CONTROL OF PHOSPHOINOSITIDE SYNTHESIS AND DEGRADATION BY THE ACYLTRANSFERASE LYCAT

by

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ABSTRACT

Phosphoinositides (PIPs) are important regulators of various cellular phenomena including intracellular signaling, membrane traffic and cell migration. PIPs are formed as a result of the regulated phosphorylation of the inositol headgroup of phosphatidylinositol (PI) on specific positions by certain lipid kinases and phosphatases. It is well appreciated that the enrichment of specific PIPs, defined by inositol headgroup phosphorylation, within specific membrane compartments plays a critical role in organelle identity and membrane traffic. However, while much attention has been given to understanding of the role of inositol headgroup phosphorylation in PIP function, much less is known about the role of dynamic incorporation of specific acyl groups into these phospholipids.

Importantly, PI and PIPs exhibit remarkable and unique selectivity for certain acyl groups. For example, about 45% of PIs (but not other phospholipids) are rich in 1-steroyl 2-arachidonyl. We recently identified that the possible control of the selective incorporation of steric acid at the sn-1 position is by the acyltransferase LYCAT, which controls the levels, acyl profile and function of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) (Bone et al. Mol Biol Cell 2017. 28:161-172).

Here we examine how perturbation of LYCAT leads to a reduction in the levels of $PI(4,5)P_2$ and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$). To measure the rate of $PI(4,5)P_2$ synthesis, we treated cells with ionomycin to first ablate this PIP, followed by washout of the drug and monitoring of rate of reappearance via localization of a fluorescent $PI(4,5)P_2$ probe. To measure the rate of $PI(4,5)P_2$ degradation, we arrested $PI(4,5)P_2$ synthesis by a pharmacological inhibitor, phenylarsine oxide (PAO) and monitored the loss of cellular $PI(4,5)P_2$. Lastly, to examine the production of $PI(3,4,5)P_3$, we treated cells with epidermal growth factor (EGF) and monitored the production of this PIP.

Together, this work provides new information about how the dynamic and selective remodeling of specific phospholipids controls their levels, localization and function.

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LIST OF ABBREVIATIONS

ABD: Adaptor-binding domain

AP2: Adaptor protein 2

ATG14: Beclin 1- associated autophagy- related key regulator

BSA: Bovine serum albumin

CB: Clathrin-binding

CCP: Clathrin coated pit

CCV: Clathrin coated vesicle

CDP-DAG: Cytidine diphosphate diacylglycerol

CDS: Cytidine diphosphate synthase

CL: Cardiolipin

CME: Clathrin mediated endocytosis

COPI: Coat protein complex I

COPII: Coat protein complex II

DAG: Diacylglycerol

DMEM: Dulbecco's modified eagle's medium

DIO: Diet-induced obesity

DLCL: Dilysocardiolipin

E-Syt2: Extended synaptotagmin 2

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ER: Endoplasmic reticulum

Gab1: Growth receptor protein 1

GC: Gas chromatography

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GPI: Glycosylphosphatidylinositol

Grb2: Growth factor receptor protein 2

GSK3β: Glycogen synthase kinase-3

GTP: Guanosine triphosphate

IP_3: Inositol triphosphate

IP3R: IP_3 receptor

LPAAT: Lysophasphatic acid acyltransferases

LYCAT: Lysocardiolipin acyltransferase

MCS: Membrane contact sites

MLCL: Monolysocardiolipin

mTOR: Mechanistic target of rapamycin

PA: Phosphatidic acid

PAO: Phenylarine oxide

PBD: $PI(4,5)P_2$ binding domain

PBS: Phosphate buffer saline

PC: Phosphatidylcholine

PDK1: Phosphoinositide- dependent kinase-1

PE: Phosphatidylethanolamine

PG: Phosphatidylglycerol

PH: Pleckstrin homology

PI: Phosphoinositides

$PI(3,4,5)P_3$: Phosphatidylinositol-3,4,5-triphosphate

$PI(4,5)P_2$: Phosphatidylinositol-4,5-bisphosphate

PI(4)P: Phosphatidylinositol-4-phosphate

PI3K: Phosphoinostide 3- kinase

PI3P: Phosphatidylinositol-3-phosphate

PI4K: Phosphoinostide 4-kinase

PIP1: Phosphatidylinositol monophosphates

PIP2: Phosphatidylinositol bisphosphates

PIP5K: Phosphoinositidylinositol-4-phosphate 5-kinase

PITPNM: Phosphatidylinositol transfer protein membrane-associated

PKB: Protein kinase B

PKC: Protein kinase C

PLA_1: Phospholipase A_1

PLA_2: Phospholipase A_2

PLC: Phospholipase C

PLCδ: Phospholipase Cδ

PLD: Phospholipase D

PS: Phosphatidylserine

PTEN: Phosphatase and tensin homolog

PX: Phox

RBD: Ras-binding domain

RPE: Retinal pigment epithelial

RTK: Receptor tyrosine kinase

SAM: Sterile α motif

SDCM: Spinning disc confocal microscopy

SH2: Src homology 2

SH3: Src homology 3

SHIP2: SH2- containing inositol 5'- phosphatase 2

SNAREs: Soluble N-ethylmalemide- sensitive factor attachment protein receptor

SOS1: Son of seven homolog 1

TIRFM: Total internal reflection fluorescence microscopy

t-SNAREs: Target soluble N-ethylmalemide- sensitive factor attachment protein receptor

TfnR: Transferrin receptor

v-SNAREs: Vesicle soluble N-ethylmalemide- sensitive factor attachment protein receptor

Vps15: Vacuolar protein sorting 15

Vps34: Vacuolar protein sorting 34

16:0: Palmitic acid

18:0: Stearic acid

20:4: Rachidonic acid

CHAPTER 1

INTRODUCTION

1.1 Introduction

In this thesis I will examine the regulation of a key class of phospholipids, phosphoinositides (PIPs) by an enzyme that controls the type of fatty acid associated with them. In this introduction, I will provide background for this research, where I will initially go through how the cell is a compartmentalized unit with different membrane bound organelles that communicate by vesicular traffic and how these vesicular traffic events are regulated by PIPs. I will then discuss the regulation of PIP dynamics by different phosphatases and kinases and how they function in a cell, specifically to PI (4,5) P_2 and PI (3,4,5) P_3. I will then move on to discuss how these PIPs are synthesized and their specificity of fatty acyl chains. Lastly, I will talk about the star enzyme of my thesis, lysocardiolipin acyltransference (LYCAT) and how it plays a role in acyl remodelling of PI (4,5) P_2 and membrane traffic.

1.2 Membrane traffic and cell organization

1.2.1 Basics of traffic and endocytosis/exocytosis

Cells are organized by compartmentalization into membrane-bound organelles. This compartmentalization is dynamic, such that cells are able to internalize materials such as nutrients, release material such as waste and exchange enzymes or hormones to and from the extracellular space. Membrane traffic is the process that allows the cells to accomplish these tasks and involves the formation of membrane-bound vesicles from various membrane compartments. These vesicles are subsequently used for transport of

protein, lipids and other macromolecules to a target compartment, following fusion of that vesicle with said compartment. Of particular interest is the membrane traffic to and from the plasma membrane, which controls the proteins found at the cell surface. Endocytosis is a term used to describe the specific formation of an intracellular vesicle from the plasma membrane, and this process serves to restrict the abundance of specific proteins at the cell surface. Exocytosis is the process that allows an intracellular vesicle(s) to be targeted to and fuse with the plasma membrane to allow release of material (such as cell surface proteins) to the cell surface or to the extracellular space[1]. Membrane traffic to and from the cell surface a critical regulator of many aspects of cell physiology, and plays a role in nutrient update, signal transduction, cell adhesion and migration, and infection and immunity (Figure 1.1)[1].

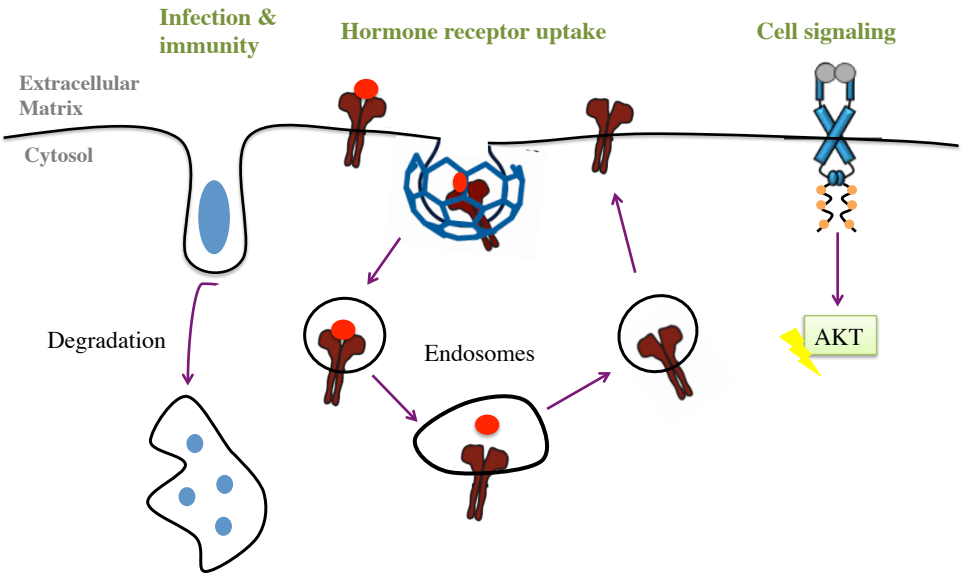

Figure 1.1: Membrane traffic controls a cell's physiology in many different ways.

Membranes are exchanged between organelles to maintain cellular homeostasis and survival. It is also important for regulating cell surface proteins that aid in hormone receptor signaling, degradation of foreign particles and cell signaling.

1.2.2 Organelle compartments

The many organelles within a cell consist of phospholipid bilayers and their embedded organelle-specific proteins. These organelles include the rough and smooth endoplasmic reticulum (ER), Golgi apparatus, lysosomes, peroxisomes, mitochondria and endosomes[1]. Collectively, these organelles play important roles in protein synthesis, protein transport, protein modification and protein sorting. For example, some proteins are synthesized in the ER and processed in the Golgi apparatus, packaged in vesicles and then subsequently delivered to the appropriate destinations, such as lysosomes, plasma membrane or endosomes[2]. Vesicles containing cargo protein that are sent and fused to the plasma membrane, release the protein outside the cell; such proteins are known as secretory proteins. Another example of membrane sorting during biosynthesis are the proteins destined for lysosomes[3]. In this case, the vesicle containing lysosomal proteins (typically lysosomal hydrolases) buds off the Golgi apparatus and eventually fuses with late endosomes/lysosomes, thus delivering these enzymes to the lysosome, which is important for lysosomal function[2]. As a result, vesicle-mediated membrane traffic plays a key role in protein transport between various membrane compartments.

1.2.3 Vesicle Transport

As mentioned above, vesicles play a significant role for transport of materials between various membrane compartments within a cell. Moreover, they are key to exocytosis for secretion, and in endocytosis for the uptake and intracellular transporting material within the cytoplasm[3]. Vesicular-transport also maintains organization in the cell by transporting material to specific locations and the movement and targeting of vesicles is not random. Vesicle transport contains four essential steps: vesicle budding, transport,

tethering and fusion[4]. Each step must be regulated to guarantee that the vesicle containing cargo protein is delivered to the correct organelle compartment. Vesicle budding typically involves coat-proteins such as clathrin, coat protein complex I (COPI) (at the Golgi) and coat protein complex II (COPII) (at ER), this then eventually leads to scission by proteins such as dynamin, and transport happens along microtubules and cytoskeleton[4].

What is responsible for vesicle tethering and fusion to the destined target membrane? The two stages are carried out by the function of proteins called Rabs and soluble N-ethylmalemide sensitive factor attachment protein receptor (SNARES), respectively. Rab proteins are peripheral membrane proteins anchored in the vesicle lipid bilayer through a prenyl group, and which are key for tethering/docking a vesicle to the target membrane. The Rab protein is active when bound to guanosine triphosphate (GTP), while in contrast, when it is bound to guanosine diphosphate (GDP) it is inactive[3, 4]. In some cases, Rabs function in vesicle tethering by binding to Rab effector proteins on the target membrane, an interaction that occurs specifically when in the Rab-GTP state. This Rab-GTP effector protein interaction allows tethering and eventual vesicle fusion to the target membrane[4]. An example of this, is the early endosome autoantigen 1 (EEA1) as an effector protein of Rab5 in endocytic membrane fusion. EEA1 consists of an N-terminal C_2H_2 zinc finger that binds to Rab5-GTP on the membrane of one organelle and a C-terminal that binds both Rab5- GTP and the FYVE finger binds to phosphatidylinositol-3-sphosphate (PI(3)P) on the membrane of the other organelle (endosomes)[5]. After this initial interaction, the vesicle and target membranes are fused together.

The vesicle containing cargo protein also consists of transmembrane vesicle soluble N-ethylmalemide- sensitive factor attachment protein receptor (v-SNAREs) that forms a coiled-coil with transmembrane target soluble N-ethylmalemide- sensitive factor attachment protein receptor (t-SNAREs) on the target membrane to allow fusion of the vesicle and the release of the cargo protein, after the association between Rab-GTP and the Rab effector protein[3,6]. The v-SNAREs and the t-SNAREs form a *trans*-SNARE complex, which is composed of three helices from the t-SNARE assembled with a helix from the v-SNARE[6,7]. This complex begins to zipper from the membrane-distal N terminal towards the membrane-proximal C terminal, generating an inward force that overcomes the large energy barrier between the negative membrane charges[6,7]. Eventually, the membrane fuse and the *trans*-SNARE complex becomes a *cis*-SNARE complex[6,7].

After fusion of the vesicle containing cargo protein, the transmembrane cargo receptor and the v-SNARE are on the target membrane. The cargo receptor and the v-SNARE, after dissociation from the t-SNAREs using ATP with different protein complexes, is associated with new vesicle formation from the transmembrane in order to go back to the originating membrane to pick up more cargo protein[3].

1.3 Regulation of Membrane Traffic from Phosphoinostide Lipids

This section will cover the importance of phosphatidylinositols (PIs) and phosphoinositides (PIPs) roles in membrane traffic in a cell. Specific PIs that will be explained are phosphatidylinositol-4,5-bisphosphate (PI (4,5) P_2), phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-3,4,5-triphosphate (PI (3,4,5) P_3). Each PI consists of unique lipid kinases and phosphatases that are important for their synthesis or

turnover. Lastly, I will discuss two cellular functions that involve $PI(4,5)P_2$ and $PI(3,4,5)P_3$.

1.3.1 Phosphoinostide function and structure

Vesicle transport helps carry cargo protein(s) from one organelle to the other, but what molecules function to regulate this process? PIs are lipids that control membrane trafficking, and in doing so, play very important roles in controlling cell and whole-body physiology including infection and immunity, hormone receptor signalling, nutrient and ion homeostasis, cell adhesion and migration[8]. As seen in Figure 1.2, PIs (parent molecule) are composed of an inositol head group, a phosphate group, a diacylglycerol (DAG) backbone and 2 fatty acid chains. Importantly, PIs have a specific profile of fatty acyl chains, such that up to 40-50% of PIs have a steric acid that is 18 carbons long on the sn-1 position (18:0) and a arachidonic acid that is 20 carbons long with 4 double bonds on the sn-2 position (20:4)[9]. Notably, other phospholipids such as phosphatidylcholine, phopsphatidylethanolamine and phosphatidylserine have very different acyl chain profiles, demonstrating the acyl chain profile specificity of PI[9,10]. Moreover, I will elaborate below, the functional significance of this PI acyl chain profile specificity, as it is poorly understood, yet represents an important consideration for understanding the role and regulation of this important class of phospholipids.

Importantly, the inositol head group of PI can be phosphorylated at positions 3, 4 and 5, and these phosphorylated derivatives of PI are collectively referred to as phosphoinositides (PIPs). The regulated phosphorylation and dephosphorylation of PI, maintained by various lipid kinases and phosphatases at one or all of these sites can result in 7 different possible PIPs, as seen in Figure 1.2[11]. Each of these 7 different species of

PIPs are enriched in different areas of the cell[5] as seen in Figure 1.3. A detailed discussion of all phosphoinositide species is beyond the scope of this work. Instead, I will discuss PI (4,5)P_2, which is the focus of the work in my thesis and in addition provide some background on PI(3,4,5)P_3.

1.3.1.1 Phosphatidylinositol-4,5-bisphosphate

PI(4,5)P_2 composition makes up about 0.5-1% on the plasma membrane and is a key lipid that interacts with many proteins at the plasma membrane, which act upon controlling membrane trafficking and remodelling events such as clathrin mediated endocytosis (CME), actin polymerization and membrane contact sites (plasma membrane-ER) [12, 13]. In addition, PI(45)P_2 also binds and/or directly regulates proteins such as small GTPases and ion channels[14]. Moreover, PI(45)P_2 also plays a role in many different metabolic pathways, in which it allows for synthesis and metabolism, and more specifically it is a specific substrate for key enzymes, such as phospholipase C (PLC), phosphatidylinositol-3 kinase (PI3K) and many 5 phosphatases (eg. OCRL, synaptojanin 1 and 2)[14]. Furthermore, in addition to direct roles in controlling various aspects of cell physiology, PI(4,5)P_2 is also an important substrate for the production of additional lipid species, such as PI(3,4,5)P_3 by the action of PI3K and the production of secondary messengers, DAG and inositol triphosphate (IP_3) by PLC[15,16].

1.3.1.2 Phosphatidylinositol-4-phosphate

As described in more detail below, PI(4,5)P_2 is generate by the phosphorylation of phosphatidylinositol-4-phosphate (PI(4)P) by specific lipid kinases[17]. PI4P makes about 0.05% of the cell and its mainly localized at the Golgi apparatus and also found at the plasma membrane and late endosomes/lysosomes[12,17]. It plays an essential role in Golgi

trafficking by recruiting coat proteins and accessory factors for vesicular transport and membrane contact sites (Golgi to ER and Golgi to plasma membrane)[18]. Moreover, it contributes to the synthesis of $PI(4,5)P_2$ in the plasma membrane by phosphoinositidylinositol-4-phosphate 5 -kinase (PIP5K).

1.3.1.3 Phosphatidylinositol-3,4,5-trisphosphate

As mentioned above, $PI(4,5)P_2$ can itself be phosphorylated to produce $PI(3,4,5)P_3$, a potent signalling lipid that is mainly localized in the plasma membrane and is the only tri-phosphorylated PIP out of the seven different species. It significantly contributes as a secondary messenger of multiple downstream targets of the PI3K pathway, and is also a target lipid for phosphatase and tensin homolog (PTEN) [19]. It has been shown that when cells are unstimulated/at resting state $PI(3,4,5)P_3$ is largely undetectable at the plasma membrane[19]. However, when PI3K is activated through a number of possible signals, such as by engagement of ligands by either receptor tyrosine kinase (RTK) or G-protein receptors, the levels of $PI(3,4,5)P_3$ increase at the plasma membrane[20]. One example of an RTK signalling pathway is the epidermal growth factor (EGF) binding to the epidermal growth factor receptor (EGFR), activating PI3K, which leads to the conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ and binding to AKT, aiding in cell survival, growth, proliferation, apoptosis and motility[20].

1.3.2 Regulation of PIP levels and dynamics

As mentioned previously, there are different, but specific phosphatases and kinases that result in the seven different species of phosphoinositides shown in Figure 1.2. The proper localization of phosphoinostide kinases and phosphatases are important for the function and localization of specific PIP species in a cell[21]. Figure 1.3 illustrates

the enrichment of different species of PIs in different membrane compartments in the cell. For example, PI(4,5)P_2 is most abundant on the plasma membrane while PI4P is most abundant on the Golgi apparatus and the plasma membrane.

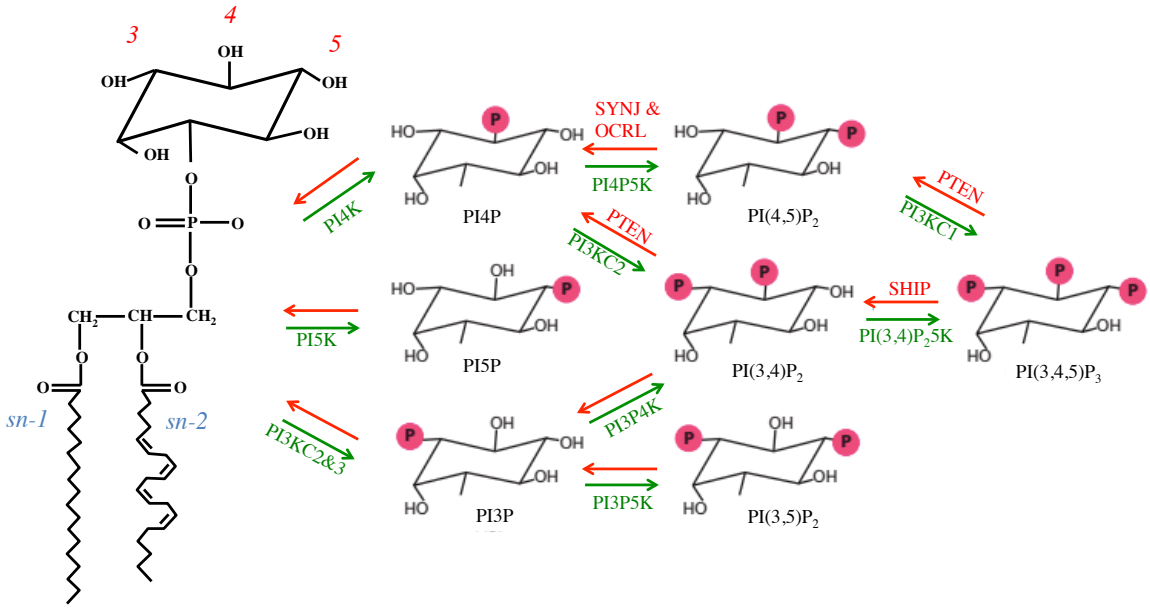

Figure 1.2: Synthesis of seven phosphatidylinositol species.

Phosphatidylinositol (PI), known as the parent, is compromised of a diacylglycerol (DAG) backbone and an inositol headgroup. The DAG backbone is attached to two acyl chains at positions, *sn-1* and *sn-2* through an ester bond and a phosphodiester bond at the *sn-3* position. The inositol head group can be phosphorylated on the 3rd ,4th or 5th positions, creating seven distinct phosphoinositides (PIP) species. PIPs are phosphorylated or de-phosphorylated by different kinases (green) and phosphatases (red).

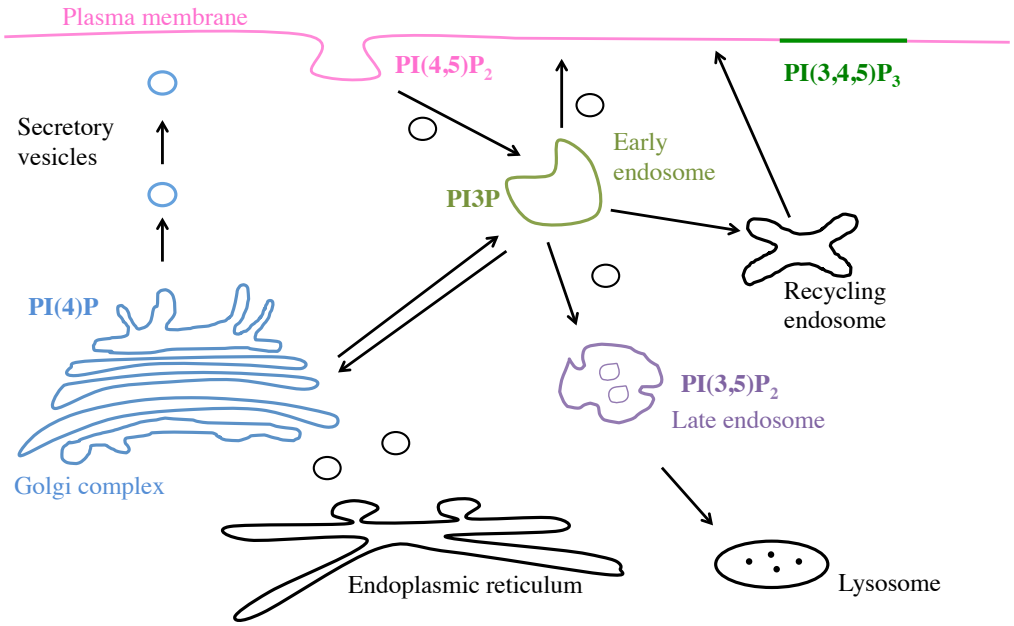

Figure 1.3: Cellular distribution of phosphoinositides.

The seven different PIP species are localized to distinct organelles in the cell, where each PIP is distinctly at a higher concentration in certain organelles compared to others.

1.3.2.1 $PI(4,5)P_2$ kinases and phosphatases

$PI(4,5)P_2$ is phosphorylated on the 4th and 5th position on the inositol head group, by the sequential action of PI4K and PIP5K, respectively, starting with PI[17,18,22]. As discussed above, $PI(4,5)P_2$ is most abundant on the plasma membrane of a cell[11,22]. Specifically, $PI(4,5)P_2$ is primarily made from (PI4P), as a result of phosphorylation at the 5th position of the inositol group by PIP5K, to make $PI(4,5)P_2$[22] . This phosphorylation of the 5-position of the inositol headgroup by PIP5K is reversible, such that the dephosphorylation is catalyzed by a number of different phosphoinositide 5-phosphatases to form PI4P[21]. Some of these specific 5-phosphatases that act on $PI(4,5)P_2$ are OCRL (inositol polyphosphate 5-phosphatase) and synaptojanin 1 (SYNJ 1). Both these

phosphatases are responsible for converting $PI(4,5)P_2$ to PI4P, and also have a preference for specific acyl chains [23].

1.3.2.1.1 Phosphoinositidylinositol-4-phosphate 5 kinase (PIP5K)

There are three different isoforms of type I PIP5Ks: α, β, and γ[24]. PIP5Kα is abundant in the heart, placenta, skeletal muscles, kidney and pancreas, PIP5Kβ is abundant in the heart, pancreas, brain, kidney, skeletal muscle and lung and PIP5Kγ is highly enriched in the brain[25]. Nonetheless, PIP5Ks are expressed ubiquitously in just about every human cell and tissue type.

An activator of type I PIP5Kα, β, and γ is a small GTPase Arf6 (ADP-ribosylation factor 6)[26]. Arf6 is mainly involved in membrane trafficking and transmembrane cargo[26]. Arf6 is recruited and associated to the plasma membrane through its myristoylated helix at the N-terminal region in a GTP-dependent manner that directly activates PIP5K, regulating many different functions such as, exocytosis, endocytosis, endosomal recycling and bacterial invasion[26,27].

In addition to control by Arf6, type I PIP5Ks are regulated by PA (phosphatidic acid), post-translational modification such as phosphorylation/ dephosphorylation and by other proteins[26]. All three isoforms of type I PIP5Ks are activated by direct binding of PA[24]. PA binding to PIP5K employs electrostatic and hydrophobic interactions inducing kinase activity[28]. Moreover, type I PIP5K kinase activity is upregulated in the presence of PA[28]. During agonist stimulation, PA is synthesized by two phospholipid- metabolizing enzymes, phospholipase D (PLD) and diacylglycerol kinase (DGK). The role of PLD is to hydrolyze phosphatidylcholine (PC) and DGK phosphorylates DAG to make PA[29].

Important for this thesis, PIP5Ks have been shown to be sensitive to the acyl chain composition of substrate PI4P[24]. Shulga et al., experimented with the three different PIP5K isoforms α, β, and γ with three different substrates, 18:0/20:4-PtdIns4P, 18:0/18:1-PtdIns4P, and 16:0/16:0-PtdIns4P. The results showed that the three different PIP5K isoforms preferred unsaturated acyl chains, 18:0/20:4-PtdIns4P, 18:0/18:1-PtdIns4P, compared with only saturated acyl chains, 16:0/16:0-PtdIns4P[24]. Thus, it was proposed that when a certain isoform of PIP5K preferentially phosphorylated 18:0/20:4-PtdIns4P, it would propose that specific PIP5K isoforms are involved in the PI cycle (discussed below) enriching 1-stearoyl-2-arachidonoyl species of PIs[24].

1.3.2.1.2 OCRL, SYNJ 1 and SYNJ2

OCRL and SYNJ 1 catalyze the dephosphorylation of 5th position of the inositol head group on PI(4,5)P_2, to create PI4P. These phosphatases have many important functions in a cell (as described below). For example, these phosphatases play an important role in different stages of clathrin coated vesicle (CCV) formation in CME, that allows for the regulation of PI(4,5)P_2 turnover[29]. CME is a critical pathway by which receptors and their ligands on the plasma membrane are internalized. CME is important for cell nutrition, cellular signalling and regulation of adhesion [30]. All SYNJs consist of a central inositol 5-phosphatase domain, with a Sac1 domain on the N- terminus and a proline-rich domain on the C-terminus. Both the inositol 5-phosphatase domain and the Sac1 domain are highly conserved in the SYNJ family and are key to polyphosphoinositide phosphatase activity[31]. In addition, the proline- rich tail aids with binding to SH3 (SRC homology 3) domain-containing proteins such as endophilin,

amphiphysin and CIN85, allows for additional control of SYNJ localization, which is important for certain functions such as in CME[32].

The Sac1 domain has specificity for substrates based on phosphorylation of the PIP headgroup, such that it acts primarily on PI(3)P, PI(4)P and PI(3,5)P_2 , whereas the inositol 5-phosphatase acts on PI(4,5)P_2 and PI(3,4,5)P_3[32]. SYNJ1 and SYNJ2 play distinct roles in CME (discussed later). OCRL is composed of a plextrin homology (PH)-like domain on the N-terminal, a 5 phosphatase catalytic domain, ASH domain and RhoGAP-like domain on the C-terminal[14]. OCRL is localized in the endolysosomal compartment, and loss of proper OCRL function leads to a significant increase in PI(4,5)P_2 on the endosomes causing a delay in recycling the receptors after endocytosis from the plasma membrane[14].

According to Schmid et al., both OCRL and SYNJ 1 have a preference of dephosphorylating PI(4,5)P_2 with an acyl chain specificity of 18:0/20:4, however, OCRL is highly more selective for that specificity[23]. Using similar methods, SYNJ 2 was found to be largely indifferent with respect to the head group phosphorylation and fatty acyl chain composition of its substrates for catalysis of the dephosphorylation of the 5-position of the inositol headgroup[23].

1.3.2.2 PI(3,4,5)P_3 kinase and phosphatase

PI(3,4,5)P_3 is the product of PI(4,5)P_2 being phosphorylated on the 3rd position on the inositol head group, by PI3K. This causes PI(3,4,5)P_3 to become abundant on the plasma membrane of a cell after stimulation with a variety of cues, including tyrosine kinase receptor or a G- protein coupled receptors [13,33,34]. Moreover, the production PI(3,4,5)P_3 is negatively regulated by the phosphatase and tensin homologue (PTEN) or

the SH2-containing inositol 5′-phosphatase 2 (SHIP2), which remove the phosphate group on the 3rd position or 5th position of the inositol group from $PI(3,4,5)P_2$ to form $PI(4,5)P_2$ or $PI(3,4)P_2$, respectively[35].

1.3.2.2.1 PI3K (3 classes)

PI3K catalyzes the phosphorylation on the 3rd position of the inositol group of a PI/PIP. The PI3K family is essential for many aspects of cell biology. In addition, it is one of the central proteins of study when it comes to human cancer, diabetes and aging[16]. Only 0.25% of PIPs are phosphorylated on the 3rd position, giving rise to the idea that PI3Ks and their 3-phosphorlated products are key for regulating and signalling functions within cells, rather than structural functions[36]. There are three different PI3K classes (I, II, III) that each contain different isoforms [16]. The PI3K family appears to be only found in eukaryotes, with the exception of class III PI3K that is conserved from yeast to humans[16]. I will focus on discussing class I PI3K since it is the most studied and is responsible for the production of $PI(3,4,5)P_3$.

PI3K Class I

In vitro, all classes can generate PI3P, however, class I is specific to the production of $PI(3,4,5)P_3$ from $PI(4,5)P_2$. Class I is comprised of a catalytic subunit p110 (p110α, β, δ, γ) and a regulatory subunit p85 [p85α (or its splice variants p55α and p50α), p85β, p55γ, p101 or p84][16,37,38]. The different catalytic isoform subunits of p110 consist of a Ras-binding domain (RBD), adaptor-binding domain (ABD) (present in all p110 subunits except p110γ) at the N-terminus and a C2 domain, helical domain and a kinase domain. Each regulatory subunit consists of two Src homology 2 domains (SH2), nSH2 and cSH2, and a p110-binding region (iSH2). The iSH2 is able to interact with the

catalytic subunits, p110α, p110β and p110δ by the ABD[16,38]. p85-p110 complex is recruited to RTKs at the plasma membrane, allowing for activation of downstream pathways[16,38]. p85 is a negative regulator for the kinase activity of the catalytic subunit p110α, through the interaction of a helical domain[16,38]. Studies have shown that the catalytic subunit, p110γ does not have an iSH2 domain/p85-binding domain, however, heterodimers of p110γ form with the regulatory subunits p101 or p84 that lack an SH2 domain and are only activated through GPCRs[16,37].

Multiple growth factor signalling pathways are controlled by the activation of RTKs and GPCRs by ligand, that allow recruitment of p85-p110 complexes to the plasma membrane. Binding of p85 to the phosphotyrosine motif by the nSH2 and the cSH2 domains causes conformational change of p110 (catalytic subunit) exposing the kinase domain, causing conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ through its kinase activity[16,39]. The presence of $PI(3,4,5)P_3$ on the plasma membrane recruits AKT, which controls multiple pathways, such as survival, growth, proliferation, apoptosis and motility. Furthermore, class I PI3K aids in cortical F-actin is involved in dynamics chemotaxis and phagocytosis. For example, $PI(3,4,5)P_3$ produced by p110γ at the leading edge in neutrophils allows recruitment of Rac GTPase, leading to F-actin polymerization, cell migration and cellular processes. Mutations in the class I PI3K or p85 adaptors are associated with human diseases from diabetes to cancer.

PI3K Class II

Class II PI3Ks are specific to the production of $PI(3,4)P_2$ (which may be activated by receptor signalling and during endocytosis) from PI4P and the production of PI3P from PI. Class II is comprised of catalytic subunits PI3KC2α, β and γ. The different

catalytic isoform subunits of PI3KC2 (except PI3KC2γ) consist of an α, β isoform of a clathrin-binding (CB) region and a RBD in the N-terminus, a C2 domain, a helical domain and a kinase domain, in addition, it consists of a Phox (PX) domain and a second C2 domain in the C-terminus that preferentially bind to $PI(4,5)P_2$[16]. The kinase activity of PI3KC2α is inhibited, until bound to clathrin, suggesting that it is implicated in CME[16,40]. PI3KC2β binds to the scaffold protein intersectin, which allows increased PI3P synthesis[16,41]. Class II PI3Ks are mainly localized near the cell cortex, since they are seen to have distinct roles in cell migration, cortical remodelling, endocytosis and exocytosis[16,42,43]. However, $PI(3,4)P_2$ is not only produced by PI3KC2, recent studies have shown that production of $PI(3,4)P_2$ can be produced downstream of $PI(3,4,5)P_3$ by the action of SHIP2[42].

PI3K Class III

Class III PI3K was initially known as vacuolar protein sorting 34 (Vps34), and is responsible for the production of PI(3)P from PI. It is essential for cargo sorting membrane trafficking, specifically endosomal protein, endosome-lysosome maturation, autophagosome formation, autophagy flux and cytokinesis[16]. For Vps34 to bind to its lipid substrate, it must interact with adaptor protein vacuolar protein sorting 15 (Vps15). The Vps34-Vps15 complex ensures that there is interaction with the correct membrane target and prevents lipid phosphorylation on other, off-target membrane compartments [44]. Binding of the Vps34-Vps15 complex with a second complex, Beclin 1 (BECN1) and Beclin 1-associated autophagy-related key regulator (ATG14) increases activation for autophagy[44]. Vps15 doesn't only interact with PI, but it is also involved with the

interaction of membrane proteins such as Rab5 GTPase that aids in directing Vps34 to endosomes/endosomal maturation[16].

1.3.2.2.2 PTEN and SHIP2

PTEN and SHIP2 are responsible for hydrolyzing the 3-phosphate and the 5-phosphate of $PI(3,4,5)P_3$, respectively. PTEN generates $PI(4,5)P_2$ from $PI(3,4,5)P_3$ and negatively regulates the PI3K/AKT pathway and is composed of 5 functional domains; an N-terminus $PI(4,5)P_2$ binding domain (PBD), a phosphatase domain (HCXXGXXR, where X is any amino acid), a C2 domain (allowing for membrane association), a carboxy-terminal tail and a PDZ-binding domain (aiding in anchoring receptor proteins)[45]. PTEN is unique in that it is one of the most important tumour suppressors. PTEN loss results in a significant increase of $PI(3,4,5)P_3$ at the plasma membrane, recruiting and activating proteins with a PH domain, such as AKT family members and phosphoinositide- dependent kinase-1 (PDK1), leading to uncontrolled cell growth and proliferation[45].

SHIP2 is essentially responsible for the production of $PI(3,4)P_2$ from $PI(3,4,5)P_3$, but also generates PI(4)P from $PI(4,5)P_2$[46]. SHIP2 plays roles in endocytosis, adhesion, proliferation, apoptosis and insulin signalling [46]. It contains an N-terminal SH2 domain, a central catalytic 5-phosphatase, a C-terminal PRD, a NPXY (Asn-Pro-X-Tyr) motif and a sterile alpha motif (SAM)[46]. The NPXY motif is contains a tyrosine phosphorylation site and the SAM motif is known to be a domain that interacts with a variety of proteins[47]. PI3K is the central protein mediating insulin metabolic effects leading glucose transporter 4 (GLUT4) translocation to the plasma membrane to allow glucose to enter the cell, and SHIP2 is involved in negatively regulating insulin signalling[48].

1.3.2.3 Phospholipase C (PLCγ and PLCδ)

PLC is a crucial enzyme in PIP turnover and produces two second messengers, IP_3 and DAG from $PI(4,5)P_2$. Cleavage of $PI(4,5)P_2$ occurs in response to activation of hormone receptors, neurotransmitters, growth factors and other molecules. IP_3 binds to an IP_3-gated Ca^{2+} channel, called the IP_3 receptor (IP3R), on the ER membrane, opening it to allow the release of Ca^{2+} into the cytosol[49]. DAG induces activation of protein kinase C (PKC), which is responsible for many signalling events and aids in regulating many cellular proteins, such as receptors, enzymes, cytoskeletal proteins, and transcription factors[50]. DAG resides in the membrane and serves as an anchor for PKC.

There are 11 PLC isozymes, divided into 4 classes; β(1-4), γ(1,2), δ(1-4), and ε(1)[51]; this section will focus on PLCγ1 and its activation by EGF and PLCδ1, and its activation by calcium. All PLCs have a highly conserved catalytic X and Y TIM (eight α-helices and eight parallel β-strands) barrel domain, as well as a PH domain, EF-hand motif and C2 domain. The X and Y TIM barrel domain is attached together by a negatively charged flexible linker that acts as an auto-inhibitory region. The X and Y linker moves away from the active site when close to the plasma membrane, since both of them are negatively charged, they will repel away from each other[15]. The EF-hand motif, a 4 tandem helix-loop-helix structure and the C2 domain are involved in calcium binding contributing to enzyme regulation and activity[15].

As previously mentioned, EGFR is a transmembrane receptor that has intrinsic protein-tyrosine kinase activity. EGFR is activated through binding of specific ligands such as EGF, by its exofacial domains, which results in activation of the EGFR kinase domain, followed by autophosphorylation of several of its tyrosine residues in the C-

terminal domain[52]. This causes a signaling cascade of several downstream molecules that stimulate pathways such as the Ras/mitogen-activated protein kinase, PI3K, signal transducers, transcription protein activators and PLCγ1. PLCγ1 is critical for growth factor-dependent signal transduction in a cell, and unlike other PLC domain structures, PLCγ1 core domains consist of 2 SH2 domains (nSH2 and cSH2) and 1 SH3 domain located between the X and Y catalytic domains. PLCγ1 is initially inactive because the TIM barrel is autoinhibited by the cSH2 domain within the X and Y linker[53]. The phosphorylated tails of activated RTKs allow the nSH2 domain to bind phosphorylated Tyr992, causing recruitment to the plasma membrane[53,54] . Afterwards, PLCγ1 is phosphorylated on several sites; however, phosphorylation on Tyr783 within the X-Y linker causes intramolecular association with the cSH2 domain. This leads to conformational change within the X-Y linker, resulting in binding and hydrolysis of $PI(4,5)P_2$ triggering a release of calcium from the endoplasmic reticulum and activation of PKC [52,53,55]. Moreover, membrane association and further stabilization of PLCγ1 is caused by the N-terminal binding to $PI(3,4,5)P_3$, a lipid secondary messenger synthesized by PI3K [51,56]. The SH3 domain has been shown to bind to the son of seven homolog 1 (SOS1) protein via proline rich domain to stimulate guanine nucleotide exchange factor (GEF) activity[51].

PLCδ1 differs from PLCγ1 in that its PH domain binds $PI(4,5)P_2$ and is most sensitive to calcium[51]. The X and Y linker contains 4 amino acid residues that bind calcium (Glu341, Asp343, Glu390 and Asn312), the C2 domain contains 3 calcium-binding sites, such that when PLCδ1 is bound to calcium, it displays substantially increased phosphodiesterase activity. Moreover, PLCδ1 also has an EF-hand domain that

binds calcium and is required for the association of the PH domain with $PI(4,5)P_2$[51]. This calcium regulation of PLCδ1 allows this isoform to be activated subsequent to an intracellular increase of Ca^{2+} triggered by initial activation of other PLC isoforms[51]. These interactions cause cleavage of $PI(4,5)P_2$, triggering a release of calcium from the endoplasmic reticulum and activation of PKC[52].

In order to study the functional outcomes of activation of PLCδ1, I used a drug called ionomycin in my experimental studies to cause a flow of calcium into the cell, which allowed experimental and transient activation of PLCδ1, thus impacting $PI(4,5)P_2$ dynamics. Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus*[57]. It has a hydrophobic periphery and carries calcium ions into the cell. When calcium moves into the cell upon ionomycin treatment, it stimulates phospholipase Cδ (PLCδ) to cleave $PI(4,5)P_2$ into inositol triphosphate and diacylglycerol (DAG), which leads to a robust and dramatic decrease of $PI(4,5)P_2$.

1.4 Functions of specific PIPs, focusing on $PI(4,5)P_2$ and $PI(3,4,5)P_3$

1.4.1 Role of $PI(4,5)P_2$ in clathrin mediated endocytosis

$PI(4,5)P_2$ has many functions inside the cell including control of endocytosis, actin remodeling and cell migration[58]. In order to illustrate some of the functions of $PI(4,5)P_2$ and how it is regulated by protein binding, I will focus on explaining how $PI(4,5)P_2$ plays a role in regulating CME. Clathrin is a coat protein that has a triskelion structure used to create vesicles containing protein, hormones, metabolites and sometimes viruses, in the cytoplasm for intracellular trafficking[59]. The triskelion is made up of three heavy chains to provide the structural backbone of the clathrin framework[59]. After clathrin coated vesicles form and bud off the membrane (scission is dependent on the

GTPase dynamin), the vesicle then loses its clathrin coat and continues on to its target membrane compartment, usually the early endosome, to undergo fusion and deliver the cargo protein. Figure 1.4 illustrates this idea of $PI(4,5)P_2$, which is abundant on the plasma membrane binding to the adaptor protein 2 (AP2), which in turn facilitates the membrane recruitment of clathrin, allowing for the formation of clathrin-meditated vesicles[60]. $PI(4,5)P_2$ is an essential regulator of clathrin-mediated endocytosis of the plasma membrane[61].

AP2 is a heterotetramer, consisting of two large subunits (α and β2), a medium subunit (μ2), and a small subunit (σ2). The N-terminals of subunits α and β2, with subunits μ2 and σ2 assemble the core of AP2. The C-terminals of subunits α and β2 form the two appendage domains attached to flexible linkers[30]. The α and β2 subunits are the appendage domains, where the β2 subunit is the one that interacts with clathrin and is required for clathrin assembly, and the α subunit binds to and recruits endocytic accessory proteins required for the maturation process[30]. AP2 binds $PI(4,5)P_2$ through 3 spatially distinct motifs found within or between three subunit, α, β2 and μ2[30]. In addition to binding $PI(4,5)P_2$, the μ2 and σ2 subunits interact with tyrosine-based (Yxxϕ, where ϕ indicates a hydrophobic residue) and dileucine-based (([DE]XXXL[LI], were X is any amino acid) sorting motifs, respectively, which are found on the integral membrane proteins that are subject to internalization from the cell surface by CME. As such, AP2 can interact with both $PI(4,5)P_2$ found throughout the plasma membrane, as well as with short linear amino acid sequences found in the cytosolic tails of transmembrane proteins, such as transferrin receptor (TfnR) and EGFR[30,62,63]. μ2 interaction with Yxxϕ motif- containing cargo efficiently regulates AP2 activation and

clathrin coated pit (CCP) nucleation[30]. However, σ2 interaction with [DE]XXXL[LI] motif-containing cargo has little effect on AP2 activation and CCP nucleation[30]. Studies have shown that cells expressing a $\mu 2^{cargo-}$mutant that is unable to bind to cargoes carrying the Yxxϕ motif, resulted in a significant decrease in the initiation rates of CCP formation, whereas cells expressing $\sigma 2^{cargo-}$mutant that is unable to bind to cargoes carrying the [DE]XXXL[LI] motif had no effect[30]. Importantly, a decrease in rates of CCP formation caused by $\mu 2^{cargo-}$mutant, results in a significant decrease in clathrin recruitment speed[30], causing major defects in internalization of cargo-receptors.

AP2 exists in two conformations: open/active and closed/inactive. In the inactive state, the subunit β2 blocks the $\mu 2$ and σ2 domains within the AP2 core, making them unable to engage ligands such as protein sorting motifs or $PI(4,5)P_2$[64]. The initial step for activation of AP2, (1) is the recruitment to the plasma membrane and binding of $PI(4,5)P_2$ through the binding pockets of α and β2 subunits, allowing it to change conformation and bind clathrin through the β2 appendage. This conformational change also causes the β2 appendage to relieve the steric hindrance that prevents the $\mu 2$ and σ2 subunits within the AP2 core from engaging receptor sorting motifs. This binding of $PI(4,5)P_2$ activates AP2 causes AP2 and initiates the formation of CCP; (2) Stabilization of the AP2 complex occurs when the $\mu 2$ and σ2 subunits interact with transmembrane cargo protein through their respective binding motifs Yxxϕ and [DE]XXXL[LI]. Further stabilization occurs through $\mu 2$-$PI(4,5)P_2$ binding (3) α, β2 and $\mu 2$- $PI(4,5)P_2$ binding sites must be sustained for CCP stabilization and efficient maturation[30]. During the multiple stages of clathrin coated vesicle (CCV) formation, various 5-phosphatses such as OCRL, SYNJ1 and SYNJ2 bind to clathrin, AP2 or other endocytic accessory proteins, and

regulate $PI(4,5)P_2$ turnover[65]. In other words, 5-phosphatases allow for negative regulation of formation of vesicles by CME. For example, knockout in SYNJ1 in mice, resulted in prolonged CCP formation, suggesting that $PI(4,5)P_2$ hydrolysis by SYNJ1 is key to uncoating clathrin-coated vesicles[66]. Whereas a knockdown in SYNJ2, caused a decrease in CCP formation, suggesting that SYNJ2 is key in the early steps of CME[32,67].

Previous studies have shown that an acute and mass depletion of $PI(4,5)P_2$ from the plasma membrane creates a significant loss of all CCPs[68]. Taken together, $PI(4,5)P_2$ is mandatory for the binding of AP2 to the plasma membrane to initiate CME.

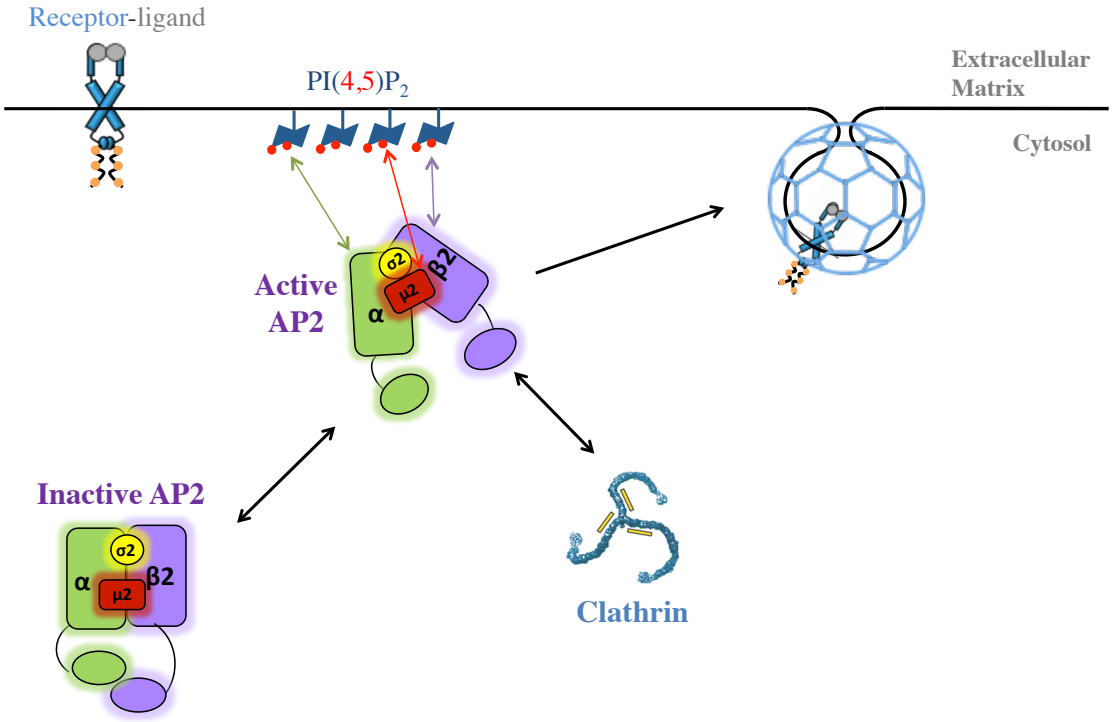

Figure 1.4: $PI(4,5)P_2$ is a major regulator in clathrin-mediated endocytosis.

CME is mediated by AP2, $PI(4,5)P_2$ and clathrin. AP2 is initially inactive and in the cytoplasm, however, after a receptor-ligand signals to be endocytosized, AP2 is recruited to the plasma membrane. AP2 is composed of 4 subunits, α β2, μ2 and σ2. AP2 becomes active, after binding to $PI(4,5)P_2$ through the α, β2, μ2 subunits, causing conformational

changes and allowing clathrin and the receptor to bind, creating a clathrin vesicle containing receptor-ligand.

1.4.2 Role of $PI(3,4,5)P_3$ in PI3K/AKT/mTOR pathway

As mentioned previously in section 1.2.2.b.1 (class I PI3K), the PI3K/ATK pathway controls proliferation, migration, cell survival and metabolism[16]. Given this broad role in controlling cell physiology, PI3K must be tightly regulated, and it is perhaps not surprising then that disruption of the normal regulation of PI3K can also contribute to the progression of different types of cancers[16,69]. In order to initiate the signalling pathway of PI3K/AKT, EGFR, must bind ligand, activating the kinase domain of EGFR and causing autophosphorylation of multiple tyrosine residues[52]. Phosphorylation of EGFR translocates an adaptor protein called growth factor receptor protein 2 (Grb2), which binds to the receptor and mediates growth receptor protein 1 (Gab1) to also associate with the receptor via binding to proline rich region on Gab1[70]. Gab1 consists of multiple tyrosine residues that allow for binding of the p85 regulatory subunit of PI3K[70]. Gab1 is also a binding partner for the tyrosine phosphatase SHP2, to inactivate PI3K recruitment to the receptor[70].

After autophosphorylation of EGFR and recruitment of PI3K, $PI(4,5)P_2$ is phosphorylated to $PI(3,4,5)P_3$ at the plasma membrane. This allows recruitment of the kinase AKT to the plasma membrane, due to the ability of the PH domain of AKT to bind to $PI(3,4,5)P_3$ (Figure 1.5)[33,34,71] . AKT, also known as protein kinase B (PKB), is a serine/threonine protein kinase that regulates cell survival and proliferation[33]. There are three isoforms in mammals located at distinct subcellular locations in the cell, (1) AKT2 is activated at the plasma membrane or endosomes sites by $PI(3,4)P_2$ and (2) AKT1 and

AKT3 have a preference for $PI(3,4,5)P_3$ at the plasma membrane[42]. All AKT isoforms contain an N-terminal PH domain, a central kinase domain and a C-terminal regulatory domain. Activation of AKT is caused by binding to $PI(3,4,5)P_3$, causing it to reveal its kinase domain to PDK1, which phosphorylates AKT at T308[34]. It is further activated by phosphorylation of the S437 by mechanistic target of rapamycin (mTOR) complex 2 (mTORC2)[34]. Moreover, phosphorylation of S437 stabilizes T308 phosphorylation and the active state of AKT[34]. Once activated, AKT phosphorylates a wide variety of substrates, such as glycogen synthase kinase-3 (GSK3β), which allows Akt to thus controlling cell proliferation, metabolism, cell survival and mTORC1. Specifically, by control and activation of mTORC1 signaling, Akt is able to promote the translation of protein mRNAs and ribosomal biosynthesis in cell growth[34,70].

I have outlined some specific functions of $PI(4,5)P_2$ and $PI(3,4,5)P_3$, which serves to highlight the many important functions of PIPs as major regulators of many signaling pathways and membrane traffic phenomena. But how are PI and, by extension, PIPs made? The next section will go through the different steps it takes to synthesize PI and PIPs, as this aspect underlies the specific acyl chain profile of these lipids, for which mounting evidence now suggests may control PI/PIP function.

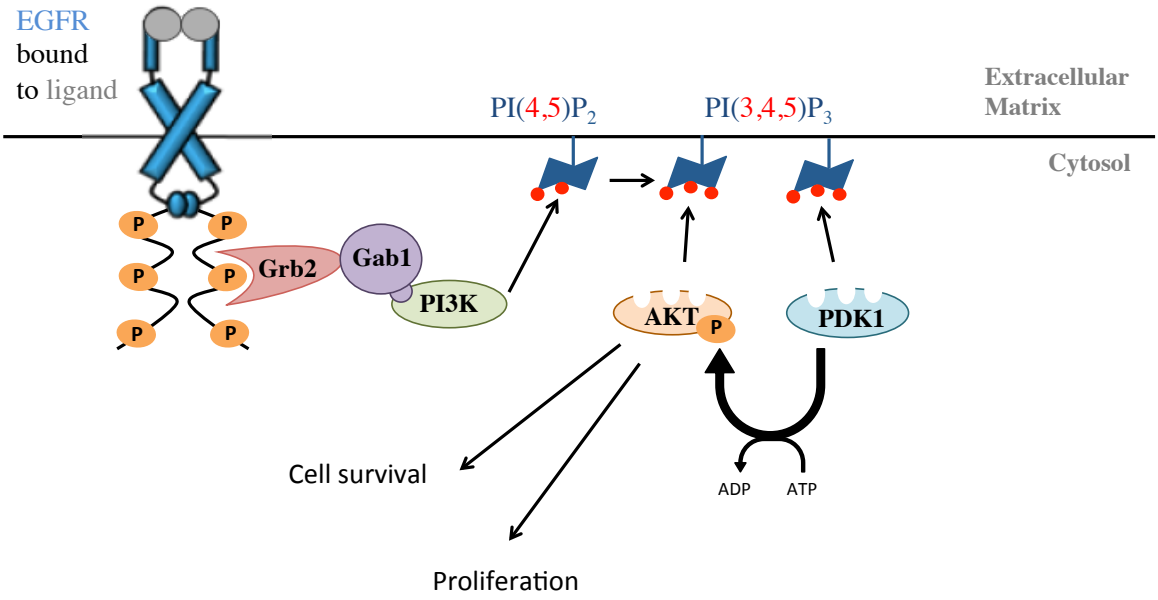

Figure 1.5: PI3K/AKT signaling pathway.

Ligand binding to EGFR causes dimerization and autophosphorylation of the receptor, causing activation of multiple intracellular signalling. To activate AKT, growth factor receptor-bound protein 2 (Grb2) binds to the phosphorylated EGFR, mediating the growth receptor protein 1 (Gab1) binding via its proline rich region. Gab1 contains a number of tyrosine residues that allow for binding of the p85 regulatory domain on PI3K, causing phosphorylation of $PI(4,5)P_2$ to $PI(3,4,5)P_3$. Production of $PI(3,4,5)P_3$, causes recruitment of AKT via binding of PI(3,4,5)P3 through its PH domain. Following this, AKT is phosphorylated by phosphoinostide-dependent protein kinase 1 (PDK1) and mechanistic target of rapamycin (mTOR) complex 2, resulting in AKT activation.

1.5 Phospholipid Synthesis

Phospholipids are initially made in the endoplasmic reticulum. The synthesis of all phospholipids starts off with glycerol-3-phosphate, resulting in the formation of lysophosphatic acid (LPA), catalyzed by the enzyme glycerol-3-phosphate acytransferase (GPAT) (Figure 1.6) [72]. LPA is then converted to phosphatidic acid (PA), which can be

carried out by several enzymes termed lysophasphatic acid acyltransferases (LPAAT). PA is then converted to DAG by DGK. DAG can be synthesized into cytidine diphosphate diacylglycerol (CDP-DAG) by cytidine-diphosphate synthase (CDS). The formation of DAG is a direct precursor for phosphatidylethanolamine (PE) and phosphatidylcholine (PC). In contrast, the formation of CDP-DAG is a precursor for phosphatidylserine (PS), phosphatidylglycerol (PG), and PI. This process is referred to as the Kennedy pathway (also known as the *de novo* lipid synthesis pathway), as depicted in the green box in figure 1.6 [72].

Given the common pathways for synthesis of phospholipids, it is likely that PIs undergo remodelling subsequent to initial synthesis to achieve acyl chain specificity that is unique to PIs. Enzymes such as phospholipase A_1 (PLA_1) (specific for sn-1) and phospholipase A_2 (PLA_2) (specific for sn-2), cleave the fatty acyl chains on the PI, generating lyso-PI molecules. Subsequently, acyltransferases function to re-form PI from lyso-PI. This process is referred to as the Lands cycle, as depicted in the purple box in figure 1.6 [72]. As I will discuss further below, I hypothesize that after cleavage of the fatty acid at the sn-1 position of PIs by PLA_1, LYCAT replaces the removed fatty acyl chain with 18:0 (steric acid), which establishes part of the acyl chain specificity of PI and PIPs (Figure 1.7)[10]. Similarly, the fatty acyl specificity of the sn-2 position of PI and PIPs is thought to be maintained by the sequential function of PLA_2 enzymes and MBOAT7, the latter being an acyltransferase that is selective for incorporation of 20:4 (arachidonic acid) into lyso-PI (figure 1.7)[73].

Hence, while the protein enzymes that are able to effect the incorporation of specific fatty acids into PI have been known and the key acyltransferase LYCAT has

been identified recently, the functional consequence of the incorporation of specific acyl groups into PIs on the function of PI and PIPs remains largely unknown.

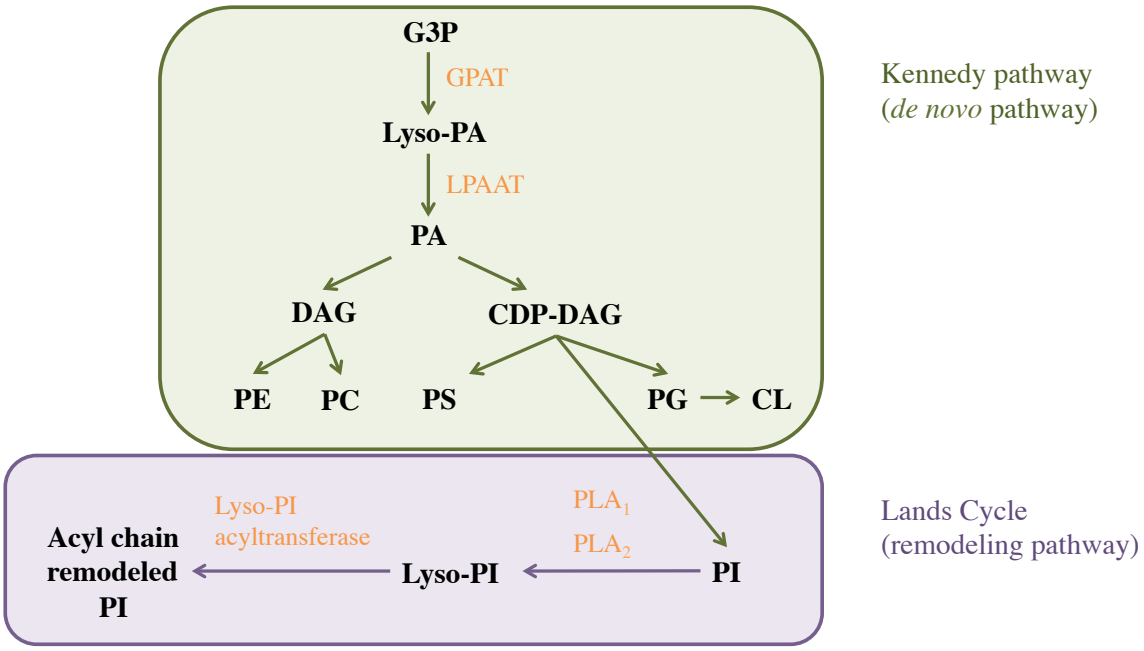

Figure 1.6: Phospholipid synthesis and acyl chain remodeling.

Phospholipids are initially synthesized in the endoplasmic reticulum through the Kennedy pathway (also known as the *de novo* lipid synthesis pathway). The pathway begins with glycerol-3-phosphate (G3P), which is synthesized to lyso-PA by G3P acyltransferase (GPAT) and then to phosphatidic acid (PA) by lyso-PA acyltransferase (LPAAT). Diacylglycerol kinase converts PA to diacylglycerol (DAG), and then DAG can be converted to cytidine diphosphate diacylglycerol (CDP-DAG) by cytidine-diphosphate synthase (CDS). CDP-DAG is a precursor for phosphatidylinositol (PI) synthesis by phosphatidylinositol synthase (PIS). Subsequently after the Kennedy pathway, PI undergoes acyl chain remodelling in the plasma membrane by phospholipase A (PLA_1/ PLA_2) in the Lands cycle (also known as the remodelling pathway). The figure shown is specific to PI synthesis in the Lands cycle.

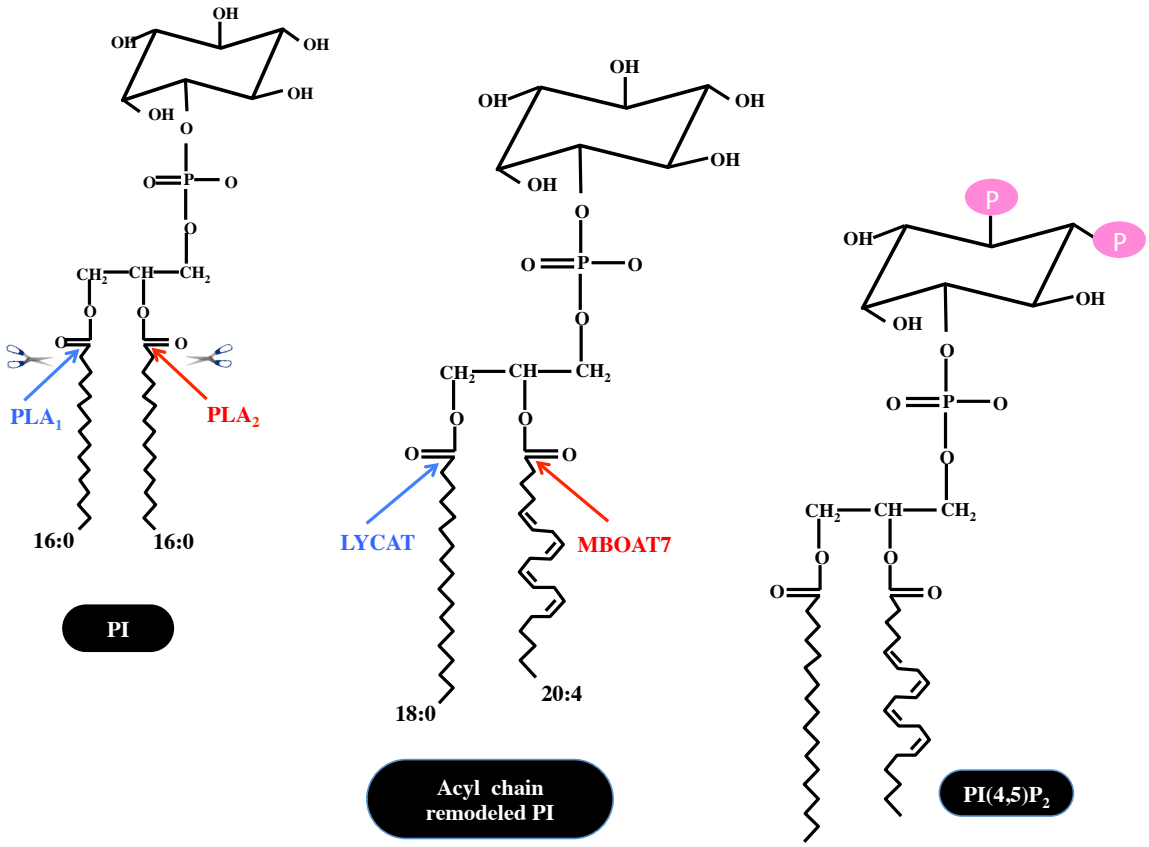

Figure 1.7: Acyl chain remodeling of PI.

Shown is a simple schematic of acyl chain remodeling of PI. PLA_1 is responsible for hydrolyzing the *sn-1* position, while PLA_2 hydrolyzes the *sn-2* position. LYCAT has a binding pocket for substrates that bind anionic lyso-PI, causing acyl chain modification to 18:0. MBOAT7 is responsible for addition of a 20:4 acyl chain on the *sn-2* position. The modified PI then becomes phosphorylated at the 4^{th} and 5^{th} positions on the inositol headgroup by PI4K and PIP5K, respectively.

1.6 Acylation specificity of PIPs

As discussed previously, PI and PIPs have remarkable specificity of fatty acyl chains. As described in detail above, phospholipids are formed as a result of an esterification reaction of fatty acids with glycerol[3]. Through mass spectrometry, its been shown that the sum of the fatty acid chains in PI and other phospholipids, measured in

retinal pigment epithelial (RPE) cells differentiate between one another[9]. It is evident that about 45% of PI is composed of a sum of acyl species of 38:4[9]. This means that in summation, there are 38 carbons and 4 double bonds. Specifically, 38:4 is the sum of the fatty acids 18:0 and 20:4. Unfortunately, this mass spectrometry approach doesn't provide information on the length of individual fatty acid chains on a phospholipid (PI, PC and PS), just the sum of the two fatty acids. However it has been shown that PIs have unique fatty acyl chain specificity that adds on an 18:0 (steric acid) carbon chain on the sn-1 position and a 20:4 (arachidonic acid) carbon chain on the sn-2 position of the PI[74]. Given the similarities in the synthetic pathway for different phospholipids seen in Figure 1.6 and explained in depth in the next section, it is possible that phospholipids may all be initially synthesized in the ER with similar incorporation of specific types of acyl groups, and that acyl chains are subsequently subject to remodelling by phospholipases and acyltransferases to achieve fatty acyl chain specificity of PIs[75]. However, the function of acyl chain specificity of PIPs is currently is still very poorly understood and thus the subject of my thesis.

1.7 PI Cycle

In addition to the Kennedy pathway for *de novo* PI synthesis and the Lands' Cycle for remodelling of the acyl groups of PI/PIPs, additional biochemical processes may also impact the fatty acid composition of PI/PIPs, in particular the PI cycle, which functions to re-generate PI following cleavage by PLC. The PI cycle consists of a variety of enzyme-catalyzed biochemical reactions; thus, the intermediates are synthesized every completed cycle. The PI cycle does not occur in a single location, as some reactions occur at the plasma membrane while others occur within the endoplasmic reticulum[76]. Therefore,

lipids are transferred between these two locations to complete the cycle[76]. At the first stage of the PI cycle, lipid-signalling intermediates such as DAG and PA are produced by PLC and PLD, respectively, upon cellular stimulation by certain agonists. One to form PA is produced by PLD hydrolysis of other phospholipids, mostly phasphatidylcholine[76]. Another pathway that relates to my project is the activation of PLC (by Ca^{2+}), which hydrolyzes PI(4,5)P_2 to produce DAG. DAG is then converted to PA by DGKε (mammalian isoform of DGK)[76]. DGKε has specificity for 18:0/20:4[77]. With the substrate PA, it is reciprocally transferred from the plasma membrane to the endoplasmic reticulum through phosphatidylinositol transfer protein membrane-associated (PITPNM) at membrane contact sites (MCS)[78]. Once within the endoplasmic reticulum or at MCS between the plasma membrane and endoplasmic reticulum, PA is then synthesized to CDP-DAG by CDS, which then produces PI by phosphatidylinositol synthase (PIS)[79]. PI is then translocated to the plasma membrane, allowing the eventual regeneration of specific PIPs such as PI(4,5)P_2 . In addition to this model involving translocation of lipids from the plasma membrane and endoplasmic reticulum, another model has emerged in which ER-derived PIS vesicles that make dynamic and transient contact with the plasma membrane, thus allowing CDS-DAG to be converted to PI, without requirement for transport of the lipid substrates from the plasma membrane to the endoplasmic reticulum and then back to the plasma membrane[9]. Once PI is re-generated in the plasma membrane, there are 2 last steps involved in remodelling PI(4,5)P_2: 1) PI to PI4P by PI4K and 2) PI4P to PI(4,5)P_2 by PIP5K[9,79].

1.8 Lysocardiolipin acyltransferase (LYCAT)

1.8.1 Study of LYCAT knockout in mice

As described above, there are several pathways that can lead to enrichment of specific fatty acids within PI, in particular the Lands' cycle and the PI cycle. Here, I will focus on the Lands' cycle, which leads to the enrichment of specific acyl chains within PI/PIPs as a result of sequential action of PLA and acyltransferase enzymes. As I indicated above, the acyltransferase that may establish the acyl specificity of the sn-1 position of PI/PIPs is LYCAT.

To study the biological function of the incorporation of specific acyl chains into PIs by LYCAT, Imae *et al*. (2012) generated a LYCAT ($LYCAT^{(-/-)}$) knockout mouse for [10]. They observed that $LYCAT^{(-/-)}$ mice presented a robust decrease of steric acid within PI in the liver (reduction of 42%), the heart (reduction of 36%) and the skeletal muscles (reduction of 66%) compared to PI from livers of $LYCAT^{(+/+)}$ mice. Furthermore, $LYCAT^{(-/-)}$ mice exhibited a large increase in palmitic acid (16:0) incorporation into PIs, with some increase also observed in oleic acid (18:2n-6), *cis*-vaccenic acid (18:1n-7) and linoleic acid (18:2n-6) in PI, presumably replacing the steric acid found in PI in $LYCAT^{(+/+)}$ mice. Arachidonic acid, located on the sn-2 position of a PI also showed a modest reduction in the incorporation into PIs in some of these tissues. However, the decrease of steric acid within PI/PIPs was much greater than that of arachidonic acid. As such, the authors concluded that LYCAT preferentially controls the incorporation of 18:0 into the sn-1 position of PI, and LYCAT is not thought to contribute directly to the acylation of arachidonic acid on PI in mouse tissues[10]. Moreover, knocking down LYCAT decreased the levels of various PIPs[9]. The molecular species of

phosphatidylinositol monophosphates (PIP_1), phosphatidylinositol bisphosphates (PIP_2) and PI were decreased by 41%, 50% and 27% respectively. In order for PI and PIPs to establish acyl specificity, LYCAT controls the incorporation of 18:0 on the sn-1 position into lyso-PI[10].

1.8.2 The biochemistry of LYCAT

Given that LYCAT controls the acyl specificity of PI and PIPs, as indicated by the changes in acyl profile specificity in the $LYCAT^{(-/-)}$ mice described above, it is possible that LYCAT does so by direct transfer of specific acyl species, specifically into lyso-PI but not other phospholipids. Cao *et al*. (2004) examined the preference of LYCAT (which is also known by the names ALCAT1, AGPAT8 and LCLAT1) toward different substrate fatty acyl-CoAs in Sf9 cells (clonal isolate of *Spodoptera frugiperda* Sf21 cells). Different acyl-CoA derivatives were studied, including *n*-octanoyl-CoA (C8:0), lauroyl-CoA (C12:0), palmitoyl-CoA (C16:0), stearoyl-CoA (C18:0), oleoyl-CoA (18:1), linoleoyl-CoA (18:2), and arachidoyl-CoA (C20:4) in order to determine if LYCAT had any preference for the transfer of specific acyl groups onto lyso-phospholipids[80]. The different acyl-CoAs were used to measure the acyltransferase activity of LYCAT *in vitro*, using either monolysocardiolipin (MLCL) or dilysocardiolipin (DLCL) acyl acceptor substrates. It was seen when either oleoyl-CoA (18:1) or linoleoyl-CoA (18:2) were used as acyl donor, a 5- to 10-fold increase was observed in LYCAT activity, suggesting that LYCAT has a preference for the unique fatty acid pattern of C_{18} found in mammalian PI/PIPs (as well as cardiolipin (CL), a lipid found enriched in mitochondria)[80]. This is consistent with LYCAT acting to selectively

transfer steric acid into the sn-1 position of lyso-PI, in order to establish PI acyl chain specificity.

After identifying that LYCAT has a strong preference for C_{18}, it was then found that LYCAT specifically determines the fatty acyl chain at the sn-1 position of PI *in vivo* [81]. PLA_1 is an enzyme that hydrolyzes fatty acids attached at the sn-1 position of PIs. *Ipla*-1, a family member of PLA_1 in *C. elegans* was studied for its ability to impact the fatty acid composition of phospholipids (PC, PE, PS and PI), by comparing the lipid composition of wild-type and *ipla-1* mutants of *C. elegans* by gas chromatography (GC). It was found that the *ipa-1* mutation had significant effects on the fatty acid composition of PI, but not PC, PE and PS. While steric acid (18:0) made up 25% of the fatty acids in PI in wild-type animals, in the *ipa-1* mutants steric acid was reduced to 6% of total PI fatty acids [81]. In contrast, the predominant fatty acid at the sn-2 position in PI was not different in wild-type versus *ipa-1* mutant animals. Moreover, mammalian LYCAT is the closest homologue of three acyltransferases, *acl-8*, *acl-9* and *acl-10* in *C. elegans*. The triple mutant *C. elegans* of *acl-8*, *acl-9* and *acl-10* was studied and it was concluded that it had the same effects on the acyl profile of PI as observed in the *ipa-1* mutant animals [81]. Therefore, both these conclusion indicate that the sequential activity of *ipla-1* and then LYCAT is responsible for incorporating an 18:0 steric acid in the sn-1 position of PIs (Figure 1.7).

Taken together, these studies indicate that for PI and PIPs to achieve their specific fatty acid composition, they must go through a PI-specific Lands' cycle, which occurs subsequently to the Kennedy cycle. Moreover, these studies collectively suggest that LYCAT plays an important role in this PI-specific Lands cycle.

Other studies also partially support the role of LYCAT in a PI-specific Lands cycle. Zhao *et al*. (2009) found that LYCAT has acyltransferase activity that shows substrate preference for lyso-phosphatidylinositol (LPI) and lyso-phosphatidylglycerol (LPG) compared to other lyso-phospholipids. This was done in human embryonic kidney 293 (HEK293) cells that overexpressed human LYCAT; cells overexpressing LYCAT catalyzed PI formation when the membranes expressing LYCAT were incubated with LPI and ^{14}C-labeled oleoyl-CoA[82]. The same results were observed as LYCAT catalyzed the formation of PG when the membranes expressing LYCAT were incubated with LPG and ^{14}C-labeled oleoyl-CoA[82]. Moreover, LYCAT also catalyzed the formation of lysocardiolipin and CL with ^{14}C-labeled oleoyl- CoA[82]. Therefore, it was concluded that LYCAT has a binding pocket for substrates that bind anionic lyso-PI, thus able to remodel the anionic PLs (PI, PG and CL) *in vitro*. However, taken together with the other studies of LYCAT specificity, it appears that LYCAT is limited to regulation of PI in cells[10].

Interestingly, a study by Li *et al*., (2010) found that LYCAT is up-regulated by oxidative stress and diet-induced obesity (DIO)[83]. They experimented with LYCAT$^{(-/-)}$ mice on a high fat diet, which presented compelling changes in the acyl chain content in CL species. Mice had weight loss and improved glucose tolerance compared to wiltd-type control mice. The results showed that LYCAT led to the synthesis of CL species that were highly sensitive to oxidative damage, mitochondrial dysfunction associated with diabetes, obesity and other metabolic diseases [83]. CL is a key mitochondrial lipid, found in the inner mitochondrial membrane that aids in oxidative phosphorylation[80,83]. Therefore, this study concluded that LYCAT causes abnormal CL remodelling, causing

mitochondrial dysfunction, and inhibiting LYCAT may be a potential treatment for obesity and other metabolic diseases[83].

LYCAT is composed of 376 amino acids, is predicted to be a transmembrane protein based on a hydropathy plot, and has a molecular weight of 44.4 kDa[7,85]. LYCAT in humans and in murine species are about 89% identical and have highly conserved motifs in the GPAT family, NHX_4D and EGTD, which are involved in enzymatic activity and substrate binding, respectively[85]. LYCAT is detected in many tissues, however it is highly expressed in the heart and kidney in human tissues and liver and brain in mice tissues, suggesting that acyl specificity is important in these locations.[85]

1.8.3 'Acyltransferase LYCAT control specific phosphoinositides and related membrane traffic' by Bone et al. (2017)

LYCAT controls the acyl specificity of various PIPs and the levels and abundance of these PIPs in cells, indicating that LYCAT has an important role to play in regulation of membrane traffic[9]. Specifically, a study by Bone *et al*. (2017) indicated that LYCAT silencing led to a significant decrease in $PI(4,5)P_2$ and PI(3)P levels, with no significant change in PI(4)P levels[9]. In addition, examination of the acyl chain profile of PIPs showed a reduction of 38:4 in PIP_2 levels, but not in PIP and PIP_1 in LYCAT-silenced cells, consistent with previous models of LYCAT controlling the specific incorporation of steric acid into the sn-1 position of PI/PIPs (as described above). Thus, both these observations suggested that LYCAT has a preference in establishing the acyl chain profile of a subset of PIPs, including $PI(4,5)P_2$[9]. *Bone et al*. (2017) also studied how silencing LYCAT disrupts membrane trafficking, finding that it disrupted CME as well as membrane traffic at the early endosome. Hence, LYCAT controls $PI(4,5)P_2$ dependent endocytosis and PI3P dependent intracellular membrane traffic[9].

Lastly, another main finding is that LYCAT is co-localized to ER-derived PIS vesicles[9]. As described above, a recent study found that PIS is localized in ER-derived vesicles and form transient contacts with various organelle membranes[86]. Bone et al. (2017) examined LYCAT localization by transfecting RPE cells with LYCAT-FLAG and with various markers of specific organelle structures and vesicles: PIS-GFP (for ER-derived vesicles); KDEL-GFP (for bulk ER) and extended synaptotagmin 2 (E-Syt2)-GFP (for plasma membrane/ER contact sites). LYCAT had poor co-localization with KDEL-GFP (ER marker), intermediate intensity correlation with E-Syt2 (membrane contact site marker) and very strong intensity correlation with PIS-GFP[9]. Therefore, these results suggested that LYCAT co-localizes with ER-derived PIS vesicles, some of which interact with ER-PM sites[9].

Therefore, *Bone et al*. (2017) concluded that LYCAT is required for regulation of membrane traffic in the cell, and for specific processes such as clathrin-mediated endocytosis and endocytic recycling. Moreover, LYCAT controls specific acyl chain profile, total levels and alters localization of PI(4,5)P_2 and PI3P, but not PI4P. Lastly, LYCAT is co-localized with ER-PIS derived vesicles[9]. Importantly, how LYCAT may control the levels, localization and dynamics of specific PIPs, in particular PI(4,5)P2 were not addressed in this previous study, and is the subject of this thesis.

1.9 Objective and Study Rationale

It is known that LYCAT regulates the acylation of the sn-1 position on phosphoinositides, and that LYCAT has a role in maintaining the levels and localization of specific PIPs such as PI(4,5)P_2. LYCAT is involved in synthesis, degradation and

membrane traffic of $PI(4,5)P_2$. However, whether or not LYCAT controls the synthesis of $PI(4,5)P_2$ by PIP5K is not known. Moreover, whether LYCAT is part of a concerted process to replenish $PI(4,5)P_2$ after stimuli that transiently reduce the levels of this lipid is not known. As mentioned before, $PI(4,5)P_2$ is known to play a part in the production of $PI(3,4,5)P_3$ after EGF stimulation, however whether or not LYCAT effects the production of $PI(3,4,5)P_3$ is not known.

Thus, my hypothesis is that LYCAT controls the synthesis of $PI(4,5)P_2$, and importantly, that LYCAT plays a role in re-synthesizing $PI(4,5)P_2$ after stimuli that cause acute depletion of this lipid. Moreover, LYCAT controls the production of $PI(3,4,5)P_3$ after EGF stimulation.

To test this hypothesis, I will undertake the following specific AIMS:

Aim 1: Determine the role of LYCAT in $PI(4,5)P_2$ synthesis

1.1 Establish an assay to measure $PI(4,5)P_2$ levels

1.2 Study $PI(4,5)P_2$ re-synthesis after depletion with ionomycin in control cells

1.3 Study $PI(4,5)P_2$ re-synthesis after depletion with ionomycin in LYCAT silenced cells

Aim 2. Determine the role of LYCAT in $PI(4,5)P_2$ degradation

2.1 Establish an assay to measure degradation of $PI(4,5)P_2$ levels

2.2 Study $PI(4,5)P_2$ degradation after inhibition with phenylarine oxide (PAO) in control cells

2.3 Study $PI(4,5)P_2$ degradation after inhibition with PAO in LYCAT silenced cells.

Aim 3: Determine the role of LYCAT in PI(3,4,5)P_3 synthesis after EGF stimulation

3. 1 Establish an assay to measure the appearance of PI(3,4,5)P_3

3.2 Study PI(3,4,5)P_3 levels after EGF stimulation in control cells

3.3 Study PI(3,4,5)P_3 levels after EGF stimulation in LYCAT silenced cells

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

The two cell lines used in this study were the wild-type ARPE-19 human pigment epithelial cell line (RPE-WT) that was cultured in DMEM/F12 media and the MDA-MB 231 epithelial human breast cancer cell line that was cultured in RPMI media. Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and cells were grown at 37°C and 5% CO_2. The media and the supplements were obtained from Thermo Fisher Scientific.

2.2 Gene silencing by siRNA

The *LCLAT1* (will be referred to as LYCAT) gene expression was silenced in both RPE-WT and MDA-MB 231 cells by transfecting with a siRNA oligonucleotide against LYCAT (siLYCAT) with the sequence of 5'- GGAAAUGGAAGGAUGACAAUU-3'. MDA-MB 231 cells were independently transfected with a siRNA oligonucleotide against synaptojanin-1 (SYNJ1) (siSYNJ1) with a nucleotide sequence of 5'- UGACAAAGCUCGAGCACUUUU-3'. In parallel to transfecting cells with siLYCAT or siSYNJ1, another cell group was transfected with a non-targeting control oligonucleotide with a nucleotide sequence of 5'- CGUACUGCUUGCGAUACGGUU-3' (siCon7). All the siRNA oligonucleotides were obtained from Dharmacon. Transfection of siRNAs was achieved, after culturing cells to reach 70- 80% confluency (usually 24 h post-seeding), using Lipofectamine RNAiMAX (Thermo Fisher Scientific), as per manufacturer's instructions. Prior to transfection, cells were washed three times with phosphate buffered saline (PBS) (Thermo Fisher Scientific). For each well of a 6-well plate, 110 pmol of

each siRNA oligonucleotide (5.5 μL of 20 μM siRNA stock), 6.25 μL transfection reagent and 200 μL of Opti-MEM medium (Thermo Fisher Scientific) were mixed at room temperature for 15-20 minutes. Subsequently, the 200 μL siRNA-reagent complex solution was added dropwise to a well of a 6-well plate containing 1.8 mL of Opti-MEM medium for 4-6 hours. Afterwards, the cells were washed three times with PBS and replaced with regular growth media, according to the cell type. siRNA transfection was done twice, 24 h and 48 h after seeding.

2.3 Transfection of plasmid and siRNA oligonucleotides

Plasmids were extracted and purified using endo-free E.Z.N.A® Plasmid Midiprep Kit (Omega Bio-Tek) as per manufacturer's instructions. Plasmids encoding PH-PLCδ GFP were from the Botelho and Antonescu labs and previously described[87,88]. Plasmids encoding GPI-GFP and PH-AKT-GFP were kindly provided by Dr. Sergio Grinstein and previously described[87]. The plasmids were transfected into RPE-WT and MDA-MB 231 cells using Lipofectamine 2000 (ThermoFisher Scientific) as per manufacturer's instruction. For one well of a 6-well plate, 2 μg of plasmid were combined with 3 μL of transfection reagent in 100 μL of Opti-MEM and incubated for 15-20 minutes at room temperature. Subsequently, the 100 μL DNA-reagent complex solution was added dropwise to the well(s) containing 900 μL of Opti-MEM medium for 4-6 h. Afterwards, the cells were washed three times with PBS and replaced with regular growth media, according to the cell type. If the experimental design only required transfection of plasmid DNA, then the cells were incubated for 24 h prior to experiment. However, if the experimental design required plasmid DNA transfection after siRNA transfection, plasmid DNA transfection occurred 72 h after seeding.

2.4 Fluorescence microscopy

Microscopy was done with either fixed or live-cells. Live-cell imaging was done by spinning disc confocal microscopy (SDCM) using a Quorum Diskovery spinning confocal microscope system equipped with a Leica DMi8, connected to an Andor Zyla 4.2 Megapixel sCMOS camera and 63x/1.47 NA objective. Imaging was done using 488-nm and 537-nm laser illumination using 525/50M and 600/50M-emission filters. System and imaging was controlled with the Quorum Wave FX powered by MetaMorph software (Quorum Technologies, Guelph, ON). Live-cell imaging was performed by incubating cells with RPMI phenol free medium (Thermo Fisher Scientific) at 37°C and 5% CO_2 using an environmental control chamber. Cells were imaged with 100 μm pinholes at 100-180 ms. The exposure time was dependent on the fluorescent intensity of the cell. When SDCM was used on fixed-cell imaging, z-stacks were taken at 0.4 μm intervals.

For total internal reflection fluorescence microscopy (TIRFM), cells were imaged after they were fixed. TIRF depth used was 100 μm at 50-100 ms. The exposure time was dependent on the fluorescent intensity of the cell.

2.5 Depletion and recovery of PI(4,5)P_2 after acute depletion with ionomycin

Ionomycin was used to acutely deplete PI(4,5)P_2 in order to measure PI(4,5)P_2 depletion and re-synthesis using two general strategies. The strategy to achieve this is summarized in Figure 2.1. First, cells were treated with 10 μM of ionomycin (Sigma Aldrich) in phenol free RPMI media for different times and washed with 1% bovine serum albumin (BSA) diluted in serum free media (Thermo Fisher Scientific) to remove ionomycin from the plasma membrane for 4 min at 37°C and 5% CO_2. Cells were then fixed with 4% paraformaldehyde 1X PBS for 20 min on ice, quenched with 100 mM

glycine in 1X PBS for 15 min, washed 3 to 5 times with 1X PBS and mounted in fluorescence mounting media (DAKO) or preserved in 1X PBS for imaging by TIRFM the next day. Alternatively, cells were treated with 10 µM of ionomycin in phenol free RPMI media for 2 min or PAO at 37°C and 5% CO_2 during live-cell imaging with SDCM to visualize the plasma membrane.

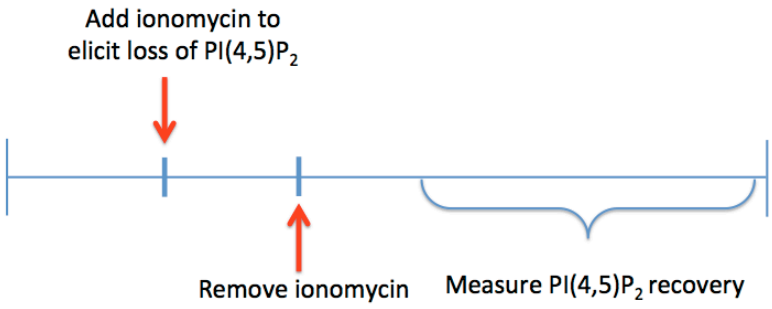

Figure 2.1: Diagram depicting experimental strategy of PI(4,5)P_2 recovery.

Cells are treated with ionomycin, which elicits a robust reduction of PI(4,5)P_2, followed by ionomycin washout and measurement of PI(4,5)P_2 recovery.

2.6 PI(4,5)P_2 turnover following depletion with phenylarsine oxide (PAO)

Cells were exposed to PAO to measure the rate of PI(4,5)P_2 degradation of by inhibiting PI4K and to deplete the precursor, PI(4)P. This was done by treating MDA-MB 231 cells with 10 µM of PAO (Sigma Aldrich) in phenol free RPMI media and performing live-cell imaging by SDCM for 10-12 min at 37°C and 5% CO_2. The general strategy is depicted in Figure 2.2.

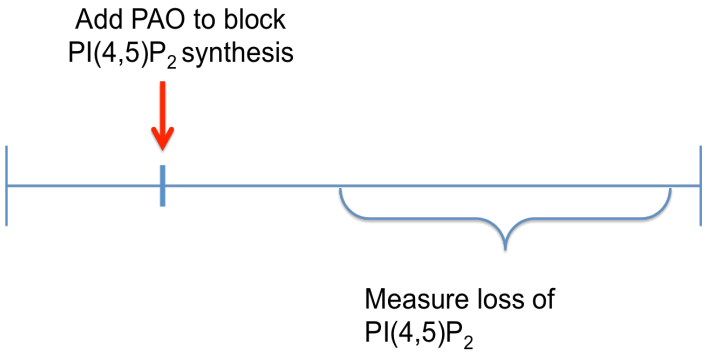

Figure 2.2: Diagram depicting experimental strategy of $PI(4,5)P_2$ degradation.

PAO is added to arrest PI4P and $PI(4,5)P_2$ synthesis, followed by measurement of $PI(4,5)P_2$ abundance, which is controlled by the rate of $PI(4,5)P_2$ turnover.

2.7 $PI(3,4,5)P_3$ synthesis following EGF stimulation

Cells were stimulated with EGF to measure the rate of $PI(3,4,5)P_3$ production on the plasma membrane by activation of PI3K, which phosphorylates $PI(4,5)P_2$ to $PI(3,4,5)P_3$. MDA-MB 231 cells were serum starved with DMEM-low glucose (Sigma Aldrich) for 1 h and treated with 20 ng/mL of EGF in phenol free RPMI media and subjected to live-cell imaging by SDCM for 5-6 min at 37°C and 5% CO_2. The general strategy is depicted in Figure 2.3.

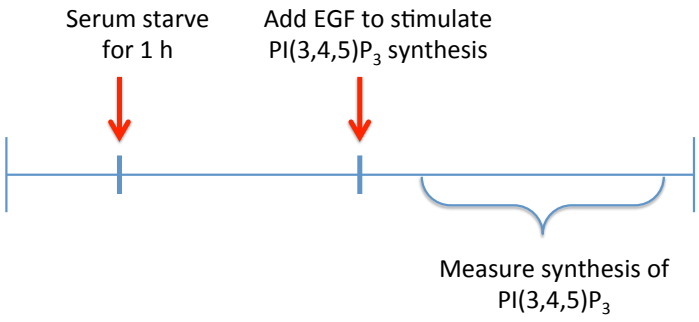

Figure 2.3: Diagram depicting experimental strategy of $PI(3,4,5)P_3$ production.

Cells are serum starved for 1 h. EGF is added to stimulate $PI(3,4,5)P_3$ synthesis, followed by measurement of PH-AKT (rim/cytosol).

2.8 Fluorescence microscopy image analysis

2.8.1 Quantification of PI(4,5)P_2 localization from TIRFM images

To measure PI(4,5)P_2 levels, the ratio of the PH-PLCδ GFP probe on the plasma membrane (TIRF) relative to the entire cell (EPI) was measured. ImageJ was used to obtain a TIRF/EPI ratio, first, the free-hand selection tool was used to trace the plasma membrane imaged by TIRF (1) and 3 random circles were placed in the extracellular space (2-4) shown in Figure 2.4 (TIRF). Second, positions 1-4 were used for the EPI image (5-8) as shown in Figure 2.3 (EPI). The formula used was:

$$\frac{TIRF}{EPI} = \frac{1 - \left(\frac{2+3+4}{3}\right)}{5 - \left(\frac{6+7+8}{3}\right)}$$

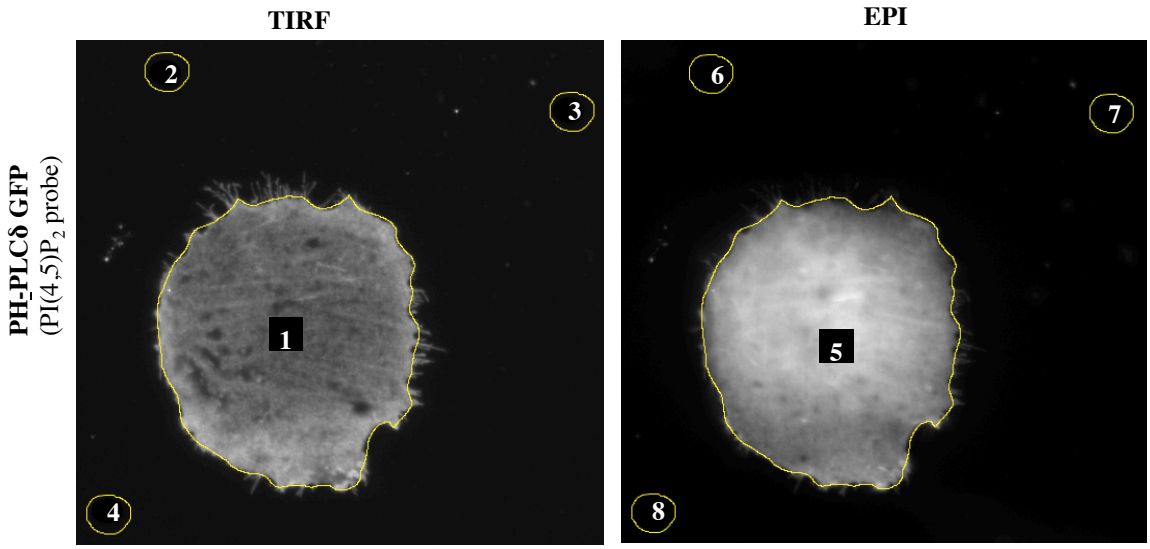

Figure 2.4: TIRF/EPI quantification of RPE-WT using ImageJ.

Shown is representative fluorescence micrograph of an RPE-WT cell, transfected with PH-PLCδ GFP and imaged with TIRFM and EPIM. The numbers shown were used in the TIRF/EPI ratio formula, shown above.

2.8.2 Quantification of PI (4,5) P_2 localization from SDCM images or videos

To measure PI(4,5)P_2 levels, the ratio of the PH-PLCδ GFP probe on the plasma membrane relative to the cytosol was measured. ImageJ was used to obtain a rim/cytosol ratio, the straight-line tool (4 pixels wide) was placed randomly on the plasma membrane (1), in the cytosol (2) and in the extracellular space (3), as shown in Figure 2.5. In order to avoid experimental bias, when choosing where to place the straight-line tool, I randomly chose the 3 positions (1-3) before looking through rest of the images. When quantifying videos, I made sure the straight-line tool stayed on the same spot on the plasma membrane, throughout the video. The formula used was:

$$\frac{rim}{cytosol} = \frac{(1-3)}{(2-3)}$$

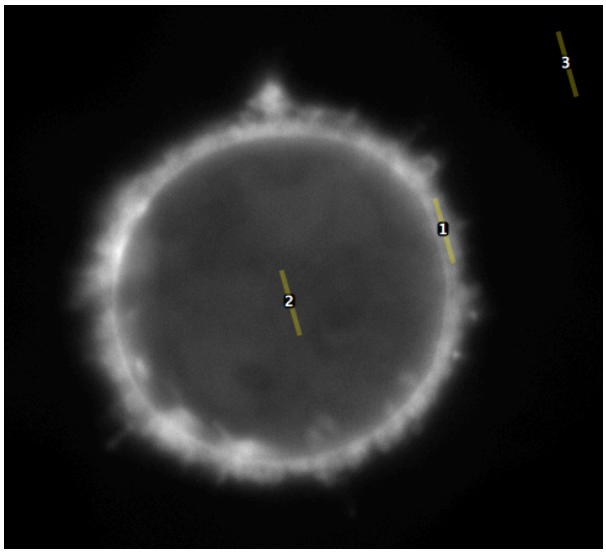

Figure 2.5: Rim/cytosol quantification of MDA-MB 231 cells using ImageJ.

Shown is representative fluorescence micrograph of an MDA-MB 231 cell, transfected with PH-PLCδ GFP and imaged with SDCM. The numbers shown were used in the rim/cytosol ratio formula, shown above.

2.9 Statistical analysis and robustness

When quantifying for TIRF/EPI or rim/cytosol ratio, ≤ 15 cells were imaged per condition per experiment, across three or more independent experiments. Fixed-cell images quantified for $PI(4,5)P_2$ recovery after ionomycin treatment using ImageJ (previously described in section 2.7.1 and 2.7.2), were averaged for each condition in siControl and siLYCAT cells, for all independent experiments, and normalized to control (before addition of ionomycin). siControl and siLYCAT conditions were analyzed by two-way ANOVA with *post hoc* Sidak's multiple comparisons test. Significant pairwise comparisons were marked on the graphs by * (siControl) and † (siLYCAT) (Figure 2.1C). Data is represented as mean ± the standard error of the mean (SEM).

Live-cell videos quantified for $PI(4,5)P_2$ levels before and after PAO treatment or $PI(3,4,5)P_3$ levels before and after EGF stimulation using ImageJ, were averaged for each time frame per condition for all independent experiments. An area under the curve (AUC) was analyzed, following a one-way ANOVA or an unpaired t-test to test for statistical significance between conditions (Figure 3.2C and 3.3B, respectively). Moreover, the baseline for the AUC graphs was set to 1. Data is represented as mean ± the standard error of the mean (SEM).

CHAPTER 3

RESULTS

Previous work from our labs showed that disruption of LYCAT expression caused a 21% ± 0.06% reduction in $PI(4,5)P_2$ levels in RPE cells[9]. The reason for this reduction in $PI(4,5)P_2$ levels remained unknown, but may occur due to impaired synthesis and/or increased turnover. To resolve this, I proposed the hypothesis that LYCAT expression is necessary to maintain the synthesis of $PI(4,5)P_2$. Moreover, given that $PI(4,5)P_2$ levels were impaired in LYCAT silenced cells, I postulated that LYCAT function is necessary for the production of $PI(3,4,5)P_3$ after EGF stimulation. To test these hypotheses, I determined the rates of $PI(4,5)P_2$ re-synthesis and degradation and $PI(3,4,5)P_3$ synthesis after EGF stimulation using microscopy assays in cells expressing GFP-based biosensors of $PI(4,5)P_2$ and $PI(3,4,5)P_3$.

3.1 LYCAT silencing does not impact overall $PI(4,5)P_2$ synthesis capacity

3.1.1 Incomplete recovery of the $PI(4,5)P_2$ probe in RPE-WT cells in the TIRF field after ionomycin removal.

To measure $PI(4,5)P_2$ synthesis on the plasma membrane, I had to first develop a method to acutely deplete $PI(4,5)P_2$ levels and track its recovery within cells. This could then be used to compare $PI(4,5)P_2$ re-synthesis in siControl and siLYCAT cells, expressing the PH PLCδ-GFP biosensor, a pleckstrin homology (PH) domain fused to GFP that is highly selective for $PI(4,5)P_2$, thus able to visualize $PI(4,5)P_2$ on the plasma membrane.

To deplete $PI(4,5)P_2$, I used a drug called ionomycin (explained previously) that allows extracellular calcium to permeate into the cell, activating PLC, cleaving $PI(4,5)P_2$ to DAG and IP_3, resulting in a significant decrease in $PI(4,5)P_2$ levels. Initially, TIRFM

was used to measure $PI(4,5)P_2$ levels by measuring the fluorescence intensity of the PH-PLCδ GFP probe at the plasma membrane (TIRF field) and total fluorescence (EPI) and taking its ratio (TIRF/EPI), before and after ionomycin treatment in RPE-WT cells (Figure 3.1A). Figure 3.1B shows different time treatments with 10 μM of ionomycin in RPE-WT cells in both conditions. There was about a 70% depletion of $PI(4,5)P_2$ levels after 5 min and about 75% depletion of $PI(4,5)P_2$ levels after 10, 15 and 20 min of ionomycin treatment (n=1). Therefore, it was concluded that 5 min of 10 μM ionomycin treatment was sufficient to deplete $PI(4,5)P_2$ from the plasma membrane.

Next, I had to determine how to remove ionomycin and how long it takes for the cells to re-synthesize $PI(4,5)P_2$. I used 1% of bovine serum albumin (BSA) diluted in serum-free Dulbecco's modified eagle's medium (DMEM) to effectively remove ionomycin, since BSA's hydrophobic properties enable it to bind to ionomycin's hydrophobic properties, extracting it from the plasma membrane. Figure 3.1D presents data showing 5 min, 10 min and 30 min of $PI(4,5)P_2$ recovery of siControl (blue bars) and siLYCAT (red bars) RPE-WT cells, after an acute depletion of 10 μM of ionomycin in RPE-WT cells. Figure 3.1A shows PH-PLCδ GFP fluorescence micrographs of siControl and siLYCAT RPE-WT cells at resting state, 0 min of recovery and 30 min recovery after 5 min of 10 μM ionomycin treatment. However, it was observed that after multiple trials, cells did not fully recover their TIRF/EPI ratio of PH-PLCδ GFP even after 30 min of ionomycin removal, suggesting that $PI(4,5)P_2$ levels were not recovering fully or an artifact existed. For example, we considered the possibility that ionomycin altered cell attachment to the coverslip, which would alter the TIRF/EPI ratio.

To test this, RPE-WT cells were transfected with a fluorescent glycosylphosphatidylinositol (GPI) (Figure 3.1C). GPI is anchored and decorates the plasma membrane and consists of GFP fused to a tetrasaccharide core and a PI anchor. Results showed GPI levels (green bars) started at steady state, decreased by about 20% (n=1) during ionomycin treatment and did not fully recover (Figure 3.1C). Therefore, TIRFM was sensitive to changes in cell shape and adhesion, creating an artifact in our approach. We thus turned to a different method that relies on spinning disk confocal microscopy (SDCM).

3.1.2 Complete recovery of PI(4,5)P_2 probe is observed after removal of ionomycin in MDA-MB 231 cells using SDCM

SDCM allows for imaging of cells along the z-axis by acquiring thin planar sections of the cell, removing out of focus light. To benefit from this, we changed the cell line to MDA-MB-231 cells (breast cancer cell line), which are rounded and taller than the flat RPE cells (Figure 3.2A). The fluorescence of PH-PLCδ GFP was then quantified using rim/cytosol ratio before and after ionomycin treatment as an indicator of PI(4,5)P2 levels. Measurements of PI(4,5)P_2 levels by PH- PLCδ GFP were subjected to two-way ANOVA.

After establishing an assay to deplete and recover PI(4,5)P_2 levels, I replicated these experiments in siControl and siLYCAT MDA-MB-231 cells (Figure 3.2A). To study whether LYCAT plays a role in re-synthesis of PI(4,5)P_2, MDA-MB-231 cells were first transfected with non-targeting (control) siRNA or siRNA targeting LYCAT, and then transfected with PH-PLCδ GFP, followed by ionomycin treatment at different time points and recovery by removing ionomycin. The fluorescence of PH-PLCδ-GFP was then monitored using SDCM and the ratio of rim/cytosol calculated. Figure 3.2B

shows a significant drop in the fluorescence of PH-PLCδ GFP from the plasma membrane in both siControl (blue bars) and siLYCAT (red bars) MDA-MB 231 cells after 5 min treatment of ionomycin. However, after removal of ionomycin with 1% BSA, there is an equivalent rate of recovery of the PH-PLCδ GFP probe for both conditions. This led me to conclude that even though there is a knockdown of LYCAT, cells are still able to recover $PI(4,5)P_2$ at the same rate of control cells. Thus, the question now is whether LYCAT plays a role in $PI(4,5)P_2$ degradation.

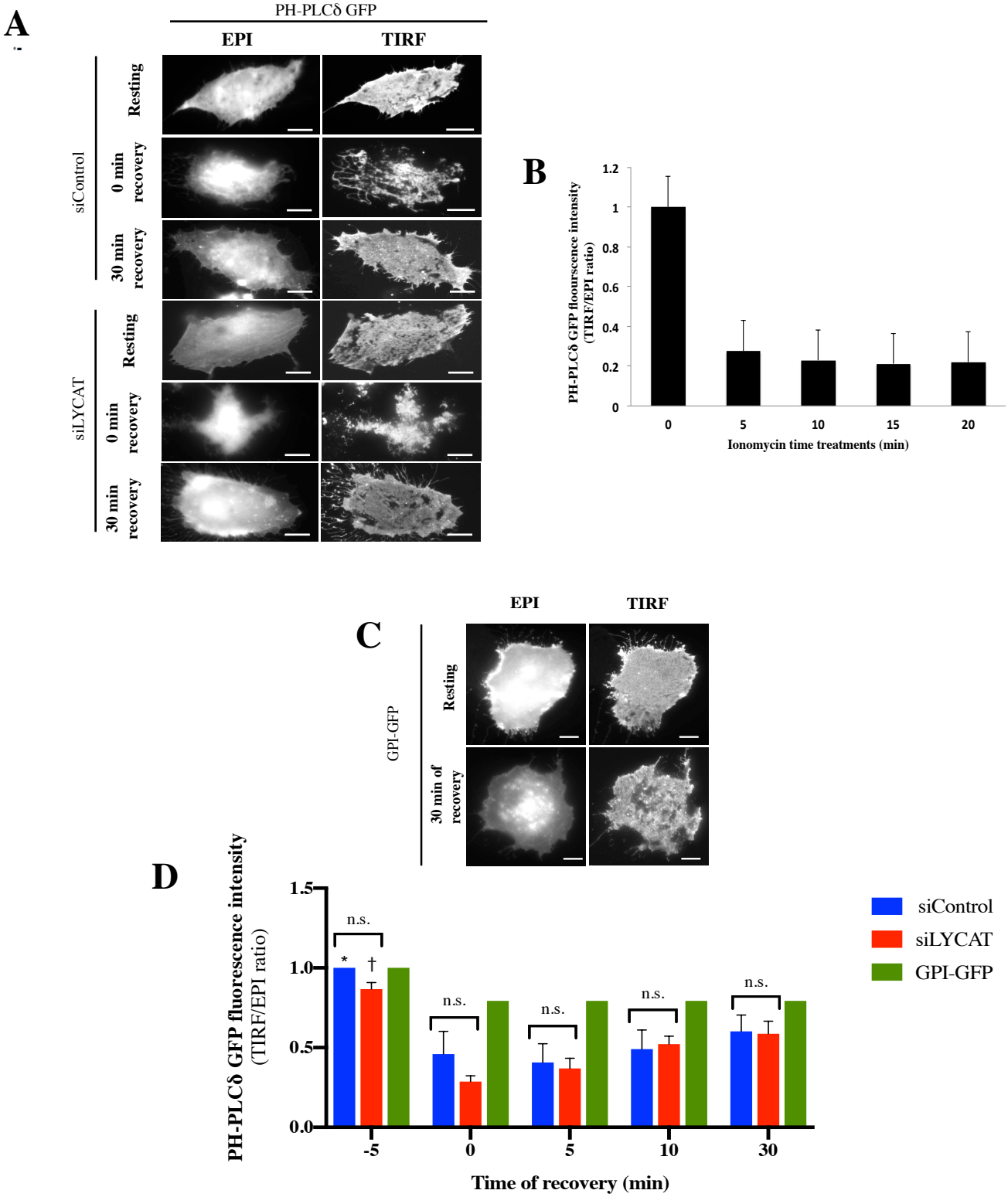

Figure 3.1: Incomplete recovery of PI(4,5)P_2 probe in RPE-WT cells after removal of ionomycin.

(**A**) Representative PH-PLCδ GFP fluorescence micrographs of siControl and siLYCAT RPE-WT cells at resting state, 0 min of recovery and 30 min recovery after 5 min of 10 μM ionomycin treatment. Cells were fixed and subjected to TIRFM and epifluorescence microscopy. (**B**) Representative GPI-GFP fluorescence micrographs imaged by TIRFM and epifluorescence of RPE-WT cells at resting state and 30 min recovery after 5 min of 10 μM ionomycin treatment. Cells were fixed and subjected to TIRFM and epifluorescence microscopy. (**C**) RPE-WT cells were transfected with PH-PLCδ GFP, followed by exposure to 10 μM ionomycin for different periods of time. n=1 (**D**) RPE-WT cells were first transfected with siRNA targeting LYCAT (siLYCAT) or non-targeting siRNA (siControl), and then transfected with PH-PLCδ GFP or GPI-GFP (n=1), followed by ionomycin treatment and removal. Data are represented as mean ± SEM and was tested with a two-way ANOVA *post hoc* Tukey's multiple comparisons test, for siControl and siLYCAT, n=5 per condition; *$p < 0.05$ relative to control siRNA treated cells.

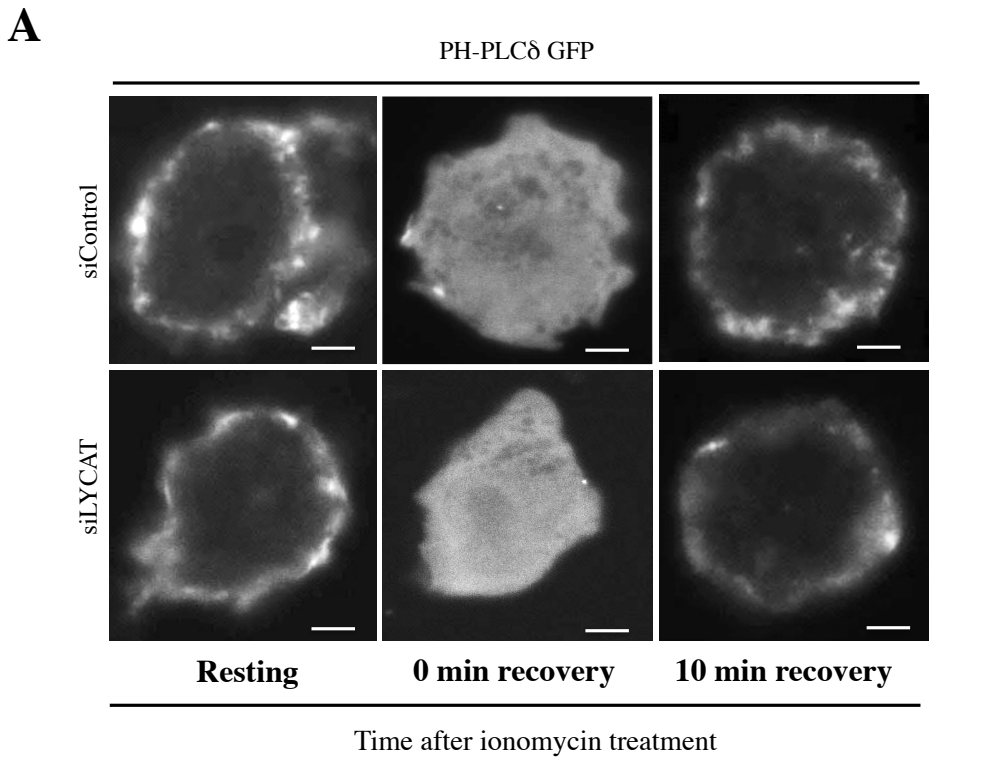

Figure 3.2: LYCAT silencing does not impact acute PI(4,5)P_2 synthesis.

(**A**) Representative PH-PLCδ GFP fluorescence micrographs of siControl and siLYCAT MDA-MB 231 cells at resting state, 0 min recovery and 10 min recovery after 5 min of 10 μM ionomycin treatment. Cells were fixed and subjected to SDCM using z-stacks. (**B**) MDA-MB-231 cells were first transfected with siRNA targeting LYCAT (siLYCAT) or non-targeting siRNA (siControl), and then transfected with PH-PLCδ GFP, followed by 10 μM ionomycin treatment and washout (recovery). Data are represented as mean ± SEM and was tested with a two-way ANOVA *post hoc* Sidak's multiple comparisons test, for siControl and siLYCAT, n=3 per condition; * and † $p < 0.05$ relative to control siRNA treated cells.

3.2 LYCAT silencing impairs $PI(4,5)P_2$ degradation

3.2.1 $PI(4,5)P_2$ degradation is impaired in siLYCAT MDA-MB 231 cells

In order to measure the degradation rate of $PI(4,5)P_2$ levels, I established an assay to inhibit the synthesis of $PI(4,5)P_2$ by treating cells with phenylarsine oxide (PAO), an inhibitor of PI4K. Briefly, PI4K phosphorylates PI on the 4^{th} position of the inositol headgroup to make PI4P, which is then converted to $PI(4,5)P_2$ by PI5K. Therefore, PAO inhibits the forward reaction from PI to $PI(4,5)P_2$, dissociating synthesis from degradation. Figure 3.3A presents SDCM images of MDA-MB 231 cells transfected with PH-PLCδ GFP before and after PAO treatment for both conditions. Figure 3.3B data show $PI(4,5)P_2$ levels (rim/cytosol) at different time treatments and dose concentrations of PAO. There was an approximate 30% ± 0.08% decrease in $PI(4,5)P_2$ levels in all the conditions. Therefore, it was concluded that 10 μM of PAO for approximately 10 min was sufficient enough to study the degradation of $PI(4,5)P_2$ levels.

To study whether LYCAT plays a role in degradation of $PI(4,5)P_2$, MDA-MB 231 cells were first transfected with non-targeting (control) siRNA or siRNA targeting LYCAT and then transfected with PH-PLCδ GFP, followed by PAO treatment, after 30 s

of rest (Figure 3.3A). PI(4,5)P_2 levels were monitored using SDCM. The data in figure 3.3C were analyzed as an area under the curve (AUC), shown in figure 3.3D, to determine whether there is a statistical significance in PI(4,5)P_2 degradation comparing siControl to siLYCAT. Results showed a significant difference in PI(4,5)P_2 levels between siControl and siLYCAT, with a larger AUC in the siLYCAT conditions. Measurements of PI(4,5)P_2 by PH-PLCδ GFP were subjected to a AUC and a one-way ANOVA.

3.2.2 Properly acylated PI(4,5)P_2 is required for PI(4,5)P_2 turnover in MDA-MD 231 cells

SYNJ1 is a 5-phosphatase that removes the 5th phosphate on the inositol headgroup of PIPs. There is evidence that SYNJ1 is responsible for a significant portion of PI(4,5)P_2 turnover and may be sensitive to the acyl specificity of PI(4,5)P_2[89]. Thus, we tested PI(4,5)P_2 turnover rate in cells silenced for SYNJ1 and expressing PH-PLCδ GFP. These cells were then exposed to PAO after a 30 s resting phase (Figure 3.3A). The data in figure 3.3C was analyzed as AUC, shown in figure 3.3D, to see whether there were statistical significance in PI(4,5)P_2 degradation comparing all three conditions. Results showed a significant difference in PI(4,5)P_2 turnover between siControl and siSYNJ1, where siSYNJ1 cells were more resistant to PI(4,5)P_2 turnover induced by PAO. However, there was no significant difference between PI(4,5)P_2 turnover rates in siSYNJ1 and siLYCAT cells. This suggested that since there is a reduction in 18:0/20:4 PI(4,5)P_2 in LYCAT silenced cells, SYNJ1 does not function effectively to turnover PI(4,5)P_2.

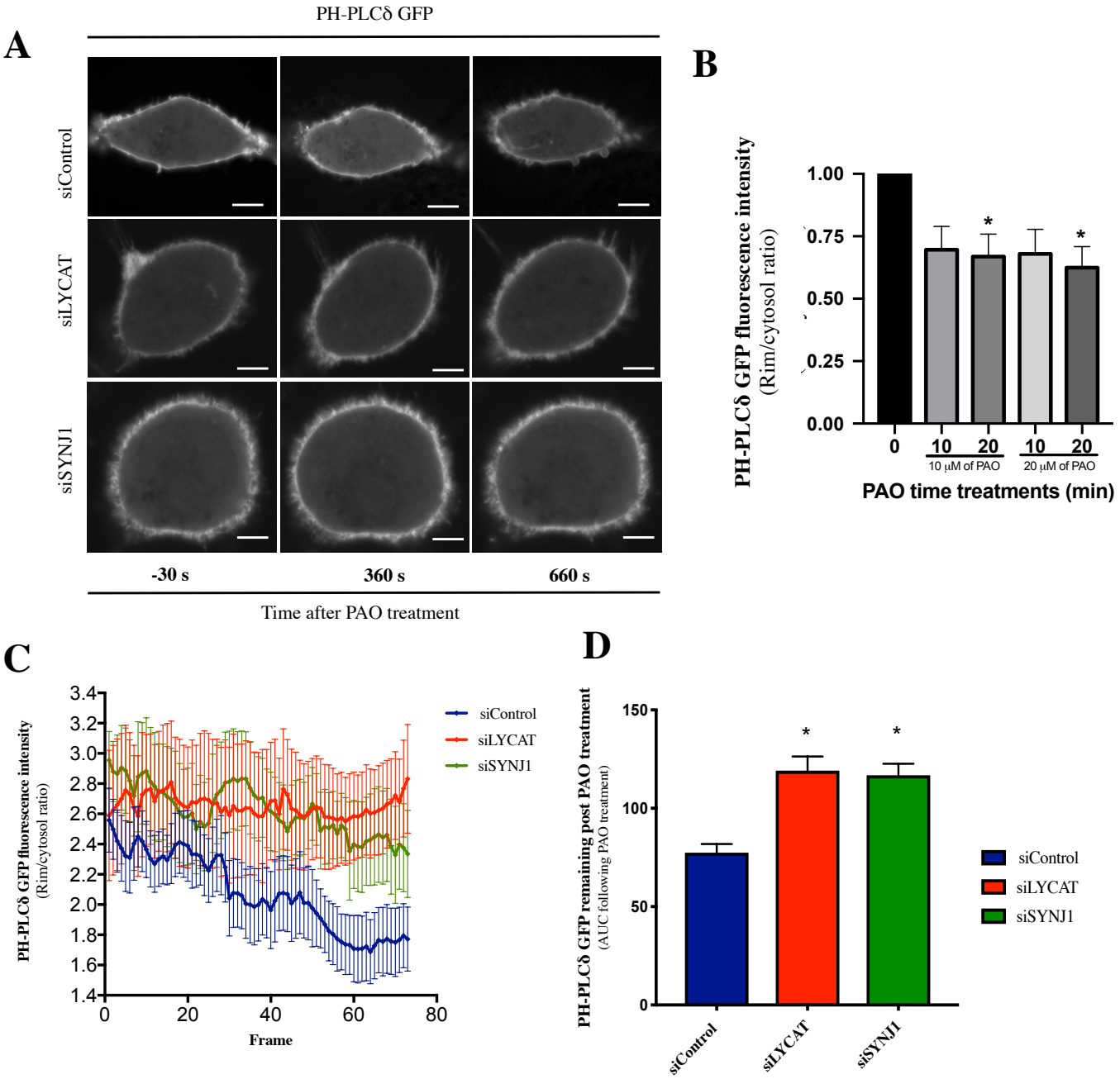

Figure 3.3: Silencing of LYCAT impairs PI(4,5)P_2 degradation.

(**A**) Representative PH-PLCδ GFP fluorescence micrographs of siControl, siLYCAT and siSYNJ1 MDA-MB 231 cells in resting state and 360 s (6 min) and 660 s (12 min) after 10 μM PAO treatment. Cells were subjected to live-imaging by SDCM (**B**) MDA-MB 231 cells were transfected with PH-PLCδ GFP, followed by different doses of PAO for 10 and 30 min. Data are represented as mean ± SEM and was tested with a one-way

ANOVA post hoc Tukey's multiple comparisons test, for siControl and siLYCAT, n=2 per condition; *p< 0.05 relative to control siRNA treated cells. (**C**) MDA-MB-231 cells were first transfected with siRNA targeting LYCAT (siLYCAT), non-targeting siRNA (siControl) or siRNA targeting SYNJ1 and then transfected with PH-PLCδ GFP followed by 10 μM PAO treatment. Frames were imaged every 10 seconds. (**D**) Shows the AUC according to data in (**C**). Data are represented as mean ± SEM and was tested with an AUC followed by one-way ANOVA and *post hoc* Tukey's multiple comparisons test. For siControl, siLYCAT and siSYNJ1 cells, n=11-12 cells per condition. *p< 0.05, relative to control siRNA treated cells.

3.3 LYCAT silencing impaired $PI(3,4,5)P_3$ production

$PI(4,5)P_2$ is a precursor of $PI(3,4,5)P_3$ production and since LYCAT silencing decreases $PI(4,5)P_2$ levels, we hypothesized that LYCAT disruption will impair $PI(3,4,5)P_3$ synthesis. In order to study whether LYCAT plays a role in $PI(3,4,5)P_3$ synthesis, I established an assay to study $PI(3,4,5)P_3$ levels in siControl and siLYCAT cells after EGF stimulation. Cells were treated with a high dose of EGF (20 ng/ml) to stimulate $PI(3,4,5)P_3$ production and increase the likelihood that the PH-AKT GFP, the $PI(3,4,5)P_3$ biosensor, would localize to the plasma membrane from the cytosol, as previously described[90]. MDA-MB-231 cells were first transfected with non-targeting (control) siRNA or siRNA targeting LYCAT and then transfected with PH-AKT GFP, followed by EGF stimulation (20 ng/ml). $PI(3,4,5)P_3$ levels were monitored by SDCM (Figure 3.4A)

Results showed a slower rate of $PI(3,4,5)P_3$ production in siLYCAT cells after EGF stimulation (Figure 3.4B). The data in figure 3.3B was analyzed as the AUC, as shown in figure 3.3C, to see whether there was a significant difference in $PI(3,4,5)P_3$ production

comparing control and LYCAT silenced cells. We observed that siLYCAT cells displayed a significantly lower AUC compared to siControl cells in response to EGF, as measured by AUC and using an unpaired Student's t-test. These results suggested that LYCAT silenced cells have a slower rate of $PI(3,4,5)P_3$ production after EGF stimulation.

Taken together that there is an overall lower level of $PI(4,5)P_2$, and a non-existent rate of degradation, this suggests that the rate of synthesis also has to be very slow, compared to control cells. Moreover, that an impairment in the synthesis and degradation of $PI(4,5)P_2$ causes a delay in $PI(3,4,5)P_3$ production after EGF stimulation.

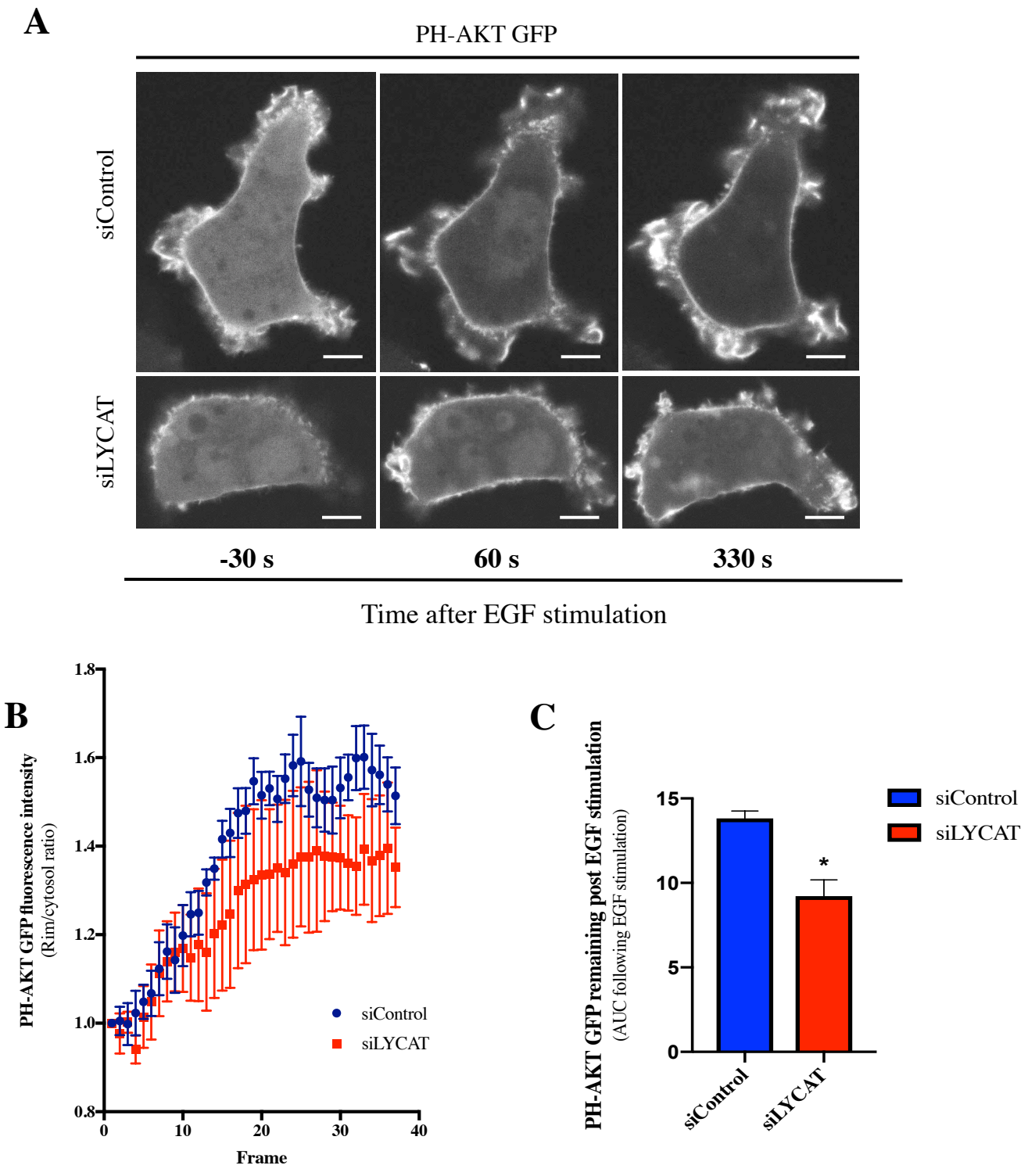

Figure 3.4: Silencing of LYCAT impairs PI(3,4,5)P_3 production.

(A) Representative PH-AKT GFP fluorescence micrographs of siControl and siLYCAT MDA-MB 231 cells in resting state and 60 s (1 min) and 330 s (5.5 min) after 20 ng/mL EGF stimulation. Cells were subjected to live-imaging by SDCM. (**B**) MDA-MB-231 cells were first transfected with siRNA targeting LYCAT (siLYCAT), non-targeting siRNA (siControl) and then transfected with PH-AKT GFP, followed by 20ng/ml EGF stimulation for 5.5 min. Frames were imaged every 10 seconds. (**C**) Shows the AUC according to data in (**B**) Data are represented as mean ± SEM and was tested with an AUC followed by an unpaired t-test and, n=3 per condition. *p< 0.05, relative to control siRNA treated cells.

CHAPTER 4

DISCUSSION

Our lab previously reported that LYCAT silenced RPE-WT cells exhibit a 20% reduction in the steady state levels of $PI(4,5)P_2$, which led me to hypothesize that LYCAT controls the synthesis/turnover of $PI(4,5)P_2$[9]. This study has shown that after an acute depletion of $PI(4,5)P_2$, cells were able to make this PIP at approximately the same rate in both LYCAT silenced and control conditions, indicating that the absolute capacity for synthesis of $PI(4,5)P_2$ is retained in LYCAT silenced cells. Importantly, impairment of steady state $PI(4,5)P_2$ synthesis via inhibition of PI4K by PAO revealed an impairment of $PI(4,5)P_2$ turnover in LYCAT silenced cells compared to control cells. Interestingly, I also observed that when SYNJ1 silenced cells were treated with PAO in a similar assay, there was also an impairment in $PI(4,5)P_2$ turnover, thus providing evidence that SYNJ1 is the inositol phosphatase directly responsible for $PI(4,5)P_2$ turnover at steady state in resting cells. Lastly, I wanted to determine whether LYCAT perturbation impacts EGF stimulated production of $PI(3,4,5)P_3$. I uncovered that LYCAT silenced cells caused impairment in $PI(3,4,5)P_3$ production compared to control cells, suggesting that LYCAT does control the rate of production of this PIP.

4.1 LYCAT silencing causes slower dynamic PI(4,5)P_2 synthesis and turnover

As seen in figure 3.3, there is an impairment in PI(4,5)P_2 turnover in MDA-MB 231 cells subjected to SYNJ1 silencing, suggesting that PI(4,5)P_2 turnover is largely due to the activity of SYNJ1 at steady state. Briefly, SYNJ1 is a 5-phosphatase that hydrolyzes the phosphate on the 5th position of the inositol headgroup of PI(4,5)P_2, resulting in production of PI4P. PI(4,5)P_2 turnover is also impaired upon LYCAT silencing, therefore, this suggests that PI(4,5)P_2 turnover by SYNJ1 exhibits a preference about acyl chain specificity of PI(4,5)P_2. This interpretation is consist with a previous study which identified that SYNJ1 preferentially selects for PI(4,5)P_2 harbouring 18:0/20:4 as its substrate compared to PI(4,5)P_2 harbouring shorter acyl chains[89]. Another suggestion I propose is that, PI(4,5)P_2 may be mislocalized to different parts of the plasma membrane or cell compartment, thus making it inaccessible to SYNJ1. Therefore, since LYCAT silenced cells showed a significant decrease in the 18:0/20:4 combination and a decrease in PI(4,5)P_2 levels in comparison to control cells, I propose that SYNJ1 inefficiently detects properly acylated PI(4,5)P_2 , thus resulting in impaired PI(4,5)P_2 turnover in LYCAT silenced cells[9].

Figure 3.2B shows that LYCAT silenced cells are capable of generating PI(4,5)P_2 at the same rate as control cells after an acute depletion with ionomycin. Interestingly, this is different than the prediction made by studies that have shown PIP5K prefers 18:0/20:4 PI(4,5)P_2. Briefly, PIP5K comes in three different isoforms, of which prefer to phosphorylate the 5th position on the inositol head group on unsaturated acyl chains, 18:0/20:4 PI4P and 18:0/18:1 PI4P[24]. Thus, this suggests that certain isoforms of PIP5K that phosphorylate 18:0/20:4 PI4P are involved in the PI cycle enriching 18:0/20:4

species of PIs[24]. My results show that that there is another mechanism in the cell that is able to synthesis PI(4,5)P_2 during stressful conditions (discussed below), or that calcium or other signals modulate the substrate preference of PIP5Ks to accommodate a variety of acyl species of PI4P.

However, taken together with the observation that LYCAT silencing decreases overall PI(4,5)P_2 levels, this indicates that at steady state, the rate of PI(4,5)P_2 synthesis and turnover is also substantially reduced in LYCAT silenced cells. As mentioned, there is evidence for acyl chain specificity for both enzymes regulating synthesis and turnover of PI(4,5)P_2, PIP5K and SYNJ1, respectively, but what about their biochemical properties? Is mis-acylated PI(4,5)P_2 recognized by these enzymes? If so, what is the mechanism by which lipid kinases and phosphatases that act on the hydrophilic headgroup at the surface of a lipid bilayer may be influenced by acyl profile, as these fatty acids are buried in the hydrophobic core of the lipid bilayer?

Phosphatidylinositol transfer proteins (PITPs) are responsible for PI delivery and PI presentation[91]. PI delivery functions to transfer lipids from the ER to non-ER membranes. There are two isoforms of PITP; PITPα and PITPβ. Both can bind to PI and PC, with a higher affinity for PI. Moreover, its been shown that both PITPs preferentially bind to highly curved membrane surfaces[91]. In contrast, PI presentation functions to present the lipids to lipid kinases for the production of PIPs. Sec14 is related to PITP, however, the significant difference between PITP and Sec14 is that when a lipid is transferred to a PITP, the headgroup is buried deep inside the hydrophobic pocket making it advantageous for transferring lipids through the cytosol. In contrast, within Sec14, the headgroup is exposed at the opening of the hydrophobic pocket, allowing it to interact

with PI kinases[91,92]. Sec14 comes in 'open' and 'closed' conformations, depending on the positioning of the α-helical gate at the lipid binding pocket[93]. When Sec14 is in an 'open' confirmation, it means that it is integrated in the membrane through its α-helix, allowing loading of a PI in to its hydrophobic pocket, subsequently presenting it to a PI kinase.

Important for my work in this thesis, Sec14 stimulates PI4K activity by performing multiple rounds of phospholipid exchange at a single membrane site[94]. However, what is not known is whether and how Sec14 may be able to distinctly detect PIs in membranes depending on acylation profile. Differences that appear subtle, such as a difference in 2 carbons in 18:0/20:4 versus 16:0/20:4 indeed affect the integration, distribution or dynamics of PI in the membrane, and thus impact how different PI species bind to Sec14. Moreover, recent studies have shown that Sec14 preferentially binds to membrane regions with a convex curved face, stimulating binding of the α-helix into the membrane[94]. It is, therefore, possible that PI/PIPs with distinct acyl groups are specifically partitioned within flat versus curved portions of the lipid bilayer, resulting in distinct interactions with Sec14 and/or PITPs, and thus distinct rates of synthesis of PI to PI4P and eventually, to $PI(4,5)P_2$.

Consistent with the model of differential partitioning of PI/PIP acyl species into distinct portions of the bilayer defined by curvature, SYNJ1 is recruited to sites of endocytosis during the latter part of CME on the plasma membrane by the SH3 domain-containing protein, endophilin[95]. Endophilin also contains a Bin1/Amphiphysin/Rvs (BAR) domain that has the important feature of sensing membrane curvature through its crescent shape structure. It has been shown that $PI(4,5)P_2$ synthesis and turnover is highly regulated by membrane curvature, where hydrolysis is achieved by SYNJ1-endophilin

partnership on highly curved membranes, rather than flat ones, facilitating membrane fission[95].

I thus propose a model in which turnover of $PI(4,5)P_2$ in LYCAT silenced cells is slow since there is a reduction in 18:0/20:4 $PI(4,5)P_2$ and an increase in shorter acyl chain of PIP_2 species, which causes these PIPs to be restricted in flatter regions of the membrane, rather then curved ones. This phenomenon would thus cause SYNJ1 to be less able to detect and access $PI(4,5)P_2$ in LYCAT silenced cells, resulting in decreased rates of $PI(4,5)P_2$ hydrolysis.

Taking all of this into account, this might suggest that since LYCAT silenced cells have a reduction in the 18:0/20:4 $PI(4,5)P_2$/PI species, they might reside in different parts of the membrane (i.e. flatter regions) or are integrated in the plasma membrane where it causes difficulty for an effector protein to bind.

4.2 LYCAT silencing may perturb effector protein binding and membrane properties

During CME, turnover of $PI(4,5)P_2$ causes proper CCP assembly and CCV formation and is dependent on 5-phosphatases, such as OCRL and SYNJ1[95,29]. Importantly, these lipid phosphatases have important roles in the turnover of $PI(4,5)P_2$ from CCV upon scission of these vesicles from the plasma membrane, ensuring that internal membranes are depleted of the plasma membrane marker $PI(4,5)P_2$. However, in LYCAT silenced cells, there is a reduction of PI3P on endosomes and altered $PI(4,5)P_2$ localization to intracellular tubules and punctate structures[9]. This is consistent with my data, where SYNJ1 presented slower rates of $PI(4,5)P_2$ turnover, suggesting a reason why

this PIP remains on intracellular tubules and punctate structures following scission of the CCV from the cell surface.

As previously mentioned, LYCAT silencing caused shorter acyl chains in PIP_2 species. In addition to the alterations in partitioning within flat versus curved membranes and/or recognition by PITPs, the differences in acyl chain profile of PIPs may affect how the lipid is positioned in the membrane, disturbing effector protein binding. Acyl chain alteration could cause 1) lipids to change their orientation in the membrane, 2) lipid packaging with ordered domains, 3) lipid enrichment in curved vs flat membrane regions (as discussed above) and 4) interactions with distinct proteins that have specific membrane inserting domains[9,96,97]. Consistent with possibility 4, there are number of potassium channels on the plasma membrane that have been shown to bind to $PI(4,5)P_2$, both through the head group and acyl chain, and therefore regulate potassium channel opening[98,99].

LYCAT has been found to regulate transferrin (TfR) internalization, but has no effect on EGFR internalization[9]. Interestingly, TfR and EGFR may rely on the same proteins for endocytosis, such as AP2, clathrin and dynamin, however, these receptors are usually found on different CCPs, and use different endocytic proteins, such as Grb2 that is essential for internalization of EGFR but not TfR[100,90]. This may indicate that the endocytic proteins that participate in the formation of CCPs that internalize TfR may be selectively sensitive to acyl chain specificity of $PI(4,5)P_2$. This is consistent with previous literature that showed that different receptors which all internalize via CME may each have unique requirements for specific factors, as our lab has recently shown with specific calcium signalling regulation of EGFR but not TfR CME[90]. This is one of many examples

that show a perturbation of LYCAT, in other words a change in acyl chain composition of PIPs, causing a disruption in membrane traffic.

4.3 LYCAT silencing disrupts PI(3,4,5)P_3 synthesis, possibly altering the PI3K/AKT pathway

PI(3,4,5)P_3 is an important regulator of the PI3K/AKT pathway, and I observed that LYCAT silencing in MDA-MB 231 cells resulted in a impairment of PI(3,4,5)P_3 production. LYCAT silencing caused a reduction in PI(4,5)P_2 levels and 18:0/20:4 acyl chain species, thus since PI(4,5)P_2 is a precursor for PI(3,4,5)P_3, this reduction in substrate for PI3K may be the reason why there is a reduction of PI(3,4,5)P_3 synthesis after EGF stimulation. Another possible explanation for the reduction in PI(3,4,5)P_3 production after EGF stimulation in LYCAT silenced cells is that PI3K, the kinase that phosphorylates PI(4,5)P_2 to become PI(3,4,5)P_3, is specific to the acyl chain profile of its substrate. Upon binding of p85α to the phosphotyrosine motif of EGFR by the nSH2 and the cSH2 domains, which causes a conformational change of the p110 (catalytic subunit), thus allowing conversion of PI(4,5)P_2 to PI(3,4,5)P_3 through its kinase domain. However, shorter acyl chains on PI(4,5)P_2 might make it difficult for PI3K to detect the inositol headgroup. Studies have shown that the headgroup of PI(4,5)P_2 is oriented at a 45° angle relative to the lipids that surround it, thus perturbations of the acyl chain may cause the headgroup to be oriented differently, causing alterations in protein recognition[101,102]. A study looked at 5 different species of PI(3,4,5)P_3 (18:0/18:2, 18:0/18:1, 18:0/20:4, 18:0/20:3, 18:0/20:2) in a human breast epithelial cell line, and observed that the 18:0/20:4 PI(3,4,5)P_3 species significantly increased after EGF stimulation[103]. This indicates that upon stimulation of EGF, PI3K is recruited to the plasma membrane and

selectively phosphorylates the 18:0/20:4 $PI(4,5)P_2$ species, although this would need to be tested by enzyme assays in a similar manner as was done to determine the acyl profile substrate preference of SYNJ1 and PIP5K described above.

Another explanation for the reduction in $PI(3,4,5)P_3$ production in LYCAT silenced cells is that since there is a delay in $PI(4,5)P_2$ turnover, the bulk of the remaining $PI(4,5)P_2$ in LYCAT knockdown cells may not be found on the plasma membrane, but instead may be present on intracellular tubules and punctate structures[9]. Therefore, if there is a reduction of $PI(4,5)P_2$ on the plasma membrane, PI3K may not be able to find its substrate to phosphorylate, as PI3K is primarily recruited to the plasma membrane upon EGF stimulation[104], resulting in a decrease in $PI(3,4,5)P_3$ synthesis. Lastly, analysis of L6 myotubes (rat skeletal muscle cells) showed that after stimulation with insulin, Class I PI3K was recruited to curved membrane regions, dorsal ruffles, and allowed for $PI(3,4,5)P_3$ synthesis, subsequently leading to AKT1 recruitment[105,106]. Moreover, the same resulted after EGF stimulation in A431 cells (human lung epithelial cells)[107]. Therefore, this suggests that shorter acyl species of $PI(4,5)P_2$ may not reside in ruffled/curved regions in the plasma membrane, making it inaccessible to PI3K, causing slower rates of $PI(3,4,5)P_3$ production.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, my work presents evidence that LYCAT silenced cells start off with lower levels of PI(4,5)P_2 because there is a delay in turnover, leading to a larger delay in synthesis. This was supported by the SYNJ1 silencing experiment (Figure 3.3 C &D) since it demonstrated that after knocking down SYNJ1 in MDA-MB 231 cells, PI(4,5)P_2 levels did not degrade as fast as control MDA-MB 231 cells after the addition of PAO to arrest PI(4,5)P_2 generation by PI4K/PIP5K. Moreover, the 18:0/20:4 PI(4,5)P_2 species are important for PI(3,4,5)P_3 synthesis after stimulation with EGF.

Further examination was conducted (data not shown) in which MDA-MB 231 cells were treated with 10 μM of ionomycin, within 2 minutes the PH-PLCδ GFP probe immediately detached from the plasma membrane and accumulated within the cytosol. This suggests that when PLCδ1 is acutely activated with calcium, it may not care about the acyl chain specificity of PI(4,5)P_2, demonstrating that perhaps not all enzymes that have PI(4,5)P_2 as a substrate exhibit preference for the specific acyl species of PI(4,5)P_2. However, it would be interesting to determine the rate of PI(4,5)P_2 cleavage in control vs LYCAT silenced cells. It may be important to determine a more subtle concentration of ionomycin (or other optimization of this treatment) to be able to properly measure the kinetics of PI(4,5)P_2 degradation by PLCδ1 in order to conclude whether LYCAT silencing, and thus the acyl chain profile of PIPs, impacts the turnover of PI(4,5)P_2 by PLCδ1. Another interesting point to determine is the recruitment of PLCδ1 to PI(4,5)P_2 and PLCγ1 to PI(4,5)P_2 and PI(3,4,5)P_3 on plasma membranes after an increase in cytosolic calcium.

Further studies of synthesis of $PI(4,5)P_2$ by PIP5K in LYCAT silenced cells can be determined. Does LYCAT prefer to phosphorylate PI4P enriched at a curved region of the membrane? A way of studying would be observing the localization of PIP5K to endophilins, since they reside in curved membrane regions. An outstanding question, is why/how do LYCAT silenced cells recover $PI(4,5)P_2$ after an increase in cytosolic calcium at the same rate as control cells? Briefly, cleavage of $PI(4,5)P_2$ produces IP_3 and DAG by activation of PLCδ1, IP_3 remains in the plasma membrane while DAG is transferred to the ER by a lipid transfer protein. DAG is then synthesized to PI through multiple reactions, then subsequently transferred back to the plasma membrane by a PITP to re-synthesize $PI(4,5)P_2$. Studies have shown that PITPα is specific to loading PI that is 18:0/20:4 and that PIP5K preferentially phosphorylates 18:0/20:4 PI4P and 18:0/18:1 PI4P[24,108]. Therefore, I propose that there must be a PITP that preferentially binds to shorter acyl chains and a PIP5K-like enzyme that synthesizes $PI(4,5)P_2$ on the plasma membrane from PI4P with shorter acyl chains as well.

An experimental limitation when measuring $PI(3,4,5)P_3$ levels, is that the PH-AKT GFP probe binds to both $PI(3,4,5)P_3$ and $PI(3,4)P_2$[87]. Therefore, this might suggest that the probe is sensitive to specific acyl chain species, and although I was still able to measure changes in the cell, this may be due to the altered binding of the PH-AKT GFP probe to lipid headgroups. Therefore, a future experiment is to use the PH-Btk (Bruton tyrosine phosphate) probe to measure $PI(3,4,5)P_3$ levels, because it specifically binds to $PI(3,4,5)P_3$[108]. If both PH-AKT and PH-Btk show a decrease in $PI(3,4,5)P_3$ levels after EGF stimulation in LYCAT silenced cells, this suggests that the probe is not acyl chain sensitive, however, if there is a difference, then the PH-AKT probe is sensitive to the acyl

chain content of its ligand. Lastly, serum starvation removes the $PI(3,4,5)P_3$ pool on the plasma membrane, however, I still noticed that some cells had a distinct rim via the PH-AKT probe on the plasma membrane in control and LYCAT silenced cells, this may suggest that the PH-AKT probe is bound to $PI(3,4)P_2$ and it seems that LYCAT silencing does not affect it. However, what is interesting is that some cells have a brighter rim than others which poses questions such as, why do some cells starts have with more $PI(3,4)P_2$? And do some cells have higher levels of the class II PI3K?

These considerations may provide more detail about LYCAT's role in PIP turnover/synthesis, effector protein binding and membrane traffic.

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