© 2015. Published by The Company of Biologists Ltd | Journal of Cell Science (2015) 128, 4047-4056 doi:10.1242/jcs.175208

COMMENTARY

Emerging roles of PtdIns(4,5)P_2 – beyond the plasma membrane

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ABSTRACT

Phosphoinositides are a collection of lipid messengers that regulate most subcellular processes. Amongst the seven phosphoinositide species, the roles for phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] at the plasma membrane, such as in endocytosis, exocytosis, actin polymerization and focal adhesion assembly, have been extensively studied. Recent studies have argued for the existence of PtdIns(4,5)P_2 at multiple intracellular compartments, including the nucleus, endosomes, lysosomes, autolysosomes, autophagic precursor membranes, ER, mitochondria and the Golgi complex. Although the generation, regulation and functions of PtdIns(4,5)P_2 are less well-defined in most other intracellular compartments, accumulating evidence demonstrates crucial roles for PtdIns(4,5)P_2 in endolysosomal trafficking, endosomal recycling, as well as autophagosomal pathways, which are the focus of this Commentary. We summarize and discuss how phosphatidylinositol phosphate kinases, PtdIns(4,5)P_2 and PtdIns(4,5)P_2-effectors regulate these intracellular protein and membrane trafficking events.

KEY WORDS: PtdIns(4,5)P_2, PIPKI, Protein and membrane trafficking

Introduction

Phosphoinositides are a small group of lipids found in all cellular membranes. Yet, these lipid messengers have crucial functions in defining localized membrane properties and modulating various subcellular biological processes, including cell signaling, protein and membrane trafficking, cytoskeleton organization, as well as gene expression (Schramp et al., 2012). The inositol ring of phosphatidylinositol can be phosphorylated at the third, fourth and fifth positions in all combinations, resulting in the generation of seven different phosphoinositide species: phosphatidylinositol 3-phosphate [PtdIns(3)*P*], PtdIns(4)*P*, PtdIns(5)*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. There has been evidence that phosphoinositides are enriched on specific subcellular membranes. For example, a substantial pool of PtdIns(4)*P* is present at the Golgi complex, where various PtdIns(4)*P* effectors have also been identified (D'Angelo et al., 2008), whereas PtdIns(3)*P* and PtdIns(3,5)P_2 are main phosphoinositide messengers at early and late endosomes, respectively, with important roles in the membrane/protein trafficking at the endosomal system (Bissig and Gruenberg, 2013).

PtdIns(4,5)P_2 is well-known as an essential lipid messenger at the plasma membrane, where its localized generation regulates endocytosis, exocytosis, actin cytoskeleton dynamics, focal adhesion assembly, ion channels and transporters (Huang, 2007; Schramp et al., 2012). However, recent progress has discovered far more widespread distributions of the different phosphoinositide species, especially PtdIns(4,5)P_2 and its primary precursor PtdIns(4)*P*, both of which have been found at multiple subcellular membrane structures with crucial roles that, previously, had not been appreciated. In this Commentary, we review the current evidence in support of intracellular PtdIns(4,5)P_2 signaling and focus on the emerging roles for PtdIns(4,5)P_2 in endolysosomal trafficking and protein recycling, as well as in autophagic membrane trafficking inside the cells.

Intracellular PtdIns(4,5)P_2 signaling

It is an established view that PtdIns(4,5)P_2 is a key lipid messenger at the plasma membrane, yet accumulating evidence supports that it also exists and has crucial roles in intracellular compartments, including – but not limited to – endosomes, lysosomes, autolysosomes, the Golgi complex, endoplasmic reticulum (ER) and nucleus (Fig. 1). Electron microscopy (EM) studies in which the pleckstrin homology domain of phospholipase C-δ (PLCδ-PH) was used as a specific PtdIns(4,5)P_2 probe (Lemmon and Ferguson, 2000) revealed that a substantial pool of PtdIns(4,5)P_2 is localized to the nucleus, Golgi complex, ER, mitochondria, recycling endosomes, as well as to the limiting membrane and intraluminal vesicles of multivesicular endosomes, although the majority of PtdIns(4,5)P_2 is found at the plasma membrane (Vicinanza et al., 2011; Watt et al., 2002). Importantly, the specificity of PtdIns(4,5)P_2 labeling by the PLCδ-PH domain in EM sections was verified by preincubation of PLCδ-PH with Ins(1,4,5)P_3 the soluble head group of PtdIns(4,5)P_2 that specifically binds PLCδ-PH in a manner that is similar to PtdIns(4,5)P_2 (Lemmon and Ferguson, 2000), and by preincubation of the ultrathin sections with an anti-PtdIns(4,5)P_2 antibody, either of which blocked labeling by PLCδ-PH (Watt et al., 2002). Immunofluorescence approaches have also revealed that the endosomal localization of PLCδ-PH correlates well with endosomal targeting of PtdIns(4,5)P_2 effectors that – upon PtdIns(4,5)P_2 binding – either change their membrane localization or protein–protein interactions (Shi et al., 2012; Vicinanza et al., 2011). However, in many cases, it has been difficult to detect the intracellular pool of PtdIns(4,5)P_2 (Li et al., 2012; Ling et al., 2007, 2002; Mellman et al., 2008; Sun et al., 2013b; Tan et al., 2015a; Thapa et al., 2012); this is not only due to the relatively low amount of PtdIns(4,5)P_2 that is generated in intracellular compartments compared to that at the plasma membrane, but also due to the lack of free PtdIns(4,5)P_2 at these sites. At these intracellular compartments, PtdIns(4,5)P_2 binds to effector proteins. There are now many examples in which PtdIns(4,5)P_2 is generated by phosphatidylinositol phosphate kinases (PIPKs) at specific membranes that are enriched in PtdIns(4,5)P_2 effectors, which themselves, typically, are PIPK-interacting proteins (Ling et al., 2002; Mellman et al., 2008; Sun et al., 2013b; Thapa et al., 2012). In

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Journal of Cell Science

Journal of Cell Science (2015) 128, 4047-4056 doi:10.1242/jcs.175208

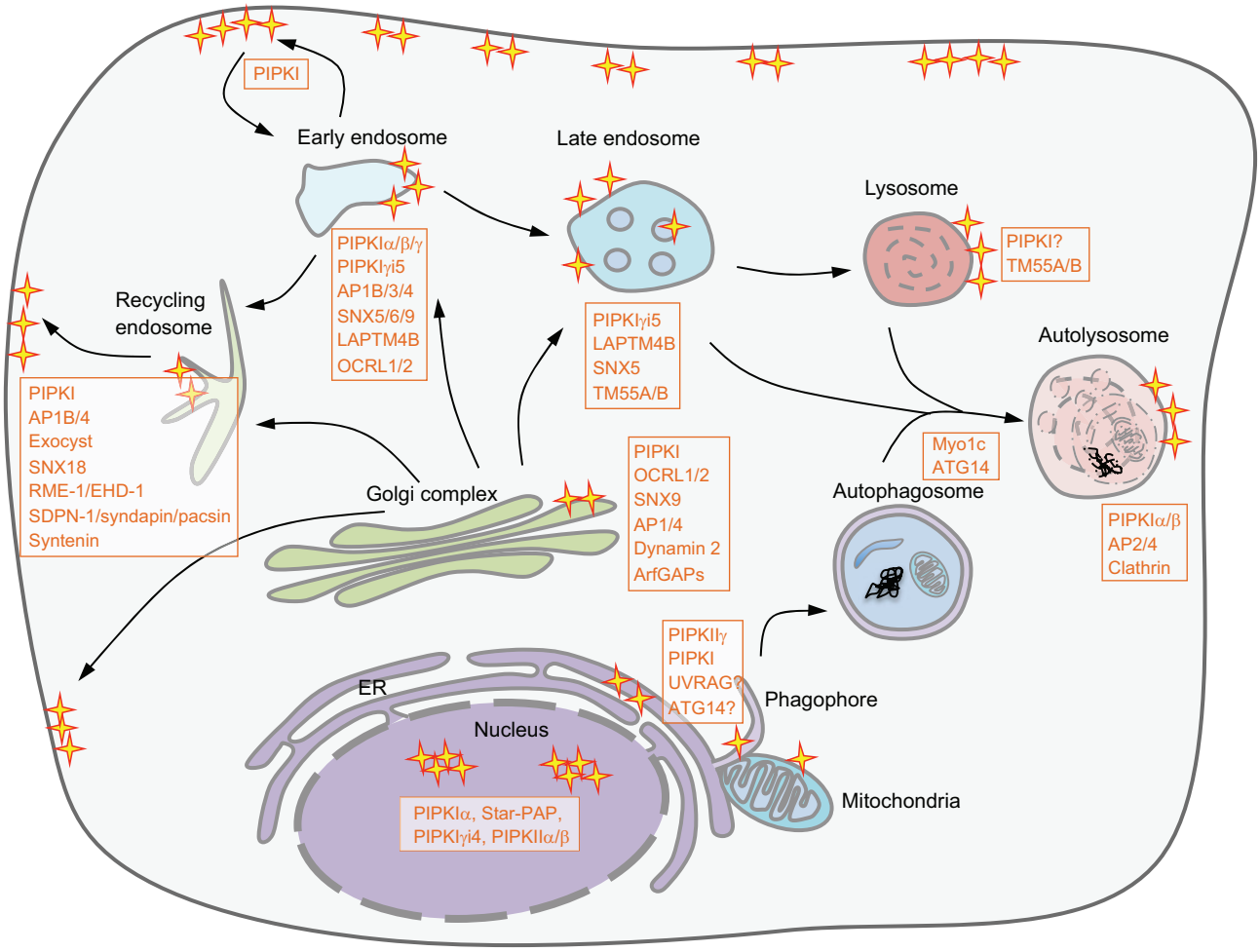

Fig. 1. Widespread subcellular distribution of PtdIns(4,5)P_2 throughout mammalian cells. Although immunofluorescence studies that have used the PLCδ-PH domain as a PtdIns(4,5)P_2 probe identified only marginal intracellular location of PtdIns(4,5)P_2, other methods revealed substantial amounts of PtdIns(4,5)P_2 within various intracellular structures, including endosomes, lysososmes, the ER, the Golgi complex, mitochondria and the nucleus, as highlighted by the orange stars. This is well-supported by the presence of the different PIPKs, PtdIns(4,5)P_2 effectors and PtdIns(4,5)P_2 phosphatases at intracellular compartments that are shown here.

other words, PIPKs usually define PtdIns(4,5)P_2 signaling by directly or indirectly associating with distinct PtdIns(4,5)P_2 effectors.

In support of intracellular PtdIns(4,5)P_2 signaling, several PIPKs that generate PtdIns(4,5)P_2 are observed at intracellular compartments (Fig. 1). In mammalian cells, most of PtdIns(4,5)P_2 is produced by type I PIPKs (PIPKIs), which use PtdIns(4)P as a substrate; a smaller pool of PtdIns(4,5)P_2 is generated by type II PIPKs (PIPKIIs), which phosphorylate PtdIns(5)P at the 4-hydroxyl group. There are several genes that encode PIPKIs and PIPKIIs, yielding PIPK isoforms α, β and γ. Each isoform has multiple splicing variants, resulting in a variety of PIPK isoforms. Among those, PIPKIα, PIPKIγi4, PIPKIIα and PIPKIIβ have been found at nuclear speckles (Li et al., 2013). PIPKIα defines a unique nuclear PtdIns(4,5)P_2 signaling pathway that controls the non-canonical 3′ processing of selected mRNAs and genome-wide gene expression, and that has been previously reviewed (Li et al., 2013). The 3′ processing is based on the mRNA 3′ polyadenylation activity of Star-PAP, a nuclear-speckle-targeted non-canonical Poly (A) polymerase (PAP), which is specifically stimulated by PtdIns (4,5)P_2 (Mellman et al., 2008). Endogenous PIPKIβ was found to primarily accumulate at perinuclear regions (Doughman et al., 2003). Furthermore, PIPKIα and PIPKIβ are also targeted to autolysosomes where they generate PtdIns(4,5)P_2 to initiate lysosomal reformation (Rong et al., 2012); consistently, PtdIns (4,5)P_2 had been previously shown to be generated by PIPKI at lysosomes (Arneson et al., 1999).

All PIPKIs have been shown to regulate endosomal trafficking (Galiano et al., 2002; Shinozaki-Narikawa et al., 2006). Our recent work has shown that PIPKIγi2 is localized to a fraction of recycling endosomes where it regulates the exocyst complex in order to facilitate transmembrane protein recycling (Thapa et al., 2012). Another isoform of human PIPKI (PIPKIγi5) is targeted to endosomal compartments and modulates endolysosomal receptor trafficking (Schill and Anderson, 2009b; Sun et al., 2013a,b; Tan et al., 2015a). PtdIns (4)P – a substrate of PIPKIs for the generation of PtdIns(4,5)P_2 – together with various PtdIns 4-kinases (PI4Ks), has also recently been found to have a wide distribution throughout the cell, including at the ER, Golgi complex, endosomes, autophagosomes, lysosomes, nucleus and the plasma membrane (D'Angelo et al., 2008; Sridhar et al., 2013; Wang et al., 2015). PI4Ks are very likely to collaborate with PIPKIs for a temporal production of PtdIns(4,5)P_2 at specific intracellular membranes, but this has not yet been demonstrated.

The relevance of intracellular PtdIns(4,5)P_2 signaling is further supported by the involvement of numerous PtdIns(4,5)P_2 effectors and phosphatases in important intracellular events (Fig. 1). In many,

perhaps most cases, these PtdIns(4,5)P_2 effectors are also PIPK-interacting proteins and their interactions are the basis for the generation of PtdIns(4,5)P_2 signals in a spatially and temporally controlled manner (Choi et al., 2015). Although the main phosphoinositides involved in endosomal trafficking are reported to be PtdIns(3)*P* and PtdIns(3,5)P_2 (Nicot and Laporte, 2008), a number of PtdIns(4,5)P_2 effectors were found at endosomes and regulate endosome and/or lysosome sorting; these include sorting nexins 5, 6, 9 and 18 (SNX5, SNX6, SNX9 and SNX18, respectively), receptor mediated endocytosis 1 (RME-1) in *C. elegans*/Eps15 homology-domain containing 1 (EHD-1) in mammals, synaptic dynamic binding protein 1 (SDPN-1) in *C. elegans*/syndapin or pacsin in mammals, syntenin, lysosomal-associated transmembrane protein 4B (LAPTM4B), adaptor protein (AP) complexes and a group of ADP-ribosylation factor (ARF) GTPase activating proteins (GAPs), also known as ArfGAPs (Lambaerts et al., 2012; Shi et al., 2012; Sun et al., 2013a,b; Tan et al., 2015a; Vicinanza et al., 2008). At the same time, PtdIns(4,5)P_2 phosphatases have also been found at endosomes and lysosomes, indicating that the generation and destruction of PtdIns(4,5)P_2 is dynamically controlled (Hsu et al., 2015; Nakatsu et al., 2015; Vicinanza et al., 2008, 2011). Finally, PtdIns(4,5)P_2, its effectors and phosphatases are also found at the Golgi complex, where they have roles in membrane trafficking and maintenance of the Golgi structure (De Matteis et al., 2002; Sweeney et al., 2002; Watt et al., 2002). Taken together, these observations clearly indicate that the cellular roles for PtdIns(4,5)P_2 go far beyond those it has at the plasma membrane.

PtdIns(4,5)P_2 and its role in the regulation of endolysosomal sorting

Cell-surface receptors define intracellular signal transduction from the extracellular environment. Internalization and lysosomal degradation of activated receptor are essential mechanisms to spatiotemporally control receptor signaling. A key step during early-to-late endosome maturation is the formation of intraluminal vesicles (ILVs), which is partially driven by the endosomal sorting complexes required for transport (ESCRT) machinery (Henne et al., 2011; Hurley et al., 2010). Together with endosomal maturation the ESCRT machinery (which comprises the cytosolic protein complexes ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) also controls the intraluminal sorting of many cell-surface receptors, which serves as a mechanism to target receptor cargos for lysosomal degradation (Eden et al., 2009). PtdIns(3)*P* is a crucial regulator of some ESCRT subunits, including Hrs (an ESCRT-0 subunit) and EAP45 (an ESCRT-II subunit) (Henne et al., 2011), whereas PtdIns (3,5)P_2 is an established canonical landmark of late endosomes and lysosomes (Nicot and Laporte, 2008). However, recent evidence also points to a role for endosomal PtdIns(4,5)P_2 in the regulation of ESCRT functions in the endolysosomal sorting of receptor cargos. Although presented at much lower levels at endosomes compared with those at the plasma membrane, PtdIns(4,5)P_2 appears to add an additional layer of control over membrane and/or protein trafficking at endolysosomes.

Human PIPKIγ mRNA is spliced into at least six isoforms, which result in the expression of six distinct enzymes that differ in their C-terminal extensions (Schill and Anderson, 2009b; Xia et al., 2011). PIPKIγi5 is a, recently identified, splicing variant of PIPKIγ that is localized to endosomal compartments where it generates PtdIns(4,5)P_2 to promote ESCRT-mediated intraluminal sorting of epidermal growth factor receptor (EGFR), a model protein for the study of endolysosomal trafficking (Fig. 2) (Schill and Anderson, 2009b; Sun et al., 2013a,b). At the endosome, PIPKIγi5 – through its unique C-tail – directly associates with the two targeting proteins SNX5 and LAPTM4B, both of which are PtdIns(4,5)P_2 effectors (Schill et al., 2014; Sun et al., 2013b; Tan et al., 2015a). SNX5 belongs to the phosphoinositide-binding Phox (PX)-homology-domain-containing protein family that is involved in various membrane trafficking processes (van Weering et al., 2010). Of

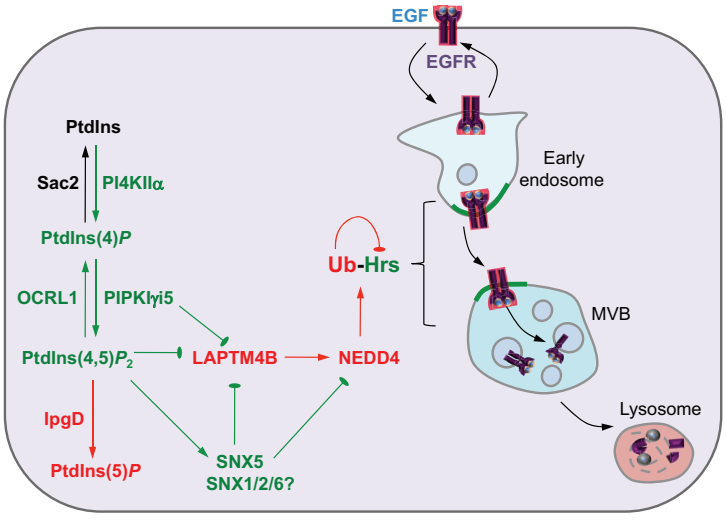

Fig. 2. Endosomal PtdIns(4,5)P_2 signaling regulates EGFR intraluminal sorting, signaling and lysosomal degradation. EGF stimulates the internalization and lysosomal trafficking of EGFR and depends on ESCRT-mediated intraluminal sorting of EGFR at endosomes. Hrs is a key subunit of the ESCRT-0 complex that directly binds to ubiquitylated EGFR and initiates its intraluminal sorting. The function of Hrs in EGFR sorting is inhibited by Nedd4, which ubiquitylates Hrs. PIPKIγi5 is localized to endosomal compartments, where it generates PtdIns(4,5)P_2 to protect Hrs from ubiquitylation through regulation of its effectors SNX5 and LAPTM4B. The red shading around EGFR indicates receptor signaling, and the areas in green on the early endosome and the late endosome containing internal vesicles, i.e. the multivesicular body (MVB), are PtdIns(4,5)P_2 microdomains. Details of the signaling pathways are provided on the left. Molecules and arrows in green promote, whereas red ones inhibit EGFR intraluminal sorting, signaling termination and lysosomal degradation. PtdIns(4,5)P_2 levels must be tightly controlled for correct EGFR sorting because disruption of either OCRL1 or PIPKIγi5 activity blocks EGFR sorting. Similarly, the bacterial PtdIns(4,5)P_2 phosphatase IpgD depletes endosomal PtdIns(4,5)P_2 and blocks EGFR degradation.

note – together with SNX1, SNX2 and SNX6 – SNX5 is also a component of the retromer complex (Wassmer et al., 2009). Each component of this complex has been shown to promote the lysosomal trafficking and degradation of EGFR (Cavet et al., 2008; Chin et al., 2001; Danson et al., 2013; Gullapalli et al., 2004), but this role appears to be independent of their functions within the retromer (Sun et al., 2013a,b); as of yet, it is not clear whether all four SNX proteins function as a complex to regulate EGFR trafficking. SNX5 contains a PX domain and a Bin–amphiphysin–Rvs (BAR) domain, and its PX domain specifically binds PtdIns(4,5)P_2 (Koharudin et al., 2009). The BAR domain of many SNX proteins has an established function in membrane tubulation but it is also important for phosphoinositide binding, as exemplified by the BAR domains within SNX5 and SNX9 (Sun et al., 2013b; Yarar et al., 2008). By generating PtdIns(4,5)P_2, PIPKIγi5 facilitates the interaction between SNX5 and Hrs that is required for protecting Hrs from being ubiquitylated by the E3 ubiquitin ligase Nedd4 (Sun et al., 2013b) (Fig. 2). The ubiquitylation of Hrs induces an intramolecular interaction between the ubiquitin-interacting motif (UIM) of Hrs and the ubiquitin molecule; this in turn, blocks association of Hrs with ubiquitylated receptors, specifically EGFR (Hoeller et al., 2006; Sun et al., 2013b; Tan et al., 2015a). Thus, PIPKIγi5, PtdIns(4,5)P_2 and SNX5 ensure the functioning of Hrs in EGFR intraluminal sorting and lysosomal degradation. Interestingly, this pathway appears to be selective for EGFR because the hepatocyte growth factor receptor Met and the G-protein-coupled receptor protease-activated receptor (PAR1) appear not to be sorted by PIPKIγi5 or SNX5 (Sun et al., 2013b).

LAPTM4B is another endosomal PIPKIγi5-interacting protein that is regulated by PtdIns(4,5)P_2 and involved in controlling EGFR sorting (Tan et al., 2015a). LAPTM4B is a member of the mammalian LAPTM family that comprises LAPTM4A, LAPTM4B and LAPTM5 (Adra et al., 1996; Hogue et al., 2002; Shao et al., 2003). All LAPTM family members are small multi-transmembrane proteins that, when overexpressed, are targeted to the late endosome and/or lysosome (Hogue et al., 2002; Milkereit and Rotin, 2011; Pak et al., 2006; Shao et al., 2003; Vergarajauregui et al., 2011); however, endogenous LAPTM4B is localized to both early and late endosomes (Tan et al., 2015a). LAPTM4B is an oncoprotein that is overexpressed in numerous human cancers of undefined oncogenic mechanisms (Kasper et al., 2005). The E3 ubiquitin protein ligase Nedd4 interacts with all LAPTM members through its WW domain and the C-terminal PY motifs within LAPTM proteins (Milkereit and Rotin, 2011; Pak et al., 2006). Nedd4 has a key role in the regulation of LAPTM5 targeting to lysosomes (Pak et al., 2006) but does not affect lysosomal sorting of LAPTM4A and LAPTM4B (Milkereit and Rotin, 2011).

Recent work has shown that the LAPTM4B–Nedd4 interaction facilitates Nedd4-mediated ubiquitylation of Hrs, which – as noted above – inhibits its ability to interact with ubiquitylated cargo proteins (Tan et al., 2015a) (Fig. 2). Hence, LAPTM4B inhibits EGF-stimulated intraluminal sorting of EGFR and, so, stabilizes endosomal EGFR signaling, which provides a potential mechanism for LAPTM4B-mediated oncogenesis. In agreement with this, a LAPTM4B mutant that is unable to interact with Nedd4 no longer inhibits EGF-stimulated EGFR degradation or promotes EGFR signaling. Interestingly, although both SNX5 and LAPTM4B are PtdIns(4,5)P_2 effectors that are targeted by PIPKIγi5 – in contrast to SNX5 that promotes Hrs-mediated EGFR degradation – LAPTM4B inhibits ESCRT-mediated EGFR degradation and its binding with PtdIns(4,5)P_2 compromises this inhibitory function. There is also crosstalk between SNX5 and LAPTM4B. Ectopic expression of PIPKIγi5 enhances the association of SNX5 with both Hrs and LAPTM4B, but at the same time SNX5 inhibits the association of Hrs with LAPTM4B. This indicates that SNX5 is incorporated into two distinct complexes to regulate Hrs function: in one complex, SNX5 directly interacts with Hrs and, in the other complex, it indirectly suppresses the inhibitory role of LAPTM4B. Therefore, the PIPKIγi5-PtdIns(4,5)P_2-SNX5-LAPTM4B nexus represents an additional layer of control for EGFR endosomal sorting and signaling (Tan et al., 2015a). Importantly, PIPKIγi5 directly binds to the N-terminus of LAPTM4B (Tan et al., 2015a); this sequence is only found in primates but not in lower vertebrates including mice (Shao et al., 2003), indicating that this endosomal PtdIns(4,5)P_2 nexus has evolved more recently in higher vertebrates. Remarkably, the PIPKIγi5–PtdIns(4,5)P_2-SNX5–LAPTM4B nexus appears selective for EGFR because other receptors are not controlled through this pathway (Tan et al., 2015a).

It appears that the endosomal pool of PtdIns(4,5)P_2 must be tightly controlled because deregulation of endosomal PtdIns(4,5)P_2 levels causes dysfunction of the endosomal system (Fig. 2). Loss of the endosomal PtdIns(4,5)P_2 5-phosphatase OCRL1, which is mutated in Lowe oculocerebrorenal syndrome (Attree et al., 1992) and Dent disease (Hoopes et al., 2005), results in the endosomal accumulation of PtdIns(4,5)P_2, which blocks EGFR endosomal sorting and lysosomal degradation (Vicinanza et al., 2011). IpgD, a phosphoinositide phosphatase that is secreted into host cells by the bacterium *Shigella flexneri*, dephosphorylates part of host PtdIns(4,5)P_2 into PtdIns(5)*P*, thereby, causing endosomal accumulation of PI5P that blocks EGFR lysosomal trafficking (Ramel et al., 2011). However, transforming PtdIns(4,5)P_2 into PtdIns(4)*P* does not block lysosomal degradation of EGFR (Ramel et al., 2011). A possible explanation is that PtdIns(4)*P* is still spatiotemporally phosphorylated to PtdIns(4,5)P_2 by PIPKIγi5 at an endosomal surface that is enriched in PtdIns(4,5)P_2 effectors. Consistently, the type IIα phosphatidylinositol 4-kinase (PI4KIIα) that generates PtdIns(4)*P*, the precursor of PtdIns(4,5)P_2, is also found at EGFR-trafficking endosomes and is required for lysosomal sorting and degradation of EGFR (Minogue et al., 2006).

Given the highly regulated and dynamic fashion of PtdIns(4,5)P_2 generation at endosomes to regulate endosomal trafficking events, PtdIns(4,5)P_2, similar to PtdIns(3)*P* (Gillooly et al., 2003), is likely to also form microdomains at the endosomal surface; the dynamic turnover of such PtdIns(4,5)P_2 microdomains, in turn, controls receptor and/or membrane trafficking and endosome maturation. Although present at high levels at the plasma membrane, after endocytosis PtdIns(4,5)P_2 is rapidly dephosphorylated (Nández et al., 2014; Zoncu et al., 2009). Thus, the endosomal PtdIns(4,5)P_2 microdomains should be strictly controlled in the context of endosomal PI4Ks, PIPKs and PtdIns(4,5)P_2 effectors. Of note, phosphoinositides, including PtdIns(4,5)P_2, diffuse rapidly within the membrane (Yaradanakul and Hilgemann, 2007). Multiple diffusion barriers have been proposed to establish localised PtdIns(4,5)P_2 concentrations, such as protein fences, protein and/or lipid clusters, and membrane bends (Hilgemann, 2007). Local PIPKIγi5 and PtdIns(4,5)P_2 effectors, such as SNX5, SNX6 and LAPTM4B, as well as inward budding of the limiting membrane during intraluminal sorting, might all contribute to the establishment of local PtdIns(4,5)P_2 microdomains at endosomal surface. To ensure a dynamic turnover of PtdIns(4,5)P_2 microdomains, endosomal phosphoinositide phosphatases, such as OCRL1 and Sac2, might also be involved (Hsu et al., 2015; Nakatsu et al., 2015; Billcliff and Lowe, 2014). However, if the generation of PtdIns(4,5)P_2 by PIPKs is spatially coordinated with

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PtdIns(4,5)P_2 effectors, then PtdIns(4,5)P_2 could be channeled to the effector without requiring a PtdIns(4,5)P_2 microdomain.

PtdIns(4,5)P_2 might also regulate the trafficking of epithelial cadherin (E-cadherin) (Schill and Anderson, 2009a). All PIPKIγ isoforms directly bind E-cadherin through the conserved kinase domain, and PIPKIγi2 binds and regulates E-cadherin trafficking to and from the plasma membrane (Schill and Anderson, 2009a), which will be discussed below. PIPKIγi5 also regulates lysosomal sorting and degradation of E-cadherin, although this is less well defined (Schill et al., 2014). In this pathway, PIPKIγi5 directly binds and promotes E-cadherin sorting to the late endosome and/or lysosome compartments, but SNX5 appears to inhibit E-cadherin degradation by an unknown mechanism (Schill et al., 2014). The ESCRT complex is also known to regulate the lysosomal sorting of ubiquitylated E-cadherin (Palacios et al., 2005), but how it collaborates with PIPKIγi5-mediated PtdIns(4,5)P_2 signaling in E-cadherin sorting needs further investigation. The roles for PIPKIγi1 and PIPKIγi4 in E-cadherin regulation, if any, have not yet been defined.

PtdIns(4,5)P_2 in the regulation of endosomal recycling

The role of PtdIns(4,5)P_2 in the regulation of clathrin-mediated endocytosis is established (Martin, 2001), but accumulating evidence has also demonstrated an involvement of PtdIns(4,5)P_2 in endosomal recycling pathways that affect cell polarization, spreading and motility (Fig. 3). For instance, a decrease in PtdIns(4,5)P_2 levels impairs both endocytosis and endosomal recycling of internalized transferrin back to the plasma membrane (Kim et al., 2006), suggesting a general role for PtdIns(4,5)P_2 in multiple membrane trafficking events. In addition, a number of PtdIns(4,5)P_2-binding proteins have been implicated in endosomal recycling, including adaptor protein (AP) complex, exocyst subunits, RME-1/EHD-1, SDPN-1/syndapin/pacsin, and syntenin, among others (He et al., 2007; Ling et al., 2007; Liu et al., 2007; Zhang et al., 2008). Moreover, several small GTPases, including ARF6, activate PIPKI at different subcellular localizations (Funakoshi et al., 2011; Honda et al., 1999). ARF6 not only regulates endocytosis at the plasma membrane but also mediates post-endocytic recycling of multiple receptors, such as transferrin receptor, integrin, EGFR and, potentially, others, from endosomes (Allaire et al., 2013; Schweitzer et al., 2011). ARF6 also regulates the recycling of syndecan from endosomes to the plasma membrane, which depends on the interaction between syndecan and its adaptor protein syntenin, the latter of which has a PDZ domain that is specific for PtdIns(4,5)P_2 binding (Zimmermann et al., 2005). In this context, it is noteworthy that the syntenin–PtdIns(4,5)P_2 interaction is crucial for ARF6-mediated recycling of syndecan (Lambaerts et al., 2012; Zimmermann et al., 2005).

ARF6 can directly interact with the exocyst complex to promote endosomal recycling (Prigent et al., 2003) and also directly activates PIPKIγ isoforms (Krauss et al., 2003). Consistently, roles for PIPKIγi2-mediated PtdIns(4,5)P_2 signaling in exocyst-modulated polarized trafficking of integrins and E-cadherin from endosomes to the plasma membrane have been described (Ling et al., 2007; Thapa et al., 2012; Xiong et al., 2012). It has been shown that PIPKIγi2 directly binds to E-cadherin and controls its targeting to the plasma membrane to form adherens junctions (AJs) (Ling et al., 2007). Of note, a mutant form of E-cadherin that has been identified in hereditary diffuse gastric cancer shows diminished PIPKIγi2 binding, which results in a strong accumulation of the E-cadherin mutant inside the cells (Ling et al., 2007), underscoring a physiologically relevant role for PIPKIγi2 and PtdIns(4,5)P_2 signaling in the sorting of

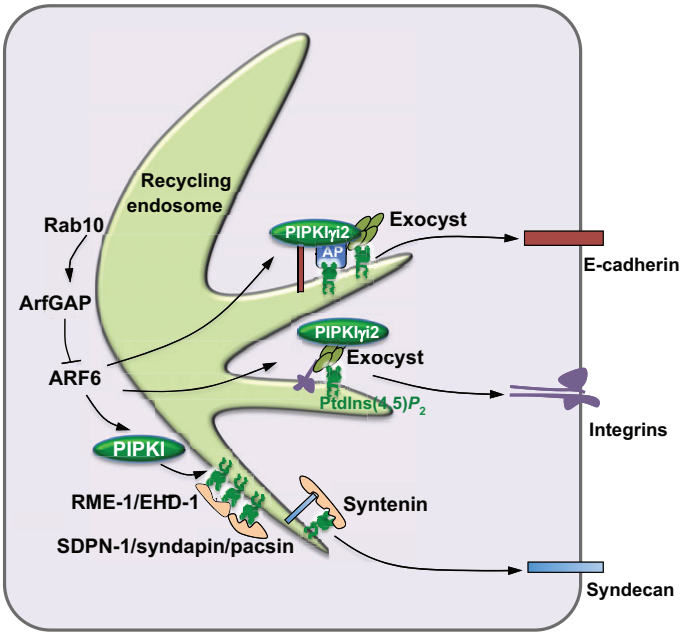

Fig. 3. PtdIns(4,5)P_2 signaling controls endosomal recycling pathways. Small GTPases, such as ARF6 and Rab10, regulate PIPKI activation and PtdIns(4,5)P_2 generation at recycling compartments. The localized PtdIns(4,5)P_2 signals mediate the endosomal recycling of multiple proteins to the plasma membrane, as exemplified by syndecan, integrins and E-cadherin. RME-1/EHD-1 and SDPN-1/syndapin/pacsin are two conserved PtdIns(4,5)P_2 effectors that mediate membrane bending at recycling endosomes, which is essential for the recycling of multiple cargos including transferrin receptor. PIPKIγi2 directly binds to the exocyst and AP complex and, so, promotes cargo association with both PtdIns(4,5)P_2-effector complexes, which mediates vesicle budding from recycling endosomes and tethering of recycling vesicles to the plasma membrane.

E-cadherin from endosomal compartments to the plasma membrane. Appropriate E-cadherin recycling also depends on a direct interaction between PIPKIγi2 and the endosomal AP complex AP1B, which recruits clathrin for the budding of endosomal recycling vesicles (Ling et al., 2007). This interaction is mediated by a YSPL motif within the unique C-tail of PIPKIγi2 that is specifically recognized by the μ1B subunit of the AP1B complex (Bairstow et al., 2006; Bonifacino and Traub, 2003; Sugimoto et al., 2002). As such, PIPKIγi2 mediates E-cadherin recycling by functioning not only as a PtdIns(4,5)P_2 generating enzyme that controls the assembly of the AP1B complex but also by acting as a scaffold protein for promoting the interaction between AP1B and E-cadherin, thereby defining the specificity of this particular recycling pathway (Fig. 3).

Besides AP1B, the exocyst complex is another essential effector of PIPKIγi2 and mediates endosomal recycling of multiple proteins (Thapa et al., 2012; Xiong et al., 2012). As is the case for AP1B, PIPKIγi2 also integrates the exocyst complex into the E-cadherin trafficking pathway and facilitates the endosome-to-plasma membrane sorting of E-cadherin for AJ assembly (Thapa et al., 2012; Xiong et al., 2012). Two exocyst subunits, Sec3 and Exo70, directly bind PtdIns(4,5)P_2, and this is crucial for their roles in polarized vesicle trafficking/tethering and endosomal recycling (He et al., 2007; Liu et al., 2007; Zhang et al., 2008). By associating with Sec6, Exo70 and integrins, PIPKIγi2 links the exocyst to the endosomal compartment that participates in integrin recycling (Thapa et al., 2012). PIPKIγi2 also interacts with talin, a focal adhesion molecule and PtdIns(4,5)P_2 effector and, so, coordinates the roles of

the exocyst and talin in the polarized trafficking of integrins to the leading edge of directionally migrating cells (Thapa et al., 2012). This appears to be crucial for highly migrating and invading tumor cells.

Rab10, another GTPase that is important for endosomal recycling, appears to modulate endosomal PtdIns(4,5)P_2 levels by acting as a negative regulator upstream of ARF6, thereby adding an additional layer of control for endosomal PtdIns(4,5)P_2 signaling (Shi et al., 2012). Downstream of ARF6 and PIPKI-mediated PtdIns(4,5)P_2 generation, RME-1/EHD-1 and SDPN-1/syndapin/pacsin are two further endosomal PtdIns(4,5)P_2 effectors that are involved in endocytic recycling (Shi and Grant, 2014; Shi et al., 2012, 2007).

PtdIns(4,5)P_2 in the regulation of autophagic membrane trafficking

Macroautophagy (referred to as autophagy from here on) is a highly conserved intracellular self-digestion process that involves the formation of double-membrane compartments called autophagosomes that engulf and degrade cytoplasmic contents by subsequent fusion with the lysosome. It is generally accepted that nutrient starvation induces the initiation of autophagosome membranes at the ER, but the membrane has been shown to come from multiple sources, including the plasma membrane, early and recycling endosomes, ER, mitochondria, Golgi, and ER-Golgi intermediate compartments (ERGIC) (Chan and Tang, 2013; Lamb et al., 2013). A number of autophagy-related (ATG) proteins function collaboratively to coordinate membrane recruitment to phagophore initiation sites at the ER, and contribute to phagophore initiation and autophagosome formation. The VPS34 complex is thought to generate PtdIns(3)*P* at the ER, where it recruits the effectors double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting 2 (WIPI2) to control phagophore initiation (Itakura et al., 2008; Itakura and Mizushima, 2010; Matsunaga et al., 2009; Polson et al., 2010; Sun et al., 2008; Zhong et al., 2009). However, other phosphoinositide species are likely to be also involved in the regulation of phagophore initiation, as exemplified by a recent report that PtdIns(5)*P* stimulates autophagy that is induced by glucose starvation (Vicinanza et al., 2015).

The roles for PtdIns(4,5)P_2 in the trafficking of autophagic membrane precursors have been recently established (Fig. 4). At the plasma membrane, PtdIns(4,5)P_2 controls clathrin-mediated endocytosis and, thus, plays a role in trafficking steps from the plasma membrane that are thought to provide source of membrane for phagophore precursors (Puri et al., 2013). In support of this concept, ARF6, which activates PIPKI at the plasma membrane for PtdIns(4,5)P_2 generation, is required for autophagy initiation (Moreau et al., 2012). By using the PLCδ-PH domain as a probe, PtdIns(4,5)P_2 has been found at early autophagic vesicles that are partially positive for the endogenous phagophore proteins ATG16L1, ATG12 and ATG5 (Moreau et al., 2012), supporting a role for PtdIns(4,5)P_2 in regulating phagophore precursor formation and, potentially, phagophore nucleation. Consistently, the Rho GTPase-activating protein 26 (ARHGAP26, also known as GRAF1), which is a marker for a clathrin-independent endocytic pathway and a PtdIns(4,5)P_2-binding protein, colocalizes with ATG16L1 and is required for autophagy initiation (Lundmark et al., 2008; Moreau et al., 2012). An additional PtdIns(4,5)P_2 effector that regulates phagophore precursor trafficking is SNX18 (Knævelsrud et al., 2013a), a PX-BAR protein belonging to the SNX9 family, which also includes SNX33 (van Weering et al., 2010). SNX18 was identified as a positive regulator of autophagy in a screen by using small interfering (si)RNAs that targeted PX-domain-containing proteins (Knævelsrud et al., 2013b). Interestingly, although SNX18 has a high sequence similarity with SNX9 and SNX33, and the three proteins have some redundant functions (Ma and Chircop, 2012; Park et al., 2010), the role of SNX18 in autophagic membrane trafficking appears to be specific and is probably owing to the interaction of SNX18 with ATG16L1 and LC3 (Knævelsrud et al., 2013b), a well-established marker for all (pre-)autophagosome membranes (Kabeya et al., 2000). It has been shown that SNX18 binds to PtdIns(4,5)P_2-containing recycling endosomal membranes and that its function in membrane tubulation is important for SNX18-mediated delivery of vesicles containing ATG16L1 and LC3 to phagophore precursors (Knævelsrud et al., 2013b). Thus, PtdIns(4,5)P_2 not only regulates clathrin-dependent endocytosis but also controls post-endocytic trafficking of phagophore precursors. However, it is still not clear which PIPK isoform regulates the generation of PtdIns(4,5)P_2 in this process. The origin of PtdIns(4,5)P_2, i.e. whether the pool of PtdIns(4,5)P_2 on phagophore precursor vesicles comes from the plasma membrane or is dynamically generated by a phagophore-precursor-targeted PIPK, also needs to be addressed in future studies.

PtdIns(4,5)P_2 also appears to have a role in phagophore nucleation at the ER. It has been reported that PtdIns(4)*P* and PtdIns(4,5)P_2 can be generated at the ER where PtdIns are synthesized (Helms et al., 1991). In addition, phosphorylation of PIPKIIγ is regulated at the ER (Itoh et al., 1998), suggesting that its activity at the ER surface is also regulated. VPS34 interacts with either ATG14 or UVRAG and both complexes are required for the initiation of autophagy (Itakura et al., 2008; Matsunaga et al., 2009; Sun et al., 2008; Zhong et al., 2009). In fact, ATG14 and UVRAG both bind PtdIns(3)*P* and PtdIns(4,5)P_2 (Fan et al., 2011; He et al., 2013); however, a role for PtdIns(4,5)P_2 in the regulation of VPS34-containing complexes and in phagophore initiation remains to be defined. It has been reported that PtdIns(4,5)P_2 is lost at the mitochondrion outer membrane upon serum starvation (Rosivatz and Woscholski, 2011). Because nutrient-starvation-induced phagophore initiation occurs at ER–mitochondrion contact sites (Hamasaki et al., 2013), it is possible that a PIPK and/or PtdIns(4,5)P_2 itself translocate from mitochondria to the ER to promote starvation-induced initiation of autophagy. Interestingly, phospholipase D 1 (PLD1), which generates phosphatidic acid to activate PIPKI-mediated production of PtdIns(4,5)P_2, has been shown to be required for autophagy initiation (Dall'Armi et al., 2010; Jenkins and Frohman, 2005). PLD1 is localized to various subcellular membrane structures, including endosomes, Golgi, plasma membrane and potentially the ER (Jenkins and Frohman, 2005). Thus, a possible PLD1–PIPKI–PtdIns(4,5)P_2 signaling axis is positioned at or in close proximity of the ER to regulate VPS34 complexes and nucleation of phagophore membranes (Fig. 4).

There is also emerging evidence that PtdIns(4,5)P_2 regulates the fusion of the autophagosome with endolysosomes, although it remains to be determined whether PtdIns(4,5)P_2 is also generated at autophagosomes. At least its precursor PtdIns(4)*P* has recently been found at autophagosomes and shown to have a crucial role in the fusion step. Upon autophagy stimulation, the γ-aminobutyric acid (GABA) receptor-associated proteins (GABARAPs) have been shown to recruit PI4KIIα to autophagosomes where it generates PtdIns(4)*P* to promote the fusion of autophagosomes with endolysosomes (Wang et al., 2015). Interestingly, delivery of exogenous PtdIns(4)*P* – but not PtdIns(4,5)P_2 – into PI4KIIα-knockdown cells rescued autophagosome–endolysosome fusion,

Journal of Cell Science (2015) 128, 4047-4056 doi:10.1242/jcs.175208

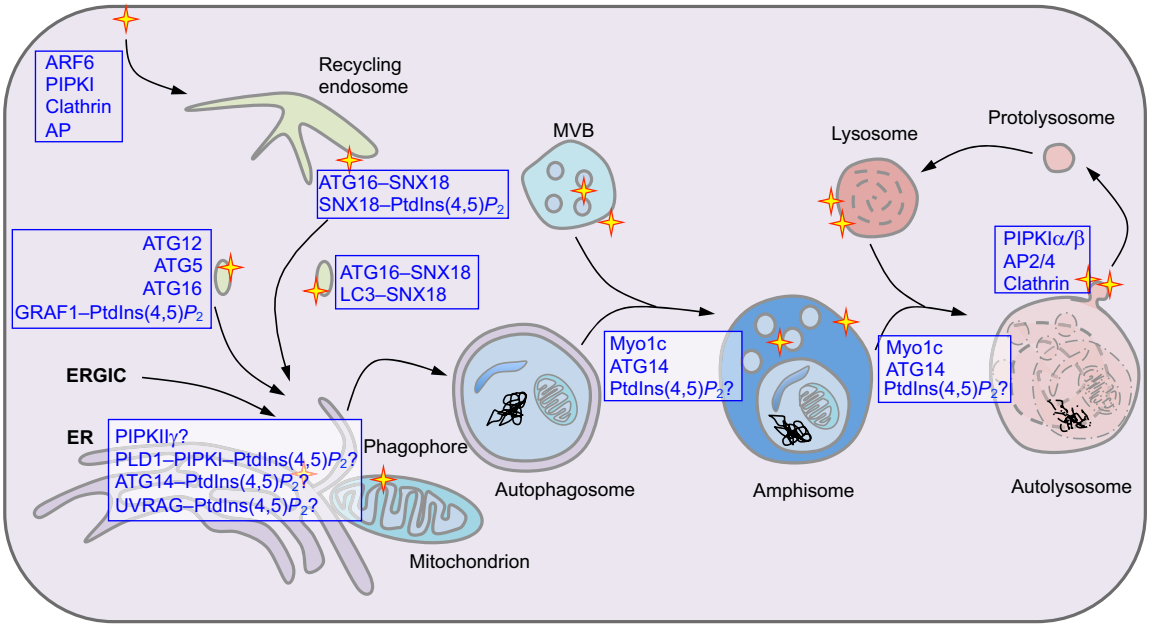

Fig. 4. PtdIns(4,5)P_2 regulates autophagic membrane trafficking. Although starvation-induced autophagy is initiated at the ER, the autophagosome membrane comes from multiple sources including the plasma membrane, early and recycling endosomes, Golgi complex, ERGIC, mitochondria and, possibly, other compartments. The roles of PtdIns(4,5)P_2 signaling in endocytosis at the plasma membrane – which provides a source of autophagosome membranes through early and recycling endosomes – and in autophagic lysosomal reformation (ALR) – which regenerates new functional lysosomes – have been recently dissected in detail. Substantial evidence also suggests roles for PtdIns(4,5)P_2 in autophagic membrane precursor trafficking and, potentially, phagophore nucleation, and autophagosome fusion with endolysosomes. The relevant factors that are involved in PtdIns(4,5)P_2-regulated autophagic membrane trafficking have been listed at each trafficking step. Multivesicular body (MVB). ER-Golgi intermediate compartments (ERGIC).

indicating that PtdIns(4,5)P_2 alone is not sufficient for fusion and, moreover, that PtdIns(4)P does not simply act as a PtdIns(4,5)P_2 precursor (Wang et al., 2015). However, this finding does not rule out the possibility that PtdIns(4,5)P_2 is also required for fusion, because exogenous PtdIns(4)P can be converted into PtdIns(4,5)P_2 in the cell. Therefore, both PtdIns(4)P and PtdIns(4,5)P_2 are likely to have key roles in the autophagosome–endolysosome fusion step. In support of this notion, another recent study reported that Myo1c, a myosin that specifically binds PtdIns(4,5)P_2 (Hokanson and Ostap, 2006), is required for the fusion of autophagosomes with lysosomes (Brandstaetter et al., 2015), suggesting that PtdIns(4,5)P_2, indeed, has a role in this step. In addition, ATG14 has been recently shown to promote autophagosome fusion with endolysosomes through interaction with the syntaxin 17 (STX17) soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex (Diao et al., 2015), although it remains to be investigated whether the binding of PtdIns(4,5)P_2 to ATG14 is involved in this process. Future studies are, thus, needed to identify the specific PIPKs and any additional PtdIns(4,5)P_2 effectors that are involved in this step of autophagy. Of note, recent studies have shown a role for the endosomally localized LAPTM4B, a PIPKIγi5-interacting protein, in both autophagy initiation and maturation (Li et al., 2011; Tan et al., 2015b). Given the dynamic nature of ER–endosome contacts (Friedman et al., 2013), it would be interesting to investigate whether the PIPKIγi5-mediated PtdIns(4,5)P_2 signaling is involved in autophagy regulation by regulating these interactions.

At the end of the autophagy pathway, after the autolysosomal degradation of autophagy substrates, PtdIns(4,5)P_2 is known to regulate autophagic lysosomal reformation (ALR), which involves membrane budding and reformation of new lysosomes from autolysosomes (Rong et al., 2012; Yu et al., 2010). Here, PIPKIβ initiates lysosomal reformation by generating PtdIns(4,5)P_2 at the autolysosomal surface, and induces membrane budding and tubule formation by recruiting the adapter protein complexes AP2 and AP4, as well as clathrin. PIPKIα is then targeted to the reforming tubules and promotes membrane fission to generate proto-lysosomes (Rong et al., 2012). Consistently, PI4KIIIβ, which generates PtdIns(4)P, has also been shown to regulate lysosomal membrane tubulation (Sridhar et al., 2013). Future studies that explore the roles of any additional PtdIns(4,5)P_2 effectors are required in order to elucidate autophagic lysosomal reformation and, thus, further expand our understanding of this autolysosomal pathway and the exact roles of PtdIns(4,5)P_2 therein.

Conclusions and future prospects

At first glance, it might be difficult to conceive why PtdIns(4,5)P_2 signaling would be functionally important at intracellular compartments where other phosphoinositide species are known to be abundant. Yet, when reconsidering that the generation of PtdIns(4,5)P_2 is coupled with the activity of PtdIns(4,5)P_2 effectors at specific locations in order to regulate their cellular functions, it becomes clear that PtdIns(4,5)P_2 provides an additional, unique layer of signaling specificity at intracellular compartments – for example at endosomes, despite the fact that the endosomal marker phosphoinositide is PtdIns(3)P. It is also reasonable to assume that not all endosomal events are only mediated by PtdIns(3)P. Similarly, at the plasma membrane, where PtdIns(4,5)P_2 has been shown to have numerous defined functions, PtdIns(4)P fulfills roles that are independent of PtdIns(4,5)P_2 (Hammond et al., 2012).

We have summarized here the recent evidence for intracellular PtdIns(4,5)P_2 signaling, and discussed our current understanding of how PtdIns(4,5)P_2 regulates endolysosomal sorting, recycling and autophagy. The current evidence supports the presence of specific intracellular PIPK isoforms at distinct intracellular compartments,

which is controlled by unique sequences within each PIPK and their targeting proteins. PIPKs, PtdIns(4,5)P_2 phosphatases and PtdIns(4,5)P_2 effectors together define the PtdIns(4,5)P_2 signaling nexus at a particular location within the cell. However, many basic questions remain in these areas. Where does the PtdIns(4,5)P_2 come from at each intracellular compartment? For example, can PtdIns(4,5)P_2 be directly transferred from one compartment to another at organelle contact sites, or are there specific PIPKs that are responsible for PtdIns(4,5)P_2 generation in each compartment? Which are the PtdIns(4,5)P_2 effector proteins and how does PtdIns(4,5)P_2 modulate their functions? One major challenge for the study of intracellular PtdIns(4,5)P_2 is to develop approaches that can effectively detect the intracellular pools of PtdIns(4,5)P_2 and identify specific PIPKs that are responsible for PtdIns(4,5)P_2 generation at different intracellular compartments. Because overexpressed PIPKs are extensively targeted to the plasma membrane in a nonspecific manner, new approaches, in which expression of PIPKs can be induced at low levels or endogenous PIPKs are tagged, need to be developed. For example, by making use of the clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas9 genome-editing technology, we, theoretically, are able to tag any PIPKs. With the identification of further intracellular PIPKs and their associating factors, which – typically – are PtdIns(4,5)P_2 effectors, it is anticipated that we will gain a much better understanding of intracellular PtdIns(4,5)P_2 signaling, and their relevance to cellular physiology and pathologies.

Acknowledgements
We apologize to those whose work could not be cited due to space considerations. We thank all the other Anderson lab members for their scientific discussions and comments on the manuscript prior to submission.

Competing interests
The authors declare no competing or financial interests.

Funding
This work is supported by National Institutes of Health grants [grant numbers: CA104708 and GM057549] to R.A.A. and a Howard Hughes Medical Institute International Student Research Fellowship [grant number 59107631] to X.T. Deposited in PMC for release after 12 months.

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