A Role for PICALM in Macroautophagy and Cellular Cholesterol Homeostasis

by

Jacob Mercer

Department of Pharmacology and Cancer Biology
Duke University

Date:_____
Approved:

_____
Daniel Wechsler, Supervisor

_____
Oren Becher

_____
Bernard Mathey-Prevot

_____
Christopher Newgard

_____
Jeffrey Rathmell

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

2015

<u>ABSTRACT</u>

A Role for PICALM in Macroautophagy and Cellular Cholesterol Homeostasis

by

Jacob Mercer

Department of Pharmacology and Cancer Biology
Duke University

Date:_____
Approved:

_____
Daniel Wechsler, Supervisor

_____
Oren Becher

_____
Bernard Mathey-Prevot

_____
Christopher Newgard

_____
Jeffrey Rathmell

An abstract of a 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

2015

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.

Contents

List of Tables

List of Figures

xiii

Acknowledgements

I would like to thank Dr. Daniel Wechsler for allowing me to work in his lab. Your ability to remain patient, focused, and poised still amazes me. Thank you for improving my lab and critical thinking skills. More importantly, thank you for always making me feel welcome.

When times were tough, Dan always made sure that I was moving in the right direction, both as a student and as a person. Thank you for your constant support. I learned many important life lessons that I will always remember.

I would also like to thank my co-advisor, Dr. Catherine Lavau. Through her actions, she taught me to remain focused and determined, while simultaneously remaining happy and confident. Thank you for providing consistent help during my time as a graduate student. I am a better person after working under your guidance.

In addition, my committee members helped me throughout my time as a graduate student. Thank you Dr. Jeffrey Rathmell, Dr. Christopher Newgard, Dr. Bernard Mathey-Prevot, and Dr. Oren Becher. Your critique and words of encouragement are appreciated.

Dr. Michael Armstrong, Dr. Corinne Linardic and all of their lab members have made my time as a graduate student a more enjoyable process. They have all helped in their unique way. Dr. Amanda Conway, Dr. Jessica Heath, and Dr. Paula Scotland (former Wechsler lab members) always encouraged me to continue to move forward. Dr.

Wait Aumann and Dr. Sei-Gyung Kim, current Wechsler lab members, have provided a fun and comfortable work environment.

I am lucky enough to have too many close friends, cousins, aunts and uncles to name in this document. All of you have helped me in more ways than you know.

My family, Mom, Dad and older brother (Aaron), have always encouraged me to work hard. They all lead by their actions. Their unconditional love and support throughout my time in graduate school has helped enormously. Without them, I would be lost. Thank you for keeping me on the right path.

Last but not least, a huge thanks to my Grandparents: Dr. Robert Mercer, Shirley Mercer, Miguel Lopez, and Consuelo Lopez. I love you all.

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 upegulation 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 <u>over</u>expression 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 Nucleocytoplasmic Shuttling of PICALM

PICALM is primarily localized to the cytoplasm. However, multiple studies have

shown that PICALM has the ability to undergo nucleocytoplasmic trafficking (6, 30).

Interestingly, studies have shown that proteins involved in CME undergo

nucleocytoplasmic 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 LRP1 (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 sectionswill 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-

58). 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).
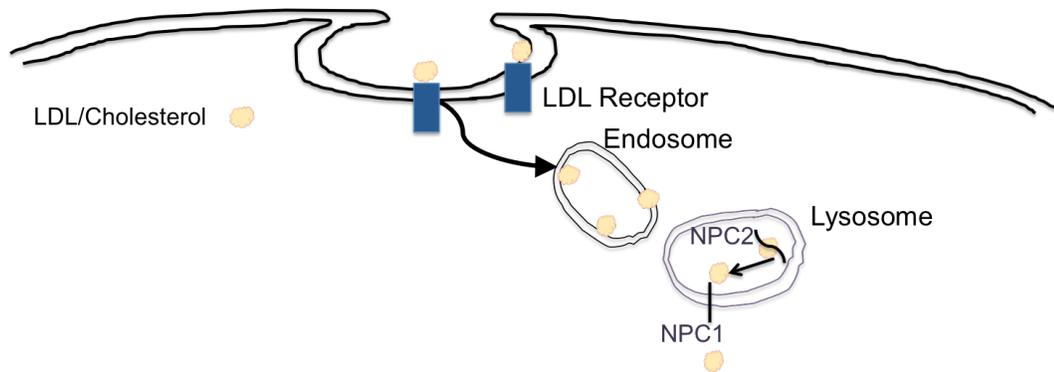
Figure 1: Cellular Cholesterol Internalization

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).
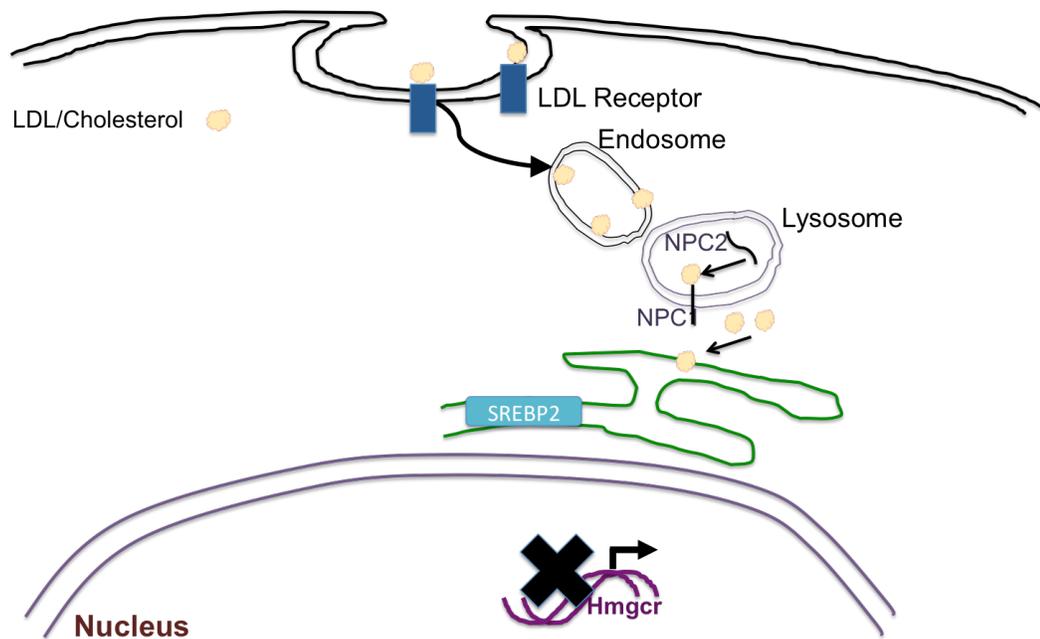
Figure 2: SREBP2 and Cellular Cholesterol Trafficking

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.
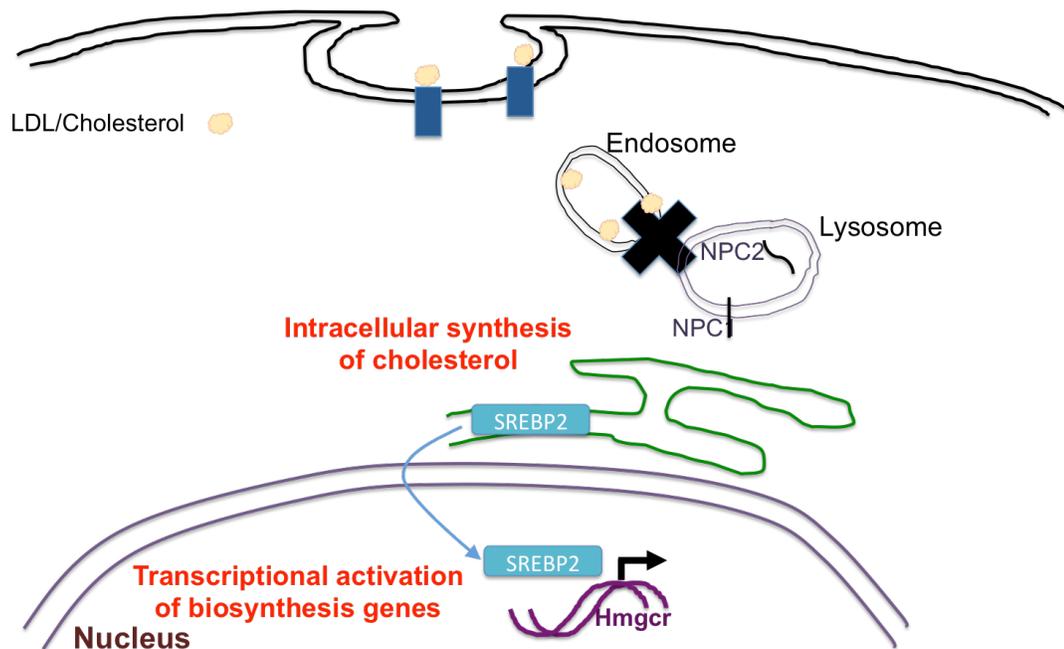
Figure 3: Low Cholesterol Levels Leads to SREBP2 Activation

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

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 *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 lipoporoteins 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, and

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[fit1-5R] mouse embryo (8). The *fit1* Picalm[fit1-5R] 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^{-/-}* (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 *BgIII* 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 log$_2$ 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 RNeasy Mini 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 $\Delta\Delta$CT method.

Table 1: Sequences of murine primers used for qPCR

Gene	Sequence (5' - 3')
Hmgcr	(fwd)-5'-TGGCACCATGTCAGGCGTCCG-3' (rev)-5'-AGCGACACACAGGCCGGGAA-3'
Hmgcs	(fwd)-5'-GTCCTGGCACAGTACTCACC-3' (rev)-5'-ACACTCCAACCCTCTTCCCT-3'
Pmvk	(fwd)-5'-CCTATGGGGCTGTGATACAGA-3' (rev)-5'-TCTCCGTGGTTCTCAATGACC-3'
Fdps	(fwd)-5'-GGGAACCAGAAATTGGATGCT-3'

30

	(rev)-5′-AGGCATTGTACTCTAGGACCTC-3′
Fdft1	(fwd)-5′-CGATTCCGCATGGGAGGCCG-3′
	(rev)-5′-TGGCGTGCCGTATGTCCCCA-3′
Cyp51	(fwd)-5′-GCTGCCCGCTGGAGCGAAAA-3′
	(rev)-5′-GCGCAGCTGCATCACTCCCC-3′
Nsdhl	(fwd)-5′-CGACCTGTGCAACCAACAGGACC-3′
	(rev)-5′-ACTGTACGGCGGAGGGGACG-3′
LDLR	(fwd)-5′-CCGGAGTTGCAGAAGACTCAT-3′
	(rev)-5′-GAGTCAGGAATGCATCGGCT-3′
SREBP2	(fwd)-5′-AGCTGCACATCACAGGGAAG-3′
	(rev)-5′- TGTGCACATCTGAACAGGCA-3′
β2M	(fwd)-5′-ACCGGCCTGTATGCTATCCAGAAA-3′
	(rev)-5′-GGTGAATTCAGTGTGAGCCAGGAT-3′

Table 2: Sequences of human primers used for qPCR

Gene	Sequence (5′ - 3′)
Hmgcr	(fwd)-5′-CAGCTCCGAGCGTGCGTAAG-3′
	(rev)-5′-CCTTGGATCCTCCAGATCTCACT-3′
Hmgcs	(fwd)-5′-CATTAGACCGCTGCTATTCTGTC-3′
	(rev)-5′-TTCAGCAACATCCGAGCTAGA-3
Pmvk	(fwd)-5′-CGCGCGGTGTCCCGATTTTA-3′
	(rev)-5-TCAGCTCCAAGTCTGCTCTGC-3′
Fdps	(fwd)-5′-ACTCGACCCACAGAGCCGAT-3′
	(rev)-5′-AGGGGCATCCTGTTCCCAGAT-3′
Fdft1	(fwd)-5′-ACTTCCCAACGATCTCCCTTG-3′
	(rev)-5′-CCCATTCTCCGGCAAATGTC-3′
Cyp51	(fwd)-5′-GAAACGCAGACAGTCTCAAGA-3′
	(rev)-5′-ACGCCCATCCTTGTATGTAGC-3′
Nsdhl	(fwd)-5′-CCGAGCTGGGCCAATCCTCT-3′
	(rev)-5′-CTCCTTATCCGTCCGTAGGCG-3′
LDLR	(fwd)-5′-CTCCCCATCGTGCTCCTCGTC-3′
	(rev)-5′-TCACGCCACGTCATCCTCCAG-3′
SREBP2	(fwd)-5′-CTGCAACAACAGACGGTAATGA-3′
	(rev)-5′-CCATTGGCCGTTTGTGTCAG-3′

β2M	(fwd)-5′-GCCTGCCGTGTGAACCATGT-3′
	(rev)-5′-TGCGGCATCTTCAAACCTCCA-3′

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

34

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

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

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 macroautopahgy 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.
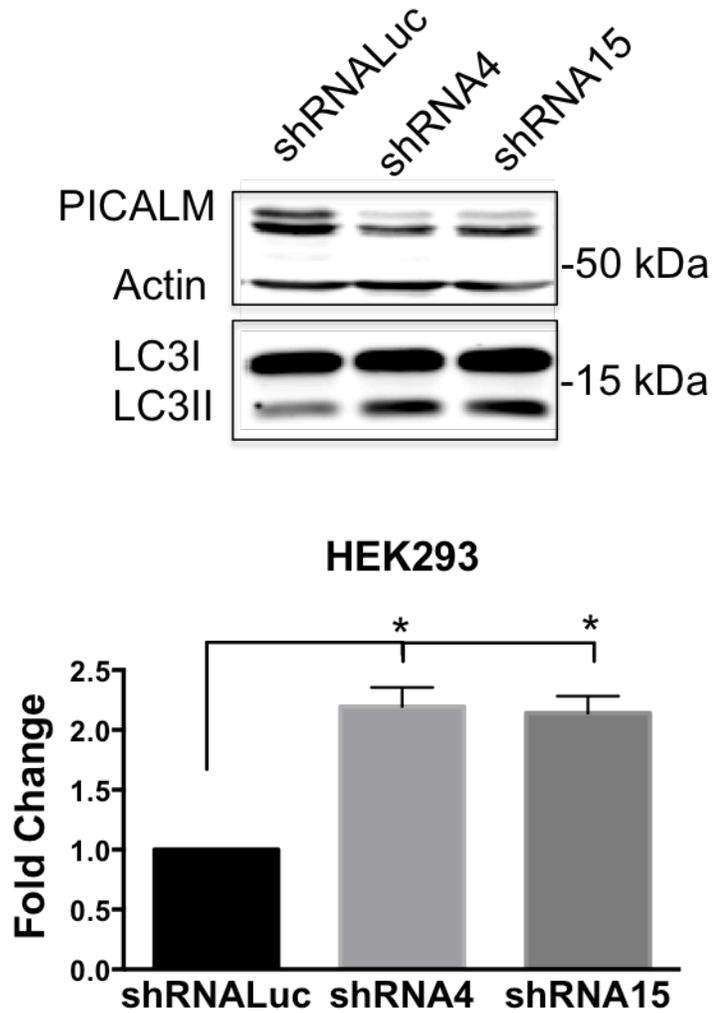
Figure 4: PICALM shRNA knockdown results in elevated LC3II Levels

Levels of LC3II measured by immunoblot analysis in HEK293 cells expressing shRNA4, shRNA15 and shRNAluc control in full serum (10% FBS) conditions. 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.
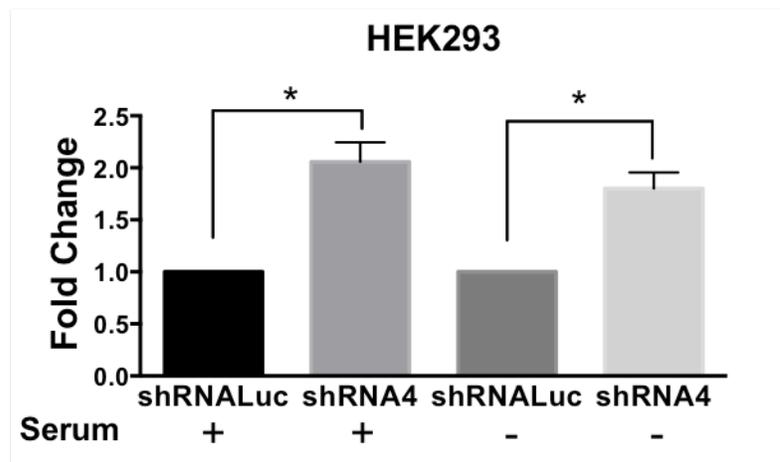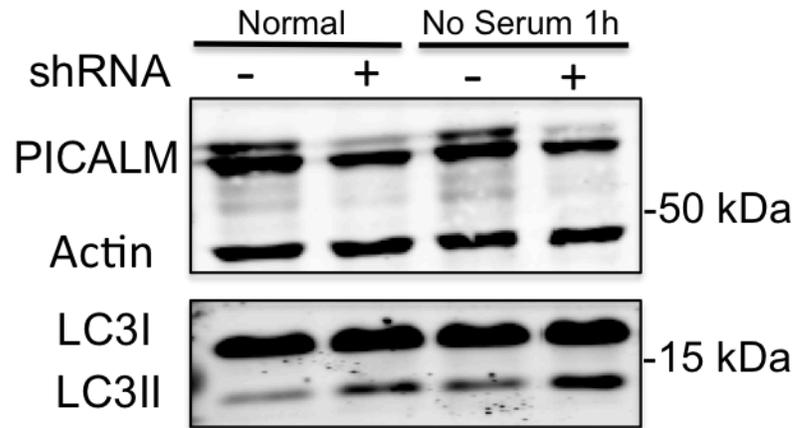
Figure 5: PICALM shRNA knockdown elevates LC3II levels in normal and stressed conditions in HEK293 cells

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.
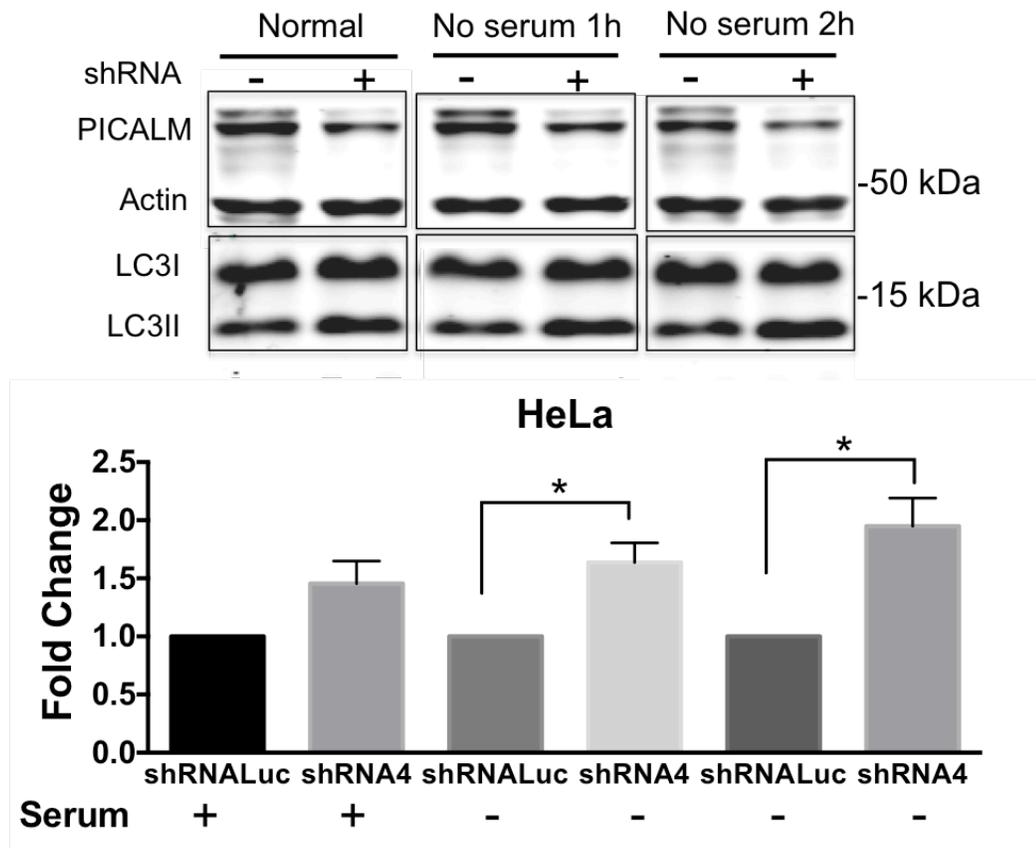
Figure 6: PICALM shRNA knockdown results in elevated LC3II levels in normal and stressed conditions in HeLa cells

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**).
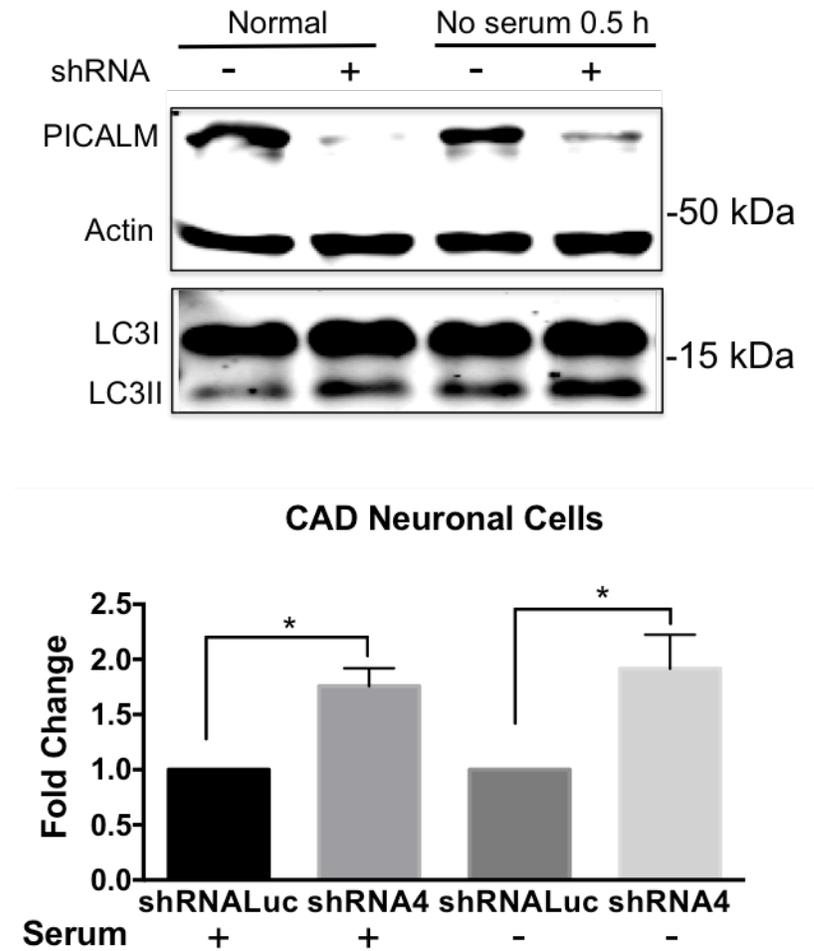
Figure 7: PICALM shRNA knockdown results in elevated LC3II levels in CAD neuronal cells

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.
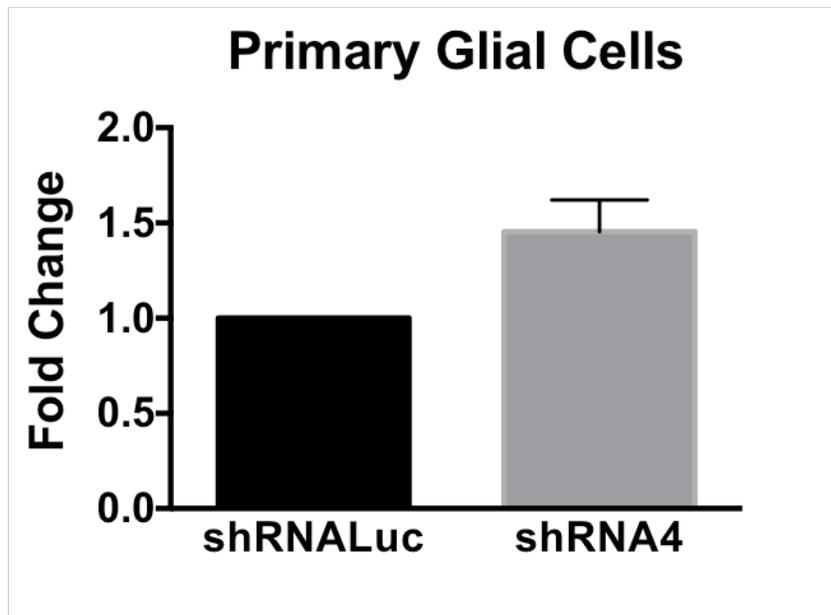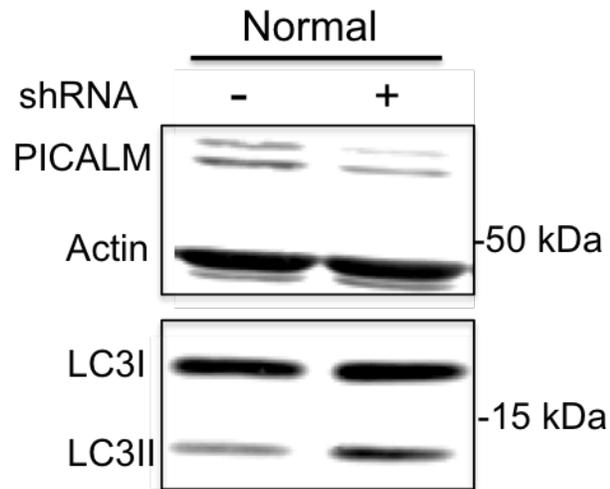
Figure 8: PICALM shRNA knockdown results in elevated LC3II levels in primary glial cells

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.

45
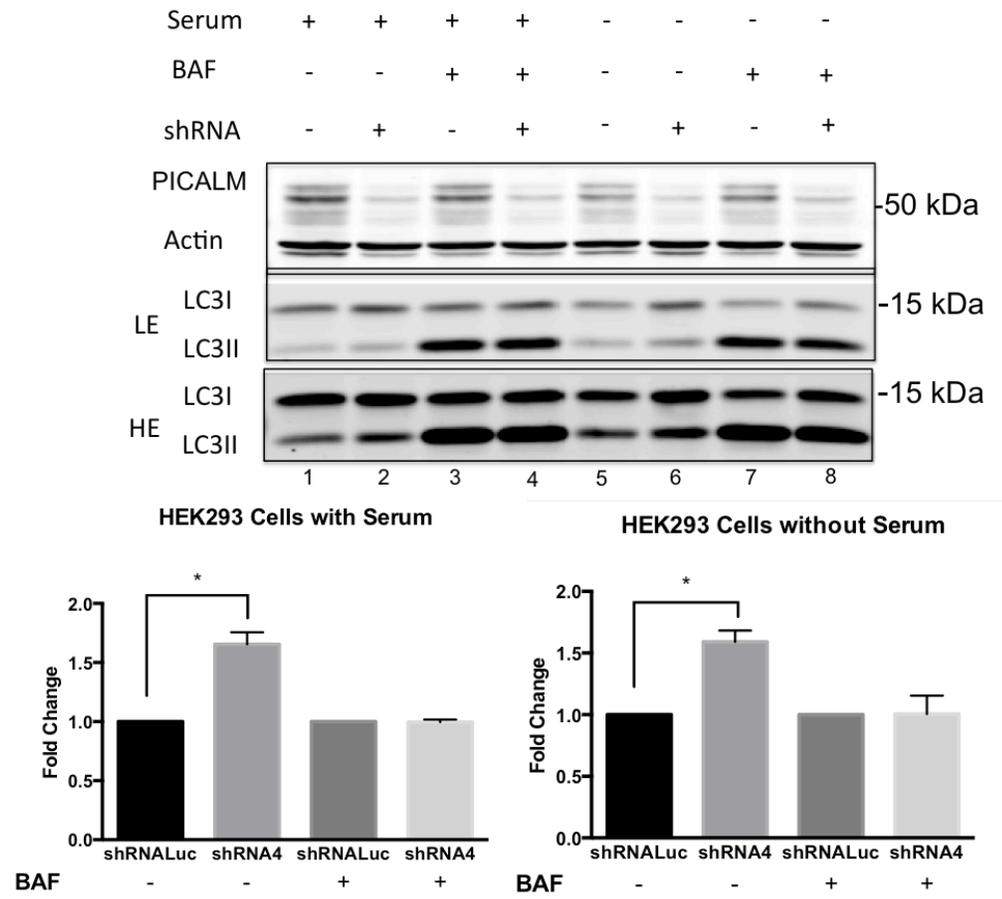
Figure 9: PICALM shRNA knockdown does not alter LC3II levels in the presence of Bafilomycin

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.
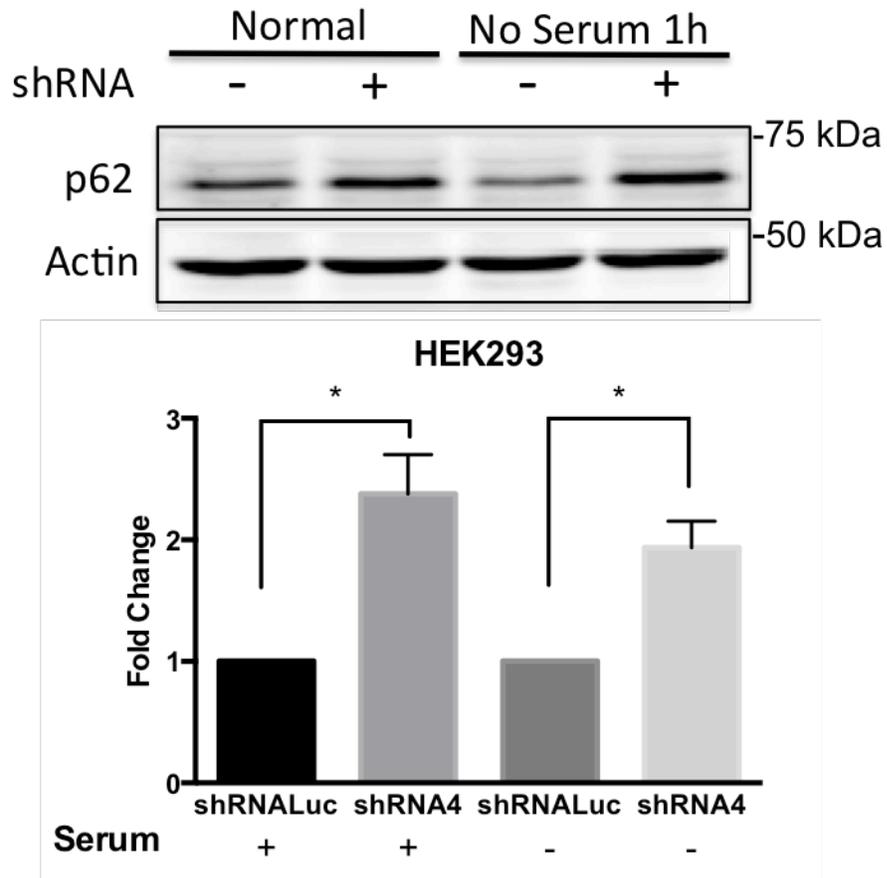
Figure 10: PICALM shRNA knockdown results in elevated p62 levels in HEK293 cells

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.
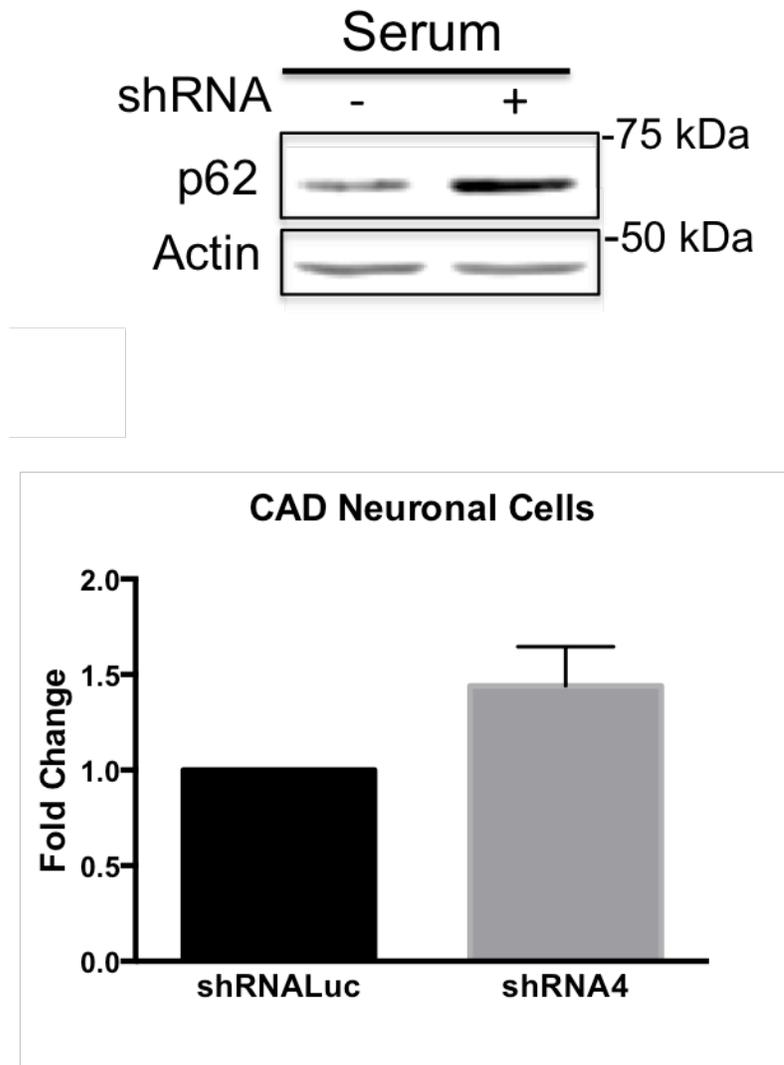
Figure 11: PICALM shRNA knockdown results in elevated p62 levels in CAD neuronal cells

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**).
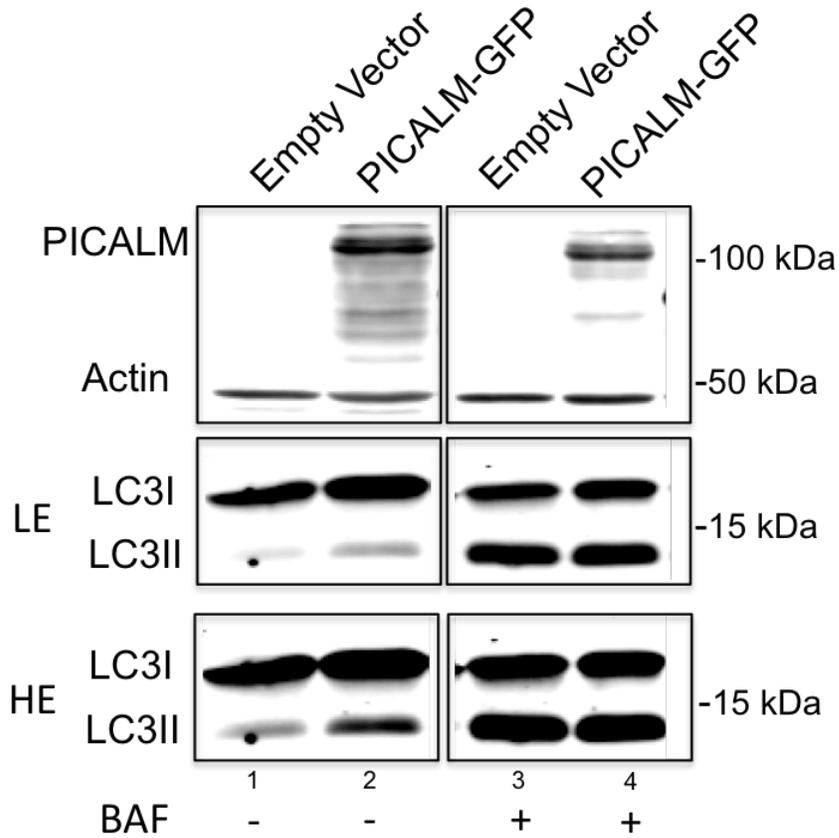
Figure 12: PICALM overexpression elevates LC3II levels in the absence of Bafilomycin

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.
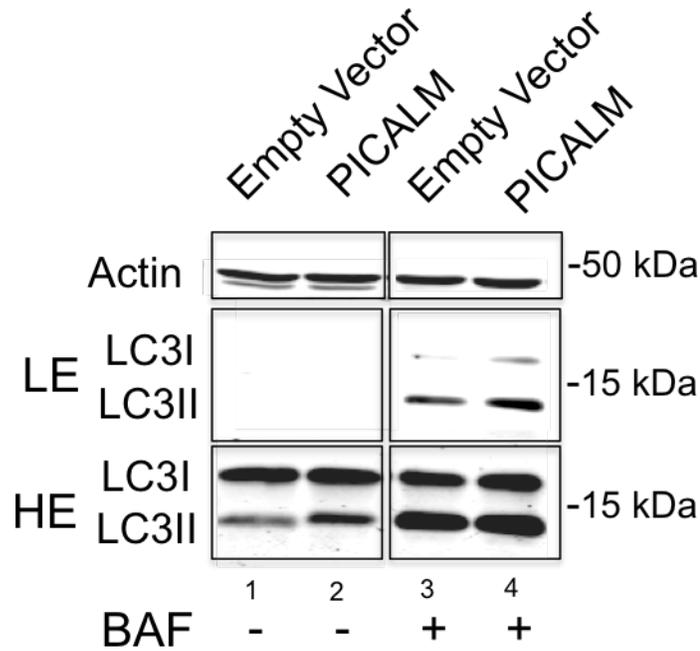
Figure 14: PICALM overexpression elevates LC3II levels in the absence of Bafilomycin

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.
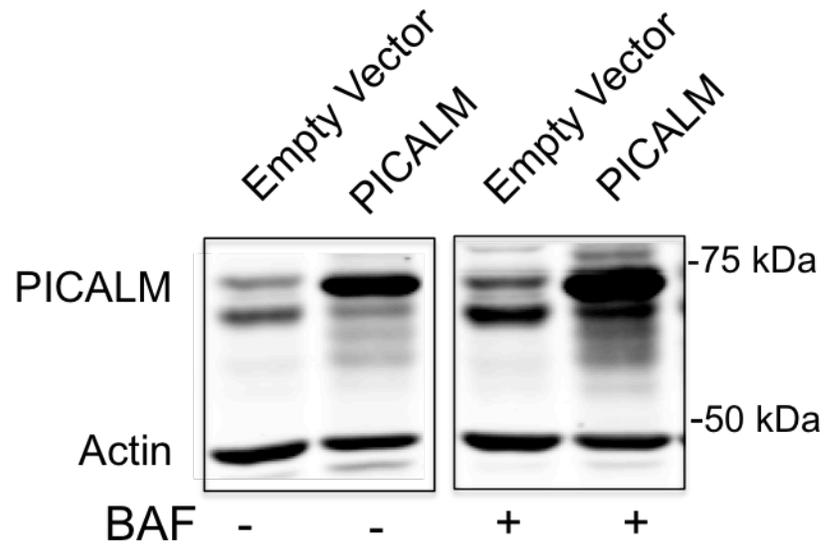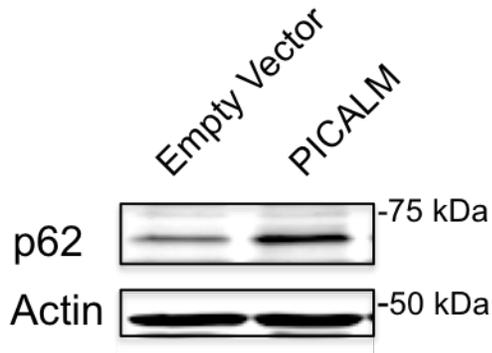
Figure 15: PICALM overexpression results in elevated p62 levels in the absence of Bafilomycin

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

57

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*$^{-/-}$ 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*$^{-/-}$ embryos were retrovirally transduced to rescue *PICALM* expression. Control *Picalm*$^{-/-}$ cells were transduced with an empty MSCVneo retroviral vector. Four biological replicates of

Picalm^{-/-} 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*^{-/-} and *PICALM*-rescued cells (**Table 4**). Expression levels of multiple genes in the cholesterol biosynthesis pathway were elevated in *PICALM*-rescued compared with *Picalm*^{-/-} 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

Number	GO term	neg ln (p value)	Genes
1	GO:0006695 [8]: cholesterol biosynthesis	10.41	Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk
2	GO:0044255 [5]: cellular lipid metabolism	10.41	Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Galc, Hmgcs1, Hsd11b1, Mogat2, Nsdhl, Pip5k1b, Pmvk, Slc27a4
3	GO:0016126 [7]: sterol biosynthesis	10.41	Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk

4	GO:0008610 [6]: lipid biosynthesis	10.41	Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Hmgcs1, Mogat2, Nsdhl, Pmvk
5	GO:0006629 [5]: lipid metabolism	10.41	Alox5ap, Cdipt, Cyp51, Dgat2, Fdft1, Fdps, Galc, Hmgcs1, Hsd11b1, Mogat2, Nsdhl, Pip5k1b, Plcb2, Pmvk, Slc27a4
6	GO:0008203 [7]: cholesterol metabolism	9.16	Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk
7	GO:0016125 [6]: sterol metabolism	8.8	Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk
8	GO:0006066 [5]: alcohol metabolism	8.62	Cyp51, Fdft1, Gpd2, Hk1, Hmgcs1, Nsdhl, Pmm1, Pmvk
9	GO:0006694 [7]: steroid biosynthesis	8.16	Cyp51, Fdft1, Hmgcs1, Nsdhl, Pmvk
10	GO:0007186 [6]: G-protein coupled receptor protein signaling pathway	7.85	Arrb2, Ccr5, Ltb4r1, Mrgpra2
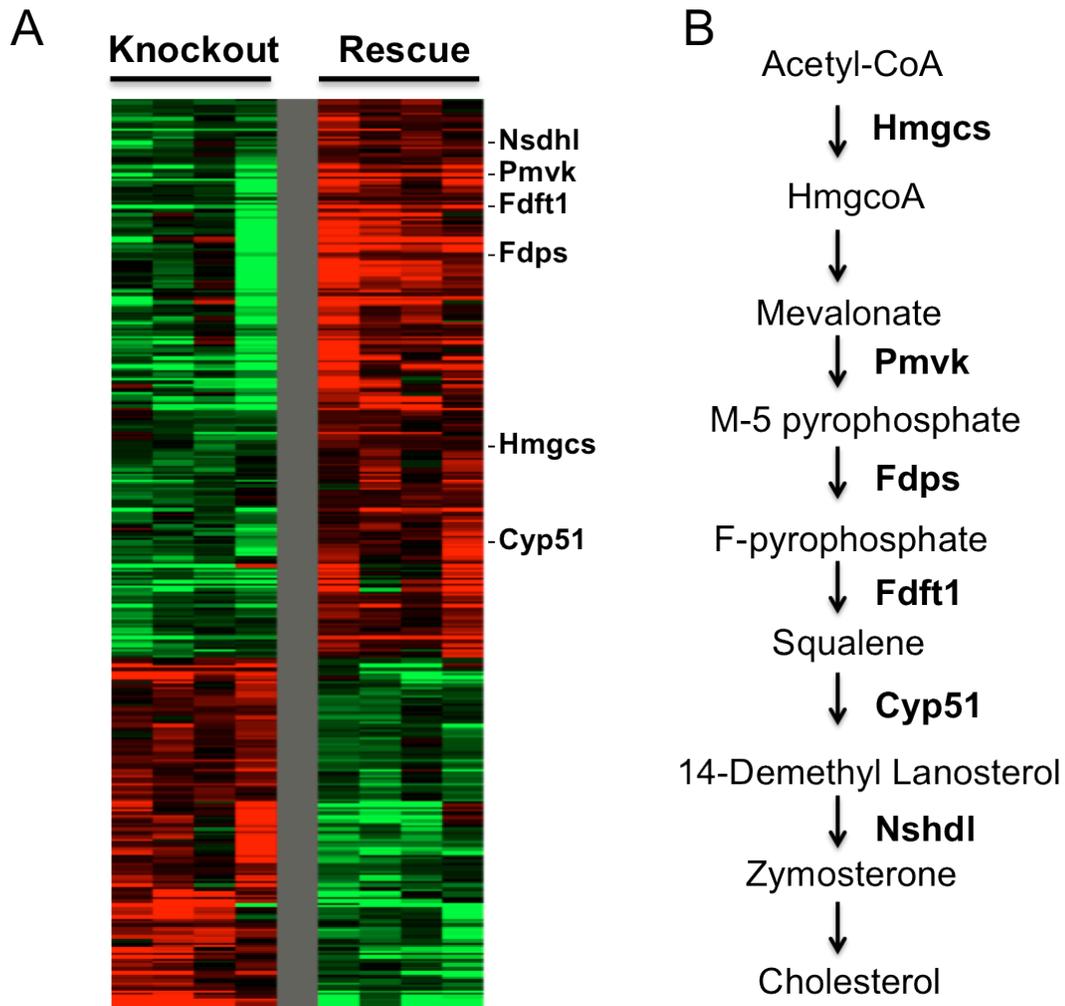
Figure 16: Microarray analysis identifies altered cholesterol biosynthesis gene expression

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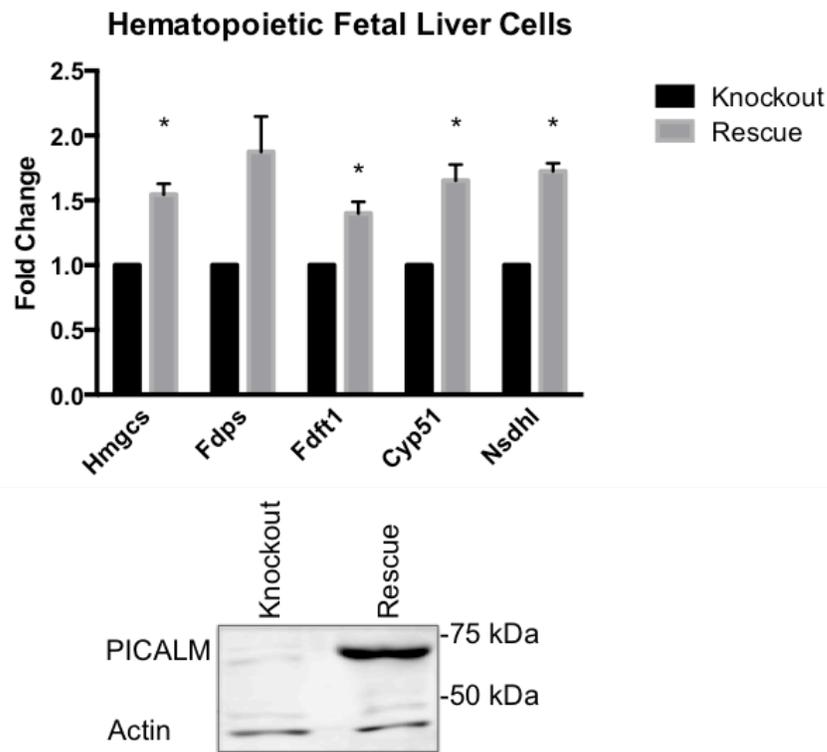
Figure 17: Validation of microarray results by qRT PCR analysis

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).
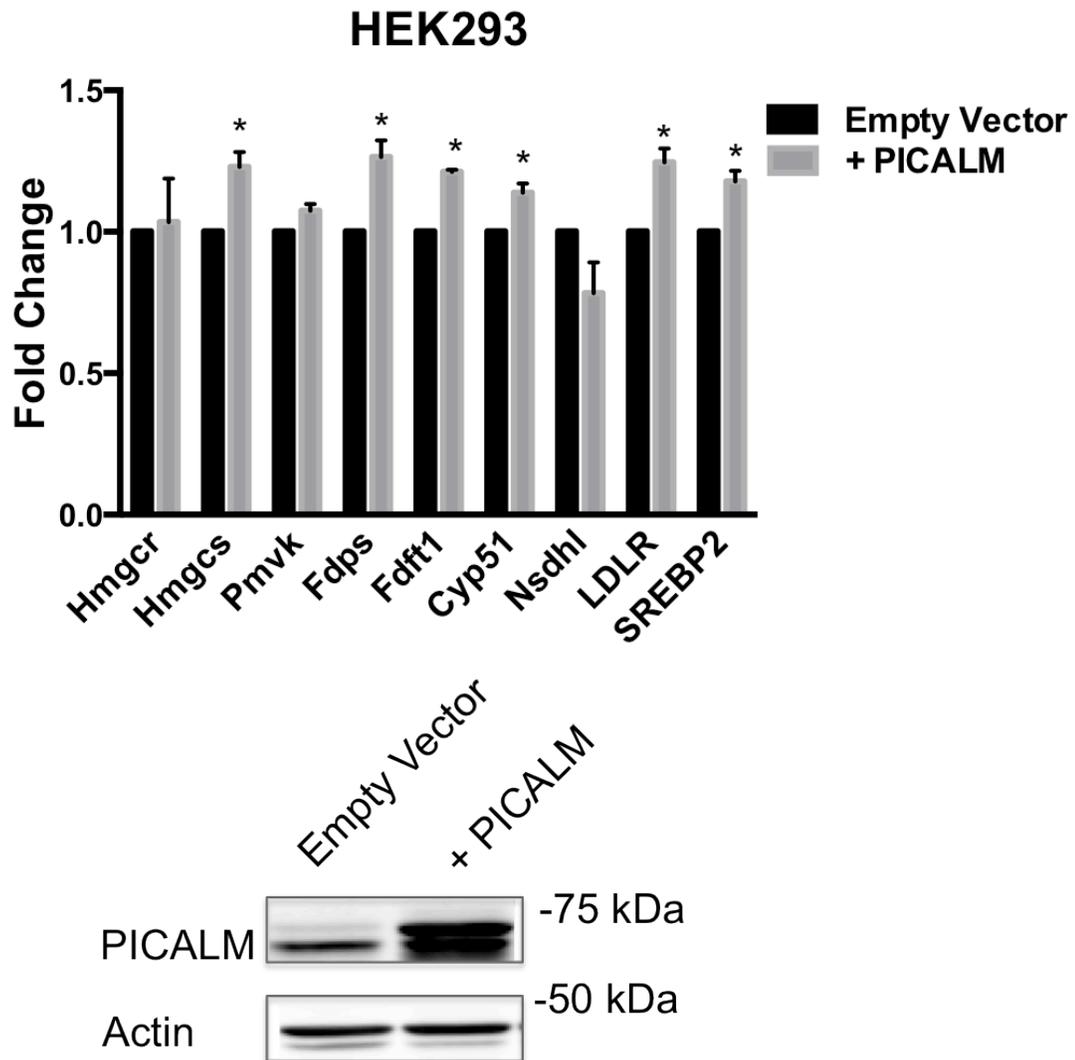
Figure 18: PICALM overexpression results in elevated cholesterol biosynthesis gene expression

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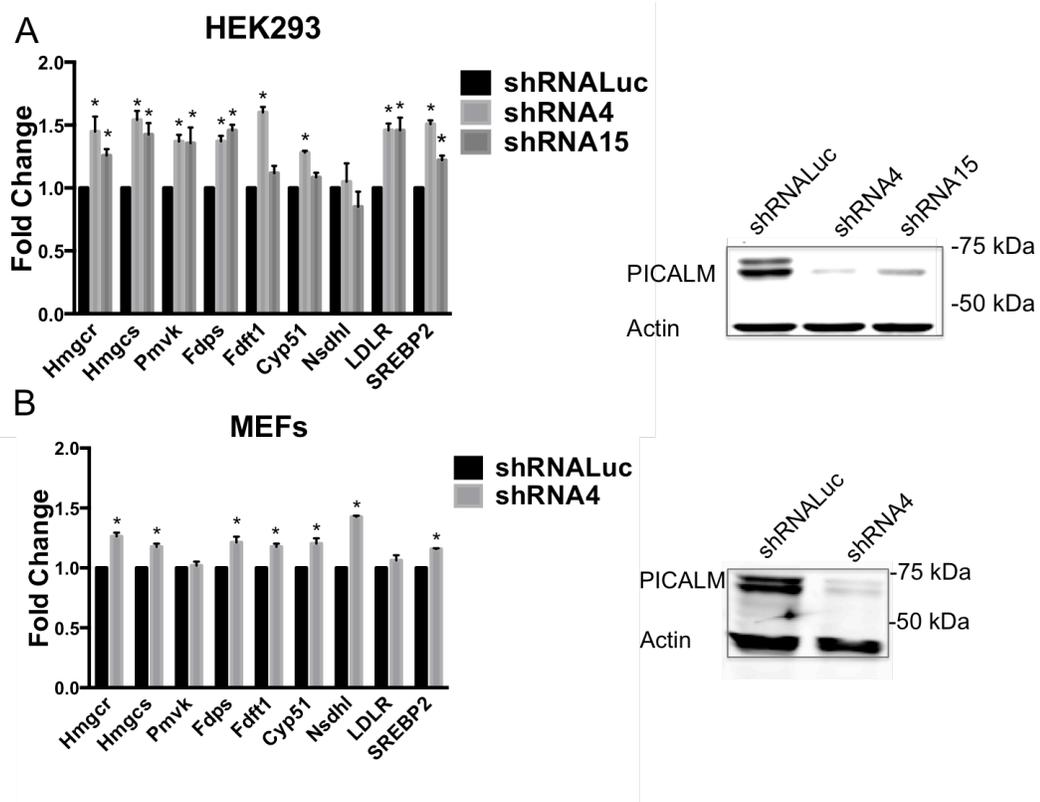
Figure 19: PICALM knockdown leads to elevated cholesterol biosynthesis gene expression in HEK293 and MEF cells

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**).
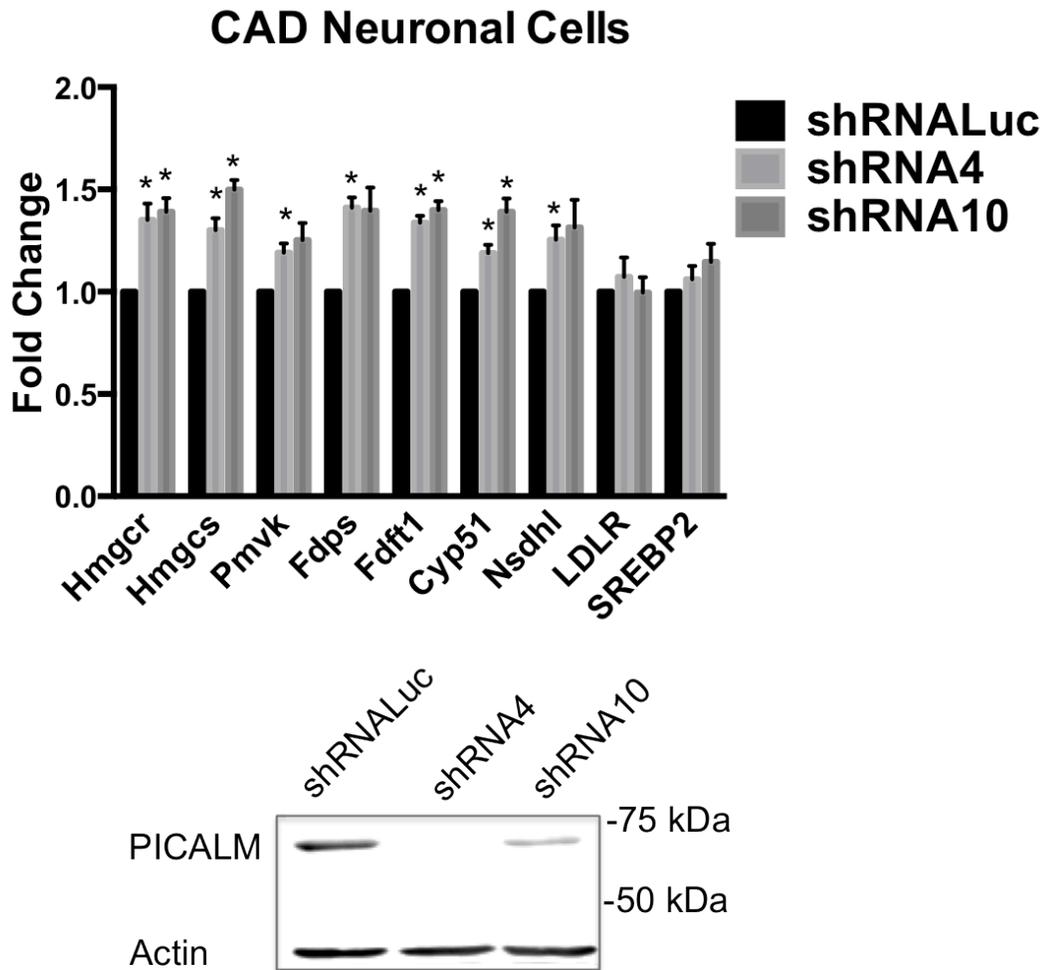
Figure 20: PICALM knockdown elevates cholesterol biosynthesis gene expression in CAD neuronal cells

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 (120

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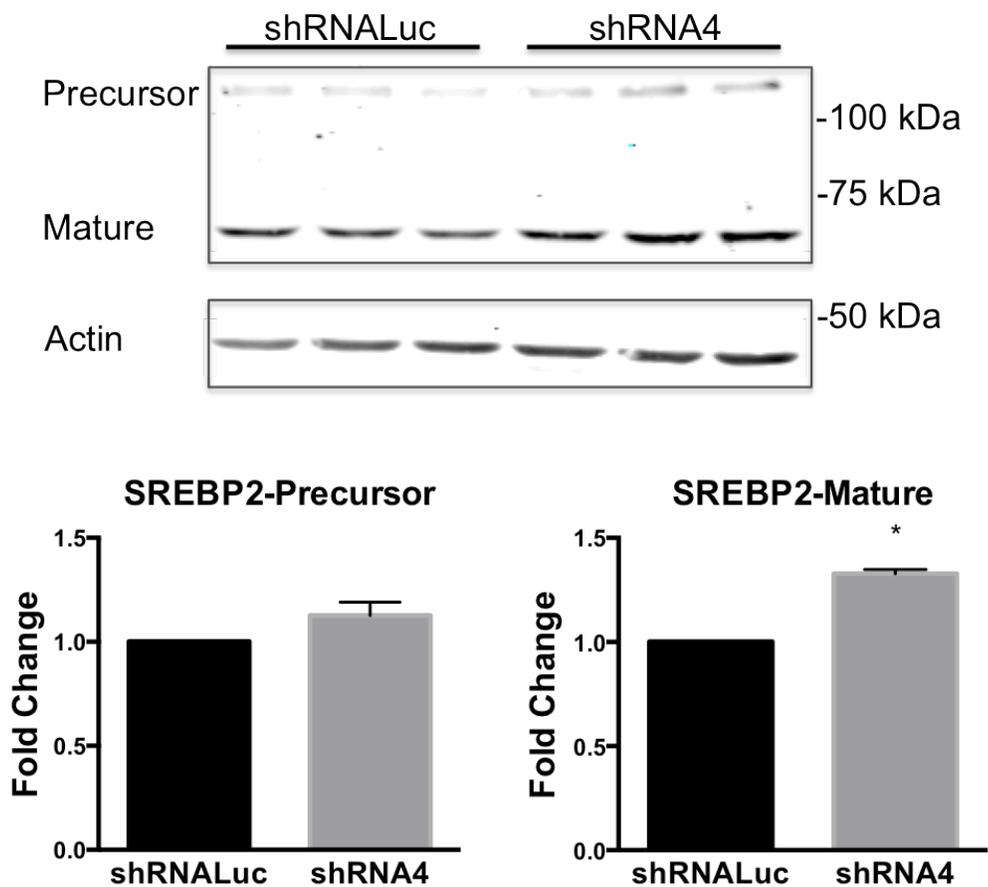
Figure 21: PICALM knockdown with shRNA 4 results in elevated SREBP2 levels

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.
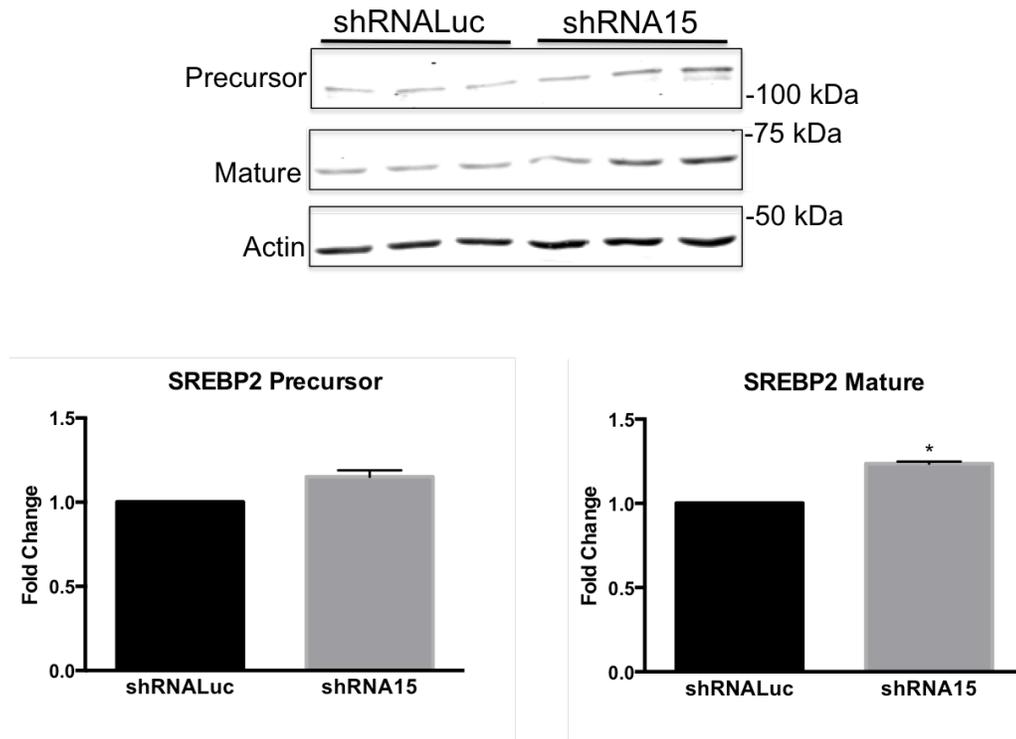
Figure 22: PICALM shRNA knockdown with shRNA 15 results in modest elevation of SREBP2

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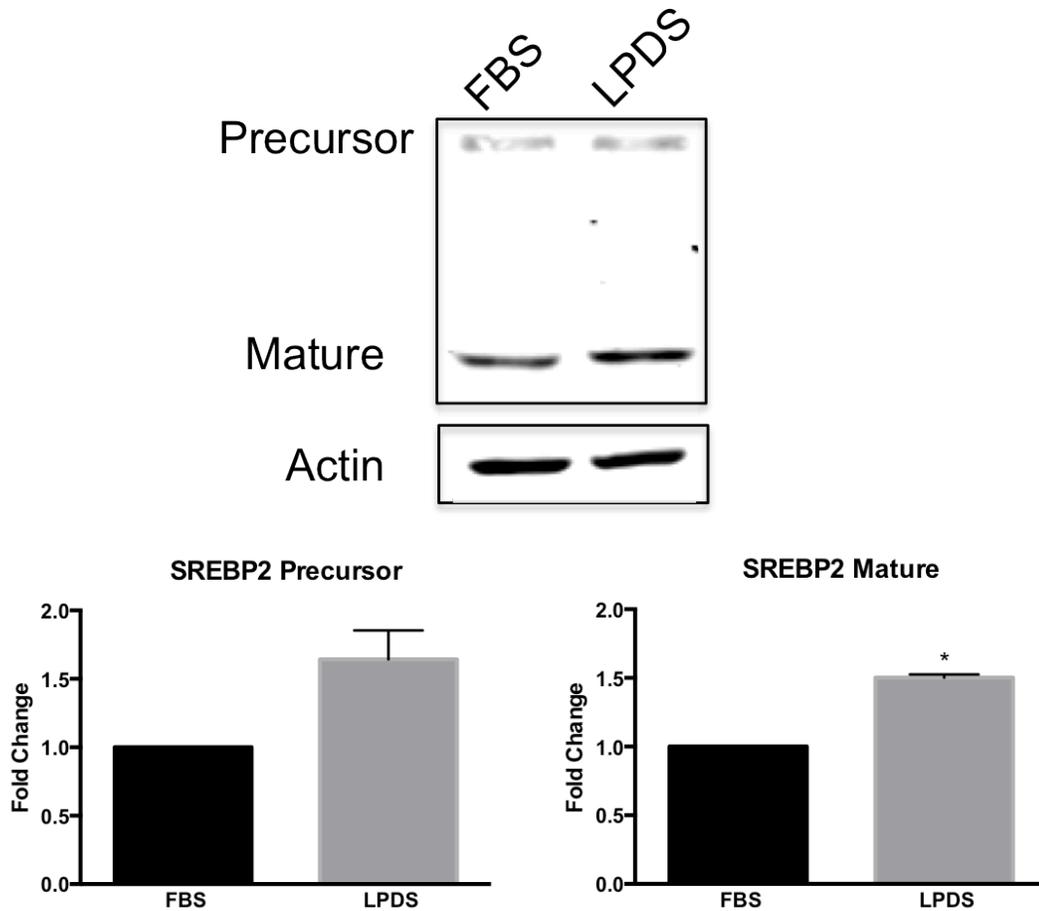
Figure 23: Lipid deprivation results in a modest elevation of SREBP2

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.

72

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**).
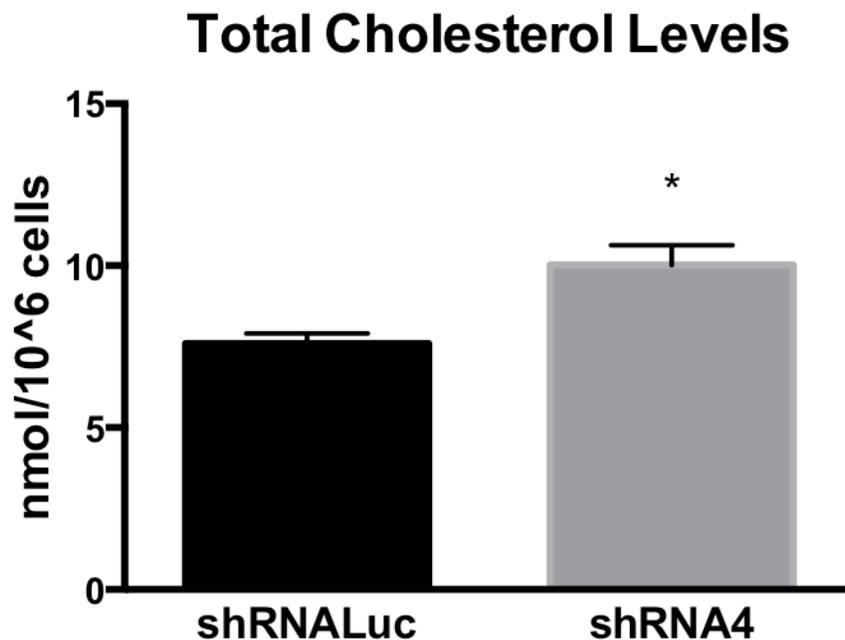
Figure 24: PICALM knockdown results in elevated cellular cholesterol levels

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 U-^{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**).
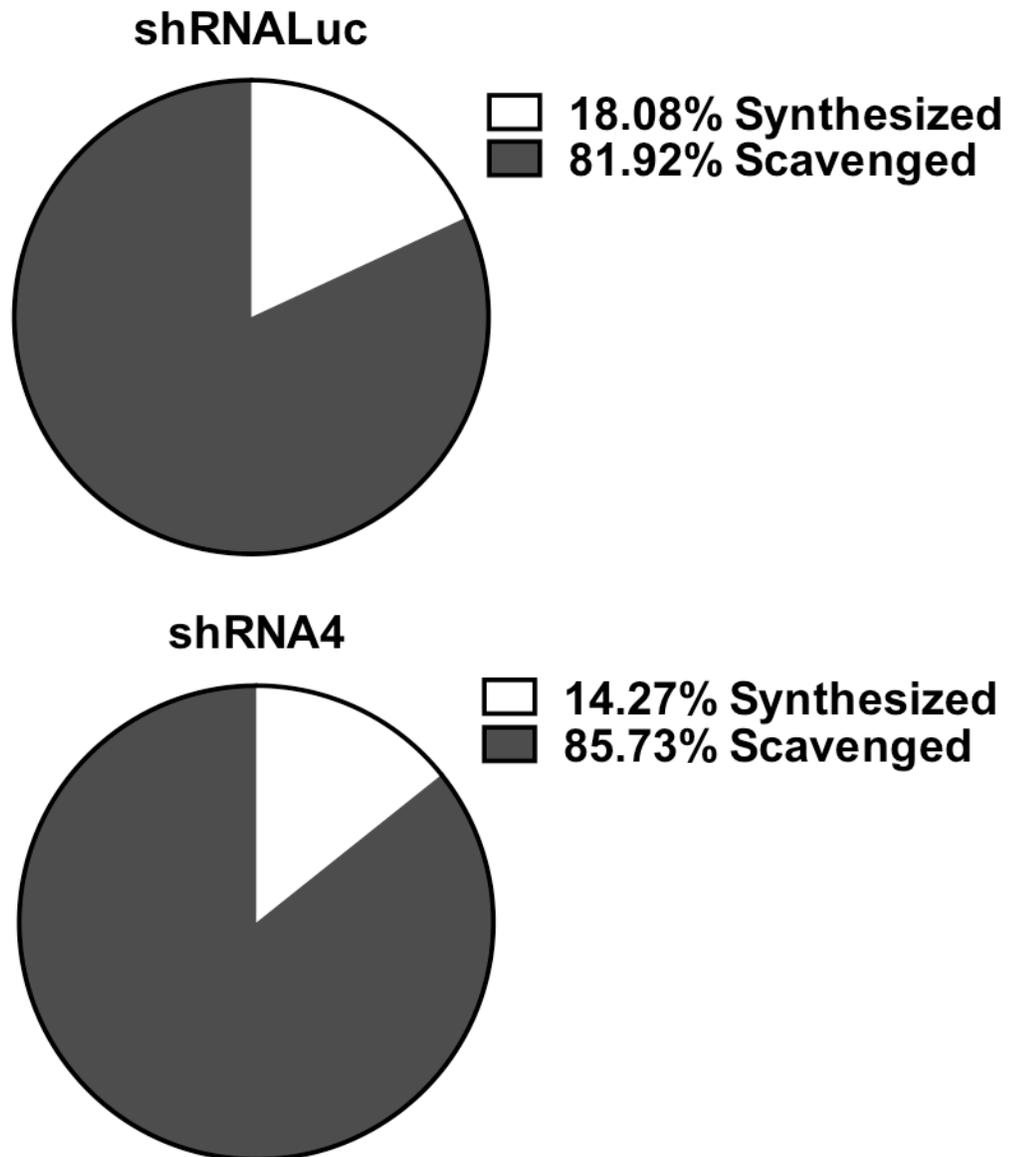
Figure 25: PICALM shRNA knockdown cells have higher abundance of scavenged cellular cholesterol

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-^{13}C$_6$-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**).
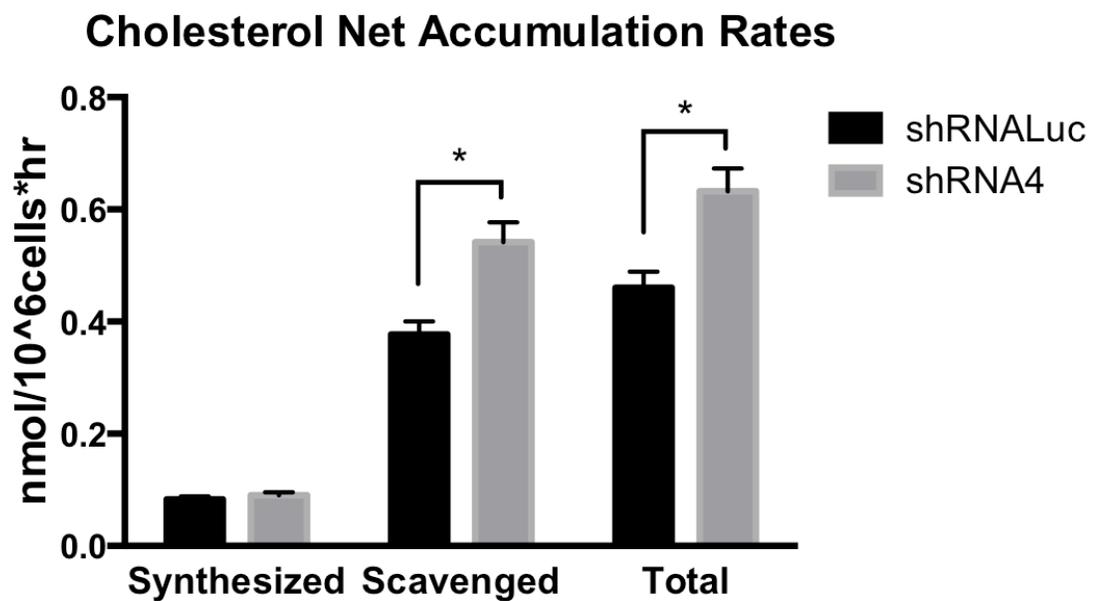
Figure 26: PICALM knockdown results in elevated net cholesterol scavenging rates

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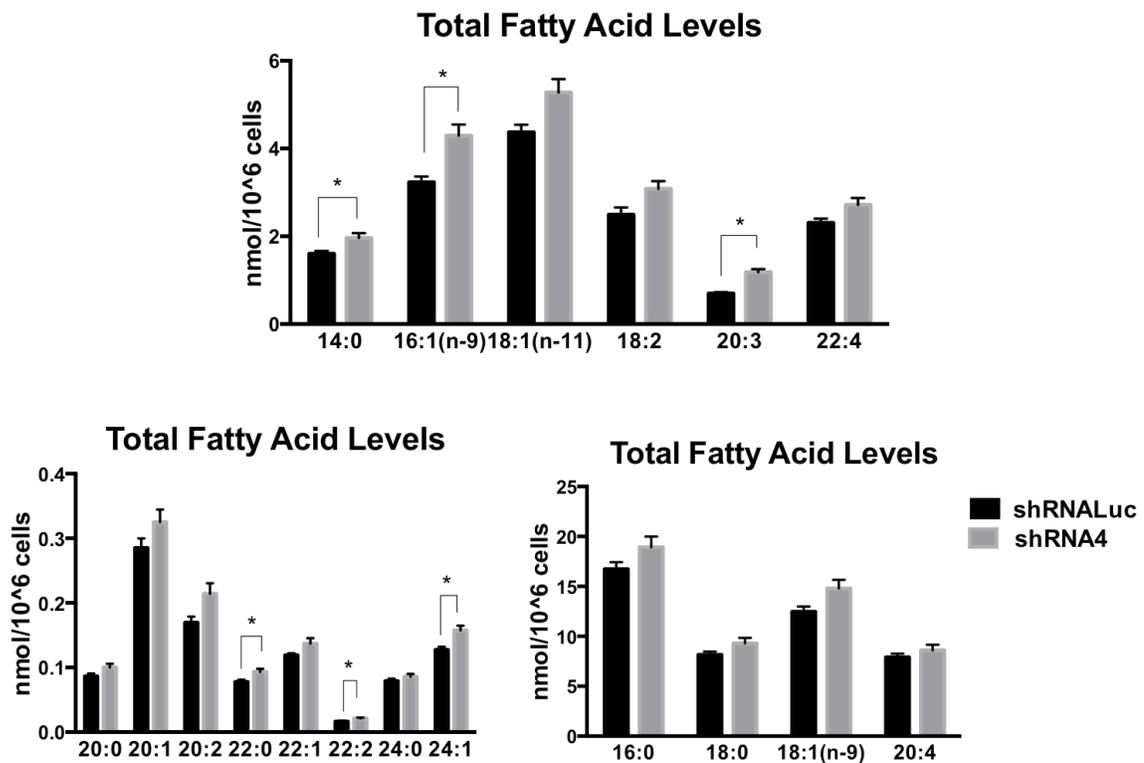
Figure 27: Total fatty acid levels are elevated or maintained in PICALM knockdown cells

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-^{13}C$_6$-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.
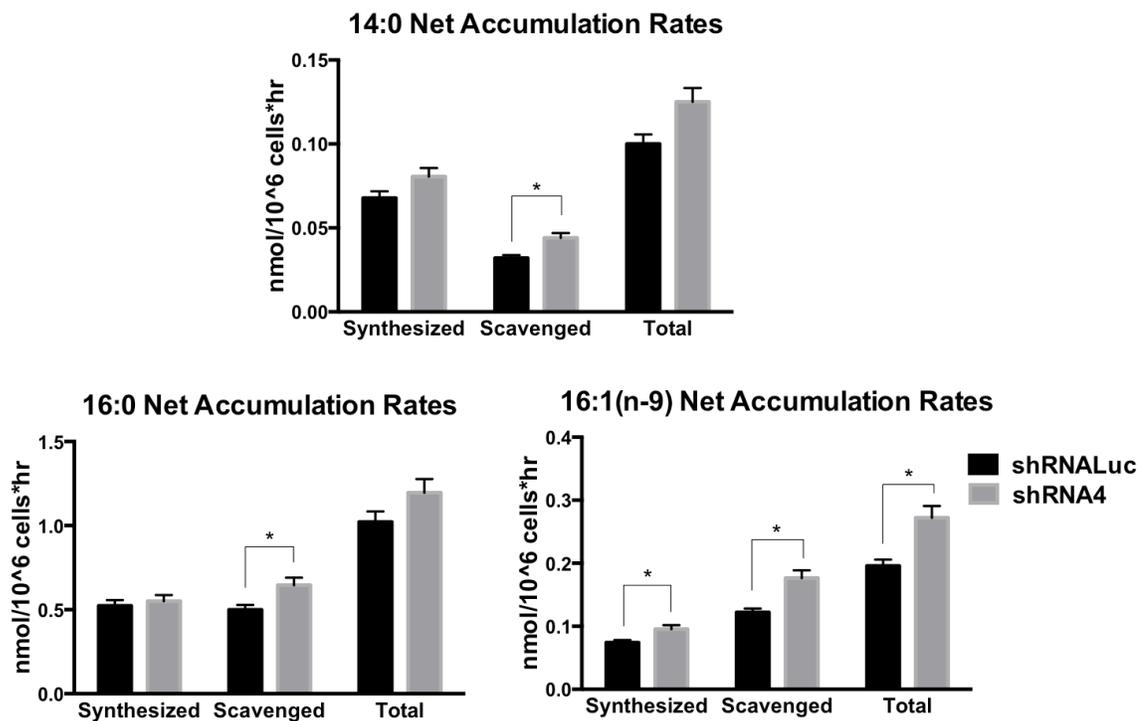
Figure 28: Net rates of fatty acid synthesis, scavenging and accumulation are elevated or maintained in PICALM knockdown cells

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-^{13}C$_6$-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
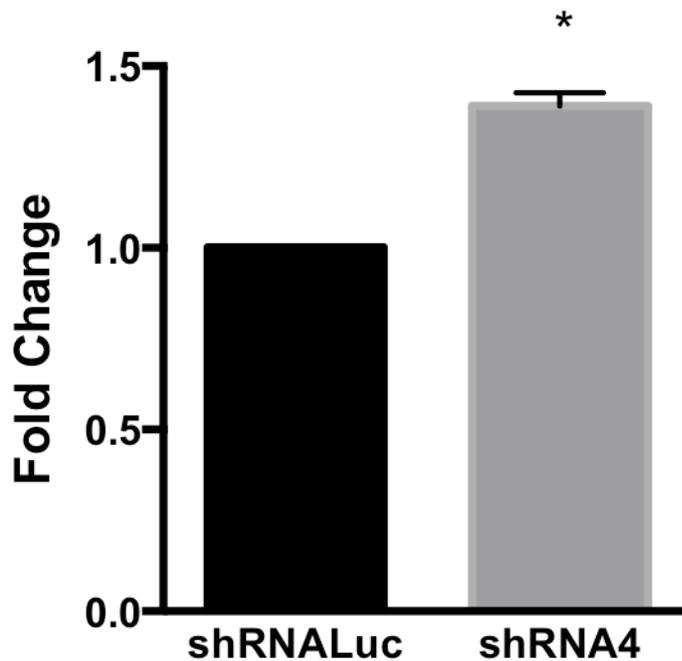
Figure 29: PICALM knockdown results in elevated LDLR surface expression

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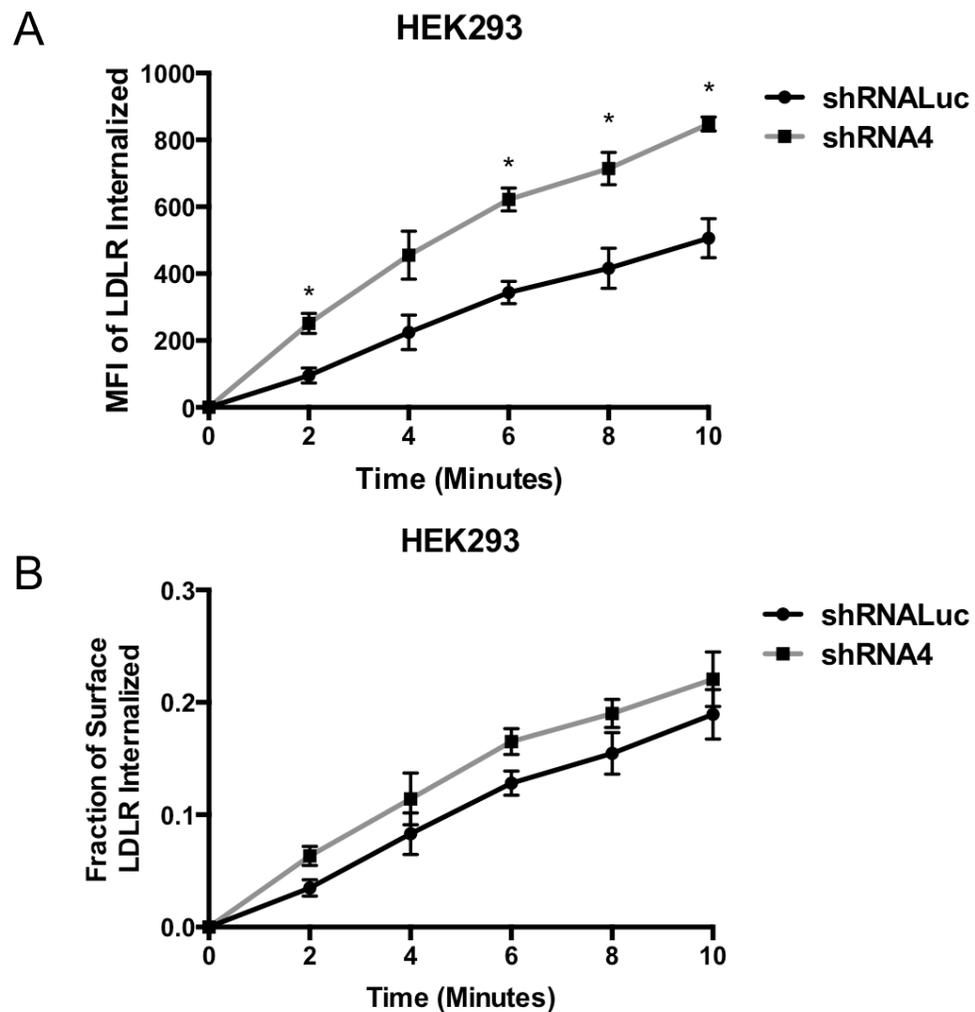
Figure 30: Total LDL receptor internalization is elevated in the absence of PICALM

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 (shRNALuc) 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

(139). 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 monooxygenase (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-lyososomal 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 macroautopagy. 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).

90

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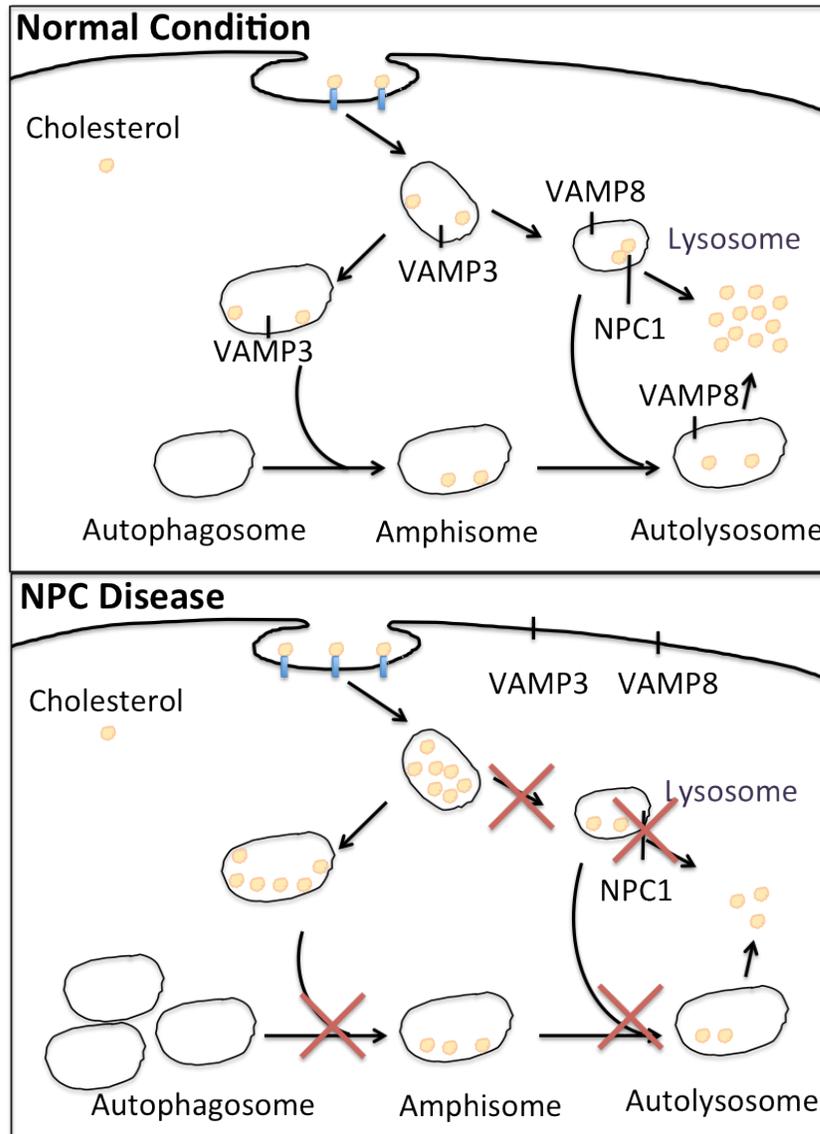
**Figure 31: Defects in the endosomal-lysosomal system and macroautophagy
contribute to altered cholesterol trafficking in NPC disease**

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**).
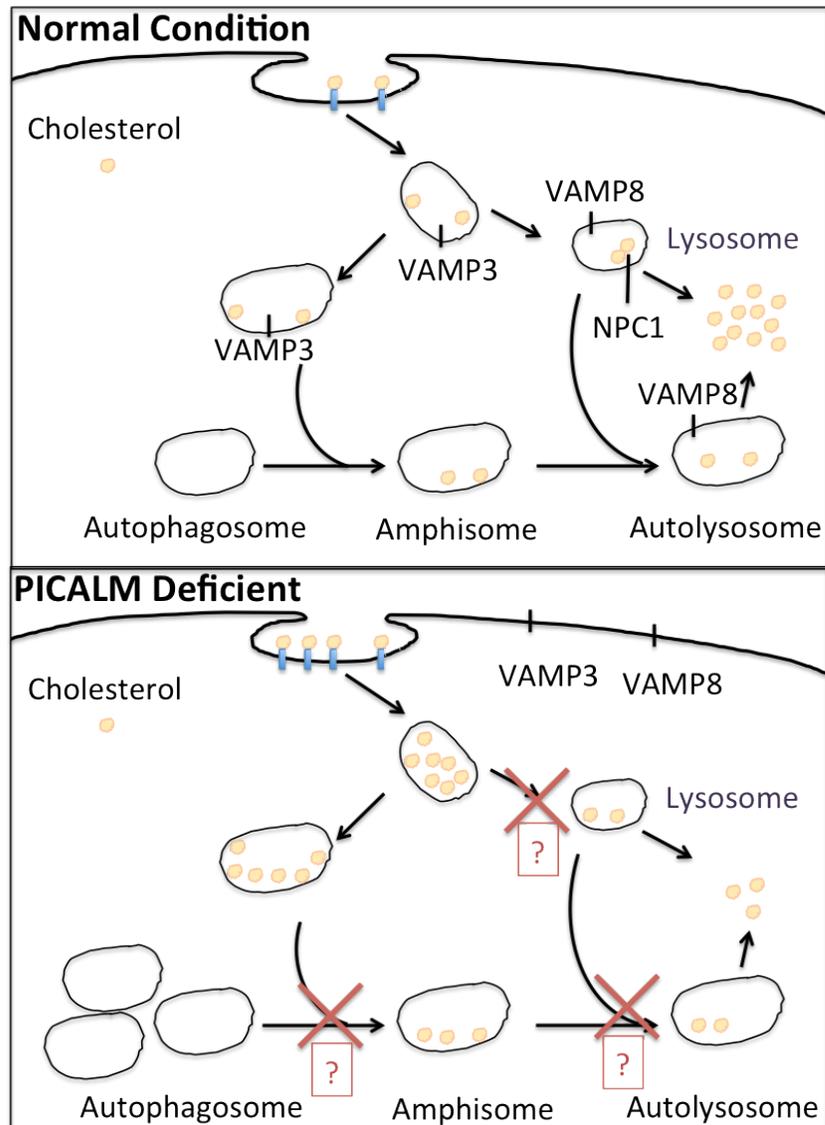
Figure 32: PICALM deficiency may result in altered SNARE protein localization, macroautophagy and cellular cholesterol homeostasis

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[c-2J]/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

Highest Fold Change	Gene ID	Gene Name	Fold Change
1	1419691_at	Camp	5.020108341
2	1419709_at	Stfa1 /// Stfa3	4.305725956
3	1418722_at	Ngp	4.017798606
4	1427747_a_at	Lcn2	3.563405709
5	1434484_at	1100001G20Rik	3.336776368
6	1450009_at	Ltf	2.905153714
7	1448756_at	S100a9	2.88053193
8	1449829_at	Itgb2l	2.707390051
9	1417290_at	Lrg1	2.686855994
10	1427256_at	Vcan	2.602486627
11	1455099_at	Mogat2	2.370766785
12	1455493_at	Syne1	2.366002339
13	1423915_at	Olfml2b	2.302044944
14	1418345_at	Tnfsf12 /// Tnfsf12-tnfsf13 /// Tnfsf13	2.281527827
15	1421811_at	LOC640441 /// Thbs1	2.205576804
16	1422013_at	Clec4a2	2.168780806
17	1426642_at	Fn1	2.140681632
18	1451858_at	LOC668727 /// Mrgpra2	2.134156538
19	1422756_at	Slc32a1	2.119415944
20	1460302_at	Thbs1	2.118788378
21	1439036_a_at	Atp1b1	2.104710053
22	1420407_at	Ltb4r1	2.072162652

23	1425538_x_at	Ceacam1	2.042613152
24	1449038_at	Foxo3	1.952038152
25	1460419_a_at	Prkcb1	1.932432471
26	1449184_at	Pglyrp1	1.925134509
27	1425407_s_at	Clec4a2 /// Clec4b1	1.923493894
28	1460682_s_at	Ceacam1 /// Ceacam2	1.90729632
29	1456603_at	1500005K14Rik	1.711995904
30	1450389_s_at	Pip5k1b	1.670486704
31	1434674_at	Lyst	1.656431737
32	1422046_at	Itgam	1.650341972
33	1451021_a_at	Klf5	1.631513019
34	1420641_a_at	Sqrdl	1.620140017
35	1448871_at	Mapk13	1.617675514
36	1434025_at	---	1.605362664
37	1422678_at	Dgat2	1.603516835
38	1425958_at	Il1f9	1.597546584
39	1450377_at	LOC640441 /// Thbs1	1.597261194
40	1449453_at	Bst1	1.565534444

Appendix B

Table 6: Top 40 Genes Downregulated in Microarray

Highest Fold Change	Gene ID	Gene Name	Fold Change
1	1437279_x_at	Sdc1	0.290652908
2	1436759_x_at	Cnn3 /// LOC100047856	0.353851269
3	1416645_a_at	Afp	0.41948489
4	1449939_s_at	Dlk1	0.427678845
5	1425923_at	Mycn	0.461652675
6	1425470_at	---	0.471932113
7	1448734_at	Cp	0.52079175
8	1437025_at	Cd28 /// LOC100048845	0.55841709
9	1425742_a_at	Tsc22d1	0.573457311
10	1419561_at	Ccl3	0.5834291
11	1454971_x_at	Tsc22d1	0.593925035
12	1421492_at	Ptgds2	0.600920586
13	1424733_at	P2ry14	0.605989835
14	1425454_a_at	Il12a	0.616812967
15	1434500_at	Ttyh2	0.62069503
16	1434149_at	Tcf4	0.63010886
17	1419387_s_at	Muc13	0.632879503
18	1418872_at	Abcb1b	0.635388992
19	1421027_a_at	Mef2c	0.643451471
20	1436917_s_at	Gpsm1	0.660352155
21	1420505_a_at	Stxbp1	0.6698788
22	1438659_x_at	Chchd6	0.682294149

23	1420824_at	Sema4d	0.69909734
24	1455090_at	Angptl2	0.699801906
25	1426543_x_at	Endod1	0.709220553
26	1417331_a_at	Arl6	0.730217171
27	1450082_s_at	Etv5	0.7355738
28	1426169_a_at	Lat2	0.73899666
29	1452679_at	Tubb2b	0.739272678
30	1433655_at	Rnf141	0.747678913
31	1454699_at	LOC100047324 /// Sesn1	0.75673627
32	1424029_at	Tspyl4	0.758446593
33	1451063_at	Stxbp4	0.759805468
34	1424242_at	Bphl	0.760188971
35	1455653_at	Ccnj	0.763337779
36	1438354_x_at	Cnn3	0.767225193
37	1426934_at	Nhsl1	0.771088042
38	1460695_a_at	2010111I01Rik	0.772714834
39	1425934_a_at	B4galt4	0.780265491
40	1451474_a_at	Parp8	0.784824373

References

1.   Moreau K, Fleming A, Imarisio S, Lopez Ramirez A, Mercer JL, Jimenez-Sanchez M, et al. PICALM modulates autophagy activity and tau accumulation. Nature communications. 2014;5:4998.

2.   Mercer JL, Argus JP, Crabtree DM, Keenan MM, Wilks MQ, Chi JT, et al. Modulation of PICALM Levels Perturbs Cellular Cholesterol Homeostasis. PloS one. 2015;10(6):e0129776.

3.   Dreyling MH, Martinez-Climent JA, Zheng M, Mao J, Rowley JD, Bohlander SK. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(10):4804-9.

4.   Abdelhaleem M, Beimnet K, Kirby-Allen M, Naqvi A, Hitzler J, Shago M. High incidence of CALM-AF10 fusion and the identification of a novel fusion transcript in acute megakaryoblastic leukemia in children without Down's syndrome. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2007;21(2):352-3.

5.   Bohlander SK, Muschinsky V, Schrader K, Siebert R, Schlegelberger B, Harder L, et al. Molecular analysis of the CALM/AF10 fusion: identical rearrangements in acute myeloid leukemia, acute lymphoblastic leukemia and malignant lymphoma patients. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2000;14(1):93-9.

6.   Conway AE, Scotland PB, Lavau CP, Wechsler DS. A CALM-derived nuclear export signal is essential for CALM-AF10-mediated leukemogenesis. Blood. 2013;121(23):4758-68.

7.   Conway AE, Haldeman JM, Wechsler DS, Lavau CP. A critical role for CRM1 in regulating HOXA gene transcription in CALM-AF10 leukemias. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2014.

8.   Klebig ML, Wall MD, Potter MD, Rowe EL, Carpenter DA, Rinchik EM. Mutations in the clathrin-assembly gene Picalm are responsible for the hematopoietic and iron metabolism abnormalities in fit1 mice. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(14):8360-5.

9.   Suzuki M, Tanaka H, Tanimura A, Tanabe K, Oe N, Rai S, et al. The clathrin assembly protein PICALM is required for erythroid maturation and transferrin internalization in mice. PloS one. 2012;7(2):e31854.

10.   Harel A, Mattson MP, Yao PJ. CALM, a clathrin assembly protein, influences cell surface GluR2 abundance. Neuromolecular medicine. 2011;13(1):88-90.

11.   Meyerholz A, Hinrichsen L, Groos S, Esk PC, Brandes G, Ungewickell EJ. Effect of clathrin assembly lymphoid myeloid leukemia protein depletion on clathrin coat formation. Traffic. 2005;6(12):1225-34.

12.   Scotland PB, Heath JL, Conway AE, Porter NB, Armstrong MB, Walker JA, et al. The PICALM Protein Plays a Key Role in Iron Homeostasis and Cell Proliferation. PloS one. 2012;7(8):e44252.

13.   Tebar F, Bohlander SK, Sorkin A. Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. Molecular biology of the cell. 1999;10(8):2687-702.

14.   McMahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nature reviews Molecular cell biology. 2011;12(8):517-33.

15.   Doherty GJ, McMahon HT. Mechanisms of endocytosis. Annual review of biochemistry. 2009;78:857-902.

16.   Popova NV, Deyev IE, Petrenko AG. Clathrin-mediated endocytosis and adaptor proteins. Acta naturae. 2013;5(3):62-73.

17.   Brodsky FM. Diversity of clathrin function: new tricks for an old protein. Annual review of cell and developmental biology. 2012;28:309-36.

18.   Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, Gibson A, et al. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science. 2001;291(5506):1051-5.

19.   Moshkanbaryans L, Chan LS, Graham ME. The Biochemical Properties and Functions of CALM and AP180 in Clathrin Mediated Endocytosis. Membranes. 2014;4(3):388-413.

20.   Legendre-Guillemin V, Wasiak S, Hussain NK, Angers A, McPherson PS. ENTH/ANTH proteins and clathrin-mediated membrane budding. Journal of cell science. 2004;117(Pt 1):9-18.

21.   Tian Y, Chang JC, Fan EY, Flajolet M, Greengard P. Adaptor complex AP2/PICALM, through interaction with LC3, targets Alzheimer's APP-CTF for terminal degradation via autophagy. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(42):17071-6.

22.   Xiao Q, Gil SC, Yan P, Wang Y, Han S, Gonzales E, et al. Role of phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia (PICALM) in intracellular amyloid precursor protein (APP) processing and amyloid plaque pathogenesis. The Journal of biological chemistry. 2012;287(25):21279-89.

23.   Morris SM, Cooper JA. Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. Traffic. 2001;2(2):111-23.

24.   Motley A, Bright NA, Seaman MN, Robinson MS. Clathrin-mediated endocytosis in AP-2-depleted cells. The Journal of cell biology. 2003;162(5):909-18.

25.   Purdy AK, Alvarez Arias DA, Oshinsky J, James AM, Serebriiskii I, Campbell KS. The ap-2 clathrin adaptor mediates endocytosis of an inhibitory killer cell Ig-like receptor in human NK cells. J Immunol. 2014;193(9):4675-83.

26.   Huang F, Khvorova A, Marshall W, Sorkin A. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. The Journal of biological chemistry. 2004;279(16):16657-61.

27.   Miller SE, Mathiasen S, Bright NA, Pierre F, Kelly BT, Kladt N, et al. CALM Regulates Clathrin-Coated Vesicle Size and Maturation by Directly Sensing and Driving Membrane Curvature. Developmental cell. 2015;33(2):163-75.

28.   Sahlender DA, Kozik P, Miller SE, Peden AA, Robinson MS. Uncoupling the functions of CALM in VAMP sorting and clathrin-coated pit formation. PloS one. 2013;8(5):e64514.

29.   Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron. 1998;21(6):1465-75.

30.   Vecchi M, Polo S, Poupon V, van de Loo JW, Benmerah A, Di Fiore PP. Nucleocytoplasmic shuttling of endocytic proteins. The Journal of cell biology. 2001;153(7):1511-7.

31.   Pilecka I, Banach-Orlowska M, Miaczynska M. Nuclear functions of endocytic proteins. European journal of cell biology. 2007;86(9):533-47.

32.   Pyrzynska B, Pilecka I, Miaczynska M. Endocytic proteins in the regulation of nuclear signaling, transcription and tumorigenesis. Molecular oncology. 2009;3(4):321-38.

33.   Jahn R, Scheller RH. SNAREs--engines for membrane fusion. Nature reviews Molecular cell biology. 2006;7(9):631-43.

34.   Harel A, Wu F, Mattson MP, Morris CM, Yao PJ. Evidence for CALM in directing VAMP2 trafficking. Traffic. 2008;9(3):417-29.

35.   Koo SJ, Markovic S, Puchkov D, Mahrenholz CC, Beceren-Braun F, Maritzen T, et al. SNARE motif-mediated sorting of synaptobrevin by the endocytic adaptors clathrin

assembly lymphoid myeloid leukemia (CALM) and AP180 at synapses. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(33):13540-5.

36.   Miller SE, Sahlender DA, Graham SC, Honing S, Robinson MS, Peden AA, et al. The molecular basis for the endocytosis of small R-SNAREs by the clathrin adaptor CALM. Cell. 2011;147(5):1118-31.

37.   Rai S, Tanaka H, Suzuki M, Ogoh H, Taniguchi Y, Morita Y, et al. Clathrin Assembly Protein CALM Plays a Critical Role in KIT Signaling by Regulating Its Cellular Transport from Early to Late Endosomes in Hematopoietic Cells. PloS one. 2014;9(10):e109441.

38.   Moreau K, Renna M, Rubinsztein DC. Connections between SNAREs and autophagy. Trends in biochemical sciences. 2013;38(2):57-63.

39.   Holtzman DM, Morris JC, Goate AM. Alzheimer's disease: the challenge of the second century. Science translational medicine. 2011;3(77):77sr1.

40.   Kumar A, Singh A, Ekavali. A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacological reports : PR. 2015;67(2):195-203.

41.   Citron M. Alzheimer's disease: strategies for disease modification. Nature reviews Drug discovery. 2010;9(5):387-98.

42.   Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nature genetics. 2009;41(10):1088-93.

43.   Corneveaux JJ, Myers AJ, Allen AN, Pruzin JJ, Ramirez M, Engel A, et al. Association of CR1, CLU and PICALM with Alzheimer's disease in a cohort of clinically characterized and neuropathologically verified individuals. Human molecular genetics. 2010;19(16):3295-301.

44.   Jun G, Naj AC, Beecham GW, Wang LS, Buros J, Gallins PJ, et al. Meta-analysis confirms CR1, CLU, and PICALM as alzheimer disease risk loci and reveals interactions with APOE genotypes. Archives of neurology. 2010;67(12):1473-84.

45.   Seshadri S, Fitzpatrick AL, Ikram MA, DeStefano AL, Gudnason V, Boada M, et al. Genome-wide analysis of genetic loci associated with Alzheimer disease. JAMA : the journal of the American Medical Association. 2010;303(18):1832-40.

46.   Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993;261(5123):921-3.

47.   Morgen K, Ramirez A, Frolich L, Tost H, Plichta MM, Kolsch H, et al. Genetic interaction of PICALM and APOE is associated with brain atrophy and cognitive impairment in Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2014;10(5 Suppl):S269-76.

48.   Zhao Z, Sagare AP, Ma Q, Halliday MR, Kong P, Kisler K, et al. Central role for PICALM in amyloid-beta blood-brain barrier transcytosis and clearance. Nature neuroscience. 2015.

49.   Kanatsu K, Morohashi Y, Suzuki M, Kuroda H, Watanabe T, Tomita T, et al. Decreased CALM expression reduces Abeta42 to total Abeta ratio through clathrin-mediated endocytosis of gamma-secretase. Nature communications. 2014;5:3386.

50.   Baig S, Joseph SA, Tayler H, Abraham R, Owen MJ, Williams J, et al. Distribution and expression of picalm in Alzheimer disease. Journal of neuropathology and experimental neurology. 2010;69(10):1071-7.

51.   Thomas RS, Lelos MJ, Good MA, Kidd EJ. Clathrin-mediated endocytic proteins are upregulated in the cortex of the Tg2576 mouse model of Alzheimer's disease-like amyloid pathology. Biochemical and biophysical research communications. 2011;415(4):656-61.

52.   Xu W, Tan L, Yu JT. The Role of PICALM in Alzheimer's Disease. Molecular neurobiology. 2014.

53.   Ando K, Brion JP, Stygelbout V, Suain V, Authelet M, Dedecker R, et al. Clathrin adaptor CALM/PICALM is associated with neurofibrillary tangles and is cleaved in Alzheimer's brains. Acta neuropathologica. 2013;125(6):861-78.

54.   Menzies FM, Fleming A, Rubinsztein DC. Compromised autophagy and neurodegenerative diseases. Nature reviews Neuroscience. 2015;16(6):345-57.

55.   Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nature cell biology. 2010;12(9):814-22.

56.   Galluzzi L, Pietrocola F, Levine B, Kroemer G. Metabolic control of autophagy. Cell. 2014;159(6):1263-76.

57.   Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012;8(4):445-544.

58.   Feng Y, Yao Z, Klionsky DJ. How to control self-digestion: transcriptional, post-transcriptional, and post-translational regulation of autophagy. Trends in cell biology. 2015;25(6):354-63.

59.   Evangelisti C, Evangelisti C, Chiarini F, Lonetti A, Buontempo F, Neri LM, et al. Autophagy in acute leukemias: a double-edged sword with important therapeutic implications. Biochimica et biophysica acta. 2015;1853(1):14-26.

60.   Schneider JL, Cuervo AM. Autophagy and human disease: emerging themes. Current opinion in genetics & development. 2014;26:16-23.

61.   Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell research. 2014;24(1):24-41.

62.   Mehrpour M, Esclatine A, Beau I, Codogno P. Overview of macroautophagy regulation in mammalian cells. Cell research. 2010;20(7):748-62.

63.   Sanchez-Wandelmer J, Reggiori F. Amphisomes: out of the autophagosome shadow? The EMBO journal. 2013;32(24):3116-8.

64.   Chen YA, Scheller RH. SNARE-mediated membrane fusion. Nature reviews Molecular cell biology. 2001;2(2):98-106.

65.   Nair U, Jotwani A, Geng J, Gammoh N, Richerson D, Yen WL, et al. SNARE proteins are required for macroautophagy. Cell. 2011;146(2):290-302.

66.   Zhu D, Zhang Y, Lam PP, Dolai S, Liu Y, Cai EP, et al. Dual role of VAMP8 in regulating insulin exocytosis and islet beta cell growth. Cell metabolism. 2012;16(2):238-49.

67.   Furuta N, Fujita N, Noda T, Yoshimori T, Amano A. Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Molecular biology of the cell. 2010;21(6):1001-10.

68.   Itakura E, Kishi-Itakura C, Mizushima N. The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. Cell. 2012;151(6):1256-69.

69.   Itakura E, Mizushima N. Syntaxin 17: the autophagosomal SNARE. Autophagy. 2013;9(6):917-9.

70.   Sarkar S, Maetzel D, Korolchuk VI, Jaenisch R. Restarting stalled autophagy a potential therapeutic approach for the lipid storage disorder, Niemann-Pick type C1 disease. Autophagy. 2014;10(6):1137-40.

71.   Ciechanover A, Kwon YT. Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. Experimental & molecular medicine. 2015;47:e147.

72.   Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nature medicine. 2004;10 Suppl:S10-7.

73.   Cheung ZH, Ip NY. Autophagy deregulation in neurodegenerative diseases - recent advances and future perspectives. Journal of neurochemistry. 2011;118(3):317-25.

74.   Yang DS, Stavrides P, Saito M, Kumar A, Rodriguez-Navarro JA, Pawlik M, et al. Defective macroautophagic turnover of brain lipids in the TgCRND8 Alzheimer mouse model: prevention by correcting lysosomal proteolytic deficits. Brain : a journal of neurology. 2014;137(Pt 12):3300-18.

75.   Inoue K, Rispoli J, Kaphzan H, Klann E, Chen EI, Kim J, et al. Macroautophagy deficiency mediates age-dependent neurodegeneration through a phospho-tau pathway. Molecular neurodegeneration. 2012;7:48.

76.   Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, et al. Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. The Journal of cell biology. 2005;171(1):87-98.

77.   Zheng L, Terman A, Hallbeck M, Dehvari N, Cowburn RF, Benedikz E, et al. Macroautophagy-generated increase of lysosomal amyloid beta-protein mediates oxidant-induced apoptosis of cultured neuroblastoma cells. Autophagy. 2011;7(12):1528-45.

78.   Dong H, Czaja MJ. Regulation of lipid droplets by autophagy. Trends in endocrinology and metabolism: TEM. 2011;22(6):234-40.

79.   Singh R, Cuervo AM. Lipophagy: connecting autophagy and lipid metabolism. International journal of cell biology. 2012;2012:282041.

80.   Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. Nature. 2009;458(7242):1131-5.

81.	Seo YK, Jeon TI, Chong HK, Biesinger J, Xie X, Osborne TF. Genome-wide localization of SREBP-2 in hepatic chromatin predicts a role in autophagy. Cell metabolism. 2011;13(4):367-75.

82.	Lamb CA, Dooley HC, Tooze SA. Endocytosis and autophagy: Shared machinery for degradation. BioEssays : news and reviews in molecular, cellular and developmental biology. 2013;35(1):34-45.

83.	Tooze SA, Abada A, Elazar Z. Endocytosis and autophagy: exploitation or cooperation? Cold Spring Harbor perspectives in biology. 2014;6(5):a018358.

84.	Maetzel D, Sarkar S, Wang H, Abi-Mosleh L, Xu P, Cheng AW, et al. Genetic and chemical correction of cholesterol accumulation and impaired autophagy in hepatic and neural cells derived from Niemann-Pick Type C patient-specific iPS cells. Stem cell reports. 2014;2(6):866-80.

85.	Sarkar S, Carroll B, Buganim Y, Maetzel D, Ng AH, Cassady JP, et al. Impaired autophagy in the lipid-storage disorder Niemann-Pick type C1 disease. Cell reports. 2013;5(5):1302-15.

86.	Ikonen E. Mechanisms for cellular cholesterol transport: defects and human disease. Physiological reviews. 2006;86(4):1237-61.

87.	Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nature reviews Molecular cell biology. 2008;9(2):125-38.

88.	Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. Nature. 2005;438(7068):612-21.

89.	Simons K, Toomre D. Lipid rafts and signal transduction. Nature reviews Molecular cell biology. 2000;1(1):31-9.

90.	Anderson RG. Joe Goldstein and Mike Brown: from cholesterol homeostasis to new paradigms in membrane biology. Trends in cell biology. 2003;13(10):534-9.

91.   Dietschy JM, Turley SD. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. Journal of lipid research. 2004;45(8):1375-97.

92.   Espenshade PJ, Hughes AL. Regulation of sterol synthesis in eukaryotes. Annual review of genetics. 2007;41:401-27.

93.   Goedeke L, Fernandez-Hernando C. Regulation of cholesterol homeostasis. Cellular and molecular life sciences : CMLS. 2012;69(6):915-30.

94.   Goldstein JL, Brown MS. The LDL receptor. Arteriosclerosis, thrombosis, and vascular biology. 2009;29(4):431-8.

95.   Li Y, Cam J, Bu G. Low-density lipoprotein receptor family: endocytosis and signal transduction. Molecular neurobiology. 2001;23(1):53-67.

96.   Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. Trends in endocrinology and metabolism: TEM. 2002;13(2):66-74.

97.   Chang TY, Chang CC, Ohgami N, Yamauchi Y. Cholesterol sensing, trafficking, and esterification. Annual review of cell and developmental biology. 2006;22:129-57.

98.   Liscum L, Sturley SL. Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. Biochimica et biophysica acta. 2004;1685(1-3):22-7.

99.   Mukherjee S, Maxfield FR. Lipid and cholesterol trafficking in NPC. Biochimica et biophysica acta. 2004;1685(1-3):28-37.

100.   Subramanian K, Balch WE. NPC1/NPC2 function as a tag team duo to mobilize cholesterol. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(40):15223-4.

101.   Yu XH, Jiang N, Yao PB, Zheng XL, Cayabyab FS, Tang CK. NPC1, intracellular cholesterol trafficking and atherosclerosis. Clinica chimica acta; international journal of clinical chemistry. 2014;429:69-75.

102.   Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell. 2006;124(1):35-46.

103.   Weber LW, Boll M, Stampfl A. Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. World journal of gastroenterology : WJG. 2004;10(21):3081-7.

104.   Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS. Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. Cell metabolism. 2008;8(6):512-21.

105.   Brown MS, Goldstein JL. Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. Journal of lipid research. 2009;50 Suppl:S15-27.

106.   Rung E, Friberg PA, Shao R, Larsson DG, Nielsen E, Svensson PA, et al. Progesterone-receptor antagonists and statins decrease de novo cholesterol synthesis and increase apoptosis in rat and human periovulatory granulosa cells in vitro. Biology of reproduction. 2005;72(3):538-45.

107.   Brusselmans K, Timmermans L, Van de Sande T, Van Veldhoven PP, Guan G, Shechter I, et al. Squalene synthase, a determinant of Raft-associated cholesterol and modulator of cancer cell proliferation. The Journal of biological chemistry. 2007;282(26):18777-85.

108.   Kwak BR, Mulhaupt F, Mach F. Atherosclerosis: anti-inflammatory and immunomodulatory activities of statins. Autoimmunity reviews. 2003;2(6):332-8.

109.   Rosenson RS. Statins in atherosclerosis: lipid-lowering agents with antioxidant capabilities. Atherosclerosis. 2004;173(1):1-12.

110.   Vaughan CJ, Gotto AM, Jr., Basson CT. The evolving role of statins in the management of atherosclerosis. Journal of the American College of Cardiology. 2000;35(1):1-10.

111.   Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, et al. Simvastatin strongly reduces levels of Alzheimer's disease beta -amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(10):5856-61.

112.   Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. Lancet. 2000;356(9242):1627-31.

113.   Kandiah N, Feldman HH. Therapeutic potential of statins in Alzheimer's disease. Journal of the neurological sciences. 2009;283(1-2):230-4.

114.   Dietschy JM. Central nervous system: cholesterol turnover, brain development and neurodegeneration. Biological chemistry. 2009;390(4):287-93.

115.   Vance JE, Hayashi H, Karten B. Cholesterol homeostasis in neurons and glial cells. Seminars in cell & developmental biology. 2005;16(2):193-212.

116.   Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. Arteriosclerosis, thrombosis, and vascular biology. 2004;24(5):806-15.

117.   Karasinska JM, Hayden MR. Cholesterol metabolism in Huntington disease. Nature reviews Neurology. 2011;7(10):561-72.

118.   Di Paolo G, Kim TW. Linking lipids to Alzheimer's disease: cholesterol and beyond. Nature reviews Neuroscience. 2011;12(5):284-96.

119.   Ehehalt R, Keller P, Haass C, Thiele C, Simons K. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. The Journal of cell biology. 2003;160(1):113-23.

120.   Grimm MO, Grimm HS, Tomic I, Beyreuther K, Hartmann T, Bergmann C. Independent inhibition of Alzheimer disease beta- and gamma-secretase cleavage by lowered cholesterol levels. The Journal of biological chemistry. 2008;283(17):11302-11.

121.   Panchal M, Loeper J, Cossec JC, Perruchini C, Lazar A, Pompon D, et al. Enrichment of cholesterol in microdissected Alzheimer's disease senile plaques as assessed by mass spectrometry. Journal of lipid research. 2010;51(3):598-605.

122.   Xiong H, Callaghan D, Jones A, Walker DG, Lue LF, Beach TG, et al. Cholesterol retention in Alzheimer's brain is responsible for high beta- and gamma-secretase activities and Abeta production. Neurobiology of disease. 2008;29(3):422-37.

123.   Bhattacharyya R, Kovacs DM. ACAT inhibition and amyloid beta reduction. Biochimica et biophysica acta. 2010;1801(8):960-5.

124.   Hutter-Paier B, Huttunen HJ, Puglielli L, Eckman CB, Kim DY, Hofmeister A, et al. The ACAT inhibitor CP-113,818 markedly reduces amyloid pathology in a mouse model of Alzheimer's disease. Neuron. 2004;44(2):227-38.

125.   Puglielli L, Tanzi RE, Kovacs DM. Alzheimer's disease: the cholesterol connection. Nature neuroscience. 2003;6(4):345-51.

126.   Du C, Redner RL, Cooke MP, Lavau C. Overexpression of wild-type retinoic acid receptor alpha (RARalpha) recapitulates retinoic acid-sensitive transformation of primary myeloid progenitors by acute promyelocytic leukemia RARalpha-fusion genes. Blood. 1999;94(2):793-802.

127.   Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, et al. A loss-of-function RNA interference screen for molecular targets in cancer. Nature. 2006;441(7089):106-10.

128.   Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. Gene therapy. 1994;1(2):136-8.

129.   Kingston RE, Chen CA, Okayama H. Calcium phosphate transfection. Current protocols in immunology / edited by John E Coligan [et al]. 2001;Chapter 10:Unit 10 3.

130.   Tang X, Lucas JE, Chen JL, LaMonte G, Wu J, Wang MC, et al. Functional interaction between responses to lactic acidosis and hypoxia regulates genomic transcriptional outputs. Cancer research. 2012;72(2):491-502.

131.   Gatza ML, Kung HN, Blackwell KL, Dewhirst MW, Marks JR, Chi JT. Analysis of tumor environmental response and oncogenic pathway activation identifies distinct basal and luminal features in HER2-related breast tumor subtypes. Breast cancer research : BCR. 2011;13(3):R62.

132.   Chang JT, Nevins JR. GATHER: a systems approach to interpreting genomic signatures. Bioinformatics. 2006;22(23):2926-33.

133.   Wong AS, Cheung ZH, Ip NY. Molecular machinery of macroautophagy and its deregulation in diseases. Biochimica et biophysica acta. 2011;1812(11):1490-7.

134.   Klionsky DJ, Elazar Z, Seglen PO, Rubinsztein DC. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? Autophagy. 2008;4(7):849-50.

135.   Rusten TE, Stenmark H. p62, an autophagy hero or culprit? Nature cell biology. 2010;12(3):207-9.

136.   Bjorkoy G, Lamark T, Pankiv S, Overvatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. Methods in enzymology. 2009;452:181-97.

137.   Qi Y, Wang JK, McMillian M, Chikaraishi DM. Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1997;17(4):1217-25.

138.   Suri C, Fung BP, Tischler AS, Chikaraishi DM. Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1993;13(3):1280-91.

139.   Williams KJ, Argus JP, Zhu Y, Wilks MQ, Marbois BN, York AG, et al. An essential requirement for the SCAP/SREBP signaling axis to protect cancer cells from lipotoxicity. Cancer research. 2013;73(9):2850-62.

140.   Eden ER, Sun XM, Patel DD, Soutar AK. Adaptor protein disabled-2 modulates low density lipoprotein receptor synthesis in fibroblasts from patients with autosomal recessive hypercholesterolaemia. Human molecular genetics. 2007;16(22):2751-9.

141.   Garuti R, Jones C, Li WP, Michaely P, Herz J, Gerard RD, et al. The modular adaptor protein autosomal recessive hypercholesterolemia (ARH) promotes low density lipoprotein receptor clustering into clathrin-coated pits. The Journal of biological chemistry. 2005;280(49):40996-1004.

142.   Girard E, Chmiest D, Fournier N, Johannes L, Paul JL, Vedie B, et al. Rab7 is functionally required for selective cargo sorting at the early endosome. Traffic. 2014;15(3):309-26.

143.   Girard E, Paul JL, Fournier N, Beaune P, Johannes L, Lamaze C, et al. The dynamin chemical inhibitor dynasore impairs cholesterol trafficking and sterol-sensitive genes transcription in human HeLa cells and macrophages. PloS one. 2011;6(12):e29042.

144.   Maurer ME, Cooper JA. The adaptor protein Dab2 sorts LDL receptors into coated pits independently of AP-2 and ARH. Journal of cell science. 2006;119(Pt 20):4235-46.

145.   Robinet P, Fradagrada A, Monier MN, Marchetti M, Cogny A, Moatti N, et al. Dynamin is involved in endolysosomal cholesterol delivery to the endoplasmic reticulum: role in cholesterol homeostasis. Traffic. 2006;7(7):811-23.

146.   Song BL, Sever N, DeBose-Boyd RA. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. Molecular cell. 2005;19(6):829-40.

147.   Zelcer N, Sharpe LJ, Loregger A, Kristiana I, Cook EC, Phan L, et al. The E3 ubiquitin ligase MARCH6 degrades squalene monooxygenase and affects 3-hydroxy-3-

methyl-glutaryl coenzyme A reductase and the cholesterol synthesis pathway. Molecular and cellular biology. 2014;34(7):1262-70.

148.   Yu L, York J, von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. Stimulation of cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5 and G8. The Journal of biological chemistry. 2003;278(18):15565-70.

149.   Liscum L, Faust JR. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. The Journal of biological chemistry. 1987;262(35):17002-8.

150.   Peake KB, Vance JE. Defective cholesterol trafficking in Niemann-Pick C-deficient cells. FEBS letters. 2010;584(13):2731-9.

151.   Sobo K, Le Blanc I, Luyet PP, Fivaz M, Ferguson C, Parton RG, et al. Late endosomal cholesterol accumulation leads to impaired intra-endosomal trafficking. PloS one. 2007;2(9):e851.

152.   Vance JE. Lipid imbalance in the neurological disorder, Niemann-Pick C disease. FEBS letters. 2006;580(23):5518-24.

153.   Mori T, Paris D, Town T, Rojiani AM, Sparks DL, Delledonne A, et al. Cholesterol accumulates in senile plaques of Alzheimer disease patients and in transgenic APP(SW) mice. Journal of neuropathology and experimental neurology. 2001;60(8):778-85.

154.   Sparrow SM, Carter JM, Ridgway ND, Cook HW, Byers DM. U18666A inhibits intracellular cholesterol transport and neurotransmitter release in human neuroblastoma cells. Neurochemical research. 1999;24(1):69-77.

155.   Chang TY, Reid PC, Sugii S, Ohgami N, Cruz JC, Chang CC. Niemann-Pick type C disease and intracellular cholesterol trafficking. The Journal of biological chemistry. 2005;280(22):20917-20.

156.   Du X, Kazim AS, Brown AJ, Yang H. An essential role of Hrs/Vps27 in endosomal cholesterol trafficking. Cell reports. 2012;1(1):29-35.

157.   Neefjes J, van der Kant R. Stuck in traffic: an emerging theme in diseases of the nervous system. Trends in neurosciences. 2014;37(2):66-76.

158.   Jin LW, Shie FS, Maezawa I, Vincent I, Bird T. Intracellular accumulation of amyloidogenic fragments of amyloid-beta precursor protein in neurons with Niemann-Pick type C defects is associated with endosomal abnormalities. The American journal of pathology. 2004;164(3):975-85.

159.   Malnar M, Hecimovic S, Mattsson N, Zetterberg H. Bidirectional links between Alzheimer's disease and Niemann-Pick type C disease. Neurobiology of disease. 2014.

160.   Nixon RA. Niemann-Pick Type C disease and Alzheimer's disease: the APP-endosome connection fattens up. The American journal of pathology. 2004;164(3):757-61.

161.   Runz H, Rietdorf J, Tomic I, de Bernard M, Beyreuther K, Pepperkok R, et al. Inhibition of intracellular cholesterol transport alters presenilin localization and amyloid precursor protein processing in neuronal cells. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002;22(5):1679-89.

162.   Tangemo C, Weber D, Theiss S, Mengel E, Runz H. Niemann-Pick Type C disease: characterizing lipid levels in patients with variant lysosomal cholesterol storage. Journal of lipid research. 2011;52(4):813-25.

163.   Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nature reviews Neurology. 2013;9(2):106-18.

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