

Chapter 2

Phosphoinositide Signaling During Membrane Transport in *Saccharomyces Cerevisiae*

Amber L. Schuh and Anjon Audhya

Abstract Phosphatidylinositol (PI) is distinct from other phospholipids, possessing a head group that can be modified by phosphorylation at multiple positions to generate unique signaling molecules collectively known as phosphoinositides. The set of kinases and phosphatases that regulate PI metabolism are conserved throughout eukaryotic evolution, and numerous studies have demonstrated that phosphoinositides regulate a diverse spectrum of cellular processes, including vesicle transport, cell proliferation, and cytoskeleton organization. Over the past two decades, nearly all PI derivatives have been shown to interact directly with cellular proteins to affect their localization and/or activity. Additionally, there is growing evidence, which suggests that phosphoinositides may also affect local membrane topology. Here, we focus on the role of phosphoinositides in membrane trafficking and underscore the significant role that yeast has played in the field.

Keywords Lysosomal/vacuolar trafficking · Autophagy · Endocytosis · Protein secretion · Actin cytoskeleton

2.1 Phosphoinositide Metabolism in the Yeast *Saccharomyces Cerevisiae*

In wild-type *Saccharomyces cerevisiae*, four major phosphorylated derivatives of PI have been identified: PI3P, PI4P, PI3,5P₂ and PI4,5P₂ (Fig. 2.1). A single soluble Class III PI 3-kinase, encoded by *VPS34*, generates the total cellular pool of PI3P, which accumulates mostly in the limiting membranes of endosomes and lysosome-like vacuoles (Schu et al. 1993; Stack and Emr 1994; Stenmark and Gillooly 2001).

A. Audhya (✉) · A. L. Schuh

Department of Biomolecular Chemistry, University of Wisconsin-Madison Medical School,
1300 University Avenue, WI, 53706 Madison, USA
e-mail: audhya@wisc.edu

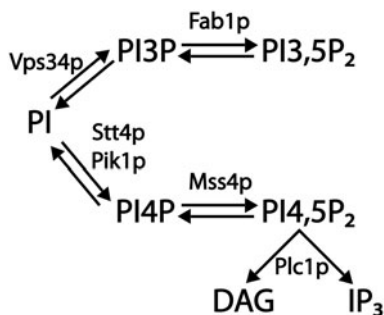


Fig. 2.1 Phosphoinositide metabolism in yeast. The synthesis pathways for the 4 phosphoinositides known to exist in yeast are shown. Major PI kinases are highlighted, as is the phospholipase C isoform Plc1p, which hydrolyzes PI4,5P₂. Not pictured are phosphoinositide phosphatases, which mediate the dephosphorylation of various PI derivatives

Vps34p functions as a subunit of at least two distinct protein complexes, both of which also contain the serine/threonine-protein kinase Vps15p. Myristoylation anchors Vps15p to membranes and assists in the recruitment of cytosolic Vps34p to appropriate intracellular membranes (Herman et al. 1991; Stack et al. 1993). Vps15p is considered to be a regulatory subunit within PI 3-kinase complexes and contains HEAT domains responsible for additional protein-protein interactions (Vanhaesebroeck et al. 2010). Despite the essential nature of the Vps15p kinase domain to Vps34p recruitment and activation, direct substrates of Vps15p remain undefined.

The stability of PI3P is regulated by both catabolic and anabolic pathways. Two major routes of PI3P degradation have been characterized. First, PI3P contained in the limiting membrane of endosomes is a substrate for proteolytic enzymes in the vacuole lumen. Inhibition of membrane transport to the vacuole thereby causes an increase in cellular PI3P levels (Wurmser and Emr 1998). Alternatively, PI3P is subject to dephosphorylation by a group of lipid phosphatases, which include the myotubularin-related enzyme Ymr1p and two synaptojanin-like proteins, Sjl2p and Sjl3p (Stolz et al. 1998; Parrish et al. 2004). Although PI3P synthesis is not essential to yeast cell viability, depletion of all three PI3P phosphatases results in lethality (Parrish et al. 2005). It remains unclear whether elevated PI3P levels are solely responsible for this effect, but the result highlights the importance of regulated phosphoinositide turnover for normal cell proliferation.

PI3P is also a substrate for the Fab1p lipid kinase, which specifically phosphorylates the D5 position in the inositol ring to generate PI3,5P₂. The majority of Fab1p localizes to the limiting membrane of the yeast vacuole, where it functions together with Vac7p, a transmembrane regulatory factor, to generate the total cellular pool of PI3,5P₂ (Bonangelino et al. 1997; Gary et al. 2002; Botelho et al. 2008). In the absence of Vac7p, levels of PI3,5P₂ become undetectable. However, deletion of Fig4p, a PI3,5P₂ 5-phosphatase, can suppress phenotypes exhibited by loss of Vac7p, indicating that Fab1p remains functional in the absence of its regulator (Gary et al. 2002). Consistent with this finding, mutant isoforms of Fab1p have been characterized, which bypass the requirement for Vac7p in PI3,5P₂ synthesis.

In contrast to the unique functions of Vps34p to generate 3' phosphoinositides and Fab1p to generate PI3,5P₂, three PI 4-kinases have been characterized in yeast and include the type IIIa PI 4-kinase *STT4*, the type IIIb PI 4-kinase *PIK1*, and the type II PI 4-kinase *LSB6*. Both Stt4p and Pik1p are essential genes in yeast, suggesting they possess non-overlapping functions and generate unique pools of PI4P, which cannot substitute for one another (Audhya et al. 2000). Consistent with this idea, Stt4p localizes primarily to the plasma membrane, while the majority of Pik1p accumulates on Golgi membranes (Walch-Solimena and Novick 1999; Audhya and Emr 2002). At the cell surface, Stt4p appear to coalesce into discrete patches that are enriched in the mother cell. Purification of Stt4p has revealed a number of interacting proteins that regulate Stt4p function. These include Sfk1p and Efr3p, two putative transmembrane proteins and Ypp1p, a soluble protein containing two tetratricopeptide repeat domains (Audhya and Emr 2002; Baird et al. 2008; Zhai et al. 2008). Inhibition of each regulator has been shown to decrease PI4P levels at the cell surface and perturb the localization of Stt4p. Unlike Sfk1p, both Efr3p and Ypp1p are essential for yeast cell viability, suggesting they may regulate unique biochemical pathways downstream of Stt4p, but further studies are required to address this issue.

Distinct from plasma membrane synthesis of PI4P, Pik1p generates an essential pool of PI4P on Golgi membranes. Proper targeting of Pik1p to the Golgi requires an interaction with Frq1p, a myristoylated regulator of Pik1p-mediated PI4P synthesis (Hendricks et al. 1999). Loss of Frq1p is lethal, but overproduction of Pik1p can bypass the requirement of Frq1p, suggesting that additional mechanisms exist to target Pik1p to Golgi membranes. In addition to its role at the Golgi, Pik1p undergoes nucleo-cytoplasmic shuttling and has been postulated to generate a nuclear pool of PI4P (Garcia-Bustos et al. 1994; Strahl et al. 2005). Frq1p is not required for Pik1p function in the nucleus, and a specific role for nuclear PI4P has yet to be defined.

Unlike Stt4p and Pik1p, Lsb6p activity is not essential for normal growth of *S. cerevisiae*, and its loss does not impact the total cellular levels of PI4P under standard conditions (Han et al. 2002; Shelton et al. 2003). However, overproduction of Lsb6p weakly suppresses the loss of Stt4p, indicating that Lsb6p can function as a PI 4-kinase *in vivo*. Localization studies have placed Lsb6p at the plasma membrane, consistent with its ability to suppress deletion of *STT4*, and the limiting membrane of the vacuole. Additionally, Lsb6p has been shown to regulate endosome motility (Chang et al. 2005). However, this function of Lsb6p is independent of its lipid kinase activity.

Similar to PI3P, metabolism of PI4P is mediated by a set of lipid phosphatases and a single lipid kinase, the PI4P 5-kinase Mss4p. The plasma membrane pool of PI4P generated by Stt4p is largely regulated by the Sac1p lipid phosphatase (Foti et al. 2001). Biochemical and localization studies indicate that Sac1p is a type II membrane protein that localizes mainly to the ER under normal growth conditions (Foti et al. 2001; Faulhammer et al. 2005). Recent findings indicate that Sac1p hydrolyzes PI4P at sites of ER-plasma membrane contact, acting from the ER in trans on its plasma membrane substrate (Stefan et al. 2011). The retention of Sac1p in the ER is dependent on the dolichol phosphate mannosyl synthase Dpm1p, and loss of Dpm1p results in the accumulation of Sac1p in the Golgi (Faulhammer et al.

2005). In the absence of Sac1p, PI4P levels on the cell surface dramatically increase in an Stt4p-dependent fashion (Roy and Levine 2004).

PI4P generated by Stt4p can also be converted into PI4,5P₂ by the action of Mss4p. Similar to Stt4p, Mss4p is largely restricted to discrete foci on the plasma membrane (Homma et al. 1998; Audhya and Emr 2002). However, patches of Stt4p and Mss4p do not overlap, suggesting that Mss4p can utilize alternative sources of PI4P at the cell surface (Audhya and Emr 2002). Consistent with this idea, elimination of Stt4p activity only diminishes PI4,5P₂ levels by ~50% (Audhya et al. 2000). The remaining pool of PI4,5P₂ is synthesized from PI4P initially generated at the Golgi by Pik1p, which is likely transported to the plasma membrane within the membranes of secretory vesicles. Golgi PI4P is further regulated by multiple phosphoinositide phosphatases, including members of the synaptojanin-like family of lipid phosphatases, Sjl2p and Sjl3p, as well as Sac1p (Guo et al. 1999a; Foti et al. 2001; Faulhammer et al. 2005).

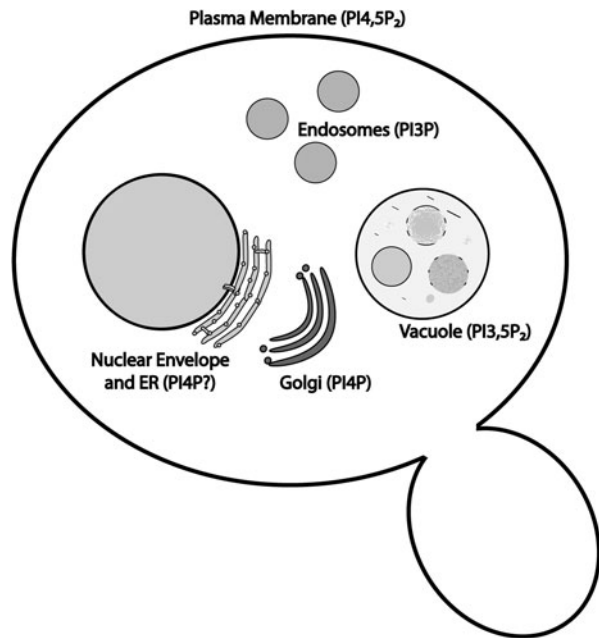
Although the majority of Mss4p localizes to the plasma membrane, the PI4P 5-kinase also undergoes nucleo-cytoplasmic shuttling similar to Pik1p (Audhya and Emr 2003). The factors that mediate nuclear import and export of Mss4p (Kap123p and Bcp1p) are distinct from those that regulate Pik1p nucleo-cytoplasmic transport (Kap95p and Msn5p) (Strahl et al. 2005). Also unlike Pik1p, inhibition of Mss4p nuclear entry fails to affect normal cellular growth, suggesting that nuclear PI4,5P₂ is not essential in yeast (Audhya and Emr 2003). Instead, targeting of Mss4p to the nucleus may function to regulate its activity on the cell surface and thereby control cytoplasmic PI4,5P₂ synthesis. Although factors required specifically for cell surface Mss4p patch formation have yet to be defined, plasma membrane targeting of Mss4p is partially dependent on its phosphorylation by yeast casein kinase I activity (Audhya and Emr 2003). Additionally, the small calcium-binding protein calmodulin appears to regulate Mss4p lipid kinase activity, although the mechanistic basis for this affect remains undefined (Desrivieres et al. 2002). Levels of PI4,5P₂ are further regulated by lipid phosphatases, including the synaptojanin-like proteins Sjl1p and Sjl2p, the ER-localized inositol 5-phosphatase Inp54p, and the phospholipase C isoform Plc1p, which specifically hydrolyzes PI4,5P₂ to generate diacylglycerol (DAG) and IP₃ (Flick and Thorner 1993; Wiradjaja et al. 2001; Stefan et al. 2002).

Through these highly regulated biosynthetic and degradation pathways, individual phosphoinositides are spatially restricted within the cell. PI3P and PI3,5P₂ are enriched in endosomal and vacuolar membranes, while PI4P and PI4,5P₂ are concentrated in the Golgi and plasma membrane (Fig. 2.2). In the following sections, we will highlight mechanisms by which phosphoinositides function to regulate virtually all of the membrane trafficking pathways that have been described in yeast.

2.2 Binding Domains and Effector Proteins of PI3P

PI3P carries out its cellular functions by recruiting and/or activating a subset of proteins to specific internal membrane compartments where PI3P is synthesized. These proteins function in signal transduction, vesicle trafficking, and cytoskeletal rearrangements. Despite these differences in function, the majority of proteins known to

Fig. 2.2 Distribution of phosphoinositides in yeast. A cartoon depicting a yeast cell, with various organelles that harbor phosphoinositides highlighted. The plasma membrane is enriched with PI4,5P₂, the vacuole with PI3,5P₂, endosomes with PI3P, and the Golgi with PI4P. The pool of PI4P synthesized on the plasma membrane is not shown for simplicity



bind to PI3P share a small subset of domains that interact directly with the phosphoinositide (Stenmark and Gillooly 2001, Seet and Hong 2006). We will first highlight roles for PI3P generated at the endosome by PI3K Complex II, which is composed of Vps15p, Vps34p, Vps30p and Vps38p (Kihara et al. 2001). In particular, effectors of PI3P in both anterograde transport from the endosome to the vacuole and retrograde transport from the endosome to the Golgi will be discussed. Additionally, we will describe alternative roles for PI3P in constitutive and starvation-induced autophagy pathways, which utilize PI3K Complex I, containing Vps15p, Vps34p, Vps30p, and Atg14p (Kametaka et al. 1998; Kihara et al. 2001).

2.2.1 The FYVE Domain

The FYVE domain is composed of ~70 conserved residues that specifically bind PI3P. This domain is cysteine rich and coordinates two zinc ions, which are essential for structural integrity and PI3P binding (Gaullier et al. 1998; Burd and Emr 1998; Kutateladze et al. 1999; Misra and Hurley 1999). Three conserved sequences have been identified in all FYVE domains: the amino-terminal WxxD motif, the central (R/K)(R/K) HHCR motif, and the carboxyl-terminal RVC motif. These three regions are crucial in forming a concave binding pocket for PI3P association. In addition, the FYVE domain contains a hydrophobic protrusion, commonly referred to as the membrane insertion loop (MIL) domain, adjacent to the (R/K)(R/K) HHCR motif (Kutateladze et al. 1999; Misra and Hurley 1999).

The FYVE domain uses a multivalent mechanism to anchor itself to a target membrane, which includes specific interactions with PI3P as well as other lipids. Based upon the calculated electrostatic properties of several FYVE domains, a strong positive potential exists around the MIL domain, which supports interactions with acidic phospholipids (Diraviyam et al. 2003). Consistent with this finding, studies have shown that the acidic phospholipid phosphatidylserine (PS) specifically enhances the affinity of FYVE domains for lipid bilayers (Stahelin et al. 2002). In addition, these nonspecific electrostatic interactions likely help to align the FYVE domain in an optimal position for membrane penetration of the MIL domain (Stahelin et al. 2002; Kutateladze et al. 2004). Insertion of the hydrophobic MIL domain is dependent upon the presence of PI3P, and this process has been shown to significantly increase the affinity between lipid bilayers and FYVE domains (Kutateladze et al. 2004). Mutagenesis of select hydrophobic residues within the MIL region drastically reduces the affinity for membrane bound PI3P and disrupts normal function of FYVE domain containing proteins (Kutateladze et al. 1999; Stahelin et al. 2002).

The association of FYVE domains with membranes also appears to be pH dependent. In a neutral buffer (pH 7.0), FYVE domain containing proteins in yeast localize to endosomal and vacuolar membranes. However, when cells are shifted into a mildly basic buffer (pH 8.0), FYVE domain containing proteins largely redistribute to the cytoplasm (Lee et al. 2005; He et al. 2009). These data support a model in which the two histidine residues within the conserved (R/K)(R/K) HHCR motif must be protonated to form hydrogen bonds with the 3' phosphate of PI3P (Dumas et al. 2001; Kutateladze 2006).

In total, five FYVE domain containing proteins have been identified in yeast: Vac1p, Fab1p, Vps27p, Pib1p, and Pib2p. Vac1p is required for the transport of vesicles from the Golgi to prevacuolar endosomes (Webb et al. 1997). Similar to other FYVE domains, the Vac1p FYVE domain binds directly to PI3P and localizes to endosomes when expressed as a GFP fusion. However, in cells lacking PI3P, Vac1p continues to accumulate on membranes, suggesting that the interaction between Vac1p and PI3P does not solely influence its localization (Tall et al. 1999). Instead, PI3P may regulate Vac1p activity, potentially by influencing its interactions with other proteins. Consistent with this idea, both the Rab-type GTPase Vps21p and the Sec1-like protein Vps45p, interact with Vac1p in a FYVE domain-dependent manner (Peterson et al. 1999; Tall et al. 1999).

Fab1p, a PI3P 5-kinase, and Vps27p, a component of the ESCRT (endosomal sorting complex required for transport) machinery, have both been implicated in the formation of multivesicular endosomes (MVEs), which are specialized organelles essential for the degradation of many transmembrane proteins (Odorizzi et al. 1998; Katzmman et al. 2003; Bilodeau et al. 2003; Raiborg and Stenmark 2009). In this pathway, ubiquitin-modified cargoes are initially sequestered within vesicles that bud into the endosome lumen. Upon MVE fusion with the vacuole, cargo-laden vesicles are transferred directly into the hydrolytic environment of the vacuole lumen, resulting in protein and vesicle degradation. In the absence of PI3P, neither Vps27p nor Fab1p localize properly, and MVE biogenesis is dramatically inhibited. Unlike the FYVE domain of Vps27p, which localizes to endosomes when expressed as

a GFP fusion, the Fab1p FYVE domain associates specifically with the vacuolar limiting membrane (Botelho et al. 2008). Although it has been shown to bind PI3P directly, these data suggest that the Fab1p FYVE domain may also bind another factor, which directs its accumulation onto the vacuole membrane. The identity of such a factor remains unknown.

In addition to the interaction between PI3P and the FYVE domains of Vps27p and Fab1p, the ESCRT-II subunit Vps36p, which also functions in MVE biogenesis, binds to PI3P through a noncanonical binding pocket within its amino-terminal GLUE (GRAM-like, ubiquitin binding in EAP45) domain (Teo et al. 2006). The GLUE domain exhibits a split PH (pleckstrin homology) domain fold with two curved beta sheets and a single long alpha helix. A highly basic pocket is delineated by three variable loops within the GLUE domain, a configuration that is distinct from most PH domains that interact with phosphoinositides. Mutations within the basic region inhibit protein sorting into MVEs, indicating association of ESCRT-II with PI3P is critical for function (Teo et al. 2006; Im and Hurley 2008).

Pib1p may also function in the MVE pathway as an E3 RING-type ubiquitin ligase. Studies indicate that Pib1p localizes to both endosomal and vacuolar membranes, and its distribution is dependent solely on its interaction with PI3P (Shin et al. 2001). Although specific Pib1p-dependent cargoes have yet to be identified, it is likely that Pib1p functions at the initial stages of cargo selection/modification in the MVE pathway. The role of Pib2p remains unknown, although localization studies indicate that it accumulates on the vacuolar membrane under steady state conditions (Huh et al. 2003).

2.2.2 *The Phox Homology (PX) Domain*

The PX domain is composed of approximately 130 amino acids and is found in proteins that function in vesicle trafficking, protein sorting, and lipid modification (Seet and Hong 2006). Typically, the PX domain folds into a compact structure composed of three beta strands followed by three alpha helices. A conserved RR(F/Y)S(D/E)F motif and three additional basic residues located nearby are proposed to form a binding pocket for PI3P (Cheever et al. 2001; Bravo et al. 2001; Sato et al. 2001). In addition, many of the PX domains contain a polyproline motif (PxxP), which is predicted to interact with SH3 domains (Xu et al. 2001). PX domains also interact with membranes by using a multivalent mechanism, which includes non-specific electrostatic interactions, hydrophobic insertion, and oligomerization. In particular, oligomerization is crucial for increasing the affinity of PX domains that otherwise would be unable to localize to membranes due to low affinities in their monomeric state (Kutateladze 2007).

In yeast, 15 proteins that harbor a PX domain have been identified, and all have an affinity for PI3P. Using surface plasmon resonance (SPR), four yeast PX domains have been classified as having a high affinity for PI3P, ranging from 0.15 to 0.5 μ M,

while the rest exhibit affinities greater than 100 μM and are classified as low affinity PX domains (Yu and Lemmon 2001). These affinity measurements suggest that only the four high affinity PX domains are capable of membrane association independently of other binding partners, while the remaining PX domains must require oligomerization or interaction with additional proteins to mediate their localization.

The four yeast proteins with high affinity for PI3P are Mdm1p, Snx3p, Vam7p and Ypt35p. Mdm1p is required for mitochondrial and nuclear inheritance, and although its PX domain has been shown to bind to PI3P with a high affinity, the function of this interaction remains unknown (Yu and Lemmon 2001). Snx3p is a member of the sorting nexin family and functions in the retrograde transport of a subset of cargoes from endosomes to the Golgi (Strochlic et al. 2007). Importantly, the retrieval of certain transmembrane receptors from late endosomes is required to maintain the proper sorting of hydrolases to the vacuole. One of the best studied receptors is the type I membrane protein Vps10p, which is essential for the normal trafficking of the soluble vacuolar hydrolase carboxypeptidase Y (CPY). At the endosome, CPY dissociates from Vps10p for ultimate delivery to the vacuole, while Vps10p undergoes recycling to the trans Golgi network (TGN), becoming available for another round of CPY transport (Marcusson et al. 1994; Stack et al. 1995). The high affinity of Snx3p for PI3P targets it to tubular endosomes where it can associate with Vps10p and mediate its retrieval. However, mutations in the Snx3p PX domain that inhibit PI3P binding result in the missorting of receptors to the vacuole, inhibiting further transport of CPY and other cargoes (Strochlic et al. 2007). These data implicate PI3P in retrograde trafficking from endosomes to the Golgi. Snx3p functions together with the retromer complex, a set of proteins also required for retrograde trafficking from endosomes to the Golgi. Two components of the retromer complex, Vps5p and Vps17p, also contain PX domains, each with a low affinity for PI3P. However, when co-assembled, the multiple low affinity interactions with PI3P maintain an endosomal distribution for the retromer complex, and further highlight a role for PI3P in orchestrating receptor recycling from the endosomal system (Burda et al. 2002).

The third high affinity PI3P interacting protein Vam7p is a target SNARE (Soluble NSF Attachment Protein Receptor) that functions during the docking and fusion steps of membrane transport to the vacuole (Cheever et al. 2001; Song et al. 2001). An intact PX domain is required for this function, implicating PI3P in the late stages of membrane transport to the vacuole. Finally, Ypt35p also binds to PI3P with high affinity and has been found to localize to endosomal membranes. However, the specific function of Ypt35p remains unknown (Yu and Lemmon 2001).

Among the other low affinity PI3P-interacting PX domains, Mvp1p, Spo14p, Snx4p, Snx41p, and Atg20p each possess characterized roles in membrane trafficking. Mvp1p is an endosomal protein required for protein sorting to the vacuole. Although its precise role has not been clearly identified, genetic studies indicate that Mvp1p functions with the dynamin-like protein Vps1p in retrograde protein transport from endosomes to the Golgi (Ekena and Stevens 1995). Spo14p is a phospholipase D isoform that has been implicated in protein secretion (Sreenivas et al. 1998). During vegetative growth, Spo14p localizes to endosomes, but its specific activity there

is poorly characterized, and it would be premature to suggest that PI3P functions directly in the secretory pathway (Sciorra et al. 2002). Snx4p (also known as Atg24p), Snx41p, and Atg20p (also known as Snx42p) all are sorting nexins that function in a common complex to sort receptors from early endosomes to the Golgi (Hettema et al. 2003). Even though the binding affinities for individual PX domains within the individual sorting nexins are low, their assembly into a complex leads to PX domain multimerization and an increased affinity for PI3P on endosomes.

In addition, both Snx4p/Atg24p and Atg20p are required for selective autophagy, a constitutive cytoplasm to vacuole targeting (CVT) pathway in which a perivacuolar phagophore assembly site (PAS) initiates the biogenesis of an autophagosome, a double-membrane vesicle (Nice et al. 2002; He and Klionsky 2009). This pathway depends on a large group of proteins that assist in the elongation of a double-membrane structure known as an isolation membrane that ultimately circularizes to form an autophagosome. The autophagosome then fuses with the vacuole, leading to the degradation of its contents (Huang and Klionsky 2002). When yeast cells are under starvation, an alternative bulk autophagy pathway is initiated to break down proteins into amino acids for energy (Burman and Ktistakis 2010). The CVT and bulk autophagy pathways share many of the same proteins, including the PI3K Complex I (Vps15p, Vps34p, Vps30p, and Atg14p). The Atg14p subunit of this complex provides specificity in directing Vps34p to PASs (Farre et al. 2009). Interactions between PI3P and the PX domains of Snx4p/Atg24p and Atg20p are required for normal CVT pathway function, directly implicating PI3P in the constitutive formation of autophagosomes (Nice et al. 2002).

Further highlighting a role for phosphoinositides in the CVT pathway are two additional PI3P effectors called Atg21p and Atg27p. Unlike other PI3P binding proteins, neither Atg21p nor Atg27p harbor a PX or FYVE domain. Instead, PI3P binding is mediated by short basic stretches of amino acids, FRRG in Atg21p and KKPAKK in Atg27p (Wurmser and Emr 2002; Stromhaug et al. 2004; Krick et al. 2006; Nair et al. 2010). Mutations in these motifs, which inhibit PI3P binding, block CVT pathway function. In addition, deletion of *VPS34* or *VPS15* has also been shown to block starvation-induced bulk autophagy (Wurmser and Emr 2002). One phosphoinositide effector potentially responsible for this effect is Atg18p, which also contains an FRRG motif capable of binding to PI3P (Barth et al. 2001; Dove et al. 2004). Although the degree to which mutations in the Atg18p PI3P-binding motif affects bulk autophagy remains controversial (Krick et al. 2006), the participation of PI3P in autophagosome biogenesis is incontrovertible.

In summary, studies in yeast have clearly illustrated the various roles PI3P plays in membrane trafficking, both in directing localization of effector molecules and regulating their activities. Although it is likely that several new PI3P effectors still await characterization, most will likely function at endosomes, vacuoles, or autophagosomes, where PI3P is highly enriched. We predict that many of these new molecules will exhibit a low affinity for PI3P, but together with other protein-protein interactions, specificity for endosomal/autophagosomal signaling pathways can be achieved.

2.3 Effectors of PI3,5P₂ and Their Roles in Membrane Trafficking

In yeast, a single PI3P 5-kinase called Fab1p has been identified and is responsible for the generation of PI3,5P₂ (Cooke et al. 1998; Gary et al. 1998). The Fab1p lipid kinase contains three conserved protein domains: an amino-terminal FYVE domain, a Cpn60/TCP-1 chaperonin family (CCT) domain, and a carboxyl-terminal lipid kinase domain (Gary et al. 1998; Efe et al. 2005). PI3,5P₂ is largely generated on the vacuolar limiting membrane, where it has a role in retrograde trafficking from the vacuole and vacuolar homeostasis (Dove and Johnson 2007). Deletion of *FAB1* leads to several phenotypes including enlarged vacuoles, defects in MVE biogenesis, a lack of vacuolar acidification and slow temperature sensitive growth (Yamamoto et al. 1995; Cooke et al. 1998; Gary et al. 1998; Odorizzi et al. 1998). Here, we will discuss the effectors of PI3,5P₂ that have been discovered and how they relate to the effects seen upon loss of Fab1p activity.

Two classes of PI3,5P₂ binding domains have been identified. The first is found in a family of seven bladed β -propeller proteins that bind phosphoinositides (PROPPINs), and the second is contained within members of the epsin family. In yeast, three PROPPIN proteins have been identified, including Atg18p, Atg21p and Hsv2p (Michell et al. 2006). As discussed earlier, both Atg18p and Atg21p harbor a FRRG motif capable of interacting with PI3P. However, SPR studies indicate they can also bind PI3,5P₂ with high affinity (~ 500 nM) (Dove et al. 2004). Moreover, loss of Atg18p causes a dramatic increase in vacuole size, similar to the phenotype observed following *FAB1* deletion, suggesting a role downstream of PI3,5P₂ signaling (Dove et al. 2004; Cooke et al. 2004). Localization studies indicate that all three proteins associate with both endosomal membranes and the limiting membrane of the vacuole. It is possible that PROPPIN proteins associate with both PI3P and PI3,5P₂ and exhibit distinct activities depending on the lipid to which they are bound. For example, interactions between PI3P and Atg18p and Atg21p are likely important for constitutive autophagy, which does not depend on Fab1p-mediated PI3,5P₂ production (Gary et al. 1998; Wurmser and Emr 2002). In contrast, interaction between PI3,5P₂ and Atg18p is probably required for normal vacuole homeostasis and retrograde trafficking from the vacuole to the Golgi via an endosomal intermediate (Dove et al. 2004). Notably, in cells lacking Fab1p, the PROPPIN proteins continue to localize to the endosome and vacuolar membranes (Efe et al. 2007). Nevertheless, PI3,5P₂ binding to these factors may regulate their function as opposed to their localization. Hsv2p is the least well characterized PROPPIN in yeast, but studies indicate that the protein participates in a unique autophagic pathway responsible for the turnover of nuclear membranes (Krick et al. 2008). It remains unclear whether PI3,5P₂ may function to regulate this activity.

As discussed earlier, Fab1p also regulates the biogenesis of luminal vesicles within endosomes (Odorizzi et al. 1998). While none of the PROPPIN proteins exhibit a function at the MVE, two other effectors of PI3,5P₂ have been implicated in MVE-mediated protein sorting. Both Ent3p, which contains a phosphoinositide binding motif called the ENTH (epsin N-terminal homology) domain, and Ent5p, which harbors a related ANTH (AP180 N-terminal homology) domain, bind to PI3,5P₂

in vitro and require Fab1p activity for localization *in vivo* (Friant et al. 2003; Eugster et al. 2004). Additionally, the simultaneous loss of Ent3p and Ent5p interferes with the trafficking of integral membrane proteins to the vacuole lumen, similar to the phenotype exhibited by *fab1* mutant cells (Eugster et al. 2004). Moreover, both Ent3p and Ent5p have been implicated in AP-1 (adaptor protein 1)-dependent sorting of chitin synthase, a process previously shown to require Fab1p activity, further suggesting a role for the epsin-like proteins downstream of PI3,5P₂ signaling (Costaguta et al. 2006; Phelan et al. 2006; Copic et al. 2007).

The phenotypes exhibited by mutant yeast cells lacking Fab1p cannot be explained by the few effector molecules that have been discovered to date. Therefore, it is highly probable that additional PI3,5P₂-binding proteins await characterization. In particular, the proteins that mediate Fab1p-dependent vacuolar acidification remain unknown. Although speculative, the vacuolar ATPase, which is known to regulate the acidification of vacuoles, is a likely candidate for this function. Presumably, there are also other PI3,5P₂ effectors, in addition to Atg18p, which participate in the retrograde transport of proteins from the vacuole to endosomes, since deletion of *ATG18* does not phenocopy the effects of *FAB1* deletion in this pathway (Efe et al. 2007). The limited production of PI3,5P₂ in yeast cells has hindered progress to identify key effectors of this lipid, but further genetic and biochemical studies will almost certainly uncover new proteins that harbor binding domains specific for PI3,5P₂.

2.4 Roles for PI4P in Membrane Transport

At least two non-redundant pools of PI4P are synthesized in yeast cells, one at the Golgi apparatus and a second at the plasma membrane. Each functions in multiple membrane trafficking pathways, involving both protein and lipid transport. The type IIb PI 4-kinase Pik1p regulates PI4P production at the Golgi, and plays critical roles in maintaining secretory protein export to the cell surface, trafficking of cargoes to the vacuole, and endocytic protein transport (Hama et al. 1999; Walch-Solimena and Novick 1999; Audhya et al. 2000). The type IIIa PI 4-kinase controls PI4P synthesis at the plasma membrane and has also been implicated in endocytic trafficking, likely through its regulation of actin cytoskeleton organization (Audhya and Emr 2002; Tahirovic et al. 2005). Additionally, roles for PI4P extend to the endoplasmic reticulum, both in protein secretion from this compartment and aminophospholipid transport (Trotter et al. 1998; Lorente-Rodriguez and Barlowe 2011). Here, we will discuss the various roles of PI4P at different intracellular compartments, specifying effectors that have been identified thus far, which function in membrane transport.

2.4.1 Functions of PI4P Synthesized by Pik1p in the Golgi

A function for PI4P in secretion from the Golgi in yeast was initially suggested by studies that focused on the PI transfer protein Sec14p, which is essential for the

biogenesis of Golgi transport vesicles (Kearns et al. 1997). In a screen for bypass suppressors of a *sec14* temperature sensitive allele, a mutant isoform of the Sac1p phosphoinositide phosphatase was identified. Loss of Sac1p activity led to an increase in PI4P that was necessary for *sec14* suppression, suggesting a role for this lipid in Golgi secretion. Consistent with this finding, overexpression of the PI 4-kinase *PIK1* partially restored the growth of *sec14* mutant cells at elevated temperature (Hama et al. 1999). Moreover, loss of Sec14p activity led to diminished production of PI4P, suggesting a role for Sec14p in regulating Pik1p activity. Since subsequent studies have demonstrated that *sac1* mutant cells accumulate PI4P mostly at the cell surface in an Stt4p-dependent manner, the precise nature of Sac1p-mediated Sec14p bypass remains unclear (Foti et al. 2001; Roy and Levine 2004). However, additional studies further confirmed an essential function for PI4P synthesis at the Golgi. Specifically, multiple *pik1* mutant alleles have been isolated, and each confers a defect in protein transport from the Golgi (Hama et al. 1999; Walch-Solimena and Novick 1999). In some cases, Golgi to plasma membrane secretion is specifically inhibited by loss of Pik1p activity, but other *pik1* mutant strains exhibit more severe defects in Golgi function that affect both secretory protein transport and the trafficking of biosynthetic cargoes from the Golgi to endosomes and the vacuole (Audhya et al. 2000; Sciorra et al. 2005; Lorente-Rodriguez and Barlowe 2011). Additionally, protein glycosylation in the Golgi is adversely affected by the absence of Pik1p activity, suggesting that multiple cisternae require PI4P synthesis for normal assembly and/or function (Audhya et al. 2000).

Several effectors of PI4P that function at the Golgi have been described in yeast. The first were members of the oxysterol binding protein (OSBP) family, which likely function in the transfer of lipids between biological membranes (Li et al. 2002). There are a total of 7 OSBPs in yeast, Osh1p-Osh7p, which share a common essential function, and each binds promiscuously to phosphoinositides (Beh et al. 2001; Schulz and Prinz 2007). Osh1p, Osh2p and Osh3p each harbor an amino-terminal PH domain, a known phosphoinositide interacting motif. In general, PH domains are composed of approximately 120 amino acids that share a common structure consisting of two perpendicular anti-parallel beta sheets, followed by a carboxyl-terminal amphipathic helix (Lemmon 2008). Although diverse in amino acid composition, a single tryptophan located within the helix serves to nucleate the core of the domain. A survey of more than 33 PH domains in yeast revealed that most bind to phosphoinositides with little specificity and low affinity, and proteins that harbor PH domains typically require additional interactions for proper intracellular targeting (Yu et al. 2004). By analyzing GFP fusions to the PH domain-containing OSBPs, Osh1p was shown to localize in part to the Golgi and is potentially involved in sterol transfer with other organelles (Levine and Munro 2001). In contrast, neither Osh2p nor Osh3p discernibly accumulated on Golgi membranes, although the PH domain of Osh2p alone binds to PI4P on the Golgi in a Pik1p-dependent fashion (Levine and Munro 2002). These data highlight the role of additional protein-protein interactions in specifying the distribution of PH domain containing proteins in yeast.

In the case of Osh4p, phosphoinositide binding is mediated by the conserved sterol binding domain, which is found in all OSBPs (Li et al. 2002). Studies indicate

that Osh4p localizes to the Golgi in a PI4P-dependent fashion and may mediate the movement of sterols to and from this organelle, thereby regulating lipid homeostasis and vesicle biogenesis. Additionally, recent findings indicate that Osh4p negatively regulates Pik1p activity (Fairn et al. 2007). These data suggest that Osh4p may “sense” PI4P levels on Golgi membranes. For example, if a high concentration of PI4P accumulated on the Golgi, Osh4p recruitment would increase to inhibit Pik1p and slow PI4P synthesis. This type of feedback inhibition may be especially important to regulate secretion in response to changing growth conditions and environmental stress (Faulhammer et al. 2007).

In addition to members of the OSBP family, the gamma-ear-containing, ADP-ribosylation factor binding protein Gga2p has also been shown to interact with PI4P at the Golgi (Demmel et al. 2008). Gga2p functions as an adaptor for clathrin recruitment and participates in transport between the Golgi and endosomes (Black and Pelham 2000; Costaguta et al. 2001). Loss of Gga2p function phenocopied several of the morphological and secretory defects observed in *pik1* mutant cells, including the accumulation of abnormal, cup-shaped membranous structures in the cytoplasm termed “Berkeley bodies.” These data suggest that Gga2p may be the most relevant Pik1p-dependent PI4P effector in Golgi to endosome trafficking (Demmel et al. 2008). PI4P binding is mediated by the VHS (Vps27p/Hrs/STAM) domain of Gga2p, which shares significant similarity to the structure of phosphoinositide-binding ANTH/ENTH domains (Demmel et al. 2008). In general, VHS domains contain approximately 150 amino acids and consist of 8 helices arranged in a super-helix (Mao et al. 2000). In Gga2p, the loop preceding helix 8 exhibits a pattern of charged and aromatic residues, similar to those found in the ANTH domain of CALM, a clathrin adaptor that interacts with PI4,5P₂ at the plasma membrane (Stahelin et al. 2003; Demmel et al. 2008). Mutations within this basic region of Gga2p disrupted its association with PI4P and diminished Gga2p association with the Golgi. However, a GFP fusion to the VHS domain of Gga2p showed that it alone was insufficient to localize to Golgi membranes. Instead, the neighboring Arf1p GTPase-interacting GAT domain was also required. Therefore, similar to most PH domain containing proteins in yeast, both lipid-protein and protein-protein interactions are required for proper Gga2p localization (Zhdankina et al. 2001; Demmel et al. 2008).

As noted earlier, protein glycosylation in the secretory pathway is sensitive to impaired Pik1p function, suggesting that PI4P may be required for the proper function or localization of Golgi glycosyltransferases. In many cases, retention of these enzymes in the Golgi requires the function of Vps74p, an oligomeric protein that binds to the cytosolic domains of glycosyltransferases and restricts their transport to other organelles (Schmitz et al. 2008; Tu et al. 2008). Based on crystallographic data, Vps74p harbors four alpha-helices that form a central core, which is surrounded by several additional amphipathic alpha-helices and four beta-strands (Schmitz et al. 2008). The recruitment of Vps74p to the Golgi is mediated by PI4P generated by Pik1p (Wood et al. 2009). In *pik1* mutant cells, Vps74p becomes cytosolic, and glycosyltransferases are no longer retained in the Golgi, thus leading to defects in secretory cargo glycosylation. The PI4P-binding motif within Vps74p is composed of a conserved basic region near the amino-terminus of helix 6, and mutations in this

domain inhibit Vps74p-mediated retention of Golgi glycosyltransferases. Although the loss of other PI4P effectors may further contribute to the defects in protein glycosylation observed in *pik1* mutant cells, these data confirm a role for PI4P in this process and further demonstrate that Pik1p-mediated PI4P production is required for retrograde transport of cargoes in the Golgi (Wood et al. 2009).

Effectors of PI4P in yeast that function during secretory membrane transport have been arguably the most challenging to identify. Genetic studies strongly suggest that Pik1p functions together with multiple components of the secretory pathway, including at least three Rab-type GTPases (Ypt31p, Ypt32p, and Sec4p), two tethering complexes (TRAPP II and exocyst), components of the actin-myosin network, and the phospholipid flippase Drs2p (Walch-Solimena and Novick 1999; Sciorra et al. 2005). In particular, Rab GTPases are known regulators of membrane trafficking, which have been shown to interface with phosphoinositide signaling during endosomal sorting (Zerial and McBride 2001). One attractive model for Golgi secretion involves a cascade of Rab activation, in which the Golgi-localized Ypt32p GTPase recruits the guanine nucleotide exchange factor (GEF) for the subsequent Rab GTPase that acts in the pathway (Mizuno-Yamasaki et al. 2010). Indeed, the active GTP-bound form of Ypt32p, generated by the GEF activity of the TRAPP II tethering complex, binds directly to Sec2p, which catalyzes GTP exchange on Sec4p that is present on secretory vesicles (Ortiz et al. 2002). Active Sec4p can then recruit components of the exocyst complex, which are necessary for vesicle fusion with the plasma membrane (Guo et al. 1999b). The Rab cascade is further regulated by Sec15p, a component of the exocyst complex, which competes with Ypt32p for Sec2p binding (Mizuno-Yamasaki et al. 2010). The precise mechanism by which Sec15p replaces Ypt32p remains unknown, but recent evidence implicates a role for PI4P in this switch. Specifically, Sec2p was found to interact directly with PI4P generated by Pik1p on Golgi membranes. Three basic patches within the Sec2p GEF were found to be important for PI4P binding *in vitro*, and a mutant isoform of Sec2p containing mutations in these regions failed to localize properly (Mizuno-Yamasaki et al. 2010). Both Ypt31p and Ypt32p were also found to be important for Sec2p localization, suggesting that a combination of protein-protein and lipid-protein interactions were necessary for proper Sec2p targeting. Consistent with this idea, Sec2p was shown to form a ternary complex with both PI4P and Ypt32p *in vitro*. In contrast, PI4P inhibited the association of Sec2p with Sec15p, suggesting a role for PI4P in stabilizing the association between Sec2p and Ypt32p at the Golgi to drive vesicle formation (Mizuno-Yamasaki et al. 2010). Subsequent to vesicle budding, Sec2p may catalyze the formation of active, GTP-bound Sec4p, initiating the recruitment of exocyst subunits including Sec15p, which ultimately displaces Ypt32p. Although this idea is speculative, the data clearly define a novel function for PI4P in secretion from the Golgi and help to explain several of the genetic interactions defined previously using *pik1* mutant cells.

In addition to the role of Rab-type GTPases, vesicle biogenesis from the Golgi also requires the action of Drs2p, a type IV P-type ATPase, which catalyzes the translocation of aminophospholipids from one leaflet of the lipid bilayer to the other. Similar to Pik1p, Drs2p has been implicated in the formation of vesicles destined

for the plasma membrane as well as the endosomal/vacuolar system (Chen et al. 1999; Hua et al. 2002; Gall et al. 2002). Recently, the flippase activity of Drs2p was shown to be dependent on PI4P generated by Pik1p (Natarajan et al. 2009). A phosphoinositide binding site within Drs2p was identified and found to exhibit similarity to the PI3P-interacting split PH domain of Vps36p, a component of the ESCRT-II complex. A basic motif within this region (RMKKQR) was critical for PI4P binding *in vitro*, and mutations in this region prohibited complementation in *drs2* mutant cells. Unlike other roles for PI4P in targeting effectors to the Golgi, these data highlight a unique function for this lipid in regulating an enzymatic activity necessary for Golgi vesicle formation.

Since secretory vesicles move from the Golgi to the plasma membrane along actin cables in yeast, it is not surprising that mutations affecting actin cytoskeleton organization are lethal to *pik1* mutant cells (Pruyne et al. 2004; Walch-Solimena and Novick 1999). Further study into this connection has demonstrated that PI4P present in secretory vesicles is critical for vesicle movement mediated by the type V myosin Myo2p. Under normal conditions, directed vesicle movement also requires interactions between Myo2p and the Rab-type GTPases Ypt31p, Ypt32p, and Sec4p. However, by enhancing the association between Myo2p and PI4P, binding to the Rab-type GTPases becomes dispensable (Santiago-Tirado et al. 2011). Although Myo2p has not been demonstrated to interact directly with PI4P, these findings strongly suggest that coincidence detection of PI4P and Rab GTPases is important for myosin-dependent transport of secretory vesicles. In the future, it will be critical to understand the mechanism by which Myo2p recognizes vesicles containing PI4P.

While the majority of studies have focused on a role for Pik1p-mediated PI4P production at the trans Golgi network, a cell free assay used to study ER to Golgi transport uncovered a critical role for PI4P in this pathway. Specifically, the presence of PI4P in the cis-Golgi was found to be necessary for COPII vesicle fusion (Lorente-Rodriguez and Barlowe 2011). Although vesicle tethering did not require the presence of PI4P, inhibitors of PI4P reduced SNARE complex assembly. Analysis of *pik1* mutant cells further suggested a role for PI4P in anterograde transport between the ER and Golgi. However, a specific effector of PI4P at this early step of the secretory pathway remains unidentified. In a similar fashion, several studies have suggested a potential role for PI4P at the endosome, but confirmation of such a function awaits the characterization of a PI4P-binding protein, which regulates endosomal trafficking (Walch-Solimena and Novick 1999; Audhya et al. 2000).

2.4.2 Functions of PI4P Synthesized by Stt4p at the Plasma Membrane

Although a significant portion of PI4P generated by Stt4p is rapidly metabolized to PI4,5P₂ (discussed in the next section), the unique phenotypes of *stt4* mutant cells suggest that the plasma membrane pool of PI4P has specific effectors. Consistent with this finding, the Cla4p protein kinase, which is involved in actin cytoskeleton

organization, polarized cell growth and cell division, harbors a PH domain that binds to PI4P generated by Stt4p (Eby et al. 1998; Wild et al. 2004). In cells lacking Stt4p kinase activity, Cla4p is mislocalized from the cell surface, suggesting that its interaction with PI4P is critical for its normal targeting. Like other yeast proteins that harbor PH domains, interaction with PI4P alone is insufficient to direct localization of Cla4p. However, together with another interacting protein, the Cdc42p Rho-type GTPase, Cla4p is able to maintain a polarized distribution at the plasma membrane (Wild et al. 2004). These data again highlight coincident roles for lipid and protein interactions to maintain the cellular distribution of phosphoinositide interacting molecules. Furthermore, these findings illustrate that PI4P directly regulates actin polarity by controlling Cla4p recruitment to the plasma membrane. Notably, in the absence of Stt4p function, directed membrane transport to the bud is disrupted by perturbations in actin organization, resulting in isotropic cell growth (Audhya et al. 2000). Similar phenotypes have been observed in cells lacking Cla4p and a related protein kinase, Ste20p (Holly and Blumer 1999). Although Ste20p has not been shown to interact with phosphoinositides, these are the first data demonstrating that PI4P generated by Stt4p exhibits functions in actin organization beyond its role as a precursor to Mss4p-mediated PI4,5P₂ synthesis.

In addition to its role at the plasma membrane, Stt4p has also been implicated in the transport of the phospholipid PS from the ER to the Golgi. Specifically, inhibition of Stt4p kinase activity leads to an accumulation of PS in the ER, which under normal conditions is metabolized to form phosphatidylethanolamine (PE) in the Golgi (Trotter et al. 1998). These data suggest that PI4P generated by Stt4p regulates the movement of phospholipids in the early secretory pathway, although an effector of PI4P in this process remains unknown. Additionally, these findings raise the possibility that PI4P generated by Stt4p at the plasma membrane can be transferred to the ER, potentially at sites of ER-plasma membrane contact. Such a process may be mediated by members of the OSBP family, which all exhibit the capacity to bind phosphoinositides. In particular, Osh3p appears to be specifically enriched at sites of ER-plasma membrane contact, and this localization is dependent on an interaction between its PH domain and PI4P (Stefan et al. 2011). Although a model in which OSBPs directly transfer phosphoinositides between organelles is highly speculative, it is supported by studies suggesting that PI4P generated by Stt4p can accumulate in the ER following inactivation of the Sac1p lipid phosphatase (Li et al. 2002; Wood et al. 2009). Further studies are clearly required to confirm or reject this possibility.

Beyond the putative role of OSBPs in lipid transfer, this family of phosphoinositide-binding proteins also regulates PI4P turnover. In mutant cells deficient in OSBP function, PI4P levels rise substantially, similar to the phenotype exhibited by cells lacking Sac1p phosphatase activity (Stefan et al. 2011). Furthermore, OSBPs have been shown to activate the phosphatase activity of Sac1p *in vitro*. Thus, PI4P autoregulates its stability by recruiting effectors that increase PI4P hydrolysis. This type of feedback inhibition may be necessary to restrict PI4P signaling on the plasma membrane and prevent the mistargeting of PI4P binding proteins required for function downstream of Pik1p at the Golgi.

As mentioned earlier, Osh3p is an effector of PI4P generated on the plasma membrane by Stt4p and localizes to sites of ER-plasma membrane contact (Stefan et al. 2011). Since PI4P accumulates homogeneously on the cell surface, these data suggest that Osh3p distribution is also regulated by additional interactions. Consistent with this idea, two ER membrane proteins that regulate phospholipid biosynthesis, Scs2p and Scs22p, also bind to Osh3p and control its accumulation at sites of ER-plasma membrane contact (Stefan et al. 2011). Notably, Scs2p has been shown to bind PI4P *in vitro*, and mutations that disrupt its ability to associate with phosphoinositides diminish its function in regulating the metabolism of ER phospholipids (Kagiwada and Hashimoto 2007). Together, these data suggest that PI4P generated by Stt4p plays an important role in generating and/or stabilizing contact sites between the plasma membrane and ER through the recruitment of multiple effector proteins. These contact sites potentially allow for the direct transfer of lipids between these distinct organelles, allowing for rapid changes in membrane content independently of vesicular transport. Further studies are necessary to determine whether inactivation of Stt4p may lead to the disruption of ER-plasma membrane contact and what affect this has on cell growth and viability.

Characterization of *stt4* mutant cells also revealed a role for Stt4p-mediated production of PI4P in vacuolar membrane homeostasis. Following loss of Stt4p activity, vacuoles undergo a dramatic reduction in volume, while their overall surface area does not appear to change significantly (Audhya et al. 2000). One possibility is that PI4P generated at the plasma membrane is required for controlling cellular osmolarity, and defects in PI4P production lead to changes in osmotic pressure within the cell that cause vacuoles to lose volume. Importantly, such an effect is not seen following loss of Mss4p-mediated PI4,5P₂ production, indicating that PI4P does not simply act as a precursor lipid in this pathway. In the future, it will be important to define specific effectors of PI4P, which control cellular osmolarity and/or vacuole size and shape.

2.5 Roles for PI4,5P₂ in Membrane Transport

Although the single yeast PI4P 5-kinase Mss4p undergoes nucleo-cytoplasmic shuttling, studies indicate that the essential cellular pool of PI4,5P₂ is synthesized on the plasma membrane (Audhya and Emr 2003). At this location, PI4,5P₂ regulates a number of distinct processes, which include endocytosis, exocytosis, cytokinesis, maintenance of cell polarity, and actin cytoskeleton organization. In several cases, PI4,5P₂ functions as a localization determinant. However, many effectors rely on PI4,5P₂ binding to regulate their activities directly. We will discuss a variety of mechanisms by which PI4,5P₂ modulates cellular function, with an emphasis on its diverse roles in membrane trafficking.

During endocytosis, a number of different factors must be recruited to the cell surface in a coordinated fashion to drive membrane invagination and scission. In the case of clathrin-mediated endocytosis, adaptor proteins arrive soon after clathrin

marks an endocytic site (Liu et al. 2010). Several of these factors require the presence of PI4,5P₂ to associate with the plasma membrane. For example, the alpha subunit of the mammalian AP-2 adaptor protein complex has been shown to bind PI4,5P₂ through a conserved amino-terminal basic region, and this interaction is required for its localization (Collins et al. 2002). Although the yeast AP-2 alpha subunit Apl3p exhibits only 30% sequence identity with its human homolog, the basic residues within the amino-terminus are almost entirely conserved, suggesting that Apl3p also binds PI4,5P₂. However, in contrast to human AP-2, the role of the yeast complex is less clear as mutant cells lacking all AP-2 adaptor subunits fail to exhibit significant defects in clathrin-mediated endocytosis (Huang et al. 1999; Yeung et al. 1999). These data suggest that alternative proteins likely function in a redundant manner to the AP-2 complex in yeast.

The epsins, Ent1p and Ent2p, are adaptor proteins, which may fulfill such a function. Unlike AP-2, epsin function is essential for yeast cell viability, and specifically plays an important role during clathrin-mediated endocytosis (Wendland et al. 1999). Both Ent1p and Ent2p harbor ENTH (epsin N-terminal homology) domains, which bind to PI4,5P₂ (Aguliar et al. 2003). In general, ENTH domains are approximately 150 amino acids in length and are composed of 9 alpha helices connected by loops of varying sizes. Based on the structure of the ENTH domain from mammalian epsin, PI4,5P₂ interacts with basic amino acids in loop 1 and helices 3 and 4 (Itoh et al. 2001). The conservation of this region in Ent1p and Ent2p strongly suggests a common mechanism for phosphoinositide binding. Additionally, genetic studies indicate that the ENTH domain from either Ent1p or Ent2p is sufficient to complement deletions of both genes (Aguliar et al. 2006). These data suggest additional functions beyond phosphoinositide binding for this region. Consistent with this idea, the ENTH domain has been found to be a coincident detector of both PI4,5P₂ and GEFs for the Rho-type GTPase Cdc42p, which play an important role in polarized cell growth (Aguliar et al. 2006). Thus, a combination of protein-protein and protein-lipid interactions coordinates spatial and temporal regulation of endocytosis with maintenance of cell polarity.

Yeast cells also express two epsin-related proteins, Yap1801p and Yap1802p, which harbor ANTH (AP180 N-terminal homology) domains. The ANTH domain exhibits a similar overall structure to the ENTH domain, containing 9 helices connected by loops (Legendre-Guillemain et al. 2004). Additionally, a PI4,5P₂ binding site within the ANTH domain has been defined (Stahelin et al. 2003), and contains the conserved sequence Kx₉Kx(K/R)(H/Y). Although initial studies failed to identify endocytic defects in cells lacking Yap1801p and Yap1802p, subsequent findings indicated that the adaptor proteins possess cargo specific roles in endocytosis (Wendland and Emr 1998; Huang et al. 1999; Burston et al. 2009). These data further implicate PI4,5P₂ in regulating endocytic trafficking in yeast.

In addition to Yap1801p and Yap1802p, a third ANTH domain containing protein Sla2p has been implicated in endocytosis. Furthermore, Sla2p also functions to regulate actin organization, connecting membrane transport from the cell surface to the underlying cytoskeleton (Wesp et al. 1997). The conserved basic region within the Sla2p ANTH domain was shown to be critical for PI4,5P₂ interaction, and mutations

in this region perturbed endocytic function in cells otherwise lacking Sla2p (Sun et al. 2005). However, loss of the basic region failed to impact localization of Sla2p, indicating that PI4,5P₂ is not essential for targeting of Sla2p to the plasma membrane. Instead, PI4,5P₂ likely regulates the activity of Sla2p during endocytosis. Since Sla2p binds to components of both the clathrin coat and the actin cytoskeleton, modulations in Sla2p function by PI4,5P₂ may be critical to appropriately couple vesicle formation with actin-dependent vesicle internalization. However, further studies are necessary to pinpoint the precise consequence of PI4,5P₂ interaction with the Sla2p ANTH domain.

The intimate connection between endocytosis and cytoskeletal organization implicates several additional PI4,5P₂ binding proteins, which modulate actin assembly, in membrane trafficking. For example in mammalian cells, the actin severing protein gelsolin is rapidly inactivated in the presence of PI4,5P₂, while PI4,5P₂ binding to the basic motif in WASP family members stimulates actin polymerization mediated by the Arp2/3 complex (Janmey and Stossel 1987; Rohatgi et al. 2000; Papayannopoulos et al. 2005). Thus, using several mechanisms, PI4,5P₂ accelerates the formation of actin networks, which play a key role in endocytic vesicle budding. In total, more than 30 actin filament binding proteins have been reported to associate directly with phosphoinositides (Yin and Janmey 2003).

Similar to gelsolin, PI4,5P₂ also exhibits an inhibitory effect on the actin depolymerizing factor (ADF)/cofilin known as Cof1p in yeast. In the absence of PI4,5P₂, Cof1p promotes rapid actin dynamics by driving the severing of actin filaments, but its ability to associate with actin and its depolymerizing activity is strongly diminished by PI4,5P₂ (Carlier et al. 1997; Gorbatyuk et al. 2006). Based on a series of *cof1* mutant alleles, a highly conserved basic helix in Cof1p, as well as other positively charged residues found throughout the molecule, appear to be important for PI4,5P₂ binding (Ojala et al. 2001). These findings suggest that Cof1p may bind multiple PI4,5P₂ molecules simultaneously. However, work using ADF/cofilin from another organism indicated that PI4,5P₂ binding was mediated by the carboxyl-terminus of the protein (Kusano et al. 1999). Additional work is necessary to define the precise mechanism by which PI4,5P₂ binds cofilin and inhibits its activity.

Actin polymerization can also be terminated by the function of capping protein, an evolutionarily conserved, heterodimeric complex consisting of alpha and beta subunits, encoded by *CAP1* and *CAP2* in yeast. Through its association with the ends of actin filaments, capping protein inhibits further actin dynamics (Saarikangas et al. 2010). Both subunits contain highly basic residues that are critical for PI4,5P₂ binding, and association with PI4,5P₂ interferes with the interaction between capping protein and actin, promoting actin dynamics necessary for endocytosis (Kim et al. 2007). The importance of the interaction between capping protein and PI4,5P₂ is further highlighted by work indicating that a partial loss of function *mss4* allele exhibits synthetic lethality with deletion of either *CAP1* or *CAP2* (Audhya et al. 2004).

Profilin, encoded by *PFY1* in yeast, is another PI4,5P₂ binding protein that interacts specifically with monomeric actin. Under certain cellular conditions, profilin can act as an actin monomer sequestering molecule, inhibiting actin filament assembly (Witke 2004). Interaction with PI4,5P₂ disrupts the association of profilin with

actin, thus inhibiting the effect of profilin on actin polymerization. Several regions of profilin have been implicated in PI4,5P₂ binding, and the interaction appears to be electrostatic in nature (Lassing and Lindberg 1985; Richer et al. 2008). In a similar fashion, PI4,5P₂ also inhibits the actin monomer sequestering activity of the yeast twinfilin Twf1p (Palmgren et al. 2001). Thus, by maintaining free pools of monomeric actin, the presence of PI4,5P₂ generates an environment conducive to actin polymerization and endocytosis.

In several cases, PI4,5P₂ regulates cytoskeletal organization through effectors that do not bind actin directly. For example, the Rho-type GTPase Rho1p is required for polarity of the actin cytoskeleton in yeast, as well as polarized secretion (Levin 2005). The Rho1p GEF Rom2p harbors a PH domain that has been shown to interact with PI4,5P₂ (Audhya and Emr 2002). Inhibition of Stt4p or Mss4p, which diminish PI4,5P₂ synthesis on the plasma membrane, results in a defect in Rom2p localization and leads to depolarization of the actin cytoskeleton, suggesting that phosphoinositide signaling regulates Rho1p activity. Furthermore, a polybasic sequence within Rho1p, which plays a role in targeting the GTPase to sites of polarized growth independent of its GEFs, has also been shown to interact with PI4,5P₂ (Yoshida et al. 2009). Together, these findings demonstrate that phosphoinositide and Rho-type GTPase signaling are tightly coupled.

Mss4p-dependent PI4,5P₂ synthesis is also required for the normal localization of Slm1p and Slm2p, two additional regulators of actin organization in yeast. Both Slm1p and Slm2p contain PH domains capable of interacting with PI4,5P₂, and mutations in the Slm1p PH domain that inhibit phosphoinositide interaction result in the mislocalization of Slm1p to the cytoplasm (Audhya et al. 2004; Fadri et al. 2005). Although the precise mechanism underlying Slm1p and Slm2p regulation of actin cytoskeleton organization remains unknown, these data further highlight the diversity of PI4,5P₂ effectors that control actin polarity. Furthermore, Slm1p and Slm2p also play roles in sphingolipid biosynthesis, which function in several membrane trafficking pathways including endocytosis (Tabuchi et al. 2006). These data suggest that phosphoinositide and sphingolipid signaling likely intersect, potentially to coordinate vesicle transport with membrane homeostasis.

In addition to its role in endocytosis, the actin cytoskeleton also performs a key function in directed protein secretion and exocytosis. As discussed earlier, PI4P generated on the Golgi by Pik1p plays several important roles in generating secretory vesicles, which move along actin cables to sites of polarized growth. Fusion of vesicles with the plasma membrane requires the activity of a tethering complex known as the exocyst. Of the 8 exocyst subunits, two have been shown to interact with PI4,5P₂ and direct their localization to the plasma membrane, Exo70p and Sec3p (He et al. 2007; Zhang et al. 2008). In the case of Exo70p, PI4,5P₂ binding is mediated by a basic patch of amino acids in the carboxyl-terminus. Similarly, a basic region in the amino-terminus of Sec3p, which has been shown to adopt a PH domain fold, also binds to PI4,5P₂. Consistent with a role for PI4,5P₂ in regulating exocyst localization on the cell surface, inhibition of Mss4p activity led to the accumulation of the exocyst complex in the cytoplasm. Notably, unlike the uniform distribution of PI4,5P₂ on the plasma membrane, the exocyst complex is

highly polarized, suggesting additional interactions are necessary for proper exocyst distribution. Indeed, several Rho-type GTPases, which localize in an asymmetric fashion, bind to components of the exocyst, and these interactions also play an important role in recruiting the complex to the plasma membrane (Zhang et al. 2008). Taken together, these data again highlight the importance of dual targeting mechanisms, which combine protein and lipid signals, in determining the localization of phosphoinositide effectors in cells.

Finally, PI4,5P₂ has also been implicated in the final stages of cell division in yeast by regulating several components of the septin complex, which orchestrate membrane remodeling events necessary for cytokinesis. The septins Cdc3p, Cdc10p, Cdc11p, and Cdc12p have been demonstrated to form filaments that can function as a diffusion barrier as cells divide (Weirich et al. 2008). Studies indicate that each septin harbors a basic motif capable of binding to PI4,5P₂. Mutations in basic residues found in Cdc3p, which mediate PI4,5P₂ binding, cause defects in cell division (Votin et al. 2009). *In vitro*, PI4,5P₂ promotes septin filament assembly and organization, suggesting that protein-lipid interactions in this case function beyond membrane recruitment (Bertin et al. 2008, 2010). Further work is necessary to understand how phosphoinositide binding may affect septin dynamics during the process of cell division.

2.6 Perspectives and Conclusions

The recognized roles of phosphoinositides in membrane transport pathways have grown significantly over the last two decades. Although many more effectors likely await characterization, it has become clear that phosphoinositide signaling plays key roles in the movement of cargoes between virtually all cellular compartments. Two major themes have emerged from the study of PI metabolism. First, the localization of phosphoinositides is highly regulated, and their distributions rarely overlap. Thus, phosphoinositides function as organelle specific labels, enabling membrane compartments to be distinguished from one another and preventing crosstalk between distinct biochemical processes within a common cytoplasm. Second, phosphoinositides infrequently function alone, and instead regulate cellular pathways in concert with other factors. Such a mechanism provides the high specificity necessary for directed membrane transport between organelles. In the future, the diversity of phosphoinositide effectors will likely continue to grow, hopefully shedding additional light on the complex mechanisms by which membrane trafficking is appropriately coordinated.

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