The acyltransferase LYCAT controls specific phosphoinositides and related membrane traffic

Leslie N. Bone, Roya M. Dayam, Minhyoung Lee, Nozomu Kono, Gregory D. Fairn, Hiroyuki Araji, Roberto J. Botelho, Costin N. Antonescu

Department of Chemistry and Biology and Graduate Program in Molecular Science, Ryerson University, Toronto, ON M5S 1A8, Canada; Department of Health Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan; Keenan Research Centre for Biomedical Science of St. Michael’s Hospital, Toronto, ON M5B 1W8, Canada; Japan Agency for Medical Research and Development–Core Research for Evolutionary Science and Technology, Tokyo 113-0033, Japan

ABSTRACT Phosphoinositides (PIPs) are key regulators of membrane traffic and signaling. The interconversion of PIPs by lipid kinases and phosphatases regulates their functionality. Phosphatidylinositol (PI) and PIPs have a unique enrichment of 1-stearoyl-2-arachidonyl acyl species; however, the regulation and function of this specific acyl profile remains poorly understood. We examined the role of the PI acyltransferase LYCAT in control of PIPs and PIP-dependent membrane traffic. LYCAT silencing selectively perturbed the levels and localization of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P$_2$] and phosphatidylinositol-3-phosphate and the membrane traffic dependent on these specific PIPs but was without effect on phosphatidylinositol-4-phosphate or biosynthetic membrane traffic. The acyl profile of PI(4,5)P$_2$ was selectively altered in LYCAT-deficient cells, whereas LYCAT localized with phosphatidylinositol synthase. We propose that LYCAT remodels the acyl chains of PI, which is then channeled into PI(4,5)P$_2$. Our observations suggest that the PIP acyl chain profile may exert broad control of cell physiology.

INTRODUCTION Phosphoinositides (PIPs) control many facets of cell physiology, such as nutrient uptake, receptor signaling, and cell adhesion by control of specific stages of membrane traffic (Di Paolo and De Camilli, 2006; Krauss and Haucke, 2007). Through the action of lipid kinases and phosphatases, PIPs can be interconverted into seven different species defined by phosphorylation of the inositol head group (Ballal, 2013). Each of the seven PIPs exhibits unique enrichment within membrane compartments and helps to recruit a variety of cognate effector proteins. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) and phosphatidylinositol-3-phosphate (PI(3)P) illustrate these concepts well.

PI(4,5)P$_2$ predominates within the plasma membrane (PM) and regulates clathrin-mediated endocytosis (referred to here as endocytosis) to control the internalization of cell surface proteins such as transferrin (Tfn) receptor (TR; Jost et al., 1998; Varnai et al., 2006; Zoncu et al., 2007; Posor et al., 2013). PI(4,5)P$_2$ binds to and recruits AP2 and other proteins, which, together with cargo molecules and clathrin, initiate the formation and assembly of clathrin-coated pits (CCPs; Gaidarov and Keen, 1999; Itoh et al., 2001; Jackson et al., 2010). CCPs couple cargo selection to membrane invagination and eventually undergo scission from the PM by the GTPase dynamin2 to yield endocytic vesicles (Conner and Schmid, 2003; McMahan and Boucrot, 2011). CCPS harbor lipid phosphatases such as synaptojanins and OCRL that mediate PI(4,5)P$_2$ turnover to control the efficiency of vesicle formation (Antonescu et al., 2011) and, after
scission, clathrin uncoating (Cremona et al., 1999; Kim et al., 2002). Disruption of PI(4,5)P₂ synthesis and turnover affects CCP initiation, size, and lifetime (Cremona et al., 1999; Nakatsu et al., 2010; Antonescu et al., 2011; Posor et al., 2013; Nández et al., 2014).

Endocytic vesicles traffic to early endosomes, which sort and send cargo molecules to specific destinations such as to the lysosomes for degradation or recycling to the PM, processes that require PI(3)P (Li et al., 1995; Puchner et al., 2013). PI(3)P binds to and recruits effector proteins like EEA1, which mediate endosome fusion, and sorting nexins, which mediate cargo sorting and vesiculation. To complete recycling of internalized cargo to the PM, PI(3)P is then dephosphorylated to phosphatidylinositol (PI) by myotubulin-1 (Ketel et al., 2016). Hence the internalization, sorting, and trafficking of membrane cargo proteins depends on the dynamic and sequential interconversion of PIP species by coordinated regimes of phosphorylation and dephosphorylation.

Although much has been revealed about the regulation and function of PIPs based on modification of the inositol head group, much less is known about the control and function of the acyl chains of these lipids, despite the fact that 50–70% of total PI is 1-stearoyl-2-arachidonoyl (18:0/20:4, combined as 38:4), an acyl chain enrichment not found in other phospholipids (Holub and Kuksis, 1978; Hicks et al., 2006; Imae et al., 2011; D’Souza and Epand, 2014). In addition, PIPs also exhibit many minor acyl combinations, generating a great molecular diversity that remains functionally unexplored. PIP acyl chains are likely to affect regulation and function because acyl chains affect the substrate preference of type I phosphatidylinositol phosphate kinases (PIPKI) and OCRL, which synthesize and degrade PI(4,5)P₂, respectively (Schmid et al., 2004; Shulga et al., 2012).

Acyl chain specificity and diversity may arise during de novo synthesis of PI in the endoplasmic reticulum (ER), where one of many acyltransferases may add acyl chains to generate the biosynthetic precursor phosphatidic acid (PA), which then is converted to cytidine diphosphate (CDP)-diacylglycerol (DAG) by CDP-diacylglycerol synthase (CDS; D’Souza and Epand, 2014). CDS2 shows preference for 38:4 PA, which may then be channeled to PI synthesis (PIS; D’Souza et al., 2014; D’Souza and Epand, 2015). Alternatively, PI/PIP acyl chains may be remodeled postsynthesis by the action of phospholipases and acyltransferases (Shindou and Shimizu, 2009; Imae et al., 2011; Kim et al., 2011). However, there is a dearth of knowledge about these PI acyltransferases.

Lysocardiolipin acyltransferase (LYCAT; also known as LCLAT1 or ALCAT1) is a lipid acyltransferase that exhibits preference for lysophosphatidylinositol and lysophosphatidylglycerol over other phospholipids in vitro, as well as preference for incorporating longer fatty acyl-CoA substrates (18:0;18:1 over 12:0;16:0) into these lysolipids (Zhao et al., 2009). L YCAT-knockout mice exhibit a reduction of stea rate and an increase in palmitate within PI/PIPs in various tissues (Imae et al., 2011). Taken together, these studies show that L YCAT is required for PI/PIP acyl chain specificity, perhaps by selectively incorporating stearate into the sn-1 position of PI/PIPs. Indeed a similar role has been proposed for the Caenorhabditis elegans homologues of L YCAT (Imae et al., 2010, 2011). L YCAT may also remodel the acyl chain profile of cardiolipin (CL; Cao et al., 2004). In mice fed a high-fat diet as a model of diet-induced obesity, L YCAT expression is enhanced, leading to CL acyl remodeling and mitochondrial defects (Li et al., 2010). L YCAT-knockout mice are protected from defects in mitochondrial function and insulin signaling induced by high-fat feeding (Li et al., 2010). Given that L YCAT-knockout mice fed a normal diet exhibited only reduced stearate content within PI without changes in other lipids (including CL; Imae et al., 2011), it is likely that the predominant function of L YCAT under normal conditions is to control PI/PIP acyl chain profile.

Although L YCAT regulates the acyl chain profile of PI/PPIPs and CL, the function of L YCAT in controlling membrane traffic remains unexplored. Using biochemical and lipidomic measurements to quantify lipids and lipid acyl species in combination with microcopy-based approaches to resolve how perturbations of L YCAT affect endomembrane traffic, we find that L YCAT exerts control over the acyl chain profile of specific PIP species and has an important but selective role in regulating multiple aspects of PIP-dependent endomembrane traffic.

RESULTS

L YCAT silencing perturbs endosomal traffic but not biosynthetic traffic

To examine whether L YCAT controls PIP-dependent endomembrane traffic, we used small interfering RNA (siRNA)-mediated silencing of L YCAT in human ARPE-19 cells (RPE henceforth), which resulted in an ~80% reduction in L YCAT protein expression (Supplemental Figure S1, A and B), and examined TfR membrane traffic, a process controlled by PI(4,5)P₂-dependent endocytosis (Varnai et al., 2006), and PI(3)P-dependent intracellular traffic (van Dam et al., 2002). L YCAT silencing significantly reduced cell surface TfR levels (Figure 1, A and B), whereas total levels of TfR were unaltered (Supplemental Figure S1, C and D). Of importance, cell surface TfR levels in L YCAT-silenced cells were rescued by expression of siRNA-resistant exogenous L YCAT, demonstrating that the altered surface levels of TfR were due to specific perturbation of L YCAT (Figure 1C).

TfR exhibits a pronounced perinuclear morphology as a result of trafficking through intracellular compartments, including recycling endosomes (Dugani et al., 2008). L YCAT silencing caused a dispersal of the perinuclear accumulation of TfR (Figure 1, D and E), further suggesting a major perturbation of intracellular trafficking upon L YCAT silencing. To contrast with endocytic trafficking, we examined the biosynthetic membrane traffic pathway, which is controlled by phosphatidylinositol-4-phosphate (PI(4)P; Szentpetery et al., 2010), using a temperature-sensitive form of vesicular stomatitis virus G (VSVG) that is trapped in and then released from the ER by temperature shift (Presley et al., 1997; Szentpetery et al., 2010). L YCAT silencing did not alter VSVG trafficking and distribution (Figure 1, F and G). Thus L YCAT silencing alters TfR traffic, which relies on PI(4,5)P₂ and PI(3)P, but has no apparent effect on the PI(4)P-dependent biosynthetic pathway.

L YCAT silencing reduces total levels and alters localization of PI(3)P and PI(4,5)P₂

Defective TfR distribution in L YCAT-silenced cells might correspond to altered PIP levels, which we tested by labeling cells with [3H]myoinositol and measuring the levels of specific PIPs by high-performance liquid chromatography (HPLC; Ho et al., 2016). L YCAT silencing reduced the levels of PI(3)P and PI(4,5)P₂ by 25% ± 0.05% and 21% ± 0.06%, respectively, but had no effect on PI(4)P levels (Figure 2A), suggesting that L YCAT silencing affects the levels of a subset of PIPs and associated functions.

We then examined the cellular localization of PIPs, using fluorescently labeled protein probes specific for PI(3)P, PI(4)P, and PI(4,5)P₂ (Stauffer et al., 1998; Stenmark et al., 2002; Hammond et al., 2014). L YCAT silencing reduced the number of 2FYVE-GFP structures, consistently with abated levels of PI(3)P (Figure 2, B and C). L YCAT silencing also altered the localization of the PI(4,5)P₂ probe PH-PLCδ-GFP (Várnai and Balla, 1998). Whereas in control cells, PH-PLCδ-GFP predominantly and uniformly decorated the PM, in L YCAT-silenced
LYCAT silencing alters clathrin-mediated endocytosis

Because LYCAT silencing altered the levels and localization of PI(4,5)P₂, we next investigated the effect of LYCAT silencing on CCP dynamics, which is controlled by PI(4,5)P₂ (Antonescu et al., 2011). We performed time-lapse total internal reflection fluorescence microscopy (TIRF-M) in RPE cells stably expressing a GFP-fusion of clathrin light chain (eGFP-CLCa; Figure 3A) followed by automated detection, tracking, and analysis of CCPs (Aguet et al., 2013). Consistent with reduction of PI(4,5)P₂ levels and perturbation of PI(4,5)P₂ localization, LYCAT silencing reduced CCP initiation density (Figure 3B) and CCP size (Figure 3C), the latter measured by the fluorescence intensity of eGFP-CLCa within diffusion-limited CCPs. We also observed that LYCAT silencing altered CCP lifetimes (Supplemental Figure S1E). Consistent with these defects in CCP dynamics, we observed that LYCAT silencing led to a significant reduction in the rate of Tf internalization (Figure 3D). Together these data indicate that LYCAT is a novel regulator of endocytosis, likely by affecting PI(4,5)P₂ levels and localization.

LYCAT silencing alters intracellular traffic of TfR

Because LYCAT suppression reduced PI(3)P levels, we predicted that PI(3)P-dependent trafficking processes would be disturbed in LYCAT-silenced cells. To test this, we monitored the arrival of fluorescently labeled Tf pulsed for various time points into EEA1-positive endosomes. LYCAT silencing did not appreciably alter the number or intensity of EEA1 puncta, indicating that we could employ EEA1 as a marker of early endosomes (Supplemental Figure S2A). LYCAT silencing substantially delayed the arrival of Tf to EEA1 compartments (Figure 4, A and B), which is consistent with the effect of reduction in PI(3)P levels by other manipulations (van Dam et al., 2002).

We next investigated whether LYCAT-silenced cells manifested a defect in TfR recycling. First, we measured the ability of internalized Tf to access the total pool of internal TfR-labeled endosomes, which include recycling endosomes. We observed a delayed arrival of Tf to the total pool of cellular TfR in LYCAT-silenced cells relative to control cells (Supplemental Figure S2, B and C). To complement this assay and measure the rate of TfR recycling, we cultured live cells with antibodies that recognize an exofacial TfR epitope to measure the rate of arrival of TfR to the PM, thus measuring TfR recycling (Antonescu et al., 2008). LYCAT silencing reduced the rate of labeling with anti-TfR antibody (Figure 4C, indicating that TfR recycling is slower in LYCAT-deficient cells. Collectively our studies reveal that LYCAT is necessary for TfR intracellular trafficking, a process that depends on PI(3)P.

LYCAT controls the acyl chain profile of specific PIPs

LYCAT regulates the specific content of stearate within PIPs in mice (Imae et al., 2011), yet LYCAT silencing did not affect PI(4)P levels or secretion from the biosynthetic pathway while abating PI(3)P and P(4,5)P₂ levels and interfering with endocytic trafficking. To better understand how LYCAT might differentially affect PIPs and trafficking processes, we analyzed the acyl chain composition of various phospholipids using liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS/MS). The acyl chain composition of each lipid is presented as a combination of the acyl chain composition of each lipid is presented as a combination of the sn-1 and sn-2 acyl groups (e.g., the 1-stearoyl-2-arachidonyl species corresponds to 38:4). As previously reported (Holub and Kuksis, 1978; Hicks et al., 2006; Imae et al., 2011; D’Souza and Epand, 2014), PI shows a unique and striking enrichment for the 38:4 acyl composition that is not seen in other phospholipids (Supplemental Figure S3A). LYCAT silencing did not appreciably disturb the acyl chain.
composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS; Supplemental Figure S3, B–D). Surprisingly, LYCAT silencing also did not affect the acyl chain profile of total PI (Supplemental Figure S3E).

We surmised that LYCAT may preferentially affect the acyl chain profile of specific PIPs instead. To address this hypothesis, we analyzed the acyl composition of PIP species using phosphate methylation and LC-ESI-MS/MS to resolve PI, monophosphorylated PIPs (PIP\(_1\)), and bisphosphorylated PIPs (PIP\(_2\); Clark et al., 2011). Of interest, the acyl chain profile of PI and PIP\(_1\) species, the latter being predominantly PIP\(_{4,5}P_2\) were unaltered in LYCAT-silenced cells (Figure 5, A and B, and Supplemental Figure S4, A and B; Stephens et al., 1993). In contrast, PIP\(_2\), which is mainly PI(4,5)P\(_2\), exhibited reduced levels of many acyl combinations in LYCAT-silenced cells, which partially reflects the reduced PI(4,5)P\(_2\) levels in these cells (Figure 5C and Supplemental Figure S4C). Of AL. N. Bone et al., 2011) and suggest that LYCAT preferentially affects the acyl chain profile of a subset of PIPs, including PI(4,5)P\(_2\), which may be necessary to maintain PI(4,5)P\(_2\) levels, localization, and function.

**LYCAT is localized to ER-derived PIS vesicles**

Previous studies suggested that LYCAT may exhibit some localization to the ER (Cao et al. 2004; Zhao et al. 2009; Imae et al., 2011). However, these studies did not perform a broader analysis of LYCAT localization with other relevant markers, an important consideration, since the ER has several subcompartments (English and Voeltz, 2013). We hypothesized that the preferential effect of LYCAT silencing on the acyl chain profile of PI(4,5)P\(_2\) but not PI/PIP\(_{1}\) may be in part due to its restricted localization to specific subcellular compartment(s). To monitor LYCAT localization, we transfected RPE cells with LYCAT-FLAG, which rescued the loss of endogenous LYCAT (Figure 1C), demonstrating that this exogenously expressed LYCAT is functional. Of interest, LYCAT-FLAG exhibited modest localization with eGFP-KDEL, a generic ER marker (Figure 6, A and B), suggesting that LYCAT may predominantly localize to other subcompartments.

PIS is found in ER-derived vesicles that rapidly move between the ER and various organelle membranes, as observed in cells expressing eGFP-PIS (Kim et al., 2011). Given the role of PIS in synthesizing PI, we hypothesized that LYCAT may localize to PIS vesicles. We found striking and extensive colocalization between LYCAT and eGFP-PIS (Figure 6, A and B). PIS vesicles make dynamic and transient contact with a number of membrane compartments, including the PM, which can be demarked by the extended synaptotagmin E-Syt2 (Min et al., 2007; Giordano et al., 2013). Indeed, LYCAT exhibited partial colocalization with E-Syt2 (Figure 6, A and B). TIRF-M showed very similar colocalization patterns between LYCAT and PIS, KDEL, and E-Syt2 at the cell periphery (Supplemental Figure S5).

To allow better understanding of the relative localization of LYCAT with eGFP-KDEL, eGFP-PIS, and eGFP-E-Syt2, we analyzed this by automated detection of LYCAT puncta followed by measurement of the localization correlation score (LCS) of the fluorescence intensities of LYCAT and that of secondary channel proteins within each object (Supplemental Figure S6). LCS is a quantitative, unbiased, and systematic analysis and revealed a very strong intensity correlation of LYCAT-FLAG with eGFP-PIS within LYCAT structures (Figure 6B). In contrast, eGFP-KDEL and LYCAT exhibited very low intensity correlation in LYCAT structures, whereas eGFP-E-Syt2 and LYCAT had intermediate intensity correlation (Figure 6B). Thus LYCAT is extensively localized to ER-derived PIS vesicles, known to transiently interact with ER-PM contact sites, but not the ER domains marked by eGFP-KDEL (Kim et al., 2011).

![Figure 2: LYCAT silencing reduces total levels and alters localization of PI(3)P and PI(4,5)P\(_2\).](image)
DISCUSSION

PIPs play essential roles in cell physiology, including regulation of membrane traffic (Di Paolo and De Camilli, 2006; Krauss and Haucke, 2007). The vast majority of studies on PIPs focused on the regulation and function of the phosphorylation state of the inositol head group. Of importance, PI and PIPs show remarkable acyl selectivity, with 50–70% of PI/PIPs harboring a 1-stearoyl-2-arachidonyl profile (Holub and Kuksis, 1978; Hicks et al., 2006; Imae et al., 2011; Holub and Xu, 2006; D’Souza and Epand, 2015). The vast majority of studies on PIPs focused on the sequential conversion of PI to PI(4)P and PI(4,5)P at the PM. However, PI(4,5)P synthesis is much more elaborate than this. Rather than depend on general ER or PM, PI and PI(4,5)P synthesis appear highly dependent on ER-PM contact sites and sub-ER membrane transport of PA, DAG, and PI between the ER and PM is also implicated in this process (Chang and Liou, 2015; Kim et al., 2015; Saheki et al., 2016). In addition, PIS was observed on dynamic ER-derived vesicles that make transient contact with a number of organelles, including with the PM (Kim et al., 2011). These observations led Kim et al. (2011) to propose that PIS vesicles form PIPERosomes, which may be involved in the localized synthesis and/or delivery of PI or PIPs at organelle contact sites. The localization of LYCAT with PIS in apparent vesicular structures (Figure 6) suggests that acyl remodeling activity may be coupled to PIS activity in these vesicles. For technical reasons related to the study of endogenous PIS, the ER-derived PIS vesicles have only been observed upon overexpression of eGFP-PIS, which is required to observe the localization of this enzyme (Kim et al., 2011). Our observation that LYCAT also localizes to punctate peripheral structures in the presence or absence of eGFP-PIS overexpression (Figure 6) is consistent with the broad formation of ER-derived vesicles under a number of physiologically relevant conditions. However, the cellular condition(s) that lead to formation of ER-derived vesicles harboring PIS and LYCAT and whether PIS vesicles indeed function to deliver lipids and/or synthesize PI or PIPs at peripheral structures in the presence or absence of eGFP-PIS overexpression (Figure 6) is consistent with the broad formation of ER-derived vesicles under a number of physiologically relevant conditions.

The acyltransferase LYCAT preferentially affects the acyl chain profile of PI(4,5)P₂

Acyltransferases may control PI/PIP acylation during de novo synthesis in the ER or by subsequent processes such as acyl or head group remodeling. The de novo synthesis of many phospholipids involves the conversion of DAG to PA (Shindou and Shimizu, 2009). Synthesis of PI results from conversion of PA to CDP-DAG by CDS, followed by conversion to PI by PIS (Shindou and Shimizu, 2009; D’Souza et al., 2014; D’Souza and Epand, 2015). CDS2, but not CDS1 or PIS, exhibits preference for 1-stearoyl-2-arachidonyl substrate, thus providing at least one mechanism for the establishment of acyl specificity of PI during de novo synthesis (D’Souza et al., 2014; D’Souza and Epand, 2015). In addition, PI acyl specificity may occur by acyl remodeling post-synthesis by the joint effort of phospholipases and acyltransferases (Imae et al., 2010). In our LYCAT-silenced cells, we did not observe an alteration in the acyl chain profile of PI or PIP₁, which is chiefly PI(4)P. This suggests that LYCAT may not be acting during bulk PI synthesis or remodeling, although we cannot exclude the possibility that other acyltransferases may act redundantly with LYCAT. On the other hand, there was a significant shift in the acyl chain profile of PIP₂, which is mainly PI(4,5)P₂. Thus LYCAT appears to be part of a pathway that preferentially acts on PI(4,5)P₂ to impart acyl chain selectivity.

How might LYCAT affect the PI(4,5)P₂ acyl profile but not bulk PI/PIP? PI(4,5)P₂ synthesis was classically believed to occur from the sequential conversion of PI to PI(4)P and PI(4,5)P₂ at the PM. However, PI(4,5)P₂ regulation is much more elaborate than this. Rather than depend on general ER or PM, PI and PI(4,5)P₂ synthesis appear highly dependent on ER-PM contact sites and submembrane layers. Nonvesicular transport of PA, DAG, and PA between the ER and PM is also implicated in this process (Chang and Liou, 2015; Kim et al., 2015; Saheki et al., 2016). In addition, PIS was observed on dynamic ER-derived vesicles that make transient contact with a number of organelles, including with the PM (Kim et al., 2011). These observations led Kim et al. (2011) to propose that PIS vesicles form PIPERosomes, which may be involved in the localized synthesis and/or delivery of PI or PIPs at organelle contact sites. The localization of LYCAT with PIS in apparent vesicular structures (Figure 6) suggests that acyl remodeling activity may be coupled to PIS activity in these vesicles. For technical reasons related to the study of endogenous PIS, the ER-derived PIS vesicles have only been observed upon overexpression of eGFP-PIS, which is required to observe the localization of this enzyme (Kim et al., 2011). Our observation that LYCAT also localizes to punctate peripheral structures in the presence or absence of eGFP-PIS overexpression (Figure 6) is consistent with the broad formation of ER-derived vesicles under a number of physiologically relevant conditions. However, the cellular condition(s) that lead to formation of ER-derived vesicles harboring PIS and LYCAT and whether PIS vesicles indeed function to deliver lipids and/or synthesize PI by acting in-trans on the PM remain to be determined. Although beyond the scope of the present study, future research should examine the dynamics of PIS and LYCAT within the ER and ER-derived vesicles, including how PIS/LYCAT vesicles interact with the plasma membrane (Kim et al., 2011) and how PI transfer proteins are required for acyl chain remodeling (Cockcroft, 2001; Chang and Liou, 2015).

Work by Hammond et al. (2012) showed that depletion of bulk PI(4)P from the PM did not reduce PI(4,5)P₂ levels, suggesting that a different pool of PI(4)P is specifically used to generate PI(4,5)P₂.
Given these emerging themes, we propose the hypothesis that LYCAT acts on a specific PI pool that is channeled toward synthesis of PI(4,5)P₂. In this model, we speculate that PIS generates two pools of PI: 1) PIS in nonspecialized ER membranes generates bulk PI, and 2) PIS and LYCAT, perhaps in distinct subdomains of the ER or in ER-derived vesicles such as PIPERosomes, regulate a specialized PI pool that is selected for PI(4,5)P₂ synthesis. The role of PIS in total PI synthesis is consistent with reduced levels in total PI, PI(4)P and PI(4,5)P₂ in PIS-silenced cells (Kim et al., 2011). We propose that the newly synthesized PI is then subject to LYCAT-dependent acyl remodeling, resulting in enrichment in 38:4 acyl chains, followed by channeling into PI(4)P and PI(4,5)P₂. Although there is no evidence that PI(4)P synthesis is affected by the acyl chains in PI, PI PKIs show preference for PI(4)P substrates containing 38:4 (Shulga et al. 2012). Thus, in the absence of LYCAT, there may be an increase in a minor pool of misacylated PI and PI(4)P, the latter being less efficiently converted into PI(4,5)P₂ due to lower PI PKI substrate preference, reducing PI(4,5)P₂ levels.

Of interest, Anderson et al. (2013) suggested the existence of a PI-specific Lands cycle upon examination of phospholipids of brains and liver of a mouse knockout of LPIAT1, a PI-specific acyltransferase that exhibits remarkable selectivity for incorporation of arachidonic acid into the sn-2 position of PI. Liver and brains from LPIAT1⁻/⁻ mice exhibited reduced levels of 38:4 PIP₁ and PIP₂, as well as of total PIP₁ and PIP₂. Taken together with our results, this may suggest a model in which several acyltransferases such as LYCAT and LPIAT1 function in a PI-specific Lands cycle for the synthesis of 38:4 PI, perhaps in a mechanism that involves coordination of several other acyltransferases.

Moreover, the 2xFYVE and PH probes that were used to determine PIP(3)P and PIP(4,5)P₂ localization and levels may themselves be selective to the acyl chain profile of the corresponding lipid. As such, it is possible that the altered localization of these probes upon LYCAT silencing may reflect both altered abundance and localization of these PIPs, as well as alterations in their acyl profile. Nonetheless, the biochemical measurements of PIP(3)P and PIP(4,5)P₂ illustrate that LYCAT silencing indeed affects the levels of both of these lipids.

LYCAT controls clathrin-mediated endocytosis

Previous studies reported that perturbation of PI(4,5)P₂ synthesis resulted in a reduction in CCP initiation rate and size, whereas increased PIP(4,5)P₂ synthesis increased CCP initiation rate and size (Antonescu et al., 2011). Our findings that LYCAT perturbation results in reduction in PIP(4,5)P₂ levels and a reduction in CCP initiation rate and size are entirely consistent with this previous work. PI(4,5)P₂ functions as a membrane ligand to many CCP proteins, including AP2, epsin, SNX9, CALM, and dynamin (Gaidarov and Keen, 1999; Itoh et al., 2001; Di Paolo and De Camilli, 2006; Krauss and Haucke, 2007; Jackson et al., 2010). Indeed, PIP(4,5)P₂ binding to AP2 contributes to conformational changes in AP2, which promotes association with membrane and cargo receptors, thus potentiating CCP assembly (Jackson et al., 2010). Of note, defects in CCP dynamics and endocytosis in LYCAT-silenced cells may result not only from reduced PIP(4,5)P₂ levels, but also from a defect in binding to effectors by misacylated PIP(4,5)P₂ (van Meer et al., 2008). The acyl chain specificity may be important to dictate the position of the phospholipid head group by controlling the axial position of the lipid in the membrane, affecting membrane curvature, or partitioning of lipids within specific membrane domains. Conversely, or in addition, endocytosis requires turnover of PIP(4,5)P₂ from nascent endocytic vesicles catalyzed by synaptojanins and OCRL (Antonescu et al., 2011; Changlieto et al., 2011). Of interest, the OCRL 5-phosphatase activity, and to a lesser extent that of synaptojanin 1, exhibits preference for 1-stearoyl-2-arachidonyl over other acyl profiles in substrates (Schmid et al., 2004). Remarkably, PIP(4,5)P₂-labeled tubules and endosomes were present in cells disrupted for 5-phosphatases such as OCRL (Nández et al., 2014), which resembles the appearance of PIP(4,5)P₂ in tubules and puncta in cells silenced for LYCAT (Figure 2B). This suggests that misacylated PIP(4,5)P₂ may affect endocytosis and be a poor substrate for OCRL/synaptojanins, causing ectopic accumulation of PIP(4,5)P₂. It is interesting that LYCAT-knockout mice did not
LYCAT controls PI(3)P and early endosomal traffic

LYCAT suppression resulted in reduced levels of PI(3)P, observed biochemically and using GFP-based probes. There are at least three possible paths to depress PI(3)P levels. First, and as suggested earlier, PI(3)P may depend on a specific precursor PI pool remodeled by LYCAT as we propose for PI(4,5)P2, since PIS vesicles form transient contacts with a number of compartments, including endosomes (Kim et al., 2011). Unfortunately, we cannot currently assess the acyl chain profile of PI(3)P, since this is a minority relative to PI(4)P in the PIP2 group. Second, defects in PI(4,5)P2 turnover upon LYCAT silencing may affect PI(3)P synthesis. Consistent with this possibility, disruption of early endosomal phosphatases causes a reduction in PI(3)P (Shin et al., 2005). Finally, the effects on PI(3)P levels and PI(3)P-dependent processes may be indirect, resulting from primary defects in clathrin-mediated endocytosis and membrane flux.

Generation of PI(3)P from PI acts as a switch responsible for the maturation of newly formed endocytic vesicles to early endosomes (Zoncu et al., 2009). In addition, depletion of PI(3)P caused these endosomes to revert to the immature endosomes (Zoncu et al., 2009). PI 3-kinase is recruited to the newly formed endosome, perhaps via interaction with Rab5-dependent membranes upon acute depletion of PI(3)P. Over, EEA1 was strongly recruited to Rab5-positive membranes upon acute depletion of PI(3)P by selective targeting of the PI(3)P phosphatase MTM1 to early endosomes (Fili et al., 2005). Finally, the effects on PI(3)P levels and PI(3)P-dependent processes may be indirect, resulting from primary defects in clathrin-mediated endocytosis and membrane flux.

show any broad phenotype with respect to viability (Imae et al., 2011). Hence it is likely that there exist compensatory mechanisms for lipid-sensitive processes. Although OCRL and synaptojanin 1 demonstrated preference for substrates with specific acyl profiles, other inositol 5-phosphatases did not (Schmid et al., 2004). Hence long-term perturbation of LYCAT, as it occurs in LYCAT−/− mice, may result in altered expression profile of these lipid phosphatases (or other enzymes) to maintain some of the physiological functions of PIPs under conditions of altered acyl profiles of these lipids.

LYCAT controls PI(3)P and early endosomal traffic

LYCAT suppression resulted in reduced levels of PI(3)P, observed biochemically and using GFP-based probes. There are at least three possible paths to depress PI(3)P levels. First, and as suggested earlier, PI(3)P may depend on a specific precursor PI pool remodeled by LYCAT as we propose for PI(4,5)P2, since PIS vesicles form transient contacts with a number of compartments, including endosomes (Kim et al., 2011). Unfortunately, we cannot currently assess the acyl chain profile of PI(3)P, since this is a minority relative to PI(4)P in the PIP2 group. Second, defects in PI(4,5)P2 turnover upon LYCAT silencing may affect PI(3)P synthesis. Consistent with this possibility, disruption of early endosomal phosphatases causes a reduction in PI(3)P (Shin et al., 2005). Finally, the effects on PI(3)P levels and PI(3)P-dependent processes may be indirect, resulting from primary defects in clathrin-mediated endocytosis and membrane flux.

Generation of PI(3)P from PI acts as a switch responsible for the maturation of newly formed endocytic vesicles to early endosomes (Zoncu et al., 2009). In addition, depletion of PI(3)P caused these endosomes to revert to the immature endosomes (Zoncu et al., 2009). PI 3-kinase is recruited to the newly formed endosome, perhaps via interaction with Rab5-dependent membranes upon acute depletion of PI(3)P. Over, EEA1 was strongly recruited to Rab5-positive membranes upon acute depletion of PI(3)P by selective targeting of the PI(3)P phosphatase MTM1 to early endosomes (Fili et al., 2005). Finally, the effects on PI(3)P levels and PI(3)P-dependent processes may be indirect, resulting from primary defects in clathrin-mediated endocytosis and membrane flux.

show any broad phenotype with respect to viability (Imae et al., 2011). Hence it is likely that there exist compensatory mechanisms for lipid-sensitive processes. Although OCRL and synaptojanin 1 demonstrated preference for substrates with specific acyl profiles, other inositol 5-phosphatases did not (Schmid et al., 2004). Hence long-term perturbation of LYCAT, as it occurs in LYCAT−/− mice, may result in altered expression profile of these lipid phosphatases (or other enzymes) to maintain some of the physiological functions of PIPs under conditions of altered acyl profiles of these lipids.

LYCAT controls PI(3)P and early endosomal traffic

LYCAT suppression resulted in reduced levels of PI(3)P, observed biochemically and using GFP-based probes. There are at least three possible paths to depress PI(3)P levels. First, and as suggested earlier, PI(3)P may depend on a specific precursor PI pool remodeled by LYCAT as we propose for PI(4,5)P2, since PIS vesicles form transient contacts with a number of compartments, including endosomes (Kim et al., 2011). Unfortunately, we cannot currently assess the acyl chain profile of PI(3)P, since this is a minority relative to PI(4)P in the PIP2 group. Second, defects in PI(4,5)P2 turnover upon LYCAT silencing may affect PI(3)P synthesis. Consistent with this possibility, disruption of early endosomal phosphatases causes a reduction in PI(3)P (Shin et al., 2005). Finally, the effects on PI(3)P levels and PI(3)P-dependent processes may be indirect, resulting from primary defects in clathrin-mediated endocytosis and membrane flux.
In conclusion, this work adds to the considerable knowledge of how phosphorylation of the inositol head group controls PI function by revealing that the acyltransferase LYCAT also regulates the dynamics, localization, and function of specific PIPs. These results suggest that control of acyl chain composition of PIPs by dynamic remodeling by specific phospholipases and acyltransferases represent an additional dimension in the control of PI function and suggest that this process may control membrane traffic and cell physiology.

**MATERIALS AND METHODS**

**Materials**

DMEM/F12, fetal bovine serum (FBS), penicillin/streptomycin solution, insulin-transferrin-selenium-ethanolamine solution, and sterile 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer were obtained from Life Technologies (Carlsbad, CA), as was Biotin-xx-Tfn. Avidin and α-phenylenediamine hydrochloride reagent were obtained from Biobasic (Markham, Canada), and biocytin was obtained from Santa Cruz Biotechnology (Dallas, TX). Superblock blocking buffer was obtained from Thermo Fisher (Rockford, IL). Antibodies and fluorescent ligands used were as follows: anti-LYCAT from Genetex (Irvine, CA); anti-EAA1 and actin from Cell Signaling Technology (Danvers, MA); anti-TfR from Santa Cruz Biotechnology; Tfn antibodies (used in Tfn uptake assay) from Bethyl Laboratories (Montgomery, TX); and Alexa 647–conjugated Tfn (A647-Tfn) from Life Technologies.

**Cell culture, transfection, gene silencing, and Western blotting**

Wild-type ARPE-19 (RPE) human retinal pigment epithelial cells (RPE-WT) and a derivative line stably expressing clathrin light chain fused to eGFP (RPE eGFP-CLCA) were previously described (Aguet et al., 2013; Garay et al., 2015). Cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2. A plasmid encoding LYCAT-my旗-FLAG (henceforth, LYCAT-FLAG) was from OriGene (plasmid MR226119, Rockville, MD). Plasmids encoding eGFP-PIS and eGFP-P4M were kindly provided by T. Balla (National Institutes of Health, Bethesda, MD; Kim et al., 2011; Hammond et al., 2014). A plasmid encoding eGFP-E-Syt2 (66831; Addgene) was kindly provided by P. de Camilli (Yale School of Medicine, New Haven, CT; Giordano et al., 2013). Plasmids expressing VSVG-GFP protein were previously characterized (Lippincott-Schwartz et al., 1997; Fain et al., 2011).

For plasmid transfection of RPE cells, we used Lipofectamine 2000 (Life Technologies) as per manufacturer’s instructions. For each well of a six-well plate, 2 μg of cDNA was precomplexed with 6 μl of transfection reagent in Opti-MEM. Cells were subsequently incubated with DNA-reagent complexes for 4 h, followed by washing and incubation of cells in regular growth medium for 24 h before the start of the experiment.

To perform LYCAT silencing, we used the following siRNA oligonucleotides (sense) from Dharmaco (Lafayette, CO): LYCAT1, 5′-GGAAALUGGAAAGAGAUCUUACAUU; LYCAT2, 5′-CCUCAAAGCGCCAUCGUUCUCAAUU; and nontargeting control siRNA, 5′-CGUACUGCUUGCAGUACCGGU. Oligonucleotides were transcribed using Lipofectamine RNAiMAX (Life Technologies), as per manufacturer’s instructions. Each siRNA construct was transfected at 220 pmol/l precomplexed to the transfection reagent in Opti-MEM for 4 h, after which cells were washed and replaced in regular growth medium. siRNA transfections were performed twice (72 and 48 h) before each experiment.

To confirm LYCAT silencing, whole-cell lysates were prepared in Laemmli sample buffer (LSB; 0.5 M Tris, pH 6.8, glycerol, 10% SDS, 10% β-mercaptoethanol, and 5% bromophenol blue; all from Bio-Shop, Burlington, Canada) supplemented with a protease and phosphatase cocktail (1 mM sodium orthovanadate, 10 nM okadaic acid, and 20 nM Protease Inhibitor Cocktail [BioShop]). Lysates were then heated at 65°C for 15 min and passed through a 27.5-gauge syringe. Proteins were resolved by glycine-Tris SDS-PAGE, followed by transfer onto a polyvinylidene fluoride membrane, and were washed, blocked, and incubated with antibodies as previously described (Garay et al., 2015). Western blot signals to detect the intensity corresponding to LYCAT protein was obtained by signal integration in an area corresponding to the appropriate lane and band for each condition. This measurement is then normalized to the loading control signal (determined with anti-actin antibodies [Cell Signaling Technology]) and subjected to statistical analysis by t test.

**Immunofluorescence staining**

To detect cell surface TFR (Figure 1, A–C), after experimental treatments as indicated, samples were immediately placed on ice and washed three times in ice-cold PBS+ (phosphate-buffered saline supplemented with 1 mM CaCl2 and 1 mM MgCl2) and incubated with anti-TFR antibodies (also on ice; Santa Cruz Biotechnology). After washing to remove unbound antibodies, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, stained with appropriate secondary antibodies, and then mounted in fluorescence mounting medium (Dako, Carpinteria, CA).

To detect specific proteins in permeabilized cells (Figures 1, D–F, 2, A and B, and 6 and Supplemental Figures S2A and S5) samples were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, probed with appropriate combinations of primary and secondary antibodies, and then mounted in fluorescence mounting medium (Dako).

**Fluorescence microscopy**

Wide-field epifluorescence microscopy experiments presented in Figures 1, A and D, and 4A and Supplemental Figure S2, A and B, were obtained using a 63× (numerical aperture [NA] 1.2) oil objective on a Zeiss Axiovert 200 M epifluorescence microscope using an ORCA-Flash 4.0 camera (Hamamatsu, Bridgewater, NJ).

Spinning-disc confocal microscopy experiments presented in Figures 2B and 6A were obtained using a Leica DMIRE2 equipped with a Yokogawa CSU X1 scan head and a 60× (NA 1.35) oil objective using a Hamamatsu C9100-13 electron-multiplying charge-coupled device (EM-CCD) camera. Excitation light was provided by 491-nm (50 mV) and 561-nm (50 mW) laser illumination, and emitted light was collected after passage through 515/40 and 594/40-nm emission filters, as appropriate.

Laser scanning confocal microscopy experiments presented in Figure 1F were performed using a Zeiss LSM700 with a 63× (NA 1.4) oil objective. Excitation light was provided by 488-nm laser illumination.

For TIRF-M, live-cell imaging (Figure 3) was performed on cells incubated in DMEM/F12 lacking phenol red supplemented with 5% fetal bovine serum and oxyrase (1/100; Oxyrase, Mansfield, OH). Cells were placed at 37°C with 5% CO2, 10% fetal bovine serum and oxyrase (1/100; Oxyrase, Mansfield, OH). Cells were placed at 37°C with 5% CO2, washed, blocked, and incubated with antibodies as previously described (Garay et al., 2015). Western blot signals to detect the intensity corresponding to LYCAT protein was obtained by signal integration in an area corresponding to the appropriate lane and band for each condition. This measurement is then normalized to the loading control signal (determined with anti-actin antibodies [Cell Signaling Technology]) and subjected to statistical analysis by t test.

**Fluorescence microscopy**

Wide-field epifluorescence microscopy experiments presented in Figures 1, A and D, and 4A and Supplemental Figure S2, A and B, were obtained using a 63× (numerical aperture [NA] 1.2) oil objective on a Zeiss Axiovert 200 M epifluorescence microscope using an ORCA-Flash 4.0 camera (Hamamatsu, Bridgewater, NJ).

Spinning-disc confocal microscopy experiments presented in Figures 2B and 6A were obtained using a Leica DMIRE2 equipped with a Yokogawa CSU X1 scan head and a 60× (NA 1.35) oil objective using a Hamamatsu C9100-13 electron-multiplying charge-coupled device (EM-CCD) camera. Excitation light was provided by 491-nm (50 mV) and 561-nm (50 mW) laser illumination, and emitted light was collected after passage through 515/40 and 594/40-nm emission filters, as appropriate.

Laser scanning confocal microscopy experiments presented in Figure 1F were performed using a Zeiss LSM700 with a 63× (NA 1.4) oil objective. Excitation light was provided by 488-nm laser illumination.

For TIRF-M, live-cell imaging (Figure 3) was performed on cells incubated in DMEM/F12 lacking phenol red supplemented with 5% fetal bovine serum and oxyrase (1/100; Oxyrase, Mansfield, OH). Cells were placed at 37°C with 5% CO2. Time-lapse image sequences were acquired at 1-s frame rate. For experiments on fixed cells (Supplemental Figure S5), samples were processed as described in Immunofluorescence staining. TIRF-M was performed using a 150× (NA 1.45) objective on an Olympus IX81 instrument equipped with CellTIRF modules (Olympus Canada, Richmond Hill, Canada) using 491-nm (50 mW) and 561-nm (50 mW) laser illumination and 520/35-nm and 624/40-nm emission filters. Images were acquired using a C9100-13 EM-CCD camera.
Fluorescence microscopy image analysis

Quantification of cell surface TfR and internal TfR distribution. Cell surface TfR (Figure 1, B and C) was quantified by measurement of the mean fluorescence intensity over the area of the whole cell. The relative concentration of TfR staining within the perinuclear region (Figure 1, D and E) was measured by kurtosis of a Gaussian model of the TfR staining intensity over the area of the whole cell. Both analyses were performed with ImageJ (National Institutes of Health, Bethesda, MD), and data were normalized to control. Measurements were subjected to t test.

Automated detection, tracking, and analysis of CCPs. Time-lapse image series obtained by TIRF-M of cells expressing eGFP-CLCa were subjected to automated detection, tracking, and analysis using custom software in Matlab (MathWorks, Natick, MA; Aguet et al., 2013). Briefly, CCPs were detected using a Gaussian-model-based approach to approximate the point spread function, followed by tracking of CCPs through each image series (Jaqaman et al., 2008). Because CCPs are diffraction-limited objects, the amplitude of the Gaussian model of the fluorescence intensity of eGFP-CLCa informs about CCP size (Figure 3C). Given the heterogeneity of CCP lifetimes, for eGFP-CLCa fluorescence intensity measurements of CCP size, objects were separated into lifetime cohorts.

LYCAT localization correlation score. Dual-channel fluorescence micrographs were subjected to automated and unbiased detection of LYP CAT structures and subsequent analysis using custom software in Matlab. Determination of the LCS was done by quantifying the correlation of fluorescence intensities of LYP CAT and a second compartment marker signal (eGFP-KDEL, eGFP-PIS, or eGFP-E-Syt2) within LYP CAT structures. In dual-channel images, LYP CAT objects were detected by a Gaussian model-based approach (Aguet et al., 2013; Garay et al., 2015), using only the LYP CAT fluorescence channel for initial object detection. Within LYP CAT objects, the fluorescence intensity corresponding to the amount of LYP CAT or other protein within each object was determined by the amplitude of the Gaussian model in each structure in each channel.

Given the variability of LYP CAT and compartment marker expression levels in each cell and image, fluorescence intensity values (f) were rescaled by converting raw fluorescence values to fluorescence standard score (z) as follows: \( z = (f - \mu)/\sigma \), where \( \mu \) and \( \sigma \) are the mean and SD of fluorescence values of that channel in that particular image, respectively. For each object, the LCS between two channels, \( z_1 \) and \( z_2 \), was determined as LCS = 1 – | \( z_1 - z_2 \) |.

To validate this method, we examined the LCS measurements in sample images, shown in Supplemental Figure S6, B–D. We performed LCS analysis on a pair of identical images (“model overlap”) and the same pair of identical images in which one of the images had undergone spatial randomization (180° rotation; “scrambled channel position”). Before LCS analysis, the correlation of raw pixel intensities of each channel in each detected LYP CAT object shows the expected result (Supplemental Figure S6B): virtually total complete correlation between “model overlap” intensities and no correlation between the “scrambled channel position” intensities. Note that the correlation in the “model overlap” images is not perfect despite the images in each channel being identical, as the detection of LYP CAT structures by Gaussian model was done assuming two distinct wavelengths for each channel (as per analysis of experimental images, as shown Figure 6), which was not the case in the sample data. Nonetheless, despite the slight offset of the Gaussian model in the secondary channel in the model images, this validation demonstrates the usefulness of this analysis.

A histogram of the calculated LCS values in these image sets (Supplemental Figure S6C) shows that the “model overlap” images have LCS values in the 0.85–1.0 range (close correlation), whereas, as expected, the “scrambled channel position” images have LYP CAT puncta with LCS values that span the entire possible range (0–1.0). LCS values were calculated for many “model overlap” and “scrambled channel position” images (Supplemental Figure S6D), showing strong median correlation scores for “model overlap” images (~0.9) and poor correlation (~0.2) for “scrambled channel position” images.

A sample analysis of LYP CAT and eGFP-PIS of the images shown in Supplemental Figure S6A shows a strong correlation of raw fluorescence intensities of LYP CAT and eGFP-PIS within detected LYP CAT objects (Supplemental Figure S6E), as well as an LCS distribution heavily skewed toward high LCS values (Supplemental Figure S6F).

Tfn colocalization with EEA1 or TfR

RPE cells were incubated with 20 μg/ml of A647-Tfn for indicated times at 37°C and then immediately placed on ice and washed three times in ice-cold PBS 2+ to remove unbound ligand, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, stained with either anti-EEA1 (Cell Signaling Technology) or anti-TfR (Santa Cruz Biotechnology) and appropriate secondary antibodies, and then mounted in fluorescence mounting medium (Dako). Colocalization of A647-Tfn with either EEA1 or TfR was performed in ImageJ using Pearson’s r. The results were subjected to two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison posttest.

VSVG biosynthetic membrane traffic assay

RPE cells were transfected with VSVG-GFP and incubated overnight at 40°C. To allow VSVG-GFP to exit the ER and accumulate in the Golgi, the cells were switched to HEPES-buffered DMEM/F12 and incubated at 20°C for 2 h. To visualize secretory vesicles in transit to the PM, after the 20°C block, the cells were transferred to medium prewarmed to 37°C and incubated for 0, 15, 30, 60, or 90 min. After incubation at 37°C for the indicated time points, cells were washed and fixed with 4% paraformaldehyde for 15 min and washed with PBS. The results were subjected to two-way ANOVA followed by Bonferroni’s multiple comparison posttest.

TfR recycling assay

TfR recycling (Figure 4C) was measured as previously described for the measurement of recycling of glucose transporters (GLUTs); these assays allow orthogonal measurement of internalization and recycling kinetics (Antonescu et al., 2008; Ishikura et al., 2010). TfR recycling was performed by incubating cells with (0.5 μg/ml) anti-TfR antibodies, which recognize an exofacial epitope on the receptor (Santa Cruz Biotechnology) in serum-free DMEM to cells, for 0, 1, 2, or 3 h at 37°C. This process results in labeling of TfR molecules upon their exposure to the extracellular milieu, as occurs during recycling to the cell surface. Subsequently cells were washed three times in ice-cold PBS 2+ to remove unbound TfR antibodies, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then probed with secondary antibodies to detect all anti-TfR antibodies bound during this assay (whether internalized or not). Samples were then mounted in fluorescence mounting medium (Dako). The amount of antibody bound during a given time interval, corresponding to TfR recycling, was normalized to total cell TfR, measured in parallel. All TfR staining intensity measurements were performed with ImageJ. These measurements were subjected to two-way ANOVA followed by Bonferroni’s multiple comparison posttest.
Measurement of TIR recycling by this assay involves labeling of TIR upon exposure to the extracellular milieu and does not subsequently distinguish between antibody-bound TIR that undergoes internalization or that which remains at the cell surface (Antonescu et al., 2008; Ishikura et al., 2010). It is possible that surface antibody labeling by TIR is partial (but similarly so in all experimental conditions), such that only a portion of TIRs is antibody labeled at each round of recycling. This may explain why this method of recycling results in longer apparent recycling time for TIR than the ~60 min previously reported (e.g., Klausner et al., 1983; Stein and Sussman, 1986). If this is the case, then conditions in which TIR internalization slowed may exhibit an overestimation of the rate of TIR recycling rate, as each TIR would have a longer cell surface residence time at each round of recycling through the plasma membrane. As such, given that LYCAT internalization is delayed in LYCAT-silenced cells (Figure 3D), the rate of TIR recycling upon LYCAT silencing may be delayed even further than what is apparent by use of this TIR recycling assay (Figure 4C).

**Tfn internalization assay**

Cells were then incubated with 10 μg/ml biotinylated transferrin (Invitrogen) for the indicated times at 37°C, after which they were washed with cold PBS* to remove excess (unbound) ligand and arrest membrane traffic. Uninternalized (surface-exposed) Tfn was quenched by sequential incubation with free avidin (3.1 μg/ml) and biocytin (5 μg/ml). Cells were then solubilized in blocking buffer (0.05% Triton X-100 and 0.05% SDS in Superblock solution; Thermo Fisher), and cell lysates were plated onto enzyme-linked immunosorbent assay plates coated with anti-Tfn antibodies and assayed for detectable (internalized) Tfn using horseradish peroxidase–conjugated streptavidin. Measurements of internalized Tfn were normalized to the total levels of surface ligand binding measured at 4°C, measured in parallel for each condition. The results were subjected to two-way ANOVA followed by Bonferroni’s multiple comparison posttest.

**Phospholipid analysis by LC-ESI-MS/MS**

Cells were washed in cold PBS, scraped, and then flash frozen. Lipids were extracted by the method of Bligh and Dyer (1959). Phospholipids in lipid extracts quantitated by using the inorganic phosphorus assay. Internal standards (13:0/15:0 PC, PE, PI, and PS) were added to the samples before LC-ESI-MS/MS analysis. The LC-ESI-MS/MS analysis was performed on a Shimadzu Nexera ultrahigh-performance liquid chromatography system (Shimadzu, Kyoto, Japan) coupled with a QTRAP 4500 hybrid triple quadrupole linear ion trap mass spectrometer (AB SCIEX, Framingham, MA). Chromatographic separation was performed on an Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, Mississauga, ON, Canada) maintained at 40°C using mobile phase a (water/methanol 50/50 [vol/vol]) containing 10 mM ammonium acetate and 0.2% acetic acid and mobile phase B (isopropanol/acetonitrile 50/50 [vol/vol]) in a gradient program (0–3 min: 30% B → 50% B; 3–24 min: 50% B → 90% B; 24–28 min: 30% B) with a flow rate of 0.3 ml/min. Neutral loss scans of 74 and 87 Da in the negative-ion mode were used to detect PC and PS, respectively. Neutral loss scan of 141 Da in the positive-ion mode was used to detect PE. Precursor ion scan of m/z = 241 in the negative-ion mode was used to detect PI. The instrument parameters for negative-ion mode were as follows: curtain gas, 10 psi; collision gas, 7 arbitrary units; ionspray voltage, −4500 V; temperature, 700°C; ion source gas 1, 30 psi; ion source gas 2, 70 psi; declustering potential, −96 V; entrance potential, −10 V; collision energy, −36 V; and collision cell exit potential, −15.4 V. The instrument parameters for positive-ion mode were as follows: curtain gas, 10 psi; collision gas, 7 arbitrary units; ionspray voltage, 4500 V; temperature, 700°C; ion source gas 1, 30 psi; ion source gas 2, 50 psi; declustering potential, 116 V; entrance potential, 10 V; collision energy, 31 V; and collision cell exit potential, 12 V. Quantification was performed by integration of the peak area of the extracted ion chromatograms for each phospholipid species.

**Phosphoinositide measurements by phosphate methylation and LC-ESI-MS/MS**

Cells (~10^6 cells) were washed with cold PBS, scraped in 1 M HCl, and centrifuged at 15,000 × g for 5 min. The pellets were resuspended in 170 μl of water and 750 μl of CHCl3/Methanol 1:1 (v/v) and incubated for 5 min at room temperature. To each sample, 725 μl of CHCl3 and 170 μl of 2 M HCl were added, followed by vortexing. After centrifugation at 1500 × g for 5 min, the lower phase was collected and washed with 780 μl of prederivatization wash solution (the upper phase of CHCl3/Methanol 0.01 M HCl (2:1:0.75 [vol/vol]). The lipid extracts were derivatized by adding 50 μl of 2 M TMS-diazomethane in hexane. The derivatization was carried out at room temperature for 10 min and stopped by adding 6 μl of glacial acetic acid. The derivatized samples were washed twice with 700 μl of postderivatization wash solution (the upper phase of CHCl3/Methanol/water 2:1:0.75 [vol/vol]). After addition of 100 μl of MeOH/H2O (9:1 [vol/vol]), the samples were dried under a stream of N2, dissolved in 80 μl of MeOH, and sonicated briefly. After addition of 20 μl of water, the samples were subjected to LC-ESI-MS/MS analysis. The LC-ESI-MS/MS analysis was performed on a Shimadzu Nexera ultrahigh-performance liquid chromatography system coupled with a QTRAP 4500 hybrid triple quadrupole linear ion trap mass spectrometer. Chromatographic separation was performed on an Acquity UPLC C4 BEH column (100 mm × 2.1 mm, 1.8 μm; Waters) maintained at 40°C using mobile phase A (water containing 0.1% formic acid) and mobile phase B (acetonitrile containing 0.1% formate) in a gradient program (0–5 min: 45% B; 5–10 min: 45% B → 100% B; 10–15 min: 100% B;
15–16 min: 100% B → 45% B; 16–20 min: 45% B) with a flow rate of 0.1 ml/min. The instrument parameters for positive-ion mode were as follows: curtain gas, 10 ps; collision gas, 7 arbitrary units; ion-spray voltage, 4500 V; temperature, 600°C; ion source gas 1, 30 ps; ion source gas 2, 50 ps; declustering potential, 121 V; entrance potential, 10 V; collision energy, 39 V; and collision cell exit potential, 10 V. Phosphoinositides were identified and quantified by multiple reaction monitoring (MRM). MRM transition parameters are shown in Supplemental Table S1.

For these measurements, an internal standard of 10 ng of 32:0 PIP2 was added to each sample. However, normalization of lipid counts to this internal standard was impractical due to the increase in 32:0 and 34:0 PIP2 in lipid samples from LYCAT-silenced cells. That the raw counts of PI and PIP between control and LYCAT-silenced cells are very similar indicates that the differences in abundance of specific acyl species of PIP2 between these silencing conditions is not due to differences in whole-sample preparation and instead reflects changes in the levels of PIP2 acyl species relative to the cellular levels of PI and PIP. Indeed, normalization of each PIP2 acyl species to the amount of 38:4 PI in each control or LYCAT-si lenced sample (Supplemental Figure S4, D and E) illustrates the alterations of PIP2 acyl species levels relative to PI upon LYCAT silencing.

ACKNOWLEDGMENTS

We thank T. Balla for the kind gifts of the plasmids encoding eGFP-PIS and eGFP-P4M and P. de Camilli for the kind gift of the plasmid encoding eGFP-E-Str2. This work was supported by an Ontario Early Researcher Award, a Canada Research Chair Award, and a Natural Sciences and Engineering Research Council Grant to R.J.B and a Ryerson Health Research Fund Award and an Operating Grant from the Canadian Institutes of Health Research (Grant 125854) to C.N.A. L.N.B was supported in part by an Ontario Graduate Scholarship.

REFERENCES

Aguet F, Antonescu CN, Mettlen M, Schmid SL, Danuser G (2013). Advances in analysis of low signal-to-noise images link dynamin and AP2 to the functions of an endocytic checkpoint. Dev Cell 26, 279–291.

Anderson KE, Kielkowska A, Durrant TN, Juvin V, Clark J, Stephens LR, Aguett F, Antonescu CN, Klip A (2010). Documenting GLUT4 exocytosis. Physiol Rev 93, 1019–1137.

Bennett CD, Randhawa VK, Cheng AW, Patel N, Klip A (2008). Selection of the perinuclear distribution of glucose transporter 4 (GLUT4) by insulin signals in muscle cells. Eur J Cell Biol 87, 337–351.

English AR, Voeltz GK (2013). Endolysosomal reticulum structure and interconnections with other organelles. Cold Spring Harb Perspect Biol 5, a013227.

Faim GD, Schieber NL, Ariotti N, Murphy S, Kuerschner L, Webb RJ, Grinstein S, Parton RG (2011). High-resolution mapping reveals topologically distinct cellular pools of phosphatidylinosine. J Cell Biol 194, 257–275.

Fili N, Calleja V, Woscholski R, Parker PJ, Larijani B (2006). Compartmental signal modulation: Endosomal phosphatidylinositol 3-phosphate controls endosome morphology and selective cargo sorting. Proc Natl Acad Sci USA 103, 15473–15478.

Gaidarov I, Keen JH (1999). Phosphatidylinositol-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. J Cell Biol 146, 755–764.

Garay C, Judge G, Lucarelli S, Bautista S, Pandey R, Singh T, Antonescu CN (2015). Epidermal growth factor-stimulated Akt phosphorylation requires clathrin or ErbB2 but not receptor endocytosis. Mol Biol Cell 26, 3504–3519.

Giordano F, Saheki Y, Idevall-Hag gren O, Colombo SF, Pirruccello M, Milosevic I, Gracheva EO, Bagriantsev SN, Borgese N, De Camilli P (2013). PIP(4,5)(P2)-dependent and Ca(2+)-regulated ER-MPR interactions mediated by the extended synaptotagmins. Cell 153, 1494–1509.

Hammond GR, Fischer MJ, Anderson KE, Holdich J, Koteci A, Balla T, Irvine RF (2012). PI4P and PI4,5P2 are essential but independent lipid determinants of membrane identity. Science 337, 727–730.

Hammond GR, Machner MP, Balla T (2014). A novel probe for phosphatidylinositol-4-phosphate reveals multiple pools beyond the Golgi. J Cell Biol 205, 113–126.

Hicks AM, DeLong CJ, Thomas MJ, Samuel M, Cui Z (2006). Unique molecular signatures of glycerolipidophospholipid species in different rat tissues analyzed by tandem mass spectrometry. Biochim Biophys Acta 1761, 1022–1029.

Ho CY, Choy CH, Botelho RJ (2016). Radiolabeling and quantification of cellular levels of phosphoinositides by high performance liquid chromatography-coupled flow scintillation. J Vis Exp 107, doi: 10.3791/53529.

Holub BJ, Kukas A (1978). Metabolism of molecular species of diacylglycerolphospholipids. Adv Lipid Res 16, 1–125.

Imae R, Inoue T, Kimura M, Kamamori T, Tomoda NH, Kage-Nakadai E, Matsui S, Arai H (2010). Intracellular phospholipase A1 and acyltransferase, which are involved in Caenorhabditis elegans stem cell divisions, determine the sn-1 fatty acyl chain of phosphatidylinositol. Mol Biol Cell 21, 3114–3124.

Imae R, Inoue T, Nakasaki Y, Uchida Y, Ohba YY, Kono N, Nakashima H, Sasaki T, Mitani S, Arai H (2011). LYCAT, a homologue of C. elegans acl-8, regulates clathrin-coated pit formation. Cell 146, 755–764.

Ishikura S, Antonescu CN, Keen JH (1999). Phosphatidylinositol-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. J Cell Biol 146, 755–764.

Itoh T, Koshiba S, Kigawa T, Kituchi A, Yokoyama S, Takenawa T (2001). Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science 291, 1047–1053.

Jackson LP, Kelly BT, McCoy AJ, Gaffry T, James LC, Collins BM, Höning S, Evans PR, Owen DJ (2010). A large-scale conformational change...
couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex. Cell 141, 1220–1229.

Jagamar K, Loerke D, Mettlen M, Kuwata H, Grinstein S, Schmid SL, Danuser G (2008). Robust single-particle tracking in live-cell time-lapse sequences. Nat Methods 5, 695–702.

Johnson EE, Overmeyer JH, Gunning WT, Maltese WA (2006). Gene silencing reveals a specific function of hVps34 phosphatidylinositol 3-kinase in late versus early endosomes. J Cell Sci 119, 1219–1232.

Jost M, Simpson F, Kavan JM, Lemmon MA, Schmid SL (1998). Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. Curr Biol 8, 1399–1402.

Ketel K, Krauss M, Nicot A-S, Puchkov D, Wieffer M, Müller R, Schultz D, Schultz D, Laporte J, Hauke V (2016). A phosphoinositide conversion mechanism for exit from endosomes. Nature 529, 408–412.

Kim WT, Chang S, Daniell L, Cremona O, Di Paolo G, De Camilli P (2002). Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptotagmin 1 knockout mice. Proc Natl Acad Sci USA 99, 17143–17148.

Kim YJ, Guzman-Hernandez ML, Balla T (2011). A highly dynamic ER-derived phosphatidylinositol-synthesizing organelle supplies phosphoinositides to cellular membranes. Dev Cell 21, 813–824.

Kim YJ, Guzman-Hernandez ML, Wsieniewski E, Balla T (2015). Phosphatidylinositol-phosphatic acid exchange by Nrr2 at ER-PM contact sites maintains phosphoinositide signaling competence. Dev Cell 33, 549–561.

Klausner RD, Van Renswoude J, Ashwell G, Kempf C, Schechter AN, Dean A, Bridges KR (1983). Receptor-mediated endocytosis of transferrin in K562 cells. J Biol Chem 258, 4715–4724.

Krauss M, Hauke V (2007). Phosphoinositides: regulators of membrane traffic and protein function. FEBS Lett 581, 2105–2111.

Lawe DC, Patki V, Heller-Harrison R, Lambright D, Corvera S (2000). The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. J Biol Chem 275, 3699–3707.

Li G, D’Souza-Schorey C, Barbieri MA, Roberts RL, Klippel A, Williams LT, Lawe DC, Patki V, Heller-Harrison R, Lambright D, Corvera S (2000). The PHD finger of endocytic Rab5 effectors regulates phosphoinositide signaling in K562 cells. J Biol Chem 275, 4715–4724.

Shin H-W, Hayashi M, Christoforidis S, Lucash-Verviai S, Hoeipnfen S, Wrenn MR, Modregger J, Uttenweiler-Joseph S, Wilm M, Nystuen A, et al. (2005). An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. J Cell Biol 170, 607–618.

Shindou H, Shimizu T (2009). Acyl-CoA:linoleoylplacid phospholipid acyltransferases. J Biol Chem 284, 1–5.

Shulga YV, Anderson RA, Topham MK, Epand RM (2012). Phosphatidylinositol-4-phosphate 5-kinase isoforms exhibit acyl chain selectivity for both substrate and lipid activator. J Biol Chem 287, 35953–35963.

Stauffer TP, Ahn S, Meyer T (1998). Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. Curr Biol 8, 343–348.

Stein BS, Sussman HH (1986). Demonstration of two distinct transferrin receptor recycling pathways and transferrin-independent receptor internalization in K562 cells. J Biol Chem 261, 10319–10331.

Stemmark H, Aslassl R, Driscoll PC (2002). The phosphatidylinositol 3-phosphate-binding FYVE finger. FEBS Lett 513, 77–84.

Stephens LR, Jackson TR, Hawkins PT (1993). Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signaling system? Biochim Biophys Acta 1179, 27–75.

Zentpeter Z, Várnai P, Balla T (2010). Acute manipulation of Golgi phosphoinositides to assess their importance in cellular trafficking and signaling. Proc Natl Acad Sci USA 107, 8225–8230.

van Dam EM, ten Broeke T, Jansen K, Spijkers P, Stoorvogel W (2002). Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. J Biol Chem 277, 48876–48883.

van Meer G, Voelker DR, Feigenson GW (2008). Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9, 112–124.

Varna P, Balla T (1998). Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol 143, 501–510.

Varna P, Thyaagarajan B, Rohacs T, Balla T (2006). Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. J Cell Biol 175, 377–382.

Zhao Y, Chen Y-Q, Li S, Konrad RJ, Cao G (2009). The microsomal cardiolipin remodeling enzyme acyl-CoA lysocardiolipin acyltransferase is an acyltransferase of multiple anionic lysophospholipids. J Lipid Res 50, 945–956.

Zoncu R, Perera RM, Balkin DM, Pirrucchini M, Toomre D, De Camilli P (2009). A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 136, 1110–1121.

Zoncu R, Perera RM, Sebastian R, Nakatsu F, Chen H, Balla T, Ayala G, Toomre D, De Camilli PV (2007). Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. Proc Natl Acad Sci USA 104, 3793–3798.