PtdIns(4,5)P₂ signaling regulates ATG14 and autophagy

Xiaojun Tan¹,¹, Narendra Thapa¹,¹, Yihan Liao⁹, Suyong Choi⁹, and Richard A. Anderson²

¹Program in Molecular and Cellular Pharmacology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53706

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Autophagy is a regulated self-digestion pathway with fundamental roles in cell homeostasis and diseases. Autophagy is regulated by coordinated actions of a series of autophagy-related (ATG) proteins. The Barkor/ATG14(L)-VPS34 (a class III phosphatidylinositol 3-kinase) complex and its product phosphatidylinositol 3-phosphate (PtdIns(3)P) play key roles in autophagy initiation. ATG14 contains a C-terminal Barkor/ATG14(L) autophagosome-targeting sequence (BATS) domain that senses the curvature of PtdIns(3)P-containing membrane. The BATS domain also strongly binds PtdIns(4,5)P₂, but the functional significance has been unclear. Here we show that ATG14 specifically interacts with type I PIP kinase isoform 5 (PIPKIγ5), an enzyme that generates PtdIns(4,5)P₂ in mammalian cells. Autophagosomes have associated PIPKIγ5 and PtdIns(4,5)P₂ that are colocalized with late endosomes and the endoplasmic reticulum. PtdIns(4,5)P₂ generation at these sites requires PIPKIγ5. Loss of PIPKIγ5 results in a loss of ATG14, UV irradiation resistance-associated gene, and Beclin 1 and a block of autophagy. PtdIns(4,5)P₂ binding to the ATG14–BATS domain regulates ATG14 interaction with VPS34 and Beclin 1, and thus plays a key role in ATG14 complex assembly and autophagy initiation. This study identifies an unexpected role for PtdIns(4,5)P₂ signaling in the regulation of ATG14 complex and autophagy.

Significance

Autophagy is a conserved lysosomal degradation pathway, the deregulation of which is found in many human diseases, including cancers, neurodegeneration diseases, and aging. Understanding the molecular mechanisms of autophagy regulation is the basis for improving clinical therapeutics. Phosphatidylinositol 3-kinase [PtdIns(3)P] is a key lipid messenger directly involved in autophagy initiation. Here we discovered that PtdIns(4,5)P₂ plays an equally important role in autophagy. The autophagy-related protein 14 (ATG14) specifically interacts with PIPKIγ5, a PtdIns(4,5)P₂ generating enzyme that controls ATG14 stability and protein–protein interactions. PtdIns(4,5)P₂ directly binds ATG14 and regulates the ATG14–VPS34 (a class III phosphatidylinositol 3-kinase) complex assembly. Our study identifies a PtdIns(4,5)P₂ signaling pathway that is directly involved in autophagosome membrane initiation and argues for a previously unappreciated role for endosomes in autophagy initiation.

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¹X.T. and N.T. contributed equally to this work.

²To whom correspondence should be addressed. Email: raanders@wisc.edu.

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To assess the subcellular localization of PIPKιγ5 and ATG14, we coexpressed EGFP–ATG14 and monomeric DsRed–PIPKιγ5 in MDA–MB-231 cells. PIPKιγ5 and ATG14 partially colocalized in punctate intracellular compartments (Fig. 1C, Upper). PIPKιγ5 has been previously shown to associate with endosomes (16, 17), but ATG14 puncta form on the ER surface (7, 21), which was confirmed here (Fig. 1C, Lower). Of note, the subcellular localization of PIPKιγ5 is sensitive to its expression levels, as high expression of PIPKιγ5 disrupted its specific targeting (Fig. S2A).

When expressed at physiologic levels, PIPKιγ5 localized to punctate structures with endosomal but not with mitochondrial or the Golgi complex markers (Fig. S2B). Late endosomes, are often in contact with the ER (22), and it is possible that PIPKιγ5 and ATG14 concentrate at these sites. Consistently, PIPKιγ5 and ATG14 associate with both ER and late endosomes, as shown by costaining with the ER marker Calnexin and the late endosome marker LAMP1 (Fig. 1 D and E). The ER targeting of PIPKιγ5 and ATG14 is also observed using ER Tracker (Fig. S2C). The ATG14 endosomal association is specific for late but not early endosomes (Fig. S3A). This is confirmed with ATG14 targeting to LAPTMB4B (Fig. S3B, Upper), another endosome marker primarily localized to late endosomes (17).

ATG14 and ATG5 puncta formation is associated with autophagy initiation (21, 23). ATG5 puncta, which is usually used as a phagophore marker, also localized to LAPTMB4B positive endosomes (Fig. S3B, Lower). Similarly, LC3 was found to have extensive contacts with LAMP1 compartments (Fig. 1 F and G) and with EGFR* endosomes (Fig. S3C) that are involved in autophagy initiation (24). PIPKιγ5 and ATG14 also extensively colocalized with LC3 and LAMP1 (Fig. 1 F and G), and so did ATG5 (Fig. S3D). The data suggest that PIPKιγ5 and ATG14 might dynamically interact at an ER–endosome interface.

**PIPKιγ5 Controls the Stability of the ATG14–Beclin 1 Complex.** Small interference RNA (siRNA)-mediated knockdown of PIPKιγ5, but not PIPKιγ2, caused a loss of ATG14, Beclin 1, and UV irradiation resistance-associated gene (UVRAG) (Fig. 2A and B), which was also observed in another cell line (Fig. S4A). To confirm that the loss of ATG14 and Beclin 1 was due to PIPKιγ5 depletion, two PIPKιγ5-knockout cell lines were generated, and this confirmed that ATG14 and Beclin 1 were lost in these cells (Fig. S4B). Whereas PIPKιγ5 is required for ATG14 levels, knockdown or knockout of ATG14 did not affect PIPKιγ5 expression (Fig. S4 C and D).

Although PIPKιγ5 associated with ATG14, Beclin 1, and VPS34 in overexpressed co-IP (Fig. S4E), PIPKιγ5 did not affect the levels of VPS34 and Rubicon (Fig. 2A). Similarly, whereas PIPKιγ5 knockdown resulted in a loss of the ATG14–Beclin 1–VPS34 complex (Fig. 2B), the Beclin 1–Rubicon complex was not down-regulated (Fig. 2B). The protein turnover of ATG14 is regulated by ubiquitination and proteasome degradation (25, 26). In cells with stable ectopic ATG14 expression, knockdown of PIPKιγ5 nonetheless caused a loss of ATG14 (Fig. 2C) that was rescued by MG132, a proteasome inhibitor, indicating that PIPKιγ5 did not control ATG14 levels by transcriptional regulation. Consistently, loss of PIPKιγ5 increased ubiquitination of ATG14 (Fig. 2D). ATG14 ubiquitination and turnover is regulated by Cullin3 complexes (25, 26); knockdown of Cullin3 rescued the ATG14 levels in PIPKιγ5-depleted cells (Fig. 2E). Thus, PIPKιγ5 controls the stability of ATG14 by regulating its ubiquitination and degradation.

**PIPKιγ5 Is Required for Autophagy.** Endocytosis and the trafficking of autophagic membrane precursors are modulated by PtdIns(4,5)P2 (18, 19), but a direct role for PtdIns(4,5)P2 in the regulation of autophagy initiation has not been demonstrated. The PIPKιγ5 interaction with and regulation of ATG14 stability suggest that PIPKιγ5-mediated PtdIns(4,5)P2 signaling might be involved in autophagy initiation. The double FYVE-containing protein 1 (DFCP1) is a PtdIns(3)P effector and early autophagic marker downstream of the ATG14 complex and upstream of LC3 lipidation (1). Consistent with a role for PIPKιγ5 in ATG14 complex stability, the puncta formation of DFCP1 was strongly suppressed in PIPKιγ5-knockdown cells (Fig. 3 A and B), consistent with a role for PIPKιγ5 in autophagy initiation. DFCP1 puncta were observed in control siRNA transfected MDA–MB-231 cells cultured in either normal or serum-free media (Fig. S4), suggesting relatively high basal autophagic activity in this cell line.

**Fig. 1.** PIPKιγ5 interacts and colocalizes with ATG14. (A) ATG14 specifically co-IPs with PIPKιγ5. HEK293T cells were cotransfected with GFP–ATG14 and Myc–tagged PIPKι isoforms. Twenty-four hours after transfection, cells were harvested and WCLs used for IP with anti-Myc. The immunocomplex was analyzed by immunoblotting. (B) Endogenous co-IP of PIPKιγ5 and ATG14 in MDA–MB-231 cells. Cells were cultured in normal medium with serum (N) or serum-free medium (S) for 24 h, and WCLs were used for IP with anti-PIPKιγ5. The immunocomplex was analyzed by immunoblotting. (C) GFP–ATG14 partially colocalizes with PIPKιγ5 and Calnexin. MDA–MB-231 cells were costained with DsRed (monomer)–PIPKιγ5 and EGFP–ATG14 or singly transfected with EGFP–ATG14. Forty-eight hours after transfection, cells were fixed and stained as indicated. (D–G) PIPKιγ5 and ATG14 puncta are localized to endosome–autophagosome and endosome–ER–associated structures. MDA–MB-231 cells were transfected with DsRed (monomer)–PIPKιγ5 or mCherry–ATG14, and 48 h later, cells were fixed and stained for indicated subcellular markers. Boxes are selected regions for magnified view. (Scale bars, 10 μm.)
autolysosomes as the EGFP signal is quenched in acidic compartments, whereas yellow puncta represent autophagosome precursors, phagophores or newly formed autophagosomes (27). In PIPKιγ5-knockdown cells, more yellow LC3 puncta were observed (Fig. 3E), indicating a block of autophagosome maturation. Containing of LC3 and the late endosome/lysosome marker LAMP1 confirmed the accumulation of immature autophagosomes, most of which have not fused with endolysosomes (Fig. S6D). Together, these results indicate that loss of PIPKιγ5 causes not only a suppression of autophagy initiation as reflected by less LC3-II accumulation upon lysosomal inhibition, but it also causes a more severe block of autophagosome–endolysosome fusion.

**PIPKιγ5 Generates PtdIns(4,5)P₂ for Autophagic Signaling.** The data suggest that PtdIns(4,5)P₂ signals are required for autophagy initiation and potentially also autophagosome maturation. To detect PtdIns(4,5)P₂ generation, GFP-PLCδ–PH, a specific PtdIns(4,5)P₂ probe, was expressed, and the cells were then costained for autophagic membrane marker LC3 and endosome marker LAMP1. Although high expression of PLCδ–PH primarily caused plasma membrane targeting, clear localization of PLCδ–PH to intracellular compartments was observed in cells expressing low levels of PLCδ–PH (Fig. S7). Remarkably, although all cells

A431 cells showed no clear LC3 or DFCP1 puncta staining in normal media, reflecting lower basal autophagy, and serum-starvation–induced DFCP1 puncta formation in this cell line was also suppressed by PIPKιγ5 knockdown (Fig. S5). The data suggest that PIPKιγ5 controls initiation of both basal autophagy and serum-starvation–induced autophagy.

To further investigate the role for PIPKιγ5 in autophagy, LC3 lipidation was analyzed. Knockdown of PIPKιγ5 strongly blocked autophagic LC3-II turnover, as indicated by the loss of LC3-II accumulation upon chloroquine (CQ) treatment (Fig. 3C). Similar results were observed in both A431 and MDA–MB-231 cells (Fig. 3D). Loss of PIPKιγ2 did not affect LC3-II turnover (Fig. S6A), consistent with a specific interaction between ATG14 and PIPKιγ5 (Fig. 1L) as well as a requirement for PIPKιγ5 but not PIPKιγ2 in ATG14 stability (Fig. 2A). Overexpression of PIPKιγ5 also strongly suppressed autophagy initiation (Fig. S6B), indicating that PIPKιγ5 levels must be tightly controlled for proper functioning in autophagy. This is generally the case for PIP kinases as they lose normal targeting when overexpressed (9). Of note, although LC3-II generation was blocked in PIPKιγ5-knockdown cells, the basal LC3-II levels without lysosomal inhibition were consistently higher than in control cells (Fig. 3C and D and Fig. S6C), implying inhibition of both autophagy initiation and maturation upon loss of PIPKιγ5. This hypothesis was confirmed using a cell line stably expressing mCherry–EGFP–LC3, in which red puncta correspond to matured and acidified autophagosomes or
showed similar levels of LAMP1 staining, the PLCδ-PH puncta were observed specifically in cells with more LC3 puncta, where they extensively associated with LC3 and LAMP1 compartments (Fig. 4A). This finding suggests that cells with more autophagic activity may require more spatial generation of PtdIns(4,5)P₂. Similar to the localization of ATG14 and PIPKIγ5, PLCδ-PH extensively associated with ER and endosomes (Fig. 4B), but it did not show evident association with Golgi or mitochondria (Fig. S7). When coexpressed in MDA-MB-231 cells, PIPKIγ5 and PLCδ-PH colocalized on microdomains of LAMP1 or LC3 compartments (Fig. 4C), indicating that the ectopically expressed PIPKIγ5 still has kinase activity and is able to produce PtdIns(4,5)P₂. Knockdown of PIPKIγ5 completely blocked the binding of intracellular puncta by PLCδ-PH, although more LC3 puncta accumulated in the knockdown cells (Fig. 4D), indicating that PIPKIγ5 is the major enzyme that produces intracellular PtdIns(4,5)P₂ signals detectable by PLCδ-PH at these sites and under these conditions.

**PtdIns(4,5)P₂ Binding Controls ATG14 Function in Autophagy.** As PtdIns(4,5)P₂, the product of PIPKIγ5, binds the ATG14–BATS domain of ATG14, the minimal required region for membrane targeting of ATG14 (8), it suggests that PtdIns(4,5)P₂ binding might regulate ATG14 function in autophagy. PtdIns(4,5)P₂ binding of the BATS domain was confirmed in liposome binding assays (Fig. 5A). The BATS domain consists of 80 aa, in which there are only four basic residues (R423, R442, K486, and R492) that could potentially bind negatively charged PtdIns(4,5)P₂. Four BATS mutants were generated, each with one basic residue mutated to alanine, but the K486A mutant was not expressed in *Escherichia coli*. The other three BATS mutants were all expressed well and purified for liposome binding assay to test PtdIns(4,5)P₂ and PtdIns(3)P binding. As shown in Fig. 5B, BATS–R492A had normal PtdIns(4,5)P₂ binding and slightly reduced PtdIns(3)P binding, whereas BATS–R423A and BATS–R442A lost PtdIns(4,5)P₂ binding and kept PtdIns(3)P binding. Thus, R423A and R442A are two point mutants that specifically lose PtdIns(4,5)P₂ binding.

When either of the two point mutations was introduced into full-length ATG14, it reduced ATG14 interaction with VPS34 and Beclin 1, but it was not the case when introducing the R492A point mutation that retains PtdIns(4,5)P₂ binding (Fig. 5C), although all three mutants showed normal puncta formation (Fig. S8A). Consistent with literature (3–6), knockdown of ATG14 strongly blocked autophagy initiation (Figs. S8B and S9). When ATG14 knockdown cells were treated with control or siRNA, and 48 h later, cells were fixed and stained for endogenous LC3 and LAMP1, we extensively associated with ER and endosomes (Fig. 4D), indicating that PIPKIγ5 controls ATG14 stability by mechanisms other than PtdIns(4,5)P₂ binding to its BATS domain. Compared with these ATG14 mutants, loss of PIPKIγ5 causes more severe defects, such as loss of ATG14, Beclin 1, and UVRAG. This finding could explain why the ATG14 mutants do not fully phenocopy PIPKIγ5 knockdown.

Our results identified PIPKIγ5 as a specific interacting protein of ATG14 and it controls ATG14 stability and autophagic activity. PtdIns(4,5)P₂, the product of PIPKIγ5, binds to the ATG14–BATS domain and controls ATG14 interactions with VPS34 and Beclin 1, which then recruit downstream effectors to initiate autophagy (28). Our results suggest that PIPKIγ5 is a key regulator of ATG14 function in autophagy.

**Discussion**

Phosphoinositides are present in all cellular membranes and regulate membrane trafficking events including autophagic membrane trafficking. PtdIns(3)P has been considered as the sole phosphoinositide lipid messenger that controls autophagy initiation (29). The VPS34 complex generates PtdIns(3)P in the ER membrane, which serves as a lipid platform for the recruitment of downstream effectors to initiate the phagophore membrane, such as DFCP1 and WD repeat domain, phosphoinositide interacting 2 (WIP1) (1). However, recent evidence has argued for a role for PtdIns(5)P, instead of PtdIns(3)P, in autophagy initiation upon glucose starvation (30). The current study further extends this lipid pool to include PtdIns(4,5)P₂, which regulates the VPS34 complex. Our results establish that the PIPKIγ5-mediated PtdIns(4,5)P₂ signaling functions upstream of PtdIns(3)P by stabilizing ATG14 and
Fig. 5. PtdIns(4,5)P₂ binding controls ATG14 function in autophagy. (A) ATG14–BATS domain binds PtdIns(4,5)P₂ in lipidosome binding assay. A total of 1 μg of purified T7-tagged BATS domain was incubated with PtdIns(4,5)P₂ liposomes for 10 min at room temperature. Liposomes with BATS domain bound were collected by centrifugation. The amount of T7-BATS in supernatants and pellets was examined by immunoblotting. (B) Identification of BATS domain mutants losing PtdIns(4,5)P₂ binding. wild-type or mutated T–BATS domain were purified and assayed in lipidosome binding as described in A. (C) Full-length ATG14 with PtdIns(4,5)P₂-binding defective BATS domain shows diminished VPS34 and Beclin 1 association. HEK293T cells were transfected with indicated ATG14 constructs, and 24 h later, cells were harvested for IP assay. (D) ATG14 mutants losing PtdIns(4,5)P₂ binding to the BATS domain are defective in autophagy. MDA-MB-231 cells stably expressing siRNA-resistant wild-type or mutated ATG14 were transfected with ATG14 siRNA. Seventy-two hours later, cells were treated as indicated with 80 μM CQ for 2 h and harvested for immunoblotting analysis of LC3-II turnover. LC3-II levels were quantified and normalized to Actin; mean ± SD; n = 4; *P < 0.05; ***P < 0.001. (E) Proposed model for PIPKγi5 and PtdIns(4,5)P₂ regulation of the ATG14 complex in autophagy. PIPKγi5 interacts with ATG14 and generates PtdIns(4,5)P₂ to regulate the stability and activity of the ATG14-VPS34 complex at endosome- and ER-associated membrane. PIPKγi5 also controls autophagosome fusion with endolysosomes. PIP2, PtdIns(4,5)P₂.

Beclin 1 and promoting the ATG14–Beclin 1–VPS34 complex assembly (Fig. 5E). These data suggest that additional PtdIns(4,5)P₂ effectors and lipid metabolizing enzymes such as PI4K, PtdIns(4,5)P₂ phosphatases, SNX18, and others (18, 19, 31) might also be involved in fine tuning autophagy initiation.

Autophagy initiates at preautophagosomal structure (PAS) near the vacuole in yeast (32), which provides a basis to couple autophagosome formation and the subsequent fusion with the vacuole. In mammalian cells, most autophagosomes initially fuse with late endosomes or multivesicular endosomes before finally fusing with the lysosome, suggesting that autophagosomes might initiate close to or in contact with late endosomes. The endosomal localized EGFR controls both basal and serum-starvation-induced autophagy initiation (24), consistent with autophagy initiation in close proximity to endosomes. In support of this finding, most LC3 puncta were found closely associated with EGFR⁺ endosomes (Fig. S3C), which are usually also positive for the lysosomal-associated protein transmembrane 4B (LAPT4B) (24), another endosome marker (17). The mammalian PAS has been represented by multiple ATG14 and ATG5 puncta that associate with the ER (7, 8, 21). However, the relationship between mammalian PAS and late endosomes/lysosomes has not been addressed to date, although late endosomes have been demonstrated to constantly associate with the ER (22). The current study suggests that PtdIns(4,5)P₂ signaling might function as a connection between the ATG14 puncta and endosomes, as both PIPKγi5 and ATG14 extensively associate with the ER and endosomes. Interestingly, both PIPKγi5 and ATG14 puncta are specifically localized to punctate but not tubular ER structures that colocalize with endosomes (Fig. 1 and Fig. S2C). These punctate ER subdomains might correlate to ER patches (33) that also contribute to the previously described autophagy-initiating ER–mitochondria contacts (21, 34). However, future studies are needed to dissect the detailed molecular basis for the contacts between ER patches and endosomes in this context.

It is noteworthy that PIPKγi5 has putative roles not only in autophagy initiation as shown by less LC3-II accumulation upon lysosome inhibition but also in the autophagosome–endolysosome fusion, as loss of PIPKγi5 blocked the acidification of autophagic compartments. ATG14 has been recently reported to promote autophagosome fusion with endolysosomes by interacting with STX17 and priming the ternary STX17–SNAP29–VAPM8 soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex formation that directly mediates the fusion step (28). Whether PIPKγi5 regulates the fusion step by controlling the role for ATG14 in STX17 complex assembly is intrinsically difficult to test because ATG14 is lost upon PIPKγi5 knockdown. In addition, the ATG14 mutants without PtdIns(4,5)P₂ binding to the BATS domain show defects only in autophagy initiation with no detected LC3-II accumulation, suggesting that either PtdIns(4,5)P₂ regulates the fusion step by binding to other regions on ATG14 outside of the BATS domain or PIPKγi5 has another effector that contributes to the fusion step, such as Myo1c, a myosin that specifically binds PtdIns(4,5)P₂ and regulates autophagosome fusion with lysosomes (20).

A debate over intracellular PtdIns(4,5)P₂ signaling is whether significant PtdIns(4,5)P₂ exists on intracellular compartments or if this PtdIns(4,5)P₂ only reflects a biological noise. Increasing evidence supports the specific and essential functions of intracellular PtdIns(4,5)P₂ (10). However, PtdIns(4,5)P₂ is poorly detected on intracellular compartments likely because of the lack of free PtdIns(4,5)P₂, as it is bound to effectors that associate with PIP kinases (9). More PtdIns(4,5)P₂ exists on the plasma membrane and high expression of PLCδ–PH could mask its intracellular targeting with only apparent localization to the plasma membrane. Interestingly, more PLCδ–PH puncta was observed in cells with more LC3 punctate staining, indicating a correlation between autophagy and intracellular PtdIns(4,5)P₂ signaling. The intracellular targeting of PLCδ–PH was lost upon knockdown of PIPKγi5, consistent with its role in PtdIns(4,5)P₂ generation at these sites. It is likely that PIPKγi5 produces more free PtdIns(4,5)P₂ signals than PIP kinases in other intracellular pathways, such as PIPKγi2 that controls the endosomal recycling of E-cadherin (13) and integrins (14).

In summary, this study has revealed an endosome- and ER-associated PtdIns(4,5)P₂ signaling pathway that controls ATG14 complex stability and assembly required for autophagy. It reveals an unexpected role for PtdIns(4,5)P₂ in autophagic membrane trafficking and argues for a role of ER–late endosome contacts in autophagy regulation as a potential mammalian PAS.

Materials and Methods

See SI Materials and Methods for detailed description.
**Reagents, Cell Culture, and Treatments.** MDA-MB-231, A431, and HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Corning) supplemented with 10% (vol/vol) FBS, DNA, and sRNA transfection was carried out using Lipofectamine 3000 (Thermo Fisher Scientific) and Oligo-fectamine RNAiMax (Thermo Fisher Scientific), respectively, following manufacturer’s protocols, except that medium was changed 2 h after transfection with Lipofectamine 3000. For serum starvation, cells were washed twice with serum-free DMEM and then cultured with serum-free DMEM for indicated periods. MG132 was dissolved in DMSO as a 20-mM stock and directly added to cell culture plates for treatments.

**Co-IP.** IP of indicated proteins was performed using non-denaturing whole cell lysates (WCLs). The immunocomplexes were isolated using indicated antibodies and protein–G-conjugated beads and separated by SDS/PAGE, followed by analysis by immunoblotting as indicated.

**Autophagy Assays.** Autophagic activity was analyzed by Western blotting detection of LC3-II turnover in the presence and absence of autophagy inhibitor CQ, which is well established in literature. For autophagosome maturation, an MDA-MB-231 cell line stably expressing mCherry–EGFP–LC3 was used to track acidification of the LC3 compartments, as the fluorescence signals from EGFP, but not mCherry, are quenched by acidic pH upon autophagosome fusion with lysosomes.

**Immunofluorescence Microscopy.** Immunostaining of endogenous and tagged proteins was carried out following standard protocols. Cells on glass coverslips were washed, fixed in paraformaldehyde (PFA), permeabilized, and blocked in BSA. Incubation with primary antibodies was performed at 37°C 2 h or 4°C overnight and secondary antibodies at room temperature for 1 h. Fluorescence images were obtained using MetaMorph with a Nikon Eclipse TE2000-U microscope and further processed and assembled in MetaMorph and Adobe Photoshop.

**Liposome Binding Assay.** PtdIns(4,5)P2 PolyPisosomes (Echelon) were used for the liposome binding assay. A total of 1 μg of purified BATS domain and 10 μL PolyPisosomes were incubated at room temperature for 10 min. The liposomes were precipitated by centrifugation at 16,000 × g for 10 min. The lipid–protein pellets were washed twice with binding buffer before being dissolved in SDS-loading buffer and analyzed by Western blot with anti-T7 tag antibody.

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Supporting Information

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SI Materials and Methods

Plasmids, siRNA, and Stable Cell Lines. The mCherry–ATG14 and mCherry–ATG5 constructs were from Michael Davidson, Florida State University, Tallahassee, FL (Addgene plasmids nos. 54989 and 54995); pEGFP–ATG14 (3), EGF–ATG5 (35), and Flag–STX17 (36) were from Noboru Mizushima, University of Tokyo, Tokyo (Addgene plasmids nos. 24295, 22852, and 45911); Myc–DFCP1 was from Qing Zhong, University of Texas Southwestern Medical Center, Dallas. GFP–PLCδ–PH (57) was from Tobias Meyer, Stanford University, Stanford, CA (Addgene plasmid no. 21179). Most PiPKβ constructs were described previously (38). Monomeric DsRed–PiPKβ5 was generated by inserting human PiPKβ5 ORF into the DsRed–monomer–C1 vector. The ATG14–BATS domain sequence was cloned into pET28b for expression of T7-tagged BATS domain in E. coli. Point mutations of the BATS domain were generated by PCR using primers containing intended point mutation. For stable expression of PiPKβ5, ATG14, and mutants, the DNA sequences were cloned into pWPT vector for viral production and infection. The pWPT vector containing the DNA sequence of interest was co-transfected with pmd2.g and pSPAX2 (Addgene plasmids nos. 12259 and 12260, from Didier Trono, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) into HEK293T cells (~70% confluency at the time of transfection) for virus production. Forty-eight hours after transfection, the culture medium with viruses was collected and flouting cells and debris were removed by centrifugation. The supernatants containing viruses were infected to MDA-MB-231 cells. Based on immunofluorescence microscopy, the infection rate has been usually over 80%. The infected cells were used as a pool to come out downstream experiments. siControl, 5′-AGG UAG UGU AAU CGC CUU G-3′; siPiPKβ5-5′-GGG UAG GAG GUA CUG GAU U-3′; and siCullin3, 5′-GUC GUA GAC AGA GCC GCA A-3′.

Antibodies. GFP [Roche, IP 2 μg per sample, Western blot (WB) 1:1,000]; Myc (4A6, Millipore, IP 2 μg per sample), Myc–HRP (9E11, Santa Cruz, WB 1:5,000); Flag (M2, Sigma, IP: 2 μg per sample; F7425, Sigma, WB 1:5,000); Flag (Sigma, WB 1:3,000); EEA1 (Cell Signaling, IF 1:200); LAMP1 (H4A3, Abcam, IF 1:200); Calnexin (Santa Cruz, IF 1:200); GM130 (BD, IF 1:100); Tomm20 (Santa Cruz, IF 1:2,000); LC3 (Cell Signaling, WB 1:2,000, IF 1:200); Actin (C4, MP Biomedicals, WB 1:100,000); GAPDH–HRP (FL-335, Santa Cruz, WB 1:1,000); ATG14 (MBL, WB 1:1,000, IP 2 μL per sample); Becn1 (Santa Cruz, clone E8, WB 1:300, IP 2 μg per sample; clone H300, WB 1:50,000); LAPTmM4B anti-sera (18895-1-AP, Proteintech, IF 1:500); EGFR (LA22, Millipore, IF 1:1,000); Rubicon (MBL, WB 1:1,000); VPS34 (Echelon, WB 1:200); GFP (clones 7.1 and 13.1, Roche, WB 1:1,000, IP 2 μg per sample); Ubiquitin (07-375, Upstate, WB 1:500); T7–HRP (69048, Novagen, WB 1:500); Cullin3 (ab75851, Abcam); and PiPKβ2 and PiPKβ5 antibodies were homologous.

CRISPR/Cas9 Knockout Cell Lines. To generate PiPKβ5 knockout MDA-MB-231 cell lines, guide RNAs targeting the 5′ region of PiPKβ5 were designed and cloned into pX330-U6-chimeric_BB-CBh-hSpCas9 vector (Add gene plasmid no. 42230) (39). The two sgRNAs target the following two sequences: 1, GGA CTG TAA GTG ACC GCA GC and 2, TCA GAC ACT GAT CTC CGG GC. For ATG14 knockout in HEK293T cells, two sgRNAs were designed to target sequences within exon 1: 1, TGT GCA ACA CTA CCC GCC CGG and 2, GGT GGA CTC GTG GGA CGA TG. The pX30 vectors containing the insertion were then cotransfected with pLPCX empty vector (providing puromycin resistance) into MDA-MB-231 cells; 24 h later, cells were treated with fresh medium containing 1.5 μg/mL puromycin for 48 h. Cells were then cultured in normal medium for another 5–7 d before monoclonal cell seeding in 96-well plates. The monoclonal cell lines were expanded and tested in Western blotting to verify knockout efficiency.

IP and Immunoblotting. Cells were harvested with the lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM MgCl2, and protease inhibitor mixture). All cells were scrapped off culturing dishes during lysing, and the WCLs were centrifuged at 20,000 × g for 10 min and supernatants were collected for IP or immunoblotting. For IP, the lysates were incubated with 2 μg of indicated antibodies and protein–G-conjugated beads at 4 °C for 2 h. The beads were then precipitated by centrifugation at 3,500 × g for 10 s and washed twice with the above lysing buffer. The co-IPed protein complexes were eluted in loading buffer containing 1% SDS and 1% 2-mercaptoethanol and separated by SDS/PAGE and analyzed by immunoblotting as indicated.

Immunofluorescence Microscopy. Cells grown on coverslips were washed twice with cold PBS, fixed in 3.7% (wt/vol) paraformaldehyde (PFA) for 5 min at room temperature, and permeabilized in 0.5% Triton X-100 in PBS for 5 min at RT, followed by blocking in 3% (wt/vol) BSA for 1 h at room temperature. Incubation with primary antibodies with indicated dilutions above was performed at 37 °C for 2 h or 4 °C overnight. Then cells were washed twice in washing buffer (0.1% Triton X-100 in PBS), and incubated with Alexa-488, -555, or Pacific blue-conjugated secondary antibodies at room temperature for 1 h, followed by washing three times with washing buffer. Coverslips were mounted on slides using VECTASHIELD Antifade Mounting Medium (Vector Laboratories) and fixed with nail coat. Images were obtained using MetaMorph with a Nikon Eclipse TE2000-U microscope and further processed and assembled in Adobe Photoshop.

Protein Purification. The T7-tagged BATS domain and mutants expressed by pET28b contain His-tag and were purified by Ni-NTA columns. Bacteria were lysed in lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor), sonicated, and centrifuged at 20,000 × g for 10 min. Supernatants were incubated with 1 mL of precharged Ni-NTA Agarose (Qiagen) for 1 h at 4 °C, and then the lysis–agarose mixture was loaded into the column tube. The column was then washed with 5 mL lysing buffer, followed by washing with 6 mL Tris buffer at pH 8.0 containing 30 mM imidazole. Then His-tagged protein was eluted with 1 mL elution buffer containing 500 mM imidazole. The eluted protein was collected and analyzed by Western blotting using an HRP-conjugated T7 tag antibody.

Liposome Binding Assay. PolyP1Posomes (Echelon) containing 65 mol% phosphatidylcholine (PC), 29 mol% phosphatidyethanolamine (PE), 1 mol% biotin-PE, and 5 mol% phosphatidylinositol (4, 5) bisphosphate were used for the liposome binding assay. No liposomes andcontrol PolyP1Posomes containing 70% of PC, 29% PE, and 1% biotin-PE were used as negative controls. A total of 1 mL binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.05% Nonidet P-40) per tube was spun at 16,000 × g for 5 min for preclearence, and the supernatant was moved into a centrifuge tube. A total of 1 μg of purified BATS domain and 10 μL PolyP1Posomes were added to each tube and incubated with rotation at room temperature for 10 min. The samples were spun at 16,000 × g for 10 min, and supernatant was saved. The lipid pellets were washed with 1 mL of binding buffer by inverting the tube several times. Samples were spun again at 16,000 × g for 5 min. The wash was repeated once more
before dissolving the lipid–protein complex in 100 μL of SDS loading buffer. A total of 20 μL of each sample was analyzed by Western blot with HRP-conjugated anti-T7 tag antibody.

**Statistics.** Statistical significance was determined by Student’s *t* test. Data were expressed as mean ± SD. All experiments were independently repeated at least three times.

Fig. S1. The PIPKIγi5 interaction with ATG14 and Beclin 1 is not affected by serum starvation. HEK293T cells were cotransfected with Myc–PIPKIγi5 and Flag–ATG14 (A and B) or Flag–Beclin 1 (C). Twenty-four hours after transfection, WCLs were harvested for IP assay using anti-Myc (A and C) or anti-Flag (B). The immunocomplexes were analyzed by immunoblotting.

Fig. S2. Subcellular localization of PIPKIγi5. (A) High expression of PIPKIγi5 disrupts its specific subcellular localization. MDA–MB-231 cells were transfected with DsRed(monomer)–PIPKIγi5, and 48 h later, cells were fixed and stained for endogenous LC3. Representative cells with low (+), medium (++), or high (+++) expression of PIPKIγi5 are shown. (B) PIPKIγi5 is localized to endosome-associated membranes but not the mitochondria or the Golgi complex. MDA–MB-231 cells were transfected with DsRed(monomer)–PIPKIγi5, and 48 h later, cells were fixed and stained for GM130, Tomm20, or LAMP1. (C) PIPKIγi5 and ATG14 colocalize with punctate ER structures. MDA–MB-231 cells were transfected with DsRed(monomer)–PIPKIγi5 or mCherry–ATG14, and 48 h later, cells were stained with ER Tracker for 30 min and fixed for microscopy. Boxes are selected regions for magnified view. (Scale bars, 10 μm.)
Fig. S3. Subcellular localization of ATG14 and ATG5. (A) ATG14 puncta primarily associates with late but not early endosomes. MDA–MB-231 cells were transfected with GFP–ATG14, and 48 h later, cells were fixed and stained for the early endosome marker EEA1 or the late endosome/lysosome marker LAMP1. (B) Both ATG14 and ATG5 associate with LAPTM4B endosomes. MDA–MB-231 cells were transfected with GFP–ATG14 or GFP–ATG5, and 48 h later, cells were fixed and stained for endogenous LAPTM4B. (C) LC3 associates with EGFR+ endosomes. MDA–MB-231 cells cultured in normal medium were fixed and stained for endogenous LC3 and EGFR. (D) ATG5 colocalizes with LC3 and LAMP1. MDA–MB-231 cells were transfected with mCherry–ATG5, and 48 h later, cells were fixed and stained for endogenous LC3 and LAMP1. Boxes are selected regions for magnified view. (Scale bar, 10 μm.)
Fig. S4. Loss of PIPKιγ5 destabilizes ATG14 and Beclin 1. (A) Knockdown of PIPKιγ5 causes a loss of ATG14 and Beclin 1 in A431 cells. Seventy-two hours after transfection of control or PIPKιγ5 siRNA, WCLs were harvested for immunoblotting analysis. (B) PIPKιγ5-knockout MDA-MB-231 cells have decreased levels of ATG14 and Beclin 1. PIPKιγ5-knockout cells were generated by CRISPR/Cas9 technology (see SI Materials and Methods for details). WCLs of parental and PIPKιγ5-knockout MDA-MB-231 cells were analyzed by immunoblotting. (C) Knockdown of ATG14 does not affect PIPKιγ5 level. Seventy-two hours after transfection of control or ATG14 siRNA, WCLs were harvested for immunoblotting analysis. (D) ATG14 knockout does not affect PIPKιγ5 and Beclin 1 levels. ATG14-knockout cells were generated by CRISPR/Cas9 technology. WCLs of parental and ATG14-knockout HEK293T cells were analyzed by immunoblotting. (E) PIPKιγ5 associates with ectopically expressed ATG14, Beclin 1, and VPS34 in a co-IP assay. HEK293T cells were cotransfected with Myc–PIPКιγ5 and indicated plasmid. Twenty-four hours after transfection, WCLs were harvested for IP assay using anti-Myc, followed by immunoblotting analysis of the immunocomplex.
Fig. S5. PIPKIγ5 regulates DFCP1 puncta formation in A431 cells. (A) Knockdown of PIPKIγ5 causes accumulation of LC3 in serum-starved A431 cells. Cells were transfected with control or PIPKIγ5 siRNA. Seventy-two hours after transfection, cells were fixed and stained for endogenous LC3. Note: PIPKIγ5 knockdown-induced LC3 accumulation was only observed after starvation, due to low basal autophagy in A431 cells. See also Figs. 3E and 4D and Fig. S6C and D for PIPKIγ5 knockdown-induced LC3 accumulation in MDA-MB-231 cells. (B) Knockdown of PIPKIγ5 inhibits serum-starvation-induced DFCP1 puncta formation in A431 cells. Cells were transfected with control or PIPKIγ5 siRNA. Forty-eight hours after transfection, cells were transfected with Myc-tagged DFCP1 and further cultured in normal or serum-free media for 24 h, followed by fixation and immunostaining of Myc-DFCP1. (Scale bar, 10 μm.) (C) Quantification of LC3 and DFCP1 staining in A and B. Mean ± SD; n = 3.
Fig. S6. PIPK\(\gamma\)i5 regulates autophagy. (A) Knockdown of PIPK\(\gamma\)i2 does not affect autophagic LC3-II turnover. Seventy-two hours after transfection of control or PIPK\(\gamma\)i2 siRNA, MDA–MB-231 cells were treated or not with 80 \(\mu\)M CQ for 2 h, followed by WCL harvest for immunoblotting analysis. (B) PIPK\(\gamma\)i5 overexpression blocks LC3-II turnover. MDA–MB-231 cells stably expressing PIPK\(\gamma\)i5 or empty vector (EV) were treated or not with 80 \(\mu\)M CQ for 2 h, followed by WCL harvest for immunoblotting analysis. (C) Knockdown of PIPK\(\gamma\)i5 causes accumulation of LC3. MDA–MB-231 cells were transfected with control or PIPK\(\gamma\)i5 siRNA. Seventy-two hours after transfection, cells were fixed and stained for endogenous LC3. (Scale bar, 10 \(\mu\)m.) (D) Knockdown of PIPK\(\gamma\)i5 causes accumulation of LC3 that is separate from LAMP1 compartments. MDA–MB-231 cells were transfected with control or PIPK\(\gamma\)i5 siRNA. Seventy-two hours after transfection, cells were fixed and stained for endogenous LC3 and LAMP1. (Scale bar, 10 \(\mu\)m.)

Fig. S7. Subcellular localization of PLC\(\delta\)–PH domain. MDA–MB-231 cells were transfected with GFP–PLC\(\delta\)–PH, and 48 h later, cells were fixed and stained for endogenous GM130 or Tomm20. Boxes are selected regions for magnified view. (Scale bars, 10 \(\mu\)m.)
Fig. S8. Subcellular localization of ATG14 mutants. (A) ATG14 mutants have normal subcellular puncta localization. MDA–MB-231 cells were transfected with GFP–ATG14 and indicated mutants, and 48 h later, cells were fixed and stained for LAMP1. (B) ATG14 knockdown strongly blocks LC3-II generation. MDA–MB-231 cells were transfected with control or ATG14 siRNA. Seventy-two hours after transfection, cells were harvested for immunoblotting analysis.
Fig. 59. ATG14 knockdown cells reexpressing mutants have no defects in autophagosome–endolysosome fusion. MDA–MB-231 cells were transfected with indicated DNA constructs, and after 48 h, cells were transfected with control or ATG14 siRNA. Seventy-two hours later, cells were fixed and costained for LC3 and LAMP1. (Scale bar, 10 μm.)
Fig. S10. ATG14 mutants with PtdIns(4,5)P$_2$-binding defective BATS domain have enhanced interaction with STX17 and PIPK$_i$$\gamma_5$. HEK293T cells were co-transfected with Flag-STX17 (A) or Myc-PIPK$_i$$\gamma_5$ (B) and indicated GFP-ATG14 constructs, and 24 h later, cells were harvested for co-IP assays with indicated antibodies, followed by immunoblotting analysis of the immunocomplexes.