GABARAPs regulate PI4P-dependent autophagosome: lysosome fusion

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The Atg8 autophagy proteins are essential for autophagosome biogenesis and maturation. The γ-aminobutyric acid receptor-associated protein (GABARAP) Atg8 family is much less understood than the LC3 Atg8 family, and the relationship between the GABARAPs’ previously identified roles as modulators of transmembrane protein trafficking and autophagy is not known. Here we report that GABARAPs recruit palmitoylated PI4KIIα, a lipid kinase that generates phosphatidylinositol 4-phosphate (PI4P) and binds GABARAPs, from the perinuclear Golgi region to autophagosomes to generate PI4P in situ. Depletion of either GABARAP or PI4KIIα, or overexpression of a dominant-negative kinase-dead PI4KIIα mutant, decreases autophagy flux by blocking autophagosome:lysosome fusion, resulting in the accumulation of abnormally large autophagosomes. The autophagosome defects are rescued by overexpressing PI4KIIα or by restoring intracellular PI4P through "PI4P shuffling." Importantly, PI4KIIα’s role in autophagy is distinct from that of PI4KIIIα and is independent of subsequent phosphatidylinositol 4,5 biphosphate (PIP2) generation. Thus, GABARAPs recruit PI4KIIα to autophagosomes, and PI4P generation on autogasomes is critically important for fusion with lysosomes. Our results establish that PI4KIIα and PI4P are essential effectors of the GABARAP interactome’s fusion machinery.

Autophagosomes Contain PI4P and PI4KIIα. We used LC3 staining to identify autophagic structures (predominantly autophagosomes) and to determine whether they contain PI4P/PI4KIIα. As expected, in growing cells (Ctrl), LC3 was mostly cytosolic and present on the occasional autophagosomes formed by basal autophagy (Fig. 2A). Unlike PI4P, it was not concentrated in the perinuclear Golgi region. Starvation induced a large increase in the number of LC3 puncta that contained PI4P (Fig. 2A). Similar results were obtained by using the PI4P reporter, GFP-OSBP-PH [the PI4P binding pleckstrin homology (PH) domain of the oxysterol binding protein], to detect PI4P (Fig. 2B).

Many of the LC3 puncta also contained myc-PI4KIIα (Fig. 2C). The extent of colocalization, estimated by analysis of confocal Z stack images, was less than 10% in Ctrl growing cells and more than 60% in starved cells (Fig. 2D). Rapamycin (Rap), a mechanistic target of rapamycin (mTOR) inhibitor that induces autophagy, also generated autophagosomes containing PI4KIIα and the PI4P reporter (Fig. S2). In conclusion, PI4KIIα and PI4P are associated with many LC3-positive autophagic structures.

Significance

Autophagy is an essential homeostatic process that is critically important for maintaining health and that is dysregulated in multiple devastating diseases. The steps in the final stages of autophagy that culminate in autophagosome:lysosome fusion are not well understood. The γ-aminobutyric acid receptor-associated protein (GABARAP) family of Atg8 (autophagy-related 8) proteins has been implicated in autophagosome maturation. Here we report that phosphatidylinositol 4-kinase IIα (PI4KIIα), a lipid kinase that generates phosphatidylinositol 4-phosphate (PI4P) and binds GABARAPs, is recruited to autophagosomes by GABARAPs. Furthermore, PI4P generation by PI4KIIα, but not by PI4KIIIα, another major mammalian PI4K, promotes autophagosome fusion with lysosomes. Our results establish for the first time to our knowledge that PI4KIIα is a specific downstream effector of GABARAP and that PI4P has a key role in the final stage of autophagy.

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nonpalmitoylable myc-PI4KIIα was not enriched in autophagosomes (Fig. 2). Significantly, dominantly cytosolic and nonraft-associated and ectopically associated with membranes as a peripheral protein (8, 9). The PI4KIIα mutant in which all of the cysteines in the CCPCC motif were replaced by serines (SSPSS) is predominantly cytosolic and nonraft-associated and ectopically associates with membranes as a peripheral protein (8, 9). Significantly, it was not enriched in autophagosomes (Fig. 2E and Fig. S2B), detected either by staining endogenous LC3 in HeLa cells (Fig. 2E) or GFP-LC3 in HeLa cells stably expressing GFP-LC3 (HeLa/GFP-LC3) (Fig. S2B). These results establish that PI4KIIα recruitment to autophagosomes is palmitoylation-dependent.

PI4KIIα Translocation to Autophagosomes Is Palmitoylation-Dependent. PI4KIIα is palmitoylated on a cysteine-rich motif (174CCPCC178) in the Golgi and behaves like an integral membrane protein that is associated with cholesterol-rich lipid raft microdomains (8, 9). The nonpalmitoylatable myc-PI4KIIα mutant in which all of the cysteines in the CCPCC motif were replaced by serines (SSPSS) is predominantly cytosolic and nonraft-associated and ectopically associates with membranes as a peripheral protein (8, 9). Significantly, it was not enriched in autophagosomes (Fig. 2E and Fig. S2B), detected either by staining endogenous LC3 in HeLa cells (Fig. 2E) or GFP-LC3 in HeLa cells stably expressing GFP-LC3 (HeLa/GFP-LC3) (Fig. S2B). These results establish that PI4KIIα recruitment to autophagosomes is palmitoylation-dependent.

PI4KIIα Depletion Generates Abnormally Large Autophagosomes. When HeLa/GFP-LC3 cells were maintained in Opti-Eagle’s minimal essential medium (MEM)/5% (vol/vol) FBS to minimize basal autophagy, GFP-LC3 was diffusely cytosolic and nuclear (Fig. 3A). EBSS or Rap induced formation of small GFP-LC3 puncta in siCtrl cells and much larger puncta in siPI4KIIα cells. Quantitative image analysis showed that the siPI4KIIα cells had five- and three-fold higher residual GFP-LC3 at the end of treatment, respectively (Fig. 3A). Because the HeLa/GFP-LC3 cells expressed similar amounts of GFP-LC3 initially before autophagy induction, we conclude that siPI4KIIα blocks GFP-LC3 degradation to increase puncta size. Likewise, siPI4KIIα also generated large autophagosomes in cells expressing only endogenous LC3 (Fig. 3A, Middle).

The GFP-LC3 puncta have a unimodal area distribution in autophagic siCtrl cells and a bimodal distribution in siPI4KIIα cells (Fig. 3A). The coexistence of normal-sized and enlarged autophagosomes in siPI4KIIα cells raises the possibility that autophagosomes may have formed normally initially, but are defective at a later stage, culminating in enlargement. Transmission electron microscopy showed that the enlarged structures were double-membrane vesicles that contained organelles and assorted cytoplasmic materials (Fig. 3B, Top). Thus, they are bona fide autophagosomes and not single-membrane amphisomes generated by endosome fusion with autophagosomes (10). Correlative light and electron microscopy confirmed that the enlarged structures contained GFP-LC3 (Fig. 3B, Bottom). Interestingly, similar to PI4KIIα depletion, GABARAP depletion also generated enlarged autophagosomes, suggesting they may be functionally related. This possibility is confirmed in experiments described here.

PI4KIIα Depletion or Kinase-Dead PI4KIIα Overexpression Inhibits Autophagy Flux. We used Western blotting to determine that siPI4KIIα cells had decreased LC3 autophagy flux (3) that could explain their enlarged autophagosome phenotype. Control growing cells had predominantly LC3-I (cytosolic) and a small amount of lipidated LC3-II (Fig. 3C), EBSS increased LC3-I conversion to LC3-II, which is degraded after A:L fusion. Bafilomycin A1 (Baf A1) increased LC3-II steady state levels in siCtrl cells by blocking its degradation in autolysosomes. In contrast, starved siPI4KIIα cells had more LC3-II than Ctrl cells, and Baf A1 did not increase LC3-II levels much further. Quantitative Western blotting showed that the starved siPI4KIIα cells had an ~150% increase in final LC3 levels and a more than 90% decrease in the extent of LC3 degradation.

Fig. 1. Autophagy induces PI4KIIα redistribution to cytoplasmic puncta. (Scale bar, 5 μm.) (A) PI4KIIα and TGN46 localization. COS cells stably expressing GFP-PI4KIIα were exposed to growth medium (Ctrl) or EBSS and stained with anti-TGN46 (red). (B) siPI4KIIα decreased peripheral PI4P puncta in starved HeLa cells.

Fig. 2. LC3 autophagosomes contain PI4P and PI4KIIα. HeLa cells were exposed to growth medium (Ctrl) or EBSS. (Scale bars, 10 μm.) (A) LC3 (red) and anti-PI4P (green). (B) LC3 (red) and GFP-OSBP-PH (a PI4P reporter). (C) LC3 (red) and myc-PI4KIIα (green). (Top) Images from a single confocal plane. (Bottom) Serial stack of an EBSS-treated cell. The red and green components in multiple planes were analyzed in the vertical (V) and horizontal (H) dimensions. (D) Imaris AutoQuant (Bitplane) colocalization analysis. Z stacks from 20 cells similar to those shown in C were analyzed for each data point. Percentages LC3 colocalization with myc-PI4KIIα or GFP-OSBP-PH and vice versa are shown (mean ± SEM; ****p < 0.0001). (E) LC3 and the nonpalmitoylatable SSPSS myc-PI4KIIα.
any potential scaffolding functions.

generation of PI4P on autophagosomes is required in addition to capitalizes on the higher sensitivity of GFP to quenching by monomeric red fluorescent protein (mRFP)-GFP-LC3, which autophagosome fusion with lysosomes (A:L fusion) at late-stage whether the block in autophagic flux is a result of decreased S3 malized against actin content and expressed relative to siCtrl cells set as 100%.

Values are percentage mean ± SEM (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Effects of PI4KIIα depletion or KD PI4KIIα overexpression on autophagosome size and autophagy flux. (A) PI4KIIα-depletion. (Left) GFP-LC3 in HeLa/GFP-LC3 cells exposed to different conditions. (Right) Endogenous LC3 in starved HeLa cells. Bar graph, quantitation of GFP-LC3 fluorescence in HeLa/GFP-LC3 cells after EBSS or Rap treatment. The y axis shows GFP-LC3 intensities remaining in siPI4KIIα relative to siCtrl (set at a value of 1) cells (mean ± SEM; n = 3; **P < 0.001). Fifty cells per condition. Area distribution, GFP-LC3 puncta areas (μm²) from 20 cells per condition were determined by ImageJ area analysis (mean ± SEM; n = 3). (B) Representative electron micrographs. EBSS-treated HeLa/GFP-LC3 cells were processed for transmission electron microscopy (Top) or for correlative light and electron microscopy (Bottom). (Scale bars, 100 nm.) The area enclosed by the rectangle in the top row is enlarged in the third panel to show the vesicle’s double limiting membranes. (Bottom, Left to Right): first panel, GFP-LC3 fluorescence in starved siPI4KIIα HeLa/GFP-LC3 cells. Scale bar, 10 μm.) Rectangle highlights a representative cell selected for correlative electron microscopy. Second panel, a low-magnification electron micrograph. (Scale bar, 5 μm.) The regions highlighted by an asterisk (i and ii) are enlarged in the last two panels. Arrows, autophagosomes. (Scale bars, 200 nm.) (C) Autophagy flux. (Left) Western blot of endogenous LC3, PI4KIIα, and actin without and with EBSS. Cells were incubated with Baf A1 (100 nM, 12 h) and exposed to EBSS for the last 2 h. (Right) Quantitation of Western blot. LC3 remaining and percentage LC3 degradation were normalized against actin content and expressed relative to siCtrl cells set at 100%.

Values are percentage mean ± SEM (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

(Wang et al. PNAS vol. 112 no. 22 | June 2, 2015) Degradation of p62/sequestome 1 (SQSTM1), another autophagy substrate, was also impaired (Fig. S3A).
The kinase dead (KD) myc-PI4KIIα Lys115Met (K115M) substitution mutant, which is raft-associated and correctly targeted to endomembranes in growing cells (8, 9), was also recruited to autophagosomes (Fig. S3A). Furthermore, high-level KD PI4KIIα overexpression generated enlarged autophagosomes and decreased degradation of GFP-LC3 and autophagy flux (Fig. 3C and Fig. S3B). Therefore, it acted as a dominant negative inhibitor to replicate the PI4KIIα depletion phenotype. We conclude that PI4KIIα generation of PI4P on autophagosomes is required in addition to any potential scaffolding functions.

PI4KIIα Depletion Inhibits Acidification and A:L Fusion. To determine whether the block in autophagic flux is a result of decreased autophagosome fusion with lysosomes (A:L fusion) at late-stage autophagy (10), we used several approaches. First, the tandem monomeric red fluorescent protein (mRFP)-GFP-LC3, which capitalizes on the higher sensitivity of GFP to quenching by acidic pH than mRFP (11), was used to determine whether there is a decreased rate of autophagosome acidification. siCtrl cells showed a time-dependent decrease in unfused (yellow) autophagosomes in EBSS, whereas siPI4KIIα cells had much slower rates of decrease (Fig. 4A). Similar results were obtained with Rap. Because there was no obvious change in LysoTracker staining (Fig. S4A), this decrease is not likely to be a result of gross lysosomal/endosomal acidification defects per se.

Second, live cell imaging was used to examine the dynamics of A:L fusion. Previous studies have shown that autophagosomes engage in homotypic and heterotypic fusions, including complete fusion with lysosomes to form autolysosomes, kiss-and-run fusion, and extension of tubules to reach targets (12). Under our experimental conditions, complete heterotypic fusions predominated. For example, in the time-lapse frames for Ctrl cells (Fig. 4B, sequence 1), the green LC3 and red lysosomal-associated membrane protein 1 (LAMP1) puncta formed a hybrid yellow autolysosome by the 130-s frame. Subsequently, the yellow puncta turned red, most likely as a result of quenching of GFP-LC3 fluorescence in the acidic autolysosomes (also see Fig. S4B and Movie S1). In contrast, in siPI4KIIα cells, the majority of autophagosomes did not fuse with lysosomes (Fig. 4B, sequence 2). In some cases, autophagosomes transiently contacted lysosomes (e.g., at 410 s) but rapidly separated (Fig. S4B and Movies S2 and S3). Particle tracking analyses showed that the contact periods between the pairs in PI4KIIα-depleted cells ranged from 5 to 35 s (mean ± SEM: 16 ± 6 s; n = 5). Interestingly, some vesicle pairs with long apparent dwell times underwent successive rounds of partial “engagement and disengagement” before completely separating (Fig. S4B and Movie S4). Occasionally, some large LC3 autophagosomes extended tubules to contact lysosomes but did not fuse (Fig. 4B, sequence 3, Fig. S4B, and Movie S3). Taken together, our results established that PI4KIIα depletion inhibits autophagosome acidification by blocking A:L fusion.
**GABARAPs Recruit PI4KIIα to Autophagosomes.** Because PI4KIIα coimmunoprecipitated with GABARAPs (4), we determined they are functionally related in autophagy. Coimmunoprecipitation assays confirmed that PI4KIIα-bound GFP-GABARAPs, but not GFP-LC3 (Fig. 5A and Fig. S5A). Furthermore, they were colocalized in autophagic cells (Fig. 5A). The shortest N- and C-terminal truncated myc-PI4KIIα tested (aa 92–397) that bound GFP-GABARAP contains both the catalytic and palmitoylation motif (8) (Fig. 5A). Unexpectedly, SSPPS myc-PI4KIIα, which is neither palmitoylated nor autophagosome-associated (Fig. 2E), also coimmunoprecipitated with GFP-GABARAP. Thus, PI4KIIα interaction with GABARAPs is necessary but not sufficient for autophagosome targeting. Additional studies will be required to determine what is required for PI4KIIα targeting, and what for preferential binding to GABARAPs.

We compared the effect of depletion either player on each other's behavior to delineate their upstream/downstream relations. siPI4KIIα generated large GFP-LC3 puncta that contained YFP-GABARAPs, but not Effects on mRFP-PI4KIIα structural features as in siCtrl cells (Fig. 5C). In contrast, although siGABARAP depletion (3) (Fig. S5C) also generated enlarged autophagosomes, these abnormal autophagosomes were deficient in both PI4KIIα and PI4P (Fig. S5C and D and Fig. S5B). Because the large autophagosomes in siGABARAP cells have similar ultrastructural features as in siPI4KIIα cells (Fig. 3B), GABARAPs are placed upstream of PI4KIIα. Thus, GABARAPs are recruited to autophagosomes independent of PI4KIIα, and they recruit PI4KIIα to autophagosomes in a palmitoylation-dependent manner.

**PI4KIIα Acts Downstream of GABARAPs.** To confirm this hierarchy, we examined whether overexpression of the putative downstream effector (PI4KIIα) compensates for the depletion of the upstream controllers (GABARAPs). Overexpression of WT myc-PI4KIIα decreased accumulation of enlarged autophagosomes and GFP-LC3 puncta intensity in siGABARAP cells (Fig. 6A and D). Rescue is dependent on PI4P generation, because KD myc-PI4KIIα did not rescue. We hypothesize that PI4KIIα overexpression may have rescued the siGABARAP phenotype by increasing promiscuous, GABARAP-independent PI4KIIα association/PI4P generation in all membranes.

PI4P "shuttling" (6) was used to determine whether the siPI4KIIα or siGABARAPs autophagy block is a direct result of PI4P depletion. PI4P shuttling decreased the size of the GFP-LC3 puncta and also promoted GFP-LC3 degradation, as assessed by GFP-LC3 staining intensity (Fig. 6C) and by LC3 Western blotting (Fig. 6D). In contrast, PI3P shuttling did not rescue. These results establish conclusively that PI4P directly promotes A:L fusion directly and independent of its subsequent conversion to PI3P.

**PI4KIIα and PI4KIIβ Have Different Roles in Autophagy.** Because we showed that PI4KIIα promotes A:L fusion and is present on autophagosomes, we next asked whether PI4KIIα is also constitutively associated with lysosomes. We found that in growing cells,
expression fails to rescue the siPI4KIIα-exposed to EBSS. Bar graph, percentage siPI4KIIα cells with large LC3 autophagosomes in PI4KIIβ-depleted cells had decreased overlap with LAMP1 (Fig. S6B). In contrast, neither siPI5Kβ nor siPI4KIIIβ affected LAMP1:GFP-LC3 colocalization to a similar extent. Significantly, myc-PI4KIIβ neither translocated to autophagosomes nor rescued the enlarged autophagosomes in siPI4KIIα cells (Fig. 7B). Thus, PI4KIIα is uniquely poised to control A:L fusion using a mechanism distinct from that of PI4KIIβ, and without a requirement for downstream PI3P generation.

**Discussion**

We establish that GABARAPs recruit palmitoylated PI4KIIα from the Golgi to autophagosomes, and that PI4P generation in situ promotes A:L fusion. Our results raise the intriguing and hitherto unexplored possibility that GABARAPs’ role in autophagosome maturation may be integrated with their established role as modulators of transmembrane protein trafficking. The requirement for GABARAPs, PI4KIIα, and PI4P for A:L fusion was established using multiple approaches. Because autophagosomes undergo homotypic as well as heterotypic fusions (12), we hypothesize that the abnormally large autophagosomes in sGABARAPα and sPI4KIIα cells may have been generated by multiple default homotypic fusion events when heterotypic fusion is blocked.

GABARAPs are placed upstream of PI4KIIα and PI4P, and our working model is summarized in Fig. 8. GABARAP depletion generates enlarged autophagosomes that are PI4KIIα- and PI4P-deficient and have decreased autophagic flux. The GABARAP depletion phenotype can be rescued either by overexpressing WT PI4KIIα (but not KD PI4KIIα) or by shutting PI4P. Thus, PI4P generation, and not simply tethering by PI4KIIα within the GABARAP interactome, is required. Because rescue occurs even in the absence of GABARAPs, GABARAPs’ primary role in this context is to increase PI4P. PI4KIIα depletion also generated enlarged autophagosomes that contain GABARAPs, but not PI4P, placing it downstream of GABARAPs. This phenotype can be rescued by PIP2, but not PIP3, shuttling. Unlike PI4KIIα, PI4KIIβ does not reside on autophagosomes and fails to rescue the enlarged autophagosome phenotype in PI4KIIα-depleted cells. Thus, PI4KIIα is uniquely responsible for promoting A:L fusion.

We further propose that GABARAPs recruit PI4KIIα to autophagosomes through vesicular trafficking from the Golgi because, first, PI4KIIα is palmitoylated primarily at the Golgi (8, 9), and only palmitoylated PI4KIIα translocates to autophagosomes. GABARAPs may direct trafficking of palmitoylated PI4KIIα from the Golgi to autophagosomes, analogous to their previously identified role as facilitators of GABAergic trafficking to the plasma membrane (2). Second, PI4KIIα has already been implicated in constitutive exocytic, endo/lysosomal trafficking (6, 15, 16), and its recruitment to autophagosomes is decreased by GABARAP depletion. Third, there is emerging evidence that lipids enriched in raft microdomains may be involved in autophagosome maturation. Finally, trafficking of Golgi and late/recycling endosomes, which are rich in PI4KIIα, is much less than that for PI4KIIβ.

**Table: siRNA Targets and siRNA Phenotypes**

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Fig. 8. Summary of results and proposed hierarchy in the GABARAP interactome. PI4KIIα or GABARAP deletion individually blocks A:L fusion, resulting in accumulation of enlarged autophagosomes. GABARAPs and PI4KIIα cooperate to ensure in situ generation of PI4P on autophagosomes to promote A:L fusion. The hierarchical relationships among GABARAPs, PI4KIIα, and PI4P are delineated by depletion and rescue approaches. Circles with double membranes are autophagosomes containing random cytoplasmic material and organelles.
raft-associated PI4KIIα, contributes to intermixing of membranes, leading to autophagosomal expansion (1).

Our results raise the fundamental question of how the GABARAP/PI4KIIα/PI4P interactor regulates the A:L fusion machinery. Previous studies have shown that autophagic clearance is independent steps (12) and that the GABARAP scaffolding network promotes cargo recruitment, tethering, and potentially membrane fusion (3). Our live cell imaging studies reveal that autophagosomes and lysosomes in PI4KIIα-depleted cells occasionally exhibit a transient and repetitive partial engagement/dissengagement behavior that is different from straightforward kiss-and-run. This unusual behavior suggests that stable docking and membrane hemifusion may be compromised.

Because shuttled PI4P rescued the effects of deleting either GABARAPs or PI4KIIα in late-stage autophagy, PI4P is strongly implicated in promoting late-stage autophagy. By analogy to PI3P, which regulates early-stage autophagy by creating a platform to recruit specific effectors, PI4P may provide a platform for assembling Rab7 modulators, and interacting proteins (10).

Shuttling PI4P, generated by the v-erbB PI4KIIIα, and its yeast homolog, Pik1, has previously been shown to directly regulate sorting from lysosomes to preserve lysosomal identity (13) and Atg9 Golgi trafficking (17), as well as indirectly by supporting PI3P synthesis to promote autophagic biogenesis (18) and autophagic lysosome reformation (13, 14). Here we show that the PI4P generated by PI4KIIα on autophagosomes has a different and unique role. We hypothesize that this PI4P pool may interact with some of the proteins recently implicated in A:L fusion, such as the novel autophagosomal SNARE syntaxin 17, Rab7 modulators, and interacting proteins (10).

Our findings provide a mechanistic model for integrating two previously unrelated GABARAP functions to promote PI4KIIα-and PI4P-dependent A:L fusion. The unique contributions of PI4KIIα to the GABARAP interactor also raise the intriguing possibility that some of the human disorders ascribed to defects in PI4KIIα (5) may arise partly as a result of dysfunctional PI4KIIα-dependent autophagic clearance.

Materials and Methods

See SI Materials and Methods for detailed description.

Reagents and Plasmids. Anti-PI4P and PI4P/IP3P "Shuttling" Kits were from Echelon Bioscience. Plasmids are as described previously. Protein overexpression was kept at a low level by using low plasmid concentrations and short periods of overexpression.

Cell Lines, siRNAs, and Rescue. HeLa cells cultured in DMEM containing 10% (vol/vol) FBS were transfected with epitope-tagged CDNAs using Lipofectamine 2000. Previously validated siRNAs that target human PI4KIIα (6), PI5P5KII (19), and PI4KIIβ (20) or Ctrl siRNA were from Sigma. The siGABARAP-GATE16 pool was directed against all members of the family (3). In some experiments, a HeLa cell line that stably expresses GFP-LC3 (HeLa/GFP-LC3) or a COS cell line that stably expresses GFP-PI4KIIα was used.

Autophagy Assays. For most experiments, cells were cultured in DMEM/10% (vol/vol) FBS, and autophagy was induced by treatment with EBBS (for 30 min to 2 h) or DMEM/Rap (1 μM, for 2–4 h). In some cases, cells were transferred to Opti-MEM/5% (vol/vol) FBS for 4 h to minimize basal autophagy and were then exposed to autophagic stimuli. Bulk autophagic flux was determined by Western blotting of LC3 or GFP-LC3, and band intensity was quantified using NIH Image software, as described previously (3). LC3 flux was calculated from the intensities of LC3-I and LC3-II bands and normalized against actin loading controls. Autophagosome acidification was determined by transfecting the tandem mRFP-GFP-LC3 fusion construct 24 h after siRNA treatment.

Cells were treated with Baf A1 (100 nM) for 12 h, washed to release the Baf A1 block, and fixed at 0, 1, and 3 h with 4% (vol/vol) paraformaldehyde. Puncta with yellow (mRFP+GFP) or red fluorescence were quantified.

Communoprecipitation. HEK-293 cells were cotransfected with myc-PI4KIIα variants, and GFP-Atg8 plasmids were processed for communoprecipitation using anti-myc or anti-GFP and protein G-agarose and subjected to Western blotting.

Microscopy and Image Analysis. Cells grown on coverslips were processed for immunofluorescence microscopy and examined by laser scanning confocal microscopy (Zeiss LSM 510 META UV/Vis). Colocalization analysis and time-lapse recordings were described in SI Materials and Methods. HeLa/GFP cells exposed to EBBS were prepared for conventional electron microscopy or correlative light and electron microscopy, as described previously (21).

PI4P/PIP2 "Shuttling." Cells transfected with siRNA for 48 h were replated on coverslips for 12–24 h. They were washed with serum-free DMEM twice, before incubation at 37 °C with or without PI4P as described previously (21). The diC16 PI4P and PIP2, and their optimized carriers were mixed at a 1:1 molar ratio for 15 min at room temperature before adding to the culture medium to a final phosphoinositide concentration of 50 μM. Autophagy was initiated at the same time as shuttle addition.

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Supplemental Information

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SI Materials and Methods

Plasmids. Epitope-tagged PI4KIIα cDNAs, myc-PIP5Kβ, GFP-OSBP-PH, mRFP-GFP-LC3, and GFP-LC3 were described previously in our publications. GFP-GABARAP, mCherry-GATE16, and DSRED-LAMP1 plasmids were gifts of Zvulun Elazar.

siRNAs. siRNAs were transfected with RNAiMAX, and cells were analyzed after 48–72 h. In rescue experiments, cells were transfected with plasmids using RNAiMAX 48 h after the initial siRNA transfection and used 24 h later.

Immunofluorescence Microscopy and Image Analysis. Colocalization in serial Z stacks of optical sections was analyzed by using the Imaris Autoquant software, version 7.6.5 (Bitplane). Colocalization in a single confocal plane was determined using the Zeiss LSM510 software. ImageJ (NIH) was used to quantify fluorescence intensity (corrected integrated density function) and particle size. Endogenous LC3 was stained after methanol fixation for 5 min at −20 °C and permeabilization in cold acetone. This procedure improves signal-to-noise ratio and reports endogenous LC3 localization more reliably than the conventional triton permeabilization procedure. PI4P was stained using a fixation protocol optimized for preserving PI4P in internal membranes.

Time-Lapse Microscopy. HeLa/GFP-LC3 cells were transfected with siCtrl or siPI4KIIα for 48 h, and then with pDSRED-LAMP1. After 24 h, cells were treated with EBSS for 1 h, placed in an environmental chamber with 5% (vol/vol) CO2, and held at 37 °C. Cells were imaged on a Zeiss LSM 510 confocal microscope, using a ×63 1.4 NA plan Apochromat oil immersion lens at 37 °C. Two hundred images (488- and 543-nm laser lines) were collected with a 5-s cycle delay and analyzed with the Zeiss LSM Image Browser software. The Imaris version 7.7.5 particle tracking algorithm (Bitplane) was used to analyze particle trajectory.

Electron Microscopy. For correlative light and electron microscopy, HeLa/GFP-LC3 cells were plated in 35-mm dishes containing a glass coverslip with grids (MatTek Corp), fixed and imaged by fluorescence microscopy, and subsequently processed for electron microscopy by the University of Texas Southwestern Electron Microscopy Core Facility.

Statistical Analyses. Data were expressed as mean ± SEM, and *P* < 0.05 is considered statistically significant. A two-tailed unpaired test was performed to compare two groups, and a one-way ANOVA analysis of variance was used to compare values among multiple groups, using the Sigma Plot software.

![Fig. S1.](image-url)

PI4KIIα depletion blocks appearance of extra-Golgi PI4P puncta after EBSS treatment. (A) Western blot with anti-PI4KIIα and anti-actin. (B) Quantitation of percentage of cells with abundant scattered PI4P puncta (mean ± SEM; n = 3; ***P < 0.001), similar to those shown in Fig. 1. Fifty cells per condition were analyzed per experiment in a blinded fashion.
Fig. S2. LC3 localization. (A and B) Z stacks from EBSS- or Rap-treated HeLa cells showing LC3 localization relative to GFP-OSBP-PH and myc-PI4KI\textalpha. Related to Fig. 2 C and D. (C) SSPSS myc-PI4KI\textalpha was expressed in HeLa/GFP-LC3 cells. Related to Fig. 2E.
**Fig. S3.** Autophagy flux. (A) Effects of si\(\text{PI4KII}\alpha\) on p62/SQSTM1 degradation. (B) Effects of KD PI4KII\(\alpha\) overexpression on autophagosomal size and autophagic flux. HeLa/GFP-LC3 cells with or without overexpressed KD myc-PI4KII\(\alpha\) were starved in EBSS and stained with anti-myc. (Scale bar, 5 \(\mu\)m.) (C) GFP-LC3 autophagic flux determined by Western blotting. Densitometry results are shown in Fig. 3C.

**Fig. S4.** Effect of si\(\text{PI4KII}\alpha\) on acidification and A:L fusion. (A) LysoTracker labeling. Starved siCtrl and si\(\text{PI4KII}\alpha\) HeLa cells were starved and incubated with LysoTracker Red DND-99 to label acidic organelles. Some cells were pretreated with Baf A1 to inhibit acidification. Related to Fig. 4A. (B) Time-lapse microscopy showing movement of GFP-LC3 particles (autophagosomes) and RFP-LAMP1 particles (lysosomes). HeLa/GFP-LC3 cells without or with si\(\text{PI4KII}\alpha\) transfection were retransfected with pH2Red-LAMP1, starved, and subjected to time-lapse microscopy. Videos generated from time-lapse images are shown in Movies S1–S4. Related to Fig. 4B.
Fig. 5A. Relation between GABARAPs and PI4KIIIα. (A) GFP-GABARAP pull-down of myc-PI4KIIIα. Reciprocal of Fig. 5A. (B) Effects of siPI4KIIIα on GFP-LC3 and mCherry-GATE-16 colocalization. (Top) siCtrl. (Bottom) siPI4KIIIα. Related to Fig. 5B and C. (C) Quantitation of GABARAP depletion. mRNA levels of GABARAP, GABARAPL1, and GATE-16 from cells transfected with a mixed pool of GABARAP siRNA was determined by quantitative PCR and expressed relative to Ctrl (mean ± SEM; n = 3).
Fig. S6. PI4KIIα has a unique role in autophagy. HeLa/GFP-LC3 cells were used. (A) Quantitation of mRNA level after siRNA targeting. mRNA levels were determined by quantitative PCR and expressed relative to Ctrl (mean ± SEM; n = 3). (B) Differential effects of siPI4KIIα, siPIP5Kβ, and siPI4KIIIβ on LAMP1 colocalization with GFP-LC3. HeLa/GFP-LC3 cells transfected with siRNAs were starved and stained with anti-LAMP1. (C) Comparison of effects of siRNA on percentage colocalization of LAMP-1 with GFP-LC3. Values were normalized against Ctrl value set at 100. Forty-five cells from three experiments were quantified per condition. *P < 0.05; ***P < 0.001.
Movie S2. Starved siPI4KIIα cell, showing enlarged autophagosomes and fewer productive A:L fusions.

Movie S3. Starved siPI4KIIα cell, showing an abnormally enlarged autophagosome extending tubules to contact lysosomes, but failing to fuse.
**Movie S4.** Particle tracking analysis in a starved siPI4KIIα cell, showing that an autophagosome and lysosome pair engage in successive rounds of “engagement and disengagement” without fusion in the time frame analyzed.