have the same function regulating lipolysis. Perilipin 3 function was first described as binding to and mediating transport of mannose 6-phosphate receptors throughout the cell (18). This cytosolic function of perilipin 3 indicates that the protein is involved in a variety of other functions.

Even though perilipin 3 function is unknown, it is the only perilipin that has a partial crystal structure and therefore confirmed structural data. The 47kDa protein was predicted to have active N- and C-termini that are separated to allow for independent functional domains (25). A fragment of the C-terminal domain has been isolated and crystallized. X-ray crystallography has a topology of novel alpha/beta domain and a four-helix bundle with a hydrophobic cleft (26) resembling a “boot”. This bundle resembles the lipoprotein binding domain of apolipoprotein E, suggesting that lipoprotein and LD binding may have similar mechanisms (26). The amino acid sequence of this motif is conserved throughout the perilipin family and perilipin 5 is proposed to have the same “boot” shaped c-terminus.

## Section 3.4 Perilipin 4

Expression of perilipin 4 is limited to adipose tissue, brain, skeletal muscle, and heart (27, 28, 29), either bound to LDs or in the cytosol. Coating nascent LDs with perilipin 4 allows for packaging of

TAG in cultured adipocytes (28). This is indicative of perilipin 4 participating in the preliminary formation of LDs. Perilipin 4's proposed function is to act as a regulator, in a hormone and substrate-dependent manner, to associate with LDs (30). The protein remains in the cytosol until increases in cellular FFA levels (30). During fat cell development, perilipin 4 appears to have a redundant function (30) and is eventually replaced by other perilipin family members when lipid droplets mature in the cell.

## Section 3.5 Perilipin 5

Perilipin 5 is the second most studied member of the perilipin family and is a major lipolytic regulator in oxidative tissues. These oxidative tissues--heart, liver, BAT, and skeletal muscle--also have the highest expression of the perilipin 5 protein and require high energy to function, utilizing FFA and glucose (31). Perilipin 5 is found on the surface of lipid droplets or bound to discrete structures resembling high density lipid droplets in the cytosol (32). Accumulation of lipid leads to perilipin 5 mobilizing from the cytosolic pool to the mature LDs to regulate lipid metabolism (32).

To regulate metabolism, perilipin 5 has been determined to independently bind to ATGL, CGI-58 and HSL (31, 32). In unstimulated conditions, perilipin 5 sequesters ATGL and CGI-58, preventing interaction for TAG hydrolysis. This function of perilipin 5 is controlled by phosphorylation mediated by PKA on Serine155 (31). Phosphorylation presumably allows for the release of CGI-58 and activation of ATGL thus initiating lipolysis (31). Serine 155 phosphorylation is not just important in the regulation of lipid metabolism but has more recently been found to regulate gene expression, proposing an uncommon function for the perilipin protein.

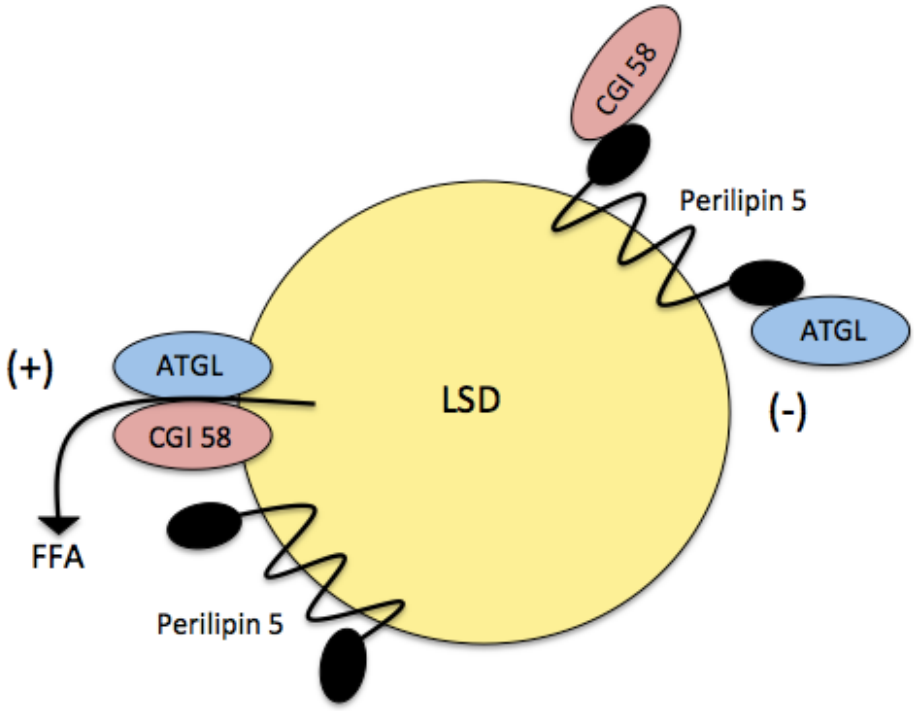



*Figure 3. Regulation of lipid metabolism, perilipin 5 interacting with ATGL, an enzyme required for TAG metabolism in stimulated and basal conditions. Proposed depiction of effect of phosphorylation of the protein by the PKA pathway.*

Nuclear staining of perilipin 5 following catecholamine treatment to stimulate the PKA pathway has long been observed by researchers but understudied until recently (33). Following treatment with isoproterenol and 3-isobutyl-1-methylxanthine (IBMX), perilipin 5 expression was found to increase overall and nucleus enrichment was observed (33). The treatment of the small molecules, IBMX and forskolin, stimulates the PKA pathway. Stimulation of PKA leads to phosphorylation of perilipin 5 and this phosphorylation alters the cellular location of the protein. The nuclear function of perilipin 5 is to complex with PGC-1α and SIRT1 and promote the PGC-1α gene program, thus matching mitochondria capacity to cellular lipid levels by regulating mitochondrial biogenesis. By complexing with PGC-1α and SIRT1, perilipin 5 is acting as a transcription factor and co-regulator. Perilipin 5 is around 63 kDa in molecular weight, lacks a clear nuclear localization sequence, and has only been characterized bound to lipid. Therefore, this function of nuclear localization and transport is of interest and is in its preliminary stages of study as the molecular mechanism for perilipin 5 nuclear trafficking is unknown at this time.

## Section 4.0 Relevant Biological Information

This thesis focuses specifically on the movement of perilipin 5 to the nucleus and related cellular pathways, components, and regulators. To appreciate the significance of finding perilipin 5 in the nucleus an understanding of the underlying cellular biology is required.

## Section 4.1 Protein Kinase A

The protein kinase A (PKA) pathway is one of the most common cell signaling pathways that transduces signals in cellular processes such as lipolysis, glycogen metabolism, and neurotransmission. Downstream effects of PKA phosphorylation is altered gene expression, changes in catabolism, membrane permeation, or altering other kinase signaling pathways. Initiation of the PKA pathway comes from external signals which can be a varying combination of ligand with receptor. Common external signals include catecholamines such as epinephrine, IMBX, and dopamine. A common PKA complex is heterotrimeric with dimer regulatory subunits, each with two cAMP binding sites.

In relation to perilipin 5, the PKA pathway has been found to phosphorylate serine 155 to alter lipolysis (31) and more recently, gene expression (33).

## Section 4.2 Calmodulin-dependent protein kinase II

Calmodulin-dependent protein kinase II (CaMKII) is a serine-threonine specific protein complex which is regulated by the $Ca^{2+}$/calmodulin complex. The minimum recognition motif of the kinase is RXXS or RXXT, due to the necessity of a serine or threonine to phosphorylate (34). Calmodulin is a multifunctional intermediate and calcium-binding messenger protein expressed in eukaryotic cells. Unsurprisingly, intracellular calcium ions ($Ca^{2+}$) are required for activation through binding. Calmodulin is important for intracellular signal transduction, modifying calcium's interactions with numerous kinases or phosphatases. Due to these interactions calmodulin plays a role in the mediation of inflammation,

metabolism, apoptosis, smooth muscle contraction, intracellular movement, memory, and the immune response.

CaMKII is a crucial regulator of physiology and pathology in cardiac tissue (34). Perilipin 1 has been predicted to be strongly downregulated by autocamtide-3 derived inhibitory peptide (AC3-I) mediated CaMKII inhibition (34). Sequence analysis of perilipin 1 indicates that the protein has a minimum recognition motif for CaMKII binding. Due to perilipin 5 being expressed in heart tissue and sharing some sequence homology with perilipin 1, this kinase is of interest for leading to altered activity or regulation of perilipin 5.

## Section 4.3 Protein Kinase C

PKC, protein kinase C, is another regulatory kinase signaling pathway which plays a large role in metabolism, memory, and control of autonomous cellular activities such as proliferation. Mammalian PKC is a superfamily that is comprised of twelve different genes and contains at least three distinct subfamilies (35). The grouping of the subfamilies is based on the second messenger requirements for activation and are grouped as conventional, novel, and atypical (36). The downstream effect of PKC is similar to that of PKA leading to phosphorylation of proteins. PKC is activated by phorbol esters, which are known tumor promotors. The minimal consensus sequence for PKC was generally determined to be RXXS/TXRX (36).

## Section 4.4 Nuclear Pore Complex

The nuclear envelope acts as a key regulator of gene expression. Nuclear pores permeate the membrane and selectively allow small molecules to pass through the membrane. The nuclear pore complex (NPC) has an eightfold rotational symmetry around the central transport channel, and the overall structure of the complex can be seen in Figure 4 (37). The cytoplasmic face of the pore has cytoplasmic filaments, which facilitate transport through the NPC. Nucleoporins contain stretches of phenylalanine-

glycine (FG) repeats which project into the central transport channel and create a barrier to prevent the passive diffusion of macromolecules larger than 40kDa but still facilitates the rapid transport of larger cargoes designated to reside within the nucleus (37).

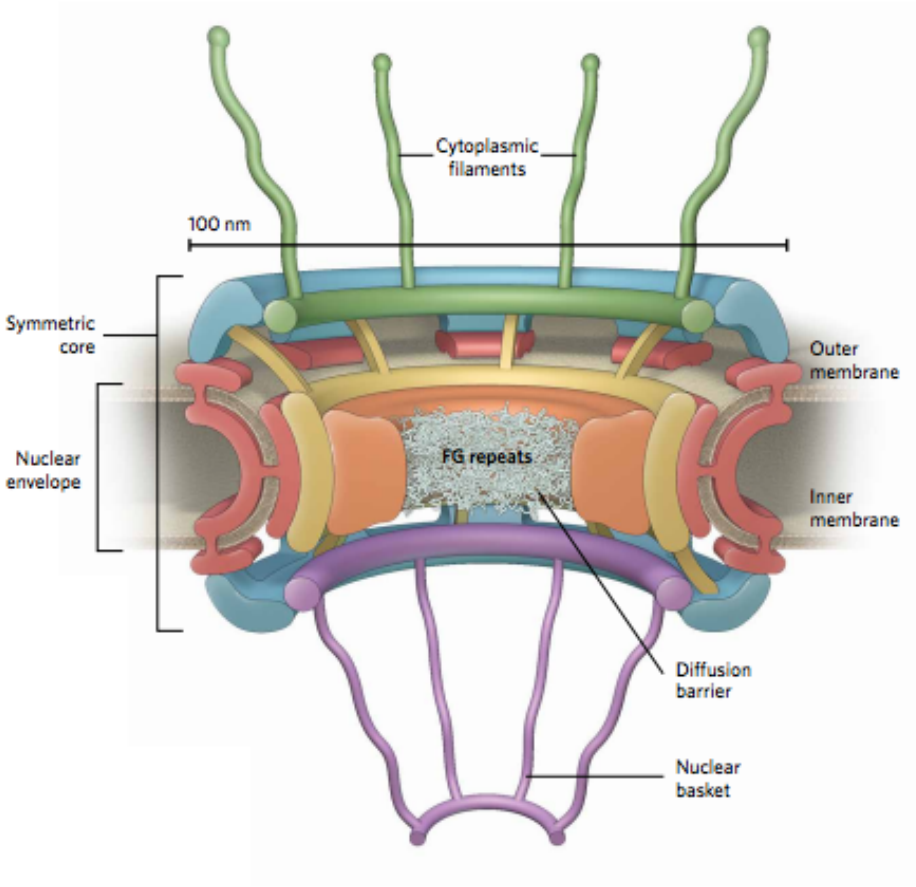



*Figure 4. Nuclear Pore Complex adapted from The Scientist 2016 (37).*

## Section 4.5 Nuclear Transport Mechanisms

Macromolecules are often transported through the NPC in their native state. Larger proteins and complexes are transported by binding to a class of transport molecules called karyopherins. Karyopherins include importins and exportins and can link to cargo by adaptor proteins. The Ran cycle is the major pathway for trafficking macromolecules across the nuclear envelope. Ran GTPases regulate importation and exportation of the macromolecule of interest. Karyoprotein complexes interact with the FG repeats

and are driven by the hydrolysis of GTP. The concentration of Ran on either side of the nuclear pore determines whether the protein will be imported, when there is a high concentration of Ran-GTP, or exported, when there is a high concentration of Ran-GDP.

Binding of a karyopherin to a specific cargo requires a nuclear localization sequence, or NLS. Putative consensus sequences of the classic NLs have been defined (38,39). Two localization sequences have been deemed classical, single (monopartite) or two (bipartite) stretches of basic amino acids. The monopartite sequence is exemplified in the SV40 large T antigen NLS ($^{126}$PKKKRRV$^{132}$). Structural and thermodynamic studies have shown that the monopartite sequence requires a lysine in the P1 position followed by residues in P2 and P4 to yield a loose consensus sequence of K(K/R)X(K/R) (39). Bipartite nuclear localization sequences are characterized by the nucleoplasmin NLS ($^{155}$KRPAATKKAGQAKKKK$^{170}$) (40). The linker region of the bipartite NLS has been limited to ten amino acids based on characterization of the nucleoplasmin NLS, but *in vitro* studies have shown up to 12 residue linkers allowing for binding to Importin-α (40). Online tools such as PSORTII, NLS mapper, and Predict Protein can be used for *in silico* analysis of localization sequences with varying degrees of accuracy (39).

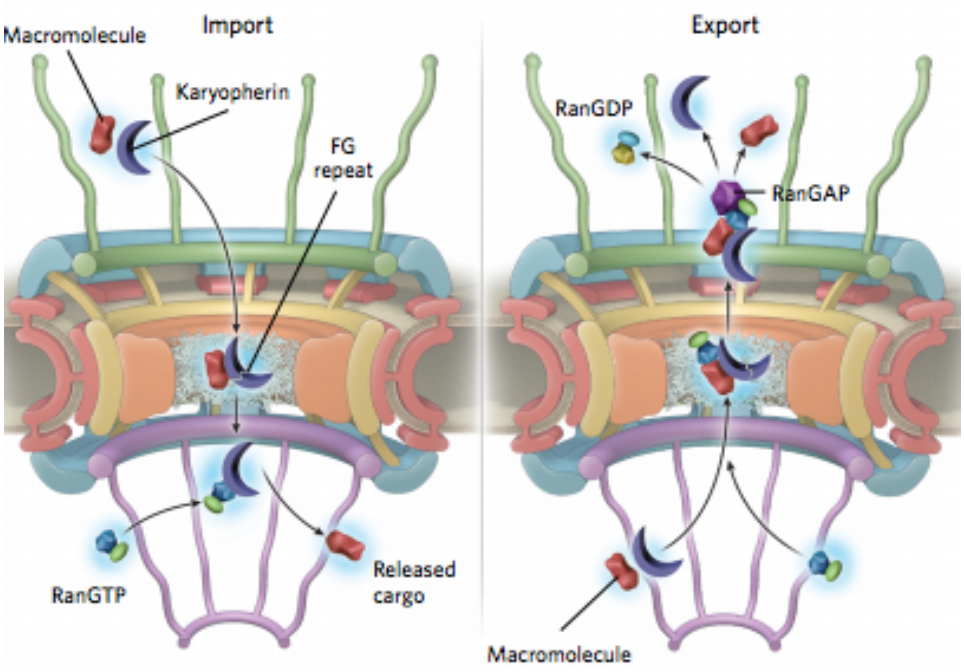



*Figure 5. Ran GTPase transport cycles adapted from The Scientist 2016 (37).*

## Section 4.6 PPARs

PPARs, peroxisome proliferator-activated receptors, are a family of nuclear receptors, with the family members being α, β/δ, and γ (41). PPAR-γ, is essential for tissue differentiation and adipogenesis and has been found to be directly linked to the perilipins (42, 43). PPAR- α, and β/δ regulate fatty acid oxidation and therefore also play a role in metabolism (41).

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α, is a transcription factor that was originally discovered as a cold-inducible coactivator of adaptive thermogenesis (41). The transcription factor has also been found to be biologically active in mitochondrial biogenesis, metabolism of glucose and fatty acids, heart development, and skeletal muscle tissue reconstruction (41, 44). PGC-1α has two putative nuclear localization sequences and is highly expressed in oxidative tissues such as brown adipose tissue (BAT), heart, and skeletal muscle. Surprisingly, it is reported that expression of the

transcription factor is low in liver (41). PGC-1α binds to PPAR- γ leading to its co-activation which stimulates the transcription of genes involved in BAT differentiation.

Perilipin 5 has been found to localize to the nucleus and interact with the PGC-1α gene program to regulate mitochondrial biogenesis (33). The proposed reasoning behind this linkage of a protein involved in lipid metabolism and a transcription factor is to tie the capacity in which cells can metabolize FFA to its lipid load, or lipid capacity. This is because excessive delivery of FFA to mitochondria leads to incomplete fatty acid oxidation, insulin resistance, and mitochondrial inefficiency (33). When stimulated by PKA, perilipin 5 expressing cells have increased lipolysis and an increase in FFA in the cell. PGC-1α acts as a transcriptional regulator of mitochondrial biogenesis and an increase in a cell's mitochondria sequesters the FFA content for complete oxidation and management (33).

Overall, this thesis aims to investigate the cellular mechanism of nuclear localization of perilipin 5. Our protein of interest, perilipin 5, regulates lipid metabolism through direct interactions with ATGL and HSL and regulation of gene expression which leads to mitochondrial biogenesis. These interactions can have implications to metabolic disease, therefore, learning more about how perilipin 5 is trafficked in the cell can lead to a greater understanding lipid related disorders and metabolic disease states.

# METHODS

## 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. For lipid loading testing, cells were treated with 400µM oleic acid complexed to fatty acid-free bovine serum albumin (FAF BSA) (lipid load media) to promote TAG storage and control cells were treated for 24 h with fresh media.

## DNA Isolation

A glycerol stock of *E. coli* containing a PLIN5 cDNA 3x-FLAG vector was used for plasmid isolation. This vector contains the cDNA sequence for the mouse full length perilipin 5 protein with 3 FLAG-tag sequence and was produced previously in the lab. Overnight cultures were grown in LB media samples and plasmid DNA was isolated using QIAGEN Plasmid *Plus* Midi Kid. The bacterial culture was lysed, DNA extracted and then purified using QIAGEN Plasmid *Plus* spin columns and buffers. DNA was eluted in 500µL of elution buffer and stored at 4º C until later use.

## Transfection

To obtain CHO cells expressing the full length perilipin 5 vector, cells were transfected with the isolated DNA using the Lipofectamine® 2000 reagent and protocol (Invitrogen). CHO K1 cells were split into a sterile six well plate and grown to approximately 80% confluency. Cells were transfected with the full length perilipin 5 sequence with the 3X-FLAG expression vector. Selection was preformed using 300µg/mL G418.

To quickly look at perilipin 5 localization and expression transient transfected coverslips were also used for initial observation. The Lipofectamine® 2000 protocol was followed using the 12µL

concentration of lipofectamine for transfection. Transfections were set to incubate for a 24 h period at 37ºC and 5% $CO_2$ before using for experimentation. This gives varying expression levels of the protein across a population but does allow for preliminary experimentation.

Cellular Lysate

Confluent cells were scraped and transferred into an Eppendorf tube. Cells were pelleted at 800 rpm for 5 minutes and washed three times with 1x PBS to remove remaining media and dead cells. Once washed, cells were suspended in 10mM TRIS to promote lysis and incubated in ice for 15 minutes. Cells were then passed through a 28-gauge needle seven times to shear. For whole body or 'crude' lysates, cellular samples these samples were used without further preparation. For samples that required a "post-nuclear supernatant", cells were centrifuged at 5,000 rpm for 5 minutes at 4º C and the supernatant was isolated from the pellet to use.

Immunoblots

Protein samples were quantified using the Pierce BCA Protein Assay kit (ThermoFisher) and following its protocol. 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 then ran for 1 h at 160 V with 1X MOPS SDS-Page running buffer. 2µL of LI-COR fluorescent molecular marker 700 CW were also loaded for size determination. When the gel finished running, the separated protein was then transferred to a nitrocellulose membrane for 16 h at 12V.

The following morning, the membrane was removed from the sandwich and dried on the bench for at least an hour and 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 on the bench with rocking to bind any nonspecific recognition sites. Following blocking, the blot was incubated in primary antibody solution with block overnight at 4º C, on a rocker. The common primary antibodies used were anti-FLAG

(SIGMA)1:5,000 concentration antibody to blocker and anti-OX PAT (developed by the Tansey lab) at a 1:1,000 concentration.

The next day, the membrane was washed three times in 1X PBS for five-minute increments at room temperature with rocking. Following washing, the blot was incubated in secondary antibody for 55 minutes at room temperature while rocking. This step was light sensitive, and the rest of the work was therefore done in the dark. Secondary antibodies were used at a 1:20,000 concentration. For the FLAG primary antibody, a secondary of goat anti-mouse 800 CW (LI-COR) is used. The OX PAT primary uses a goat anti-rabbit 600 CW secondary when detecting with multiple probes or 800 CW (LI-COR) for single antibody detection methods. Three final washes of 1X PBS for 5 minutes at room temperature were done to remove nonspecific antibody following incubation. Target signal 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 full length perilipin 5 protein were plated on coverslips in a six-well tissue culture dish. Cells were washed with PBS three times and 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 1h at room temperature. Coverslips were incubated with primary antibody in 1x PBS, 5% Goat Serum, 0.1% Triton X-100, 0.2% FLAG anti-mouse antibody overnight in a humidified chamber at 4ºC. The next day the coverslips were washed three times with PBS and Triton-X100 and incubated with secondary antibodies in antibody dilution buffer. Excess antibody is then removed by blotting on a Kimwipe, and coverslips were mounted with Prolong Gold anti fade reagent with DAPI (Invitrogen). Coverslips cured overnight on the bench before imaging using Olympus B201 BX40 microscope.

Bioinformatics

The perilipin 5 amino acid sequence (mouse) was run through motif recognition software that are readily available online. To predict nuclear localization importation and exportation recognition sites

cNLS mapper (45) and NetNES (46) were used. For kinase recognition motif prediction, PROSITE (47) was used. This tool can be easily located on ExPASY.

# RESULTS AND DISCUSSION

The overarching objective of this work is to elucidate aspects of the cellular trafficking of perilipin 5. The protein is typically found on larger LD structures and acting as a regulator of lipid metabolism in the cell. Recent observations by the Tansey lab and other groups (33) proposed a new role and localization for perilipin 5 in the nucleus regulating gene expression, especially those involved in mitochondrial biogenesis. This work largely focuses on the determination of the nuclear localization mechanism of perilipin 5.

Perilipin 5 is found on larger mature lipid droplets and on dense lipid droplets in the cytosol resembling high density lipid droplets (HDLDs), which are similar in size and structure. Bartholomew, et al (32) characterized the cytosolic fraction of perilipin 5 using native gradient gel electrophoresis. The small structures were described as being around 15nm in diameter and containing perilipins 3 and 5 (32). The top band of the blot, indicated by a single *, is the perilipin 5 protein associated to the discreet HDLDs while the free perilipin 5 protein is seen in the lower band (**) at approximately 70kDa (Figure 6). This data shows the distinctive pools of cellular perilipin 5 which can be visualized and are not limited to large LDs. To traffic to the nucleus, perilipin 5 has to dissociate from the larger lipid droplet for import. The nuclear pore complex is a highly selective and small port that molecules can pass through. As discussed previously, structures over 40 kDa in molecular weight cannot pass through the nuclear pore complex. This suggests that the small HDLD-like structures that perilipin 5 is found on in the cytosol are not likely to be transported into the nucleus due to their ~575kDa molecular weight, but it is unknown if perilipin 5 is trafficked through the nuclear pore complex bound to lipid or other small molecules since the protein is not reported to be free in the cytosol.

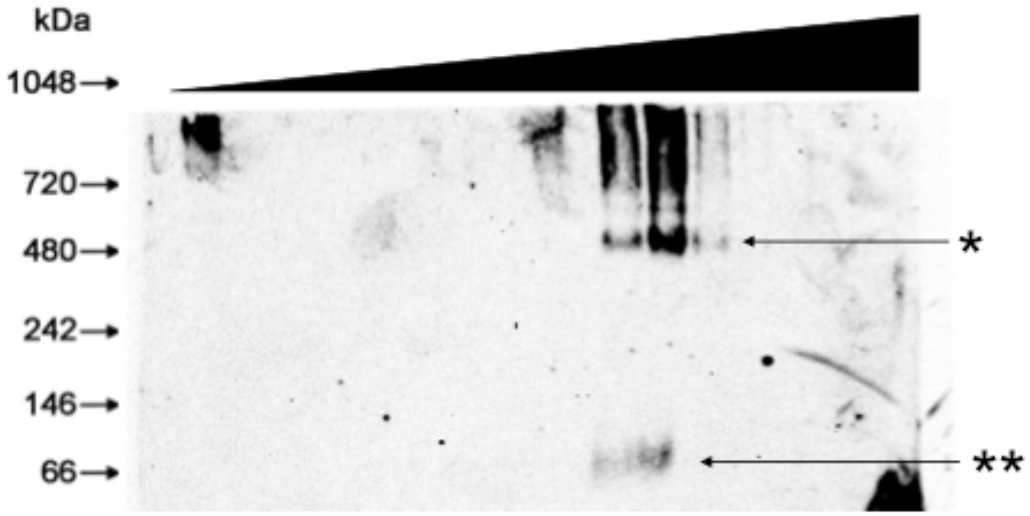



*Figure 6. From Bartholomew et al 2010 (32). Perilipin 5 is found on discrete structures resembling HLDL in nature*

To be able to study perilipin 5, we used CHO cells that were transfected with the full length perilipin 5 protein with 3X FLAG tag. The full-length protein with the 3X FLAG tag allows the use of either FLAG antibody at the carboxy terminus of the protein or the OXPAT antibody at the amino terminus to be used to look at the protein using immunofluorescence (Figure 7).

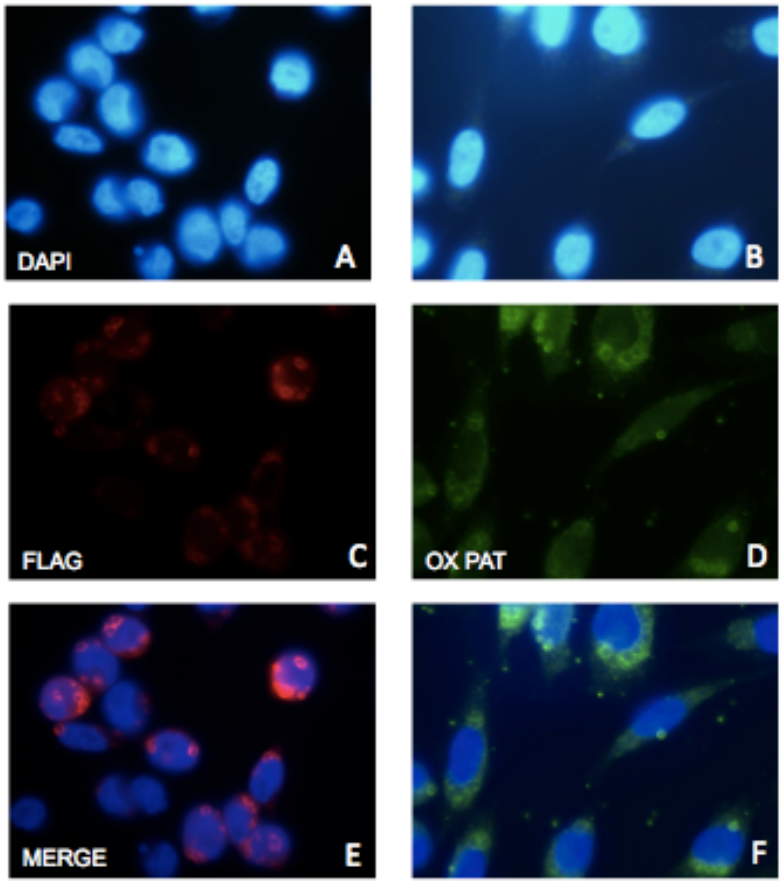



*Figure 7. Fluorescent micrographs of CHO cells expressing perilipin 5*

The two antibodies tag different terminuses of the protein and are produced in different animals allowing for both antibody systems to be used within the same experiment. OXPAT, produced in rabbit, binds to the amino (N)- terminus of perilipin 5, while FLAG, produced in mouse, binds to the 3X FLAG tag motif located at the carboxy terminus. Labeling of CHO cell samples using the two immunofluorescent antibodies can be seen in Figure 7. OX PAT, uses a secondary antibody which excites at 488nm and emits at 525nm (green) and is seen in panels D and F of Figure 7. The secondary used with FLAG excites at 594nm and emits at 617nm (red), seen in panels C and E of Figure 7. Panels A and B are of nuclear staining using the DNA stain DAPI. This is to be able to accurately visualize and determine the number of cells in the field of view and tell how many of the cells are expressing the protein of interest. As can be seen with both antibody stained micrographs, perilipin 5 is typically seen encompassing lipid droplets in its unstimulated state. The dual tag antibody system can also be used to predict if there is

cleavage before localization or if the full-length protein is translocating following stimulation of the PKA pathway, due to the opposite ends of the protein being tagged. The PKA pathway is stimulated using IBMX and forskolin, which are known up-regulators of the kinase leading to increased phosphorylation.

The nuclear staining of perilipin 5 has been observed by multiple groups throughout the years of studying the protein. The phenomenon was typically written off as an artifact of staining because other questions were being investigated at the time it was observed. In 2016, the nuclear localization of perilipin 5 was observed in multiple sets of immunofluorescent coverslips following stimulation of the PKA pathway. The rationale behind stimulating PKA was to determine if the upregulation and subsequent phosphorylation of perilipin 5 lead to the fragmentation of lipid droplets as seen with perilipin 1 (15). While fragmentation of lipid droplets was not observed, staining was observed in nuclei. Following deliberation and troubleshooting of the staining, it was determined that it was best to move forward with the phenomenon as the excitation spectrum of DAPI does not extend past the 450nm range and therefore would not cause fluorescence at 488nm, and it was unlikely that anything else would be causing background staining other than protein localization.

Not long after this decision was made, Perry Bickel's group at The University of Texas Southwestern published on the PKA dependent nuclear localization of perilipin 5 and its subsequent interaction with PGC-1α to regulate mitochondrial biogenesis (33).

While the Bickel group did lay the foundation for nuclear perilipin 5, the larger picture is still missing. Time dependency of localization and whole cell lipid concentration were two conditions of interest in preliminary mechanism studies. As observed with other perilipin family members, individual perilipin concentration and distribution throughout the cell can vary due to concentration of neutral lipid (15, 18, 32).

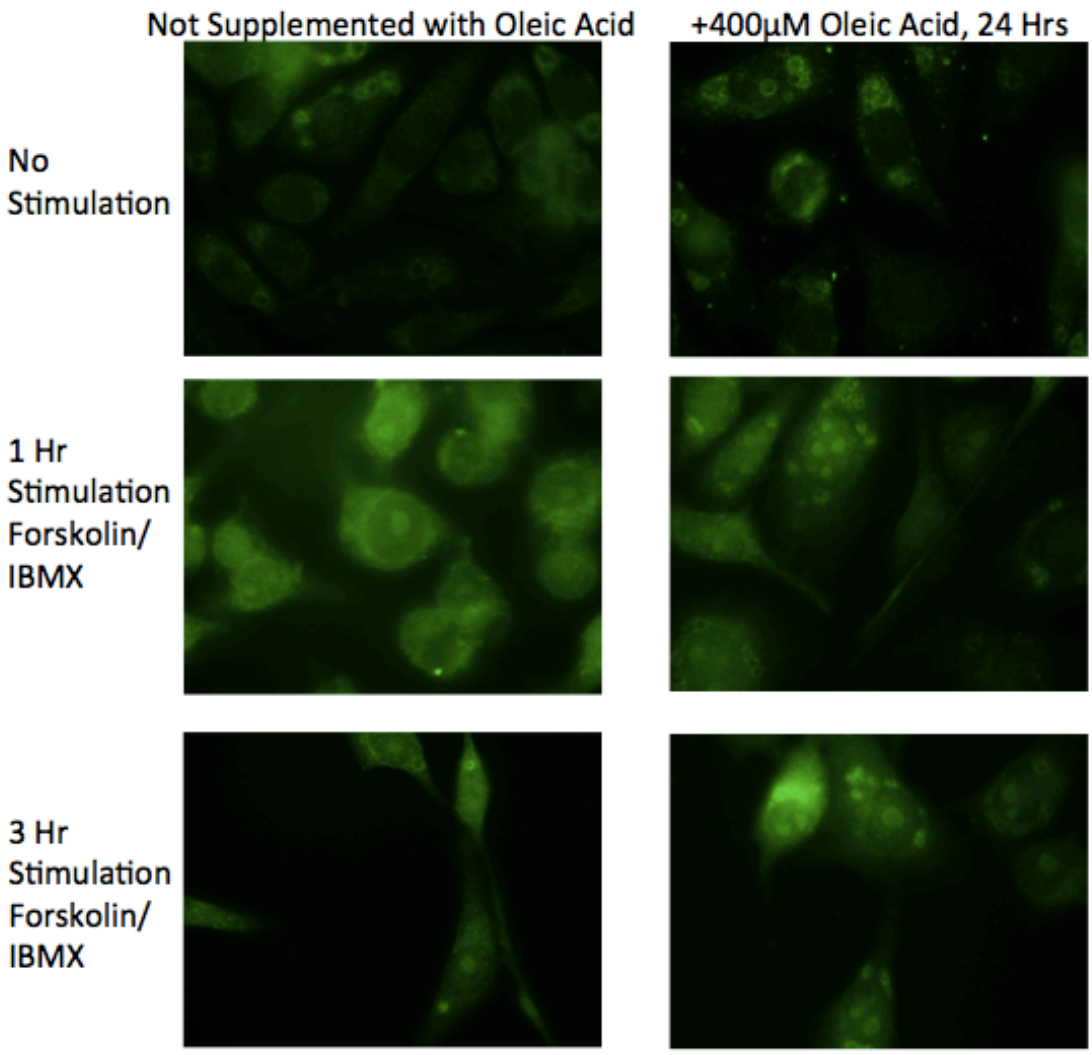
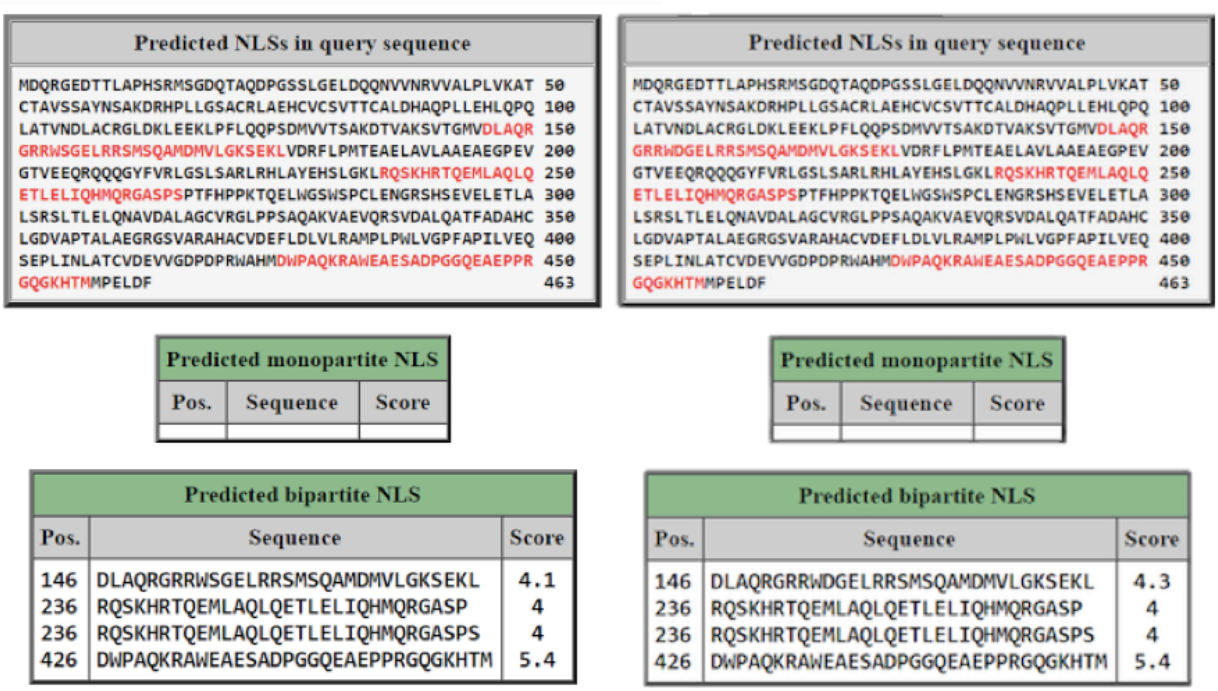



*Figure 8. CHO OX PAT Coverslips following PKA stimulation*

CHO cells expressing perilipin 5 were treated with oleic acid and phosphorylation stimulants, IBMX and forskolin (stimulating the PKA pathway). The supplementation of oleic acid increases the cell's concentration of neutral lipid and promotes accumulation of lipid in LDs. Overnight incubation ensures that this accumulation of lipid occurs. Altering the lipid concentration in cells can lead to varied expression levels or localization of the perilipin proteins. One and three-hour time points were then used to look at effects of stimulation on perilipin. Following one hour of treatment, there is clear localization of perilipin 5 in the nucleus as seen in Figure 8. The nuclear localization appears as shaded pockets within the nucleus and is clearly distinct from LD staining of perilipin 5 which appears as rings around lipid

droplets instead of shaded regions. This localization appears to be independent of cellular lipid concentration as the phenomenon was observed with and without treatment of oleic acid.



**Predicted NLSs in query sequence**

```
MDQRGEDTTLAPHSRMSGDQTAQDPGSSLGELDQQNVVNRVVALPLVKAT 50
CTAVSSAYNSAKDRHPLLGSACRLAEHCVCSVTTCALDHAQPLLEHLQPQ 100
LATVNDLACRGLDKLEEKLPFLQQPSDMVVTSAKDTVAKSVTGMVDLAQR 150
GRRWSGELRRSMSQAMDMVLGKSEKLVDRFLPMTEAELAVLAAEAEGPEV 200
GTVEEQRQQQGYFVRLGSLSARLRHLAYEHSLGKLRQSKHRTQEMLAQLQ 250
ETLELIQHMQRGASPSPTFHPPKTQELWGSWSPCLENGRSHSEVELETLA 300
LSRSLTLELQNAVDALAGCVRGLPPSAQAKVAEVQRSVDALQATFADAHC 350
LGDVAPTALAEGRGSVARAHACVDEFLDLVLRAMPLPWLVGPFAPILVEQ 400
SEPLINLATCVDEVVGDPDPRWAHMDWPAQKRAWEAESADPGGQEAEPPR 450
GQGKHTMMPELDF                                       463
```

**Predicted monopartite NLS**

| Pos. | Sequence | Score |
|---|---|---|

**Predicted bipartite NLS**

| Pos. | Sequence | Score |
|---|---|---|
| 146 | DLAQRGRRWSGELRRSMSQAMDMVLGKSEKL | 4.1 |
| 236 | RQSKHRTQEMLAQLQETLELIQHMQRGASP | 4 |
| 236 | RQSKHRTQEMLAQLQETLELIQHMQRGASPS | 4 |
| 426 | DWPAQKRAWEAESADPGGQEAEPPRGQGKHTM | 5.4 |

**Predicted NLSs in query sequence**

```
MDQRGEDTTLAPHSRMSGDQTAQDPGSSLGELDQQNVVNRVVALPLVKAT 50
CTAVSSAYNSAKDRHPLLGSACRLAEHCVCSVTTCALDHAQPLLEHLQPQ 100
LATVNDLACRGLDKLEEKLPFLQQPSDMVVTSAKDTVAKSVTGMVDLAQR 150
GRRWDGELRRSMSQAMDMVLGKSEKLVDRFLPMTEAELAVLAAEAEGPEV 200
GTVEEQRQQQGYFVRLGSLSARLRHLAYEHSLGKLRQSKHRTQEMLAQLQ 250
ETLELIQHMQRGASPSPTFHPPKTQELWGSWSPCLENGRSHSEVELETLA 300
LSRSLTLELQNAVDALAGCVRGLPPSAQAKVAEVQRSVDALQATFADAHC 350
LGDVAPTALAEGRGSVARAHACVDEFLDLVLRAMPLPWLVGPFAPILVEQ 400
SEPLINLATCVDEVVGDPDPRWAHMDWPAQKRAWEAESADPGGQEAEPPR 450
GQGKHTMMPELDF                                       463
```

**Predicted monopartite NLS**

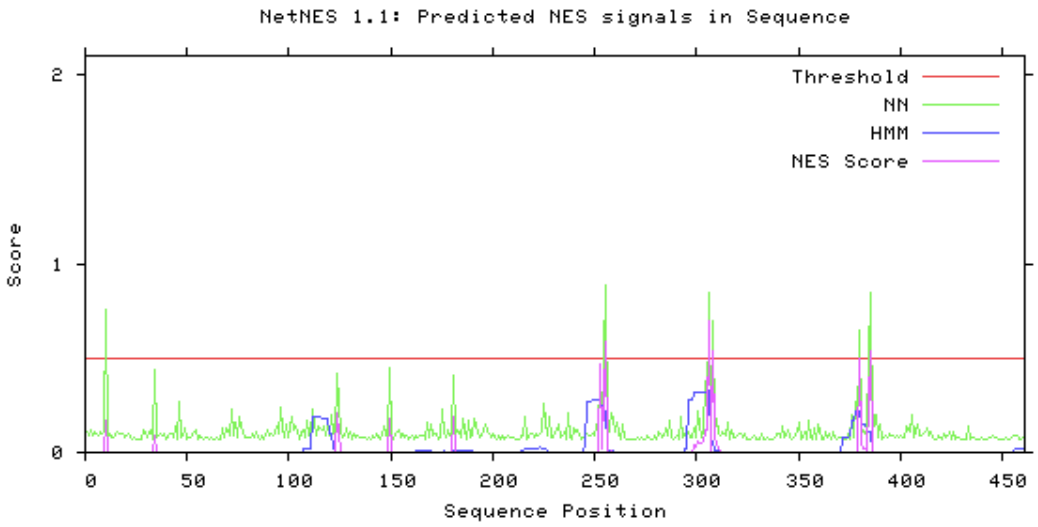
| Pos. | Sequence | Score |
|---|---|---|

**Predicted bipartite NLS**

| Pos. | Sequence | Score |
|---|---|---|
| 146 | DLAQRGRRWDGELRRSMSQAMDMVLGKSEKL | 4.3 |
| 236 | RQSKHRTQEMLAQLQETLELIQHMQRGASP | 4 |
| 236 | RQSKHRTQEMLAQLQETLELIQHMQRGASPS | 4 |
| 426 | DWPAQKRAWEAESADPGGQEAEPPRGQGKHTM | 5.4 |

*Figure 9. Proposed perilipin 5 nuclear localization sequence based on sequence analysis using cNLS Mapper.*

Conventionally, proteins that are trafficked to the nucleus have some sort of nuclear localization sequence within their amino acid code which allows for importins to bind and shuttle through the nuclear pore complex using the RAN cycle. To determine if perilipin 5 contains a nuclear localization sequence (NLS), cNLS mapper was used (45) to predict putative NLSs. While this mapper is more commonly used for yeast amino acid sequences, it can be used as a predictive tool for other mammalian sequences. The mouse amino acid sequence for perilipin 5 was used since this is the protein isoform expressed in the cell model. Figure 9, the left query is of the native amino acid sequence, while the query to the right is of a phosphomimetic mutant sequence (S155D). A higher predicted score was observed for the mutated sequence for the bipartite NLS at the phosphorylation site with a score of 4.3 instead of 4.1 suggesting that phosphorylation of S155 does lead to a stronger NLS sequence and therefore could lead to importin binding and successive nuclear enrichment.




| Position | Residue | ANN | HMM | NES |
|---|---|---|---|---|
| 255 | L | 0.889 | 0.218 | 0.590 |
| 306 | L | 0.843 | 0.325 | 0.692 |
| 308 | L | 0.695 | 0.064 | 0.540 |
| 380 | L | 0.647 | 0.221 | 0.501 |
| 385 | L | 0.850 | 0.109 | 0.537 |

*Figure 10. Nuclear export sequence prediction using NetNES to determine potential sites signaling for nuclear export of perilipin 5 following enrichment.*

Nuclear enrichment of perilipin 5 has been found to decrease and reverse when stimuli is removed (33). Also, due to the fact that this phenomenon is not observed consistently and continually when imaging cells it is not likely that the protein mobilized from a cytosolic pool to alter transcription. For perilipin 5 to be exported from the nucleus, it requires a nuclear export signal to bind to an exportin. NetNES (46) was used to look for predicted nuclear exportation sequences which could be allowing for the removal of perilipin 5 from the nucleus after stimulus. Four potential leucine residues are of interest for nuclear exportation, with L306 being the strongest predicted candidate, as hydrophobic residues are important in defining the signal as seen in Figure 10. Predominately, the export sequences are predicted to be further downstream from the NLS. From a regulatory standpoint this separation of import and export motifs is unsurprising, as it is desirable to be able to clearly expose importation motifs and bury exportation motifs when translocating to the nucleus and vice versa for exportation.

| Protein Kinase C Phosphorylation Site | |
|---|---|
| Amino Acid Numbers | Residues |
| 60-62 | SaK |
| 132-134 | SaK |
| 173-175 | SeK |
| 220-222 | SaR |
| cAMP and cGMP-dependent Protein Kinase Phosphorylation Site | |
| Amino Acid Numbers | Residues |
| 152-155 | RRwS |

*Table 1. PROSITE results for perilipin 5 (mouse) predicting frequently occurring signature protein motifs. Only results from PKC and PKA included in this table, motifs predicting phosphorylation by CaMKII were not found in the sequence.*

With phosphorylation being a driving factor of nuclear localization, it is of interest to examine other kinases to determine if they have the same effect as PKA. PROSITE (47), was used to predict common motifs found within the perilipin 5 amino acid sequence. Specifically, CaMKII and PKC are kinases of interest to the lab at this time. CaMKII plays a large role in cardiac tissue, one of the major tissues that perilipin 5 is expressed in and therefore could be altering the protein function to lead to physiological changes in cardiac muscle tissue. The other kinase, PKC, is another regulatory kinase signaling pathway which plays a large role in metabolism and is similar to PKA. The PKC family of kinases is highly variable but has a standard recognition motif that is widely accepted for predicting interactions.

Using PROSITE, multiple PKC motifs were determined suggesting that there is a high chance that in some way PKC does alter the protein. While a recognition site for phosphorylation by CaMKII was not found by the software this does not mean that the kinase will not phosphorylate perilipin 5. Due to the minimum recognition motif found in the literature, CaMKII could also interact with perilipin 5 at residues 152-155 where PKA is a known regulator. The *in silico* work does need to be validated with *in vitro* studies as PROSITE and the other sequence mappers are only predictive tools which can be used to guide initial phases of studies.

# CONCLUSIONS AND FUTURE WORK

The nuclear localization of perilipin 5 has large implications for metabolism and regulation of gene expression due to its interaction with the PGC-1α gene program. Current work is being done to investigate perilipin 5 stimulation and how that affects nuclear localization. The overall proposed mechanism behind perilipin 5 nuclear localization can be broken down into three main categories of questions. The first looks at how the protein leaves the lipid droplet surface, followed by how the protein is trafficked throughout the cell and lastly how it enters the nucleus to interact with the PGC-1α gene

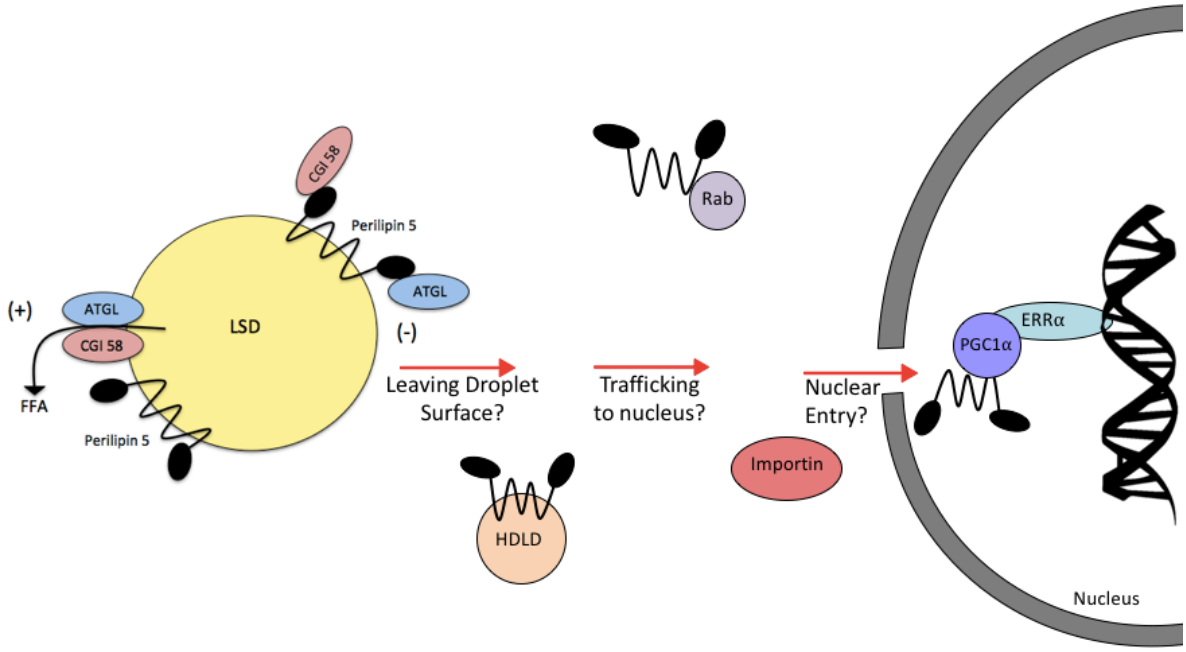



*Figure 11. Schematic of how perilipin 5 trafficks to the nucleus to interact with the PGC-1α to regulate transcription.*

program regulating mitochondrial biogenesis. Studies on the phosphorylation state of perilipin 5 begin to answer the first question. Further investigations into this question include using immunofluorescence microscopy in the presence and absence of stimulants and inhibitors of kinases, especially the kinases PKC and CaMKII. Mass spectrometry and site directed mutagenesis can be used to determine if the protein is phosphorylated, and at which residues, and how this alters function

This thesis also begins to dissect the question of nuclear entry of perilipin 5 through bioinformatics techniques. The predictions made by cNLS Mapper and NetNES should be analyzed *in*

*vitro* to determine if importins or exportins recognize and bind to the protein. PGC-1α is also known to have a cytosolic pool that translocates to the nucleus under stimulating conditions (48). Therefore, it would be interesting to determine if perilipin 5 is binding to PGC-1α in the cytosol and is then translocating to the nucleus as a complex to regulate transcription or if the association of the coactivator and perilipin 5 are independently trafficking and then associating.

Addressing the trafficking process via interaction partners is also a line of inquiry which still needs addressed. Pull down assays, computer modeling, and immunofluorescent western blotting and immunofluorescence can be used to look a co-localization of trafficking partners such as Rabs. This work is preliminarily scratching the proverbial surface of the nuclear transport mechanism of perilipin 5. Further experimentation to tease apart the major questions of the trafficking process can be continued to determine how this phenomenon occurs and regulates mitochondrial biogenesis. This line of inquiry can have greater implications for understanding metabolic disease states.

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# APPENDIX 1: ABBREVIATIONS USED IN THIS WORK

| | |
|---|---|
| AC3-I | autocamtide-3 inhibitory peptide |
| Akt | protein kinase B |
| ATGL | adipose triglyceride lipase |
| BAT | brown adipose tissue |
| BSA | bovine serum albumin |
| C-terminal | carboxy-terminus |
| CaMKII | calmodulin-dependent protein kinase II |
| cAMP | cyclic-adenosine monophosphate |
| CGI-58 | comparative gene identification 58 |
| CHO | Chinese hamster ovary |
| DAG | diacylglycerol |
| DNA | deoxyribonucleic acid |
| FABP4 | fatty acid binding protein-4 |
| FAF | fatty acid free |
| FFA | free fatty acid |
| FG repeats | phenylalanine-glycine repeats |
| HDL | high density lipoprotein |
| HDLD | high density lipid droplet |
| HSL | hormone sensitive lipase |
| IBMX | 3-isobutyl-1-methylxanthine |
| kDa | kilo Dalton |
| LDs | lipid droplets |
| MAG | monoacylglycerol |
| MGL | monoglyceride lipase |
| N-terminal | amino-terminal |
| NAFLD | nonalcoholic fatty liver disease |
| NASH | nonalcoholic steatohepatitis |

| | |
|---|---|
| NLS | nuclear localization sequence |
| NPC | nuclear pore complex |
| PBS | phosphate-buffered saline |
| PDE3B | phosphodiesterase 3B |
| PGC-1α | peroxisome proliferator-activator receptor-γ coactivator-1α |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PPARs | peroxisome proliferator-activated receptors |
| SIRT1 | silent mating type information regulation 2 homolog 1 |
| TAG | triacylglycerol |
| TRIS | Tris(hydroxymethyl)aminomethane |
| US | United States |
| WAT | white adipose tissue |