The PH domain from the *Toxoplasma gondii* PH-containing protein-1 (TgPH1) serves as an ectopic reporter of phosphatidylinositol 3-phosphate in mammalian cells

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Abstract

The PH domain from the *Toxoplasma gondii* PH-containing protein-1 (TgPH1) serves as an ectopic reporter of phosphatidylinositol 3-phosphate in mammalian cells

Krishna Chintaluri, Master of Science, Molecular Science, Ryerson University, 2017

Phosphoinositides (PtdInsPs) lipids recruit effector proteins to membranes to mediate a variety of functions including signal transduction and membrane trafficking. Each PtdInsP binds to a specific set of effectors through characteristic protein domains such as the PH, FYVE and PX domains. Domains with high affinity for a single PtdInsP species are useful as probes to visualize the distribution and dynamics of that PtdInsP. The endolysosomal system is governed by two primary PtdInsPs: phosphatidylinositol-3-phosphate [PtdIns(3)P] and phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P_2], which are thought to localize and control early endosomes and lysosomes, respectively. While PtdIns(3)P has been analysed with mammalian-derived PX and FYVE domains, PtdIns(3,5)P_2 indicators remain controversial. Thus, complementary probes against these PtdInsPs are needed, including those originating from non-mammalian proteins. Here, we characterized in mammalian cells the dynamics of the PH domain from PH-containing protein-1 from the parasite *Toxoplasma gondii* (TgPH1), which was previously shown to bind PtdIns(3,5)P_2 *in vitro*. However, we show that TgPH1 retains membrane-binding in PIKfyve-inhibited cells, suggesting that TgPH1 is not a viable PtdIns(3,5)P_2 marker in mammalian cells. Instead, PtdIns(3)P depletion using pharmacological treatments dissociated TgPH1 from membranes. Indeed, TgPH1 co-localized to EEA1-positive endosomes. In addition, TgPH1 co-localized and behaved similarly to the PX domain of $p40^{phox}$ and tandem FYVE domain of EEA1, which are commonly used as PtdIns(3)P indicators. Collectively, TgPH1 offers a complementary reporter for PtdIns(3)P derived from a non-mammalian protein and that is distinct from commonly employed PX and FYVE domain-based probes.

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AKT1	RAC-alpha serine/threonine protein kinase
ALS	Amyotrophic lateral sclerosis
AP-2	Clathrin adaptor protein 2
Arf1	ADP-ribosylation factor-1
ARF6	GTPase ADP ribosylation factor 6
BODIPY	Boron-dipyrromethene
Btk	Bruton’s tyrosine kinase
CISK	Cytokine-independent survival kinase
DAG	Diacylglycerol
EEA1	Early endosome antigen 1
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
FAPP1	Four-phosphate-adaptor protein 1
FYVE	Fab1, YOTB, Vac1 and EEA1
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
GTPase	Guanosine triphosphate hydrolyzing enzymes
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
IP3	Inositol triphosphate
IR	Insulin receptor

LDL	Low-density lipoprotein
LSDs	Lysosomal storage diseases
MTM1	Myotubularins
MTMR2	MTM1-related protein-2
mTOR	Mechanistic/mammalian target of rapamcyin
MVBs	Multivesicular bodies
NBD	Nitrobenzoxadiazole
PDK1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology
Phox	Neutrophil NADP oxidase complex
PI3K	Phosphatidylinositol-3-kinase
PI3K	Phosphoninositide-3-kinase
PLCδ-1	Phospholipase Cδ-1
PLD	Phospholipase D
PTB	Phosphotyrosine binding domain
PtdIn(3)P	Phosphatidylinositol 3-phosphate
PtdIns(3,4,5)P3	Phosphatidylinositol 3,4,5-trisphosphate
PtdIns(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(4)P-5K	Phosphatidylinositol 4-phosphate 5-kinase
PtdIns(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(4)K	Phosphoinositide 4-kinases
PtdInsK	Phosphoinositide kinases

PtdInsPs	Phosphoinositides
PTEN	Phosphatase and tensin homology
PX domain	Phox homology domain
SHIP1/SHIP2	SH2 containing inositol 5-phosphatase
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SNX	Soring nexins
Tfn	Transferrin
TGN	Trans-Golgi network
TgPH1	Toxoplasma gondii PH-containing protein-1

Introduction

Organelles and Trafficking

One of the distinguishing features of an eukaryotic cell is the presence of organelles in the cytosol. Organelles are membrane enclosed compartments each with a specific function. The compartmentalization is necessary for supporting the metabolic requirements of eukaryotic requirements and sequestering processes that are incompatible with each other. Compartmentalization helps provide specific environments to facilitate the cell carrying out specific functions more efficiently. For example, the lysosome is highly acidic which is optimal for many of the hydrolytic enzymes that reside within. Sequestering these hydrolytic enzymes prevents components in the cytosol from being degraded by these lytic enzymes and processes to take place elsewhere simultaneously such as protein synthesis in the endoplasmic reticulum (ER). Organelles such as the ER are found in direct contact with other organelles such as the nucleus with multiple tubules extending into the cytosol. Organelles such as the Golgi apparatus, in contrast is near to the nucleus. Regardless, organelles are part of a dynamic and integrated network within the cell (Karp, 2013).

Materials are shuttled between organelles through small membrane-bound vesicles that bud from a donor membrane compartment and fuse with an acceptor membrane compartment resulting in deposition of the soluble cargo. For example, proteins synthesized in the ER are post-translationally modified as they traffic through the Golgi complex and are transported from there to destinations such as the plasma membrane (see Figure 1.1), endosomes, lysosomes or the cytosol. In the case of endocytosis, materials from outside of the cell also rely on vesicular traffic to be

delivered into appropriate compartments such as the endosomes or lysosomes. Different cargo such as proteins and lipids are transported through vesicles. Many of the proteins that are responsible for trafficking between organelles are recruited to the cytosolic surfaces of membranes. Examples include coat proteins that generate and surround the vesicle, motor proteins such as dynein or kinesin that are responsible for movement of vesicles along the cytoskeleton and tethering factors such as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) that facilitate fusion of vesicles to the acceptor membrane. The accuracy of the vesicular traffic is reliant on organelles being correctly recognized (Karp, 2013).

Many of the peripheral membrane proteins correctly recognize the organelle by binding to specific lipids such as phosphonisitides (PtdInsPs) and guanosine triphosphate hydrolyzing enzymes (GTPases). Two families of GTPases, mainly the Rab and Arf GTPases are responsible for maintaining the identity of organelles playing a role in recruiting the appropriate peripheral proteins to cytosolic surfaces (Behnia and Munro, 2005). These GTPases act as coordinators of vesicular traffic. The restriction of certain GTPases and PtdInsPs helps with the accuracy of vesicular transport and governs organelle identity. The role of some PtdInsPs and GTPases in traffic will be highlighted in the following sections.

The Endosomal Pathway

Eukaryotic cells meet their nutritional requirements through different pathways such as the import of glucose and amino acids via plasma membrane transporters or ingestion of larger extracellular molecules through different modes of endocytosis. Endocytosis describes the process by which segments of the plasma membrane

invaginate towards the interior of the cell and by doing so, internalize extracellular cargo (nutrients, growth factor receptors etc.) and subsequent release in endolysosomes (Doherty and McMahon, 2009; Grant and Donaldson, 2009).

Endocytosis can be divided into two main types, clathrin-dependent/receptor mediated and clathrin-independent endocytosis. Processes that require receptor mediated endocytosis include the internalization of iron through Transferrin (Tfn) receptors, low-density lipoprotein (LDL) which is a carrier of cholesterol or growth factor signaling through epidermal growth factor receptor (EGFR) (Grant and Donaldson, 2009; Palm and Thompson, 2017). What is common to all forms of endocytosis is the delivery of cargo to early endosomes and the cargo's subsequent routing to late endosomes and lysosomes for degradation, or to the trans-Golgi network (TGN) or to recycling endosomes (Grant and Donaldson, 2009). Endosomes essentially act as sorting centers for membrane traffic. Receptor-mediated endocytosis of EGFR will be used as examples to describe the endosomal pathway (see Fig. 1 for a summary and overview of the endosomal pathway). Receptor-mediated endocytosis begins with binding of a ligand to a receptor followed by recruitment of intracellular proteins that begin the process of internalization.

Binding of extracellular EGF to EGFR results in dimerization of the receptor, resulting in autophosphorylation of its cytoplasmic domain. Autophosphorylation results in recruitment of proteins activating signal transduction pathways including the MAPK signaling cascade, the phosphonisitide-3-kinase (PI3K) pathway and the phospholipase Cγ pathway (Arteaga and Engelman, 2014; Delos Santos, Garay and Antonescu, 2015). Following activation, EGFR is internalized through clathrin-coated vesicles after

recruitment of clathrin and clathrin adaptor protein 2 (AP-2). The recruitment of clathrin and AP-2 is dependent on the small GTPase ADP ribosylation factor 6 (ARF6) which recruits phosphatidylinositol 4-phosphate 5-kinase [PtdIns(4)P-5K] for phosphorylation of phosphatidylinositol 4-phosphate [PtdIns(4)P] to yield phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] (Honda *et al.*, 1999). Recruitment of PLC hydrolyses PtdIns(4,5)P2 to produce inositol trisphosphate (IP3) and diacylglycerol (DAG) which activates protein kinase C and phosphatidylinositol-3-kinase (PI3K) that in turn converts PtdIns(4,5)P2 into PtdIns(3,4,5)P3 (Keough and Thompson, 1972; Akhtar and Abdel-Latif, 1978; Meldrum, Parker and Carozzi, 1991). PtdIns(3,4,5)P3 is recognized by phosphoinositide-dependent kinase-1 (PDK1) that goes on to activate Akt that is involved in a number of downstream signals (Alessi *et al.*, 1997).

Following endocytosis, the vesicles fuse with early endosomes. The small GTPase Rab5 is found on early endosomes and recruits class III phosphoinositide 3-kinase (Vps34) which results in generation of PtdIns(3)P on endosomes (Murray *et al.*, 2002). Vesicle fusion occurs in conjunction with Rab5, early endosome antigen 1(EEA1) and SNAREs (Mills, Jones and Clague, 1998; Stenmark *et al.*, 1998; McBride *et al.*, 1999).The two major routes for EGFR trafficking from early endosomes is recycling of the receptor back to the cell surface or lysosomal degradation. In the case of transferrin, transferrin receptors can either be rapidly recycled to the cell surface through early endosomes or recycling endosomes (Frederick R. Maxfield & Timothy E. McGraw, 2004).

The ubiquitination of EGFR receptor is critical for its down regulation and for sorting from early endosomes to late endosomes. Grb2 recruits the E3 ubiquitin ligase

Cb1 which results in monomeric and polymeric ubiquitination of lysine residues of the EGFR receptor (Duan *et al.*, 2003; Huang *et al.*, 2006). Specifically, mono-ubiquitination and polyubiquitination are associated with trafficking of EGFR to multivesicular bodies (MVBs). The endosomal sorting complexes required for transport (ESCRT) machinery are several groups of protein complexes that are responsible for sorting ubiquitinated cargos into multivesicular bodies (MVBs) (Raiborg and Stenmark, 2009). Four ESCRT complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III are recruited to early endosomes after interaction with ubiquitinated cargo. The recruitment of ESCRT complexes is dependent on initial recognition of PtdIns(3)P by Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) a component of the ESCRT-0 complex (Raiborg *et al.*, 2002). MVBs eventually fuse with late endosomes and lysosomes where the material is degraded. The role of PtdIns(3)Ps in membrane trafficking and their regulation is discussed in more detail in the section on phosphatidylinositol 3-phosphates.

Lysosomes

Delivery of cargo from endosomes to lysosomes is dependent on generation of phosphatidylinositol 3,5- bisphosphate [PtdInsP(3,5)P2] where it plays an important role in trafficking from endosomes to lysosomes. Phosphatidylinositol 3-phosphate 5-kinase, PIKfyve (Fab1 is the yeast homolog) acts on PtdIns(3)P on early endosomes (Gary *et al.*, 1998). Pharmocological inhibition of PIKfyve has been shown to block the lysosome from degrading EGFR, the idea being that MVBs containing EGFR are prevented from fusing with lysosomes due to PtdIns(3,5)P2 depletion (de Lartigue *et al.*, 2009).

Lysosomes are organelles which are found in most animal cells and are responsible for degradation of polysaccharides, proteins, and lipids. The highly acidic environment of the lysosome (pH 4.5-5) is optimized for the activity of the more than 60 enzymes which reside there. The breakdown of molecules by these enzymes yields monosaccharides, amino acids and fatty acids respectively. These products can then be exported out of the lysosome to be used by the cell to replenish energy stores or for reuse in biosynthetic pathways. Lysosomes breakdown both intracellular and extracellular material. In the latter case, the lysosome is the terminal organelle in the endosomal pathway with the result being the degradation of the extracellular material. The intracellular degradation of material mediated by lysosomes is termed autophagy. Autophagy is the recycling of the cytoplasmic material in the lysosome, a process most often linked to low energy, nutrient starvation, and the need for repair after cellular damage. The integration of lysosomes in cellular homeostasis is dependent on their association with nutrient sensing machinery such as proteins like mammalian/mechanistic target of rapamycin(mTOR)(Puertollano, 2014; Xu and Ren, 2015).The protein mTOR is often termed the master regulator of growth that senses nutrient levels and energy and acts to integrate these diverse signals in the maintenance of the cell. Consequently, the proper functioning of lysosomes is extremely important and deregulation can result in lysosomal storage diseases (LSDs) and neurodegenerative diseases such as Alzheimer's (Xu and Ren, 2015). A more detailed role of PtdIns(3,5)P2, their regulation, and the critical role they play in maintaining lysosomal function are expanded in the section titled phosphatidylinositol 3,5, bisphosphate.

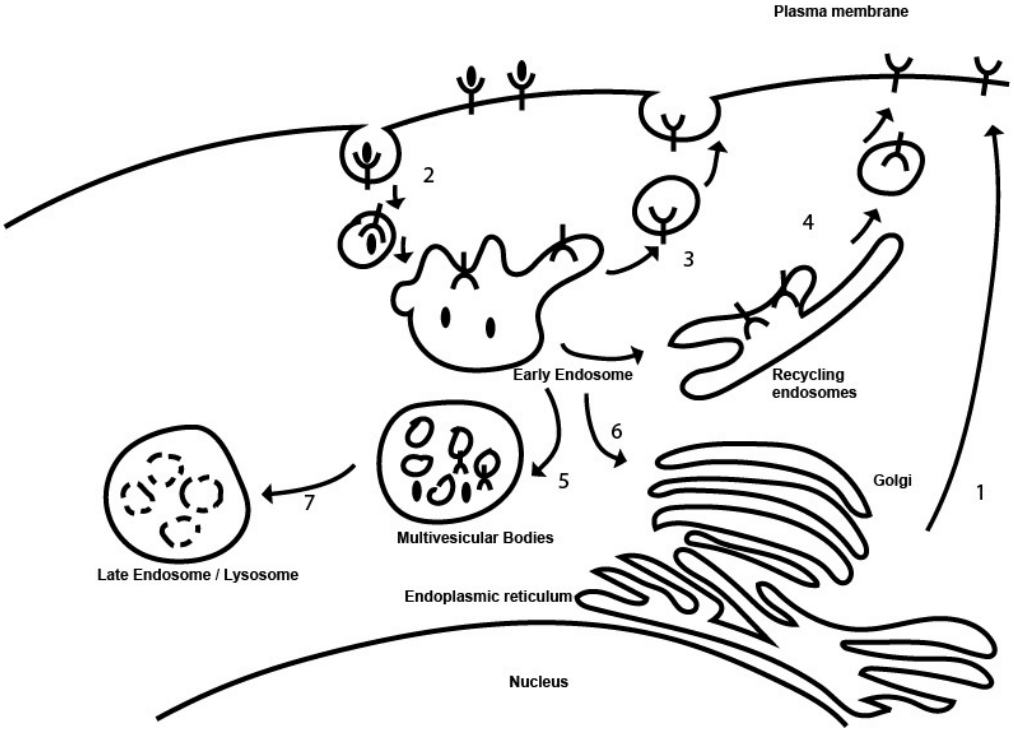

Figure 1. The Endosomal Pathway. Proteins synthesized in the ER are post-translationally modified as they traffic through the Golgi complex and are transported from there to destinations such as the plasma membrane (1). Binding of ligand to a receptor (for example extracellular EGF to EGFR) results in activation of signal transduction pathways and recruitment of cytosolic proteins such as AP-2 and clathrin to initiate endocytosis and formation of vesicles (2). The endocytosed vesicle fuses with early endosomes or in certain cases, endocytosed cargo such as transferrin receptor is rapidly recycled back to the plasma membrane (3) or makes its way into recycling endosomes for slower recycling (4). From early endosomes, material can traffic to the Golgi (6) or material meant for degradation is sorted into multivesicular bodies (MVBs) which then fuse with late endosomes/lysosomes where proteases degrade the material (7).

Phosphoinositides

Phosphoinositides (PtdInsPs) are generated through the phosphorylation of the inositol head group at the 3, 4 or 5 positions of the base molecule phosphatidylinositol (PtdIns), with the phosphorylation being reversible through the action of lipid kinases and phosphatases(see Figure 2 A) Phosphatidylinositol makes up somewhere between 10-20% of all phospholipids depending on cell type, while PtdInsPs such as phosphatidylinositol 4,5 bisphosphate [PtdIns(4,5)P2], Phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] or Phosphatidylinositol 3-phosphate [PtdIns(3)P] being several magnitudes less abundant than the base molecule (Di Paolo and De Camilli, 2006a; Balla, 2013). The seven-different species of PtdInsPs (see Figure 2 B) are found embedded in the cytosolic leaflet of cellular membranes with the head group oriented towards the cytosol acting as a docking site to facilitate interactions between proteins and effectors (Figure 1). Some PtdInsPs are constitutively present on membranes while others are synthesized or turned over because of cell surface activators or other stimuli. Each species of PtdInsPs binds to a unique set of cognate effectors and displays a characteristic subcellular distribution. The spatial restriction of PtdInsPs coupled to the recruitment of species-specific PtdInsP effector proteins endows the host membrane or organelle with their attributable functions and ‘identity’ (Behnia and Munro, 2005; Di Paolo and De Camilli, 2006b; Kutateladze, 2010; Balla, 2013). The interplay between PtdInsPs, enzymes responsible for their synthesis and turnover, and interaction with proteins that recognize PtdInsPs leads to complex signaling networks within the cells.

Phosphoinositides are interconverted through the actions of kinases and phosphatases through recognition of the inositol headgroup. There are several classes

of kinases and they are sorted into three categories, phosphoinositides kinases (PtdInsK) phosphonsitide 3-kinases (PtdIns3K), phosphoinositide 4-kinases (PtdIns4K)(Sasaki *et al.*, 2009). Specific kinases in each class are responsible for phosphorylating and generating different species of PtdInsPs. Using PtdIns3Ks as example, Class I PtdIns3Ks preferentially phosphorylate PtdIns(4,5)P2 to yield PtdIns(3,4,5)P3, Class II PtdIns3Ks are responsible for generating PtdIns(3)P from PtdIns or PtdIns(3,4)P2 from PtdIns(4)P, and Class III PtdIns3Ks generate PtdIns(3)P from PtdIns (Sasaki *et al.*, 2009). Phosphatases are responsible for removal of phosphates from the 3,4, or 5 positions of the inositol headgroup PtdInsPs. Phosphatases are characterized into three types, PtdIns(3)P phosphatases, PtdIns(4)P phosphatases and PtdIns(5)P phosphatases (Sasaki *et al.*, 2009).

Each PtdInsP species can have dozens of effectors that behave differently. This suggests that PtdInsPs control these effectors together with additional factors to increase specificity of each effector.This concept is referred to as coincidence detection and follows the idea that a single PtdInsP can control two effectors differently because these effectors bind differently to a second ligand. For example, PtdInsPs recruit many effectors that can also bind to GTPases. To further illustrate, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 in co-ordination with Cdc42, a small GTPase, recruit actin regulatory proteins for cytoskeletal rearrangement. For example N-WASP binding to PtdIns(4,5)P2 and GTP-bound Cdc42 results in a conformational change that allows binding to the ARP2/3 complex which is involved in actin polymerization (Rozelle *et al.*, 2000). Another example of coordination between GTPases and PtdInsPs is the role of the four-phosphate-adaptor protein (FAPP1) in secretory transport from the trans-Golgi network

(TGN) to the plasma membrane (He *et al.*, 2011; Liu, Kahn and Prestegard, 2014). The protein FAPP1 must bind to PtdIns(4)P and ADP-ribosylation factor-1 (Arf1), a small GTPase. Knockdown of FAPP1 results in impaired cargo transfer from the TGN to the plasma membrane (He *et al.*, 2011).

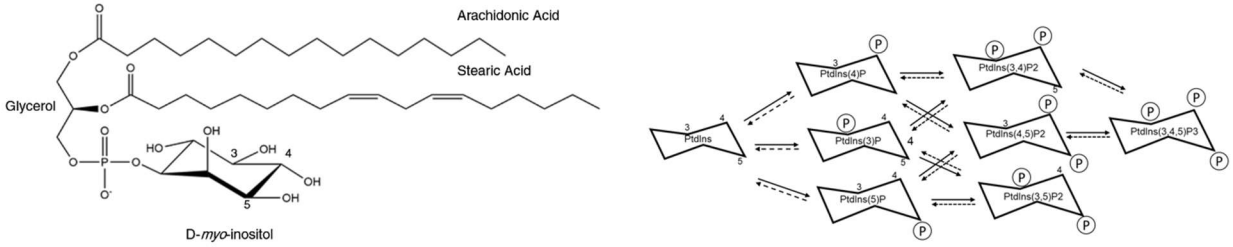

Figure 2. Phosphatidylinositol and their phosphoinositide derivatives. Phosphatidylinositol is comprised of a D-*myo*-inositol headgroup attached to a glycerol backbone and two fatty acid chains: arachidonic acid and stearic acid (A). Phosphatidylinositol can be phosphorylated at the 3, 4 or 5 positions to yield the 7-different species of phosphoinositides (B) that can be interconverted dephosphorylated through the actions of kinases (black arrows) or phosphatases (dotted arrows). Only the inositol headgroups are shown for B.

PtdInsPs play an important role in signal transduction including that of growth factors like EGF and of hormones like insulin. In the case of insulin signaling, insulin causes the dimerization of the two cytosolic β-domains activates the kinase property of the insulin receptor (IR) which results in phosphorylation of several tyrosine residues.

The phosphorylated sites on the β-domain are then recognized by insulin-receptor substrates (IRS-1 and IRS-2). IRS proteins have a pleckstrin homology (PH) domain adjacent to a phosphotyrosine binding (PTB) domain and C-terminal tail with multiple phosphorylation sites. The PH domain mediates interaction with PtdIns(4,5)P2 and the PTB domain recognizes phosphorylated residues on the insulin receptor. The multiple tyrosine, serine and threonine sites present at the C-terminus of IRS-1 are phosphorylated by the activated insulin receptor. The lipid kinase phosphoinositide 3-kinase (PI3K) recognizes phosphotyrosine residues on IRS-1 through its regulatory domain and converts PtdIns(4,5)P2 to PtdIns(3,4,5)P3. The resulting conversion to PtdIns(3,4,5)P3 is recognized by PtdIns(3,4,5)P3-dependent protein kinase (PDK1). PDK1 then phosphorylates and activates proteins such as RAC-alpha serine/threonine-protein kinase (AKT1). The active AKT1 can then go on to phosphorylate and activate downstream targets that can result in increased trafficking of glucose receptors (GLUT4) to the cell surface promoting glucose uptake, or stimulating glycogen synthesis. The activity of the PI3K/AKT pathway is regulated by PtdIns(3,4,5)P3 3-phosphatase, phosphatase and tensin homolog (PTEN) and PtdIns(3,4,5)P3 5-phosphatase, SH2 containing inositol 5-phosphatase (SHIP1 and SHIP2). The dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(3,4)P3 by SHIP1 attenuates PI3K/AKT activation. Mutations in SHIP2 have been associated with type 2 diabetes in humans(Shi *et al.*, 2006). Similar to insulin receptor signaling, PtdIns(4,5)P2 conversion to PtdIns(3,4,5)P3 by PDK1 results in AKT activation with in EGFR signaling. The attenuation of the signaling is through PTEN. Dysregulation of PTEN has been implicated in many cancers. Provided here are a few examples of the important role of

PtdInsPs in cytoskeletal rearrangement, maintenance of vesicle trafficking and in receptor signaling. Dysregulation of PtdInsPs can drive many diseases such as cancer, diabetes, obesity and rare genetic disorders (Vicinanza *et al.*, 2008; McCrea and De Camilli, 2009; Jean and Kiger, 2012; Balla, 2013). The next few sections will focus on more detailed discussions on PtdIns(3)P and PtdIns(3,5)P2 (see Fig.3) which play an important role in the endosomal system.

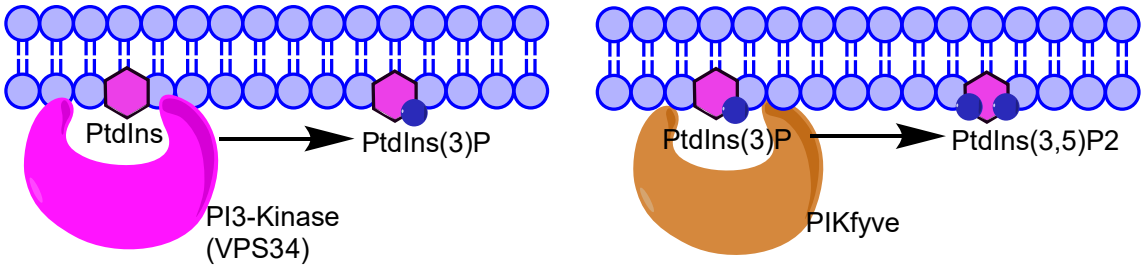

Figure 3. Endosomal phosphoinositides.

Phosphatidylinositol 3-phosphate [PtdIns(3)P] is converted from phosphatidylinositol [PtdIns] through the actions of the kinase VPS34. PtdIns(3)P is present mainly on early endosomes. The kinase PIKfyve converts PtdIns(3)P to phosphatidylinositol 3,5-bisphospe [PtdIns(3,5)P2] which is present mainly on endosomes.

Phosphatidylinositol-3-phosphate

Synthesized by Vps34 Class III PI 3-kinase, PtdIns(3)P is the predominant species of PtdInsPs found on endosomes, where it governs early endosome fusion, maturation and cargo sorting (see Fig.3) (Gillooly, 2000; Lawe *et al.*, 2000; Raiborg *et al.*, 2001; Vieira *et al.*, 2001; Bago *et al.*, 2014). Myotubularins (MTM1) are the main antagonists of PtdIns(3)P signaling since they are PtdIns(3)P 3-phosphatases that hydrolyze PtdIns(3)P to PtdIns. Mutations in MTM1 have been implicated in myotubular myopathy

and mutations in MTM1-related protein-2 (MTMR2) in Charcot-Marie-Tooth Disease (Gillooly, Simonsen and Stenmark, 2001).

Many proteins which bind to PtdIns(3)P contain FYVE domains such as EEA1, HRS and PIKfyve. The protein EEA1 is an effector of the small GTPase Rab5 and PtdIns(3)P and is important for endosome docking and fusion (McBride *et al.*, 1999; Zerial *et al.*, 1999; Murray *et al.*, 2002). The protein HRS as part of the ESCRT complex plays an important role in sorting cargo for degradation through multivesicular bodies for eventual degradation in the lysosome (Raiborg *et al.*, 2002; Hirano *et al.*, 2006). Other proteins that bind to PtdIns(3)P with high specificity are proteins containing the Phox homology domain (PX domain) such as $p40^{phox}$ subunit of NADPH oxidase complex and sorting nexins (SNX). SNX proteins play a role in intracellular trafficking, for example SNX1 is involved in EGFR trafficking to endosomes for degradation(Kurten, Cadena and Gill, 1996). Many other SNX proteins have since been identified all playing roles in intracellular trafficking and protein sorting (Worby and Dixon, 2002).

Using the PX domain from $p40^{phox}$ and the FYVE domains from Hrs and EEA1 as probes, PtdIns(3)P is thought to primarily localize to early endosomes, though it is also detectable in omegasomes, which are precursors of autophagosomes and early phagosomes (Gillooly, 2000; Ellson, Anderson, *et al.*, 2001; Ellson, Gobert-Gosse, *et al.*, 2001; Vieira *et al.*, 2001; Axe *et al.*, 2008). Recruitment of PIKfyve to endosomes results in conversion of PtdIns(3)P to PtdIns(3,5)P2 (de Lartigue *et al.*, 2009)

Phosphatidylinositol 3,5-bisphosphate

Phosphatidylinositol 3,5 bisphosphate is a low abundance phosphoinositide that is found on endosomes and lysosomes and generated from PtdIns(3)P through the actions of the kinase PIKfyve (Fab1 being the yeast homolog). PtdIns(3,5)P2 only makes up 0.1% of total phosphoinositides in yeast and only 0.04% in mammalian fibroblasts (Mccartney, Zhang and Weisman, 2014). The activity of PIKfyve is positively regulated through association with the adaptor protein Vac14 and the phosphatase Sac3 (see Fig.4) (Shisheva, 2008; de Lartigue *et al.*, 2009). The kinase PIKfyve is a PtdIns(3)P 5-kinase, and Sac3 is a PtdIns(3,5)P2 5-kinase responsible for converting PtdIns(3,5)P2 back into PI(3)P (see Figure 4). PtdIns(3,5)P2 can also be converted into PtdIns(5)P through myotubularin phosphatases (Robinson and Dixon, 2006).

Deficiency in levels of PtdIns(3,5)P2 as a result of mutations in the above mentioned proteins leads to enlargement of vacuoles in yeast cells and enlargement of endolysosomes in mammalian cells (Ikonomov, Sbrissa and Shisheva, 2001; Ikonomov *et al.*, 2002; Rutherford, 2006; Ho, Alghamdi and Botelho, 2012; Lenk and Meisler, 2014; Mccartney, Zhang and Weisman, 2014; Kim *et al.*, 2016). Mutations in Sac3 (Fig4), results in extensive degeneration and neuronal vacuolization in the brain of mice (Chow *et al.*, 2007).

An important role of PtdIns(3,5)P2 is its interaction with TRPML1, a lysosomal Ca^{2+} channel that is present on endolysosomes that permits release of calcium. The release of calcium is thought to be important for vesicle fusion and impairment of TRPML1 results in enlargement of lysosomes(Dong *et al.*, 2010). Inhibition of PIKfyve and silencing of TRPML1 also prevents phagosome maturation (Dayam *et al.*, 2015)

Deficiency in levels of PtdIns(3,5)P2 have been implicated in diseases such as amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth syndrome and other rare neurological diseases((Lenk and Meisler, 2014; Lenk *et al.*, 2016).

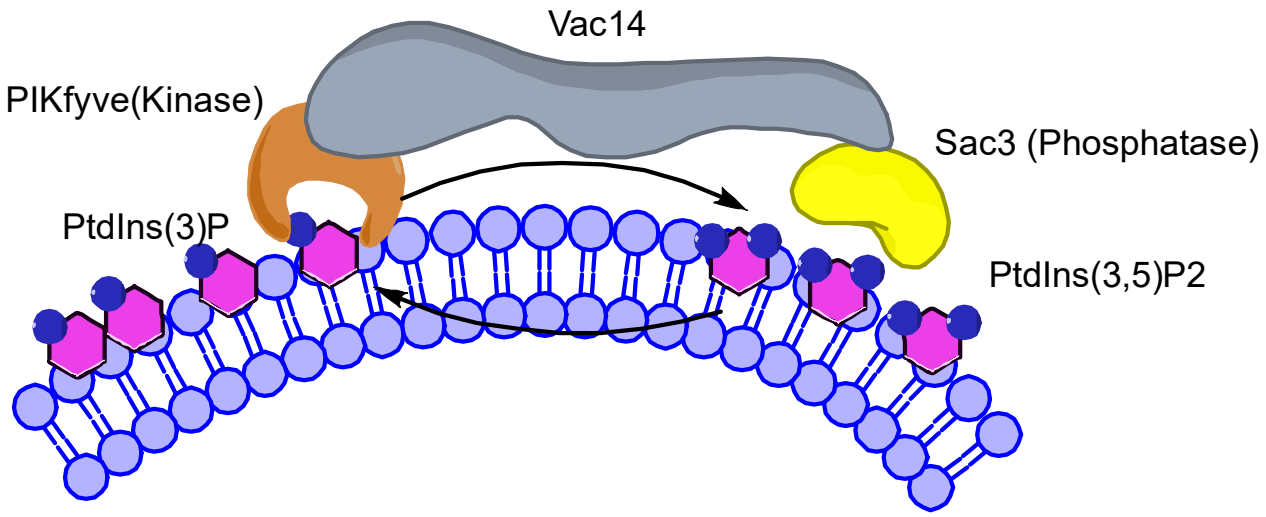

Figure 4. Regulation of PtdIns(3,5)P2. The kinase PIKfyve phosphorylates PtdIns(3)P at the 5 position to generate PtdIns(3,5)P2, and Sac3 the phosphatase responsible for dephosphorylating PtdIns(3,5)P2. The adaptor Vac14 interacts with both PIKfyve and Sac3.

Visualization of Phosphoinositides

Traditionally, our knowledge of cellular distribution of phospholipids or any other lipid for that matter was reliant on fractionation studies. However, owing to the dynamic nature of the cell, phospholipids are quickly interconverted which necessitates the need for methods to be able to quickly capture these changes, ideally by visualization using microscopy. One strategy is to conjugate PtdInsPs with a fluorescent tag. These are

essentially analogues of PtdInsPs but conjugated with fluorescent dyes such as boron-dipyrromethene (BODIPY) or nitrobenzoxadiazole (NBD) (Halet and Viard, 2008). These fluorescently tagged PtdInsPs are incorporated by the cell and microscopy can be used to report their intracellular localization. It remains unclear what the effects of adding these exogeneous PtdInsPs on the physiology of the cell and how the fluorophores affect the properties of those PtdInsPs. Additionally, there needs to be a transmembrane carrier that facilitates the delivery of fluorescent PtdInsPs and it is not know how exactly they detach from this carrier (Halet and Viard, 2008).

Alternatively, PtdInsPs may be visualized with antibodies specific to a single PtdinsP species. The use of anti-PtdInsP antibodies offers some advantages over addition of exogenous lipids with the main advantage of antibodies are they can bind to PtdInsPs with a high level of specificity and accuracy. Antibodies require the samples to be fixed and processed prior to imaging. The focus of this thesis will be mostly on the use of fluorescently-conjugated phosphoinositide binding domains which will explained in the following section.

Phosphoinositide Binding Domains

Our knowledge of the subcellular localization of PtdInsPs is partly informed through effector proteins and their inositol-lipid binding domains (Kutateladze, 2010; Hammond and Balla, 2015; Várnai *et al.*, 2017). A diverse number of PtdInsP-interacting protein domains have been discovered and include the ENTH, GRAM, FYVE, PH, PHD, and PX domains, among many others (Hammond and Balla, 2015). The affinity and specificity of these domains towards each PtdInsP can differ greatly; some domains exhibit superb specificity and high affinity for a single PtdInsP species, while others are

promiscuous and/or exhibit low affinity (Kavran *et al.*, 1998; Balla, 2005; Lemmon, 2008; Hammond and Balla, 2015). For this thesis, the domains that will be explored in most detail will be the PH, FYVE and PX domains with the PH domain being the best characterized. See Table 1 for several different protein domains, their specificity to PtdInsPs and examples of proteins containing those domains. This list is not exhaustive but just represents the best characterized PtdInsPs binding domains. Importantly, chimeras of fluorescent proteins and PtdInsP-binding domains that show high specificity and affinity for a single PtdinsP species can reveal the localization of the target PtdInsPs via fluorescence microscopy (Stauffer, Ahn and Meyer, 1998; Oatey *et al.*, 1999; Gillooly, 2000; Balla, 2007; M. A. Lemmon, 2007; Várnai *et al.*, 2016). There is now a large array of widely employed PtdInsPs reporters based on this strategy (Hammond and Balla, 2015; Idevall-Hagren and De Camilli, 2015; Várnai *et al.*, 2016). Figure 5 shows a simplified cartoon diagram of the abovementioned PtdInsP reporters. However, these protein domain-based probes are not without caveats since they may also interact with additional endogenous ligands, thus preferentially detecting PtdInsP pools that co-exist with the additional ligand (Lawe *et al.*, 2000; Raiborg *et al.*, 2001; Karathanassis *et al.*, 2002; He *et al.*, 2011; Lucas and Cho, 2011; Liu, Kahn and Prestegard, 2014; Stahelin, Scott and Frick, 2014). In the next section, I will expand further on PH domains, FYVE domains and PX domains which are important for my thesis.

Domains	PtdInsPs Specificity	Examples of Proteins
ANTH/CALM	PtdIns(4,5)P2	AP180
ENTH	PtdIns(4,5)P2	Epsin
C2	PtdIns(3,4,)P3	PTEN
FERM	PtdIns(4,5)P2	Ezrin, moesin, radixin, talin
FYVE	PtdIns(3)P2	PIKfyve, EEA1, Hrs, SARA
GRAM	PtdIns(3,5)P2	Myotubularin
PDZ	PtdIns	
PH	PtdIns(4)P PtdIns(3,4)P2 PtdIns(4,5)P2 PtdIns(3,4,5)P3	FAPP1/2, OSBP AKT/PKB, TAPP1,2 PLCδ1, dynamin BTK, AKT/PKB, ARNO, GRP1
PTB	PtdIns(4,5)P2	Dab1, ARH, Shc
PX	PtdIns(3)P PtdIns(5)P PtdIns(4,5)P2 PtdIns(3,4,5)P3 PtdIns	SNX 2,3,7,13 SNX13 Class II PI(3)kinase CISK VPS34

Table 1. A list of protein domains known to bind to PtdInsPs and the parent proteins. The above list has been compiled from several different sources (Balla, 2005; Di Paolo and De Camilli, 2006a; Lemmon, 2008)

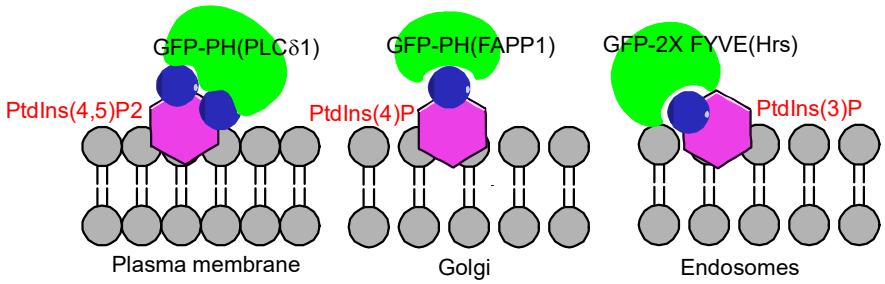

Figure 5. Fluorescent protein-based probes for visualization of phosphoinositides. Examples of some protein based probes including the domain and the parent protein containing the domain that is responsible for binding to PtdInsPs at cellular membranes.

PH domains

The most well characterized PtdInsP-binding domain is the pleckstrin homology domain (PH domain), a ~120 amino acid residue domain first identified in pleckstrin and subsequently in other proteins thought to be involved in signalling (Haslam, Koide and Hemmings, 1993). The isolated PH domain from pleckstrin was shown to bind specifically to PtdIns(4,5)P2 (Harlan *et al.*, 1994). The PH domains in general were thought to help facilitate localization of proteins to membranes. However, PH domains could also bind to proteins since it was determined experimentally that PH domains could bind to subunits of G proteins such as the PH domain of beta-adrenergic receptor kinase (Harlan *et al.*, 1994).

Subsequent characterization of PH domains from several proteins revealed that only two out of ten PH domains exhibited a strong propensity to bind only one species of PtdInsP, whereas the majority were promiscuous in binding to more than one species of PtdInsPs (Kavran *et al.*, 1998). The PH domain from phospholipase Cδ-1(PLC-δ1)

and GRP1 (for general receptor for phosphoinositides-1) were shown to respectively bind to PtdIns(4,5)P2 and PtdIns(3,4,5)P3 specifically (Kavran *et al.*, 1998). PH domains have been identified in many different proteins such as kinases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and phospholipases to name a few (Balla, 2005). It's important to note that not all proteins containing PH domains bind to PtdInsPs. In fact, most PH domain containing proteins in *Saccharomyces cerevisiae* do not bind strongly or specifically to PtdInsPs (M. a Lemmon, 2007; Lemmon, 2008).

Most PH domains consist of ~120 aa residues arranged with an anti-parallel β-sheet followed by one or two α-helices (see Fig.6) (Yamamoto *et al.*, 2016). PH domains with a strong specificity towards PtdInsPs have a similar structure in that they are made up of seven-stranded β-sandwich with the β1-β4 strands and their adjoining loops (or some variation of) mediating the interaction with the head group of phosphoinositides (Balla, 2005). For example, the loop between the β1- β2 strands of PLCδ1 PH contains a sequence motif of lysine and arginine residues that interact with phosphates (Lemmon, 2008). More specifically, any PH domains that bind to PtdInsPs via the motif with the consensus sequence KXn(K/R)XR within the loop joining the β1-β2 strands are considered to have a 'canonical' lipid binding site (Yamamoto *et al.*, 2016). Examples of proteins containing PH domains with 'canonical' lipid-binding sites include GRP1, ARNO, PLC-□1, DAPP1, PDK1, PEPP1, PKB/Akt, C-PH, Kindlin-2, and Btk PH domains (Yamamoto *et al.*, 2016). Proteins which have alternate lipid-binding sites include ArhGAP9 and β-spectrin, and ASAP1 which is thought to have both 'canonical' and secondary lipid-binding sites (Yamamoto *et al.*, 2016). It's important to

note that some PH domains bind more strongly to cleaved head groups of PtdInsPs without the diacylglycerol backbone. For example, the PH domain of PLCδ1 binds more strongly to Ins(1,4,5)P3 than its membrane bound counterpart PtdIns(4,5)P2(Kavran *et al.*, 1998)

Since, some PH domain have shown to have specificity towards PtdInsPs they have been utilized as intracellular reporters for those specific PtdInsPs. The PH domain of PLC-δ1 was fused to GFP and has been used as an intracellular reporter for PtdIns(4,5)P2 (Stauffer, Ahn and Meyer, 1998; Várnai and Balla, 1998). The PH domains from ARNO and GRP1 have also been fused with GFP to generate intracellular reporters for PtdIns(3,4,5)P3 (Oatey *et al.*, 1999).

FYVE Domains

In comparison to PH domains, the FYVE domain (Fab1, YOTB, Vac1 and EEA1) is a 60-70 amino acid zinc-finger domain comprised of two β-hairpins and a C-terminal α-helix held together by two Zn^{2+} ions (see Fig.6) (Balla, 2005; Lemmon, 2008). FYVE domains bind to PtdIns(3)P with the interaction mediated by a conserved motif RRHHCRQCGNIF in the first β-strand. Proteins with FYVE domains are recruited primarily to endocytic compartments which are particularly enriched in PtdIns(3)P and critical for a number of different functions. Many FYVE domains exhibit weak affinity towards isolated headgroups compared to their membrane bound counterparts. This contrasts with other inositol binding domains such as PH domains which have shown robust affinity towards isolated head groups (Gillooly, Simonsen and Stenmark, 2001). The weak affinity is reflected in the fact that isolated FYVE domains do not bind strongly to PtdIns(3)P. This weak affinity necessitated the need for generation of constructs

expressing FYVE domains fused in tandem to report PtdIns(3)P both in vivo and in vitro (Gillooly, 2000). In fact, the FYVE domains from Hrs and EEA1 have been fused to GFP to be utilized as intracellular reporters for PtdIns(3)P (Gillooly, 2000)

PX domains

Another domain that binds to PtdIns(3)P typically is the Phox-homology domains (PX) domains. PX domains were first characterized in $p40^{phox}$ and $p47^{phox}$ subunits of the neutrophil NADPH oxidase (phox) complex (Ponting, 1996). PX domains are made up of 120 amino acids arranged in a N-terminal three-stranded β-sheet and four α-helices and importantly the inositol binding pocket is located between the loop connecting the β1-β2 strands(see Fig. 6) and one of the α-helix (Balla, 2005). Most PX domains preferentially bind to PtdIns(3)P but some have been identified to bind to other PtdInsPs (see Table 1). PX domains have been found in proteins such as class II phosphoinositide 3-kinases (PI3Ks), sorting nexins (SNX), Vps proteins in yeast, phospholipase D (PLD), cytokine-independent survival kinase (CISK), and SNAREs (Bravo *et al.*, 2001). The PX domain of $p40^{phox}$ has been fused with GFP and acts as an intracellular reporter of PtdIns(3)P (Kanai *et al.*, 2001).

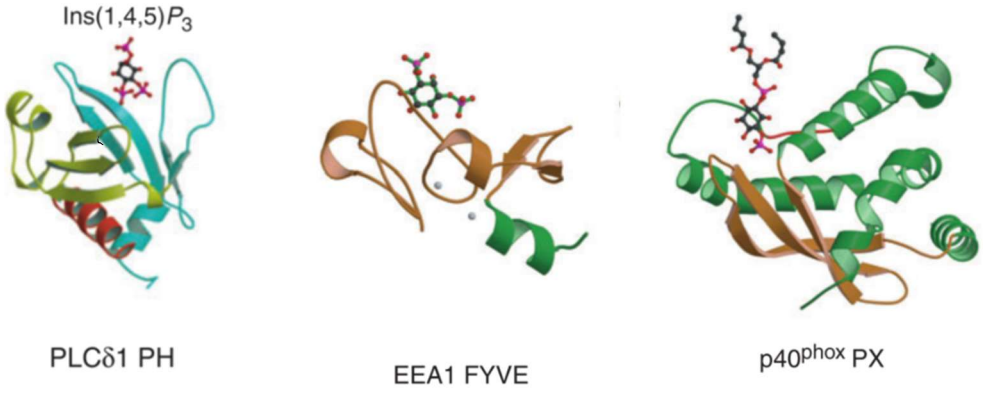

Figure 6. Structure of PH, FYVE and PX domains binding to inositol headgroups. The PH domain from PLC δ1 is shown here binding Ins(3,4,5)P3 between the β1-β4 sheets. The EEA1 FYVE domain is shown here binding to Ins(3)P via the RRHHCRQCGNIF sequence in the first β-strand. The p40^{phox} PX domain is shown binding to Ins(3)P via a binding pocket located between the β1- β2 strands. Images are taken from (Balla, 2005)

Rationale, Hypothesis and Objectives

A tandem fusion of the N-terminal of TRPML1, a region of TRPML1 shown to be regulated by PtdIsn(3,5)P_2, was reported to localize this lipid (Dong *et al.*, 2010; Li *et al.*, 2013). Nevertheless, the specificity of this probe towards PtdIns(3,5)P_2 was questioned in at least some cell types (Hammond *et al.*, 2015). The importance of PtdIns(3,5)P2 notwithstanding, it seems that a highly specific probe does not exist for that species. Recently, TgPH1, a protein from the parasite *Toxoplasma gondii* was shown to have specificity towards PtdIns(3,5)P2 in that parasite (Daher *et al.*, 2016). TgPH1 was isolated during affinity precipitation with PtdIns(3,5)P_2-beads from *T. gondii* lysates and shown to interact with PtdIns(3,5)P_2 using in vitro assays (Daher *et al.*, 2016). Interestingly, TgPH1 contains a PH domain, a well-documented inositol-binding domain that is found in many proteins (see section on inositol-binding domains and PH domains).

Hypothesis: given this information, *we hypothesized that constructs of TgPH1 fused with GFP may be a suitable probe for PtdIns(3,5)P2 when expressed in mammalian cells*.

To test this hypothesis, I had the following objectives:

1) Generate constructs expressing in mammalian cells single and tandem GFP fusion of TgPH1, transfect mammalian cells to see whether both probes localize to lysosomes (where PtdIns(3,5)P2 is thought to be enriched) and whether binding of probes is sensitive to depletion of PtdIns(3,5)P2

2) If TgPH1 probe is specific for PtdIns(3,5)P2, asses the probe further in different mammalian cell types including HeLa cells and RAW macrophages. If probe is not specific for PtdIns(3,5)P2, assess whether binding of the probe is sensitive to depletion of other PtdInsPs such as PtdIns(3)P

3) See whether TgPH1 co-localizes with endosomal markers such as EEA1 and existing probes for PtdIns(3)P.

Materials and Methods

Nucleic acids

Plasmids encoding 2FYVE-RFP and p40PX-mCherry were kindly provided by Dr. Sergio Grinstein. Two plasmids encoding GFP-fusion proteins of TgPH1 were generated here, eGFP-TgPH1, eGFP-2x-TgPH1 as follows. The eGFP-TgPH1 and GFP-2xTgPH1 constructs were synthesized in pcDNA 3.1::N-eGFP backbone (Genscript). For pcDNA 3.1::N-eGFP-2x-TgPH1, a GSGN linker was inserted between the two tandem copies of TgPH1. The sequence of TgPH1(kindly provided by Wassim Daher) as follows were synthesized into the pcDNA 3.1::N-eGFP vectors using the KpnI and NotI sites.

TgPH1:
TATGAACGCCTTCCAGTCGCCCAGTTTGGACATCACCATCAGCCGGGGTGATGTC
GTGAAGGAAGGGTGGCTATGCAAGCAGTCAAAGTTTCTGAAGGACTGGAGACGAC
GCTGGTTCGTTTTGACTCCCTACTGCTTGTGCAGCTTCAAAACGTCGGACATCTAC
CATTCCAAGCCAACTGAGATCCTCTTTCTAAGGGACTGCAGCACTGTCAAGAGCGC
TGACGAGGACATCCAGAAAGAGAACGCATTCCGGGTCGATACTCCTAATCGTGTG
TTTTTCCTGATAGCTGACAACAACCAAGAAAAGGAATCGTGGATTGGTCACATCGG
AAGGCAGATGGTGCGCCCGTCAGTCATGGTTAATGACTCTTACGGCCAGGACAGT
GACTAA

Plasmids were prepared with a midi-preparation plasmid kit (E.Z.N.A.® Endo-Free Plasmid Maxi Kit)

Cell culture and transfection

RAW 264.7 cells (ATCC TIB-71) and HeLa cells (ATCC CCL-2). RAW and HeLa cells were maintained in 25 cm^2 filter-cap flasks (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco). Passaging was done by scraping RAW cells, or using Trypsin-EDTA (0.25% Trypsin with EDTA 4NA; Life Technologies) for HeLa cells. For experiments with RAW and HeLa cells, cells were seeded at ~25 to 30% confluency onto 12-mm square glass coverslips (VWR) or 18-mm circular glass coverslips (Electron Microscopy Sciences) RAW and HeLa cells were transfected for 24 h with 1 µg of plasmid DNA using FuGENE HD (Promega) as per manufacturer's instructions

Pharmacological depletion of phosphoinositides

To deplete PtdIns(3,5)P_2, cells were treated with 20 nM apilimod (Toronto Research Chemicals) for 1 h (Cai *et al.*, 2013). Alternatively, cells were exposed to Vps34-IN1 at 1 µM or 5 µM for 1 h to deplete PtdIns(3)P (Bago *et al.*, 2014).

Immunofluorescence

After experimental treatment, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 20 min, followed by a 10-min incubation with 0.1% Triton-X100 in PBS to permeabilize cells. Cells were then blocked with 5% milk in PBS, followed by incubation with rabbit anti-mouse EEA1(1:100, Cell Signaling) for 1 h. This was followed by staining with fluorescent secondary antibodies used at 1:1000 (Bethyl Laboratories). Cells were then mounted onto slides using mounting media (Dako).

Live and fixed cell imaging

For live cell imaging with RAW cells, cells were pre-loaded with a 1.5 h pulse of 150 µg/mL fixable, anionic dextran conjugated to Alexa Fluor™ 647, 10,000 MW (ThermoFisher), followed by 1 h chase with fresh medium. Cells were then manipulated with pharmacological treatments as described above and then subjected to live-cell imaging. Imaging was performed at ambient CO_2 with cells submerged in HEPES-buffered RPMI supplemented with 5% FBS. Imaging was performed by spinning disc confocal microscopy using one of two systems: an Olympus IX81 spinning disk confocal microscope equipped with a Hamamatsu C9100-13 EMCCD camera and using a 100X 1.4 NA super apochromatic oil-immersion objective or with a Quorum DisKovery spinning disc confocal microscope system connected to an Andor Zyla 4.2 Megapixel sCMOS camera and using a 63 X 1.4 NA oil-immersion objective. Standard excitation and emission filter cubes and lasers were then employed.

Image and statistical analysis

To quantify membrane-associated to cytosolic ratio of TgPH1 probes, images were imported into Image J, region of interests (ROI) were drawn around randomly selected puncta and cytosolic regions, background corrected and the ratio of puncta to cytosol was calculated. This was performed across twenty cells per condition, across three independent experiments (Fig. 8). For each condition, averages were calculated from the three trials, and those averages were presented with standard error of the mean.

Results

TgPH1 binds to intracellular membranes independently of PtdIns(3,5)P2 in mammalian cells

TgPH1 was shown to bind PtdIns(3,5)P_2 using *in vitro* affinity precipitation with PtdIns(3,5)P_2-beads, liposomes and lipid blots, though the lipid blot also showed significant binding to PtdIns(3)P (Daher *et al.*, 2016). Thus, we speculated that TgPH1 could serve as an indicator of PtdIns(3,5)P_2 in mammalian cells. To assess this, we generated mammalian expression vectors expressing GFP chimeras of single (GFP-TgPH1) and tandem TgPH1 (GFP-2x-TgPH1) domains and transfected into RAW macrophages. RAW cells were then labelled with fluorescent dextran to demarcate lysosomes, followed by treatment with DMSO-only or 20 nM apilimod for 1 h to inhibit PIKfyve activity (Cai *et al.*, 2013). We have previously shown that apilimod depletes >80% of PtdIns(3,5)P_2 in RAW macrophages (Cai *et al.*, 2013; Ho *et al.*, 2015). In untreated RAW cells, GFP-TgPH1 associated with membranes, albeit weakly and being mostly cytosolic (Fig. 7A). By contrast, GFP-2x-TgPH1 displayed strong association with punctate and vesicle-like membranes (Fig. 7C). In addition, neither GFP-TgPH1 or GFP-2x-TgPH1 co-localized significantly with dextran-loaded lysosomes. Importantly, PIKfyve inhibition with apilimod caused extensive vacuolation in RAW macrophages (Fig. 7B, D). Strikingly, GFP-2x-TgPH1, and even GFP-TgPH1, retained their ability to associate with membranes, including extensively enlarged vacuoles (Fig. 7B, D). Finally, few GFP-2x-TgPH1 labelled vacuoles were co-stained with dextran, suggesting the presence of at least two populations of vacuoles in these cells (Fig. 7D). Collectively, these data show that TgPH1 does not depend on PIKfyve activity for

membrane association in mammalian cells, suggesting that TgPH1 is not a viable probe for PtdIns(3,5)P_2 in these cells.

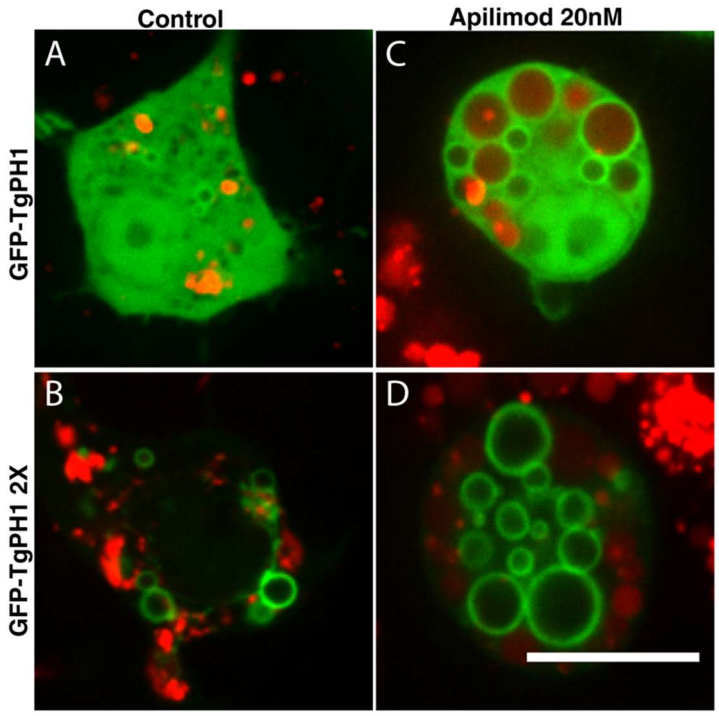

Figure 7. Subcellular distribution of GFP-fused TgPH1 proteins in PIKfyve inhibited RAW cells. RAW macrophages were transfected with GFP-TgPH1 and GFP-TgPH1 2X as described in materials and methods. Cells were pulsed and chased with red dextran which is being used here as a lysosomal marker. Cells were then left either untreated (control) or exposed to 20nM Apilimod and then imaged via live cell microscopy. TgPH1 single variant remains mainly cytosolic (A) but TgPH1 2X exhibits punctate/membrane bound localization (C) in control conditions. Both TgPH1 and TgPH1 2X remain membrane bound under Apilimod treatment (B and D respectively). Neither probe siginificantly co-localizes with red dextran which is being used here as lysosomal marker (A-D). Scale bars represent 10μm. Images are representative of 20 or more cells from 1 independent experiment.

TgPH1 becomes cytosolic in Vps34 inhibited cells

We then speculated that TgPH1 may bind to membranes in mammalian cells via PtdIns(3)P instead. To test this, we transfected both RAW and HeLa cells with GFP-2x-TgPH1 and assessed its membrane association in cells exposed to VPS34-IN1, a selective inhibitor of Vps34 class III PI3K. VPS34-IN1 was previously shown to deplete cells of PtdIns(3)P and disperse PtdIns(3)P probes from endosomes into the cytosol (Bago *et al.*, 2014). First, GFP-2x-TgPH1 exhibited membrane-bound distribution in both RAW and HeLa cells treated with vehicle alone or exposed to apilimod (Fig. 8). By contrast, cells treated with VPS34-IN1 exhibited mostly cytosolic GFP-2x-TgPH1 distribution (Fig. 8). As before we quantified the membrane to cytosol distribution using F^H/F_L ratio. Consistent with visual inspection of images, control and apilimod-treated cells displayed high F^H/F_L ratio for GFP fluorescence, while VPS34-IN1-exposed cells exhibited the converse ratio (Fig. 8B, D). Overall, these data indicate that TgPH1 associates to intracellular membranes in a PtdIns(3)P-dependent manner in mammalian cells.

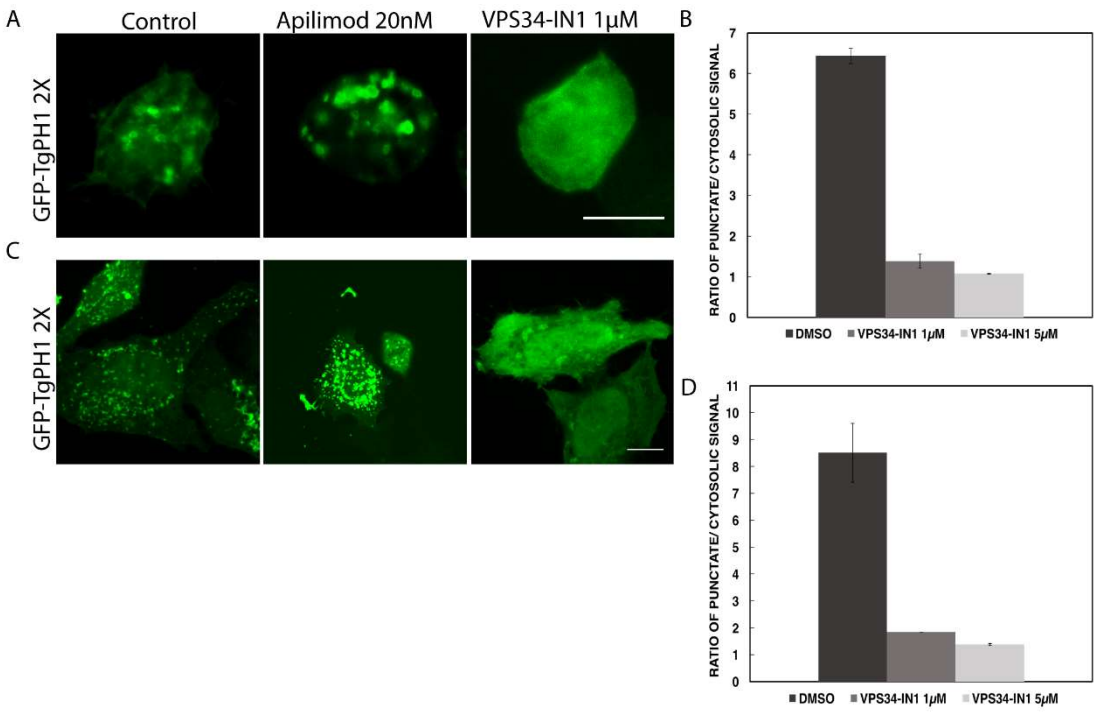

Figure 8. GFP-TgPH1 2X is sensitive to PtdIns(3)P depletion in RAW and HeLa cells. RAW macrophages (A) and HeLa (C) cells were transfected with GFP-TgPH1 2X as described in materials and methods. Figures for quantification of the sensitivity of the probe to PtdIns(3)P depletion are shown in B for RAWs and D for HeLa cells. Changes in distribution of the probe was assessed via randomized selection of punctate/membrane bound probe and comparing their signal intensities against the cytosol. Error bars represent standard error of the mean derived from analyzing 20 cells per condition for a n of 3. Images for treatment with 5µM VPS34-IN1 is not shown here. Images for Apilimod treatment are shown just to provide a comparison. Scale bars represent 10 µm.

TgPH1 associates with early endosomes

Our observations suggest that TgPH1 binds to membranes via PtdIns(3)P rather than PtdIns(3,5)P_2 in mammalian cells. This suggests that the TgPH1 associates with early endosomes, which are thought to be enriched in PtdIns(3)P, rather than late endosomes and lysosomes, which are thought to contain PtdIns(3,5)P_2 (Kutateladze *et al.*, 1999; Gillooly, 2000; Ellson, Gobert-Gosse, *et al.*, 2001; Li *et al.*, 2013). To examine this hypothesis, we expressed GFP-2x-TgPH1 in RAW macrophages and immunostained with EEA1 which demarcate early endosomes. We observed strong co-localization with EEA1-positive organelles (Fig. 4). Collectively, the distribution of TgPH1 to early endosomes is consistent with PtdIns(3)P-dependent membrane association.

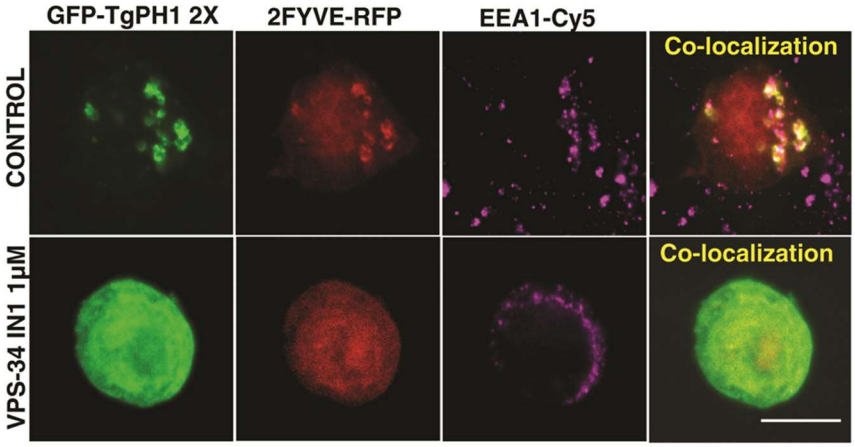

Figure 9. GFP-TgPH12X co-localizes with 2FYVE-RFP, an existing probe for PI3P in RAW cells. RAW macrophages were co-transfected with GFP-TgPH1 2X and 2FYVE-RFP as described in materials and methods. Cells were left untreated (control, top row) or treated with 1μM VPS34-IN1 (bottom row). Cells were then fixed using PFA and methanol. Endosomes (where PI3P is thought to be particularly enriched) were stained with EEA1 antibody.GFP-TgPH12X is shown co-localizing with 2-FYVE RFP and EEA1(top row, co-localization panel). In cells treated with VPS34-IN1, there is a significant shift towards a more cytosolic distribution of both probes (bottom row, co-localization panel). The scale bar represents 10μm. Images are representative of 25 or more cells from 3 independent experiments.

TgPH1 co-localizes with other PtdIns(3)P fluorescent probes.

Our data suggest that TgPH1 is a potential novel reporter for PtdIns(3)P in mammalian cells that is of non-mammalian origin and is distinct from the most commonly employed PtdIns(3)P-binding probes, which tend to carry a FYVE or PX domain (Gillooly, 2000; Ellson, Gobert-Gosse, *et al.*, 2001; Raiborg *et al.*, 2001; Song *et al.*, 2001). Thus, we assessed the co-localization of GFP-2x-TgPH1 relative to the mCherry-fusion of the PX domain of $p40^{phox}$ or mRFP chimera of a two-tandem fusion of the FYVE domain of EEA1 by co-transfection. Lastly, all three probes became cytosolic when cells were treated with VPS34-IN1 to deplete PtdIns(3)P (Fig. 9).

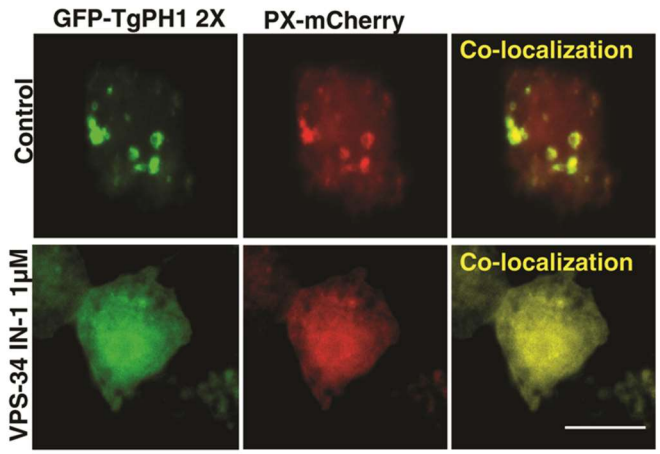

Figure 10. GFP-TgPH12X co-localizes with PX-mCherry an existing probe for PI3P in RAW cells. RAW macrophages were co-transfected with GFP-TgPH1 2X and PX-mCherry as described in materials and methods. Cells were left untreated (control) or treated with 1μM VPS34-IN1. GFP-TgPH12X is shown co-localizing with PX-mCherry (top row). In the treatment with VPS34-IN1, there is a significant shift towards a more cytosolic distribution of both probes (bottom row). The scale bars represent 10μm. Images are representative of 25 or more cells from 3 independent experiments.

Discussion

In this study, we expressed and characterized the PH domain derived from the parasite *T. gondii* PH-domain containing protein-1 (TgPH1). This domain was previously suggested to bind to PtdIns(3,5)P_2 in *T. gondii* using *in vitro* assays (Daher *et al.*, 2016). We thus, postulated that TgPH1 could report PtdIns(3,5)P_2 in mammalian cells. However, our data negate this hypothesis. Instead, we show that TgPH1 expressed in various mammalian cells, RAW and HeLa, either as a single or as a tandem-fusion, localizes to early endosomes in a PtdIns(3)P-dependent manner. This conclusion is supported with pharmacological depletion of PtdIns(3)P and PtdIns(3,5)P_2. Specifically, TgPH1 dissociated from membranes only in cells treated with inhibitors of Vps34, but not of PIKfyve. It is important to note that TgPH1 bound both PtdIns(3)P and PtdIns(3,5)P_2 when using lipid blots, though the protein only bound to PtdIns(3,5)P_2 when using liposome-binding assays (Daher *et al.*, 2016). The reason for this discrepancy remains unclear. While TgPH1 is not a viable probe for PtdIns(3,5)P_2 in mammalian cells, we provide evidence that TgPH1 behaves very similarly to FYVE and PX-based probes for PtdIns(3)P.

Given our observations, we propose that TgPH1 can serve as a complementary reporter of PtdIns(3)P in mammalian cells. This is an important consideration since PtdInsP-binding domains carry caveats when employed to investigate PtdInsP distribution and dynamics (Idevall-Hagren and De Camilli, 2015; Várnai *et al.*, 2016). Even well-characterized probes like the PH domains of FAPP1, PLCδ1 and Akt, which respectively track PtdIns(4)P, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 carry caveats (Balla, 2005; Hammond and Balla, 2015; Várnai *et al.*, 2016). For example, the PH domain of

PLCδ1, while commonly employed as a reporter for PtdIns(4,5)P_2 also binds with higher affinity to soluble inositol-1,4,5-bisphosphate (IP_3) (Quinn, Behe and Tinker, 2008; Szentpetery *et al.*, 2009). In another example, the PH domain of FAPP1 reports Golgi-associated PtdIns(4)P because it also binds to the Golgi-associated Arf1 GTPase (He *et al.*, 2011; Liu, Kahn and Prestegard, 2014). Alternative probes for these lipids respectively include the PH domain of Tubby1 that binds PtdIns(4,5)P_2 but not IP_3 and the Legionella-derived P4M, which detects multiple pools of PtdIns(4)P including in late endosomes (Quinn, Behe and Tinker, 2008; Szentpetery *et al.*, 2009; Hammond, Machner and Balla, 2014). Similarly, the PX and FYVE-based probes for PtdIns(3)P have potential flaws. For example, the FYVE domain of EEA1 carried an adjacent motif that associated with GTP-bound Rab5 (Simonsen *et al.*, 1998; Gillooly, 2000; Lawe *et al.*, 2000). In fact, in yeast, PtdIns(3)P was initially thought to be restricted to early endosomes, but subsequent use of other probes including FYVE domain of Fab1 show that PtdIns(3)P exists in vacuoles (Botelho *et al.*, 2008). In plant cells, expression of Thus, it is important to develop complementary probes for PtdIns(3)P.

TgPH1 has several advantages as a complementary tool to study PtdIns(3)P. First, the majority of PtdIns(3)P probes tend to be FYVE or PX domain based (Gillooly, 2000; Ellson, Gobert-Gosse, *et al.*, 2001; Hammond and Balla, 2015; Idevall-Hagren and De Camilli, 2015; Várnai *et al.*, 2016). TgPH1 is wholly distinct as a PH domain. Second, TgPH1 is unique among other PH domains since the vast majority of PtdInsP-binding PH domains bind to PtdIns(4)P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3,not PtdIns(3)P. Lastly, TgPH1 is not mammalian derived and may reduce the chances that it reports specific pools of PtdIns(3)P due to lack of an

endogenous protein ligand (Balla, 2005; Stahelin, Scott and Frick, 2014; Idevall-Hagren and De Camilli, 2015; Várnai *et al.*, 2016).

Our observations have also been corroborated by Dr.Gerry Hammond's group. Using a rapamycin inducible system (Fig. 11), the phosphatases MTM1 and INPP5E are recruited to Rab5-positive membranes and act upon PtdIns(3)P and PtdIns(3,5)P2 respectively. In COS-7 cells, TgPH1 remained membrane bound in cells depleted of PtdIns(3,5)P2 but was displaced when MTM1 was recruited to membranes resulting in depletion of PtdIns(3)P. Together with pharmacological treatment and a rapamycin-inducible system we demonstrate that TgPH1 is a probe that binds to PtdIns(3)P.

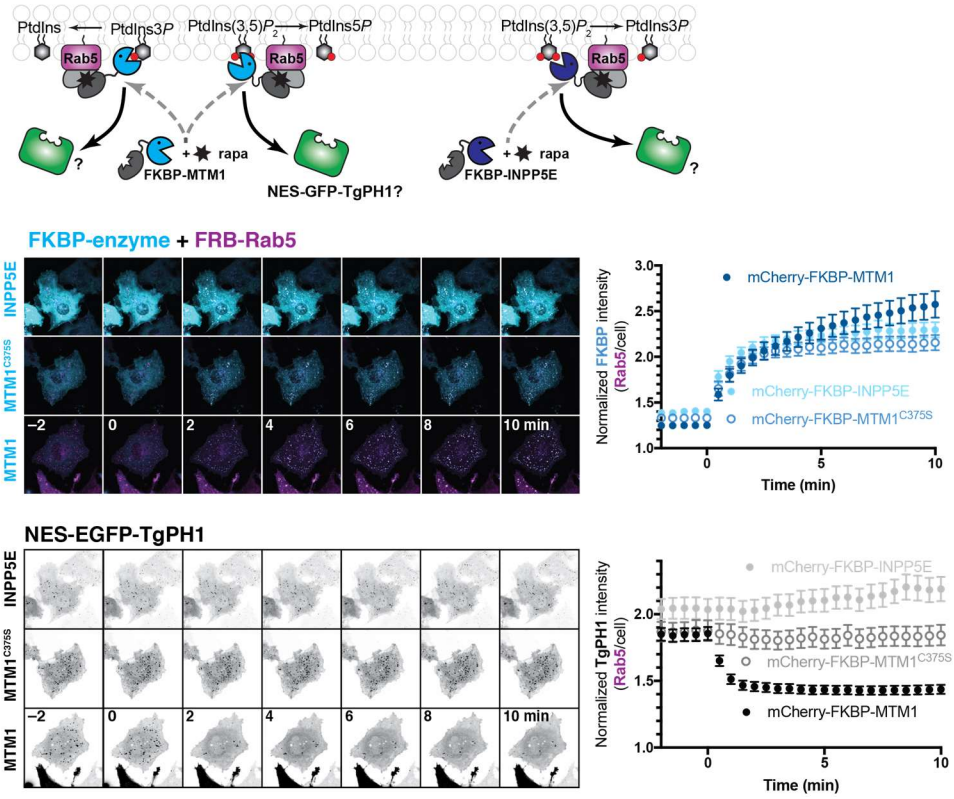

Figure 11. Depletion of PtdIns3P but not PtdIns(3,5)P_2 causes TgPH1 to dissociate from Rab5-positive membranes in COS-7 cells. Top panels show a schematic of the rapamycin-induced dimerization system and the enzymatic activities of the FKBP-conjugated phosphatases. Middle panels show time-lapse imaging of COS-7 cells expressing iRFP-FRB-Rab5 (magenta) and mCherry-FKBP fused to the indicated enzyme (cyan), whereas the bottom panels show NES-EGFP-TgPH1 (grayscale) in the same cells. Images show 2 min intervals with rapamycin (1 μM) added after time 0. The graphs at right show the normalized intensity at Rab5-positive membranes relative to the whole cell for FKBP-tagged enzymes (top) and NES-EGFP-TgPH1 (bottom). Data are means ± s.e. of 41 (MTM1), 23 (C375S) or 27 (INPP5E) cells from 3 or 4 independent experiments. The above image is unpublished data by Brady Goulden, Camilyn Celmenza and Gerald R. V. Hammond.

Future Work and Conclusion

The images presented in this thesis so far been mostly qualitative. However, a more robust statistical analysis will be employed. To quantify membrane-associated to cytosolic ratio of TgPH1 probes, we will import images into ImageJ, and then assign 3-pixel wide lines measuring 20-30-pixels in length along areas of transfected cells that exclude the nucleus. Plot profiles will be obtained and exported into spreadsheet program, values ordered according to fluorescent intensity and the ratio of the highest 10 pixels over the lowest 10-pixel values will be calculated with the expectation that cytosol-distributed signal will produce values approximate to 1, while signal that accumulates in puncta relative to cytosol will produce values greater than 1.

We also need to confirm that TgPH1 co-localizes to EEA1-positive endosomes (sites of PtdIns(3)P enrichment) but not LAMP1-positive late endosomes and lysosomes. Only TgPH1 co-localization with EEA1 is presented (see Figure 9). Statistical analysis will involve comparing TgPH1 fluorescence against EEA1 compared to TgPH1 fluorescence against LAMP1 using Pearson's coefficient or Mander's coefficient. Additionally, TgPH1 seems to co-localize with existing probes for PtdIns(3)P, the PX and FYVE based probes (see Figures 9 and 10). We still need to determine whether the probes are recognizing the same pools of PtdIns(3)P or to different pools of PtdIns(3)P. For example, PtdIns(4)P was mainly thought to localize to the Golgi as reported by GFP-PH(FAPP1) but using a reporter based on the P4M domain from *Legionella pneumophila* protein SidM revealed pools of PtdIns(4)P at the plasma membrane and late endosomes/lysosomes (Hammond, Machner and Balla, 2014). Co-expressing PX-based probes, FYVE probes and TgPH1 probes and imaging via super-

resolution microscopy may reveal whether the different probes recognize the same pools of PtdIns(3)P or different pools.

After completing the characterization of TgPH1, future steps may involve introducing mutations into the residues involved in PtdInsP binding. Mutations have been introduced into FYVE domains to pinpoint the critical residues involved in phosphoinositide binding (Burd and Emr, 1998). Naturally occurring mutations have also been identified in PH domains of proteins such as Bruton's tyrosine kinase (Btk) and AKT1 that interfere with phosphoinositide binding resulting in X-linked gammaglobulinaemia and cancer respectively (Baraldi *et al.*, 1999; Carpten *et al.*, 2007). Therefore, it is plausible that mutations could be introduced to the residues that are involved in phosphoinositide binding with the idea this would modulate the specificity and strength of interactions. It may be worthwhile to browse protein databases and identify putative PH domain-containing proteins and generate GFP-fusion chimeras to assess their viability as probes for PtdInsPs.

The development of protein based probes is for PtdInsPs is important since they offer some advantages over other methods such as exogenously added lipids or antibodies. The use of antibodies requires chemical fixatives and the integrity of the plasma membrane and other compartments might be compromised because of the unknown effect of chemical fixatives and permeabilization agents (Halet and Viard, 2008; Várnai *et al.*, 2017). The use of antibodies would not be permissible for rapid or transient changes in levels of PtdInsPs that are observed during live cell experimentation. This necessitates that phosphoinositide binding domains be used when studying the dynamics of PtdInsPs.

Overall, while TgPH1 did not fulfill our original intention of serving as a complementary tool for PtdIns(3,5)P_2, we propose that it is a useful complementary tool to investigate the location, sub-domain distribution and dynamics of PtdIns(3)P in mammalian cells.

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