Cinderella story: PI4P goes from precursor to key signaling molecule

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Abstract
Phosphatidylinositol lipids are signaling molecules involved in nearly all aspects of cellular regulation. Production of phosphatidylinositol 4-phosphate (PI4P) has long been recognized as one of the first steps in generating poly-phosphatidylinositol phosphates involved in actin organization, cell migration, and signal transduction. In addition, progress over the last decade has brought to light independent roles for PI4P in membrane trafficking and lipid homeostasis. Here, we describe recent advances that reveal the breadth of processes regulated by PI4P, the spectrum of PI4P effectors, and the mechanisms of spatiotemporal control that coordinate crosstalk between PI4P and cellular signaling pathways.

Phosphoinositides
Eukaryotic cells are compartmentalized into organelles that engage in specialized functions. These subcellular functions are coordinated by signaling proteins and phospholipids such as phosphatidylinositol (PI; Figure 1A) and its derivatives. PI lipids are minor membrane components containing a cytosolic myo-inositol head group amenable to phosphorylation at the D-3, D-4 and D-5 positions to produce seven different PI phosphates (PIPs or phosphoinositides). Specific PIPs are enriched on particular organelles, contributing to their identity and function. In addition, the ability of PIPs to be rapidly interconverted through the action of lipid kinases and phosphatases means that PIPs can be precisely regulated in space and time. Moreover, PIPs can relay changes in membrane status by recruitment of cellular effectors that recognize newly synthesized PIPs, or by activation of signaling pathways that utilize PIPs as substrates.

Generation of PI 4-phosphate (PI4P) by PI 4-kinases (PI4Ks) is the first reaction in forming PI 4,5-bisphosphate (PI(4,5)P2) and PI 3,4,5-trisphosphate (PIP3), two phosphoinositides that participate in well-studied signaling pathways, making PI4P a critical precursor in the PIP pathway (Figure 1B). Cleavage of PIP2 by phospholipase C (PLC) produces the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), initiating downstream signal transduction cascades through protein kinase C (PKC), thereby controlling growth factor signaling, cytokine induction, neurotransmitter release, muscle contraction and other responses. Phosphorylation of PIP2 by class I PI 3-kinases (PI3Ks) produces PIP3, which activates Akt and other signaling proteins to control cell proliferation and survival.

Because of the importance of PIP2- and PIP3-dependent signaling pathways in animal cells, PI4P has mainly been regarded as essential only for its role as a precursor to these PIPs. However, the discovery of conserved roles for PI4P across yeast, plants and mammals suggests that the non-precursor, signaling roles of PI4P are ancient and fundamental. For this reason, it has been proposed that PI4P became essential in its own right, with PIP2 and PIP3 acquiring specialized roles (Delage et al., 2012a). This idea partly stems from the observation that PI4P is the most abundant cellular PI (Lemmon, 2008). Indeed, PIP2 is 10- to 100-fold less abundant and PI4P 5-kinases (PIP5Ks) are much less active in plant cells compared to animal cells. Also, class I PI3Ks are absent from yeast and plants. In addition, the abundance and distribution of PI4P vary less than for PIP2 and PIP3 across species (Delage et al., 2012a) (Figure 2).

Importantly, studies of PI4Ks and the PI4P phosphatase Sac1, as well as tools to visualize and perturb cellular PI4P levels, have revealed prominent roles for PI4P in a wide range of basic cellular processes, most notably in membrane trafficking, sphingolipid metabolism and regulation of the cytoskeleton.

PI-4 kinases
In the late 1960s–80s, biochemical studies identified PI4K activity in membrane fractions from animal tissues (Behar-Banneler & Murray, 1980; Collins & Wells, 1983; Cooper & Hawthorne, 1976; Harwood & Hawthorne, 1969;
Lefebvre et al., 1976). It became apparent that there were two types of PI4K activity based on the repertoire of inhibitors to which they were sensitive, leading to characterization of type II and type III PI4Ks. Cloning of mammalian PI4Ks led to identification of two type II PI4Ks approximately 55 kDa in size, PI4KIIα and PI4KIIβ (Balla et al., 2002; Barylko et al., 2001; Minogue et al., 2001; Wei et al., 2002), and two type III PI4Ks, PI4KIIIα (230 kDa), and PI4KIIIβ (92 kDa) (Nakagawa et al., 1996a,b). The type II PI4Ks are inhibited by adenosine, micromolar calcium, submillimolar phenylarsine oxide (PAO) and the 4C5G monoclonal antibody, whereas type III PI4K activity is inhibited by low amounts of PAO, with PI4KIIIα being more sensitive (IC50 = 1–5 μM) than PI4KIIIβ (IC50 = 30 μM), and PI3K inhibitors such as wortmannin (Wm) and LY294002 (Balla & Balla, 2006; Endemann et al., 1991). Cloning of PI4Ks from the budding yeast Saccharomyces cerevisiae revealed three conserved PI4Ks – the PI4KIIIα Stt4, the PI4KIIIβ Pik1, and a single type II PI4K, Lsb6 (Flanagan et al., 1993; Garcia-Bustos et al., 1994; Han et al., 2002; Yoshida et al., 1994). These same three enzymes are also found in the fruit fly Drosophila melanogaster.

In addition to their different biochemical properties, PI4Ks affect discrete pools of cellular PI4P and have distinct cellular functions. In yeast, Stt4 and Pik1 play essential, non-overlapping roles in the cell (Audhya et al., 2000). Stt4 localizes to cortical patches at the plasma membrane (PM) and regulates actin organization as well as cell wall integrity. In contrast, Pik1, which functions in secretion, localizes to the Golgi and nucleus. In mammalian cells and flies, PI4KIIIα controls a PM pool of PI4P (Balla et al., 2008; Tan et al., 2014). Mammalian PI4KIIIα localizes to the endoplasmic reticulum (ER), dynamically to the PM (Nakatsu et al., 2012), and has also been detected at the Golgi, nucleolus, multivesicular body (MVB), and outer mitochondrial membrane (Balla et al., 2000; Kakuk et al., 2006; Wong et al., 1997) (Figure 3A). As with yeast Pik1, mammalian PI4KIIIβ primarily localizes to the Golgi and nucleus, and is required for anterograde trafficking (de Graaf et al., 2002; Godi et al., 1999; Weisz et al., 2000). PI4KIIIβ has also been reported on ER, outer mitochondrial membranes (Balla et al., 2000) and, recently, lysosomes (Sridhar et al., 2013) (Figure 3B). The Drosophila PI4KIIIβ, Fwd, localizes to the Golgi, where it is required for male germ cell cytokinesis (Brill et al., 2000; Polevoy et al., 2009). Interestingly, unlike yeast Pik1, Drosophila Fwd is non-essential (Brill et al., 2000), suggesting it carries out a redundant function with another PI4K.

The type II PI4Ks examined so far appear to be dispensable for viability in yeast, flies, and mice, yet carry out specific cellular functions. Yeast Lsb6 regulates endosome
motility in a kinase-independent manner (Chang et al., 2005). In contrast, catalytic activity of Drosophila PI4KII is required for sorting of secretory granule and endosomal cargo in the larval salivary gland (Burgess et al., 2012). PI4KIIα mutant mice are viable, yet develop late onset neurodegenerative disease (Simons et al., 2009). In mammalian tissue culture cells, PI4KIIα is required for endosomal sorting (Craigie et al., 2008; Jovic et al., 2012; Mossinger et al., 2012) and its catalytic activity is needed for post-Golgi trafficking at the trans-Golgi network (TGN) (Wang et al., 2003, 2007). In contrast, mammalian PI4KIIβ is found on endosomal compartments and translocates to the PM upon Rac signaling (Balla et al., 2002; Wei et al., 2002).

Challenges of PI4P biology

Common methods to visualize cellular PI4P include antibody detection and expression of fluorescent proteins fused to PI4P-binding domains (Hammond et al., 2009; Varnai & Balla, 2008). However, determining which pools of PI4P are produced by individual PI4Ks has been difficult due to the lack of fluorescent probes that bind only PI4P, and the lack of complete and isoform-specific PI4K pharmacological inhibitors. Pleckstrin homology (PH) domains from oxysterol binding protein (OSBP) and four-phosphate adaptor proteins (FAPPs) are frequently used to detect PI4P, but they also bind ADP-ribosylation factor 1 (Arf1). At high expression levels, these probes titrate the lipid away from its normal function. Existing enzyme inhibitors do not distinguish between type II PI4Ks and, at the concentration of PAO that is specific for PI4KIIIα, only 80% of its activity is reduced (Balla et al., 2008). Additionally, the essential functions of PI4Ks mean that treatment with short interfering RNA (siRNA) either eliminates cells entirely or only moderately affects PI4P levels because of incomplete knockdown in surviving cells.
Nevertheless, cell biologists have found clever ways to overcome these obstacles in order to detect and perturb PI4P. Chief among these is a method to acutely deplete a specific phosphoinositide on a membrane of interest without caveats associated with enzyme inhibition or knockdown (Filiet al., 2006; Heo et al., 2006; Varnai et al., 2006). FRB (FK506 binding protein 12 [FKBP12]- and rapamycin-binding protein) is tethered to a transmembrane protein on the membrane of interest. Recruitment of a PIP phosphatase fused to FKBP12 to this site is controlled by acute rapamycin-induced heterodimerization of FRB and FKBP12. This technique has been used successfully to deplete PI4P on the PM and Golgi by targeted recruitment of a PI 4-phosphatase domain from Sac1 (Hammond et al., 2012; Salcedo-Sicilia et al., 2013; Szentpetery et al., 2010). Importantly, this technique is effective in identifying the subcellular location where PI4P is important, but does not identify the PI4K responsible for its production. Other efforts to disrupt specific pools of PI4P include the pursuit of new drugs to inhibit specific PI4Ks, which resulted in identification of PIK93, a panspecific PI3K p110 subunit inhibitor that also selectively targets PI4KIIIb over PI4KIIIα (Knight et al., 2006). The search for compounds with anti-viral activity has led to the discovery of isoform-specific PI4K inhibitors such as AL-9 (for PI4KIIIα)
Table 1. Methods to detect cellular PI4P.

| Method                              | Advantages                                    | Disadvantages                                      | Recent References                   |
|-------------------------------------|-----------------------------------------------|----------------------------------------------------|-------------------------------------|
| Metabolic labeling with $^{32}$P or $^{3}$H-inositol and measuring incorporation into PIPs | Quantitative; direct lipid analysis; high level labeling can detect low abundance PIPs and distinguish between regioisomers; allows examination of membrane fractions | Difficult to examine subpopulations of cells within a tissue | Wood et al., 2012; Stefan et al., 2011; Cheong et al., 2010; Balla et al., 2008 |
| Expression of PI4P-binding modules fused to fluorescent proteins | Amenable to live imaging; information on organelle and spatial distribution | Modules often harbor second site that binds other proteins; high-level expression may titrate lipid away from normal function | Santiago-Tirado et al., 2011; Stefan et al., 2011; Mizuno-Yamasaki et al., 2010; Balla et al., 2008 |
| Antibody detection                  | Detection of endogenous PIPs                  | Results are sensitive to experimental conditions    | Forrest et al., 2013; Hammond et al., 2009 |
| High performance lipid chromatography followed by mass spectrometry | High sensitivity; can be applied to total tissue; allows examination of membrane fractions; information on fatty acid composition | Difficult to examine subpopulations of cells within a tissue | Forrest et al., 2013; Hammond et al., 2012; Clark et al., 2011 |

and T-00127 HEV1 and GW5074 (for PI4KIIIβ) (Arita et al., 2011; Vaillancourt et al., 2012). In addition, combinatorial evidence gathered from multiple inhibitors can provide valuable information. For example, a pool of PI4P that is dispersed by the application of Wm and <10 µM PAO, but not PIK93, is likely to be regulated by PI4KIIIδ (Balla et al., 2008). Lastly, optimization and modification of earlier methods has helped overcome inconsistencies seen with PI4P antibody staining (Hammond et al., 2009), and revealed a previously undetected cyan fluorescent protein (CFP)-FAPP-PH signal at the PM (Wuttke et al., 2010). Our current understanding of the many cellular roles for PI4P is a direct result of these advances (Tables 1 and 2).

In this review, we present an overview of this current understanding of PI4P biology and the evidence from which it was derived. In the first half, we review the molecular roles and physiological goals that are accomplished with PI4P signaling. In the second half, we examine the mechanisms that fine-tune the production of PI4P in these contexts. We also emphasize the current gaps in our knowledge and suggest worthwhile directions to fuel the next chapter of discovery.

**Cellular roles of PI4P**

**Signal transduction**

Shortly after the discovery of phosphoinositides, it was observed that ligand stimulation can induce cellular phosphoinositide metabolism. Radiolabeled phosphate or inositol was incorporated into the phosphoinositide lipid pool after incubation of guinea pig brain cortex slices with acetylcholine (Hokin & Hokin, 1955, 1958), a result termed “the phospholipid effect”. Through a series of agonist stimulation experiments where specific phosphoinositides were monitored (Berridge, 1983), the idea of a ‘phosphoinositide cycle’ emerged: ligand receptors and GTP stimulate hydrolysis of PIP2 by PLC, and the resulting second messenger products are recycled to regenerate PI and PIP2 (Akhtar & Abdel-Latif, 1984; Dunlop & Larkins, 1986). Receptors that stimulate PLC include receptor tyrosine kinases and G-protein coupled receptors (GPCRs) such as rhodopsin, chemokine and ATP purinergic receptors, angiotensin II receptor, and the muscarinic acetylcholine receptor. Because PI4P is much more abundant than PI 5-phosphate (PI5P) (Lemmon, 2008), PI2P is likely generated through phosphorylation of PI4P rather than PI5P. Support for the idea that PI4P is the major precursor of PI2P comes from experiments in insulin-secreting MIN6 β-cells, which show PIP2 levels closely following those of PI4P (Wuttke et al., 2010). Cells were permeabilized with *Staphylococcus aureus* α-toxin, which produces small pores, allowing depletion and re-addition of ATP to inhibit and activate PI4Ks. Using OSBP-PH-CFP to follow PI4P and the PI2P-binding PH domain of phospholipase C δ (PLCδ-PH) fused to yellow fluorescent protein (YFP) to monitor PI2P, re-addition of low concentrations of ATP induced the appearance of PM PI2P without a concomitant generation of PM PI2P until higher concentrations of ATP were added. Inhibition of PI4K activity with 200 µM LY294002 then decreased PI4P, with PI2P levels following after a 25 second delay. This suggests that the majority of PM PI2P undergoes substantial turnover which requires prior formation of PI4P, and that this route of PI2P synthesis is more likely than others (Wuttke et al., 2010), such as synthesis from PI5P, trafficking of PI2P from intracellular membranes or dephosphorylation of PI3P.

PI4P is thought to play a critical role in PLC signaling because of its function as a PI2P precursor. Indeed, PI4K activity is required for a sustained PLC response under signaling conditions (Balla et al., 2005; Creba et al., 1983). Continued second messenger production leads to prolonged stimulation of ER-localized IP3 receptors by IP3, releasing intracellular calcium stores and promoting sustained PKC activity, which is stimulated by DAG. PLC activation also leads to opening of transient receptor potential (TRP) cation channels, which aids in signal amplification (Montell, 2012). Activated PKC initiates downstream signal transduction pathways, including those propagated by MAPK/ERK.

With some exceptions, PI4KIIIβ is generally thought to produce the PI4P precursor for PI2P that is consumed by PLC. Indeed, in mammalian cells, PI4KIIIβ is needed to replenish PI2P during continued PLC, PKC and MAPK signaling (An et al., 2011; Balla et al., 2008; Hammond et al., 2012).
Table 2. Methods used to manipulate PI4P levels.

| Method                                      | Advantages                                                                 | Disadvantages                                                                                                                                                                                                 | Recent References                                                                 |
|---------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| Pharmacological inhibitors                  | Inhibit enzyme without affecting structural roles; identify contribution by PI4K class; can use in combination with other methods | Lack of complete and specific inhibition of specific PI4K isoforms (Wortmannin, LY294002, PAO); some inhibitors with high selectivity for PI4Ks isoforms also inhibit PI3Ks (PIK93, GW5074) | Bianco et al., 2012; Hammond et al., 2012; Jovic et al., 2012; Vaillancourt et al., 2012; van der Schaar et al., 2012; Arita et al., 2011; Zakharian et al., 2011; Wuttke et al., 2010; Hsu et al., 2010; Balla et al., 2008; Toth et al., 2006 |
| Wortmannin, LY294002                          |                                                                           |                                                                                                                                             |                                                                                   |
| PAO                                         |                                                                           |                                                                                                                                             |                                                                                   |
| PIK93                                       |                                                                           |                                                                                                                                             |                                                                                   |
| Adenosine                                    |                                                                           |                                                                                                                                             |                                                                                   |
| AL-9                                         |                                                                           |                                                                                                                                             |                                                                                   |
| T-001.27 HEV1                                 |                                                                           |                                                                                                                                             |                                                                                   |
| GW5074                                       |                                                                           |                                                                                                                                             |                                                                                   |
| Compound A, Compound B                        |                                                                           |                                                                                                                                             |                                                                                   |
| Inhibition of type II PI4K with 4C5G monoclonal antibody | >90% inhibition of type II PI4K activity with little effect on type III | Effect on type IIβ activity unknown; requires protein transfection                                                                                                                                           | Minogue et al., 2006; Endemann et al., 1991                                       |
| Inhibition of PI4Ks by ATP depletion          | Reversible                                                                | Requires cell permeabilization; may deplete small molecules of interest; may inhibit other kinases that affect PI4K activity | Wuttke et al., 2010                                                              |
| Titration by PI4P-binding modules            | Second-site binding allows sequestration of specific pools                 | Toxic effects of overexpression                                                                                                           | Salcedo-Sicilia et al., 2013; Lorente-Rodriguez & Burlowe, 2011; Dippold et al., 2009; Blumental-Perry et al., 2006 |
| Direct delivery with lipid carrier           | Relatively acute action in cultured cells                                 | Lack of membrane-specific targeting                                                                                                       | Pan et al., 2008                                                                   |
| Water-soluble PIP analogs                    | Technical convenience for in vitro/patch assays                           | Do not mimic native conformation of cellular PIPs                                                                                             | Zakharian et al., 2011; Caromile et al., 2010                                      |
| Knockdown of PI4Ks or Sac1                   | Incomplete silencing may reduce activity without causing lethality        | Residual activity from incomplete silencing may mask phenotype; RNAi off-targets                                                           | Sridhar et al., 2013; Jovic et al., 2012; Yavari et al., 2010                      |
| Overexpression of PI4Ks or Sac1              | Manipulate PIP levels to varying degrees                                  | May produce artificial phenotype                                                                                                           | Daboussi et al., 2012; Zhang et al., 2012b; Santiago-Tirado et al., 2011          |
| Genetic ablation of PI4Ks or Sac1            | Complete and specific removal of enzyme                                   | Essential nature of gene may necessitate genetic tools to examine effects in specific cells; cell/organism may compensate for loss of enzyme | Nakatsu et al., 2012; Yan et al., 2011; Burgess et al., 2007; Simons et al., 2009; Wei et al., 2003; Fori et al., 2001; Brill et al., 2000; Audhya et al., 2000 |
| FKBPI2-FRB rapamycin-inducible lipid phosphatase recruitment | Acute disruption; reversible; depletes PIPs without generating second messengers; membrane-specific | Does not reveal enzymes responsible for depleted lipid pool; dependent on specificity of phosphatase recruited | Hammond et al., 2012; Szentpetery et al., 2010; Varnai et al., 2006; Fili et al., 2006; Heo et al., 2006 |
| Fusion of Sac1 phosphatase domain to PLCβ-PH membrane-targeting element | Rapamycin treatment not required (e.g. for expression in animals)         | Does not reveal enzymes responsible for deleted lipid pool; cell/organism may compensate for loss of PHP                                         | Jesch et al., 2010                                                                |

For a comprehensive list of recent references, please refer to the supplementary material or cited literature cited in the table.
In Arabidopsis thaliana, PI4KIIIβ1/2 contributes to PLC signaling induced by cold exposure, although the role of PI4KIIIz, which is essential, could not be directly assessed (Delage et al., 2012b). This role for PI4KIIIβ is surprising, given that it is not thought to contribute substantially to PMPIP2. However, it is unclear whether plant PLC uses PI4P as a substrate, in which case production of PIP2 would not be required. Yeast Stt4 is essential for PKC and MAPK signaling, although this is not mediated by PLC. Instead, Stt4-dependent formation of PI4P, and subsequent production of PIP2 by the PIP5K Mss4, recruits protein effectors that stimulate PKC (Audhya & Emr, 2002). The Rho guanine nucleotide exchange factor (GEF) Rom2 is recruited to the PM in part by its PIP2-binding PH domain. PM localized Rom2 activates Rhoh1, which in turn stimulates PKC in a MAPK pathway known as the cell wall integrity pathway. Stt4 and Mss4 are also required for recruitment of the Ste5 scaffolding protein that induces MAPK signaling and cell polarization in response to mating pheromone (Garrenton et al., 2010).

PI4P has also been implicated in PLC signaling as a direct substrate. Mammalian PLC has been shown to cleave PI4P in vitro (Seifert et al., 2004; Smrcka et al., 1991; Wilson et al., 1984), but until recently this has not been demonstrated in cells. Activation of the Gα-coupled muscarinic receptor in tsA-201 human embryonal kidney cells leads to PIP2 depletion and a rise in DAG and IP3 levels (Falkenburger et al., 2012a). Expression of a 5-phosphatase effectively depleted PIP2, and thus strongly inhibited IP3 formation by PLC during receptor activation. However, the level of DAG remained unchanged even with 80% depletion of PIP2, suggesting that PLC may cleave PI4P to produce DAG and inositol 1,4-bisphosphate (IP2). Computational modeling using observed time courses and empirical rate constants supported this scenario. Indeed, when the authors attempted to model a scenario in which PLC does not cleave PI4P, no adjustment of parameters could reproduce the experimental data. In addition, strong evidence for PLCε cleavage of perinuclear PI4P comes from neonatal rat ventricular myocytes (Zhang et al., 2013). A perinuclear Golgi population of PI4P was depleted, and DAG signal was increased, when this specific PLC was activated. This effect is blocked by PLCε siRNA or disruption of the interaction of PLCε with muscle-specific A kinase anchoring protein, which anchors it to the nuclear envelope. The decrease in PI4P is unlikely to result from its conversion to PIP2 because fluorescent markers such as GFP-PLCδ-PH did not detect perinuclear PI4P and because targeting of a 5-phosphatase to the Golgi did not restore PI4P levels following PLCε activation at the nuclear envelope. In contrast, targeting of Sac1 to the Golgi during PLCε stimulation prevented nuclear protein kinase D (PKD) activation, an event that requires DAG production. Thus, in some contexts, PI4P is a PLC substrate, although how widespread this is remains to be seen.

In animal cells, PI4P also contributes to other signaling pathways. A PI4KIIz-dependent surge in PI4P and PIP2 levels is required for aggregation and phosphorylation of the Wnt3a co-receptor Lrp5/6 and for β-catenin stabilization during canonical Wnt signaling (Pan et al., 2008; see below). Hedgehog (Hh) signaling in Drosophila requires PI4P for translocation of the GPCR Smoothened to the PM (Yavari et al., 2010). In addition, PI4KIIz is required for regulation of the Hippo signaling pathway and for apical localization of the upstream signaling component Merlin (Yan et al., 2011). However, exactly how PI4P contributes to these pathways is currently unclear.

A potential role for PI4KIIz in generating the pool of PI4P that feeds into PI3P signaling has not been widely explored. A study in zebrafish showed that PI4KIIz knockdown by morpholino injection produced a pectoral fin development defect similar to that caused by treatment with a PI3K inhibitor (Ma et al., 2009). In addition, PI4KIIz morphants showed reduced expression of the PI3K-Akt target genes fgf10 and mkk3, which are required for FGF signaling in limb bud development. Studies of PI4KIIz and PI4KIIβ in COS-7 cells suggested that kinase activity was dispensable for PI3K signaling and Akt phosphorylation (Chu et al., 2010). However, PI4KIIz was not tested, raising the possibility that this enzyme has a conserved role in PI3P signaling.

ER and Golgi trafficking

In addition to its role in PIP2- and PIP3-dependent cell signaling, a major function for PI4P lies in its ability to recruit cytosolic signaling molecules that contain PI4P-binding motifs (D’Angelo et al., 2008; Vicinanza et al., 2008). Often, these proteins also require the presence of a coincident protein. Many membrane trafficking components have been identified as PI4P effectors, implicating PI4P as a key executor of events at multiple stages of trafficking. Indeed, depletion of PI4P by dynamic recruitment of Sac1 to the Golgi virtually eliminated trafficking of cargo from the TGN to the PM and to endosomes (Szentpetery et al., 2010).

Bud formation and cargo sorting

Vesicle formation begins with extrusion of donor membrane to generate a bud. Bud formation is achieved either through a clathrin-mediated process, through processes dependent on other coatomer complexes, or through coat-independent pathways. At the ER and Golgi, PI4P plays a role in examples from each of these categories.

Clathrin adaptors enriched at the TGN include the tetrameric adaptor protein-1 (AP-1) complex, the Epsin-related (EpsinR) protein, and the Golgi-localized, gamma-ear-containing, Arf-binding (GGAs) family of proteins. These adaptors have clathrin and specific cargo recognition sites to promote formation of cargo-containing clathrin-coated vesicles. In addition, these adaptors all contain PI4P- and Arf1-binding motifs (Ren et al., 2013). AP-1 binds PI4P in vitro, likely through the γ-adaptin subunit (Heldwein et al., 2004; Wang et al., 2003). Kinase activity of PI4KIIz is required for AP-1 recruitment and for TGN to PM transport in mammalian cells (Wang et al., 2003). However, in Drosophila, PI4KII is dispensable for AP-1 and EpsinR localization (Burgess et al., 2012). In yeast, modulation of PI4P levels by Pik1 exerts control over AP-1 localization (Daboussi et al., 2012). EpsinR binds PI4P through its ENTH domain; aids in the assembly of AP-1 and soluble n-ethylmaleimide sensitive factor adaptor protein receptor...
(SNARE) proteins into clathrin-coated vesicles (Hirst et al., 2003, 2004; Miller et al., 2007; Mills et al., 2003); and regulates retrograde trafficking of clathrin-coated vesicles to the TGN independently of AP-1 (Saint-Pol et al., 2004).

GGA proteins are recruited to the TGN by Arf1, the Arf1 GEF Golgi brefeldin A-resistant factor 1 (GBF1), a ubiquitin sorting signal, and PI4P (Lefrancois & McCormick, 2007; Wang et al., 2007), although each GGA varies in its reliance on each of these factors (Boman et al., 2002; Shiba et al., 2004; Wang et al., 2007). At the TGN, GGAs sort proteins through interaction with acidic-cluster dileucine sequences found in cytosolic tails of cargoes such as the mannose-6-phosphate receptor (Shiba et al., 2002). GGA proteins also promote trafficking of ubiquitinated cargoes to the endosomal pathway, including the yeast general amino acid transporter Gap1 and the human glucose transporter GLUT4 (Lamb et al., 2010; Scott et al., 2004). In mammalian cells, GGA Golgi localization depends on PI4KIIβ (Wang et al., 2007), and acute enzymatic depletion of Golgi PI4P leads to rapid dissociation of clathrin, GGA1 and GGA2, but curiously not GGA3, from the Golgi (Szcntpetery et al., 2010). Knockdown of PI4KIIIβ diminishes GGA3 presence at the Golgi (Dumaresq-Doiron et al., 2010). However, this is likely an indirect effect due to mislocalization of GBF1, rather than a direct effect on PI4P-dependent recruitment of GGA3 to the Golgi (see below).

In addition to binding PI4P, yeast GGAs control Golgi PI4P to mediate two waves of clathrin adaptor assembly. Using live imaging of clathrin adaptors fused to fluorescent proteins, appearance and disappearance of Gga2 puncta preceded AP-1 assembly and disassembly on the same TGN membrane by approximately 10 sec (Daboussi et al., 2012). Decreasing PI4P levels using a pik1 temperature-sensitive (ts) (pik1ts) mutant or increasing PI4P by overexpression of Pik1 had no effect on Gga2 bud formation but lengthened or shortened the time between GGA and AP-1 assembly, respectively, suggesting that the level of Golgi PI4P dictates timing of progression from GGA- to AP-1-dependent trafficking. Proper timing is important because a shortened window between GGA and AP-1 adaptor assembly leads to TGN-endosome trafficking defects. Interestingly, adaptor progression is regulated by binding and recruitment of Pik1 to the VHS domain of Gga2, leading to local production of PI4P. In accordance with this, localization of Pik1 and the PI4P sensor Osh1-PH peaks at the TGN approximately 5 seconds after the Gga2 peak, and depletion of GGA proteins delays Golgi association of Pik1, PI4P and AP-1.

Trafficking to and from the ER and Golgi is mediated by the conserved cytoplasmic coat protein (COP) complexes I and II. The COPII coat mediates budding of vesicles from the ER at specific locations termed ER exit sites (ERES), or transitional ER (tER), and begins with recruitment of the small GTPase Sar1 by its GEF Sec12. Activated Sar1 induces membrane curvature by insertion of a hydrophobic helix (Lee et al., 2005), and recruits the first coat of proteins, the Sec23-24 concave heterodimer, to further deform the membrane. Sec24 recognizes ER sorting signals while Sec23 recruits the Sec13-31 heterotetramer which forms a cage to stabilize the bud and promote fission (Bhattacharya et al., 2012). Additional contacts between the core COPII components and Sec16 are also important for ERES stabilization and fission (Yorimitsu & Sato, 2012).

Sar1 stimulation of PI4P production at ERES is necessary for nucleation of COPII coats (Blumental-Perry et al., 2006). However, it is unclear which PI4K is responsible for regulating this process, or how multiple PI4K activities would be coordinated at these sites. In ER fractions from normal rat kidney cells, Sar1 induced a membrane-associated PI4K activity that is insensitive to Wm. Such an activity is more likely to represent a type II PI4K (Blumental-Perry et al., 2006). Despite this, knockdown of PI4KIIIα significantly reduced ERES formation in HeLa cells (Farhan et al., 2008). This discrepancy may reflect cell-type specificity. Interestingly, PI4P does not appear to be required for COPII budding in yeast (Lorente-Rodriguez & Barlowe, 2011). In animal cells, specialized exit sites may facilitate clustering of specific cargo, lipids and proteins required for vesicle formation, or to spatially target released vesicles to the ER-Golgi intermediate complex (ERGIC), a compartment not present in yeast (Zanetti et al., 2012).

In yeast and mammalian cells, the COP complex retrieves vesicles from the cis-Golgi back to ER or ERGIC membranes, and mediates retrograde transport within Golgi cisternae (Emr et al., 2009). Yeast COPI mutants show defects that resemble a block in anterograde trafficking, but this effect is restricted to specific cargo proteins and can be suppressed by overexpression of vesicle-associated SNAREs (v-SNAREs) that are known to be recycled (Gaynor et al., 1998). Hence, the block in anterograde trafficking may be due to defects in retrieving COPII cargoes for recycled use at the ER. However, in mammalian cells, COPI-dependent formation of tubules at the Golgi appears to be important for intra-Golgi anterograde transport (Yang et al., 2011). Assembly of COPI-coated vesicles involves simultaneous binding of COPI to activated Arf1 (Yu et al., 2012), GBF1 (Deng et al., 2009) and to dimers of the Golgi resident transmembrane proteins p23 and p24 (Popoff et al., 2011).

Recently, the ER GTPase Rab1, PI4KIIIα, and PI4P were proposed to function together to recruit GBF1 to Golgi membranes (Dumaresq-Doiron et al., 2010). Activated Rab1b binds directly to GBF1 and promotes GBF1 and COP1 localization to the Golgi (Monetta et al., 2007). Rab1a also recruits GBF1, and constitutively active Rab1a can co-immunoprecipitate PI4KIIIα and increase the amount of the GDP-FAPPI1-PI4P marker at the Golgi (Dumaresq-Doiron et al., 2010). PI4KIIIα colocalizes significantly with GBF1. Inhibition of PI4KIIIα with Wm or PAO greatly reduces Golgi localization of GBF1 and GGA3, without affecting GBF1 or GGA3 protein levels or Golgi morphology. Knockdown of PI4KIIIα has a slightly stronger effect on GBF1 localization, which may be due to dispersal of the cis-Golgi, as seen by staining for the marker gigantin. PI4P may affect COPI assembly through recruitment of GBF1 via a polybasic domain (Dumaresq-Doiron et al., 2010), or through recruitment of the PI4P-binding Golgi phosphoprotein 3 (GOLPH3), a COPI-binding partner (Tu et al., 2008, 2012). Importantly, none of these effects were seen in cells treated with PI4KIIIβ siRNA. Thus, PI4KIIIα may promote COPI assembly in response to Rab1 stimulation. This unexpected role for PI4KIIIα may explain its long-recognized localization
at the ER in mammalian cells. It will be interesting to test this model by examining COPI localization and function in PI4KIIIz-depleted cells, the effect of Rab1 on PI4K activity, and whether GBF1 is a bona fide PI4P effector.

Additional evidence for involvement of PI4P in COPI trafficking comes from the observation that knockdown of PI transfer protein β (PITPb), which catalyzes non-vesicular transport of PI from the ER to the cis-Golgi, reduces overall PI4P levels and specifically affects COPI-mediated retrograde transport of the KDEL receptor, but not anterograde trafficking from the TGN in HeLa cells (Carvou et al., 2010). This is consistent with the idea that a unique pool of PI4P in the cis-Golgi is dedicated to retrograde trafficking. This pool may be generated specifically by PI4KIIIz because (1) PI4KIIIz affects COPI assembly proteins (Rab1 and GBF1), but does not impair overall levels of Golgi PI4P (Balla et al., 2005), and (2) despite its effect on GBF1 and GGA localization to the TGN (Dumaresq-Doiron et al., 2010), PI4KIIIz is not thought to regulate anterograde trafficking. Lack of an effect of PI4KIIIb on TGN trafficking could be explained by the presence of PI4KIIIβ and PI4KIz, which may help recruit sufficient populations of GGA and other clathrin adaptors. Indeed, AP-1 distribution is normal in PI4KIIIz knockdown cells (Dumaresq-Doiron et al., 2010). Thus, the ER and cis-Golgi may be sensitive to PI4P produced by PI4KIIIz.

Relatively little is known about the formation of clathrin-independent vesicles at the TGN. The Arf1-binding glycolipid transfer protein FAPP2 generates PI4P-dependent membrane tubules in vitro, and has been suggested to mediate clathrin-independent trafficking (Cao et al., 2009; Valente et al., 2012). In vivo, FAPP2 regulates apical cargo transport in polarized and non-polarized cells (Godi et al., 2004; Vieira et al., 2005). Whether the lipid transfer activity of FAPP2 is connected to its role in membrane trafficking is not clear. However, it is possible that FAPP2-mediated flipping of glucosylceramide lipids to the inner leaflet of the TGN creates asymmetry across the membrane, leading to curvature (De Matteis & Luini, 2008). Alternatively, glycosphingolipid self-organization into liquid-ordered domains may facilitate sorting of lipid raft-containing carriers (Surma et al., 2012). FAPP2 may also initiate budding and tubulation through insertion of a hydrophobic wedge into the bilayer after it forms extensive contacts with PI4P (Lenoir et al., 2010).

While PI4P is clearly important for recruitment of proteins that initiate vesicle formation, it is also important for sorting of cargo destined for these vesicles, as evidenced by missorting of proteins under reduced PI4K activity (Burgess et al., 2012; Jovic et al., 2012). This makes sense, given that many adaptors localize through multiple low-affinity interactions with PI4P, ARF-1 and cargo recognition sequences that act synergistically to target them to the correct membranes and increase cargo-binding affinity (Wang et al., 2007). Thus, the role of PI4P in sorting is intimately associated with adaptor recruitment.

Vesicle formation and structural integrity

Filamentous (F-) actin facilitates vesicle trafficking by powering endosome motility and by remodeling the cell cortex during endo- and exocytosis (Lanzetti, 2007). For example, the F-actin regulator WASP homologue associated with actin, membranes, and microtubules (WHAMM) interacts with microtubules or Arp2/3 to promote membrane tubulation or tubule elongation, respectively, thereby regulating Golgi structure and anterograde trafficking (Campellone et al., 2008; Shen et al., 2012). However, by and large, the role of F-actin in Golgi and post-Golgi trafficking has not been well defined. Only recently has Golgi PI4P been linked to actin via myosin motors that pull newly formed vesicles away from the TGN. The highly conserved PI4P effector GOLPH3 (Vps74 in budding yeast) binds PI4P with high affinity and specificity through its 34 kDa Golgi phosphoprotein (GPP34) domain (Dippold et al., 2009; Wood et al., 2009). In HeLa cells, GOLPH3 simultaneously binds PI4P and the unconventional myosin Myo18A, providing the tensile force necessary to separate vesicles from TGN. Interestingly, the vertebrate-specific GOLPH3 paralog GOLPH3L also binds PI4P, but counters forces exerted by GOLPH3 (Ng et al., 2013). The actions of both proteins need to be regulated, as unchecked activity of either GOLPH3 or GOLPH3L impedes anterograde secretion and disrupts Golgi morphology. Indeed, GOLPH3L knockdown leads to excessive stretching and vesicle formation by GOLPH3 as well as Golgi fragmentation. In contrast, GOLPH3 knockdown leads to Golgi compaction and loss of its characteristic ribbon-like appearance. Thus, in mammalian cells, Golgi architecture depends on the balance between GOLPH3 and GOLPH3L. This may be particularly important in secretory tissues, where GOLPH3L is highly expressed. The mechanism by which GOLPH3L inhibits GOLPH3 is currently unknown. Moreover, it is unclear whether GOLPH3 is regulated in an analogous manner in flies and yeast, which lack both GOLPH3L and the ribbon-like Golgi morphology found in vertebrate cells.

In budding yeast, Pik1 is needed for proper localization of the GOLPH3 homologue Vps74, suggesting that PI4KIIIβ may regulate GOLPH3/GOLPH3L in mammalian cells. Indeed, PI4KIIIβ plays a critical role in regulating Golgi structure and function, since loss of this enzyme leads to Golgi fragmentation in a number of systems (Daboussi et al., 2012; Godi et al., 1999; Strahl et al., 2005; Polevoy et al., 2009). Golgi structure also relies on a continuous network of spectrin proteins that form a scaffold to shape and support the cell. PI4P and Arf1 jointly recruit βIII spectrin to the TGN, where it maintains the Golgi’s ribbon-like appearance and facilitates secretory trafficking (Salcedo-Sicilia et al., 2013). Knockdown of βIII spectrin or depletion of PI4P through rapamycin-mediated recruitment of Sac1 to the Golgi results in Golgi fragmentation. Thus, PI4P is essential for structural integrity of the Golgi complex.

Vesicle fission

Since PI4P is important for multiple steps leading to generation of post-Golgi vesicles, adding vesicle fission to its repertoire of functions would be an intuitive way to link all of the events in this process. Indeed, at the PM, PIP₂...
recruits the clathrin adaptor AP-2, actin remodeling factors, and the GTPase dynamin to promote endocytosis and vesicle scission (Ramachandran, 2011; Rohde et al., 2002; Yarar et al., 2007). However, there is currently little evidence for involvement of PI4P in procurement of fission machinery for coated vesicles at the Golgi. Although dynamin and dynamin-like proteins have been implicated in scission of clathrin-coated carriers at the TGN, they have been reported to bind PIP2, not PI4P (Bonekamp et al., 2010; Weller et al., 2010). COPI and COPII vesicles, which require PI4P for their formation, do not require dynamin (Campelo & Malhotra, 2012). Interestingly, PI3Kα kinase activity is required in macrophages for recruitment of dynamin 2 and release of cytokine carriers from the TGN (Low et al., 2010), suggesting that PIP3, rather than PI4P, regulates fission of at least one class of vesicles at the Golgi.

A role for PI4P, however, has been reported in fission of both lysosomal vesicles (Sridhar et al., 2013) and coat-independent vesicles termed post-Golgi carriers (PGCs) that traffic from the TGN to the basolateral cell surface (Valente et al., 2012). Formation of PGCs requires the brefeldin A-dependent ADP-ribosylation substrate (BARS) protein, also known as C-terminal binding protein (CtBP) 1-short, a cytosolic protein originally identified for its ability to regulate Golgi tubulation and fragmentation during mitosis (Corda et al., 2006). Other trafficking steps that do not require dynamin, such as COPI-mediated retrograde transport and fluid-phase endocytosis, similarly utilize BARS (Bonazzi et al., 2005; Yang et al., 2005).

Evidence suggests that PI4P is involved in BARS-mediated PGC fission. For example, in rat brain cells, BARS co-purifies with 14-3-3γ, a member of a PI4KIIIβ-interacting family of scaffolding proteins (Valente et al., 2012). In COS-7 cells, BARS also pulls down PI4KIIIβ itself, along with its activating kinase PKD. PKD-mediated phosphorylation of PI4KIIIβ triggers binding of 14-3-3γ, resulting in sustained PI4P generation (Hausser et al., 2006). The discovery that 14-3-3γ, PI4KIIIβ and PKD form a complex with BARS suggests that PI4KIIIβ is stabilized and activated at sites of vesicle fission. With no predicted role for BARS as an energy-coupled molecular motor, it is unclear how it is able to sever tubules growing from the TGN. However, since BARS acts as a scaffold for lipid-modifying enzymes such as PI4KIIIβ and PLD (Haga et al., 2009), it may help create a local lipid environment that is amenable to fission (Haga et al., 2009; Valente et al., 2012). In addition, PKD phosphorylates the PI4P effectors ceramide transfer protein (CERT) and OSBP (a sterol transerfase) to remove them from the Golgi (Olayioye & Hausser, 2012). Although it seems contradictory that PKD-stimulated production of PI4P recruits these effectors only to have them phosphorylated and removed, this may represent a negative feedback loop to turn off PKD action, since CERT helps generate DAG, a PKD activator. This may also represent a mechanism to ensure optimal PI4P, ceramide and sterol levels for fission, or to eliminate proteins that sequester PI4P. With regard to the latter possibility, dephosphorylation of PIP2 to PI4P facilitates dynamin-mediated fission of endocytic vesicles in COS-7 cells (Chang-Ileto et al., 2011). This is thought to induce dynamin disassembly from the membrane, although it is also possible that electrostatic properties of concentrated PI4P are more amenable to the final stages of membrane separation.

**Vesicle fusion**

After liberation of vesicles from one organelle, they are transported and fuse with target membranes at a different site. Vesicles are first tethered by landmark proteins such as the exocyst components Sec3 and Exo70 at the plasma membrane (He & Guo, 2009), or by vesicular mediators such as the TRAPPi complex (Sztul & Lupashin, 2006) at the cis-Golgi. This is followed by fusion through assembly of a trans complex of SNARE proteins derived from vesicular and target membranes. Recently, PI4P was shown to be dispensable for vesicle budding and tethering of COPII vesicles, but critical for SNARE complex formation in fusion with the cis-Golgi during ER-to-Golgi trafficking (Lorente-Rodriguez & Barlowe, 2011). In *in vitro* assays, pre-treatment of acceptor membranes with Sac1 prevented fusion of transport vesicles. Similarly, sequestration of Golgi PI4P with the PH domain of FAPP1 *in vitro* or depletion of PI4P using a pik1 mutant in *vivo* prevented transfer of radiolabeled cargo from ER-derived COPII vesicles to Golgi acceptor membranes. Since overexpression of vesicle and cis-Golgi SNARE proteins suppressed these fusion defects, it was postulated that PI4P may interact directly with SNARE machinery.

**PI4P Gradient in Golgi trafficking and function**

Although GOLPH3 is a PI4P effector at the TGN, evidence from yeast suggests a reciprocal arrangement whereby GOLPH3/Vps74 also regulates PI4P levels at the Golgi. PI4P is enriched in the TGN, whereas Vps74 localizes primarily to early Golgi compartments (Schmitz et al., 2008) and Sac1 resides at the ER and Golgi (Whitters et al., 1993). Interestingly, vps74 and sac1 interact genetically and physically, and deletion of either gene results in greater colocalization of the PI4P marker FAPP-PH with the medial-Golgi marker Aur1 (Wood et al., 2012). Bimolecular fluorescence complementation experiments showed that Vps74 and Sac1 interact *in vivo* at the medial-Golgi, suggesting that Vps74 detects PI4P and recruits Sac1 to this compartment, thereby depleting PI4P in the early Golgi.

Sac1 also localizes to the cis-Golgi in mammalian cells, where it maintains a clear distinction between early and late compartments (Cheong et al., 2010). Here, knockdown of Sac1 resulted in increased GFP-FAPP-PH-positive structures, mislocalization of medial-Golgi glycosylation enzymes to intracellular and cell surface membranes, and alterations in N- and O-linked glycosylation patterns. Thus, expansion of PI4P-rich domains in the Golgi leads to aberrant entrance of resident Golgi proteins into the secretory pathway. The authors of this study proposed that ectopic PI4P in early Golgi compartments may recruit trafficking machinery normally assembled at the TGN. Therefore, Sac1 serves a conserved role in confining PI4P enrichment to the TGN, which ensures proper Golgi organization and function.

A local requirement for high levels of PI4P suggests that PI4P-dependent functions in the TGN would be more sensitive to PI4P disruption than those at the cis-Golgi. Evidence for this idea comes from examination of yeast...
carboxypeptidase Y (CPY), which is transported and modified through the secretory pathway and can be monitored en route by differences in molecular weight at the ER, Golgi and vacuole. *pik1* mutants kept at the non-permissive temperature for a total of 25 min delayed transport of CPY only from the TGN to the vacuole (Audhya et al., 2000). In contrast, a temperature shift of 57 minutes led to a complete block of PI4P-dependent transfer of CPY from the ER to the cis-Golgi without significantly affecting Golgi structure or function (Lorente-Rodriguez & Barlowe, 2011). Collectively, these data indicate that a gradient of Golgi PI4P is integral to cisternal identity and function.

**Non-vesicular transport and lipid metabolism**

PI4P regulates lipid homeostasis through a number of effectors that have lipid binding and/or transferase activity required for biogenesis of complex modified lipids such as sphingomyelin and glycosphingolipids (Graham & Burd, 2011). These effectors include the COF family proteins CERT, OSBP and OSBP-related proteins (ORPs), and FAPP2, which transfer ceramide, oxysterol, and glucosylceramide, respectively (D’Angelo et al., 2008). CERT-mediated movement of ceramide from ER to Golgi requires PI4KIIIβ (Toth et al., 2006). OSBP stimulates CERT-dependent sphingomyelin synthesis through an unidentified mechanism that requires PI4P generated by PI4KIIζ (Banerji et al., 2010; Perry & Ridgway, 2006). Importantly, CERT and FAPP2 transfer ceramide and glucosylceramide to the appropriate Golgi leaflet for sphingolipid biosynthesis in response to PI4P binding (Yamaji et al., 2008).

CERT and OSBP are thought to promote ER-Golgi contact sites through interaction with ER integral VAP (VAMP [vesicle-associated membrane protein]-associated protein) proteins via their FFAT (two phenylalanines followed by acidic tract) motifs, and through association with Golgi PI4P and Arf1 via their PH (De Matteis et al., 2007) or ORD domains (Li et al., 2002). ER-Golgi contact sites may allow non-vesicular lipid transfer between the two organelles. OSBP, yeast ORPs (Osh proteins), and several human ORPs transfer sterols between liposomes in vitro (Du et al., 2011; Ngo & Ridgway, 2009; Raychaudhuri et al., 2006; Schulz et al., 2009). Osh proteins lacking PH domains and FFAT motifs have been proposed to mediate sterol transfer by acting as diffusible sterol carriers (de Saint-Jean et al., 2011). Although Osh ORD domains alone can tether membranes (Schulz et al., 2009), this is somewhat controversial (de Saint-Jean et al., 2011), indicating that the mechanism of sterol transfer remains to be further defined.

PI4P also regulates and is regulated by other lipid transfer proteins. The yeast integral membrane ATPase Drs2 requires Pik1-generated PI4P to stimulate its flipase activity, which transfers phosphatidylserine and phosphatidylethanolamine from the luminal to the cytosolic leaflet of TGN and endosomes (Jacquot et al., 2012; Natarajan et al., 2009). Localization of OSBP and CERT is also dependent on Nir2 (Peretti et al., 2008), a VAP-binding PI/phosphatidylcholine transfer protein that moves PI from the ER to the Golgi, providing substrate for PI4Ks. Indeed, knockdown of VAP proteins reduces Golgi PI4P and sphingomyelin synthesis.

Unlike most PI4P effectors, Osh4 decreases PI4P levels and inhibits secretion (Fain et al., 2007; LeBlanc & McMaster, 2010; Li et al., 2002; Mousley et al., 2012). Indeed, sterol binding by Osh4 is required to keep PI4P levels in check (Stefan et al., 2011). Some insight was shed on this relationship with the demonstration that, in vitro, Osh4 can mediate rapid exchange of PI4P for dehydroergosterol, transferring these lipids in opposite directions between liposomes (de Saint-Jean et al., 2011). Hence, in vivo, Osh4 may transfer PI4P from the Golgi to the ER, exchanging it for sterol in the process. Interestingly, the authors note that Pik1 and Sac1 would maintain this directionality of transfer by producing a gradient of PI4P that is high at the Golgi and low at the ER. Consequently, Osh4 enhances a sterol gradient that is low at the ER, where it is synthesized, and high at the TGN. This gradient may help drive anterograde membrane trafficking, as sterols and sphingolipids are selectively enriched in secretory vesicles that bud from the TGN (Klemm et al., 2009). Depletion of other ORPs in HeLa cells or in *C. elegans* leads to sorting and trafficking defects in the endo-lysosomal system (Du et al., 2011; Kobuna et al., 2010), and overexpression of Osh4 induces autophagy (LeBlanc & McMaster, 2010; Mousley et al., 2012). However, it remains to be seen whether functions of ORPs at these other organelles are regulated by PI4P.

Consistent with Osh4 mediating exchange of PI4P for sterols, sterol binding promotes dissociation of Osh4 from TGN and endosomes (Mousley et al., 2012). An Osh4 sterol-binding mutant showed increased membrane association in fractionation studies and, when fused to GFP, appeared more punctate than wild-type Osh4-GFP, which is both cytosolic and punctate. Enhanced recruitment of Osh4 to TGN and endosomes impairs secretory trafficking as well as endocytic trafficking to the vacuole, suggesting that sterol deficiency, or an inability to sense sterol, induces a brake on PI4P-dependent trafficking. Interestingly, Osh4 sterol-binding mutants fail to traffic amino acid permeases to the PM, which leads to disruption of the general amino acid control pathway and to cell cycle arrest. Thus, the sterol regulatory functions of Osh4 are intimately related to PI4P-dependent trafficking and cell physiology.

**Trafficking at endosomes and lysosomes**

PI4P is detected on membranes of the endo-lysosomal system and is required for various steps of endosomal trafficking. PI4P and PIP2 are found on tubules emanating from the endocytic recycling compartment, a compartment containing endocytosed material destined for return to the PM (Jovic et al., 2009). PI4P, but not PIP2, is instrumental in tubule localization of Eps15 homology (EH) domain-1 (EHD-1), a protein known to regulate recycling of transmembrane cargo internalized by clathrin-dependent and -independent endocytosis. Indeed, mutation of a key lysine in the EH domain of EHD-1 that reduces binding to PI4P (K483E) renders it unable to associate with tubules and delays recycling (Jovic et al., 2009). PI4P may also regulate recycling endosomes through Rab11. PI4KIIIβ binds and recruits Rab11, a regulator of recycling endosomes, to the Golgi (de Graaf et al., 2004). Although *Drosophila*
Fwd/PI4KIIIβ kinase activity is dispensable for Rab11 binding, it is required for full rescue of spermatocyte cytokinesis, presumably because of the need for PI4P in restoring full secretory function (Polevoy et al., 2009).

Whereas PI4KIIIβ is localized predominantly at the Golgi, PI4KIIz has been detected at the PM, early and late endosomes, synaptic vesicles, immature secretory granules, as well as at the TGN, where it produces PI4P required for recruitment of the clathrin adaptors AP-1 and GGA1-3 (Figure 3C; Wang et al., 2003, 2007). While it remains unclear how the pools of PI4P generated by PI4KIIIβ and PI4KIIz are partitioned at the Golgi such that each enzyme recruits specific PI4P effectors, the two PI4Ks clearly play separate, sequential roles at this organelle. This was shown recently for the trafficking of the lysosomal hydrolase β-glucocerebrosidase (GBA) and its sorting receptor lysosomal integral membrane protein type 2 (LIMP-2) (Jovic et al., 2012). Inhibition of PI4KIIIβ prevented exit of this cargo from the Golgi, a block that was mirrored by acute depletion of Golgi PI4P. In contrast, knockdown of PI4KIIz resulted in accumulation of LIMP-2 in late endosomes and increased missorting of GBA to the extracellular medium. This latter effect was reversed by simultaneous inhibition of PI4KIIIβ, consistent with it acting at an earlier step. Thus, GBA/LIMP-2 transport relies on both PI4KIIIβ and PI4KIIz, albeit at different stages of trafficking.

Studies in Drosophila, mice and human cell lines have also given insight to a role for PI4KIIz at endosomes. In the Drosophila larval salivary gland, loss of PI4KII leads to mislocalization of secretory granule cargo to late endosomes as well as accumulation of the lysosomal enzyme sorting receptor (Lerp) in this compartment (Burgess et al., 2012). PI4KII mutants exhibit aberrant Retromer dynamics, indicating that PI4KII may regulate retrieval of cargo or membranes from late endosomes to the TGN. Interestingly, PI4KII localizes to tubules emanating from late endosomes. Although kinase-dead PI4KII also localizes to late endosomes in a wild-type background, catalytic activity is required for tubule formation, pointing to a key role for PI4P in this trafficking step. In HEK293 cells, PI4KIIz kinase activity is needed for assembly of clathrin adaptor AP-3 onto late endosomes. In addition, PI4KIIz, which is palmitoylated, acts as membrane cargo via a canonical dileucine AP-3 sorting motif (Craigie et al., 2008). Both catalytic activity and the dileucine motif are required for PI4KIIz localization to late endosomes as well as rescue of PI4KIIz knockdown endosomal phenotypes, indicating mutual regulation between PI4KIIz and AP-3. Moreover, biogenesis of lysosome-related organelle complex 1 (BLOC-1) mediates PI4KIIz–AP-3 interaction, and all three are needed for proper trafficking of lysosomal-associated membrane protein 1 (LAMP1) in HEK293 cells (Salazar et al., 2009). In mouse primary cortical neurons, AP-3 and BLOC-1 traffic synaptic vesicles carrying PI4KIIz from the cell body to the neurite tips (Larimore et al., 2011). Additionally, when PI4KIIz is not bound to AP-3, it can interact with BLOC-1 and the WASP and SCAR homologue (WASH), an F-actin nucleation-promoting factor specifically associated with endosomes (Ryder et al., 2013). Knockdown of WASH produces long tubules containing PI4KIIz that emanate from normal size endosomes, whereas knockdown of the BLOC-1 subunit pallidin results in enlarged endosomes with no tubules. This suggests BLOC-1 may act downstream of PI4KIIz–AP-3 interaction to sort PI4KIIz into tubules, and that WASH may regulate scission of PI4KIIz-containing carriers. In addition, PI4KIIz may indirectly recruit WASH, as GBA/LIMP-2 binds PI4P in vitro (Jia et al., 2010; Ryder et al., 2013).

An unexpected role for PI4KIIIβ at the lysosome was recently uncovered using siRNA in cultured cells. Small fractions of PI4KIIIβ and PI4P were detected at the lysosome, and the presence of wild-type, catalytically active PI4KIIIβ was required to prevent abnormal tubulation of this organelle as well as efflux of missorted resident lysosomal proteins such as LAMP1 into these tubules (Sridhar et al., 2013). This effect was unrelated to the role of PI4KIIIβ in the Golgi, as disruption of Golgi function by brefeldin A or nocodazole showed no effect on LAMP1 dynamics. These data suggest PI4KIIIβ normally facilitates cargo sorting and fission of lysosomal vesicles, preventing tubule formation. Increased association of clathrin and the clathrin adaptor AP-2 with lysosomes was observed in the absence of PI4KIIIβ, leading to the suggestion that PI4KIIIβ may prevent ectopic recruitment of these proteins. Interestingly, this report followed another that identified PI5P and PI3P as being necessary for tubule formation to create de novo lysosomes from autolysosomes following starvation-induced autophagy (Rong et al., 2012). Since tubules form in PI4KIIIβ and PI5P doubly-depleted cells, Sridhar et al. propose that PI5P is not necessary for vesicle or tubule formation per se, but that in special circumstances, such as those surrounding regeneration of lysosomes, PI5P is required to deplete PI4P through its conversion to PI2P in order to inhibit vesicle fission and favor bulk efflux through tubulation. However, important questions remain, such as what drives tubule formation in the absence of PI4P, and whether PI4P and PI3P are coordinately regulated to recruit AP-2 and scission machinery.

**PI4P and Rabs confer compartment identity**

Because PI4P is found in multiple intracellular membranes, additional factors must be required to recruit organelle-specific effectors. In addition to simultaneous detection of Arf1 by PI4P effectors such as FAPPs (Godi et al., 2004), Rab family proteins can help determine distribution of PI4P effectors. Similar to organelle-specific distribution of phosphoinositides, the secretory and endosomal systems are decorated with compartment-specific Rabs. Together, phosphoinositides and Rabs define compartment identity, and synergistically recruit downstream effectors to perform compartment- and stage-specific functions. This is important because proper trafficking requires sequential events, including disassembly of fission machinery, assembly of fusion machinery, and cargo-specific events such as protein processing. Evidence suggests PI4Ps and Rabs participate in a finely tuned assembly line for moving cargo through sequential steps in the secretory pathway. This “Rab cascade” unfolds through Rab-mediated recruitment of the GEF that activates the subsequent Rab and, in some cases, the
GTPase-activating protein (GAP) that inactivates the Rab that defines the current compartment (Jean & Kiger, 2012; Mizuno-Yamasaki et al., 2010).

PI4P has been shown to play an integral role in this identity switch. Sec2, the yeast GEF that activates the Rab protein Sec4/ Rab8 on secretory vesicles, is involved in both budding from the TGN and recruitment of the exocyst component Sec15 for docking at the plasma membrane. Interestingly, the level of PI4P on the membrane to which Sec2 is bound determines which role it plays at a given point during secretion (Mizuno-Yamasaki et al., 2010). Sec2 is recruited to the trans-Golgi by binding to a yeast Rab11 ortholog (either Ypt31 or Ypt32) and PI4P via its Ypt31/32 binding site and three polybasic patches, respectively. During budding, the exocyst machinery is prevented from prematurely assembling because binding to Ypt32 occludes the Sec15-binding site on Sec2, and because PI4P itself inhibits Sec2-Sec15 interaction. Indeed, in vitro assays show that PI4P inhibits this association in a dose-dependent manner. However, once secretory vesicles are formed, Sec15 is able to outcompete Ypt32 for Sec2 binding. Since the PI4P probe mCherry-FAPP-PH colocalizes with Sec2 only at the Golgi and not at vesicular sites, it is proposed that low levels of PI4P on secretory vesicles allow assembly of docking proteins at the expense of budding machinery. Similarly, it has been proposed that Sac1 decreases PI4P on forming exocytic vesicles (Alfarpo et al., 2011). Thus, PI4P regulates Sec2 binding partners and the switch in identity from TGN to secretory membrane. Whether other factors lead to a drop in PI4P, i.e. a sorting out of PI4P at the Golgi or enzymatic depletion on vesicles, remains to be determined.

Although reduction in PI4P levels is necessary for progression along the secretory pathway, another report in yeast demonstrates that PI4P also plays an important role on secretory vesicles, and that modulating its levels can affect compartment identity and function (Santiago-Tirado et al., 2011). The myosin V motor Myo2 transports Golgi membranes and secretory vesicles along actin cables into the nascent bud. Myo2 is recruited by Rabs of the late Golgi (Sec7), the trans-Golgi (Ypt31/32), and secretory vesicles (Sec4). GFP-FAPP-PH and mCherry-Osh2-PH colocalize with Sec7 and Ypt31, respectively, and mCherry-Osh2-PH colocalizes with Sec4-containing vesicles that accumulate at the bud tip and neck of small budded cells. Sec4 and PI4P did not overlap in wild-type mother cells, but a small degree of overlap was seen in mother cells from myo2 transport mutants, which accumulate secretory vesicles. This suggests that PI4P concentration is high on Golgi membranes and low on smaller Sec4-positive secretory vesicles, such that secretory vesicle PI4P is difficult to visualize without vesicle aggregation. These results are consistent with the hypothesis of Mizuno-Yamasaki et al. (2010) that a drop in PI4P levels occurs during the transition from Golgi to secretory vesicle. Sec4 polarization and Myo2 transport of secretory organelles depends on PI4P (Santiago-Tirado et al., 2011). Increasing Golgi PI4P by Pkh1 overexpression or sac1 deletion results in recruitment of Myo2 mutant proteins unable to bind either Ypt31/32 or Sec4, rescuing growth and transport defects. Likewise, fusion of mutant Myo2 proteins with a FAPP-PH domain also rescues Rab-binding myo2 mutants, indicating that a bridge between Myo2 and PI4P is necessary for transport. This is thought to occur via an as yet unidentified factor. Hence, under normal conditions, PI4P and a Rab synergistically recruit Myo2, with PI4P acting as a general marker for secretory membranes, and Rabs further defining specific secretory stages. However, upon disruption of Myo2-Rab association, enhancement of Myo2-PI4P interaction can compensate, showcasing the regulatory power of modulating PI4P.

Plasma membrane effectors

While much has been learned about Golgi PI4P, roles for PI4P at the PM are only beginning to be revealed. This is because PI3P and PI(4,5)P2 are also found on this membrane, and traditional genetic or biochemical techniques to disrupt PI4P made it difficult to assess whether resulting phenotypes were due to loss of PI4P or its downstream metabolic derivatives. With the advent of technologies to detect and manipulate specific pools of lipids (Clark et al., 2011; Heo et al., 2006; van den Bogaart et al., 2011; Varnai & Balla, 2008), new data suggest that despite its many essential roles at the Golgi, the majority of cellular PI4P is on the PM (Hammond et al., 2009). The classical view is that PM PI4P allows for rapid replenishment of PI(3,4)P2 after acute signaling events. PI4KIIζ appears to be the isoform that generates this pool of PI4P, as pharmacological inhibition with Wm or 10 μM PAO abolished replenishment of both PM PI4P and PI(3,4)P2 after hormone-activated PLC-coupled signaling in COS-7 cells (Balla et al., 2008; Hammond et al., 2012). Knockdown of PI4KIIζ gave a less robust effect, presumably because of incomplete silencing. Further, in permeabilized β-cells, parallel changes in PI(3,4)P2 levels in response to decreases or increases in PI4P depend on a type III PI4K (Wuttke et al., 2010).

Independent roles for PM PI4P are beginning to be defined, although the extent to which PI4P influences PI(3,4)P2 and PI(3,4,5)P3 levels remains unknown. New data suggest that, in some cells, PI4P and PI(3,4)P2 may be less intimately coupled than previously thought. Stimulation of β-cells through Gαq-protein-coupled receptors leads to opposite effects on PM PI4P and PI(3,4)P2 levels: PI3P decreases as expected after activation of PLC; however, PM PI4P levels increase, suggesting that the two lipids may be independently regulated in this cell type (Wuttke et al., 2010). More strikingly, in COS-7 cells, acute depletion of all PM PI4P by rapamycin recruitment of the Sac1 phosphatase domain to the PM had no effect on PI(3,4,5)P3 resynthesis after PLC activation (Hammond et al., 2012). Since previous results on PI4KIIζ inhibition and knockdown differ from those obtained through acute PI4P depletion, this suggests that PI4KIIζ produces a small but essential pool of PI4P at specific PM sites that is not susceptible to acute PI4P depletion. PI4KIIζ and its associated proteins may protect this pool through steric hindrance (see below). Alternatively, PI4KIIζ may produce a pool of PI4P that is used immediately for PI(3,4,5)P3 resynthesis. Indeed, kinetic studies suggest PI(3,4,5)P3 replenishment occurs so quickly that both PI4K and PI(4,5)P2 activity need to be stimulated to satisfy the observed concentration response and time course (Falkenburger et al., 2010, 2013). Additionally, PI(4,5)P2 may be replenished from other membranes. On this front, it is noteworthy that Golgi-specific depletion of PI4P moderately affects PM PI(3,4,5)P3 replenishment, suggesting that Golgi PI4P can contribute to
PM PIP$_2$ (Szentpetery et al., 2010). In either case, although P14KIIIz appears to play a critical role in PIP$_2$ replenishment after signaling, it is unclear whether it has a major role in regulating steady state PIP$_2$ levels in mammalian cells. While inhibition of P14KIIIz in COS-7 cells under normal growth conditions did decrease steady state PM PIP$_2$, it had little to no effect on steady state PIP$_2$, suggesting that PIP$_2$ normally experiences low turnover in these cells, or that it is made from a distinct or redundant pool of PI4P (Balla et al., 2008; Hammond et al., 2009).

On the other hand, genetic ablation of P14KIIIζ in yeast and in animal models suggests that this pool of PI4P is tightly linked to the functions of PM PIP$_2$ and PM identity (Audhya & Emr, 2002; Murray et al., 2012; Nakatsu et al., 2012). Indeed, in Drosophila, P14KIIIζ mutant female germ cells exhibit defects similar to those depleted of available PIP$_2$ (Tan et al., 2014), and mouse embryonic fibroblasts (MEFs) mutant for P14KIIIz upregulate expression of the PIP$_2$-generating PIP5K enzymes as a compensatory mechanism. Perhaps the different effects of P14KIIIz on steady state PIP$_2$ reflect differential requirements for P14KIIIz-dependent synthesis of PM PI4P in different cell types or in cells within living tissues.

Although previous studies identified few specific functions for PM PI4P aside from its role as a precursor to PIP$_2$, Hammond et al. (2012) found that PI4P makes a substantial contribution to the total negative charge that defines the inner leaflet of the PM. Seven membrane-targeting protein domains fused to GFP were monitored before and after dynamic depletion of PM PI4P or PIP$_2$. Rapamycin was used to recruit the phosphatase domains of Sac1 and inositol polyphosphate-5-phosphatase E (INPP5E) to deplete PI4P and PIP$_2$, respectively. For those proteins that were predicted to localize via non-specific polyanionic lipid interaction through a polybasic domain, elimination of both PM PI4P and PIP$_2$ was required to abolish PM localization, whereas depletion of either lipid alone had minimal effect. For example, while stimulation of the heat and capsaicin-activated transient receptor potential vanilloid 1 (TRPV1) cation channel was previously associated with binding to PIP$_2$, activation was achieved in the presence of either PM PI4P or PIP$_2$, with channel activity being inhibited only when both lipids were absent. However, not all cation channels operate on a general requirement for polyanionic lipids, as activation of the menthol-activated transient receptor potential melastatin 8 (TRPM8) channel specifically requires PIP$_2$. Therefore, PM PI4P functions in processes that require a general polyanionic lipid pool. With this in mind, it is possible that PI4P fulfillment of this function is especially important when PIP$_2$ is rapidly consumed, which may explain why PI4P levels increase during PLC signaling in some cells (Wuttke et al., 2010), although this would not be the case in cell types where PI4P and PIP$_2$ react in parallel (Balla et al., 2008). Whether PI4P specifically targets any protein for PM localization or activation remains to be seen. With new methods for perturbing membrane-specific pools of PI4P, analysis of lipids in sub-membrane microdomains (van den Bogaart et al., 2011), and imaging of PM dynamics (Nakatsu et al., 2012; Wuttke et al., 2010), it is an exciting time for this avenue of research.

**Dynamic regulation of PI4P signaling: mechanisms of spatiotemporal control**

Thus far, we have discussed steady-state roles for PI4P in replenishing other signaling phosphoinositides, and in recruiting organanelle-specific effector proteins, for which little phosphoinositide turnover is apparently required. However, the ability of phosphoinositides to be rapidly phosphorylated and dephosphorylated makes them ideal candidates to regulate dynamic processes in time and space. Therefore, it is not surprising that physiological cues requiring rapid cellular responses, such as nutrient availability and cell signaling, invoke pathways that regulate PI4P.

**Response to nutrients**

An early report describes modulation of PI4K activity in response to nutrients. Secretion of insulin granules by pancreatic β cells in response to increased glucose concentration requires type III PI4K activity, and is stimulated by injection of PI4P (Olsen et al., 2003). Given the relatively low concentration of the pharmacological inhibitor PAO required to inhibit insulin exocytosis (Balla & Balla, 2006), the bulk of this PI4K activity is likely provided by P14KIIz. Indeed, PI4Ks were proposed to act as metabolic sensors because reduced levels of cellular ADP, which mimic what occurs upon glucose stimulation, correspond with increased PI4K activity. Addition of ATP to permeabilized β cells stimulates PI4P production in a dose-dependent manner, consistent with a mechanism for activating PI4Ks by increasing substrate availability (Wuttke et al., 2010). In these cells, stimulation with glucose also provoked an increase in PM PI4P that was dependent on increased cytoplasmic Ca$^{2+}$, although the exact mechanism by which energy status stimulates PI4Ks is still unclear.

Another example of regulation of PI4P signaling in response to nutrients involves Sac1. The largely ER-localized Sac1 reversibly localizes to the Golgi during serum starvation in mammalian cells (Blagoveshchenskaya et al., 2008) and upon glucose deprivation or when cell growth is slowed in late log phase cultures in yeast (Faulhammer et al., 2005, 2007). The benefit of this relocation during starvation appears to be twofold. First, ER-localized Sac1 is required for biosynthesis of oligosaccharides for N-glycosylation. Removal of Sac1 from the ER when nutrients are scarce limits the use of these resources and slows passage of glycosylated proteins through the secretory pathway. Second, depletion of Golgi PI4P by Sac1 halts global secretion, thereby conserving cellular material. Nutrient status also appears to regulate Ptk1, since it dissociates from the Golgi during glucose starvation, coinciding with Sac1 relocation to the Golgi and a decrease in Golgi PI4P (Faulhammer et al., 2007). In late log phase or with nutrient deprivation, Ptk1 shifts from the TGN to the nucleus or to cytoplasmic puncta, where it forms a complex with 14-3-3 proteins (Demmel et al., 2008).

Sac1 retention in the ER depends on its interaction with the ER transmembrane protein dolichol phosphate mannosyltransferase (Dpm1). Sac1 translocation to the Golgi upon starvation requires COPII-mediated exit, and in mammalian cells, also requires prior oligomerization of Sac1 in the
ER (Blagoveshchenskaya et al., 2008). Retrieval of Sac1 to the ER upon addition of nutrients requires COPI, and in mammalian cells, requires prior dissociation of Sac1 oligomers in the Golgi. Curiously, in yeast, the Rer1 adaptor for COPI retrograde transport is also required for ER exit of Sac1, although its role in this context is in disruption of the Sac1-Dpm1 interaction (Faulhammer et al., 2007).

This effect of nutrient status on PI4P regulators in the ER and Golgi appears to be mediated by MAPK signaling. Treating NIH3T3 mouse embryonic fibroblasts with FGF and PDGF simulates nutrient addition and promotes relocation of Sac1 from the Golgi to the ER, an effect eliminated by addition of a p38 MAPK inhibitor (Blagoveshchenskaya et al., 2008). In yeast, retrieval of Sac1 from the Golgi to the ER upon glucose stimulation of starved cells requires COPI, and as decreased degradation of EGFR over a two hour period (Minogue et al., 2006). The concomitant loss of EGFR localization to late endosomes, which are large LAMP1-positive perinuclear structures, suggested that EGFR signaling complexes fail to traffic along the endocytic pathway in the absence of PI4KIIz. Thus, PI4KIIz regulates trafficking downstream of early endosomes. The yeast homologue Ls6b has similarly been implicated in actin-based endosome motility. Unlike in mammals, however, its PI4K activity is not required and it is still unclear whether Ls6b facilitates early endosome movement away from sites of endocytosis or later stages of endosome motility (Chang et al., 2005; Kim et al., 2006).

Much less is known about PI4KIIβ, whose properties differ significantly from PI4KIIz in that approximately 75% of the enzyme is unpalmitoylated and shows either cytoplasmic or peripheral membrane-association (Jung et al., 2008). Only palmitoylated, membrane-bound PI4KIIβ is catalytically active and only 25-30% of type II PI4K activity is contributed by this enzyme in resting cells (Balla et al., 2002; Jung et al., 2008). The cytosolic pool of PI4KIIβ is sensitive to proteasome degradation and is stabilized by binding Hsp90 (Jung et al., 2011). This sequestration is released upon stimulation by EGF or PDGF, which results in palmitoylation of a subset of cytosolic PI4KIIβ. This membrane-bound PI4KIIβ subsequently translocates to the PM, where it becomes active (Jung et al., 2011; Wei et al., 2002). Relocation of cytosolic PI4KIIβ to PM ruffles is also seen with overexpression of constitutively active Rac. The pool of PI4P stimulated by growth factor may regulate early steps of endocytic trafficking since PI4KIIβ can be detected on clathrin- and AP-2-containing vesicles (Li et al., 2012b). Thus, it is tempting to speculate that PI4P synthesis in endocytosis of some receptors is subject to dynamic regulation by PI4KIIβ and that, as endosomes mature, their cargo is sorted in a manner that depends on PI4KIIz.

PI4KIIz has also been implicated in Wnt signaling in HEK293 cells and in Xenopus laevis embryos (Pan et al., 2008; Qin et al., 2009). Activation of the canonical Wnt signaling pathway by Wnt3a leads to Dishevelled (Dvl) binding to PI4KIIz and PI5PKI, and increased cellular PI4P and PIP2. The rise in PIP2 is required for phosphorylation of the Wnt co-receptor, low density lipoprotein receptor-related protein (Lrp) 5/6; co-aggregation of Wnt3a and Lrp5/6 into signalosomes; and recruitment of clathrin and AP-2 for receptor endocytosis (Kim et al., 2013). Importantly, knockdown of type IIz PI4K prevented PI4P and PIP2 elevation, Lrp5/6 phosphorylation, and β-catenin stabilization (Pan et al., 2008). Dvl itself can stimulate PI4KIIz activity in vitro, suggesting that PI4KIIz may produce the PI4P.
precursor to PIP2 in this context. Indeed, kinase-dead PI4KIιz greatly reduced endocytosis of the Wnt receptor Frizzled 4 (Fz4) (Mossinger et al., 2012). PI4KIιz undergoes ubiquitination at multiple sites, and expression of PI4KIιz that is unable to bind the E3 ubiquitin ligase Itch only moderately restored Fz4 colocalization with early endosome markers. This suggests that PI4KIιz has a ubiquitin-mediated role in Fz4 internalization or sorting into early endosomes. Furthermore, siRNA of either PI4KIιz or Itch delayed lysosomal degradation of Fz4, suggesting that these proteins act together in late endosome trafficking. Itch also inhibits PI4KIιz kinase activity independently of ubiquitination, which may help to limit Wnt signaling at the cell surface; indeed, Itch knockdown increased Lrp6 phosphorylation. It would be interesting to know whether PI4KIιz kinase activity is required for PIP2 elevation and/or Lrp5/6 phosphorylation.

The small size of type II PI4Ks and their membrane association by palmitoylation allow for dynamic regulation of their localization. Indeed, PI4KIιz may act as a spatial landmark connecting Wnt signaling, PIP5KI activity, and PI4P-dependent trafficking, rather than directly providing PI4P for PIP2 synthesis. Alternatively, complex formation between PI4KIιz and a PIP5K facilitated by a core signaling pathway component such as Dvl may result in production of a pool of PM PIP2 reserved for Wnt signaling, thereby mediating crosstalk between the Wnt and PIP signaling pathways. A similar mechanism occurs at the Golgi, where PKD forms a complex with PI4KIιι and PIP5KI (Nishikawa et al., 1998) that may be important for producing a Golgi-specific pool of PIP2, which is known to be present at low levels at this organelle (Watt et al., 2002). Monitoring where PIP increases occur in response to signaling in vivo should prove useful in deciphering the precise involvement of PI4KIιz in this pathway, and will add to our understanding of spatiotemporal control of PI4P.

**PI4P regulation by enzyme localization**

In addition to PI4KIιι, other phosphoinositide enzymes have also been found to shuttle between sites. For example, Sac1 is ER-localized during periods of cell growth, but relocates to the Golgi during periods of starvation where it halts PI4P-dependent secretion (Faulhammer et al., 2005; see above). The C-terminus of Sac1 is responsible for its ER localization, whereas its N-terminus localizes Sac1 to the Golgi. Both type II and type III PI4K activities have been detected in the nucleus (Fiume et al., 2012), although little is known about the nuclear type II enzymes. Budding yeast Pik1 contains a nuclear localization signal (NLS) and a nuclear export signal (NES). Pik1 cycles through three different locales. Phosphorylation at S396 during times of nutrient limitation decreases Golgi localization and increases its association with 14-3-3 proteins in the cytoplasm, as well as its accumulation in the nucleolus (Demmel et al., 2008). A S396D phosphomimetic mutant accumulates to a greater extent in the nucleus, suggesting that dephosphorylation may be required for nuclear exit. The Golgi and nuclear functions of Pik1 are both essential, since restriction of the enzyme to either the Golgi (by addition of a CAAX box) or the nucleus (by deletion of the NES) results in lethality that can be rescued by expression of the reciprocal mutant (Strahl et al., 2005). Mammalian PI4KIιι and PI4KIιιz have also been found in the nucleus. Nucleolar localization of endogenous PI4KIιιz was abolished with DNase or RNase treatment of permeabilized rat B50 cells, suggesting that PI4KIιιz complexes with nucleic acids (Kakuk et al., 2006). The targets of nuclear PI4P are unknown, although undoubtedly a proportion is used to produce nuclear PIP2. The budding yeast PIIP5K Miss4 (Audhya & Emr, 2003) and its mammalian and *Drosophila* homologues undergo nucleocytoplasmic shuttling and have nuclear functions (Cheng & Shearn, 2004; Schill & Anderson, 2009). PIP2 has been implicated in a number of nuclear processes, including RNA processing, nuclear export, regulation of nuclear actin and chromatin remodeling (Barlow et al., 2010). In mammals, PIP2 and PIP5Ks are detected in so-called nuclear speckles, interchromatin granule clusters that are enriched for pre-mRNA splicing machinery (Boroncovsk et al., 1998). Notably, the PI4Ks involved in these processes have yet to be identified.

A requirement for some phosphoinositide kinases in multiple organelles can be explained by shuttling. However, in the case of PI4KIιιz, until recently there has been a disconnect between its apparent location and its site of action. As described above, experiments in yeast and mammals point to PI4KIιιz controlling PM PI4P (Audhya & Emr, 2002; Balla et al., 2008). In yeast, localization of the PM PI4P marker GFP-Osh2-PH is dependent on St4 (Roy & Levine, 2004). In mammalian cells, PI4KIιιz is required for replenishment of PM PI4P and PIP2 in response to PLC activation. Yeast Stt4 localizes to PM PIK patches, which are stable complexes of Stt4 molecules with the accessory proteins Ypp1 and Efr3 (Baird et al., 2008). Nonetheless, epitope tagging and immunoelectron microscopy indicated that mammalian PI4KIιιz localizes to the Golgi, nucleolus, vacuoles, and pericentriolar regions, but not the PM (Balla & Balla, 2006). Immunocytochemistry and cell fractionation experiments consistently identified endogenous PI4KIιιz at the ER (Balla et al., 2000; Wong et al., 1997), but it was still unclear how an enzyme at this location could mediate acute responses to signaling at the PM. These discrepancies were recently resolved when a conserved ~50 amino acid sequence upstream of the reported translational start site was shown to confer targeting of mammalian PI4KIιιz to the PM (Nakatsu et al., 2012). Total internal reflection fluorescence (TIRF) microscopy revealed that full length GFP-PI4KIιιz exhibits dynamic localization at the cell surface, with abundant puncta transiently appearing at the PM. Consistent with a role for PI4KIιιz at the PM, ER-PM contact sites known to be dependent on PI4P and the ER protein stromal interacting molecule 1 (STIM1) were greatly reduced in PI4KIιιz-knockout MEFs. In addition, pre-association of mouse Ypp1 (TTC7B) and Efr3 (EFR3B) at the PM was necessary for targeting of PI4KIιιz to this site.

In mammals, regulation of PI4P signaling at the PM is shaping up to be more dynamic than expected, requiring continuous recruitment of PI4KIιιz, perhaps from the ER (see below). One potential moderator of PI4KIιιz dynamics is With no lysine 1 (WNK1), a serine/threonine protein kinase that regulates many ion transporters. WNK1 was shown to stimulate DAG-activated TRPC6 channels in a
kinase-independent but PLC- and PI4KIIıııı-dependent manner (An et al., 2011). Interestingly, WNK1 promotes PI4P production as well as association of PI4KIIıııı with membrane, suggesting that WNK1 regulates PI4KIIıııı localization.

Mammalian PI4KIIıııı localization is reminiscent of findings for PI synthase (PIS), the enzyme that catalyzes addition of myo-inositol to CDP-DAG to generate PI as part of the phosphoinositide cycle. Although its synthesis is associated with the ER, PI must be made available to PI4Ks in different cellular compartments. Recently, the Balla laboratory discovered that the vast majority of enzymatically active PI4P is found on a novel system of highly mobile vesicles, formation of which is dependent on the ER membrane remodeling GTPase and COPII nucleator Sar1 (Kim et al., 2011). This population of vesicles can be separated from heavier ER membranes using a shallow fractionation gradient. In addition, by cell imaging, the vesicles did not colocalize with typical ER, Golgi or endosomal markers. Photoactivation of PIS fused to photoactivatable GFP in the perinuclear ER reveals a highly mobile pool of vesicles emanating from this compartment. In addition, PIS-positive vesicles were seen to make contact with STIM1-positive ER-PM sites, although no fusion events were detected. Interestingly, this dynamic PIS pool is also associated with CEPT1, an enzyme that converts DAG to phosphatidyethanolamine or phosphatidylcholine (English & Voeltz, 2013). PI4P and CEPT1 colocalize with Rab10 at the tips of ER tubules, which are reduced upon treatment with siRNA directed against Rab10 or expression of GDP-locked Rab10. Thus, phospholipid synthesis may be coordinated on a common, dynamic membrane platform. Colocalization of PI and PI4KIIıııı on ER-derived vesicles has yet to be demonstrated, but it is possible that synthesis and delivery of PI may be coupled to the provision of PI4KIIıııı in a common mobile compartment. It is currently unknown how PI, or PI4P, would be transferred from these vesicles into the PM, or whether as yet unidentified PITPs are required for this process.

ER-PM contact sites are emerging as important locales for PI4P metabolism. Research from fission yeast suggests that the iconic, reticular nature of ER results from the necessity of ER-PM contacts (Zhang et al., 2012a). At these sites in mammalian cells, PI4P regulates calcium entry through the STIM1/Orai1 complex when intracellular stores are depleted (Korzeniowski et al., 2009; Walsh et al., 2010). Recent data in budding yeast suggest Sac1 is involved in forming ER-PM contact sites. Sac1 is associated with the ER and Golgi and, similar to the conundrum for PI4KIIıııı, it was not clear how it controls PM PI4P (Foti et al., 2001). Osh proteins are thought to mediate this process by detecting PM PI4P, either via PH domains (for Osh1-3) or ORD domains, and tethering peripheral ER membranes to these PI4P patches through interaction of their FFAT motifs with ER VAP proteins Scs2/22 (Stefan et al., 2011). Yeast cells mutant for Osh proteins or Scs2/22 accumulate excess PM PI4P, suggesting these proteins may promote Sac1 activity at ER-PM contact sites, where Sac1 could potentially act in trans on PM PI4P. A possible role for Sac1 in dephosphorylating PI4P in trans has also been postulated for ER-

early Golgi contact sites (Wood et al., 2012). In support of this idea, the ORD domains of Osh3 and Osh4 stimulate Sac1 turnover of PI4P in trans when incubated with PI4P-containing liposomes (Stefan et al., 2011). However, since Osh4 transfers PI4P between liposomes (de Saint-Jean et al., 2011), an alternative possibility is that in vivo Osh3/4 could deliver PI4P to the ER, where it could be consumed by Sac1 in cis. In any case, PI4P-binding by Osh3/4 is a prerequisite for ORD stimulation of Sac1, suggesting that a key role for Osh proteins is to mediate substrate presentation to Sac1 (Stefan et al., 2011). Importantly, Sac1-containing ER-PM sites form in response to high PM PI4P. Hence, these interactions constitute feedback regulation. A functional relationship between Drosophila Sac1 and DVAP in modulating PI4P levels was also identified in the control of neuromuscular morphology and neurotransmission (Forrest et al., 2013). It will be interesting to see if this interaction requires Drosophila OSBP.

As it turns out, mechanisms regulating PM PI4P may have more in common than originally anticipated. Although Stt4 is stably localized to PIK patches on the yeast PM, it contains an FFAT motif (Nakatsu et al., 2012) that could potentially bind to ER VAP proteins, thereby regulating ER-PM junctions. Indeed, Stt4 co-precipitates with Scs2 (Gavin et al., 2002). Mammalian PI4KIIıııı contains a partially conserved FFAT motif, leaving open the question of whether its association with the PM is similar to ER-PM contact sites in yeast, involving tethering of tubular ER, or more similar to that of mammalian PIS, via delivery by a highly mobile ER-derived vesicular population.

These developments highlight the regulation of PI4P at specific organelle contact sites and suggest that organelle-specific enrichment of phosphoinositides is not simply achieved by restricted localization of phosphoinositide-generating enzymes. A dynamic method of PM PI4P accumulation holds important implications for PI4P signaling. Whether the sites of PI4P deposition are regulated and how this regulation is coordinated with PI4P effectors remain to be seen.

Regulation by calcium

Support for the idea of dynamic regulation of PI4P production comes from the ancient and conserved physical interaction between homologues of PI4KIIıııııı and members of the neuronal calcium sensor (NCS) family of proteins, Frequenin (Frq)/NCS-1. Intracellular calcium levels frequently rise in response to signaling downstream of receptor stimulation. Thus, coupling PI4K activity with calcium suggests a mechanism to control PI4P production in response to physiological changes. In yeast and mammalian cells, this interaction is required for many types of PI4P-dependent secretion.

Frq was first identified in Drosophila due to its ability to increase neurotransmitter release when overexpressed (Pongs et al., 1993). This effect was also demonstrated in frogs (Olausson et al., 1995) and mammals (McFerran et al., 1998; Pan et al., 2002). The high level of conservation between Frq/NCS-1 homologues was shown in rescue experiments, where yeast frq1 mutants could be suppressed.
through expression of frog or human NCS-1 (Hendricks et al., 1999; Strahl et al., 2003). NCS family proteins are less than 30 kDa in size and contain four calcium-binding EF-hand motifs. Direct binding and stimulation of PI4KIIIβ activity by Frq1/NCS-1 has been demonstrated in yeast and mammalian cells (Haynes et al., 2005; Hendricks et al., 1999; Weisz et al., 2000; Zhao et al., 2001). Overexpression of Pik1 rescues frq1<sup>ts</sup> mutants at restrictive temperature and vice versa, whereas pik<sup>ts</sup> and frq<sup>ts</sup> show synthetic lethality (Hendricks et al., 1999; Huttner et al., 2003). Removing elements required for optimal binding to each other (myristoylation of Frq1 or the N-terminal LKU domain of Pik1) reduced the ability of one protein to rescue a temperature-sensitive mutant of the other.

In mammalian cells, the NCS-1–PI4KIIIβ interaction has been studied in models of regulated and constitutive exocytosis. One example is the ATP-dependent activation of purinergic receptor signaling that results in release of dense core granules in PC12 neuroendocrine cells. The purinergic receptor is coupled to PLC, which, through hydrolysis of PIP<sub>2</sub> and formation of second messengers following stimulation, leads to a rise in intracellular calcium. In these cells, NCS-1 normally binds and stimulates PI4KIIIβ activity to promote secretion when intracellular calcium levels rise. Overexpression of NCS-1 that cannot be myristoylated prevents the stimulatory effect of overexpressed PI4KIIIβ on secretion, and siRNA knockdown of PI4KIIIβ prevents NCS-1-stimulated exocytosis in evoked cells (de Barry et al., 2006). In addition, the PI4K inhibitor PAO prevents ATP-evoked exocytosis, but overexpression of NCS-1 overcomes this effect (Rajebhosale et al., 2003). Indeed, PI4KIIIβ has been suggested to act downstream of NCS-1 in both regulated and constitutive exocytosis in a variety of cell types (de Barry et al., 2006; Gromada et al., 2005; Kapp-Barnea et al., 2003; Koizumi et al., 2002; Weisz et al., 2000).

Calcium regulation of PI4KIIIβ-dependent secretion by Frq1/NCS-1 is thought to occur via a calcium-to-myristoyl switch, as initially proposed for the related NCS family protein recoverin (Ames et al., 1997; Ames & Lim, 2012). Ca<sup>2+</sup> binding to NCS-1 induces a large conformational shift in the protein (Cox et al., 1994; McFerran et al., 1999), allowing for increased membrane association via a more extruded N-terminal myristoyl group (Ames et al., 2000). The conformational shift also exposes two large hydrophobic crevices that interact with PI4KIIIβ (Lim et al., 2011), thus making membrane-bound NCS-1 more efficient in anchoring PI4KIIIβ. Indeed, PI4KIIIβ activity was stimulated by Frq1/NCS-1 three- to ten-fold in yeast (Hendricks et al., 1999), and in a dose-dependent manner in COS-7 cells (Zhao et al., 2001). Oddly, in contrast to what is predicted by the calcium-to-myristoyl switch, localization of myristoylated NCS-1 and its interaction with PI4KIIIβ does not depend on Ca<sup>2+</sup> binding in vivo (Hendricks et al., 1999; Zhao et al., 2001). However, Ca<sup>2+</sup> binding does enhance PI4K activity (Haynes et al., 2005; Zhao et al., 2001), possibly by forcing a conformational change in PI4KIIIβ (Strahl et al., 2007).

Additional roles for calcium in fine-tuning PI4P accumulation and localization come from identification of Arf1, a PI4KIIIβ interactor, as an NCS-1 binding partner in bovine brain cells (Haynes et al., 2005). Although both proteins partially colocalize with PI4KIIIβ at the TGN, a complex of all three could not be detected under conditions that allow Arf1–NCS-1 binding, which occurs with or without Ca<sup>2+</sup>. Curiously, although Arf1 increases PI4KIIIβ activity by 125% above basal levels in <i>in vitro</i> kinase assays, and NCS-1 increases PI4KIIIβ activity by 70%, when both regulators are incubated in the presence of Ca<sup>2+</sup>, kinase activity was reduced below the level elicited with either protein alone. This suggests a regulatory network in which PI4KIIIβ is efficient at producing PI4P only in the presence of either Arf1 or Ca<sup>2+</sup>-bound NCS-1, but not both. Therefore, membrane sites occupied by both regulators serve as PI4KIIIβ-inactive zones so that Arf1-<i>and</i> Ca<sup>2+</sup>-dependent pathways do not interfere with each other, and these zones act as boundaries demarcating pools of PI4P dedicated to either Arf1-mediated secretion or Ca<sup>2+</sup>-dependent NCS-1 signaling. In support of this, overexpressed NCS-1 interferes with formation of the activated Arf1<sup>Q71L</sup> tubular Golgi phenotype, and overexpression of Arf1 abolishes Ca<sup>2+</sup>-dependent NCS-1 stimulation of secretion in PC12 cells (Haynes et al., 2005). Thus, precise and dynamic regulation of separate pools of PI4P is crucial even within the same organelle and when produced by the same PI4K. Importantly, effective mechanisms exist to link spatial regulation of PI4P to different signaling pathways.

Adding another layer of regulation to Ca<sup>2+</sup>-induced PI4P-mediated secretion is the inhibitory effect of calcium sensors protein calneuron-1 and calneuron-2 on PI4KIIIβ activity (Mikhaylova et al., 2009). Under low Ca<sup>2+</sup> conditions, the calneurons outcompete NCS-1 for binding to PI4KIIIβ and strongly inhibit PI4P production. At high Ca<sup>2+</sup> concentrations, calneurons relinquish PI4KIIIβ to NCS-1, possibly due to unfolding of calneurons upon Ca<sup>2+</sup> binding. Calneuron-1 affects secretory traffic in a manner consistent with its effects on PI4P levels because in primary cortical neurons, RNA interference (RNAi) mediated knockdown of calneuron-1 partially colocalizes with PI4KIIIβ at the TGN, a complex of all three could not be detected under conditions that allow Arf1–NCS-1 binding, which occurs with or without Ca<sup>2+</sup>.
physically binds EYFP-RabA4b. PI4KIIIβ/31/32 double mutant plants have short and aberrant root hairs and fewer distinct TGN budding profiles visualized by transmission electron microscopy. Thus, a model emerges whereby the tip-focused calcium gradient, via AtCBL1, directs PI4KIIIβ to sites of membrane growth, where it produces PI4P required for directed post-Golgi trafficking.

Extracellular PI4P

Curiously, in plants, treatment with fungal xylanase, a potent activator of plant defenses, induces a rise in extracellular PI4P in tomato cell suspensions, which is then responsible for an intracellular burst of reactive oxygen species (Gonorazky et al., 2008). Oxidative burst is an early response to pathogen recognition and is thwarted with a PLC inhibitor (Gonorazky et al., 2008). Although instead of conversion to PIP2, metabolism to PI was detected, leading the authors to suggest that PLC may hydrolyze PI4P in this system. Extracellular PI4P, along with several other phospholipids, was also found in the intercellular space of tomato plants under basal conditions (Gonorazky et al., 2012). Microarray analysis showed that application of extracellular PI4P to Arabidopsis induced expression of genes required for environmental defense responses (Alvarez-Venegas et al., 2006), together suggesting that PI4P may function in cell-to-cell communication in plants under both normal and stress conditions.

PI4P in health and disease

Phosphoinositide regulation is involved in numerous aspects of human health and disease (McCrea & De Camilli, 2009; Skwarek & Boulianne, 2009). PI4KIIIα is situated at chromosomal 22q11.2, deletion of which has been associated with higher susceptibility to psychiatric conditions such as bipolar disorder, autism and schizophrenia (Clayton et al., 2013). Reduced PI4KIIIz activity has been correlated with Alzheimer’s disease (Wu et al., 2004; Zubenko et al., 1999). Various PI4Ks are upregulated in polycystic kidney disease (Cuozzo et al., 2002), malignant melanoma, breast ductal carcinoma, pancreatic cancer and others (Waugh, 2012). Also, the PI4P effector GOLPH3 is an oncogene (Li et al., 2011; Kunigou et al., 2011; Scott et al., 2009; Zeng et al., 2012) associated with poor clinical outcome (Hu et al., 2013; Hua et al., 2012; Li et al., 2012a; Wang et al., 2012). Thus, an optimal balance of PI4K activity is of great physiological importance.

PI4P regulation and membrane trafficking are key targets in pathogen invasion. PI4P metabolism is subverted during bacterial infections. Type I PI4Ks and AP-1 are required for Listeria monocytogenes phagocytosis, and PI4P is found at the entry site in HeLa cells (Pizarro-Cerda et al., 2007). Formation of intracellular vacuolar replication complexes by various Chlamydia species requires PI4KIIz and Arf1, which are detected on the vacuole along with GFP-OSBP-PH (Moorhead et al., 2010). Upon infection, Legionella pneumophila establishes a replication vacuole, the Legionella-containing vacuole (LCV), and avoids fusion with lysosomes by intercepting and fusing with ER-derived vesicles, disguising the LCV with host markers. The Legionella proteins SidC and DrrA/SidM are released into the host cytoplasm via the type IV Icm/Dot secretion system. Once cytoplasmic, these proteins bind PI4KIIIβ-dependent PI4P on the LCV (Brombacher et al., 2009). The Rab1 GEF domain of DrrA and the N-terminal region of SidC are then able to misdirect ER vesicles en route to the Golgi by binding to Rab1 and calnexin, respectively (Brombacher et al., 2009; Ragaz et al., 2008).

Cellular PI4Ks and PI4P are also co-opted by positive-sense RNA viruses, including hepatitis C virus (HCV), coxsackievirus, and poliovirus (Alvisi et al., 2011; Bishe et al., 2012a; Delang et al., 2012). These viruses induce formation of PI4P-enriched “membranous webs” derived of ER and other cellular membranes, which serve as platforms for viral replication. In genome-wide and targeted siRNA screens, PI4KIIIz was identified as a host factor required for HCV replication and membranous web formation (Berger et al., 2009; Borawska et al., 2009; Reiss et al., 2011; Vaillancourt et al., 2009). The HCV nonstructural protein 5a (NS5A) interacts with and stimulates PI4KIIIz activity at replication sites (Berger et al., 2011; Lim & Hwang, 2011; Reiss et al., 2011). Knockdown of PI4KIIIz abrogates replication and leads to clusters of NS5A instead of the reticular distribution seen during competent infections.

Involvement of PI4KIIIβ in HCV replication is debated, with some groups reporting that it is required (Borawska et al., 2009; Zhang et al., 2012b) and others that it is not (Arita et al., 2011; Berger et al., 2011). This inconsistency may be due to the study of distinct HCV genotypes and the use of different assays in each case (Bishe et al., 2012a). PI4KIIIβ may be hijacked at other time-points in the virus life cycle (Tai & Salloum, 2011). Assembled and infectious viral particles are thought to be released via the very low density lipoprotein (VLDL) secretion pathway, and HCV transit through the Golgi, tightly coupled with Apolipoprotein E, has been visualized in live cells (Coller et al., 2012). Knockdown of PI4KIIIβ, Rab11a, GOLPH3, or MYO18A leads to retention of HCV particles in the cell, as does expression of Golgi-targeted Sac1, suggesting that Golgi PI4P and its effectors are required for virus secretion (Bishe et al., 2012b; Coller et al., 2012). It has also been suggested that PI4KIIIβ is required for clathrin-mediated endocytosis of some HCV strains, although PI4KIIIz was similarly implicated in this process (Trotard et al., 2009). Compounds that inhibit PI4KIIIz and HCV web formation show promise as potential therapeutics (Bianco et al., 2012; Vaillancourt et al., 2012), as do those targeting PI4KIIIβ to halt poliovirus and coxsackievirus replication (Hsu et al., 2010; van der Schaar et al., 2012). However, viruses were still able to evolve resistance to compounds targeting these host factors (Arita et al., 2011; van der Schaar et al., 2012; van der Schaar et al., 2012), which remains a current challenge in deploying PI4K inhibitors as antiviral therapeutic agents.

Concluding remarks and future perspectives

The wealth of new information regarding the versatile roles of PI4P has brought us a long way from the notion that this lipid functions mainly as a precursor to higher phosphorylated phosphoinositides in regulation of cellular functions.
inositides. Certainly, PI4P conversion to PIP2 is essential for PLC signaling, and likely also for PIP3 signaling; however, the bona fide independent roles for PI4P are more numerous than previously suspected, involving many aspects of membrane trafficking, including budding, tubulation, scission, linkage to motors, docking, fusing, sorting and establishing membrane identity. These discoveries bring new questions, and the next wave of research will focus on regulators that govern specific PI4P-dependent processes. Furthermore, whereas many of the cellular roles for PI4P have been identified at the ER and Golgi, independent roles for PI4P on other membranes, such as endosomes, lysosomes and the PM, are emerging.

A broad but important question is what determines functional specificity of PI4P pools derived from different PI4Ks at the same organelle. Aside from the catalytic commonality of their kinase domains, the N-termini of PI4K isoforms are unique, and a non-overlapping set of PI4K binding partners may preclude compensation by other isoforms. Unique partnerships with Rabs present ways to turn PI4P signaling on or off, as do interactions with activators that are mutually exclusive and jointly inhibiting, as in the case of Arf1, NCS-1 and PI4KIIIβ. In addition, because recent developments illustrate the interdependencies of phosphoinositide and sphingolipid metabolism, PI4K isoform-specific control of cholesterol and sphingolipid composition may be relevant in feedback mechanisms defining compartments for PI4K-specific signaling events and regulation of PI4K activity. Uncovering the full web of proteomic and lipid interactions for each PI4K will help elucidate isoform-specific mechanisms and provide insight into the development and treatment of diseases resulting from their dysfunction.

Future studies on PI4P regulation in animal systems will be valuable to this end, as roles for PIP enzymes revealed by RNAi approaches in tissue culture do not always predict the phenotypes revealed by knockout in cells or organisms. The phenotypes can be either milder or stronger than predicted. For example, whereas essential trafficking roles at the TGN were reported for PI4KIIα in cultured cells, PI4KIIα mutant mice are born healthy and survive without obvious phenotypes prior to developing late-onset neurodegeneration. In contrast, a crucial role for PI4KIIα in PM integrity and steady state PIP2 (similar to what was observed in PI4KIIα mutant flies) was discovered using a knockout approach in the MEF cell line, after knockdown of PI4KIIα in other cell lines had little effect. The observed differences are likely due to off-target effects or incomplete knockdown associated with siRNA. Studies in whole organisms will be of critical importance as new roles for PI4P and PI4Ks in human diseases continue to be revealed, and as drug candidates targeting these pathways need to be validated in physiologically relevant contexts.

Although PI4P regulation is known to affect many signaling pathways, including Wnt, FGF, EGF, Hh and Hippo signaling, we are only beginning to uncover mechanisms governing crosstalk and regulation. Recent discoveries of dynamic regulation of PI4P levels, including frequent transient contacts of PI4KIIα with the PM, hint that this system may be more complicated than expected. Future research focusing on intersection of pathways regulating PI4P will lead to greater mechanistic understanding of a multitude of cellular processes, as well as a greater understanding of the molecular role for PI4P in cell homeostasis and organismal health.

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Note added in proof

A new article published while this review was in preparation expands on the role of OSBP in PI4P metabolism and sterol trafficking.* The authors describe the process by which OSBP may promote formation of a sterol gradient, with higher levels of sterol at the Golgi as compared to the ER: OSBP tethers VAP-A and PI4P-containing membranes via its FFAT and PH domains. This membrane tethering permits the ORD domain of OSBP to transfer sterol to PI4P-containing membranes. ORD-mediated transfer of PI4P in the opposite direction then permits PI4P consumption in cis by the ER resident protein Sac1. Hence, PI4P provides both the signal and the energy to drive directional sterol transfer.

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