PI(5)P Regulates Autophagosome Biogenesis

Highlights

- PI(5)P positively regulates autophagy
- PI(5)P is associated with autophagy effectors that bind PI(3)P
- PI(5)P sustains noncanonical autophagy in PI(3)P-depleted cells
- PI(5)P is essential for VPS34-independent, glucose-starvation-induced autophagy

In Brief

PI(3)P, the product of VPS34, regulates canonical autophagy; however, mammalian cells can produce autophagosomes through enigmatic noncanonical VPS34-independent pathways. Vicinanza et al. show that PI(5)P can regulate autophagy, even in cells where VPS34 is compromised and acts via PI(3)P effectors. This provides a mechanistic explanation for forms of noncanonical autophagy.
PI(5)P Regulates Autophagosome Biogenesis

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SUMMARY

Phosphatidylinositol 3-phosphate (PI(3)P), the product of class III PI3K VPS34, recruits specific autophagic effectors, like WIPI2, during the initial steps of autophagosome biogenesis and thereby regulates canonical autophagy. However, mammalian cells can produce autophagosomes through enigmatic noncanonical VPS34-independent pathways. Here we show that PI(5)P can regulate autophagy via PI(3)P effectors and thereby identify a mechanistic explanation for forms of noncanonical autophagy. PI(5)P synthesis by the phosphatidylinositol 5-kinase PIKfyve was required for autophagosome biogenesis, and it increased levels of PI(5)P, stimulated autophagy, and reduced the levels of autophagic substrates. Inactivation of VPS34 impaired recruitment of WIPI2 and DFCP1 to autophagic precursors, reduced ATG5-ATG12 conjugation, and compromised autophagosome formation. However, these phenotypes were rescued by PI(5)P in VPS34-inactivated cells. These findings provide a mechanistic framework for alternative VPS34-independent autophagy-initiating pathways, like glucose starvation, and unravel a cytoplasmic function for PI(5)P, which previously has been linked predominantly to nuclear roles.

INTRODUCTION

Macroautophagy (henceforth autophagy) is a cellular process that delivers damaged organelles, invasive bacteria, and long-lived or aggregate-prone proteins to lysosomes for degradation (Boya et al., 2013). These substrates are engulfed along with bulk cytoplasm by double-membraned, cup-shaped phagophores, which form autophagosomes after their edges extend and fuse. After autophagosome-lysosome fusion, the resulting degradation products are recycled back to the cytosol and are reused to enhance cell survival during nutrient deprivation. Autophagy impacts the pathogenesis of diverse diseases, including neurodegenerative conditions, cancers, and infectious diseases (Nixon, 2013).

Many proteins (ATGs) regulate the membrane remodelling and trafficking events in autophagy, but comparatively little is known about the roles of lipids and their metabolizing enzymes in this process (Nakatogawa et al., 2009). Phosphoinositides (PIs) are low-abundance lipids that are interconverted by highly regulated sets of PI kinases and phosphatases. The interconvertibility of PIs enables rapid local changes in the identity of intracellular compartments to dynamically recruit effector proteins to specific membranes at the right time. The class III phosphatidylinositol 3-kinase (also known as VPS34) and its product phosphatidylinositol 3-phosphate (PI(3)P) are critical for autophagosome formation (Boya et al., 2013; Nakatogawa et al., 2009). Local increases of PI(3)P recruits proteins associated with autophagy initiation, such as DFCP1 (double FYVE-containing protein 1) and the WIPI proteins (WD-repeat protein interacting with PI), of which WIPI2 has been characterized as an effector of autophagy (Poison et al., 2010).

While autophagy has been classically considered to be PI(3)P-dependent, noncanonical VPS34-independent autophagy has been reported (Codogno et al., 2012; Plowey et al., 2008; Scarlatti et al., 2008; Zhu et al., 2007), as autophagosomes are seen in T lymphocytes and sensory neurons from Vps34−/− mice (Zhou et al., 2010) and in glucose-starved cells treated with the VPS34 inhibitor Wortmannin (Wm) (McAlpine et al., 2013). However, it is unclear whether these phenomena may be explicable by VPS34-independent source(s) of PI(3)P (Devereaux et al., 2013) or by other PIs.

PI(5)P remains the most enigmatic of the PIs due to its low abundance (Shisheva, 2013). The pathways regulated by PI(5)P in mammalian cells and its effectors/binding proteins are still poorly understood. So far these include chromatin organization in the nuclei, bacteria invasion, and cytoskeletal remodelling (Shisheva, 2013). Here, we describe a role for PI(5)P as a regulator of autophagosome biogenesis that can also regulate autophagy when VPS34 is inhibited.

RESULTS

PI(5)P Regulates Autophagosome Biogenesis

Autophagosome numbers can be measured by assessing phosphatidylethanolamine-conjugated ATG8/LC3 (LC3-II) levels...
**Figure 9.**

**A.** Western blot analysis of LC3 II and Tubulin levels in cells treated with DMSO or BAF in the presence of 1 or 10 μM of PIP5P.

**B.** Quantification of LC3 II and Tubulin levels from panel A.

**C.** Representative images of GFP-LC3 expression in cells treated with 1 or 10 μM of PIP5P in Basal and HBSS conditions.

**D.** Bar graph showing the number of GFP-LC3 spots per cell for Basal and HBSS conditions with 1 or 10 μM of PIP5P.

**E.** Bar graph showing the number of AVs and ALs per cell for cells treated with 1 or 10 μM of PIP5P.

**F.** Western blot analysis of ATG5 and ATG12 levels in cells treated with 1 or 10 μM of PIP5P.

**G.** Quantification of ATG5/ATG12 ratio from panel F.

**H.** Representative images of GFP-PHD3X and RFP-LC3 expression in cells treated with PIP5P in Basal condition.

**I.** Representative images of GFP-PHD3X and RFP-LC3 expression in cells treated with PIP5P in HBSS condition.

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versus a loading control (e.g., tubulin), or by scoring the number of LC3-positive vesicles, since LC3-II is specifically targeted to autophagosomal membranes (Kabeya et al., 2000). In the same way that PI(3)P alone is sufficient to stimulate autophagy in mammalian cells (Petiot et al., 2000), we found that addition of exogenous PI(5)P di-C16 significantly increased LC3-II levels and LC3-positive autophagic vesicle numbers (Figures 1A–1D). While LC3-II levels correlate with autophagosome numbers, these can increase due to enhanced formation or decreased degradation (Rubinstein et al., 2009). Consistent with a role in autophagosome synthesis, PI(5)P increased LC3-II levels, in an apparently dose-dependent manner, when we blocked LC3-II cleavage by inhibiting autophagosome-lysosome fusion using Bafilomycin A1 (BAF) (Figures 1A and 1B and Figures S1A and S1B available online), and resulted in increased numbers of GFP-LC3 puncta (autophagosomes) in both nutrient-replete media and Hank’s balanced salt solution (HBSS) (amino-acid/serum starvation and 1 g/D-glucose, compared to 4.5 g/l in Dulbecco’s modified Eagle’s medium [DMEM]) (Figures 1C and 1D). The effects of PI(3)P (a positive control) were stronger at 0.1 μM compared to PI(5)P, while the effects of PI(5)P were more noticeable at 1 and 10 μM (Figures S1A and S1B). PI and PI(4)P did not obviously stimulate autophagy, and PI(4)P loading of cells treated with BAF suggested possible inhibition of autophagy (Figure S1C). PI(5)P loading in cells stably expressing mRFP-GFP tandem fluorescent-tagged LC3 (see Supplemental Experimental Procedures) increased the numbers of autophagosomes and autolysosomes (Figures 1E and S1D). Furthermore, addition of PI(5)P increased the conjugation of ATG5 with ATG12 (Figures 1F and 1G), a critical event in phagophore biogenesis that appears to be regulated by PI(3)P (Ravikumar et al., 2008).

Exogenous PI(5)P was delivered to autophagic structures, as we observed fluorescent BODIPY-labeled PI(5)P on RFP-LC3 puncta (Figure S1E). BODIPY-labeled PI(5)P loading of living cells increased the number of RFP-LC3 dots dose dependently (Figure S1E). One difficulty in studying the intracellular localization of endogenous PI(5)P is the uncertain specificity of PI(5)P biosomes. While the plant homedomain (PHD) of ING2 (Bua et al., 2013; Gozani et al., 2003) and the pleckstrin homology (PH) domain of Dok proteins (Guittaud et al., 2009, 2010) show strong preference for PI(5)P and have been used for intracellular localization and manipulation of PI(5)P (Viaud et al., 2014a), we cannot exclude that they do not bind other lipids to some extent. With this caveat in mind, we detected GFP-tagged PHD3X (three tandem repeats of PHD of ING2) (Bua et al., 2013; Gozani et al., 2003; Guittaud et al., 2010) localization at the nucleus and plasma membrane (as previously described) and on discrete puncta, 25% of which were RFP-LC3 positive (Figure 1H). When cells were starved, GFP-PHD3X-positive structures increased from 4–6 to >12 spots per cell, 50% of which colocalized with RFP-LC3 (Figure 1H). A PHD mutant defective in PI(5)P binding (PHD3X Znmut) (Bua et al., 2013) failed to localize on discrete structures (Figure 1H). Superresolution structured illumination microscopy (SR-SIM) confirmed the confocal localization of PHD3X on LC3-positive vesicles/autophagosomes (Figure 1I; Movie S1).

Oversexpression of GFP-PHD3X, which sequesters intracellular PI(5)P, dramatically decreased the percentage of cells with more than 10 LC3 dots (both endogenous LC3 and RFP-tagged LC3 were assessed), compared to GFP-empty vector or GFP-PHD3X Znmut (Figures S1F–S1I), an effect mimicked by overexpression of GFP-PH-Dok-5, an alternative PI(5)P binding module (Guittaud et al., 2010; Figures S1F and S1G). As we could not exclude that some of the effects of these probes may be due to binding to other lipids, we next manipulated intracellular PI(5)P levels by targeting enzymes relevant for its biogenesis and turnover.

PI(5)P Synthesized by PIKfyve Regulates Autophagosome Formation

Type III PtdInsP 5-kinase PIKfyve appears to regulate PI(5)P biosynthesis (Figure 2A; Sbrissa et al., 1999), as reduced PI(5)P levels are seen in PIKfyve hypomorph and heterozygous mice and in cells silenced by small interfering RNA (siRNA), overexpressing a dominant-negative mutant or treated with pharmacological inhibitor of the kinase (Ikonomov et al., 2011; Sbrissa et al., 2002, 2012; Zolov et al., 2012). Low doses (100 nM) of the PIKfyve inhibitor YM-201636 (Sbrissa et al., 2012) decreased LC3-II levels (Figures 2B, 2C, and S2A), the number of ATG16L1

![Figure 1. PI(5)P Regulates Autophagosome Biogenesis](image-url)
(autophagosome precursor) and WIPI-2 vesicles (Figures 2D and 2E), and ATG5–ATG12 conjugation (Figure 2F).

Autophagosomes may contain PI(5)P from early stages of their biogenesis, since GFP-PHDX was associated with mStrawberry-ATG16L1 structures (which label phagophores or prephagophore structures) in both nutrient-replete and starved conditions (50% and 70% colocalization, respectively) (Figure S2B). A 3D analysis of PHD3X-labeled structures assessed by super-resolution structured illumination microscopy (SR-SIM) revealed PI(5)P on ATG16L1-positive vesicles in autophagy-stimulating conditions (amino-acid starvation, HBSS media) (Figure 2G; Movie S2). Low doses of YM-201636 (100 nM) or PIKfyve silencing selectively depleted GFP-PHDX on ATG16L1 vesicles, consistent with a specific reduction of PI(5)P on autophagosome precursors (Figures S2B and S2C). To further validate the idea that PI(5)P is present on early autophagic membranes, we expressed the GFP-PHDX probe, in cells where phagophores accumulated and autophagosome completion was impaired by the overexpression of proteolytic-activity-deficient mutant of ATG4B (ATG4BC74A), that prevents LC3 lipidation and inhibits autophagosome formation (Fujita et al., 2008). ATG4BC74A increased the numbers of ATG16L1 structures labeled with GFP-PHDX, in both basal and starvation conditions (Figure S2D), with some forming large ring-shaped structures.

PIKfyve is also the primary kinase responsible for production of PI(3,5)P2, a key regulator of early-to-late endosome membrane trafficking (Shisheva, 2013; Figure 2A). Note that higher concentrations and longer treatment with YM-201636, which inhibits PI(3)P conversion to PI(3,5)P2, causing elevation of PI(3)P (de Lartigue et al., 2009; Zolov et al., 2012), do not reduce autophagosome numbers (Figures 2B, 2C, and 2A), possibly due to defects in the endosomal/lysosomal compartment that block autophagosome degradation (Figures 2A, 2A, and 2A). Consistent with this, a similar block in autophagosome degradation is seen with PIKfyve siRNA knockdown (Figure S2F). To parse out the effects of impaired autophagosome formation and degradation, we treated cells with BAF for 2 hr in the presence of HBSS (an autophagy stimulus), and we found less autophagosome formation in the PIKfyve knockdown cells (Figure S2G), consistent with reduced ATG5–ATG12 conjugation after PIKfyve knockdown (as seen with short-term low-dose YM-201636 treatment) (Figure S2H). Importantly, the addition of exogenous PI(5)P di-C16 to cells treated with low concentrations of YM-201636 (Figures 2H and 2I) or treated with PIKfyve siRNA (Figures S2G and S2H) significantly rescued LC3-II levels and ATG5–ATG12 conjugation, arguing that PI(5)P is the relevant signaling molecule in PIKfyve-dependent autophagosome biogenesis.

Several in vivo studies suggest that PIKfyve may indirectly control PI(5)P levels by producing PI(3,5)P2, which is then transformed into PI(5)P by 3-phosphatases of the myotubulinar family (Figure 2A; Oppelt et al., 2013, 2014; Viaud et al., 2014b; Zolov et al., 2012). If this is the case, then the production of PI(5)P is likely localized to internal membranes where PI(3,5) P2 is located. We investigated the localization of PI(3,5)P2 using the tandem repeats of the N-terminal domain of mucolipin1 (ML1N*2), recently described as a reliable and specific probe for PI(3,5)P2 (Li et al., 2013). GFP-ML1N*2 associated with early autophagosomal structures labeled with mStrawberry-ATG16L1 under nutrient-starvation conditions (80% of ATG16 structures contain GFP-ML1N*2 during starvation versus 20% in complete media), and this association was sensitive to YM-201636 treatment (Figure S2I). Consistent with PI(3,5)P2 being a precursor of PI(5)P, we found that overexpression of myotubulinar-related phosphatase 3 (MTMR3), one of the myotubulinar PI 3-phosphatase enzymes previously implicated in PI(3,5)P2 production along with PIKfyve (Oppelt et al., 2013, 2014; Viaud et al., 2014b; Zolov et al., 2012), increased numbers of GFP-PHDX structures, while its knockdown had the reverse effect (Figure S2J) during nutrient starvation (HBSS). Various myotubulinar family members have been linked to autophagy, mainly in the regulation of PI3P metabolism (Taguchi-Atarashi et al., 2010; Vergne et al., 2009; Zou et al., 2012). Thus, myotubulinars other than MTMR3 may also regulate PI(5)P levels during autophagy induction, like PI(3)P. PI(3,5)P2 localization during starvation resembles what we found for PI(5)P, and raises the possibility that PI(3,5)P2 may contribute as a precursor for the PI(5)P pool required for autophagy (Figure 2A). PI(5)P and its precursor PI(3,5)P2 localize on autophagosome precursors, and inhibition of their synthesis results in autophagy inhibition. Our data provide clear evidence that PI(5)P synthesis occurs during early stages of autophagosome formation and is modulated by nutrient state.

Kinases that Convert PI(5)P to PI(4,5)P2

Regulate Autophagy

The major route for PI(5)P removal is attributed to type II PI5PK kinases (phosphatidylinositol 5-phosphate 4-kinases, PI5PK2K2) (Figure 2A; Clarke et al., 2010; Shisheva, 2013; Viaud et al., 2014a). Mammalian genomes contain three genes, PI5PK2K2,
PI(5)P Sustains Autophagy in Cells Depleted of PI(3)P

We tested whether PI(5)P could sustain autophagy in cells depleted of PI(3)P with the VPS34 inhibitor Wm. We validated the specificity of Wm for VPS34 activity during autophagy induction by treating VPS34-null MEFs (Figure S5A) and HeLa or GFP-LC3 HeLa stable cell lines with Wm for 2 hr in HBSS (Figures S5B and S5C). Consistent with previous studies, Wm inhibited ATG5-ATG12 conjugation, LC3 lipidation, and prevented autophagosome formation (Blommaart et al., 1997; Ravikumar et al., 2008; Figures S5A–S5D; Figures S5A–S5D). VPS34-null MEFs are insensitive to Wm compared to their WT counterparts (Figure S5A), and autophagy inhibition caused by Wm in HBSS-treated cells was rescued by PI(3)P supplementation (Figures S5B and S5C). VPS34-null cells did not have obviously decreased autophagy in HBSS (Figure S5A) like Wm-treated cells, suggesting that long-term VPS34 depletion may be compensated for by alternative pathways or by additional defects in autophagosome degradation (Devereaux et al., 2013). PI(3)P also regulates proteins crucial for sorting to vacuoles or lysosomes (Schink et al., 2013), and long-term VPS34 inhibition can impair endocytic trafficking (Petiot et al., 2003; Siddhanta et al., 1998) and cause vacuolation of late endosomal compartments (Futter et al., 2001) and mis trafficking of cathepsin D from late endosomes to lysosomes (Row et al., 2001).

To test whether PI(5)P was able to sustain autophagy in cells depleted of PI(3)P, we used acute Wm treatment and loaded cells with exogenous PI(5)P-dii-C16. Under these conditions, the requirement for the generation of PI(3)P appeared to be abrogated, as we detected LC3 lipidation (Figures 5A and 5B), increased LC3 vesicles under starvation (Figures 5C and 5D), increased double-membraned autophagosomes by electron microscopy (Figure S5D), and ATG5-ATG12 conjugation (Figure 5D), even in the presence of Wm. Similarly, increasing endogenous PI(5)P levels by PI5P4K2s knockdown resulted in an increase in LC3-II levels (Figures 5E and 5F), ATG5-ATG12 conjugation (Figures 5G and 5H), and numbers of LC3 vesicles (Figures 5I and 5J) that were resistant to Wm.

As a genetic alternative to Wm, we performed a short-term siRNA knockdown of VPS34, which does dramatically impact autophagy in HBSS (in contrast to longer-term knockdowns or Cre-mediated gene excision in the MEFs) (Figure S5A). In such VPS34-siRNA-treated cells, PI(5)P was able to rescue autophagy and ATG5-ATG12 conjugation. (Figure S5F).

We also manipulated PI(5)P and PI(3)P levels by overexpressing MTMR3, which would decrease PI(3)P levels but increase PI(5)P levels (Figures 2A and 2G). MTMR3 overexpression (but not MTMR3C413S catalytic mutant overexpression) in...
GFP-LC3 HeLa cells incubated in HBSS impaired autophagosome formation (BAF for 4 h), as previously reported (Taguchi-Atarashi et al., 2010; Figures 5J and 5K). This effect is likely due to the effects of MTMR3 on PI(3)P catabolism, which would be expected to blunt the HBSS-induced autophagy-stimulating levels of this lipid. To measure the effect of MTMR3 overexpression in a context where the role of PI(3)P can be excluded (Figure 2A), to allow us to largely focus our attention on the ability of this enzyme to generate PI(5)P, we treated cells with BAF and Wm for 4 hr in the presence of HBSS, and we found that overexpression of WT, but not of catalytic dead MTMR3 (Figures 5J and 5K), could rescue LC3 vesicle numbers in Wm-treated cells. Conversely, increasing PI(3)P (by loading of exogenous PI(3)P di-C16) restored LC3 dots in cells depleted of PI(5)P by overexpression of active PI5P4K2a, b, and γ (Figures 5L and 5M). Thus, our results point toward a common mechanism of action for PI(3)P and PI(5)P during autophagy.

PI(5)P and PI(3)P Share Common Effectors during Autophagosome Formation

Since PI(5)P can rescue autophagosome formation in PI(3)P-depleted cells, we tested if PI(5)P regulates autophagosome formation similarly to PI(3)P, through the recruitment of the WIPI2 and DFCP1 proteins. GFP-WIPI2 and DFCP1 puncta, which disappeared in starved cells treated with Wm, remained when such Wm-treated cells were preloaded with PI(5)P (Figures 6A and S6A), and the membrane association of GFP-WIPI2 and GFP-DFCP1 was dramatically impaired by Wm, but was Wm-insensitive in PI5P4K2 knockdown cells (Figures 6B–6D and S6B). Thus, WIPI2 and DFCP1 membrane binding can be preserved by PI(5)P in Wm-treated cells.

We examined whether WIPI2 proteins bind to PI(5)P using extracts from cells expressing GFP-tagged WIPI2B and WIPI2D, which we then precipitated with beads coated with different PIs (Figures 6E and S6C). WIPI2 proteins were associated with PI(5)P beads, but not with PI or uncoated beads (Figures 6E and S6C). This association seemed to be specific because the PI(5)P binding was inhibited when cells were preincubated with PI(5)P-containing but not PS-containing liposomes (Figure 6F). Similar effects also occurred for the binding of WIPI2B to PI(3)P beads and for the competition of PI(3)P-containing liposomes with PI(5)P beads (Figures 6E and 6G). WIPI2B protein pull-down by the PI(3)P beads was strongly affected by preincubation with PI(5)P-containing liposomes (Figure 6H). Likewise, WIPI2B protein pull-down by the PI(5)P beads was strongly affected by preincubation with PI(3)P-containing liposomes (Figure 6H). Furthermore, a WIPI2 mutant that was predicted not to bind to PI(3)P (Dooley et al., 2014) had similarly reduced binding to PI(5)P (Figure 6I). Thus, PI(5)P binds a PI(3)P effector involved in autophagosome biogenesis; we tested if PI(5)P was required for PI(5)P-independent autophagy.

**Autophagy Activation following Glucose Starvation Is Dependent upon PI(5)P, but Not PI(3)P**

Since we found that PI(5)P can sustain autophagy in Wm-treated cells, we considered that PI(5)P may regulate autophagy pathways where PI(3)P is dispensable, such as glucose-starvation-induced autophagy (McAlpine et al., 2013), a hypothesis strengthened by our observation that HBSS or glucose starvation increase the phosphorylation of PIKfyve at residues where this enhances its catalytic activity (Er et al., 2013; Hill et al., 2010; Liu et al., 2013; Figure S7A). Glucose deprivation in HeLa cells stably expressing GFP-LC3 increased the number of autophagosomes in DMSO- and Wm-treated cells, but not when PIKfyve was inhibited by YM-201636 treatment (Figures 7A and 7B). Conversely, both Wm and YM-201636 strongly affected but did not completely ablate autophagosome formation under amino-acid/serum starvation (HBSS, Figures 7A and 7B). We confirmed that YM-201636 reduced LC3-II levels in both glucose-starved MEFs and SKNSH cells, and that this phenomenon was not seen with Wm (Figure 7C). Consistent with a specific requirement for PI(5)P for autophagy induction during glucose withdrawal, we observed a significant YM-201636-sensitive increase in the number of ATG16-positive autophagosomes containing PI(5)P in glucose-depleted cells (Figure 7D). When PI(5)P was sequestered by GFP-PHD3X overexpression or removed by overexpression of active PI5P4K2a, b, and γ, the appearance of LC3 dots following glucose starvation was abolished (Figures 7E, 7F, and S7B). Consistent with the different effects of Wm treatment under nutrient starvation, we did not detect an increased number of PI(3)P-containing autophagosomes (using the GFP-FYVE2X probe or anti-PI(3)P antibodies) in cells depleted of glucose compared to HBSS media (Figures S7C and S7D).

Supporting the idea that PI(3,5)P2 contributes to the generation of PI(5)P during nutrient starvation, we observed increased colocalization of GFP-ML1N2 with RFP-LC3 under glucose and amino-acid/serum starvation (Figure S7E). Indeed, MTMR3 knockdown significantly reduced PI(5)P on autophagosomes (Figure 7G), inhibited autophagosome formation in glucose-starved cells (where PI(3)P is dispensable), and this effect could be reversed by adding back PI(5)P (Figures S7F and S7G). Collectively, our results argue that PI(5)P generated by PIKfyve...
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and MTMR3 is the relevant lipid species for autophagosome generation during glucose starvation. Resveratrol induces autophagy in noncanonical manner, independent of Beclin1 and partially resistant to Wm (Mauthe et al., 2011). The autophagy stimulation of resveratrol was impaired upon PI(5)P depletion due to overexpression of PISP4K2α, β, and γ (but not the catalytic-dead mutant of PISP4K2γ or GFP-empty vector) (Figure S7H).

When we labeled cells in basal, HBSS, or glucose starvation for PI(3)P (with an antibody) and for PI(5)P (with GFP-PHD3X), it was notable that the glucose-starved cells had fewer PI(3)P- and more PI(5)P-containing ATG16L1 structures, some of which appeared devoid of PI(3)P (Figure 7H), consistent with a dominant role for PI(5)P during glucose starvation. Our findings suggest that both PI(5)P and PI(3)P are important regulators of autophagy induced by HBSS. However, PI(5)P synthesis, but not PI(3)P synthesis, is required for autophagy induction by glucose withdrawal.

**DISCUSSION**

The present work identifies a role for PI(5)P as a regulator of autophagosome biogenesis. PI(5)P synthesis is required for autophagosome formation and this effect is similar to PI(3)P, as both lipids regulate recruitment of the WIPI2 and DFCP1 proteins and ATG5-ATG12 conjugation. PI(5)P and kinases acting on PI(5)P (particularly PISP4K2γ) are associated with autophagosomes and autophagosome precursors, suggesting that local alterations in the levels of this lipid are important for regulating autophagy. While we cannot exclude that alterations in PI(5)P may impact autophagosome biogenesis through its metabolites, the observations that PI(5)P binds to WIPI2, impacts PI(3)P-related phenotypes like ATG5-ATG12 conjugation, and can rescue autophagy in Wm-treated HBSS-starved cells strongly argue that it can serve as an alternative to PI(3)P. It is worth bearing in mind that PI(5)P’s presence in cells is ~100 times lower than those of the most abundant PIs, PI(4,5)P2 and PI(4)P (Sarkes and Rameh, 2010). The major source of PI(4,5)P2, which we previously have shown to be a positive regulator of autophagy (Moreau et al., 2012), is PI(4)P in resting cells (Rameh et al., 1997; Whiteford et al., 1997). Thus, reductions of PI(5)P levels hardly affect PI(4,5)P2.

The ability of PI(5)P to regulate autophagy in cells depleted of PI(3)P suggests that PI(5)P may account for the previously enigmatic concept of PI(3)P-independent, noncanonical autophagy. While we cannot exclude that a small amount of cellular PI(3)P may be generated via VPS34-independent routes, as suggested recently (Devereaux et al., 2013; McAlpine et al., 2013; Zhou et al., 2010), we consciously used acute depletion of PI(3)P using Wm to reduce the likelihood of such minor pathways having overt effects (compared to using genetic approaches, which would be necessarily more chronic and would allow redundant pathways to become more apparent). Indeed, Wm completely ablated the presence of WIPI2 and DFCP1 vesicles in our experiments. Since these effects and the concomitant ATG5-ATG12 conjugation and autophagosome formation were completely rescued by elevating PI(5)P in Wm-treated cells, we suggest that PI(5)P can account for the previously mysterious phenomenon of PI(3)P-independent autophagy. While this would be compatible with the similar binding of PI(3)P and PI(5)P to WIPI proteins (Baskaran et al., 2012; Jeffries et al., 2004), it is possible that the two lipids may have some nonredundant properties in the autophagy context. Indeed, this appeared to be the case, as we found PI(5)P (and its precursor PI(3,5)P2) localized on nascent and mature autophagosomes during glucose starvation, and this is highly dependent on the activity of the type III PtdInsP 5-kinase PIKfyve. Furthermore, glucose-starvation-induced autophagy appeared to be dependent on PI(5)P and not PI(3)P. The Wm resistance of glucose-starvation-induced autophagy was seen in HeLa cells, SKNSH cells, and MEFs. While we do not know why this is the case, given that one would expect VPS34 to be activated by upstream kinases under these conditions, one may speculate that it is not sufficient simply to activate the enzyme, but one also needs the enzyme to be in the right place, and perhaps the mechanism governing the correct localization of VPS34 to autophagosome precursors may be inefficient in glucose-starved cells.

Figure 5. PI(5)P Triggers Autophagy in the Absence of VPS34 Activation

(A and B) Western blot analysis of LC3-II and tubulin levels and quantification of LC3-II/tubulin ratio in HeLa cells pretreated with Wm (200 nM, 2 hr), then loaded with 10 μM PI(5)P for 1 hr (in the presence of Wm and BAF) (mean ± SEM).

(C) Quantification of numbers of GFP-LC3 vesicles in HeLa cells stably expressing GFP-LC3 treated as in (A) and then shifted to starvation media (HBSS) or complete media (Basal) for 2 hr (in the presence of Wm) (mean ± SEM).

(D) HeLa cells transfected with HA-ATG12 and ATG5 treated with 200 nM Wm as in (A) and subjected to western blot analysis with anti-HA antibody to detect free ATG12 antibody (bottom).

(E and F) Western blot analysis of LC3-II and tubulin levels and quantification of LC3-II/tubulin ratio in HeLa cells transfected with control, PISP4K2A, 2B, and 2C siRNA and treated with Wm (200 nM, 2 hr in the presence of BAF) (mean ± SEM).

(G and H) Western blot analysis of free ATG12 and ATG5-ATG12 complex levels with anti-HA antibody in HeLa cells treated with control, PISP4K2A, 2B, and 2C siRNA, transfected with HA-ATG12 and ATG5 for 16 hr and treated for Wm for 2 hr (mean ± SEM).

(i) HeLa cells stably expressing GFP-LC3 treated with control, PISP4K2A, 2B, and 2C siRNA, were pretreated with Wm for 2 hr in complete medium and then shifted in HBSS media for 2 hr (in the presence of Wm) (mean ± SEM).

(J and K) HeLa cells stably expressing GFP-LC3 were transfected for 30 hr with myc-tagged empty vector, myc-MTMR3WT, and myc-MTMR3C413S, incubated for 4 hr in HBSS in the presence of BAF and in the presence or absence of Wm. Cells were fixed, stained with anti-myc antibodies, and imaged by confocal microscope. Asterisks indicate transfected cells. Quantification of numbers of GFP-LC3 vesicles per cell is shown in (K) (mean ± SEM).

(L and M) HeLa cells transfected with GFP-PISP4K2A, 2B, 2C, and RFP-LC3 for 30 hr were loaded with 10 μM PI(3)P for 1 hr in complete medium, and then fixed and imaged on confocal microscope. Bar, 10 μm. Quantification of cells (percentage of total) showing more than 10 RFP-LC3 vesicles in the different conditions (L) is shown in (M); n = 200 cells (mean ± SEM). See also Figure S5.
A

Carrier 10 μM
PI-SP 10 μM
Carrier 1 μM
PI-SP 1 μM
GFP-WIPI2B puncta (% T0)

Wm treatment (min)

GFP-DHCP1 puncta (% T0)

Wm treatment (min)

B

CTR
PI-SPK2A KD
PI-SPK2B KD
PI-SPK2C KD
GFP-WIPI2B puncta (% T0)

Wm treatment (min)

C

GFP-WIPI2B puncta (% T0)

Wm treatment (min)

D

GFP-DHCP1 puncta (% T0)

Wm treatment (min)

E

beads
Input PI PI-SP PI-SP none

I.B. anti-GFP

GFP-WIPI2B

GFP

F

PI-SP beads + + +
PI-SP liposomes - + -
PS liposomes - - +

GFP-WIPI2B

G

PI-SP beads + + +
PI-SP liposomes - + -
PS liposomes - - +

GFP-WIPI2B

H

PI-SP beads - - + +
PI-SP beads + + - -
PI-SP liposomes - + - -
PI-SP liposomes - - + +

GFP-WIPI2B

I

Input PI-SP beads PI-SP beads

FTTG Wt FTTG Wt FTTG Wt

GFP-WIPI2B

(legend on next page)
Our observations may have therapeutic potential, because PIKfyve and PI3P4K2 enzymes are drug-treatable targets (Davis et al., 2013; Jefferies et al., 2008), and our data indicate that suppression of PI3P4K2’s activity increased the clearance of disease-associated autophagic substrates. Autophagy induction via other signaling effectors has benefits in a wide range of neurodegenerative diseases caused by aggregate-prone intracytoplasmic proteins, like Huntington’s and Parkinson’s diseases (Harris and Rubinsztein, 2011). Thus, PI3P4K2’s inhibition may provide a tractable therapeutic target for neurodegenerative diseases.

**EXPERIMENTAL PROCEDURES**

**Addition of Exogenous Lipids to Cells**
Unlabeled PI, PI(3)P, and PI(5)P di-C6; BODIPY-labeled PI(5)P and PI(3)P di-C6; and carrier (Echelon) were reconstituted in H2O:tert-BuOH (9:1) solution. After 1 min bath sonication, carrier and lipids were combined at a 1:1 ratio for 10 min at room temperature. The mixture of lipids and carrier was diluted in media and used for 1–2 hr incubations on cells. The final concentrations used were 0.1–10 μM. For the negative control; DMEM was combined with carrier only and added to the cells.

**Lipid Beads Pull-Down Assay**
HeLa cells (stably expressing GFP alone or GFP-WIPI2B or transiently expressing WIPi2D) were suspended in lipid-binding buffer (20 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA [pH 7.5]). The cells were passed ten times through a G25 needle and sonicated on ice. After insoluble debris was removed by high-speed centrifugation at 13,000 x g for 1 hr at 4 °C, a 50 μl slurry of PI, PI(3)P, PI(5)P, or unbound beads (Echelon Bioscience) was added to the tube and incubated for 4 hr at 4 °C under rotary agitation. The beads were washed five times with lipid-wash buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 0.25% NP40), and the bound proteins were subjected to immunoblotting. For competitive inhibition with liposomes, cell extracts were incubated on ice with 50 μM PS/PI(5)P, PS/PI(3)P liposomes for 3 hr before the addition of PI(5)P or PI(3)P beads. Preincubation of the cell lysates with liposomes containing only PS was used as a control with the same negative net charge.

**SR-SIM**
Samples were processed for conventional fluorescence microscopy and mounted on high-resolution 1.5 coverslips (Carl Zeiss). Coverslips were mounted with ProLong Gold antifade medium (P36934, Life Technologies), mounted on high-precision size 1.5 coverslips (Carl Zeiss). Coverslips were imaged on a confocal microscope equipped with a 63 × 1.4NA plan-apo Carl Zeiss objective lens and Immersol 518F (1.47 C) immersion oil. Image acquisition was carried out using ZEN 2012 Elvira edition software, in which data sets were collected with five grating phases, five rotations, and sufficient z positions spaced 110 nm apart to form an approximately 2 μm deep volume of raw SR-SIM data. Optimal grating frequencies were selected for each wavelength used. Structured illumination postprocessing was performed in ZEN using parameters determined by automated analysis of the data sets. Reconstructed images were then corrected for spherical and chromatic aberrations using channel alignment information, which was created using a 3D array of multispectral beads previously imaged with the same instrument settings. The average final image resolution was calculated to be 110 nm in x and y dimensions and 240 nm in the z dimension, which represents a 2-fold lateral and axial improvement in resolution compared to conventional microscopy. Final visualization and video production was performed in Velocity 6.3 software using isosurface rendering of selected cropped regions of the data sets.

**Statistics**
Protein levels were expressed as arbitrary units and numbers of vesicles or aggregate formation were expressed as percentages from three independent experiments carried out in triplicate, and the error bars denote SEM. Significance levels for comparisons between two groups were determined with two-tailed Student’s t test (Microsoft Excel); *p < 0.05; **p < 0.01; ***p < 0.001.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, seven figures, one table, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.12.007.

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**Figure 6. PI(5)P Recruits Proteins Required for the Initiation of Autophagosome Formation**

(A) HeLa cells transfected with GFP-WIPI2B or GFP-DFCP1 preloaded with indicated concentrations of PI(5)P for 1 hr, starved in HBSS for 1 hr, and then incubated with Wm in HBSS were tracked by time-lapse microscopy for 10 min after the addition of Wm. Quantification of WIPI2B or DFCP1 vesicles (percentage of those at the starting time [T0]) during the treatments are shown in the graphs.

(B–D) HeLa cells treated with control, PS54KGA, 2B, and 2C siRNA were transfected with GFP-WIPI2B (B), GFP-WIPI2D (C), or GFP-DFCP1 (D); starved in HBSS (1 hr); and then incubated with Wm in HBSS. WIPI2 or DFCP1 structures were tracked and quantified as in (A).

(E) Lysates from HeLa cells stably expressing GFP-WIPI2B were incubated with agarse beads coated with PI, PI(5)P, and PI(3)P, eluted with SDS-PAGE sample buffer, and recovered proteins were assessed by western blotting using antibodies against GFP. Uncoated beads and lysates from HeLa cells stably expressing GFP alone were used as internal controls.

(F–H) Cell extracts from HeLa cells stably expressing GFP-WIPI2B were incubated for 3 hr with PI(5)P-containing liposomes (F and H) or PI(3)P-containing liposomes (G and H) before a pull-down experiment using the indicated beads. PS-containing liposomes were used as internal controls for both competition assays in (F and G).
REFERENCES


Figure 7. PI(5)P Is a Master Regulator of Autophagy in Response to Glucose Starvation

(A) HeLa cells stably expressing GFP-LC3 were shifted to starvation media (HBSS or glucose-free DMEM) for 4 hr (in the presence of 200 mM Wm or 100 mM YM-201636).

(B) Quantification of vesicles per cell in HeLa cells stably expressing GFP-LC3 treated as in (A) is shown in the graph (mean ± SEM).

(C) Western blot analysis of LC3-II and tubulin levels in MEF and SKNSH cells were treated as in (A) in complete media or glucose-free media in the presence of 200 nM Wm, 100 nM YM-201636, then fixed and imaged on a confocal microscope.

(E and F) HeLa cells transfected with GFP-PI3P4K2A, 2B, 2C, or PI3P4K2C catalytic dead and RFP-LC3 for 30 hr (E) were starved for glucose for 4 hr, then fixed and imaged on a confocal microscope. Bar, 10 μm.

(G) HisGFP cells were transfected with PI3P4K2A, 2B, 2C, or PI3P4K2C catalytic dead and RFP-LC3 for 30 hr (F) were starved for glucose for 4 hr, then fixed, stained with anti-myc antibodies, and imaged on a confocal microscope. Bar, 10 μm.

(H) HeLa cells transfected for 16 hr with GFP-LC3 and RFP-LC3 for 30 hr (G) were starved for glucose for 4 hr, then fixed, stained with anti-myc antibodies, and imaged on a confocal microscope. Bar, 10 μm. See also Figure S7.


