A Role for PICALM in Macroautophagy and Cellular Cholesterol Homeostasis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

The dissertation will focus on deciphering novel roles for PICALM in cellular biology. PICALM (Phosphatidyl Inositol Clathrin Assembly Lymphoid Myeloid Protein) is a ubiquitously expressed protein that was initially identified as a partner for AF10 in a chromosomal translocation in a lymphoma cell line. Since its identification, PICALM has been shown to act as an accessory adaptor protein in clathrin-mediated endocytosis and to regulate the internalization of proteins involved in vesicular trafficking (SNARE proteins). In addition, mutations in the PICALM gene have been shown to be linked to the development of leukemia and Alzheimer’s Disease. As a result of our studies, we have determined that PICALM is involved in two previously unappreciated cellular processes: macroautophagy and cellular cholesterol metabolism. This dissertation will address each of these processes in turn.

The thesis begins with an introduction to PICALM, including a description of PICALM’s known cellular functions and its relationship to disease. In addition, general aspects of macroautophagy and cellular cholesterol metabolism will be introduced (Chapter 1). Chapter 2 will describe the materials and methods that were used in the experimental analysis.

Chapter 3 describes our observation of a novel role for PICALM in macroautophagy. PICALM regulates SNARE protein internalization and localization.
Intriguingly, SNARE proteins (VAMP3 and VAMP8) are involved in vesicular trafficking and macroautophagy. Thus, we sought to determine a role for PICALM in regulating macroautophagy by experimentally reducing or overexpressing PICALM. Our studies show that both reduction and overexpression of PICALM can modulate macroautophagy. In addition, our work indicates that PICALM modulates macroautophagy by altering autophagosome breakdown, without having an effect on autophagosome formation. This section of the thesis concludes with a possible mechanism by which PICALM may modulate macroautophagy. A substantial portion of this Chapter appeared in Moreau et al, Nature Communications, 2014 (1).

Chapter 4 focuses on PICALM’s ability to modulate cellular cholesterol homeostasis. We initially performed a microarray experiment using picalm-deficient and PICALM-expressing cells in order to obtain biological insight into possible novel roles for PICALM. This study suggested that modulating the level of PICALM expression alters cellular cholesterol homeostasis. We went on to demonstrate that PICALM reduction and overexpression result in altered cholesterol metabolism gene expression. In addition, we examined the effect of PICALM deficiency on cholesterol flux, and unexpectedly showed that PICALM reduction results in elevated cholesterol internalization, and cellular cholesterol levels. The LDL receptor is the primary route by which cholesterol is internalized. Thus, we measured LDL receptor internalization by flow cytometry. We showed that internalization of the LDL receptor is elevated in the
absence of PICALM. This portion of the thesis concludes with a possible mechanism by which PICALM alters cellular cholesterol metabolism. The majority of this Chapter appeared in Mercer et al, PLoS ONE, 2015 (2).

Finally, Chapter 5 summarizes our observations and discusses the relationship among PICALM, macroautophagy and cellular cholesterol metabolism. In addition, future directions of these projects and how these studies are relevant to disease will be discussed.
Dedication

I dedicate this document to my family and my dog, Rambis. They are giants and I stood on their shoulders.
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1. Introduction

We will first provide background information about PICALM (Chapter 1). In addition, we will examine two key cellular processes that are relevant to our studies: macroautophagy and cellular cholesterol metabolism. Chapter 3 will describe the relationship between PICALM and macroautophagy, and PICALM’s link to cellular cholesterol metabolism will be discussed in Chapter 4. The dissertation will conclude with a summary of our findings, future directions, and an explanation of how our findings are relevant to disease (Chapter 5).

1.1 PICALM Background

This section will explore the PICALM gene and its initial identification. In addition, PICALM and its cellular functions will also be described. PICALM refers to the human protein, Picalm refers to the mouse protein, PICALM refers to the human gene and Picalm refers to the mouse gene. Unless the text is specifically discussing the mouse protein, PICALM will be used.

1.1.1 Identification of PICALM

The Phosphatidyl-Inositol binding Clathrin Assembly Lymphoid Myeloid (PICALM) gene was initially identified in a chromosomal translocation in a lymphoma cell line (3). PICALM (also termed CALM), is fused to AF10 (also termed MLLT10) as a
result of a t(10;11) chromosomal translocation found in 10% of T-cell acute lymphoid leukemias (4, 5). The specific role and contributions of PICALM in leukemogenesis are still not fully understood. However, recent work from our laboratory has identified a NES (nuclear export signal) within PICALM that is necessary and sufficient for the development of leukemogenesis when it is fused to AF10 (6). In addition, this region of PICALM (in the context of the CALM-AF10 chromosomal translocation) has been shown to be crucial for the upregulation of HOXA gene family members (6). HOXA genes are well characterized as drivers of leukemogenesis, and thus PICALM may influence the development of leukemogenesis by interacting with the nuclear export receptor CRM1/XPO1 through its NES (7).

A mouse model of Picalm deficiency was generated by N-ethyl-N-nitrosurea (ENU) saturation mutagenesis (8). The mutant mice displayed decreased fitness, hence the mutated locus was named fitness1 (fit1); this locus was subsequently shown to coincide with the murine picalm gene. The mutation results in a nonsense mutation that leads to a severe truncation of the murine Picalm protein (8). The truncated form of Picalm has not been documented to have any known function. As a result, these mice have been used as a tool to study the function of Picalm in a ‘knockout’ setting.

fit1 homozygous mice have a shortened life span, severe growth retardation, lowered white blood cell counts, reduced erythroid and myeloid progenitor cell
populations, and severe anemia (8). In addition, these mice also display reduced iron deposition in the liver, and abnormal tissue iron distribution (8). Recently, a constitutive Picalm knockout mouse line was generated and characterized (9). The perturbed endocytosis observed in cells derived from fit1 mice was verified in these constitutive Picalm knockout mouse lines (9). These results further establish that the fit1 mouse model is an appropriate model with which to study PICALM’s basic biological functions.

1.1.2 PICALM and Clathrin Mediated Endocytosis (CME)

PICALM has been well characterized to function as an accessory adaptor protein in clathrin-mediated endocytosis (CME) (10-13). CME is a process by which cells internalize important nutrients such as low-density lipoprotein (LDL), transferrin and growth factors (14). In this process, various adaptor proteins bind to the plasma membrane (14-16). These adaptor proteins help recruit clathrin triskelia in order to form clathrin coated vesicles (16). Following invagination, scission of the vesicles from the plasma membrane occurs. As clathrin coated vesicles mature, the clathrin triskelia and adaptor proteins are removed from the vesicles (14, 15, 17). Newly developed vesicles can ultimately form endosomes, which can be recycled within the cell and move back to the plasma membrane. Alternatively, vesicles can be trafficked through the endosomal-
lysosomal system, where internalized cellular nutrients are processed and degraded (14, 15, 17).

The PICALM protein includes multiple domains that allow it to act as an adaptor protein in CME. These domains have been shown to be important for binding to membrane lipids, and other proteins involved in CME (12, 18, 19). Specifically, PICALM contains an N-terminal region, known as the ANTH domain. This domain allows PICALM to bind to phosphatidyl-inositol 4,5-bisphosphate (PIP5). PIP5 is a phosphatidyl-inositol lipid that is primarily found within the plasma membrane; suggesting that this allows PICALM to bind to the plasma membrane (18-20). PICALM also contains regions that allow it to recruit and interact with AP-2 (12, 21, 22), another important and well-characterized CME protein. Studies have shown that inactivation of AP-2 can result in perturbed CME (23-25). Along with these defined domains, PICALM contains poorly defined regions that mediate binding to clathrin (12). Clathrin recruitment to the plasma membrane enables the formation of clathrin-coated vesicles, ultimately facilitating the internalization of specific cargo proteins (14, 15, 17).

Studies have shown that reduced PICALM levels result in perturbed CME. Specifically, surface expression of the Transferrin Receptor (TfR) is increased when PICALM levels are modulated (9, 12), suggesting that PICALM is an important mediator of clathrin-dependent internalization. In addition, multiple studies identified that the
rate of TfR and EGF receptor internalization from the cell surface is decreased in the absence of PICALM. These results imply that cells are less efficient in clathrin-mediated internalization when PICALM levels are reduced (9, 12, 26).

Other investigations have shown that PICALM overexpression results in perturbed internalization of specific receptors (12, 13), yielding results similar to those seen with PICALM reduction. The exact mechanism by which PICALM overexpression modulates CME is not well understood. However, it is postulated that PICALM overexpression perturbs CME via a dominant negative effect (12, 13). Excess of PICALM may result in the ability of PICALM to bind, squelch and mislocalize clathrin and other adaptor proteins, resulting in perturbed CME (12, 13).

Intriguingly, several studies have shown that PICALM may also play a role in regulating the size and shape of clathrin coated vesicles that form along the plasma membrane. Specifically, PICALM deficiency results in enlarged and elongated endocytic vesicle formation (27-29). These results indicate that PICALM plays a crucial role in the regulation of shape and size of clathrin coated vesicles. In addition, it is speculated that this may involve PICALM’s ability to sense and regulate the curvature of the membrane (27).
1.1.3 Nucleocytoplastic Shuttling of PICALM

PICALM is primarily localized to the cytoplasm. However, multiple studies have shown that PICALM has the ability to undergo nucleocytoplastic trafficking (6, 30). Interestingly, studies have shown that proteins involved in CME undergo nucleocytoplastic shuttling and have transcriptional activity (30-32). Whether or not PICALM has an underappreciated role within the nucleus remains to be understood. However, GAL4 Luciferase assays have shown that PICALM may also have the ability to directly regulate transcription (30). In addition, unpublished GAL4 studies from our laboratory indicate that PICALM has some transcriptional activity. More in-depth studies are necessary in order to gain a better understanding of PICALM’s potential role in transcription.

1.1.4 PICALM and SNARE Proteins

SNARE proteins are membrane-anchored proteins that provide the required specificity and energy that is needed in order for intracellular vesicular fusion to occur (33). Multiple reports have shown that PICALM modulates the internalization of SNARE proteins. PICALM plays a crucial role in the proper internalization of the Vesicle Associated Membrane Protein (VAMP) class of SNARE proteins: VAMP2, VAMP3, VAMP4, VAMP7 and VAMP8. When PICALM levels are reduced, SNARE proteins
remain localized to the plasma membrane. This mislocalization is thought to alter the function of SNARE proteins (1, 28, 34-36).

It is likely that mislocalization of SNARE proteins, in the absence of PICALM, results in defective cellular trafficking. Indeed, studies have shown that PICALM deficiency results in defects in trafficking of the c-kit receptor (37). In addition, VAMP3 and VAMP8 are crucial for the vesicular fusion events involved in cellular trafficking events: endosomal-lysosomal function and macroautophagy (38).

More studies are required to elucidate the cellular effects that occur as a result of impaired SNARE protein internalization and localization in the absence of PICALM. Based on PICALM's ability to modulate the cellular localization of these SNARE proteins, we hypothesized that modulating PICALM levels could alter macroautophagy. Results of these studies will be discussed in more detail in Chapter 3.

**1.1.5 Alzheimer’s Disease and PICALM**

Alzheimer’s Disease is characterized by toxic amyloid beta plaque formation, increased intracellular Tau protein accumulation, and resultant neuronal cell loss (39, 40). Because of these defects, Alzheimer’s Disease patients often exhibit decreased memory and behavior that eventually accelerates death (39, 40). However, the contribution of the amyloid beta proteins and intracellular Tau proteins to Alzheimer’s Disease is still not well understood (39-41).
There are two types of Alzheimer’s Disease: a rare form, known as early-onset Alzheimer’s Disease (EOAD), and the more common form, late-onset Alzheimer’s Disease (LOAD) (39-41). Three genes, the amyloid precursor protein (APP), presenilin 1 and 2 (PSEN1, PSEN2), have been shown to be associated with EOAD. Prior to 2009, Apolipoprotein E (APOE) was the only validated genetic risk factor that was linked to the development of LOAD (42).

Recent Genome Wide Association Studies (GWAS) have reported that multiple single nucleotide polymorphisms (SNPs) in the PICALM allele are associated with the development of LOAD. These SNPs are located upstream of the coding region of PICALM, in exons, and also within PICALM introns (42-45).

The most well characterized risk factor for the development of Alzheimer’s disease is APOε4 (39, 46). This protein is an apolipoprotein that helps regulate the extracellular circulation of cholesterol in the bloodstream (39, 46). Interestingly, meta-analysis has shown that PICALM and APOε4 may synergistically interact to promote the development of LOAD (44, 47). However, more studies are necessary in order to determine how these two risk factors may synergize to promote the progression of LOAD.

The mechanism by which PICALM plays a role in the development of LOAD is not understood and is understudied. Recent reports suggest that PICALM may be
involved in the production of the toxic amyloid beta plaque (21, 22, 48). Specifically, PICALM may facilitate production of the toxic amyloid beta protein by enhancing the internalization of its precursor protein, APP (amyloid precursor protein). APP is processed to form the amyloid beta protein as it enters into the cell by clathrin-mediated endocytosis. Reduction of PICALM levels may lead to lower levels of amyloid beta protein formation by decreased internalization of APP (22).

A recent study has shown that PICALM may be important for modulating the internalization of LRP (Low density lipoprotein receptor) within the blood brain barrier (48). This receptor interacts with the toxic amyloid beta protein and facilitates its removal from the brain. Defects in the internalization of this receptor have been shown to lead to elevated amyloid beta protein formation within the brain. Interestingly, PICALM depletion resulted in altered clearance of amyloid-beta protein from the blood brain barrier, which may be due to defective internalization of LRPI (48).

In addition, studies have shown that PICALM has the ability to modulate the internalization of gamma secretase (49). This enzyme is important for the formation of amyloid beta proteins (49). Moreover, PICALM expression is elevated in human and mouse Alzheimer’s Disease brain tissue (50-52). However, some studies have shown that PICALM expression may be reduced in Alzheimer’s Disease brain tissue (53). More
studies are needed in order to provide a comprehensive analysis of the expression levels of PICALM in Alzheimer’s Disease brain tissue.

More in-depth analyses are necessary to understand how alterations in PICALM can modulate the progression of LOAD. Multiple studies indicate that PICALM plays a role in cellular trafficking, and possibly lipid biology through its ability to modulate SNARE protein internalization and CME, respectively (36, 37, 52). As a result, we set out to define PICALM’s basic biological roles in macroautophagy and cellular cholesterol metabolism. The next sections will provide an introduction to macroautophagy and cellular cholesterol homeostasis and their relationship to disease.

1.2 Macroautophagy

Chapter 3 will focus on the role of PICALM in macroautophagy, thus we will first provide some background information on macroautophagy. The relationship of macroautophagy to SNARE protein biology, lipid metabolism and Alzheimer’s Disease will also be examined in the following sections (1.2.1, 1.2.2, and 1.2.3).

Macroautophagy is an important cellular process that helps cells degrade damaged proteins (54, 55). Macroautophagy is a constitutive and inducible process (55-57). The removal of nutrients (e.g. via serum deprivation) results in the upregulation of macroautophagy, in order to recycle essential nutrients such as proteins and lipids (56-
It has also been shown that excessive macroautophagy can lead to cellular dysfunction and possibly death (59). In addition, Macroautophagy has been linked to disease. Specifically, perturbed macroautophagy has been linked to the development of a variety of diseases such as cancer, metabolic disorders and neurodegeneration (55, 60).

Macroautophagy involves the formation of cytoplasmic double-membrane vesicles, known as autophagosomes (55, 61). Autophagosomes enable the engulfment of proteins, lipids and organelles that are ultimately degraded within the autophagosomal/lysosomal compartment. Lipids and proteins that are used for the formation of autophagosomes come from a variety of sources and organelles such as the plasma membrane and the endoplasmic reticulum (55, 61, 62).

Autophagosomes have the ability to fuse directly to lysosomes. Alternatively, autophagosomes can also fuse to late endosomes. This results in the formation of larger vesicles known as amphisomes, which can then fuse to lysosomes (63). Once autophagosomal/amphisomal lysosome fusion has occurred, degradation of engulfed proteins and lipids occurs via lysosomal hydrolases. Degraded products can be recycled to replenish the cell with specific nutrients. In addition, macroautophagy also helps degrade toxic proteins that may result in the formation of protein aggregates (55, 61).
1.2.1 Macroautophagy and SNARE Proteins

In order for intracellular vesicular fusion events to occur, SNARE (Soluble NSF Attachment Protein Receptor) proteins must be present (33, 64, 65). SNARE proteins are membrane-anchored proteins that provide the energy and specificity that is necessary to mediate vesicular fusion between vesicles and organelles within the cell. SNARE proteins are important for a variety of cellular processes such as fusion of vesicles within the endocytic pathway and fusion of vesicles to the plasma membrane for exocytosis (33, 64, 66). In order for SNARE proteins to function properly, they require proper cellular localization. Without proper SNARE protein localization and function, vesicular fusion events are perturbed (36, 67-70).

As mentioned previously, PICALM is essential for the localization of specific SNARE proteins (VAMP2, VAMP3 and VAMP8) (28, 36). VAMPs (Vesicle associated membrane proteins) are specific SNARE proteins that are essential for mediating vesicular fusion events (33, 36, 64). VAMP3 is localized primarily within the early endosomal compartment within the cell (36). In contrast, VAMP8 is localized to the late endosomal/lysosomal compartment (38, 68). Interestingly, studies indicate that defects in the function and localization of these proteins may contribute to altered trafficking events (38, 67-70). For this dissertation, I will focus on a specific subset of SNARE
proteins (VAMP3 and VAMP8) and their ability to regulate autophagosome and lysosome function in macroautophagy.

1.2.2 Macroautophagy and Alzheimer’s Disease

Macroautophagy is an important process that helps regulate the degradation of damaged proteins, organelles and protein aggregates within the cell (54, 55). The formation of toxic protein aggregates is a common observation that is seen in a wide variety of neurodegenerative diseases (71, 72). Specifically, Alzheimer’s Disease is associated with the formation of two main protein aggregates: amyloid beta protein aggregates that form within the extracellular space of the cell, and intracellular Tau protein aggregates (39).

Macroautophagy perturbation has been shown to occur in Alzheimer’s Disease (54, 73, 74) but its contribution to pathogenesis is not understood. Intriguingly, several studies suggest that perturbed macroautophagy may be linked to the formation of the toxic amyloid beta and Tau neurofibrillary tangles that are commonly associated with the development of Alzheimer’s Disease (73, 75-77). Targeting macroautophagy, with drugs such as Rapamycin, which induces macroautophagy, has been proposed as a possible therapeutic option for the treatment of neurodegenerative diseases such as Alzheimer’s Disease (54, 73). Future studies are warranted in order to determine if
inhibiting or inducing macroautophagy is a viable therapeutic option to treat neurodegenerative diseases such as Alzheimer’s Disease.

### 1.2.3 Macroautophagy and Lipid Homeostasis

Recent studies have shown that macroautophagy and cellular lipid metabolism are interconnected (78-80). Lipid droplets are organelles that store cellular lipids such as cholesterol (cholesterol esters) and fatty acids when nutrients are readily available (79). Numerous recent studies have shown that macroautophagy promotes the breakdown and release of lipids from lipid droplets in order to help restore cellular lipid homeostasis. This process, often referred to as lipophagy, has been shown to be important for regulating lipid metabolism within the liver, as defects in this pathway have been proposed to be linked to the development of liver steatosis and steatohepatitis (79).

In addition, a recent study has shown that SREBP2, a master regulator of cholesterol metabolism, is intimately linked to the regulation of macroautophagy (81). These investigators showed that SREBP2 regulates the expression and induction of macroautophagy genes in lipid-deprived conditions. In the absence of SREBP2 under cholesterol-depleted conditions, the ability of the cell to induce macroautophagy and lipid droplet breakdown was reduced. This highlights the interconnection between the regulation of lipid metabolism and macroautophagy (81).
Lastly, defects in macroautophagy and lipid droplet breakdown have been linked to Alzheimer’s Disease. A recent study has shown that macroautophagy is perturbed in a mouse model of Alzheimer’s Disease (74), and that this perturbation was linked to defects in lipid turnover. In addition, recent studies have shown that the autophagosomal system may be interconnected with the endosomal-lysosomal system to regulate the trafficking of lipids (82, 83). This is clearly seen in lysosomal storage diseases such as Niemman Pick C (NPC) disease which shows defects in both macroautophagy and lipid metabolism (70, 84, 85).

1.3 Cellular Cholesterol Homeostasis

Chapter 4 will focus on our novel findings regarding the ability of PICALM to modulate cellular cholesterol homeostasis. Here, we present a background on cholesterol metabolism (cellular cholesterol internalization and cellular cholesterol biosynthesis) and its relationship to Alzheimer’s Disease (sections 1.3.1, 1.3.2, and 1.3.3).

Cells require cholesterol for survival. About 90% of cellular cholesterol is primarily found within the plasma membrane (86-88). Plasma membrane cholesterol primarily helps to regulate the rigidity and permeability of the membrane. This is essential to prevent the diffusion of specific molecules across the plasma membrane (87, 88). In addition, cholesterol can be found in specific regions of the plasma membrane,
known as lipid rafts. Lipid rafts are primarily composed of cholesterol and sphingolipids. Research has shown that lipid rafts may play important roles in multiple signaling processes (87, 89).

Cells can obtain cellular cholesterol through two primary mechanisms: internalization of cholesterol from the extracellular space, or de novo cholesterol biosynthesis (87, 90-93). In addition, cholesterol can be removed from the cell via cholesterol efflux transporters (87). This overview of cellular cholesterol metabolism will focus on cholesterol internalization and cholesterol biosynthesis.

1.3.1 Cellular Cholesterol Internalization

Cells internalize cholesterol primarily through CME of LDL (low density lipoprotein) (90, 93, 94). The receptors responsible for the internalization of LDL-cholesterol constitute the LDL receptor family that includes at least 10 members (94-96). Since cholesterol, in the form of LDL, is internalized, it is moved within the cell through the endosomal-lysosomal system (86, 93).

When LDL reaches the lysosomal compartment, it is broken down by lysosomal hydrolases, which allow cholesterol to be released within the lysosome (93, 97, 98). This allows the Niemann-Pick C (NPC) proteins to bind to cholesterol, and act in tandem to efficiently release cholesterol from the lysosome (Figure 1) (98-100). Specifically, NPC2 binds to cholesterol in the lumen of the lysosome. It then transfers cholesterol to the
NPC1 protein. This allows efficient transfer of cholesterol from the lysosome to the cytoplasm (Figure 1). Proper trafficking of cholesterol through this system is essential, as defects can lead to altered cholesterol metabolism and disease (98-101).

**Figure 1: Cellular Cholesterol Internalization**

LDL lipoproteins contain cholesterol that is internalized by the LDL receptor. This internalization is mediated by clathrin-mediated endocytosis. Once internalized, LDL is trafficked through the endosomal-lysosomal system. The LDL is broken down in the lysosome by lysosomal hydrolases. This enables cholesterol to be released into the lumen of the lysosome where it will be bind to NPC2. NPC2 transfers cholesterol to NPC1. Upon this transfer, NPC1 facilitates the release of cholesterol into the cytoplasm.

Once cholesterol is in the cytoplasm, it is trafficked via vesicular and non-vesicular trafficking to different regions of the cell. Nonetheless, some cellular cholesterol is trafficked to the endoplasmic reticulum, which houses key components of cholesterol regulation (87, 92, 93, 101).
1.3.2 SREBP2 and Cellular Cholesterol Biosynthesis

The main proteins within the endoplasmic reticulum that help regulate cholesterol internalization and biosynthesis are the SREBP (Sterol Regulatory Element Binding Protein), SCAP (Sterol Cleavage Activating Protein), and INSIG (Insulin-Induced Gene Protein) proteins (92, 102).

SREBPs encode for three different isoforms. SREBP1 encodes for SREBP1a and SREBP1c from the same promoter. SREBP2 is regulated by a promoter that is distinct from SREBP1 (92, 102, 103). SREBP1a primarily activates the transcription of genes involved in fatty acid synthesis. In addition, it has the ability to regulate genes involved in cholesterol metabolism (102). SREBP1c exclusively regulates genes involved in fatty acid synthesis (102, 103). SREBP2 is the main transcription factor that regulates cellular cholesterol metabolism (92, 102, 103). The remainder of the dissertation will focus specifically on SREBP2.

When cholesterol is efficiently internalized, it moves to the endoplasmic reticulum as discussed above (Figure 2) (section 1.3.1) (101, 102). The levels of cholesterol within the endoplasmic reticulum play a key role in regulating cellular cholesterol homeostasis (104). Brown and Goldstein showed that when cholesterol comprises more than 5% of the total lipid levels within the endoplasmic reticulum, SREBP2 trafficking and activation are prevented (Figure 2). However, when cholesterol...
levels fall below 5% of total endoplasmic reticulum lipid levels, SREBP2 is trafficked to the nucleus. This allows it to activate the transcription of cholesterol biosynthesis genes and genes involved in cholesterol internalization (Figure 3) (104).

**Figure 2: SREBP2 and Cellular Cholesterol Trafficking**

When cholesterol is internalized by clathrin-mediated endocytosis, it is trafficked through the Endosomal-Lysosomal system. When cholesterol is efficiently trafficked to the Endoplasmic Reticulum, this prevents SREBP2 from being trafficked to the nucleus. This reduces cholesterol biosynthesis gene expression and as a result, controls cholesterol biosynthesis to help maintain cellular cholesterol levels.
Figure 3: Low Cholesterol Levels Leads to SREBP2 Activation

When cellular cholesterol trafficking is perturbed, this results in reduced cholesterol levels within the Endoplasmic Reticulum. As a result, SREBP2 is cleaved and trafficked to the nucleus to activate cholesterol biosynthesis gene expression. This increases cellular cholesterol biosynthesis to restore cellular cholesterol levels.

The proteins that are essential for the trafficking of SREBP2 to the nucleus include the SCAP and INSIG proteins (92, 102). When cholesterol levels within the endoplasmic reticulum are high (above 5% of the total ER lipid levels), SCAP remains bound to cholesterol. In addition, INSIG binds to SREBP2 and SCAP, forming a ternary complex. This complex formation prevents specific COPII proteins from being able to recognize SCAP, and prevents the transport of SREBP2 from the ER to the Golgi apparatus and nuclear regions. However, when endoplasmic reticulum cholesterol

20
levels are low (below 5% of the total ER lipid levels), SCAP and INSIG undergo conformational changes that prevent the formation of the INSIG, SCAP and SREBP2 complex. This enables the region of SCAP that binds to COPII proteins to be recognized. This allows SCAP and SREBP2 to move to the Golgi apparatus via COPII transport. Upon arrival at the Golgi, SREBP2 is cleaved by two proteases (protease 1 and 2) in order to release its transcriptionally active form. Once this mature and active form of SREBP2 has been released from the Golgi, it is trafficked to the nucleus in order to regulate transcription of cellular cholesterol genes (92, 102-104).

When cellular cholesterol internalization is insufficient, SREBP2 nuclear localization is induced in order to activate the transcription of the cholesterol biosynthesis genes as described above, resulting in increased rates of cholesterol biosynthesis (102, 105). All cells synthesize cholesterol from Acetyl-CoA. The conversion of Acetyl-CoA to cholesterol requires 20 or more steps (92, 93).

In order for cells to efficiently convert Acetyl-CoA to Cholesterol, the enzymes in the pathway must be active. Most notably, HMGCR (HMG-CoA Reductase) is the rate-limiting step of this pathway (92, 106). HMGCR is targeted by a class of drugs known as statins. Statins are prescribed in order to help prevent and treat atherosclerosis, which is associated with high blood cholesterol levels (106-110). This inhibition has been shown to reduce cholesterol biosynthesis and requires cells to elevate LDL receptor
internalization to restore normal cholesterol levels. The increased internalization of the LDL receptor helps reduce circulating cholesterol within the bloodstream (110). Intriguingly, some studies suggest that statins could be used as a therapy to treat Alzheimer’s Disease. However, more studies are necessary in order to confirm these results (111-113).

1.3.3 Cholesterol Metabolism and Alzheimer’s Disease

It is estimated that 25 percent of the total body cholesterol is localized within the brain (114). In addition, cholesterol within the brain is derived primarily from \textit{in situ} synthesis, since plasma lipoproteins are unable to cross the blood brain barrier (91). The lipoproteins that circulate within the brain are composed of cholesterol, phospholipids and fatty acids. These lipoproteins are primarily synthesized by glial cells (astrocytes). Glial cells (oligodendrocytes) also synthesize cholesterol, as it is a key component of the myelin sheath that surrounds and insulates neuronal axonal membranes (115). Neuronal cells are thought to mostly rely on circulating lipoproteins within the brain, and not synthesize their own cholesterol (116).

Perturbations of cholesterol metabolism are associated with a wide variety of diseases such as atherosclerosis, cancer, obesity, and dementias, including Alzheimer’s Disease (86, 117). As indicated previously, Alzheimer’s Disease is characterized by extracellular amyloid beta (Aβ) plaques, intracellular Tau hyperphosphorylation,
neuronal cell loss (39). A number of observations point to a role for cholesterol in the pathogenesis of AD: (a) increased levels of plasma cholesterol promote the amyloidogenic processing of APP; (b) increased intracellular cholesterol levels modulate the formation of extracellular Aβ plaques; (c) SNPs in APOE have been identified as susceptibility loci for late-onset AD; and (d) Aβ plaques found in the brains of AD patients are highly enriched in cholesterol. Furthermore, treating animals with cholesterol-lowering statins, or adding these agents to cell culture, results in decreased Aβ production (118-122). Finally, inhibition of ACAT, which plays a role in cholesterol homeostasis by converting free cholesterol to esterified cholesterol, has been shown to reduce the formation of Aβ plaques (123-125).

1.4 Overarching Hypothesis and Significance

PICALM has been described to act as a regulator of VAMP3 and VAMP8 SNARE protein internalization and localization (28, 36). These SNARE proteins play roles in cellular trafficking and macroautophagy (36, 38, 68). Based on this analysis, we sought to determine if altering levels of PICALM result in perturbed macroautophagy, as a result of SNARE protein mislocalization.

Understanding PICALM’s potential role in macroautophagy can provide insight on pathogenesis. GWAS studies have shown that PICALM SNPs are associated with the development of Alzheimer’s Disease (42-44) and defects in macroautophagy have also
been related to Alzheimer’s Disease (54, 73, 74). As a result, studying a novel role for PICALM in macroautophagy may identify a potential mechanism by which PICALM contributes to the progression of Alzheimer’s Disease.

In addition to studying macroautophagy, we conducted a microarray analysis using PICALM-deficient and PICALM expressing cells. The microarray analysis showed that modulating levels of PICALM results in perturbed cholesterol biosynthesis gene expression, indicating that cholesterol biology is altered as a result of the modulation of PICALM levels. As a result of these studies, we hypothesized that modulating PICALM expression levels may perturb cellular cholesterol homeostasis. To gain a more in-depth appreciation for how PICALM modulates cholesterol homeostasis, we studied the ability of PICALM to modulate cholesterol biosynthesis gene expression, cellular cholesterol levels and the internalization and synthesis of cellular cholesterol.

Defects in cholesterol metabolism have been linked to the development of Alzheimer’s Disease (125). Studying PICALM’s role in cellular cholesterol homeostasis may provide an additional mechanism by which PICALM perturbation might contribute to the development of Alzheimer’s Disease.
2. Materials and Methods

2.1 Ethics Statement

The *in vivo* and euthanasia procedures in this study were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize animal suffering.

2.2 Animal Welfare

The animal studies described here have been approved by the Duke University Institutional Animal Care and Use Committee (IACUC) (Protocol # A021-13-01). Adult mice were euthanized by exposing them to CO$_2$ and fetuses were euthanized by decapitation. None of the experiments performed involved animal suffering.

2.3 Cell Culture

HEK293 cells (ATCC, catalog #CRL-1573), HeLa, and retroviral packaging Plat-E cells (Reference Kitamura paper) were maintained in DMEM (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 1% penicillin/streptomycin (Gibco, Life Technologies) and glutamine (?). CAD neuronal cells (a gift from Dona Chikarishi, Duke University) were cultured in
DMEM/F12 (Gibco, Life Technologies) supplemented with 8% fetal bovine serum (FBS), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). HEK293 and Plat-E cells were transfected by calcium chloride transfection. The HEK293 cells transfected with pRSMX vectors were selected with puromycin, 5ug/ml. (Sigma-Aldrich, St. Louis, MO, USA). HeLa cells were transfected with the pRSMX vectors using Lipofectamine (Life Technologies) and were selected with puromycin (5ug/ml). The CAD and primary glial cells were infected by co-culture with filtered (0.45 uM filter) Plate-E supernatant in the presence of polybrene (Sigma-Aldrich) (2 ug/ml).

PICALM overexpression experiments in HEK293 cells were performed using human PICALM cDNA, subcloned into the bicistronic MSCV-IRES-eGFP (MIE) retroviral vector. Control HEK293 cells were transfected with the MIE empty vector. Primary glial cells were isolated from the cortex of E14 fit1 Picalm<sup>fit1-5R</sup> mouse embryo (8). The fit1 Picalm<sup>fit1-5R</sup> mice have a mutation that codes for a non-functional PICALM protein (8). However, only PICALM wild type embryos were analyzed in the PICALM shRNA knockdown experiments. Cells were dissociated, plated and grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All experiments using Bafilomycin (Enzo Life Sciences) were conducted for 4 hours at 400 nM concentration. All experiments involving serum removal to induce macroautophagy were conducted in the absence of FBS (0% FBS).
Hematopoietic cells were generated by immortalizing fetal liver cells that were harvested from E14 fit1 Picalm^{fit1-5R} mouse embryos (12). The fit1 Picalm^{fit1-5R} mice have a mutation that codes for a non-functional PICALM protein missing 82% of its amino acid sequence, herein referred to as Picalm^{fit1-5R} (8). Fetal liver cells were retrovirally transduced with MLL-ENL using the MIE vector (126) and serially replated in methylcellulose culture to select immortalized hematopoietic progenitors as previously described. Cells were maintained in liquid culture in RPMI medium supplemented with 10% FBS, glutamine, penicillin and streptomycin (Invitrogen) with recombinant murine interleukin-3 and stem cell factor (5 ng/ml and 50 ng/ml, respectively, Peprotech, Rocky Hill, NJ, USA).

2.4 DNA Constructs and Vectors

Picalm shRNA were expressed using the pRSMX retroviral vector (kindly provided by Louis Staudt, NIH) (127). An shRNA directed against luciferase (5'-GTGGATTTCGAGTCGTCTTTAAT-3') was used as a negative control. Two shRNAs were used to knock down PICALM in human cells. Picalm shRNA4 (5'-GCCTTAATGTTGACTTTGAAT-3'), Picalm shRNA15 (5'-GAGCACAGATACAGTTTAT-3') were used in HEK293 cells. Only shRNA4 was used to knockdown PICALM in HeLa and primary glial cells. Picalm shRNA10 (5'-GAAATGGAACCACTAAGAA-3') was used in CAD neuronal cells in addition to
shRNA4. All shRNAs were cloned into HindIII and BglII sites or pRSMX. Picalm knockout fetal liver hematopoietic cells were rescued by retroviral transduction of human PICALM cDNA using the MSCVneo retroviral vector (128). Control cells were infected with the MSCVneo empty vector. Transduced cells were selected in G418 ( Geneticin; Gibco, Life Technologies). Transfections into HEK293 cells were performed using calcium chloride transfection (129). PICALM overexpression experiments in HEK293 cells were performed using human PICALM cDNA (12) subcloned into the bicistronic MIE retroviral vector.

2.5 Antibodies and Reagents

The following reagents were used: rabbit anti-PICALM antibody (HPA019061, Sigma-Aldrich, 1:1,000), rabbit anti-actin antibody (AC-40, Sigma-Aldrich, 1:1,000), rabbit anti-LC3II antibody (2775, Cell Signaling, 1:1,000), rabbit anti-p62 antibody (NBP1-49954, Novus Biologicals, 1:2,000), rabbit anti-SREBP2 antibody (ab28482, 1:1,000, Abcam, Cambridge, MA, USA), IRDye680-conjugated donkey anti-rabbit antibody (926-68073, 1:5,000, LI-COR, Biosciences, Lincoln, NB, USA). PE-conjugated mouse anti-human LDLR antibody (FAB2148P, 1:20, R&D Systems, Minneapolis, MN, USA), PE-conjugated mouse IgG Isotype antibody (IC002P, 1:20, R&D Systems).
2.6 Western Blot Analysis

Cells were lysed in Laemmli buffer and lysates were resolved by SDS–PAGE electrophoresis (using 7.5% acrylamide gels) and transferred to polyvinylidene difluoride membranes (IPFL00010, Millipore, Billerica, MA, USA). Membranes were incubated with the appropriate antibody overnight in Odyssey Blocking Buffer (Li-Cor) with 0.1% Tween-20. Membranes were washed in TBS with 0.1% Tween-20. Secondary antibody incubation was performed in Odyssey Blocking Buffer with 0.1% Tween-20 and 0.02% SDS for 30 min. Membranes were washed and proteins were quantitated using an Odyssey Infrared fluorescence imaging system (Li-Cor).

2.7 Microarray

Total RNA was isolated using Qiagen RNeasy® Micro kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol and analyzed by Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA). Array data were then normalized by RMA for further analysis. To identify differentially expressed genes, we applied the SAM 4.01 Excel Add-In that provides the estimate of False Discovery Rate (FDR) for multiple testing. Using an FDR threshold of 12.3%, we identified 343 probe sets, whose expression values were extracted, mean-centered (130) and clustered by Cluster 3.0 and displayed by Treeview v1.6 as done in (131). These selected genes were then deposited into GATHER (gather.genome.duke.edu) (132) to determine the enrichment for the Gene
Ontology (GO) and KEGG (KEGG). Specifically, KEGG pathway analysis was used in this study. All array analysis was conducted using log₂ scale. The data discussed in this document have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE64855.

2.8 Quantitative RT-PCR Analysis

Total RNA was isolated from MEFs using RNase QiaMini Kit (Qiagen). RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol. Real time quantitative PCR was performed using iQ Sybr-Green Mix (Bio-Rad) according to the manufacturer’s protocol. Amplification data were collected using the iQ5 Optical System (Bio-Rad). The expression levels of cholesterol biosynthesis genes were normalized to beta 2-microglobulin (β2M) and then to empty vector or shRNA luciferase control cells by the ΔΔCT method.

Table 1: Sequences of murine primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hmgcr</strong></td>
<td>(fwd)-5’-TGGCACCATGTCAGGCGTCCG-3’&lt;br&gt; (rev)-5’-AGCGACACACAGGCGGAAA-3’</td>
</tr>
<tr>
<td><strong>Hmgs</strong></td>
<td>(fwd)-5’-GTCTTGCCACAGTACTCACTACC-3’&lt;br&gt; (rev)-5’-ACACTCCAACCTCTCCCT-3’</td>
</tr>
<tr>
<td><strong>Pmvk</strong></td>
<td>(fwd)-5’-CTATGGGGCTGTGATACAGA-3’&lt;br&gt; (rev)-5’-CTCCTGGTGTGGCCCTTACC-3’</td>
</tr>
<tr>
<td><strong>Fdps</strong></td>
<td>(fwd)-5’-GGGAACCAGAATTGGATGCT-3’</td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence (5’ - 3’)</td>
</tr>
<tr>
<td>------</td>
<td>-------------------</td>
</tr>
</tbody>
</table>
| Fdft1 | (fwd)-5’-AGGCATTGTACTCTAGGACCTC-3’  
(rev)-5’-TGCGGTCCGTATGTCACCTCCCA-3’ |
| Cyp51 | (fwd)-5’-GCTGGCCTGGGACCGGACGAAA-3’  
(rev)-5’-CCGGAGCTGCATCTCAGGACG-3’ |
| Nsdhl | (fwd)-5’-CGACCTTGCAAACCACGGACC-3’  
(rev)-5’-ACTGTACGGCGGAGGGGACG-3’ |
| LDLR  | (fwd)-5’-CCGGAGTTGCAGAAGACTCAT-3’  
(rev)-5’-GAGTCAGGAATGCATCGGCT-3’ |
| SREBP2| (fwd)-5’-AGCTGCACATCAAGGGAAG-3’  
(rev)-5’-TGTGCACATCTGAACAGGCA-3’ |
| β2M   | (fwd)-5’-ACCGGCTCTGATGTCTATCCAGAAA-3’  
(rev)-5’-GGTGAATTCACTGTGAGCCAGGAT-3’ |

**Table 2: Sequences of human primers used for qPCR**
2.9 Surface Expression and Internalization Assays

HEK293 cells growing exponentially were trypsinized with 0.05% Trypsin/EDTA (Invitrogen) until approximately 30% of cells lifted off the plate. Trypsinization was stopped with serum-containing ice cold media. Cells were washed with cold PBS and resuspended in HFN (Hanks Balanced Salt Solution, FBS (2%), Sodium Azide 0.02%) containing rat IgG (1 µg/ml) for 20 min. Cells were then stained with anti-human LDLR-PE or mouse PE-conjugated IgG Isotype control for 30 minutes on ice. Cells were washed twice with cold PBS and then directly analyzed by flow cytometry (C6, Accuri, Ann Arbor, MI, USA) to measure LDL receptor surface expression by quantification of the mean fluorescence intensity. For internalization assays, cells stained with antibodies and rinsed with PBS were divided into multiple tubes and incubated at 25°C for indicated time points to induce internalization. Cells were incubated at 25°C rather than 37°C to slow down LDL receptor internalization and improve accuracy of measurements. Ice-cold acid wash buffer (0.5 M NaCl/0.2 M acetic acid) was added to halt receptor internalization and strip residual LDL receptor from the cell surface. Cells were washed twice with cold PBS and flow cytometry was used to measure the mean fluorescence intensity emitted by internalized LDL receptor molecules. The rate of LDL
receptor internalization was calculated by dividing the LDL receptor internalized at each time point by the initial LDL receptor surface expression.

### 2.10 Lipid Quantitation and $^{13}$C Tracer Analysis

HEK293 cells were cultured in glucose-free DMEM (Gibco, Life Technologies) supplemented with 10% FBS, 1% pen/strep, and 25 mM glucose. Unlabeled cells were cultured in natural glucose (Sigma-Aldrich), while labeled cells were cultured in a 1:1 molar ratio of natural to U-$^{13}$C$_6$-glucose (110187-42-3, Cambridge Isotopes, Andover, MA, USA). To reach steady state, cells were cultured in natural or label-containing medium for 6 d. The medium was changed every 12 h. Cells were grown at the same cell density and were maintained in a subconfluent state by splitting every 48 h. After 6 d (144 h) in natural or label-containing medium, cells were collected, counted, washed, and frozen at -80°C. To obtain growth curves, HEK293 shRNALuc and shRNA4 cells were grown in quadruplicate in the natural and label-containing medium after splitting the cells on d4. Cells were counted 24 and 48 h after the initial plating in order to measure cellular growth rates. Doubling time was determined by fitting cell counts at 24 h (120 h – d5) and 48 h (144 h – d6) to an exponential curve using Excel (Microsoft). Calibration curves were made using the GLC-96 fatty acid methyl ester (FAME) mix (Nu-Chek Prep), methyl cis-vaccenate (Nu-Chek Prep), and cholesterol (Sigma). 4 μg of trinonadecanoin (Nu-Chek Prep) and stigmastanol (Sigma-Aldrich) were added in 400
µL of toluene to each sample as internal standards. 3 mL of methanol and 600 µL of 8% (w/v) HCl in methanol were added to each sample before incubating at 45°C for 16 h. 2 mL of 0.04 M aqueous NaCl was added to the reaction mix and FAMEs and free sterols were extracted twice using 2 mL of n-hexane. The combined organic phases were dried under vacuum (EZ-2 Elite) and dissolved in 300 µL n-hexane. One hundred µL of this solution was used directly for FAMEs analysis via GC/MS. 150 µL of this solution was dried again under vacuum and dissolved in 25 µL of 1:1 (v/v) pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane 99:1 (Sigma-Aldrich). The resulting trimethylsilyl derivatives were directly analyzed via GC/MS.

Data was collected on an Agilent 5975C MSD (Agilent Technologies, Santa Clara, CA, USA) connected to an Agilent 7890A Gas Chromatograph (Agilent). FAMEs were analyzed on the Agilent DB-WAX column (122-7032). Trimethylsilyl ether sterols were analyzed on a ZB-MR-1 column (7HG-G016-11, Phenomenex, Torrance, CA, USA). GC/MS settings and oven programs are available upon request. Ions monitored for each analyte are provided in table #3. Area under the curve (AUC) quantitation was conducted on ChemStation software (Agilent). Absolute quantitation of fatty acids and cholesterol was achieved by first normalizing the analyte AUC (sum of all collected isotopologues) to the internal standard AUC (M+0 only). This ratio was fit to the appropriate calibration curve and then normalized to cell number. The relative contributions of synthesis and scavenging were determined by fitting the isotopologue
distributions at steady state (>5 divisions in label media) by modeling the isotopologue distribution as described. Note that for 16:1(n-7), the “scavenged” population includes scavenged 16:0 desaturated by stearoyl-CoA desaturase 1 (SCD1) inside the cell. Net accumulation rates (R) were calculated using pool size (p), doubling time (d), and percent contributions (c) using equation 1. “Net rate” for a given metabolic parameter (e.g. synthesis) is defined as the gross rate minus the rate of loss due to modification/breakdown/export.

Equation 1:

\[ R = \ln(2) \frac{cp}{d} \]

Table 3: List of Analytes and Internal Standards for GC/MS

<table>
<thead>
<tr>
<th>Analyte or Internal Standard</th>
<th>Fatty Acid Notation</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl myristate</td>
<td>14:0</td>
<td>242-254</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>16:0</td>
<td>270-284</td>
</tr>
<tr>
<td>Methyl palmitoleate</td>
<td>16:1(n-7)</td>
<td>268-282</td>
</tr>
<tr>
<td>Methyl stearate</td>
<td>18:0</td>
<td>298-314</td>
</tr>
<tr>
<td>Methyl oleate</td>
<td>18:1(n-9)</td>
<td>296-312</td>
</tr>
<tr>
<td>Methyl cis-vaccenate</td>
<td>18:1(n-7)</td>
<td>296-312</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>18:2(n-6)</td>
<td>292-300</td>
</tr>
<tr>
<td>Methyl nonadecanoate</td>
<td>19:0</td>
<td>312</td>
</tr>
<tr>
<td>Methyl arachidate</td>
<td>20:0</td>
<td>326-342</td>
</tr>
<tr>
<td>Methyl cis-11-eicosenoate</td>
<td>20:1(n-9)</td>
<td>324-340</td>
</tr>
<tr>
<td>Methyl cis-11,14-eicosadienoate</td>
<td>20:2(n-6)</td>
<td>318-326</td>
</tr>
<tr>
<td>Methyl dihomo-γ-linolenate</td>
<td>20:3(n-6)</td>
<td>318-326</td>
</tr>
<tr>
<td>Methyl arachidonate</td>
<td>20:4(n-6)</td>
<td>316-324</td>
</tr>
<tr>
<td>Methyl behenate</td>
<td>22:0</td>
<td>354-370</td>
</tr>
<tr>
<td>Methyl erucate</td>
<td>22:1(n-9)</td>
<td>352-368</td>
</tr>
<tr>
<td>Methyl cis-13,16-docosadienoate</td>
<td>22:2(n-6)</td>
<td>350-366</td>
</tr>
<tr>
<td>Methyl lignocerate</td>
<td>24:0</td>
<td>382-398</td>
</tr>
<tr>
<td>Methyl nervonate</td>
<td>24:1(n-9)</td>
<td>380-396</td>
</tr>
<tr>
<td>Cholesterol trimethylsilyl</td>
<td>n/a</td>
<td>456-477</td>
</tr>
</tbody>
</table>
ether & Stigmastanol trimethylsilyl ether & n/a & 488

### 2.11 Statistical Analysis

Standard, two-tailed Student’s t-Test analysis was used for all statistical analysis.

Values represent means ± SEM from at least three independent experiments. p values less than 0.05 were considered statistically significant.
3. PICALM Modulates Macroautophagy

This chapter will describe the role of PICALM in regulating macroautophagy, and contains results that were obtained in collaboration with David Rubinsztein’s laboratory at Cambridge University (Cambridge, UK). This collaboration resulted in a manuscript that was published in *Nature Communications*: Kevin Moreau et al. PICALM Modulates Autophagy Activity and Tau Accumulation. Nature Communications. 22 September 2014 (1).

All data shown below were produced by me the experiments that were performed by collaborators are not included here. The following text is distinct from that of the published manuscript.

3.1 Introduction

Macroautophagy is a cellular process that helps regulate cell survival under conditions of nutrient deprivation (55-57). It is essential for the removal of dysfunctional organelles and proteins to prevent the formation of toxic aggregates within cell (54, 73). Macroautophagy involves the formation of double membrane vesicles known as autophagosomes. These autophagosomes form within the cytoplasm and include various cargoes, such as proteins, lipids and organelles. Autophagosomes eventually fuse with lysosomes to promote the degradation of the engulfed cargoes. This allows for
the cell to recycle important cellular nutrients, or remove damaged proteins and organelles (55-57, 62).

The perturbation of macroautophagy is well described in disease and involved in the development of cancer, atherosclerosis, and neurodegenerative diseases such as Alzheimer’s Disease (54, 55, 59, 133). This warrants investigations to better understand how macroautophagy is regulated. The discovery of novel regulators of macroautophagy may lead to the identification of therapeutic targets to treat different disease states.

PICALM is an accessory adaptor protein involved in clathrin-mediated endocytosis of specific receptors such as those for Transferrin (TfR) and EGF (EGFR) (9, 12, 26). GWAS studies have shown that SNPs in PICALM are risk factors for the development of Alzheimer’s Disease (42-44). Recent studies have shown that altered expression of PICALM is found in Alzheimer’s Disease mouse models and Alzheimer’s Disease patients (50-53). In addition, PICALM may alter the formation of the toxic amyloid beta protein thought to contribute to the development of Alzheimer’s Disease (22, 48). However, more studies are necessary in order to determine possible mechanisms by which PICALM mutations contribute to Alzheimer’s Disease.

Recent investigations have shown that PICALM modulates the internalization and localization of SNARE proteins that play essential roles in vesicular trafficking within the cell (28, 36). Specifically, PICALM modulates the localization of VAMP3 and
VAMP8. In the absence of PICALM, these SNARE proteins remain on the plasma membrane (36). In addition, both VAMP3 and VAMP8 have been shown to be important in macroautophagy. These SNARE proteins play roles in regulating the vesicular fusion of autophagosomes to endosomes and lysosomes (38, 67, 68, 85), and altered function of VAMP3 and VAMP8 can lead to perturbed macroautophagy (38, 67, 68, 85). Since PICALM reduction alters the localization of VAMP3 and VAMP8, we sought to determine whether modulation of PICALM levels results in altered macroautophagy. Studying PICALM’s role in macroautophagy will help delineate novel mechanisms by which PICALM may contribute to disease.

3.2 PICALM Reduction Alters LC3II Levels

We performed shRNA knockdown experiments in HEK293 cells using two separate shRNAs targeting PICALM. We measured levels of LC3II as a marker of autophagosome levels, as changes in LC3II levels are an indication of altered numbers of autophagosomes (57). Interestingly, cells with reduced PICALM levels displayed increased LC3II levels, indicating that macroautophagy is altered (Figure 4). To show that this was not limited to particular growth conditions and cell type, we induced macroautophagy by starving cells of serum for 1 hour in both HEK293 (Figure 5) and HeLa cells (Figure 6). LC3II levels in PICALM deficient cells were elevated in both normal and serum starved conditions (Figures 5 and 6). These results indicate that
PICALM affects macroautophagy both at baseline conditions and also when macroautophagy is induced.

Figure 4: PICALM shRNA knockdown results in elevated LC3II Levels

Levels of LC3II measured by immunoblot analysis in HEK293 cells expressing shRNA4, shRNA15 and shRNA Luc control in full serum (10% FBS) conditions. All western blot analysis was conducted in three independent experiments. Quantitation of the analysis, normalized to shRNA Luc control cells, is shown below each western blot, p*<0.05.
Figure 5: PICALM shRNA knockdown elevates LC3II levels in normal and stressed conditions in HEK293 cells

Levels of LC3II measured by immunoblot analysis in full serum (10% FBS) and serum deprived conditions (no FBS) for 1h. Measurements were conducted in HEK293 cells expressing shRNA4 and shRNALuc control. All western blot analysis was conducted in three independent experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below each western blot, p*<0.05. Figure 1A, Moreau, et al. Nature Communications, 2014.
Levels of LC3II measured by immunoblot analysis in full serum (10% FBS) and serum deprived conditions (no FBS) for the indicated time points in HeLa cells. All western blot analysis was conducted in at least three independent experiments. Quantitation of the analysis is shown, normalized to shRNALuc control cells, below each western blot, p*<0.05. Figure 1A, Moreau, et al. Nature Communications, 2014.

To gain insight into PICALM’s ability to modulate macroautophagy in cell types of the central nervous system, we used CAD neuronal cells (Figure 7) and primary glial cells isolated from mice at embryonic day 14 (Figure 8). As expected, reduction of PICALM expression results in elevated LC3II levels in CAD neuronal cells (Figure 7) and primary glial cells (Figure 8).
Figure 7: PICALM shRNA knockdown results in elevated LC3II levels in CAD neuronal cells

Levels of LC3II measured by immunoblot analysis in full serum (8% FBS) and serum deprived conditions (no FBS) for 0.5h in CAD neuronal cells. All western blot analysis was conducted in three independent experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below each western blot, p*<0.05. Figure 1A, Moreau, et al. Nature Communications, 2014.
Figure 8: PICALM shRNA knockdown results in elevated LC3II levels in primary glial cells

Levels of LC3II measured by immunoblot analysis in full serum in primary glial cells. All western blot analysis was conducted in three independent experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below each western blot, p*<0.05.
3.3 PICALM Perturbation Does Not Alter LC3II Levels in the Presence of Bafilomycin

A change in LC3II levels indicates that autophagosome levels and macroautophagy are being altered. However, this does not discriminate whether elevation of LC3II results from increased autophagosome formation or reduced breakdown. In order to gain a better understanding of how PICALM modulates autophagosome levels, we used Bafilomycin to inhibit the breakdown of autophagosomes. Bafilomycin inhibits lysosomal proteases, which prevents the breakdown of autophagosomes from occurring (57, 134); thus enabling the study of autophagosome formation (57). Elevation of LC3II in the presence of Bafilomycin suggests perturbation of autophagosome formation. On the other hand, elevation of LC3II levels in the presence of Bafilomycin implies that autophagosome breakdown, and not autophagosome formation, is altered (57).

Knockdown of PICALM caused an elevation of LC3II levels in normal serum conditions (Figure 9, lanes 1 and 2), as previously observed (Figure 4). However, in the presence of Bafilomycin (400 nM for 4 hours), there was no change in LC3II levels (Figure 9, lanes 3 and 4). This was true for both normal and serum starvation (no serum x 1 hour) conditions (Figure 9, lanes 5 through 8). These results suggest that PICALM modulates autophagosome breakdown, but does not have a detectable effect on autophagosome formation in HEK293 cells.
Figure 9: PICALM shRNA knockdown does not alter LC3II levels in the presence of Bafilomycin

LC3II levels were measured by immunoblot analysis in the presence or absence of Bafilomycin (400 nm). Experiments were performed in full serum (10% FBS) and serum removed conditions (no FBS) for 1 hour. All western blot analysis was conducted in three separate experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below the western blot, p*<0.05. HE=High Exposure. LE=Low Exposure.

3.4 PICALM Reduction Leads to Defects in the Clearance of p62

Autophagosome clearance within cells can be studied by evaluating the levels of the p62 protein (57, 135). The p62 protein binds to and recruits ubiquinated proteins to the autophagosome to promote their degradation. As the autophagosome fuses with the
lysosome, p62 is degraded, leading to lower levels of p62. When macroautophagy occurs efficiently, levels of p62 should be reduced. However, defects in autophagosome breakdown are associated with elevated p62 levels as a result of inefficient p62 degradation (1, 57, 136).

To determine the impact of PICALM on p62, we knocked down PICALM expression in HEK293 and CAD neuronal cells using shRNAs. As expected, p62 levels were increased in HEK293 cells (Figure 10). This was true for both normal and serum starved conditions (1 hour). Similarly, reduction of PICALM levels in CAD neuronal cells resulted in elevated p62 levels (Figure 11). These results provide additional evidence that loss of PICALM leads to defects in autophagosomal breakdown.
Figure 10: PICALM shRNA knockdown results in elevated p62 levels in HEK293 cells

p62 levels were measured by immunoblot analysis in HEK293 cells. Experiments were performed in full serum (10% FBS) and serum removed conditions (no FBS) for 1h in HEK293 cells. All western blot analysis was conducted in three separate experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below the western blot, p*<0.05. Figure 2B, Moreau, et al. Nature Communications, 2014.
Figure 11: PICALM shRNA knockdown results in elevated p62 levels in CAD neuronal cells

p62 levels were measured by immunoblot analysis in CAD neuronal cells expressing shRNA4 and shRNALuc control in full serum (8% FBS) in CAD neuronal cells. All western blot analysis was conducted in three separate experiments. Quantitation of the analysis, normalized to shRNALuc control cells, is shown below the western blot, p*<0.05.
3.5 PICALM Overexpression Modulates Macroautophagy

To determine if PICALM overexpression results in perturbed macroautophagy, we overexpressed PICALM using two different approaches. First, we overexpressed PICALM fused to GFP (PICALM-GFP) in HEK293 cells. This resulted in elevated levels of LC3II (Figure 12, lanes 1 and 2). In addition, we also performed these experiments in the presence of Bafilomycin (400 nM for 4 hours; Figure 12, lanes 3 and 4). Overexpression of PICALM in the presence of Bafilomycin did not result in changes in LC3II levels. These results indicate that PICALM overexpression does not alter autophagosome formation, but rather affects autophagosome breakdown (Figure 12).
Figure 12: PICALM overexpression elevates LC3II levels in the absence of Bafilomycin

LC3II levels were measured in HEK293 cells overexpressing PICALM fused to GFP (PICALM-GFP) and were compared to Empty Vector control cells. All experiments were performed in full serum (10% FBS) conditions in the presence and absence of Bafilomycin (400 nM) for 4 hours. Western blot analysis was performed in three independent experiments. HE=High Exposure. LE=Low Exposure.

In order to rule out any non-specific effects related to the presence of GFP, we overexpressed PICALM that was not fused to GFP (PICALM) (Figure 13). As expected, this also resulted in elevated LC3II levels (Figure 14, lanes 1 and 2). However, when this
experiment was performed in the presence of Bafilomycin, the altered LC3II levels were no longer present (Figure 14, lanes 3 and 4).

**Figure 13: PICALM overexpression in HEK293 cells**

PICALM was overexpressed in HEK293 cells. PICALM was not fused to GFP. Western blot analysis was performed in three independent experiments. All experiments were performed in full serum (10% FBS) conditions in the presence and absence of Bafilomycin (400 nM) for 4 hours. Western blot analysis was performed in three independent experiments. HE=High Exposure. LE=Low Exposure.
LC3II levels were measured in HEK293 cells overexpressing PICALM that was not fused to GFP (PICALM) and were compared to Empty Vector control cells. All experiments were performed in full serum (10% FBS) conditions in the presence and absence of Bafilomycin (400 nM) for 4 hours. Western blot analysis was performed in three independent experiments. HE=High Exposure. LE=Low Exposure.

To gain a better understanding of how PICALM overexpression might alter macroautophagy, we overexpressed PICALM and probed for p62. As expected, p62 levels were elevated in the presence of PICALM overexpression (Figure 15). These results provide additional evidence to suggest that PICALM overexpression may alter autophagosome breakdown.
P62 levels were measured in HEK293 cells overexpressing PICALM and were compared to Empty vector control cells. All experiments were performed in full serum (10% FBS) conditions in the presence and absence of Bafilomycin (400 nM) for 4 hours. Western blot analysis was performed in three independent experiments. HE=High Exposure. LE=Low Exposure.

3.6 Discussion

Macroautophagy can be induced by depriving cells of essential nutrients (lipids, serum removal) proteins and organelles in order to recycle essential cellular nutrients (1, 56-58). In addition, macroautophagy has been shown to occur at basal levels and is essential for the removal of damaged proteins and organelles. This process is important for the removal of these potentially toxic products to prevent the formation of protein aggregates (54, 55, 73). The buildup of toxic protein aggregates is a common characteristic that is seen in neurodegenerative diseases such as Huntington’s Disease and Parkinson’s Disease (71, 72). Several studies suggest that altered macroautophagy may contribute to the build up of toxic proteins involved in the pathogenesis of Alzheimer’s Disease (54, 73, 76).
Alzheimer’s Disease is associated with the formation of toxic amyloid beta plaques and Tau protein accumulation (39-41). Importantly, perturbation of macroautophagy has been shown to dramatically alter the levels of these proteins (54, 73, 76, 77). As a result, it is speculated that defects in macroautophagy may help promote the formation of these toxic structures and the development of Alzheimer’s Disease (54, 73, 76, 77). Gaining a better understanding of how macroautophagy is regulated is essential in order to shed light on possible mechanisms by which it may be related to Alzheimer’s Disease development.

Recent GWAS studies have shown that SNPs within the PICALM gene are associated with an increased risk for the development of Alzheimer’s Disease (42-44). PICALM has been recently shown to be essential for the internalization and localization of VAMP3 and VAMP8. Both VAMP3 and VAMP8 are essential for the regulation of macroautophagy (38). Since PICALM plays a role in promoting the correct localization of these proteins, we sought to determine whether perturbation of PICALM protein levels leads to defects in macroautophagy.

Our studies clearly show that PICALM reduction leads to elevated LC3II levels in HEK293 cells (Figure 4). This is true when macroautophagy is occurring at basal levels (full serum conditions), and when macroautophagy is induced by serum withdrawal (Figures 5, 6 and 7). This could be replicated in HeLa cells (Figure 6), CAD neuronal cells (Figure 7), and primary glial cells (Figure 8). These results show that
PICALM’s ability to regulate macroautophagy is not a cell-specific phenomenon, and that PICALM may play a role in modulating macroautophagy in the central nervous system.

Elevation in LC3II levels is indicative of increased number of autophagosomes. However, since autophagosomes are eventually degraded by lysosomes, the elevation of LC3II levels can result from either elevated autophagosome formation or decreased autophagosome breakdown. In order to better understand how autophagosome levels are regulated, investigators commonly use lysosomal inhibitors (Bafilomycin and chloroquine) to prevent the breakdown of the autophagosomes (57, 134). This allows autophagosome formation to be studied. Persistent elevation of LC3II levels in the presence of a lysosomal inhibitor indicates that autophagosome formation is increased. In contrast, correction of LC3II levels in the presence of a lysosomal inhibitor suggests that autophagosome breakdown may be defective (57, 134).

Our studies show that PICALM reduction leads to elevated LC3II levels (Figure 9, lanes 1, 2, 5 and 6). However, in the presence of Bafilomycin, similar levels of LC3II are seen in PICALM-deficient cells and in control cells (Figure 9, lanes 3, 4, 7 and 8). These results suggest that PICALM plays a more prominent role in the breakdown of autophagosomes rather than their formation. This was corroborated by the elevation of p62 in the presence of PICALM reduction in HEK293 (Figure 10) and CAD neuronal cells (Figure 11).
Studies have shown that PICALM overexpression results in a similar phenotype to PICALM reduction (12, 13). The literature suggests that this may be due to a dominant negative effect of PICALM (12, 13). Thus, we sought to determine if PICALM overexpression results in perturbed macroautophagy. We found that PICALM overexpression caused elevation in LC3II levels, indicating a perturbation in macroautophagy (Figures 12 and 14). In addition, when PICALM was overexpressed in the presence of Bafilomycin, there was no change in LC3II levels (Figures 12 and 14). These results suggest that PICALM overexpression may alter autophagosome breakdown rather than autophagosome formation. This is similar to the results that were shown with PICALM reduction. To corroborate this data, we also measured p62 levels in the presence of PICALM overexpression. As expected, PICALM overexpression resulted in elevated p62 levels (Figure 15). This provides more evidence to suggest that PICALM overexpression may alter autophagosome breakdown.

Our results show that PICALM plays a role in the regulation of macroautophagy. In addition, PICALM appears to play a more prominent role in the regulation of autophagosome breakdown rather than autophagosome formation. More in-depth analysis is needed in order to determine whether perturbed macroautophagy in the presence of PICALM perturbation results in altered amyloid beta protein formation and Tau accumulation. Indeed, studies have already shown how PICALM’s modulation of macroautophagy may alter the formation of these toxic proteins (1, 21). This is discussed
in more detail in Chapter 5.1. Additional studies into how PICALM regulation of macroautophagy contributes to these toxic products will help shed light on a possible mechanism by which PICALM contributes to Alzheimer’s Disease.
4. Modulation of PICALM Levels Perturbs Cellular Cholesterol Homeostasis

This chapter will describe the role of PICALM in cellular cholesterol homeostasis.

The work presented here is the result of a collaboration with the Bensinger lab at UCLA, which has been published in PLoS ONE: Mercer, et al. Modulation of PICALM Levels Perturbs Cellular Cholesterol Homeostasis, PLoS ONE 10(6): e0129776, 2015. Dr. Ashley Chi (Duke University) assisted with analysis of the microarray results that led to identification PICALM’s involvement in cholesterol biosynthesis (Table 4 and Figure 16).

4.1 Introduction

PICALM is a ubiquitously expressed accessory adaptor protein that functions in Clathrin-Mediated Endocytosis (CME) (11, 13, 20). PICALM also regulates the internalization and localization of specific soluble NSF attachment protein receptors (SNAREs), including VAMP2, VAMP3, and VAMP8 (1, 28, 29, 34-36). These SNAREs play important roles in vesicular trafficking, including the endosomal-lysosomal system and macroautophagy (1, 28, 29, 34-36). The effect of PICALM on the cellular localization of SNAREs may impact other cellular processes involving trafficking. Indeed, a recent study indicated a role for PICALM perturbation in endosomal-lysosomal maturation affecting the intracellular trafficking of the c-KIT receptor (37). However, more studies
are necessary in order to gain insight into other biological roles that PICALM may regulate.

To gain insight into the biology of PICALM, we performed a microarray study comparing gene expression in Picalm-knockout and PICALM-rescued cells. Pathway analysis suggested that PICALM perturbation results in altered expression of genes in the cholesterol biosynthesis pathway. Using quantitative PCR, we confirmed that PICALM overexpression and PICALM loss results in elevated cholesterol biosynthesis pathway gene expression in multiple cell types. Isotopic labeling studies demonstrated that reduction of PICALM levels results in both increased cellular cholesterol levels and net scavenging rate. Collectively, these studies suggest that PICALM plays a role in cellular cholesterol metabolism, highlighting a novel mechanism by which alterations of PICALM might contribute to disease.

4.2 PICALM Affects Cholesterol Biosynthesis Gene Expression

To better understand PICALM’s basic biological role, we established cell lines from Picalm<sup>−/−</sup> mice (8, 12) to conduct microarray analyses. We reasoned that the PICALM knockout cells would allow us to analyze PICALM’s function in a genetically pure background. Immortalized fetal hematopoietic cells from E14 Picalm<sup>−/−</sup> embryos were retrovirally transduced to rescue PICALM expression. Control Picalm<sup>−/−</sup> cells were transduced with an empty MSCVneo retroviral vector. Four biological replicates of
*Picalm*<sup>+</sup> cell lines, either transduced with *PICALM* or the empty vector, were used to perform the microarray.

We used the GATHER (Gene Ontology Tool to Help Explain Relationships) gene ontology tool to identify common pathways that are altered in *Picalm* knockout versus *PICALM*-rescued cell lines (132). This analysis revealed that nine GO terms involving lipid and cholesterol synthesis (including GO:0006695, 0044255, 0016126, 0008610) were among the ten most significantly altered pathways when comparing *Picalm*<sup>+</sup> and *PICALM*-rescued cells (Table 4). Expression levels of multiple genes in the cholesterol biosynthesis pathway were elevated in *PICALM*-rescued compared with *Picalm*<sup>+</sup> cells. These included *Hmgcs* (1.2 fold), *Fdps* (1.3 fold), *Fdft1* (1.3 fold), *Cyp51* (1.2 fold) and *Nsdhl* (1.2 fold) (Figure 16A). The order of the genes within the cholesterol biosynthesis pathway is shown in (Figure 16B). The top 40 genes (based on fold change) that were upregulated and downregulated in the microarray are presented in Appendix A (Table 5) and Appendix B (Table 6), respectively.

**Table 4: Top Ten Biological Pathways Upregulated in the Presence of PICALM**

<table>
<thead>
<tr>
<th>Number</th>
<th>GO term</th>
<th>neg ln (p value)</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GO:0006695 [8]: cholesterol biosynthesis</td>
<td>10.41</td>
<td>Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk</td>
</tr>
<tr>
<td>2</td>
<td>GO:0044255 [5]: cellular lipid metabolism</td>
<td>10.41</td>
<td>Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Galc, Hmgcs1, Hsd11b1, Mogat2, Nsdhl, Pip5k1b, Pmvk, Sll27a4</td>
</tr>
<tr>
<td>3</td>
<td>GO:0016126 [7]: sterol biosynthesis</td>
<td>10.41</td>
<td>Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk</td>
</tr>
<tr>
<td></td>
<td>GO:0008610 [6]: lipid biosynthesis</td>
<td>10.41</td>
<td>Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Hmgcs1, Mogat2, Nsdhl, Pmvk</td>
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<tr>
<td>---</td>
<td>----------------------------------</td>
<td>-------</td>
<td>---------------------------------------------------------------------</td>
</tr>
<tr>
<td>5</td>
<td>GO:0006629 [5]: lipid metabolism</td>
<td>10.41</td>
<td>Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Galc, Hmgcs1, Hsd11b1, Mogat2, Nsdhl, Pip5k1b, Plcb2, Pmvk, Slc27a4</td>
</tr>
<tr>
<td>6</td>
<td>GO:0008203 [7]: cholesterol metabolism</td>
<td>9.16</td>
<td>Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk</td>
</tr>
<tr>
<td>7</td>
<td>GO:0016125 [6]: sterol metabolism</td>
<td>8.8</td>
<td>Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk</td>
</tr>
<tr>
<td>8</td>
<td>GO:0006066 [5]: alcohol metabolism</td>
<td>8.62</td>
<td>Cyp51, Fdft1, Gpd2, Hk1, Hmgcs1, Nsdhl, Pmm1, Pmvk</td>
</tr>
<tr>
<td>9</td>
<td>GO:0006694 [7]: steroid biosynthesis</td>
<td>8.16</td>
<td>Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk</td>
</tr>
<tr>
<td>10</td>
<td>GO:0007186 [6]: G-protein coupled receptor protein signaling pathway</td>
<td>7.85</td>
<td>Arrb2, Ccr5, Ltb4r1, Mrgpra2</td>
</tr>
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</table>
Figure 16: Microarray analysis identifies altered cholesterol biosynthesis gene expression

(A) Heatmap comparing microarray gene expression profile of four Picalm-knockout (left panels) and four Picalm-rescue (right panels) murine hematopoietic cell lines. Red indicates genes that are upregulated and green indicates those that are downregulated. Genes of the cholesterol pathway are indicated to the right of the heat map. (B) A simplified diagram of cholesterol biosynthesis pathway to position enzymes shown to be overexpressed in PICALM-rescued cells by the microarray study. Figure 1A and 1B in Mercer, et al. PLoS ONE, 2015.

Several genes involved in basic cellular lipid metabolism were also increased in the presence of PICALM, including Dgat2 (1.5 fold), Mogat2 (2.4 fold), Slc27a4 (1.3 fold)
and *Pip5k1b* (1.5 fold). Although some of the inductions were modest, the concordant changes in numerous genes of the cholesterol biosynthesis pathway strongly suggest that PICALM expression levels play a role in cholesterol metabolism. Validation of the induction of cholesterol biosynthesis gene expression in *PICALM*-rescued cells was performed by quantitative real time PCR (qPCR) and confirmed elevations in cholesterol pathway gene expression (Figure 17).

![Validation of microarray results by qRT PCR analysis](image)

**Figure 17: Validation of microarray results by qRT PCR analysis**

Validation of microarray results by qPCR in hematopoietic fetal liver cells (upper panel). Expression is normalized to *Picalm*-knockout cells. Data represent the mean of 3 independent experiments. *p<0.05. Immunoblot (lower panel) shows PICALM and actin protein levels in representative *Picalm*-knockout and *PICALM*-rescued hematopoietic cells. Figure 1C, Mercer et al, *PLoS ONE*, 2015.
The comparison of Picalm with PICALM-rescued cells demonstrates that PICALM gene dosage affects the expression of cholesterol biosynthesis genes. However, one caveat of this experimental approach is that the level of PICALM expression in rescued cells may exceed physiological amounts; this could potentially squelch PICALM binding partners and have a dominant negative effect that exceeds the effect resulting from mere loss of PICALM (12, 13).

To examine this possibility, we compared wild type HEK293 cell, to cells transiently overexpressing PICALM. We performed qPCR to compare the expression of cholesterol biosynthesis genes in the empty vector control cells to that in the PICALM overexpressing cells. The results show that PICALM overexpression induces an increase of cholesterol metabolism gene expression (Figure 18) suggesting that PICALM overexpression perturbs cholesterol homeostasis. This is consistent with studies showing that PICALM overexpression results in a dominant negative effect on multiple processes, including clathrin mediated endocytosis (12, 13), iron metabolism (12) and macroautophagy (our unpublished data).
Cholesterol biosynthesis gene expression was measured in HEK293 cells transiently transfected with a control retrovirus (Empty Vector) or PICALM expressing retroviral vector (+PICALM). Results shown are normalized to control cells. *p<0.05. Immunoblot (lower panel) shows levels of PICALM and actin proteins in representative Picalm-knockout and PICALM-rescued hematopoietic cells. Figure 1D, Mercer, et al. *PLoS ONE*, 2015.

To elucidate and further validate the role of PICALM in cholesterol homeostasis, we knocked down PICALM with shRNAs and compared cholesterol biosynthesis gene
expression in cells expressing a control shRNA. Using two separate shRNAs to knockdown PICALM in HEK293 cells, we found a 20 to 50% increase in expression of cholesterol biosynthesis genes in PICALM-deficient cells (Figure 19A). To extend this observation to additional cell lines, we knocked down Picalm in murine embryonic fibroblasts (MEFs). Similar to observations in HEK293 cells, we found that cholesterol biosynthesis gene expression increased by 10-50% in Picalm-deficient MEFs (Figure 19B).

Figure 19: PICALM knockdown leads to elevated cholesterol biosynthesis gene expression in HEK293 and MEF cells

Cholesterol biosynthesis gene expression assessed by qPCR in cells expressing a control shRNA (shRNALuc) or various PICALM shRNAs (shRNA4 or 15). Analysis was done in HEK293 cells.
(A) and MEFs (B). Expression levels are normalized to those in shRNALuc control cells. Results shown are a mean of three or more independent experiments. *p<0.05. Expression analysis of PICALM and actin in the various cell lines are shown by immunoblot. Figure 2A and 2B, Mercer, et al. PLoS ONE, 2015.

We also studied the effect of Picalm knockdown in murine CAD (Cath-a-differentiated) neuronal cells, a central nervous system catecholaminergic cell line established from a mouse brain tumor (137, 138). Reduction of Picalm expression in CAD neuronal cells resulted in a 20-50% increase in cholesterol biosynthesis gene expression (Figure 20).
Figure 20: PICALM knockdown elevates cholesterol biosynthesis gene expression in CAD neuronal cells

Cholesterol biosynthesis gene expression assessed by qPCR in CAD Neuronal cells expressing a control shRNA (shRNALuc) or various PICALM shRNAs (shRNA4 or 10). Expression levels are normalized to those in shRNALuc control cells. Results shown are a mean of three or more independent experiments. *p<0.05. Expression analysis of PICALM and actin in the various cell lines are shown by immunoblot analysis. Figure 2C, Mercer, et al. PLoS ONE, 2015.

Finally, to further examine the effect of PICALM perturbation on cholesterol homeostasis, we studied its effect on expression of SREBP2, a key regulator of cholesterol metabolism gene expression (104). SREBP2 is present in a precursor
kDa) inactive form, and a mature (65 kDa) active form that activates the transcription of cholesterol biosynthesis genes. To complement the gene expression analysis, we found that PICALM knockdown (with shRNA4) is associated with a modest elevation of the mature 65 kDa SREBP2 form in HEK293 cells (Figure 21).

**Figure 21: PICALM knockdown with shRNA 4 results in elevated SREBP2 levels**

The precursor (120 kDa) and SREBP2 mature form (70 kDa) were measured by immunoblot in HEK293 cells expressing shRNA4 or a control shRNA. Triplicate cell lysates were analyzed in 3
separate experiments. Quantitation of SREBP2 precursor and mature forms were normalized to actin, with values shown relative to levels in shRNA control cells. p<0.05. Figure 2D, Mercer et al, *PLoS ONE*, 2015.

Similar results were seen with a second shRNA targeting *PICALM* (shRNA15, **Figure 22**). Of note, we have observed that changes in levels of SREBP2 mature/active form seen by immunoblotting are subtle; even upon growing HEK293 cells in the absence of lipids for 24 hours – conditions that would be expected to dramatically increase SREBP2 levels – we only see a 45% increase in the mature SREBP2 product (**Figure 23**). The gene expression analysis along with a modest elevation of SREBP2, indicate that cholesterol homeostasis is altered when PICALM expression is perturbed.

![Image of immunoblot and bar charts showing changes in SREBP2 levels](image_url)

**Figure 22**: PICALM shRNA knockdown with shRNA 15 results in modest elevation of SREBP2
The precursor (120 kDa) and SREBP2 mature form (70 kDa) were measured by immunoblot in HEK293 cells expressing shRNA15 or the control shRNALuc. The precursor form is shown at a higher exposure than the mature form to facilitate its visualization. Triplicate cell lysates were analyzed in 3 separate experiments. Quantitation of SREBP2 precursor and mature forms were normalized to actin, with values shown relative to levels in shRNALuc control cells. *p<0.05. Figure S2A, Mercer et al, PLoS ONE, 2015.

![Image of immunoblot results]

**Figure 23: Lipid deprivation results in a modest elevation of SREBP2**

The precursor (120 kDa) and mature form of SREBP2 (70 kDa) were measured by immunoblot in HEK293 cells grown in normal, 10% FBS or 10% Lipid deficient serum (LPDS) for 24 hours, n=3 *p<0.05. Figure S2B, Mercer, et al. PLoS ONE, 2015.
4.3 PICALM Loss Enhances Cellular Cholesterol and LDL Receptor Internalization

Our studies demonstrate that loss of PICALM results in elevated expression of genes that are involved in both cholesterol biosynthesis and lipoprotein uptake. We reasoned that this gene signature might be the result of decreased cellular cholesterol content leading to a compensatory increase in cholesterol biosynthesis or increased cholesterol scavenging. To address this possibility, total cellular cholesterol was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Unexpectedly, these studies revealed that knockdown of PICALM in HEK293 cells results in increased total cellular cholesterol levels (Figure 24).

![Total Cholesterol Levels](image)

**Figure 24:** PICALM knockdown results in elevated cellular cholesterol levels
Total cellular cholesterol levels were measured in HEK293 cells expressing a control (luciferase) shRNA or PICALM shRNA (shRNA4). All results were normalized to total cell number, n=4, p*0.05. Figure 3A, Mercer, et al. *PLoS ONE*, 2015.

We therefore performed $^{13}$C tracer analysis to determine the relative contributions of synthesis and scavenging to the cellular cholesterol pool. PICALM knockdown and control cells were cultured to steady state in complete media containing a 1:1 molar ratio of $^{13}$C$_6$ glucose and natural glucose (139) and total cellular cholesterol was extracted, derivatized and measured by GC-MS. The isotope distribution for cholesterol was assessed, and relative contribution of synthesis versus scavenging was determined by modeling the isotopologue distribution (see Methods for detailed protocol) (139). We observed that PICALM knockdown cells had a higher percent abundance of unlabeled cholesterol, suggesting an increased relative reliance on scavenging compared to synthesis (Figure 25).
Figure 25: PICALM shRNA knockdown cells have higher abundance of scavenged cellular cholesterol

The relative contribution of synthesis and scavenging to cellular cholesterol was calculated at steady state by modeling isotopologue distribution of cholesterol in indicated cells cultured in 1:1 molar ratio of U-13C6-glucose (n=4 in each experimental group). Figure 3B, Mercer, et al. *PLoS ONE*, 2015.
To better understand the relative importance of scavenging and synthesis, we calculated the net rates of accumulation (hereafter referred to as “net rates”) for synthesis and scavenging, which represent the gross rate minus losses due to modification, breakdown, or export (see Methods). PICALM knockdown did not appear to affect the net rate of cholesterol biosynthesis (Figure 26). In contrast, we found that PICALM knockdown results in significantly elevated net rates of cholesterol scavenging and accumulation (Figure 26).

Figure 26: PICALM knockdown results in elevated net cholesterol scavenging rates

Net rates of cholesterol synthesis, scavenging and total accumulation were calculated using cholesterol pool size, relative contribution of synthesis and scavenging to cholesterol pool, and doubling time of cells as described in materials and methods. n=4 in each experimental group; *p<0.05. Figure 3C, Mercer, et al. PLoS ONE, 2015.
Several species of fatty acids also showed increased cellular pool sizes (Figure 27) and increased net scavenging rates (Figure 28) in the absence of PICALM. Taken together, these results indicate that reduced PICALM expression is associated with increased cellular cholesterol content and a relative increase in the contribution of lipoprotein scavenging to cholesterol homeostasis.

**Figure 27:** Total fatty acid levels are elevated or maintained in PICALM knockdown cells

Total fatty acid levels were measured in HEK293 cells expressing a control shRNA (shRNALuc) or PICALM shRNA (shRNA4), in media containing a 1:1 molar ratio of U-13C6-glucose. Fatty acid abbreviations are indicated in table 3. All results are normalized to cell number, n=4 in each experimental group, *p<0.05. Figure S3, Mercer, et al. *PLoS ONE*, 2015.
Figure 28: Net rates of fatty acid synthesis, scavenging and accumulation are elevated or maintained in PICALM knockdown cells

Net rates of fatty acid synthesis, scavenging and accumulation were calculated for shRNA luciferase (shRNALuc) control and PICALM knockdown (shRNA4) HEK293 cells in media containing a 1:1 molar ratio of natural to U-13C6-glucose using cellular pool size, relative contribution of synthesis and scavenging, and doubling time of cells as described in Materials and Methods, n=4 in each experimental group; *p<0.05. Figure S4, Mercer, et al, *PLoS ONE*, 2015.

The LDL receptor is one of the principal routes by which cholesterol enters into the cell; its internalization is dependent on clathrin-mediated endocytosis (CME) (90, 93, 94). Since PICALM plays a well-established role in CME, we sought to determine how reduced PICALM expression affects LDL receptor internalization. As shown in (Figure 29), knockdown of PICALM in HEK293 cells is associated with a significant increase in LDL receptor expression at the cell surface. This increase in surface LDL receptor protein
correlates with the increase in LDL receptor mRNA transcript seen by qPCR (Figure 19A).

**LDLR Surface Expression**

![Graph showing LDLR surface expression](image)

**Figure 29: PICALM knockdown results in elevated LDLR surface expression**

LDL receptor surface expression measured by flow cytometry in HEK293 cells expressing a control shRNA (shRNALuc) or PICALM shRNA (shRNA4). Expression levels are normalized to shRNALuc control cells. Results represent the mean of at least three independent experiments. *p<0.05. Figure 4A, Mercer, et al. *PLoS ONE*, 2015.

Endocytosis assays showed an increase in the net amount of LDL receptor internalized in PICALM knockdown cells (Figure 30A). The efficiency of LDL receptor internalization, measured as the ratio of LDL receptor internalized to the amount initially present at the cell surface, was not significantly altered in PICALM knockdown
cells. Although, there is a trend that shows higher rates of LDL receptor internalization (Figure 30B), the increase in net LDL receptor internalization seen in PICALM knockdown cells likely predominantly results from the elevated expression levels of LDL receptor.

![Figure 30](image)

**Figure 30**: Total LDL receptor internalization is elevated in the absence of PICALM

Endocytosis assays show the net amount of LDL receptor internalized (A) and the fraction of internalized LDL receptor relative to the initial amount of surface LDL receptor (B), as a function
of incubation time at 25°C in control (shRNA-Luc) or PICALM shRNA (shRNA4) cells. Each data point is the mean of at least three different experiments. MFI = mean fluorescence intensity. Figure 4B and 4C, Mercer, et al. PLoS ONE, 2015.

4.4 Discussion

Cholesterol is essential for cell survival. The majority of cellular cholesterol is found within the plasma membrane, where it regulates membrane rigidity and permeability, and modulates specific aspects of cell signaling (87, 89, 90, 92, 93, 97). Cholesterol homeostasis controls internalization of extracellular cholesterol and endogenous synthesis. Cells scavenge cholesterol when complexed with lipoproteins. This internalization occurs predominantly through clathrin-mediated endocytosis (CME) of LDL, via a variety of receptors belonging to the LDL receptor family (90, 93, 94). Once cholesterol is internalized, it is trafficked through the endosomal-lysosomal system, and released from the lysosome to the cytoplasm and various cellular compartments within the cell. If cholesterol internalization is blocked, or if there are defects in cholesterol trafficking, SREBP2, the main regulator of cholesterol biosynthesis gene expression, can activate expression of cholesterol biosynthesis genes to restore cellular cholesterol levels (90, 93, 94). Importantly, maintaining cellular cholesterol homeostasis is essential, since defects can lead to a wide range of diseases such as cancer, atherosclerosis, and neurodegenerative disease (86, 90, 91).

We performed microarray analysis of Picalm-deficient and PICALM-rescued murine hematopoietic cells and showed that PICALM perturbation appears to modulate
cellular cholesterol biosynthesis gene expression (Table 4 and Figure 16A). In addition, we performed qPCR to validate the microarray analysis (Figure 17). However, in this overexpression model, PICALM levels in rescued cells may have been supraphysiologic. Previous studies have shown that PICALM overexpression can result in PICALM having a dominant negative effect on multiple cellular processes (12, 13). Thus, both PICALM overexpression and PICALM reduction can result in a similar phenotype. To show that excess PICALM levels alter cellular cholesterol homeostasis, we overexpressed PICALM in HEK293 cells. This resulted in elevated cholesterol metabolism gene expression (Figure 18), indicating that PICALM overexpression may exert a dominant negative effect on cholesterol homeostasis. To demonstrate that physiological levels of PICALM regulate cholesterol homeostasis, we knocked down PICALM expression using shRNAs. The results of Figure 19A show that PICALM deficiency leads to elevated expression of cholesterol biosynthesis genes. We observed similar results in multiple cell types, MEFs (Figure 19B) and CAD neuronal cells (Figure 20) indicating that this phenotype is not cell type specific.

Multiple studies have shown that proteins involved in CME and endosomal-lysosomal trafficking can play key roles in maintaining normal cellular cholesterol homeostasis (140-145). Given PICALM’s well-established contribution to CME and its possible role in cellular trafficking (11, 37, 49) we explored how PICALM perturbation might modulate cellular cholesterol metabolism. To this end, we used $^{13}$C tracer analysis
Our studies demonstrate that PICALM knockdown results in increased total cellular cholesterol levels (Figure 24) and elevated net cholesterol scavenging (Figure 26). Furthermore, several species of fatty acids also showed increased cellular pool sizes (Figure 27) and increased net scavenging rates in the absence of PICALM (Figure 28). These results indicate that PICALM may play a broader role in cellular lipid homeostasis. Interestingly, loss of PICALM did not result in a significant change in the net rate of cholesterol synthesis (Figure 26). This result could be explained by a variety of factors. It is known that increased cellular cholesterol can inhibit flux through the mevalonate cholesterol biosynthetic pathway via posttranscriptional mechanisms, such as degradation of HMG-CoA reductase (146) and squalene monoxygenase (147).

Alternatively, it is also possible that increased cholesterol levels result in heightened cholesterol efflux (148) in PICALM-deficient cells. In this scenario, heightened cholesterol efflux could result in an underestimation of the magnitude of change in gross synthetic rate between PICALM-sufficient and -deficient cells. Nevertheless, the concomitant increase of net cholesterol import, with activation of cholesterol biosynthesis pathway gene expression, suggests that cholesterol homeostasis is profoundly perturbed in Picalm-deficient cells (149, 150).

Our studies further demonstrate that reduced PICALM results in elevated LDL receptor surface expression and internalization (Figure 29 and 30). However, PICALM reduction does not appear to dramatically affect the efficiency of LDL receptor
internalization. In fact, we observed a trend towards superior rates of LDL receptor internalization in shRNA knockdown compared to control cells (Figure 30B). These results suggest that PICALM does not play an essential role in the internalization of the LDL receptor from the cell surface, but rather that PICALM may be required for the proper intracellular trafficking of internalized cholesterol. We propose that the elevated levels of LDL receptor surface expression and internalization are a result of increased expression of the LDL receptor, caused by perturbation of cholesterol homeostasis.

Several reports indicate that PICALM affects cellular trafficking through the endosomal-lysosomal system (1, 37, 49). PICALM modulates the localization and activity of SNARE proteins such as VAMP3 and VAMP8, both of which are important for endosomal-lysosomal function and macroautophagy (38, 64). Our previous studies have shown that PICALM’s ability to modulate the localization of VAMP3 and VAMP8, results in perturbed macroautophagy (1). Intriguingly, these SNARE proteins have been shown to be mislocalized in Niemann-Pick Type C (NPC) disease, which is characterized by perturbed endosomal-lysosomal trafficking of cholesterol and altered cholesterol homeostasis (70, 150-152). It has also been demonstrated that macroautophagy is altered in NPC disease (84, 85). Collectively, this suggests that PICALM alteration can lead to altered cellular cholesterol homeostasis by mislocalization of VAMP3 and VAMP8 SNARE proteins that contribute to both improper cholesterol trafficking and macroautophagy (70, 85). Perturbation of both
macroautophagy and cholesterol trafficking may contribute to elevated cholesterol biosynthesis gene expression, LDL receptor surface expression and internalization, and cholesterol uptake, because cells are unable to sense cellular cholesterol in the absence of PICALM. However, deciphering the precise mechanism by which this occurs is beyond the scope of the current studies.

Our observations suggest that PICALM plays a regulatory role in maintaining cellular cholesterol homeostasis. In addition, they support a novel mechanism by which PICALM might contribute to disease. Multiple independent Genome Wide Association Studies (GWAS) indicate that PICALM SNPs play a role in the development of late-onset Alzheimer’s Disease (AD) (42-45). However, how these SNPs affect PICALM expression levels or function is poorly understood; some studies suggest that PICALM is overexpressed, while others indicate that a reduction in PICALM activity can play a pathogenic role in AD (50-53). Alterations in PICALM levels may modulate amyloid beta protein formation and Tau accumulation (1, 22, 48). The present work suggests that the ability of PICALM to modulate cholesterol biology might be an additional mechanism by which PICALM contributes to AD. This is consistent with a number of studies that have linked perturbed cholesterol metabolism to late-onset AD, as well as other neurodegenerative diseases (88, 111, 116-119, 123, 153).

The expression of APOE, a key lipoprotein and carrier of cholesterol in the brain, has been shown to be a highly significant risk factor in the development of late-onset AD
(42, 46). Meta-analysis studies suggest that PICALM and APOEε4 may synergistically interact to help promote the development of AD (44, 47). However, the manner by which APOE and PICALM might act together to promote the development of AD is not understood. Our work suggests that these risk factors may cooperate to promote the development of AD through the modulation of cellular cholesterol metabolism.

In summary, the studies presented here provide evidence to suggest that PICALM modulates cellular cholesterol metabolism. Loss of PICALM activates cellular cholesterol biosynthesis gene expression, increases LDL receptor surface expression and internalization, enhances cholesterol uptake, and increases total cellular cholesterol. This phenotype suggests that cholesterol sensing is abnormal when PICALM activity is perturbed. This novel role for PICALM in cellular cholesterol homeostasis has implications in understanding how PICALM might contribute to disease.
5. Conclusions and Future Directions

The work described here helps enhance our understanding of PICALM’s biological roles within mammalian cells in a cell culture environment. In this chapter, we will summarize our findings related to PICALM’s roles in macroautophagy (5.1) and cellular cholesterol metabolism (5.2). In addition, the relationship and the connection between macroautophagy and cholesterol homeostasis will be examined (5.3). To conclude chapter 5, future lines of investigation that stem from our studies and their relevance to disease will be described (5.4).

5.1 Conclusions: PICALM’s Role in Macroautophagy

In Chapter 3, we detailed a previously unappreciated and novel role for PICALM in macroautophagy. We used western blot analysis to show that PICALM modulates the levels of LC3II and p62. LC3II and p62 are both characterized markers used to study alterations in macroautophagy (57, 136). Intriguingly, our results showed that PICALM reduction results in the elevation of LC3II in multiple mammalian cell types (Figures 4-8). In addition, when we performed these experiments in the presence of Bafilomycin, a compound that inhibits the breakdown of autophagosomes (57, 134, 136), there was no elevation of LC3II levels in PICALM knockdown cells (Figure 9). This suggests that PICALM reduction does not alter the formation of autophagosomes. Instead, PICALM reduction is likely to have a more significant effect on autophagosome breakdown.
Indeed, when we probed for p62, a characterized marker of autophagosome flux (57, 136), we detected elevated p62 levels when PICALM levels were reduced (Figures 10 and 11). These results provide more evidence to suggest that autophagosome breakdown is altered in the absence of PICALM.

We also overexpressed PICALM in order to determine its effect on macroautophagy. It is described in the literature that PICALM overexpression can result in a dominant negative effect (12, 13). Studies have shown that PICALM overexpression results in phenotypes identical to those observed when PICALM levels are reduced (12). In fact, when PICALM was overexpressed, we observed increased LC3II levels (Figures 12 and 14), and p62 levels (Figure 15). We also overexpressed PICALM in the presence of Bafilomycin, and elevation of LC3II and p62 levels was not detected (Figures 12, 14 and 15), findings similar to what is seen when PICALM levels are reduced. These results indicate that both PICALM reduction and overexpression can result in altered macroautophagy, possibly due to defects in the breakdown of autophagosomes.

We were also interested in studying possible mechanisms by which PICALM alters macroautophagy, through a collaboration with the laboratory of David Rubinsztein at Cambridge University (1). We hypothesized that PICALM modulates macroautophagy through its ability to regulate VAMP3 and VAMP8 cellular localization. Indeed, studies performed by our collaborators showed that PICALM reduction results in altered VAMP3 and VAMP8 cellular localization (1). These results
suggest that this could be a mechanism by which PICALM modulates macroautophagy. In addition, these studies included rescue experiments, in which PICALM re-expression facilitated the restoration of normal macroautophagy. A form of PICALM that contains a mutation in the SNARE binding domain was also used to attempt to rescue macroautophagy. This mutation in impairs its ability to bind VAMP3 and VAMP8 (1, 36). Strikingly, re-expression of this PICALM mutant construct (in the setting of PICALM knockdown) did not restore macroautophagy. These results provide further evidence that PICALM may modulate macroautophagy through its ability to regulate SNARE protein internalization (1). However, additional studies are necessary to determine if PICALM modulates macroautophagy by other mechanisms.

5.2 Conclusions: PICALM’s Role in Cholesterol Homeostasis

In Chapter 4, we described a novel role for PICALM in cellular cholesterol homeostasis. In order to gain a better understanding of PICALM’s basic cell biological role, we performed a microarray using PICALM-expressing and Picalm-deficient cells. Surprisingly, our microarray analysis indicated that PICALM levels result in modulation of cholesterol biosynthesis gene expression (Figures 16 and 17). Similarly, when we analyzed different cell types, we showed that both PICALM overexpression and reduction lead to elevated cholesterol biosynthesis gene expression (Figures 18, 19 and 20). Our studies also showed that PICALM reduction results in enhanced LDL
cholesterol levels (Figure 24), cholesterol internalization (Figure 26), and LDL receptor uptake (Figure 30). These findings provide additional evidence to show that modulation of PICALM levels has a profound effect on cellular cholesterol homeostasis.

These results indicate that in the absence of PICALM, elevated cholesterol internalization helps contribute to elevated cellular cholesterol levels. In addition, in contrast to the PICALM-sufficient state, Picalm-deficient cells demonstrate elevated cholesterol biosynthesis gene expression, even as high levels of cholesterol are being internalized. However, the mechanism by which PICALM alters cholesterol metabolism is unknown, and remains to be explored. A possible mechanism by which PICALM may alter cellular cholesterol metabolism is described in section 5.3.

5.3 The Relationship between Macroautophagy and Cholesterol Homeostasis

The precise mechanisms by which PICALM alters cellular cholesterol homeostasis remain uncertain. We hypothesize that these defects may be the result of altered trafficking of cellular cholesterol. Recent studies have demonstrated that altered trafficking of cholesterol can dramatically alter cellular cholesterol homeostasis in a disease state. This is clearly seen in Niemann-Pick Type C (NPC) Disease (152, 154). NPC Disease is a neurodegenerative disorder that is characterized by altered cholesterol trafficking and homeostasis. Specifically, NPC cells show elevated cholesterol within the endosomal-lysosomal system due to a mutation in the NPC1 gene (84, 85, 150, 152, 155).
The NPC1 protein plays a key role in regulating the release of cholesterol from the lysosome as seen in (Figure 31) (98, 100, 101). In addition, studies have shown that NPC cells display elevated SREBP2 activity, cholesterol internalization and cholesterol biosynthesis gene expression (84, 149, 152, 155, 156).

This altered cholesterol metabolism is often accompanied by degeneration within the central nervous system of NPC patients (70, 152, 155, 157). Of note, NPC patients also have similar phenotypes to those that are observed in Alzheimer’s Disease. Specifically, some NPC patients show altered APP processing and toxic amyloid beta formation, both of which are common markers of Alzheimer’s Disease (158-161). However, it remains to be determined whether the altered APP processing seen in NPC contributes to disease development.

Recent studies have shown that the expression levels and cellular localization of VAMP3 and VAMP8 are altered in NPC disease (70, 85). This altered SNARE protein localization is proposed to contribute to the perturbed cholesterol trafficking within the endosomal-lysosomal system (70, 85). These studies also show that macroautophagy is perturbed along with altered cholesterol metabolism in NPC cells. Specifically, autophagosome breakdown is altered (84, 85). The authors propose that altered VAMP3 and VAMP8 localization help contribute to both altered cellular cholesterol trafficking through the perturbation of endosomal-lysosomal system and macroautophagy (70, 85).
As a result of these defects, cellular cholesterol homeostasis is predicted to be profoundly perturbed (Figure 31).

**Figure 31: Defects in the endosomal-lysosomal system and macroautophagy contribute to altered cholesterol trafficking in NPC disease**

In a normal state (upper panel), cholesterol is internalized through clathrin-mediated endocytosis. Internalized cholesterol is moved through the endosomal-lysosomal system. Ultimately, NPC1 binds to cholesterol, to help facilitate its release from the lysosome. In addition,
Macroautophagy converges with the endosomal-lysosomal system to help promote cholesterol trafficking. However, in the NPC disease state (lower panel), SNARE proteins VAMP3 and VAMP8 are mislocalized. This mislocalization may contribute to perturbed endosomal-lysosomal function, resulting in altered cholesterol trafficking. Macroautophagy is perturbed as well in the NPC disease state, and may also contribute to altered cholesterol trafficking and homeostasis commonly seen in NPC disease.

Our data regarding cholesterol homeostasis suggest that cellular cholesterol trafficking may be altered when PICALM levels are modulated. In a normal state, enhanced internalization of cholesterol should lower cholesterol biosynthesis gene expression through decreased SREBP2 activity in order to help restore normal cholesterol levels (90, 92-94, 105). However, our results show that PICALM reduction results in enhanced cholesterol biosynthesis gene expression, internalization and cellular cholesterol levels. These results indicate that the cellular machinery that regulates cellular cholesterol metabolism is not able to properly sense cellular cholesterol and as a result, elevated cholesterol biosynthesis gene expression is observed.

We hypothesize that PICALM may modulate cholesterol homeostasis via mechanisms that are similar to those seen in NPC cells: regulation of the endosomal-lysosomal and macroautophagy function. As described in the Introduction and in Chapter 3, PICALM modulates the internalization and localization of specific SNARE proteins (1, 28, 36). In particular, PICALM has been shown to be important for regulating the localization and internalization of VAMP3 and VAMP8: The same SNARE proteins that are important for cellular trafficking and are found to be mislocalized in the NPC studies (1, 28, 36, 38, 64, 85).
Based on PICALM’s ability to regulate VAMP3 and VAMP8 localization (28, 36), we hypothesize that this will result in altered endosomal-lysosomal function when PICALM expression levels are modulated. We predict that this would dramatically alter cholesterol trafficking and lead to elevated cholesterol within the endosomal-lysosomal system. As a result, cholesterol would be unable to be sensed by SREBP2. The inability to sense cellular cholesterol may contribute to the elevated SREBP2 nuclear activity and cholesterol biosynthesis gene expression that is seen when PICALM levels are altered.

Furthermore, macroautophagy may also contribute to the altered cholesterol homeostasis that is seen in the absence of PICALM. As mentioned previously, perturbed macroautophagy has been shown to contribute to altered lipid and cholesterol metabolism (78-80, 84, 85). Specifically, NPC cells display decreased autophagosome breakdown that helps contribute to the altered cholesterol trafficking and homeostasis (70, 84, 85). Importantly, our results (Chapter 3) demonstrate that PICALM modulates macroautophagy, possibly through altered autophagosome breakdown.

In summary, altered PICALM expression may modulate cholesterol metabolism through a mechanism that is similar to that observed in NPC cells (70, 84, 85). This may result in altered VAMP3 and VAMP8 cellular localization, perturbed macroautophagy and endosomal-lysosomal function. As a result, these defects may contribute to disrupted cellular cholesterol trafficking and metabolism (Figure 32).
Figure 32: PICALM deficiency may result in altered SNARE protein localization, macroautophagy and cellular cholesterol homeostasis

In normal conditions (upper panel), cholesterol is internalized through clathrin-mediated endocytosis. This internalized cholesterol is trafficked through the endosomal-lysosomal system. In addition, macroautophagy also converges with the endosomal-lysosomal system to help facilitate cholesterol trafficking. Cholesterol will ultimately be released from the lysosome, allowing cellular cholesterol homeostasis to be restored. However, in Picalm-deficient conditions, we propose that SNARE protein localization (VAMP3 and VAMP8) is altered. This mislocalization may help facilitate defects in cholesterol trafficking and macroautophagy, both of
which may contribute to altered cellular cholesterol homeostasis observed in Picalm-deficient cells.

## 5.4 Future Directions

Our results and observations warrant more in-depth studies to gain an enhanced appreciation for how PICALM modulates both macroautophagy and cholesterol metabolism. Specifically, our studies do not directly demonstrate altered cholesterol trafficking when PICALM is overexpressed and reduced. Future studies might include the use of microscopic techniques that would permit direct visualization of cholesterol buildup within the endosomal-lysosomal system. One approach to visualize cholesterol within the endosomal-lysosomal pathway uses Filipin staining (84, 151, 156, 162). Filipin is a fluorescent polyene antibiotic which can bind specifically to free cholesterol within the plasma membrane and endosomal-lysosomal system (162). This is a common technique that is used to visualize the accumulation of cholesterol within the endosomal-lysosomal pathway in cells (84, 85, 156, 162). Future studies may include the use of Filipin in order to detect cellular cholesterol localization in the presence and absence of PICALM.

Our studies highlight that PICALM modulates macroautophagy and cellular cholesterol metabolism in an in vitro setting. However, we have not shown whether PICALM modulates these processes in vivo (8). Picalm knockout mice were initially described as having a decreased life span. However, in the context of the B6(Cg)-Tyr<sup>c2</sup>/J genetic background, which the mice have since been bred into, the Picalm homozygous
mutation is lethal shortly after birth (12). This precludes studying the effect of Picalm deficiency after birth. Nevertheless, studies of embryos could be performed to determine PICALM’s role in macroautophagy and cholesterol metabolism in an in vivo setting. Future studies may include the isolation of specific regions of embryonic mouse brains to determine whether macroautophagy and cholesterol metabolism are altered between Picalm-knockout and Picalm wild type mice. In order to gain a better understanding of PICALM’s role in macroautophagy and cholesterol metabolism, it would be interesting to alter PICALM expression within the neuronal and glial cells using a conditional and tissue-specific Picalm knockout mouse. Future work on PICALM’s basic biological roles, both in vitro and in vivo, are necessary in order to better understand how this complex protein functions within the cell, and how the processes may be relevant to disease.

Our studies do not directly elucidate how PICALM’s ability to modulate macroautophagy and cholesterol metabolism relates to disease. It is noteworthy that PICALM SNPs are related to the development of Alzheimer’s Disease (42-44). It is also important to note that altered macroautophagy and cholesterol metabolism are linked to the development of Alzheimer’s Disease (54, 74, 118, 125), as described in the Introduction. Since our studies have shown that altered PICALM levels result in both perturbed macroautophagy and cholesterol metabolism, we hypothesize that these may be two mechanisms by which PICALM may modulate the development of Alzheimer’s Disease. Of course, PICALM mutations may promote the development of Alzheimer’s
Disease through multiple mechanisms, but our results warrant future studies that focus on the potential role of PICALM, macroautophagy, and cholesterol metabolism and their relationship to the development of Alzheimer’s Disease.

Future studies could also include the use of conditional and tissue specific Picalm knockout mice, specifically in neuronal and glial cells. These studies could be performed in an Alzheimer’s Disease mouse model in order to determine whether PICALM loss results in altered macroautophagy, cholesterol metabolism, and whether this contributes to the progression of Alzheimer’s Disease.

It is interesting to note that meta-analysis of Alzheimer’s Disease risk factors has provided more evidence that PICALM may promote the development of Alzheimer’s Disease by modulating cholesterol metabolism. Specifically, studies have shown that APOε4 and PICALM may synergistically interact to promote the development of Alzheimer’s Disease (44, 47). APOε4 is a primary cholesterol transport protein that is essential for the trafficking of cholesterol (163). In addition, our studies show that PICALM has the ability to modulate cellular cholesterol homeostasis. A possible mechanism by which PICALM and APOε4 interact to promote the development of Alzheimer’s Disease is through their ability to perturb cholesterol homeostasis. More studies are necessary in order to determine whether PICALM and APOε4 function together at the cellular level, and if these functions are related to the regulation of cholesterol metabolism. Exploring PICALM and APOε4’s functional cellular interactions...
may shed light on possible mechanisms by which they may contribute to Alzheimer’s Disease development. Future studies could focus on whether PICALM and APOε4 interact along similar pathways. Specifically, co-localization studies of PICALM with APOε4, could be performed, since APOε4 is internalized via clathrin-mediated endocytosis. It would also be interesting to study whether specific PICALM SNPS and APOε4 alter cholesterol biology.

Our studies have uncovered novel functions for PICALM in both macroautophagy and cholesterol metabolism. Additional experiments are necessary in order to gain a better understanding of PICALM’s role in regulating macroautophagy and cellular cholesterol homeostasis. Future studies regarding PICALM’s ability to modulate these specific cellular processes will help shed light on potential mechanisms by which PICALM contributes to disease.
### Appendix A

#### Table 5: Top 40 Genes Upregulated in Microarray

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### Appendix B

Table 6: Top 40 Genes Downregulated in Microarray

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References


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**Biography**

Jacob Leib Mercer was born (January 3rd, 1987) and raised in Santa Barbara, California. He grew up with a loving family: Jerome Mercer (Father), Cecilia Mercer (Mother), and Aaron Mercer (Brother). Basketball was his passion in his early years. He and his brother often dreamed about teaming up as the starting backcourt for the Los Angeles Lakers.

While attending San Marcos High School, Jacob was a starting Point Guard on the Varsity Boy’s Basketball Team (2002-2005). As a Senior, he was All-League (1st Team), All-County (1st team) and All-CIF (Southern section, 2nd team).

He decided to continue to pursue his dream of playing basketball at a Division I University. As a result, he decided to attend Santa Barbara City College, where he planned to play for two years, while attempting to be recruited by a Division I Men’s Basketball team. He participated as a point guard with the SBCC Men’s Basketball Team (2005-2006). After the 2006 basketball season, he decided to major in Biology, and as a result, stepped away from basketball to focus on his academics.

When he transferred to UC Santa Barbara, he decided to attempt to join the team as a ‘walk on’ (non-scholarship player) with the UCSB Men’s Basketball Team (2008-2009). He made the team, and mainly participated as a point guard on the scout team. His biggest accomplishment during this time was being a part of the “most talented scout team” in coach Bob William’s tenure. This team included: Orlando Johnson, who
played two years in the NBA (Indiana Pacers) and continues to play professional basketball in various professional leagues around the world, Jon Pastorek (San Diego State transfer), Justin Joyner, and Seth Kamphoefner. He decided to step away from basketball during his last year at UCSB (2009-2010) in order to focus on his studies and undergraduate research. He happily watched his friends and former teammates compete against Ohio State University in the first round of the 2010 Division 1 Men’s NCAA basketball tournament.

After pursuing basketball, Jacob decided to focus on his second passion: Biology. In the Spring of 2010, he decided to attend the Pharmacology and Cancer Biology program at Duke University to obtain a PhD in Pharmacology.

**Education and Training**

**No Degree obtained, Biology Focus**
Santa Barbara City College, Santa Barbara CA, 2005-2008

**B.S. Biology, Molecular Cellular and Developmental Biology Department (MCDB)**
UC Santa Barbara, Santa Barbara CA, 2008-2010

**PhD Pharmacology, Department of Pharmacology and Cancer Biology**
Duke University, Durham NC, 2010-2015
Laboratory of Daniel S. Wechsler, MD PhD