Review

Phosphatidylinositol 3-phosphates—at the interface between cell signalling and membrane traffic

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Abstract

Phosphoinositides (PIs) form a minor class of phospholipids with crucial functions in cell physiology, ranging from cell signalling and motility to a role as signposts of compartmental membrane identity. Phosphatidylinositol 3-phosphates are present at the plasma membrane and within the endolysosomal system, where they serve as key regulators of both cell signalling and of intracellular membrane traffic. Here, we provide an overview of the metabolic pathways that regulate cellular synthesis of PI 3-phosphates at distinct intracellular sites and discuss the mechanisms by which these lipids regulate cell signalling and membrane traffic. Finally, we provide a framework for how PI 3-phosphate metabolism is integrated into the cellular network.

Keywords autophagy; endolysosomal system; membrane traffic; phosphatidylinositol 3-phosphate; signalling

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Introduction

Phosphoinositides (PIs) are a minor class of comparably short-lived membrane phospholipids that mediate crucial cellular and organis- nal functions including signalling, gating of ion channels, cytoskele- ton regulation and motility, development, as well as the regulation of intracellular membrane traffic (Di Paolo & De Camilli, 2006; Balla, 2013). PI 4-phosphates such as phosphatidylinositol 4-phos- phate [PI(4)P] and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] are concentrated along both the exocytic pathway and the plasma membrane. PI 3-phosphates can be produced at the cell surface during signalling, but they are mainly found as prominent components and hallmarks of the endosomal system (Balla, 2013; Raiborg et al., 2013). Phosphatidylinositol 3-phosphates are generated via the phosphorylation of the inositol ring of phosphatidylinositol and its derivatives at the 3-position, giving rise to phosphatidylinositol 3-phosphate [PI(3)P], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2], and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3]. The distinctive subcellular localization displayed by the different phosphatidyli- nositol 3-phosphate species, as well as the rapidly reversible nature of phosphorylation, positions them as key regulators of processes at intracellular membranes, from signalling cascades downstream of extracellular receptor activation to intracellular membrane traf- ficking (Balla, 2013; Raiborg et al., 2013) (Fig 1). At the plasma membrane, PI(3,4,5)P3 and PI(3,4)P2 are generated upon extracel- lular stimulation (Cantley, 2002; Vanhaesebroeck et al., 2010a), and PI(3,4)P2 synthesis was recently described as an essential mediator of the late stages of clathrin-mediated endocytosis (CME) (Posor et al., 2013). Additionally, there is a pool of PI(3,4)P2 on endome- branes, the function of which has yet to be established (Watt et al., 2004). PI(3)P is the predominant species found on early endosomes and plays an important role in autophagy, while PI(3,5)P2 is present on late endocytic/multivesicular bodies, lysosomal and autophagic compartments (Mayinger, 2012). The generation of these distinctive pools of PI 3-phosphates (Fig 1) occurs via three classes of PI 3-kinases (PI3Ks) that exhibit different substrate specificities as well as subcellular localizations, and via a single PI 5-kinase, PIKfyve, that catalyses PI(3,5)P2 synthesis from PI(3)P (Balla, 2013; Raiborg et al., 2013). Conversely, turnover of PI 3-phosphates is accom- plished by several types of PI phosphatases, most notably the myotubularin family of PI 3-phosphatases (Hsu & Mao, 2015).

Getting started: Synthesis of PI 3-phosphates

Phosphorylation of the phosphatidylinositol D3 OH group depends on distinct classes of PI3Ks either at the plasma membrane or at endosomes, thereby generating a membrane identity tag for the endosomal system. All PI3Ks contain a “signature motif” consisting of the catalytic kinase domain, a helical domain as well as a membrane-binding C2 domain (Vanhaesebroeck et al., 2010a; Vadas et al., 2011; Balla, 2013). There are eight different PI3Ks, grouped into three classes based on the conserved domains found outside the signature motif, their association with regulatory subunits, as well as their preferred lipid substrates (Fig 2). Class I PI3Ks mainly produce PI(3,4,5)P3 in vivo, class II generates PI(3,4)P2 and PI(3)P,
and class III exclusively forms PI(3)P. A single orthologue of each class can be found in *C. elegans* and *D. melanogaster*, while yeast contain only a class III PI3K (Vanhaesebroeck et al., 2010a; Vadas et al., 2011; Jean & Kiger, 2014).

**Class I PI3-kinases**

Class I PI3Ks are tightly regulated enzymes that predominantly produce PI(3,4,5)P3. They function as heterodimers, consisting of one of four possible catalytic subunits (p110α, β, γ, and δ isoforms). PI(3,4,5)P3 is a substrate for the PI3-kinase PH domains and for the PI5-phosphatases SHIP1/2 and OCRL. Class II PI3Ks generates a plasma membrane pool of PI(3,4)P2 necessary for clathrin-coated pit (CCP) maturation and formation of free clathrin-coated vesicles (CCV) during endocytosis. PI(3)P is a defining and essential feature of early endosomes and is generated primarily by the class III PI3K Vps34 complex II with a possible contribution of class II PI3Ks (encircled by dashed line), either by direct PI(3)P synthesis or indirectly via PI(3,4)P2 hydrolysis by the PI4-kinase. As endosomes mature into late endosomes/multivesicular bodies (MVBs), PI(3)P is converted into PI(3,5)P2 via the PI4-kinase PIKfyve. Endosomes and lysosomes also harbour a poorly understood pool of PI(3,4)P2. In the liver, this endosomal PI(3,4)P2 pool is synthesized by class II PI3Ks. Autophagosomes contain PI(3)P produced by the autophagosome-specific Vps34 complex I, and possibly by the class II PI3Ks (dashed line). Endosomal recycling to the cell surface requires PI(3)P hydrolysis by myotubularin phosphatases (MTMs) such as MTM1 and the concomitant generation of PI(4)P (not shown) to enable exocytosis PI(3,5)P2 turnover at MVBs and/or lysosomes is mediated by MTMs together with the PI(3,5)P2 5-phosphatase Fig4.

**Figure 1. Localization of 3-phosphoinositides in the cell, and their relevant kinases and phosphatases.**

At the plasma membrane, receptor tyrosine kinase (purple) activation results in the production of PI(3,4,5)P3 via class I PI3Ks (with α, β, γ, and δ isoforms). PI(3,4,5)P3 is a substrate for the PI3-kinase PH domains and for the PI5-phosphatases SHIP1/2 and OCRL. Class II PI3Ks generates a plasma membrane pool of PI(3,4)P2 necessary for clathrin-coated pit (CCP) maturation and formation of free clathrin-coated vesicles (CCV) during endocytosis. PI(3)P is a defining and essential feature of early endosomes and is generated primarily by the class III PI3K Vps34 complex II with a possible contribution of class II PI3Ks (encircled by dashed line), either by direct PI(3)P synthesis or indirectly via PI(3,4)P2 hydrolysis by the PI4-kinase. As endosomes mature into late endosomes/multivesicular bodies (MVBs), PI(3)P is converted into PI(3,5)P2 via the PI4-kinase PIKfyve. Endosomes and lysosomes also harbour a poorly understood pool of PI(3,4)P2. In the liver, this endosomal PI(3,4)P2 pool is synthesized by class II PI3Ks. Autophagosomes contain PI(3)P produced by the autophagosome-specific Vps34 complex I, and possibly by the class II PI3Ks (dashed line). Endosomal recycling to the cell surface requires PI(3)P hydrolysis by myotubularin phosphatases (MTMs) such as MTM1 and the concomitant generation of PI(4)P (not shown) to enable exocytosis PI(3,5)P2 turnover at MVBs and/or lysosomes is mediated by MTMs together with the PI(3,5)P2 5-phosphatase Fig4.

**Class I PI 3-kinases**

Class I PI3Ks are tightly regulated enzymes that predominantly produce PI(3,4,5)P3. They function as heterodimers, consisting of one of four possible catalytic subunits (p110α, β, δ or γ) (Hiles et al., 1992) that associate with one of two classes of regulatory subunits, the p85 class (p85α and its splice variants p55α or p50α, p85β and p55γ) and the p101/p87 class. The catalytic subunits are grouped based on the regulatory subunit they associate with: class IA subunits (p110α, β and δ) associate with the p85 class, while the class IB subunit (p110γ) associates with p101 or p87 (Vadas et al., 2011; Jean & Kiger, 2014) (Fig 2). The assembled class I PI3Ks act downstream of activated receptor tyrosine kinases (RTKs) and heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) to control signalling cascades involved in multiple processes such as cell growth, metabolism, proliferation, survival and repression of autophagy (Vanhaesebroeck et al., 2010a; Jean & Kiger, 2014). The class IA catalytic subunits (p110α and δ in vivo) are activated following RTK stimulation, resulting in the production of PI(3,4,5)P3 at the plasma membrane and the...
**A** Conversion of PI3-Phosphates

- PI(3)P
- PI(4,5)P2
- Class III (+II) PI3K
- MTMs
- PTEN+TPiP
- INPP4A/B
- OCRL+SHIP1/2
- PI(4)P
- Class II (+I) PI3K
- PI(3,5)P2
- PI(3,4)P2
- PI(3,5)P2

**B** PI3-Kinases

- **Class I PI3K** – lipid product PI(3,4,5)P3 and/or PI(3,4)P2
  - PI3K signature motif
  - ABD: RBD: C2: Helical: Catalytic
  - Regulatory subunit: p85α, β, p55α, p50α, p55γ
- **Class II PI3K** – lipid product PI(3,4)P2 and/or PI(3)P
  - PI3K-C2α, β
  - CB and PxxP
- **Class III PI3K** – lipid product PI(3)P
  - Vps34

**C** PI3-Phosphatases

- **PTEN** – lipid substrate PI(3,4,5)P3
  - PTP: C2: PEST: PDZ-B
- **Myotubularins** – lipid substrate PI(3)P and/or PI(3,5)P2
  - MTM1
  - MTMR1
  - MTMR2
  - MTMR3
  - MTMR4
  - MTMR5
  - MTMR6
  - MTMR7
  - MTMR8
  - MTMR9
  - MTMR10
  - MTMR11
  - MTMR12
- **SHIP1/2** – lipid substrate PI(3,4,5)P3
  - SH2: Pro rich: SAM
- **Fig4** – lipid substrate PI(3,5)P2
  - Sac

**D** Other PI Kinases and Phosphatases acting on PI3-Phosphatases

- **PI4-Phosphatases**
  - INPP4 – lipid substrate PI(3,4)P2
  - INPP4A
  - INPP4B
- **PI5-Phosphatases**
  - OCRL – lipid substrate PI(3,4,5)P3 and PI(4,5)P2
  - PH: ASH: RhoGAP
- **SHIP1/2** – lipid substrate PI(3,4,5)P3
  - SH2: Pro rich: SAM
- **Fig4** – lipid substrate PI(3,5)P2
  - Sac

**PI5-Kinase**

- **PIKfyve** – lipid product PI(3,5)P2
  - FYVE: DEP: TCP-1: Kinase

Figure 2.
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PI 3-phosphates in traffic & signalling
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recruitment of effectors that bind this PI, notably the protein kinase Akt (also referred to as protein kinase B, PKB). Active Akt controls many downstream pathways such as the mechanistic target of rapamycin complex-1 (mTORC1) pathway, the pro-apoptotic factor BAD and the FOXO transcription factors (Vanhaesebroeck et al., 2010a; Dibble & Cantley, 2015). Contrary to this, the p110γ and p110β catalytic subunits are activated by the Gβγ heterodimers downstream of GPCR activation (Stephens et al., 1994; Stoyanov et al., 1995; Vanhaesebroeck et al., 2010a). Other inputs that can activate class I PI3Ks include small GTPases, for example active Rac and its family members, which bind the Ras binding domain (RBD) of the p110α, δ and γ catalytic subunits, or Rab5 that associates with the RBD of p110β. The four catalytic subunits have distinct yet overlapping functions and exhibit some degree of tissue specificity: p110α and β are expressed ubiquitously, while p110γ and δ are mainly found in immune cells (Vanhaesebroeck et al., 1997; reviewed in Vanhaesebroeck et al., 2010a). Because of their crucial role in controlling growth and proliferation, activating mutations targeting the class I PI3K signalling cascades are associated with cancer, and PI3Ks therefore represent important drug targets (Vanhaesebroeck et al., 2010b).

Class II PI 3-kinases

Class II PI3Ks are monomeric enzymes and represent the least studied class of PI3Ks with functions in both signalling and membrane traffic. A single orthologue is found in Drosophila and C. elegans, while three isoforms are present in mammals. The α and β isoforms are expressed ubiquitously, while γ is restricted mainly to the liver. The main difference between the three mammalian isoforms resides in divergent N-terminal extensions that are likely autoinhibitory and mediate protein–protein interactions (Fig 2). Unlike the class I and class III PI3Ks, class II enzymes have no known regulatory subunits but are instead regulated by interacting proteins such as clathrin, which binds to the inhibitory N-terminal region of PI3KC2α and β, or small GTPases that associate with the RBD (e.g. Rab5 in the case of PI3KC2γ). Class II PI3Ks are also sensitive to signalling inputs, such as phosphorylation, ubiquitination, and possibly calcium (Kok et al., 2009; Sasaki et al., 2009; Vanhaesebroeck et al., 2010a; Jean & Kiger, 2014; Braccini et al., 2015).

Accumulating evidence implicates class II PI3K isoforms in coupling cell signalling to membrane traffic. This involves a role for PI3K intracellular localization or the presence of additional regulatory factors in triggering the formation of PI(3)P (Yoshioka et al., 2012; Franco et al., 2014) and/or PI(3,4)P2 (Leibiger et al., 2010; Posor et al., 2013; Braccini et al., 2015). The α isoform has been demonstrated to preferentially synthesize PI(3,4)P2 both in vitro and at plasma membrane endocytic pits in vitro, a site where PI(3,4)P2 is required for membrane constriction prior to endocytic vesicle fission (Posor et al., 2013). Defective CME and/or endosomal trafficking may also underlie impaired angiogenesis and vascular barrier function in endothelial-specific PI3KC2α knock-out mice (Yoshioka et al., 2012). In agreement with a role for class II PI3Ks in PI(3,4)P2 synthesis, it was recently shown that the liver-specific γ isoform produces an endosomal pool of PI(3,4)P2 required for sustained Akt2 activation following insulin stimulation (Braccini et al., 2015). The class II α (Brown et al., 1999; Leibiger et al., 2010) and β isoforms (Arcaro et al., 2002) have also been linked to growth factor and nutrient signalling, though their precise function as well as the lipid product involved in these pathways remains to be determined. In addition to their function in PI(3,4)P2 synthesis at the cell surface and at endosomes, class II PI3Ks either directly or indirectly contribute to the maintenance of intracellular PI(3)P pools that regulate endocytic trafficking and autophagy (Jean et al., 2012; Devereaux et al., 2013; Franco et al., 2014). Further understanding of the diverse roles of the class II PI3Ks will be aided by the identification of new effectors and roles for their poorly understood lipid product, PI(3,4)P2.

Class III PI 3-kinase

Vps34 is the only PI 3-kinase in yeast (Schu et al., 1993) and was initially identified in a yeast screen for genes regulating sorting from the endosome to the lysosome (Herman & Emr, 1990). Membrane targeting of the catalytic subunit Vps34 is largely mediated via a constitutive interaction with the N-terminally myristoylated adaptor protein Vps15/p150 (Fig 2). Vps34 is present in two complexes in both yeast and mammalian cells, each complex having separate locations and functions. Complex I is involved in autophagy and contains Vps15/p150, Beclin-1 (equivalent to yeast Vps30), ATG14L (Atg14 in yeast) and NRBFF2 (Atg38 in yeast) (Cao et al., 2014; Lu et al., 2014), while the endosomal complex II contains Vps15/p150, Beclin-1/Vps30 and UVRAG (termed Vps38 in yeast) (Kihara et al., 2001; Funderburk et al., 2010; Rostislavleva et al., 2015).

The formation of these distinct complexes underlies the multitude of functions exerted by class III PI3K in intracellular trafficking, for example in endosomal fusion and sorting, trafficking from endosomes to lysosomes, phagocytosis, autophagy, as well as cytokinesis. For example, recruitment of complex II to early endosomes involves the association of Vps15/p150 via its WD40 repeat domain with Rab5-GTP, resulting in the production of a pool of PI(3)P that drives endosomal association of effectors such as the endosomal sorting complex required for transport (ESCRT). In contrast, complex I—which contains the autophagy protein ATG14L—is responsible for the role of Vps34 in PI(3)P production on autophagosome precursor membranes (Kistakos et al., 2012; Raiborg et al., 2013; Jean & Kiger, 2014). Contrary to its ability to positively regulate autophagy at the level of the autophagosome membrane, Vps34 also has a putative function in the negative regulation of autophagy via its involvement in amino acid sensing (Nobukuni et al., 2005;
Gulati et al., 2008) and mTORC1 activation through the PI(3)P-dependent recruitment of phospholipase D1 (PLD1) (Yoon et al., 2011; Bridges et al., 2012). Providing a further layer of complexity, mTORC1 activity specifically inhibits the autophagy promoting Atg14L-containing complex I (Yuan et al., 2013), while it activates complex II via phosphorylation of UVRAG during the process of autophagosome–lysosome reformation following recovery from starvation (Munson et al., 2015). Consequently, the different Vps34 complexes not only have opposing effects in the cell but can potentially negatively regulate one another.

**Fab1/FIKfyve**

In addition to PI(3)P, endosomes, late endosomes/multivesicular bodies (MVBs) and likely lysosomes contain a functionally important pool of PI(3,5)P$_2$ on their limiting membranes synthesized by the PI 5-kinase PIKfyve (termed Fab1 in yeast) (Sbrissa et al., 1999; Ikonomov et al., 2002; McCartney et al., 2014). Fab1/FIKfyve, an evolutionary conserved protein, is recruited to endosomes by its FYVE (Fab1, YOTB, Vac1 and EEA1) domain-mediated interaction with PI(3)P and forms part of a multiprotein complex that tightly controls lipid kinase activity (Fig 2). Mutations in PIKfyve and thus PI(3,5)P$_2$ production have been linked to neurological disorders such as amyotrophic lateral sclerosis (ALS) and Charcot–Marie–Tooth disease (McCartney et al., 2014).

**Turning over: Hydrolysis of PI 3-phosphates by PI phosphatases**

PI 3-phosphate turnover is generally accomplished by PI 3-phosphatases (Fig 2) that comprise the myotubulin family, PTEN, and TPIP, all of which contain a catalytic CX$_5$R motif in their active site (Hsu & Mao, 2015). Interestingly, all of these PI 3-phosphatases have been linked to human diseases as outlined below.

**Myotubulin 3-phosphatases**

Myotubulin (MTM1) was first identified as a gene mutated in patients with X-linked myotubular myopathy (also called X-linked centronuclear myopathy, XCNM) and, despite sequence similarity with protein tyrosine phosphatases, was subsequently demonstrated to act as a PI 3-phosphatase that hydrolyses PI(3)P and/or PI(3,5)P$_2$ (Blondeau et al., 2000; Taylor et al., 2000). In addition to myotubular myopathy, mutations in MTMR2 and MTMR13 are found in two forms of Charcot–Marie–Tooth neuropathy (CMT4B1 and 4B2) (Nicol & Laporte, 2008). The myotubulins are restricted to eukaryotes with 15 family members in mammals (MTM1 and MTMR1–14) (Fig 2), six of which are inactive pseudophosphatases lacking the catalytic cysteine within the CX$_5$R motif. Heterodimers composed of active and inactive isoforms have commonly been observed, with the phosphatase-inactive form being able to modulate the activity and localization of the active form (Laporte et al., 2003; Hnia et al., 2012; Hsu & Mao, 2015). In addition, the phosphatase-inactive MTMR5/13 proteins harbour a DENN domain Rab guanine-nucleotide exchange factor (GEF) that functionally activates Rab21 on PI(3)P endosomes, while their association with an active MTM allows for the hydrolysis of endosomal PI(3)P (Jean et al., 2012). Myotubulins function in a broad range of endolysosomal trafficking events. MTM1 and MTMR2 are found on early and late endosomes, respectively, where they can act on partially distinct pools of PI(3)P (Cao et al., 2007, 2008; Ketel et al., 2016). Another key site of action of the myotubulins is the autophagosomal system with the myotubulins MTMR3, the MTMR8/9 complex and MTMR14/JUMPY regulating the pools of PI(3)P and PI(3,5)P$_2$ that are necessary for the initiation and progression of autophagy (Vergne et al., 2009; Taguchi-Atarashi et al., 2010; Zou et al., 2012; Wu et al., 2014).

Myotubulins have also been implicated in regulating signalling events. As an example, MTM1-deficient muscles from knock-out mice display elevated mTORC1 activity that persists after starvation resulting in a defect in autophagy (Fetalvero et al., 2013). Contrary to this, MTM1 knock-down in HeLa cells and primary human skeletal myotubes results in defective growth factor stimulus-dependent Akt activation and reduced mTORC1 activity, leading to activation of caspase-dependent pro-apoptotic signalling (Razidlo et al., 2011). The reason for these conflicting results is not clear; however, it does appear that MTM1 acts both upstream and downstream of the Akt pathway. Laporte and colleagues found an increase in both phospho- and total Akt in muscles of MTM1-null mice and an increase in phospho-mTOR. The fact that the increase in mTOR phosphorylation only occurred at a later stage in the disease pathology than alterations in Akt suggests that these branches of the pathway are uncoupled. The authors also found dysregulation of autophagy with an increase in autophagosome formation at both early and late disease stages, while autophagy-mediated degradation was only perturbed at late disease stages, suggesting this may be caused by the increased mTOR activity also only seen at late stages (Al-Qusairi et al., 2013).

**PTEN and TPIP 3-phosphatases**

PTEN (phosphatase and tensin homologue located on chromosome 10) is a tumour suppressor that dephosphorylates the D3 position of PI(3,4,5)P$_3$ (Fig 2), thus antagonizing class I PI3K signalling by preventing Akt activation. PTEN suppression of Akt leads to reduced mTORC1 activity and, hence, crucially regulates energy metabolism, cell survival and proliferation. PTEN is also one of the most commonly mutated tumour suppressors with the loss of the lipid phosphatase activity driving tumorigenicity (Di Cristofano et al., 1998; Song et al., 2012). Recent data indicate that in addition to the plasma membrane, PTEN can operate at endocytic vesicles to inactivate Akt via a mechanism that involves PI(3)P binding to the PTEN C2 domain (Naguib et al., 2015). These data thus illustrate that the production of one species of PI 3-phosphate (i.e. PI(3)P) can mediate the removal of another (i.e. PI(3,4,5)P$_3$).

The function of PTEN must be tightly controlled as even small variations in activity can have dramatic effects on cell growth and therefore on tumorigenicity (Salmena et al., 2008; Song et al., 2012). The function of PTEN is regulated by a variety of post-transcriptional and post-translational mechanisms, for example by multiple C-terminal phosphorylation sites, ubiquitination, acetylation, SUMOylation and regulation by microRNAs (_fragoso & Barata, 2015). In addition to PTEN, some cells and tissues express the poorly characterized PTEN homologue TPIP (TPTE and PTEN homologous inositol lipid phosphatase) (Sasaki et al., 2009). Despite their conserved phosphatase activity, unlike PTEN, TPIP does not affect Akt function (Walker et al., 2001). As TPIP displays sequence homology to voltage-sensing phosphatases (VSPs), its phosphatase activity could potentially be regulated by changes in membrane potential (Kurokawa et al., 2012).
Other phosphatases acting on PI 3-phosphates

Multiple phosphorylated PI 3-phosphates such as PI(3,4)P2, PI(3,5)P2 and PI(3,4,5)P3 are also substrates for PI phosphatases other than the PI 3-phosphatases (Hsu & Mao, 2015). For example, PI(3,4)P2 is converted to PI(3)P by the closely related C36R motif-containing PI(3,4)P2-specific 4-phosphatases INPP4A and INPP4B (Figs 2 and 3B). INPP4A/B negatively regulates Akt signalling and INPP4B acts as a tumour suppressor in human cancers (Woolley et al., 2015), while loss of INPP4A leads to neurodegeneration in mutant mice (Sasaki et al., 2010). Furthermore, PI(3,4,5)P3 is subject to hydrolysis by the Lowe syndrome-associated PI 3-phosphatases OCRL, which also acts on PI(4,5)P2, and SHIP1/2, resulting in the production of PI(3,4)P2 on endosomes or the plasma membrane, respectively (Pirruccello & De Camilli, 2012). Lastly, the HEAT repeat protein Vac14 acts as a scaffold for the assembly of an endosomal complex (Zhang et al., 2012) comprising PIKfyve and the PI(3,5)P2-specific phosphatase Fig4 (Fig 2), whose activity appears to be the main source of cellular PI(5)P. Interestingly, mutations of Fig4 are responsible for Yunis-Varo`n syndrome, a familial epilepsy with polymicrogyria, and Charcot–Marie–Tooth type 4J neuropathy (CMT4J) (Menezes et al., 2014; Vaccari et al., 2015) due to altered PI(3,5)P2 metabolism (see below).

Collectively, the cellular PI 3-kinase and phosphatase activities allow for a rapid conversion of PI 3-phosphate identities between the plasma membrane and the endolysosomal system.

Manipulating PI 3-phosphate synthesis and turnover

Recent advances have allowed for the discovery of new pools of PI 3-phosphates, as well as new methods to modulate subcellular PI 3-phosphate levels in a spatiotemporally controlled manner. We will discuss some of the most recent advances regarding the acute manipulation of PI 3-phosphate levels in Boxes 1–3. For a more

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**Figure 3. Interplay of PI 3-phosphates in regulating signalling and membrane traffic.**

(A) Schematic representation of the role of PI 3-phosphates (3-PI) in the reciprocal regulation of cell signalling and membrane traffic. Signals (i.e. extracellular ligand that binds to its receptor) lead to activation of PI 3-kinases and the 3-PIs generated recruit effector proteins that often regulate membrane traffic, leading to the generation of a secondary signal. Such a downstream secondary signal may then regulate traffic and/or the response to the primary signal (e.g. by altering receptor levels or receptor-associated signalling components). (B) Endocytosis of activated signalling receptors (purple) from the cell surface via clathrin-mediated endocytosis (CME, left) or fast-endophilin-mediated endocytosis (FEME, right) involves PI(3,4)P2 and PI(3,4,5)P3 synthesis by class II PI3Ks (PI3Kc2a) and class I PI3Ks. In FEME, SHIP2/2 converts PI(3,4,5)P3 to PI(3,4)P2, which serves to recruit lamellipodin and thereby endophilin. PI(3,4)P2 is hydrolysed to PI(3)P on endosomes by the PI(3,4)P2-specific 4-phosphatase INPP4A. Activation of Akt signalling by PI(3,4,5)P3 and PI(3,4,5)P3 stimulates CME via dynamin 1, while dysregulated CME causes Akt overactivation. Endosomal sorting of internalized signalling receptors to internal vesicles of multivesicular bodies (MVB) terminates signalling. (C) mTORC1 activation on the lysosome is downstream of PI(3,4,5)P3, generated via class I PI3Ks and activated Akt. mTORC1 signalling represses PI(3)P generation via distinct Vps34-containing class III PI3K complexes, thereby suppressing autophagosome formation and late endosome maturation.
Box 1: PI 3-phosphate analogues

Recent advances in chemical synthesis has resulted in the development of membrane-permeable PI 3-phosphate analogues whereby the charged phosphate groups are masked with a hydrolase-sensitive acetoxymethyl ester group that is cleaved inside living cells within minutes (Laketa et al, 2009). These PI analogues have been successfully used in vitro and in vivo: for example, to delineate a requirement of PI(3,4,5)P3 for receptor tyrosine kinase recycling, the discovery that PI(3)P2 is an essential mediator of CME, and the finding that the PI(4,5)P2/PI(3,4,5)P3 ratio determines actomyosin contractility and plasma membrane expansion during tissue morphogenesis in Drosophila larvae (Posor et al, 2013; Laketa et al, 2014; Reversi et al, 2014). Caveats include the fact that modified PI 3-phosphates insert non-specifically into membranes and therefore may not reflect the heterogeneous spatial effects of endogenous PI 3-phosphates, as well the fact that their activation inside the cell by ester hydrolysis is comparably slow, requiring several minutes. The recent development of photoactivatable PIs that can be deprotected by UV light in 30 s now allows for the possibility of both temporal and spatial control of PI 3-phosphate levels. For example, it has been demonstrated that photo-uncaging of PI(3)P is a sufficient signal to induce endosomal fusion (Subramanian et al, 2010; Mentel et al, 2011).

detailed overview, the reader is referred to excellent recent reviews (Idevall-Hagren & De Camilli, 2015; Maekawa & Fairn, 2015).

Coupling PI 3-phosphate synthesis and turnover by co-regulation of phosphatase and kinase activities

While the presence of antagonistic PI kinases and phosphatases in the same complex may seem counterintuitive, they often act in concert to regulate the conversion of different PIs during membrane trafficking. Such a regulated PI kinase/phosphatase complex, in which the active partner changes in a spatiotemporally controlled manner, would allow for the switch in PI identity needed to terminate one event and commence another. This would allow a switch between the trafficking of a vesicle requiring one PI species and its fusion with its target membrane that may require another (Botelho, 2009). Several examples of such complexes have indeed been discovered. The perhaps best studied case is the conserved protein complex formed between the PI(3)P 5-kinase PIKfyve and the PI(3,5)P2-phosphatase Fig4, held together by the scaffolding protein Vac14 that is responsible for the biosynthesis and turnover of PI(3,5)P2 (Jin et al, 2008; Ikonomov et al, 2009). Loss of PIKfyve or Vac14 causes a loss or decrease in PI(3,5)P2 levels, respectively (Doux et al, 2006b; Zhang et al, 2007; Ikonomov et al, 2011). As the phosphatase Fig4 is required for PIKfyve activation, its deletion also results in PI(3,5)P2 depletion (Doux et al, 2006a; Chow et al, 2007). These data argue that the presence of both a kinase and a phosphatase in the same complex allows for tight regulation of endosomal PI(3,5)P2 levels. Another example is the association of the myotubularin PI 3-phosphatases MTM1 and MTMR2 with the WD40 domain of the Vps15 subunit of the class III PI3K complex II on endosomes, which inhibits their phosphatase activity. The association of the Vps15-containing class III PI3K complex with MTM1 or with endosomal GTP-bound Rabs, required for PI3K activation, is mutually exclusive. These data suggest that the Rab GTPases together with MTMs serve as molecular switches for controlling the sequential synthesis and degradation of endosomal PI(3)P (Cao et al, 2007, 2008). Interestingly, the related MTMR13, a pseudophosphatase that interacts with and regulates active MTMs, has been found to associate with the class II PI3K (PI3Kδ68D) in Drosophila and with the mammalian class II PI3Kα, thereby coordinating the turnover of a PI(3)P pool that regulates cargo exit from endosomes (Jean et al, 2012). Similar modules comprising coupled PI kinase and phosphatase activities likely underlie regulated PI conversion within the endosomal system, for example to enable cargo exit from PI(3)P-containing endosomes to the plasma membrane enriched in PI(4)P and PI(4,5)P2. Indeed, recent data demonstrate that surface delivery of endosomal cargo requires hydrolysis of PI(3)P by the phosphatidylinositol 3-phosphatase MTM1. Removal of endosomal PI(3)P by MTM1 was shown to be accompanied by phosphatidylinositol 4-kinase 2a-dependent generation of PI(4)P and recruitment of the exocyst tethering complex to enable membrane fusion. These data suggest a close coupling between PI(3)P turnover and PI(4)P synthesis that is driven by both

Box 2: Inducible acute manipulations by chemical dimerization and optogenetics

The acute activation of enzymes, for example PI 3-kinases or phosphatases, can be accomplished via the utilization of dimerization systems. Inducible acute manipulations provide a powerful tool in dissecting the spatial function of PI 3-phosphates, notably how one species of PI 3-phosphate can exert different functions on different membranes by specifically modulating its level at a given subcellular site, which is often difficult, sometimes impossible, to achieve via genetic approaches. For example, PI(3)P is produced both on early endosomes and autophagosomes via distinct, yet partially overlapping Vps34-containing class III PI3K complexes (Kihara et al, 2001; Funderburk et al, 2010). PI3Kζ2α can act on both the plasma membrane to synthesize PI(3,4)P2 (Posor et al, 2013) and on endosomes, where it may synthesize PI(3)P (Falasca & Maffucci, 2012; Franco et al, 2014) and/or PI(3,4,5)P3.

- An established method to acutely manipulate PI 3-phosphate levels at defined subcellular sites is via the dimerization between FKBP12 with the FRB domain of mTOR, whereby a membrane-targeting sequence can be fused to one domain and the enzyme to another, allowing the translocation to a specific membrane upon addition of rapamycin or analogues. Application of this technology established a crucial role for early endosomal PI(3)P in controlling endosome size and receptor sorting (Fili et al, 2006). Drawbacks, however, include the effects of rapamycin on mTOR signalling and the irreversibility of the dimerization. Recently, a reversible chemical dimerization system using a competitive ligand has been developed, allowing for the activation and subsequent arrest of PI 3-kinase activity to determine the turnover of PI(3,4,5)P3 and its downstream effectors (Feng et al, 2014).

- An independent rapidly reversible dimerization approach is based on optogenetics whereby protein heterodimerization is induced by light of specific wavelengths, and, hence, is reversible as protein heterodimers rapidly dissociate following cessation of the light pulse. This technique has been used to study the recruitment of class I PI 3-kinases to the plasma membrane and subsequent recovery from their activation (Toettcher et al, 2011; Idevall-Hagren et al, 2012). A great advantage of this approach is the ability to apply focal light pulses to rapidly induce PI 3-phosphate synthesis or turnover locally within cells. On the other hand, the application of optogenetics limits the use of fluorescent proteins to red and far-red variants, thereby putting restrictions on live imaging approaches.
Box 3: Chemical inhibitors of PI 3-phosphate signalling

The development of specific and selective inhibitors of the different classes and isoforms of PI3Ks is of great interest as a biological tool to better understand their diverse function, as well as for their potential clinical applications, most notably in anti-cancer therapy and the treatment of inflammatory diseases.

- Potent pan-PI3K inhibitors wortmannin and LY294002 inhibit the p110 catalytic subunit of the class I PI3K; however, they inhibit other kinases (e.g. Vps34-class III PI3K, PI3KCI2β) at higher concentrations.

- Idelalisib is a p110-δ-specific inhibitor used in the treatment of chronic lymphocytic leukemia (Zhu et al., 2015). Several other isoform-selective class I PI3K inhibitors exist.

- Specific Vps34 inhibitors, PIK-III, SAR405, VPS34-IN1, are also promising for therapeutic applications as potential anti-cancer drugs. These inhibitors have also provided additional evidence for Vps34-mediated PI(3)P production during vesicle trafficking, autophagy and signalling (Bago et al., 2014; Dowdle et al., 2014; Ronan et al., 2014).

- Lead compounds for compounds that selectively inhibit class II PI3Kγ is a promising first step in the development of specific inhibitors of the class II PI3Ks, the understanding of which has been hampered by a lack of structural information on these kinases, their substrate interactions and their regulation by allosteric activators or inhibitors (Freitag et al., 2015).

- Inhibitors of the PI(3,4,5)P3 3-phosphatase PTEN (e.g. SF1670, bpV (phen), bpV(pip) or VO-DHPip) have been reported and shown to cause elevated PI(3,4,5)P3 levels and overactivation of the Akt pathway with corresponding effects on cell polarization, migration, akin to genetic PTEN loss-of-function models (Di Cristofano et al., 1998; Song et al., 2012; Mak & Woscholski, 2015), however that may inhibit other enzymes such as the PI 4-phosphatases INPP4A/B (Spinelli et al., 2015).

coincident detection and reciprocal interactions between PI 3-phosphatase and PI 4-kinase activities to allow for local PI conversion at endosomes. Defects in this PI conversion module likely underlie the muscle and non-muscle defects observed in patients suffering from X-linked centronuclear myopathy (myotubular myopathy) due to mutations in the MTM1 gene (Ketel et al., 2016) (Fig 1).

While these examples focus on PI(3)P conversion in endosomal trafficking, given the importance of PI conversion in all aspects of membrane trafficking, the identification of new PI kinase and phosphatase complexes will provide valuable insight into how these conversion cascades are regulated. There is also evidence for the formation of regulatory complexes between PI3Ks and phosphatases involved in signal transduction, as seen for the PI(3,4,5)P3 3-phosphatase PTEN and the p85 regulatory subunit of class I PI3Ks. This interaction positively regulates the phosphatase activity of PTEN, providing a mechanism to couple Akt activation via p85-PI3K-mediated synthesis of PI(3,4,5)P3 with Akt inactivation via p85-PTEN-triggered PI(3,4,5)P3 hydrolysis (Barber et al., 2006; Rabinovisky et al., 2009; Chagpar et al., 2010).

And action! PI 3-phosphate-binding modules and effectors

PIPs mediate their effects at membranes via the recruitment of effector proteins containing PIP-binding domains. The incorporation of common binding domains into proteins with otherwise diverse structures and activities allows PIPs to have a broad range of functions inside the cell. Effector proteins can have direct effects on the nanoscale organization and shape of membranes, for example via tubulation, fusion or fission of membranes. Moreover, they can be involved in intracellular transport, can coordinate or regulate the activation or inhibition of other proteins such as small GTPases, kinases or phosphatases and can serve as platforms for intracellular signalling cascades (Lemmon, 2003, 2008; Kutiladze, 2010). Among the major PI 3-phosphate recognizing domains are the PH, the FYVE and the PX domains, though many other domains exist that can associate with PI 3-phosphates, often in conjunction with other proteins or membrane lipids (Table 1).

Pleckstrin homology (PH) domains with high affinity binding to PI(3,4,5)P3 and PI(3,4)P2 play a key role in PI3K signalling from the plasma membrane following agonist stimulation of cell surface receptors, with Akt as the best studied example of such a PI3K downstream effector. Growth factor activation of receptors stimulates class I PI3Ks, resulting in the synthesis of PI(3,4,5)P3, that may be further converted to PI(3,4)P2 by SHIP1/2 during the sustained phase of signalling. Both PI(3,4,5)P3 and PI(3,4)P2 bind with high affinity to the PH domain of Akt, thereby triggering its plasma membrane recruitment and activation as well as the phosphorylation of downstream targets, that is components and regulators of mTORC1 signalling (Pearce et al., 2010; Hemmings & Restuccia, 2012). Apart from Akt, other known PH domain-containing effectors of PI(3,4,5)P3 (and possibly PI(3,4)P2) comprise members of the guanine-nucleotide exchange factors (GEFs) for Rho and ADP ribosylation factor (Arf) family GTPases that are activated downstream of class I PI3K stimulating receptors. Rho-GEFs often contain a PH domain found in tandem with a Dbl homology (DH) domain that harbours GEF activity. Membrane recruitment by PH binding of the PH module stimulates the GEF activity of the DH domain, for example to promote cortical actin assembly among other effector pathways (Snyder et al., 2001; Rossman et al., 2002; Baumeister et al., 2003, 2006). One example is the Ras-GEF P-Rex1, a large scaffold protein synergistically activated by PI(3,4,5)P3 and Gβγ (Welch et al., 2002). PH domains can also regulate GEFs by controlling their localization, as seen with Ar6-GEF ARNO, which is recruited to the plasma membrane by binding of its PH domain to Ar6 and to PI(3,4,5)P3 (Venkateswarlu et al., 1998; Cohen et al., 2007). Furthermore, PH domains are found in some GTPase-activating proteins (GAPs), suggesting a role in the inactivation of GTPases (Vitale et al., 2000; Venkateswarlu et al., 2004).

FYVE domains specifically associate with PI(3)P and have been found in a large variety of endosomal and autophagosomal proteins (Stenmark et al., 1996; Burd & Emr, 1998). FYVE domains mediate or contribute to endosomal or autophagosomal protein localization, frequently as part of coincidence detection. For example, membrane targeting of the early endosomal scaffold protein EEA1 (early endosomal antigen 1) requires coincidence detection of both PI(3)P and Rab5-GTP to facilitate tethering and fusion of early endosomes (Simonsen et al., 1998; Christoforidis et al., 1999). Similar mechanisms mediate recruitment of other PI(3)P-binding proteins. These include the degradative sorting adaptor Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Raiborg et al., 2002), the FYVE domain-containing adaptor SARA (Smad anchor for receptor activation), a mediator of transforming growth factor (TGF) β-Smad...
signalling at endosomes (Miura et al., 2000; Itoh et al., 2002), the cytokinesis factor FYVE-CENT (Sagona et al., 2010), the PI(3)P phosphatase MTMR4 (Lorenzo et al., 2006; Naughtin et al., 2010), ALFY (autophagy-linked FYVE protein), a protein that targets ubiquitin-containing protein aggregates for autophagic degradation (Simonsen et al., 2004) and the omegasome protein DFCP1 (double FYVE containing protein 1) (Polson et al., 2010).

Phox homology (PX) domains often preferentially associate with PI(3)P on endosomes; however, some family members exhibit a broader lipid specificity and localization (Seet & Hong, 2006; Teasdale & Collins, 2012). The largest group of PX domain-containing proteins are the members of the sorting nexin (SNX) family of trafficking and signalling adaptors found at the plasma membrane and throughout the endosomal system (Cullen, 2008). A subset of SNXs in addition to their PI 3-phosphate-binding PX domain also contain a Bin-Amphiphsin-Rvs (BAR) domain, a dimeric helical protein module that can sense or induce membrane curvature, in particular when containing additional amphipathic helices (Peter et al., 2004). Other proteins containing PX domains include the class II PI3Ks and the endosomal kinesin KIF16B. The latter has been shown to link Vps34-mediated PI(3)P production to the peripheral dispersion of endosomes (Hoefnagel et al., 2005).

Other domains that can recognize and bind to PI 3-phosphates are summarized in Table 1. These include the PH-related GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins) domain found in endosomal myotubularins (Doersk et al., 2000; Begley et al., 2003; Berger et al., 2003; Tsujita et al., 2004; Lorenzo et al., 2005) and the PI(3)P-binding GLUE (GRAM-like, ubiquitin binding in EAP45) domain present in the Vps36 subunit of the ESCRT-II complex (Slagsvold et al., 2005; Teo et al., 2006). β-propeller PROPPIN (β-propellers that bind phosphoinositides) and WD40 domains found in phosphatases such as Atg18/WIP12 (WD-repeat PtDlns(3)P effector protein) can bind to PI 3-phosphates as well (Stramhaug et al., 2004; Polson et al., 2010; Baskaran et al., 2012; Dooley et al., 2014). The Raptor subunit of mTORC1 associates via its WD40 domain with PI(3,5)P2 on lysosomes (Michell et al., 2006; Bridges et al., 2012). PI 3-phosphate binding has also been reported for subsets of C2 domains, often in response to elevated intracellular calcium (Corbalan-Garcia & Gómez-Fernández, 2014). PI3KC2α contains a C2 domain that binds to PI(3,4)P2 and PI(4,5)P2, while the C2 domain of INPP4B has been shown to associate with PI(3,4,5)P3. These examples further illustrate how the localized production or consumption of one PI species can be regulated by another (Ferron & Vacher, 2006; Liu et al., 2006).

In many cases, the exact mode by which PI 3-phosphates are recognized and exert their physiological effect is unknown. For example, the fusion of late endosomes, lysosomes and autophagosomes requires both PI(3)P and PI(4,5)P2 to promote SNARE complex assembly, as well as membrane tethering by the heterotrimeric HOPS complex (homotypic fusion and vacuole protein sorting) (Mima & Wickner, 2009; Xu & Wickner, 2010; Balderhaar & Ungermann, 2013; Solinger & Spang, 2013). Furthermore, the Rab7-GEF Mon1-Ccz1 binds and is recruited to early endosomes via PI(3)P, and the GEF activity itself is stimulated by membranes and PI(3)P (Nordmann et al., 2010; Poteryaev et al., 2010; Cabrera et al., 2014), though the precise PI(3)P-binding determinants within Mon1-Ccz1 remain to be unravelled.

These examples illustrate that nature has evolved mechanisms of PI 3-phosphate recognition and function that extend well beyond the known repertoire of PI 3-phosphate-binding domains. In support of this view, recent proteomic studies have revealed a much larger PI-binding interactome than predicted from the simple analysis of known PI-binding domains (Junghmicel et al., 2014), suggesting that many more PI 3-phosphate-associated domains and effectors remain to be uncovered.

**Integrating PI 3-phosphate function: From signalling to membrane traffic and return**

While PI 3-phosphates—as discussed above—act as crucial second messengers activating signal transduction in response to extracellular stimuli, they also exhibit key roles in exo- and endocytic membrane traffic to and from the plasma membrane and within the
endolysosomal system. During recent years, it has, however, become clear that the roles of PI 3-phosphates in signalling from both the cell surface and from endomembranes (Vanhasebroeck et al., 2010a) and in membrane traffic (Jean & Kiger, 2012; Balla, 2013) are intertwined and, hence, often cannot be separated (Fig 3A).

Receptor endocytosis in the regulation of signalling and cellular metabolism

Upon binding their ligand, activated plasma membrane receptors such as RTKs and GPCRs undergo endocytosis, either via CME or via clathrin-independent internalization routes including macropinocytosis (Schmees et al., 2012; Levin et al., 2015) and fast-endophilin-mediated endocytosis (FEME) as described recently (Boucrot et al., 2015). Endocytosis most often serves to terminate receptor signalling and to modulate the cellular responsiveness to a given ligand by adjusting receptor surface levels. Following their traffic to early endosomes, internalized receptors can be sorted to intraluminal vesicles of MVBs or late endosomes. This results in termination of signalling via receptor segregation from the cytoplasm and via their subsequent degradation in lysosomes, thereby downregulating the future responsiveness of the cell to signalling receptor ligands (Fig 3B). Alternatively, receptors can be recycled back to the plasma membrane enabling cellular re-sensitization to receptor ligands (Grant & Donaldson, 2009; Parachoniak & Park, 2012). Receptor endocytosis can in some cases also serve as a means to transmit extracellular signals over long distances via signalling endosomes, for example in neurons, where growth factor signals need to travel retrogradely from the synapse back to the soma over long distances (Sorkin & von Zastrow, 2009; Platta & Stenmark, 2011). Commencement of a signalling event at the plasma membrane, internalization of the receptor, its delivery to early endosomes, and subsequent sorting into the degradative or recycling pathways rely on the spatiotemporally controlled synthesis and turnover of defined pools of PI 3-phosphates by the different classes of PI3Ks and phosphatases.

A major effect of the activation of RTKs and GPCRs is the generation of plasma membrane PI(3,4,5)P3 via class I PI3Ks (see above). Following their activation and downstream signalling events, activated receptors are internalized and PI(3,4,5)P3 is hydrolysed to prevent receptor hyperactivation and hyperproliferation of cells as seen in many forms of cancer (Sheppard et al., 2012). Recent data suggest differential roles for PI 3-phosphates in coordinating receptor signalling, endocytosis and downstream receptor trafficking. A prime example is the epidermal growth factor receptor (EGFR), which following activation with different concentrations of ligand—at least in some cell types—can be internalized via CME, FEME (Boucrot et al., 2015) and macropinocytosis (Sorkin & Goh, 2009; Maekawa et al., 2014). EGFR internalization via CME can be driven either by low concentrations of its ligand or by exogenous supply of PI(3,4,5)P3 in the absence of EGF. In the latter case, receptors are exclusively recycled to the cell surface rather than sorted to MVBs for degradation. Recycling requires both PI(3,4,5)P3 and members of the PAR polarity complex proteins such as PAR3 (Laketa et al., 2014), suggesting that PI(3,4,5)P3 regulates receptor tyrosine kinase trafficking and may thereby enhance the cellular responsiveness to growth factors. How exactly elevated levels of PI(3,4,5)P3 promote CME of activated EGFR, while constitutive CME of transferrin appears to be unaffected, is unknown. One possibility is that PI(3,4,5)P3 serves as a substrate for 5-phosphatases such as SHIP2 to generate a local pool of PI(3,4)P2, a lipid required for clathrin-coated pit (CCP) maturation, at the plasma membrane (Posor et al., 2013). A similar mechanism may operate in FEME of activated EGFR and other RTKs and GPCRs, where class I PI3K-mediated synthesis of PI(3,4,5)P3 downstream of receptor activation followed by its subsequent dephosphorylation into PI(3,4)P2 by SHIP1/2 has been shown to mediate recruitment of the PI(3,4)P2-binding PH domain protein lamellipodin. Lamellipodin then engages the membrane-deforming BAR domain protein endophilin and the actin cytoskeleton to drive clathrin-independent receptor internalization (Fig 3B). Endophilin-depleted cells display elevated mitogen-associated protein kinase (MAPK) activity, while the response to high doses of growth factor (e.g. EGF) is dampened, perhaps as a result of compensatory macrophinocytic EGFR internalization (Boucrot et al., 2015), a process that requires sequential breakdown of PI(3,4)P2 and PI(3)P by INPP4B and the myotubularin-related 3-phosphatases MTMR6 and 9 (Maekawa et al., 2014). Knock-down of clathrin also results in elevated MAPK signalling, because the recruitment of the MAPK kinase MEK2 to endosomes is perturbed in clathrin-depleted cells (Galperin & Sorkin, 2008). The work of Galperin and Sorkin indicates that the spatial segregation of MEK2 from EGFR signalling complexes at the level of endosomes depends on CME. These data imply that distinct endocytosis pathways differentially regulated by PI 3-phosphates affect RTK signalling. Conversely, recent data suggest that crosstalk between RTK-mediated Akt/GSK3β signalling and isoforms of dynamin regulates CME (Reis et al., 2015).

How specific receptors are internalized and signal appears to be at least in part caused by their direct association with components of the machinery for PI 3-phosphate synthesis and turnover. Apart from the well-studied signalling adaptor-mediated association of class I PI3Ks with both RTKs and GPCRs (Naga Prasad et al., 2002), other members of the PI3K family have also been linked to specific receptors. In endothelial cells, the class II PI3KC2β is specifically required for the internalization of the receptors for vascular endothelial growth factor (VEGF) and sphingosine 1-phosphate (SIP). As a consequence, VEGF and SIP signalling as well as endothelial function are impaired in endothelial cell-specific PI3KC2β knockout mice, due to defective formation of VEGF receptor 2- and SIPR-positive signalling endosomes (Yoshioka et al., 2012; Biswas et al., 2013). Similar mechanisms may explain the role for PI3KC2α in TGFβ (Aki et al., 2015) and insulin signalling. In pancreatic β cells, PI3KC2α has been shown to generate PI(3,4)P2 downstream of insulin receptor activation, allowing for the selective activation of Akt1 to promote glucose-stimulated insulin release (Leibiger et al., 2010). Although the exact role of PI3KC2α-mediated PI(3,4)P2 synthesis has not been studied in all of these cases, it is tempting to speculate that receptor signalling and PI3KC2α activation are intimately connected to receptor internalization and/or endosomal trafficking. Consistent with this hypothesis, recent work has shown that CME of various receptors requires the local generation of PI(3,4)P2 at endocytic coated pits by PI3KC2α (Posor et al., 2013) to promote membrane constriction prior to endocytic vesicle fission (Dauwe et al., 2014).

Receptor sorting within the endosomal system

While the production of PI(3,4)P2 is necessary for the completion of CME, it is the generation of PI(3)P that is essential for the
termiation of signalling events at the plasma membrane mediated via PI(3,4,5)P3. PTEN is recruited to endosomal vesicles via binding of its C2 domain to PI(3)P (Naguib et al., 2015), providing an example of how a downstream 3-PI (PI(3)P) can negatively regulate an upstream one (PI(3,4,5)P3) via the regulation of a PI 3-phosphatase. PTEN-mediated turnover of PI(3,4,5)P3 may be linked to the formation of APPL endosomes. This subpopulation of signalling early endosomes contains the Akt-interacting Rab5 effector proteins APPL1/2 (Miaczynska et al., 2004; Schenck et al., 2008) but is devoid of endosomal PI(3)P effectors such as EEA1 (Zoncu et al., 2009; but see Kalaidzidis et al., 2015 for a different view).

PI(3)P generated by class III PI3K Vps34 and possibly class II PI3Ks (Devereaux et al., 2013; Franco et al., 2014) is a defining feature of endosomes and of key importance for their function. Within the endosomal system, cargo may either be recycled to the cell surface, trafficked retrogradely to the trans-Golgi network (TGN), or sorted to MVBs/late endosomes for lysosomal degradation. Surface recycling and retrograde traffic to the TGN both involve the formation of recycling tubules and the sorting of cargo into these tubules by lipid-binding proteins of the sorting nexin (SNX) family. Tubule formation by SNX proteins depends on the ability of their PX-BAR domain module to bind to PI(3)P and to form oligomeric self-assembly that remodel the endosomal membrane (van Weering & Cullen, 2014). Some SNXs are actively involved in cargo selection: SNX17, 31 and 27 harbour a PX and a FERM (4.1/ezrin/radixin/moesin) domain, the latter associating with and sorting PDZ-domain-containing cargo, such as β1-integrin and β2-adrenergic receptors (Lauffer et al., 2010; Böttcher et al., 2012; Tseng et al., 2014). Endosomal recycling of signalling and adhesion receptors thus regulates receptor surface expression and thereby the cellular responsiveness to ligands. Moreover, PI(3)P-binding PX domain proteins including SNXs play a role in directing endosomal trafficking via the recruitment of molecular motors. For example, endosomal progression from early to recycling endosomes involves recruitment of the dynein adaptor KIBRA by the PI(3)P-binding PX domain protein SNX4 (Traer et al., 2007). Outward traffic of cargo from endosomes to the cell surface depends on the plus end-directed endosomal motor kinesin KIF16B, which is tethered to endosomes via a PI(3)P-binding PX domain (Hoepfner et al., 2005).

Transmembrane cargo (e.g. receptors) degradation occurs via sorting into intraluminal vesicles (ILVs) of maturing endosomes to generate MVBS/late endosomes that will ultimately fuse with lysosomes. Endosomal maturation is accompanied by Rab conversion from early endosomal Rab5 to late endosomal Rab7, a process that depends on the recruitment and stimulation of the Rab7 GEF Mon1-Ccz1 by PI(3)P (Nordmann et al., 2010; Poteryaev et al., 2010; Cabrera et al., 2014) as well as active cargo sorting into ILVs by the ESCRT complex. The ESCRT machinery contains 4 protein complexes, ESCRT 0, I, II and III that are recruited and work in a sequential manner (Raiborg & Stenmark, 2009). ESCRT 0 is recruited to the membrane via the PI(3)P-binding FYVE domain of its Hrs subunit, which recognizes ubiquitinated cargo such as activated growth factor receptors destined for degradation (Raiborg et al., 2001). The ubiquitinated receptors are then passed off to the ESCRT-I and II complexes, with ESCRT-II also binding to PI(3)P via the GLUE domain of its Vps36/Eap45 subunit. Finally, ESCRT-II recruits the ESCRT-III complex, which forms spirals that drive membrane deformation and fission of ILVs (Slagsvold et al., 2005; Chiaruttini et al., 2015). It is the balance between receptor degradation in lysosomes and recycling to the cell surface that modulates the sensitivity of cells to receptor ligands.

Receptor signalling from early endosomes
In addition to regulating membrane traffic within the endolysosomal system, PI(3)P also regulates signalling from early endosomes. TGFβ signalling relies on the recruitment of the PI(3)P-binding FYVE domain-containing proteins SARA (SMAD anchor for receptor activation) or endofin to early endosomes, allowing for the phosphorylation of the receptor target SMAD2 and the translocation of the SMAD2–SMAD4 complex to the nucleus to regulate gene transcription (Tsukazaki et al., 1998; Chen et al., 2007). Similarly, mitogenic signalling has been shown to involve the APPL subpopulation of early endosomes as an intermediate relay station en route to the nucleus (Miaczynska et al., 2004; Schenck et al., 2008). Conversely, signalling events can control the production of PI(3)P by Vps34, in turn modulating signalling. For example, pheromone activation of the GPCR Ste2p in S. cerevisiae causes the α subunit of its trimeric G protein complex Gpa1 to bind to and activate the Vps15 subunit of class III Vps34–PI3K. The resulting increase in endosomal PI(3)P levels serves to recruit the FYVE domain adaptor bud emergence protein 1, an essential component of MAPK signalling from endosomes (Slessareva et al., 2006).

Recent data indicate that early endosomes in addition to PI(3)P also capitalize on a local pool of PI(3,4)P2 synthesized by class II PI3Ks such as PI3KC2γ for signalling, PI3KC2γ associates with active Rab5 to control endosomal Akt2 activation downstream of insulin signalling in hepatocytes (Braccini et al., 2015). Whether similar mechanisms operate in all cell types remains an interesting question for future studies.

Nutrient signalling and the control of autophagy by PI 3-phosphates
Perhaps the most prominent example of the integration of membrane traffic and signalling within the endolysosomal system is the close association of lysosomal function with the mechanistic target of rapamycin (mTOR) signalling pathway and the control of autophagy by mTOR signalling (Fig 3C). The lysosome is a master regulator directing the cell into either an anabolic or catabolic state by serving as an integrator of the cellular nutrient and energy status. Disruptions in lysosomal signalling are increasingly found to be causative in diseases such as diabetes, cancer and various neurological disorders (Zoncu et al., 2011b; Ferguson, 2015). The surface of the lysosome serves as the site of action for two evolutionarily ancient, key signalling complexes, the mTORC1 and the AMP-activated protein kinase (AMPK). These complexes are antagonistically regulated to promote anabolic or catabolic processes, respectively (Hardie, 2014; Zhang et al., 2014). mTOR is a serine/threonine protein kinase that when active promotes growth by positively regulating protein translation through the activation of S6 kinase, as well as by inhibiting autophagy via the inhibition of ULK1 (Unc-51-like kinase) (Laplante & Sabatini, 2012). mTORC1 acts as a signal integrator at the lysosome, ensuring that all the components necessary for growth are present prior to its activation. The recruitment of mTORC1 to the lysosome, where it will be activated, is mediated via the cellular amino acid status (Sancak et al., 2008; Zoncu et al., 2011a; Bar-Peled et al., 2013). Once at the lysosome, the activation of mTORC1 is under control of growth factor
signalling. The generation of PI(3,4,5)P3 by class I PI3K with the subsequent activation of Akt (see above) results in the downstream activation of mTORC1 on the lysosome (Inoki et al., 2002; Vander Haar et al., 2007). In addition to the role of the class I PI3K in mTORC1 regulation, both class II and III PI3Ks have been implicated in mTORC1 regulation, though their exact roles merit further study. Amino acid stimulation of Vps34 results in the generation of PI(3)P, which in turn recruits phospholipase D1 (PLD1) via its PI(3)P-binding PX domain. PLD1 then generates a pool of phosphatidic acid that binds to the FKBPI2-ramapycin-binding domain of mTOR, triggering dissociation of the inhibitory DEPTOR subunit of mTORC1 (Byfield et al., 2005; Nobukuni et al., 2005; Gualti et al., 2008; Yoon et al., 2011, 2015; Bridges et al., 2012). Both the alpha isoform of the class II PI3K and PIKfyve have also been implicated in mTORC1 activation through their sequential activity in generating PI(3,5)P2 that binds the WD40 domain of the Raptor subunit of mTORC1 (Bridges et al., 2012). Furthermore, at least in yeast, PI(3,5)P2 has an additional role in the activation of TORC1 via the recruitment of the TORC1 substrate Sch9 (the homologue of mammalian S6K), which contains a PI(3,5)P2-binding C2 domain (Jin et al., 2014). While much is known about the activation of mTORC1, it remains unclear how its inactivation is accomplished, although the process is likely to be actively controlled (Demetriades et al., 2014; Menon et al., 2014). Given the prominent role of PI 3-phosphates in the endolysosomal system, it will be interesting to further explore whether and how these molecules may control the mTORC1 activation status and how such control may be linked to the late endosomal/lysosomal localization and function (Korolchuk et al., 2011).

A key process controlled by lysosomal signalling is macroautophagy (also referred to as autophagy), a major pathway for the degradation of cytoplasmic material. Autophagy is initiated by the engulfment of bulk cytosol (containing protein aggregates) and/or organelles by a cup-shaped double-membrane sheet known as the phagophore. The phagophore closes on itself to form the autophagosome, which delivers its contents to the lysosome for degradation by lysosomal hydrolases (Ravikumar et al., 2010). Autophagy is reciprocally regulated by class I and class III PI3Ks (O’Farrell et al., 2013). Receptor-mediated class I PI3K signalling activates mTORC1 during times of nutrient abundance, leading to an inhibitory phosphorylation on ULK1 and, thus suppression of autophagy. The serine/threonine kinase ULK1 (termed Atg1 in yeast) together with ATG13, FIP200 and ATG101 form part of a multiprotein complex, which plays a pivotal role in the earliest steps of the autophagy process (Stanley et al., 2014). ULK1 also regulates early trafficking steps in autophagy, such as the activation of Rab12 (Xu et al., 2015), and stimulates Vps34 complex I to generate a local pool of PI(3)P on autophagic membranes. The latter is required for formation of the phagophore and for membrane expansion via phosphorylation of the Beclin-1 subunit (Russell et al., 2013). mTORC1 also inhibits later stages of autophagy via an inhibitory phosphorylation on UVRAG, a component of Vps34 complex II, thereby suppressing autophagosome and endosome maturation (Kim et al., 2015). However, another study found that mTORC1 phosphorylation of UVRAG at different sites activates Vps34, thus generating a pool of PI(3)P on lysosomes necessary for autophagosome—lysosome reformation following starvation (Munson et al., 2015), indicating that the regulation of Vps34 activity by mTORC1 activity is complex. Together, these findings illustrate how class III PI3K Vps34 is regulated downstream of class I PI3K signalling to integrate endolysosomal membrane traffic and autophagic protein turnover with mTORC1 signalling from the lysosomal surface.

A number of PI(3)P effectors on autophagosomes have been identified. During early stages of autophagy, the FYVE domain-containing PI(3)P-binding protein DFCP1/TAFF1 associates with the nascent limiting membrane (termed “omesome”), although the exact role of DCP1/TAFF1 is unknown (Axe et al., 2008). A major class of PI 3-P effectors in autophagy are the Atg18/WIPI/PROPPIN proteins, which are defined by a 7-bladed β-propeller that binds PI(3)P and PI(3,5)P2 (Nair et al., 2010; Baskaran et al., 2012). There are 4 human WIPI proteins, which have essential but incompletely understood roles in autophagy that may at least be partially distinct (Proikas-Cezanne et al., 2015). WIPI2 was recently identified as the missing link between PI(3)P production and autophagosome formation as it recruits the ATG12–ATG5–ATG16L complex necessary for the conjugation of the essential autophagy protein LC3 to phosphatidylethanolamine at the omesome (Dooley et al., 2015). Another FYVE domain-containing PI(3)P effector is Alfy, which has a role in selective autophagy by linking ubiquitinated protein aggregates destined for degradation to the autophagic machinery (Simonsen et al., 2004; Clausen et al., 2010; Filimonenko et al., 2010). Furthermore, autophagosome biogenesis is promoted by the PI(3)P-binding PX-BAR domain protein SNX18, which has been shown to mediate delivery of Atg16L1- and LC3-positive membranes to autophagosome precursors (Knævelsrud et al., 2013). While a crucial role for Vps34 in autophagy is undisputed, there is also evidence for Vps34-independent autophagy, whereby PI(3)P could be produced either directly or indirectly by other lipid kinases and phosphatases, for example by the class II PI3Ks (Devereaux et al., 2013). Furthermore, a recent study suggests that PI(5)P synthesized by PIKfyve can promote autophagy via known PI(3)P effectors in glucose starvation-induced autophagy (Vicianza et al., 2015).

Given the importance of dysregulated autophagy in diseases such as cancer and neurodegenerative disorders, a better understanding of the roles of PI kinases, phosphatases, as well as their products and effectors, provides an exciting avenue for future research.

Future directions

We are only beginning to understand that the seemingly diverse functions of PI 3-phosphates in the regulation of membrane traffic and cell signalling pathways are owed at least in part to their spatiotemporally controlled formation and turnover at defined subcellular sites. Hence, the same PI 3-phosphate can exhibit different effects when present at distinct intracellular locations, perhaps even within distinct nanodomains present in the same organelle. Classical genetic loss-of-function approaches may therefore not be suited to capture this level of complexity as PI3Ks and phosphatases may also be recruited to several locations and/or activated locally. With the advent of new chemical (e.g. caged PI 3-phosphates, Box 1) and optogenetic approaches (Box 2), paired with genome engineering to enable expression of tagged proteins at near endogenous levels, the tools are emerging to tackle this complexity and to pave the way for a new era of research on these fascinating lipids. A continuing challenge that remains is the analysis and visualization of such local pools of PI.
3-phosphates. It is likely that the development of ever more sophisticated fluorescent reporters as well as the direct visualization of tagged PI 3-phosphate effector proteins will allow us to unravel more of nature’s PI 3-phosphate secrets. Finally, we predict that the development of specific inhibitors (Box 3) and sophisticated compounds for local inhibition of defined PI 3-phosphate-metabolizing enzymes will allow to untangle the complex interplay between the various PI 3-phosphates and their roles in integrating cell signalling and membrane traffic between the plasma membrane and the endolysosomal system. Hopefully, these tools will also enable the treatment of some of the most devastating disorders linked to altered PI 3-phosphate metabolism and signalling ranging from cancer to hereditary muscle disease and neurodegeneration.

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Conflict of interest

The authors declare that they have no conflict of interest.

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