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Control of Rab7a activity and localization through endosomal type Iγ PIP 5-kinase is required for endosome maturation and lysosome function

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Abstract

The small GTPase Ras-related protein Rab-7a (Rab7a) serves as a key organizer of the endosomal-lysosomal system. However, molecular mechanisms controlling Rab7a activation levels and subcellular translocation are still poorly defined. Here, we demonstrate that type Iγ phosphatidylinositol phosphate 5-kinase i5 (PIPKIγi5), an endosome-localized enzyme that produces phosphatidylinositol 4,5-bisphosphate (PI4,5P₂), directly interacts with Rab7a and plays critical roles in the control of endosomal-lysosomal system. The loss of PIPKIγi5 blocks Rab7a recruitment to early endosomes, which prevents the maturation of early to late endosomes. PIPKIγi5 loss disturbs retromer complex connection with Rab7a, which blocks the retrograde sorting of Cation-independent Mannose 6-Phosphate Receptor (CI-MPR) from late endosomes. This leads to the decreased sorting of hydrolases to lysosomes and reduces the autophagic degradation. By modulating retromer-Rab7a connection, PIPKIγi5 is also required for the recruitment of the GTPase-activating protein (GAP) TBC1 domain family member 5 (TBC1D5) to late endosomes, which controls the conversion of Rab7a from the active state to the inactive state. Thus, PIPKIγi5 is critical for the modulation of Rab7a activity, localization, and function in the endosomal-lysosomal system.

Keywords

PIPKIγi5; Rab7a; endosome maturation; lysosome; autophagy

Introduction

As major protein-sorting stations in the cell, endosomes are critical to maintaining cellular homeostasis (1). Endosomes play an essential role in determining the fate of membrane proteins that enter the endocytosis pathway. Proteins internalized from the plasma membrane, including signaling receptors, nutrient transporters, and adhesion molecules, first accumulate in endosomes (2). In the endosomes, these protein cargos are either sorted to

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Author contributions

Y. Sun designed the research; M. Sun, G. Luong, and F. Plastikwala performed research; Y. Sun and M. Sun analyzed data; Y. Sun wrote the paper.

lysosomes for final degradation or spared from degradation. The retrieved proteins are sorted and recycled to the plasma membrane or delivered to the secretory pathway (3). In this way, the endosomal system controls the abundance and spatial organization of cell membrane proteins, which is critical for regulating fundamental functions such as cell signaling, nutrient uptake, membrane turnover, cell movement, and development (4).

Endosomes also mediate the transport of lysosomal components from the trans-Golgi network (TGN) to lysosomes. The majority of soluble acid hydrolases are sorted from the TGN to endosomes by Cation-independent Mannose 6-Phosphate Receptor (CI-MPR) (5). The hydrolases are ultimately delivered to lysosomes by late endosome-lysosome fusion (6, 7). CI-MPRs are recycled from late endosomes/lysosomes to the TGN to mediate another round of hydrolase sorting (8). Therefore, the functions of endosomes and lysosomes are tightly linked. Abnormalities in both endosomes and lysosomes are highly involved in various human pathologies, such as neurodegenerative disorders and cancer (9, 10).

The small GTPase Ras-related protein Rab-7a (Rab7a) is one of the master regulators that organize trafficking events through the endosomal and lysosomal systems (11, 12). As a GTPase, the activation of Rab7a is controlled by binding with GTP or GDP. The shuttling of Rab7a between the inactive state (GDP-bound) and the active state (GTP-bound) controls its subcellular localization and modulates its interactions with effector proteins to affect its biological function. Active Rab7a is mainly located in late endosomes (13). It can also be recruited to early endosomes, in which it is required for mediating the maturation of early endosomes into late endosomes (14, 15). Furthermore, Rab7a controls transport from endosomes to lysosomes, the biogenesis of lysosomes, and the fusion of late endosomes with lysosomes (12). Rab7a also plays a critical role in the control of the autophagic pathway (16, 17). Rab7a associates with the membranes of autophagic vesicles and mediates autophagosome maturation. The expression of dominant-negative Rab7a causes the accumulation of larger autophagosomes (16). The control of Rab7a functioning is critical for the organization of these important trafficking events. However, the molecular mechanisms that control Rab7a activation levels and subcellular translocation are still poorly defined. Here, we identified PIPKI γ 5 as a novel regulator of Rab7a.

PIPKI γ 5 is a splicing variant of type I γ phosphatidylinositol phosphate (PIP) kinase (PIPKI γ), an enzyme that synthesizes phosphatidylinositol 4,5-bisphosphate (PI4,5P₂) by the phosphorylation of phosphatidylinositol-4-phosphate (PI4P) (18, 19). The human PIPKI γ gene is alternatively spliced, leading to the production of at least six PIPKI γ variants that are named PIPKI γ 1-i6 (19, 20). These variants have identical N-termini and kinase domains, and they contain unique extensions just at the C-terminus (19, 20). By interacting with specific binding partners via their unique C-termini, these PIPKI γ variants can be recruited to distinct subcellular compartments and play different biological roles. PIPKI γ 5 is the only variant that can be recruited to the membranes of endosomes. By cooperating with Sorting Nexin 5 (SNX5) and Lysosomal-associated transmembrane protein 4B (LAPTM4B), PIPKI γ 5 controls epidermal growth factor receptor (EGFR) sorting from endosomes to lysosomes for degradation (21–23). In this way, PIPKI γ 5 is critical for down-regulating EGFR-mediated signaling.

Here, we show that PIPKI γ 5 plays a critical role in regulating the whole endosomal-lysosomal system. Because of its function in modulating Rab7a, PIPKI γ 5 is required for the maturation of early endosomes into late endosomes. PIPKI γ 5 also controls lysosome function by modulating hydrolase sorting to lysosomes.

Materials and Methods

Cell lines and culture conditions.

The human head and neck squamous carcinoma cell line UMSCC1 was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% MEM nonessential amino acids, and 0.5 μ g/mL hydrocortisone. HEK-293FT and MEF cells were cultured in DMEM supplemented with 10% FBS.

PIPKI γ 5^{-/-} Mice and MEF cells.

PIPKI γ 5^{-/-} mice (*Pip5k1c* exon 17-del) were generated by the Virginia Commonwealth University (VCU) Transgenic/Knockout Mouse Core in a C57BL/6 genetic background. To generate the PIPKI γ 5^{-/-} mice, a CRISPR/Cas9 approach was employed (24). Two guide RNA protospacer sequences (Fig. S2), located at the 5' end of exon 17 and just beyond the 3' end of exon 17, were selected using the Broad Institute sgRNA design tool (25). Homology-directed repair at the Cas9 cleavage sites utilized a 200-base sense-strand single-stranded oligodeoxynucleotide (ssODN) (Fig. S2) to delete a 102-bp region that included exon 17 and its splice acceptor and donor sites.

For the genotyping of PIPKI γ 5^{-/-} mice, DNA was purified from 5-mm tail snips and genotyped by a PCR protocol to detect the 102-bp deletion in the *Pip5k1c* exon 17-del allele. The protocol employed an intron 16 forward primer (5'-CACCTGTATCCCCTGCCTGGTTC-3') and an intron 17 reverse primer (5'-GTCACAGGGCTTCTCTGTCTCTG-3') to generate 304-bp wild-type and 202-bp exon 17-del PCR products. To further confirm the deletion of the targeted region, a 572-bp region in mice homozygous for the *Pip5k1c* exon 17-del allele was sequenced.

MEF lines were produced from harvested embryos from female mice on 14th days after the appearance of plug.

siRNA.

The following siRNAs were ordered from Dharmacon (Lafayette, CO, USA): VPS26 siRNA, SNX5 siRNA, TBC1D5 siRNA, VPS35 siRNA, Rab7a siRNA. The siRNAs ordered from Santa Cruz Biotechnology (Dallas, TX, USA): the control scramble siRNA, CD-MPR siRNA, CI-MPR siRNA. Human PIPKI γ 5 siRNA and PIPKI γ 2 siRNA were synthesized from Dharmacon (Lafayette, CO, USA). The siRNA sequences for human PIPKI γ 5 are PIPKI γ 5 siRNA_1 5'-GGAUGGGAGGUACUGGAUU-3' and PIPKI γ 5 siRNA_2 5'-CAGAAGGGCUUUGGUAA-3'. PIPKI γ 5_1 siRNA was used for all the experiments using PIPKI γ 5 siRNA including Fig. S5C. Human PIPKI γ 2 siRNA is 5'-GAGCGACACAUAUUUCUA-3'. Oligofectamine (ThermoFisher Scientific, Waltham, MA, USA) was used for siRNA transfection.

Antibodies.

The following antibodies were ordered from Cell Signaling Technology (Danvers, MA, USA): LC3, p62, GST, CI-MPR, CTSB, Rab7a, Rab5, LAMP1, anti-Myc-Tag. Antibodies ordered from Santa Cruz Biotechnology (Dallas, TX, USA): tubulin, CD-MPR, TBC1D5, SNX6. Antibodies ordered from Abcam (Cambridge, United Kingdom): SNX5, VPS26, VPS35, EEA1, RILP. Anti-HA antibody was from BioLegend (San Diego, CA, USA).

Lysosome enrichment.

A lysosome isolation kit (LYSISO1, Sigma, St. Louis, MO, USA) was used to isolate the enriched lysosomal fraction by density gradient centrifugation according to the manufacturer's instructions. UMSCC1 cells were resuspended in ice-cold PBS and then lysed in a 7 ml Dounce homogenizer. After 20 strokes, the samples were centrifuged at 1000 g for 10 minutes at 4°C. The supernatant was transferred into a new tube and centrifuged at 20,000 g for 20 minutes, and the pellets were collected for further purification with density gradient medium. The pellets were diluted with 19% Optiprep solution, and a step gradient with 8% medium at the top and 27% medium at the bottom was generated. After centrifugation at 150,000 g for 4 hours in a SW50.1 rotor, multiple floating bands were observed in the gradient, and the fractions were withdrawn for quantification by a BCA assay kit.

Immunoprecipitation and immunoblotting.

Immunoprecipitation was performed as previously described (26). Briefly, cells were harvested and lysed in 25 mM HEPES (pH 7.2) with 150 mM NaCl, 0.5% NP-40, 1 mM MgCl₂, and protease inhibitor cocktail, and then the cells were centrifuged and incubated with protein G-sepharose and 2 µg antibody as indicated at 4 °C for 4 hours. The immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed as indicated.

Rab7a-GTP pulldown assay.

Cells were lysed in reaction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 10% glycerol, 5 mM MgCl₂, and 1 µM DTT). Two micrograms of HA-RILP was mixed with 500 µg of cell lysate at 4°C overnight, and then 30 µL of resin slurry consisting of HA magnetic beads was added and incubated for one hour. The HA magnetic beads were separated by a magnetic rack and washed three times with reaction buffer. The beads were then resuspended in Western blot loading buffer. The protein complexes pulled down by HA magnetic beads were separated by SDS-PAGE and analyzed as indicated.

Immunofluorescence.

Cells were resuspended and plated on coverslips. After 24 hours, the cells were rinsed with PBS and then fixed with 4% paraformaldehyde (PFA). The cells were permeabilized with 0.5% Triton-X100 and blocked with 3% BSA in PBS for 90 minutes at room temperature (RT), after which they were incubated with primary antibody overnight at 4°C, washed with 0.1% Triton X-100 in PBS, and incubated with fluorescently labeled secondary antibody for 60 minutes at RT. The cell nuclei were stained with DAPI solution for 1 minute and then

washed with 0.1% Triton X-100 in PBS. The cells were examined using a 60x-plan oil immersion lens on an inverted microscope (Axio Observer, Carl Zeiss, Oberkochen, Germany) or by a confocal laser microscope (Zeiss LSM 700 confocal microscope system, Carl Zeiss, Oberkochen, Germany) with a 63x oil immersion objective. The images were processed with Zen software provided by Zeiss.

Transmission electron microscopy (TEM).

Cells were grown on Permanox dishes (Electron Microscopy Sciences, Hatfield, PA, USA) initially fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and rinsed with 0.1 M cacodylate buffer. Then, the cells underwent secondary fixation in 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer, followed by rinses in 0.1 M cacodylate buffer, en bloc staining with 0.5% uranyl acetate, and dehydration with acetone (50%, 70%, 80%, 90%, and 3 changes of 100%). The cells were embedded in EMBED 812 resin, sectioned with a Leica UC6 Ultramicrotome (the sections were 65 nm thick), and stained with uranyl acetate and lead citrate. The images were acquired with a JEOL JEM-1400 Plus transmission electron microscope (JEOL USA, Inc. Peabody, MA, USA) and a Gatan OneView CMOS camera (Gatan Inc., Pleasanton, CA, USA).

Immunofluorescence colocalization analysis:

The colocalization analysis was performed by the Image-Pro platform (Image-Pro Plus 10, Media Cybernetics, Rockville, MD, USA) or ImageJ. Briefly, images of at least 50 cells per condition were obtained with the same parameters in terms of laser power and photomultiplier gain. Single channel images were converted into 8-bit grayscale images, and the threshold was adjusted to subtract the background. The ImageJ “Analyze Particles” function was used to identify and count the total number of structures in channel 1 (Green), and then the structures in channel 2 (Red) were used to build a mask that was then overlapped with the structures in channel 1. All of the statistical details of the experiments including the statistical tests used, the number of replicates and precision measures are shown in each figure legend. All data analysis was performed using Prism (GraphPad, San Diego, CA, USA).

Quantification of the protein interactions via coimmunoprecipitation assay:

Indicated proteins in the immunoprecipitated complex were detected by Western blot, and the amounts of these proteins were quantified via ImageJ. The amount of co-IPed proteins were normalized to the amount of protein available for coimmunoprecipitation (co-IP) (as compared in the lysate input panels). This value was then normalized to the amount of immunoprecipitated protein. At least three independent experiments were performed for each assay, and the data were quantified as the mean $\pm$ SEM. All of the statistical details of the experiments including the statistical tests used, the number of replicates and precision measures are shown in each figure legend. All data analysis was performed using Prism (GraphPad, San Diego, CA, USA).

Results

PIPKI γ 5 interacts with Rab7a.

The endosomal PI4,5P₂-producing kinase PIPKI γ 5 is required for the sorting of EGFR from endosomes to lysosomes for degradation (21). This suggests that PIPKI γ 5 plays an important role in endosomal trafficking. By exploring the possible effectors of PIPKI γ 5 that control the endosomal system, we identified the interaction between Rab7a and PIPKI γ 5. As shown in Fig. 1A, endogenous PIPKI γ 5 can be coimmunoprecipitated with Rab7a, indicating the interaction between PIPKI γ 5 and Rab7a. Other PIPKI γ splicing variants, such as PIPKI γ 1 and PIPKI γ 2, cannot bind Rab7a, which indicates the specificity of the PIPKI γ 5-Rab7a interaction (Fig. S1A and S1B). The PIPKI γ splicing variants have differences only in their C-termini (19), which suggests that the specific C-terminal tail of PIPKI γ 5 is responsible for the interaction with Rab7a. A series of PIPKI γ 5 C-terminal truncation mutants was made (Fig. S1C), and the capability of the mutants to interact with Rab7a was examined by co-IP assays. As shown in Fig. S1D and S1E, the amino acids between 645 and 654 in PIPKI γ 5 are required for its interaction with Rab7a. To further determine whether the PIPKI γ 5-Rab7a interaction is direct or indirect, an *in vitro* pulldown assay was performed. His₆-Rab7a was loaded with the nonhydrolyzable GTP analogue GMP-PNP or loaded with GDP, and subjected to *in vitro* pulldown assay with PIPKI γ 5. Both Rab7a-GTP and Rab7a-GDP bind PIPKI γ 5 similarly *in vitro*, which demonstrated that the PIPKI γ 5-Rab7a interaction is direct (Fig. 1B). This also suggests that the activation state of Rab7a does not affect its binding with PIPKI γ 5. To further confirm this, a constitutively active Rab7a mutant Rab7a-Q67L and a dominant-negative mutant Rab7a-T22N were used. As shown in Fig. S1F, both Rab7a-Q67L and Rab7a-T22N can bind PIPKI γ 5 similarly to wild-type Rab7a. This further supports that the Rab7a-PIPKI γ 5 interaction is not modulated by Rab7a activation state. Rab7b is another Ras-like GTPase that shares approximately 50% identity and 65% similarity with Rab7a (27). Unlike Rab7a, Rab7b cannot bind with PIPKI γ 5 (Fig. S1G). This indicates the specificity of Rab7a-PIPKI γ 5 interaction.

Immunofluorescence (IF) assays were performed to further detect the subcellular localization of PIPKI γ 5 and Rab7a. These two proteins show strong colocalization in endosomal vesicles (Fig. 1C), suggesting that they may function together in the endosomal system.

PIPKI γ 5 controls the subcellular localization and activation level of Rab7a.

The effects of the loss of PIPKI γ 5 on Rab7a subcellular localization were examined. The colocalization of Rab7a with the late endosome/lysosome marker LAMP1 was monitored to measure the translocation of Rab7a to late endosomes/lysosomes. As shown in Fig. 1D and 1E, the translocation of Rab7a to late endosomes/lysosomes was significantly enhanced when PIPKI γ 5 expression was knocked down. The translocation of Rab7a to late endosomes/lysosomes requires the conversion of Rab7a from an inactive state (GDP-bound) to an active state (GTP-bound). To determine whether PIPKI γ 5 affects the levels of active Rab7a, the levels of Rab7a-GTP were detected by a pulldown assay with the Rab-interacting lysosomal protein (RILP) (28). The RILP protein specifically binds to Rab7a-GTP but not

Rab7a-GDP. As shown in Fig. 1F and 1G, the loss of PIPKI γ 5 significantly increased the levels of active Rab7a (Rab7a pulled down by RILP). These results suggest that PIPKI γ 5 is an important regulator of Rab7a.

The effect of PIPKI γ 5 loss on Rab7a activation levels was further validated in PIPKI γ 5 knockout mouse embryonic fibroblasts (MEF). Exon 17 of the mouse *Pip5k1c* gene (encoding PIPKI γ) uniquely encodes the distinct C-terminus of PIPKI γ 5. Therefore, the specific deletion of exon 17 can knock out PIPKI γ 5 expression without affecting the expression of other PIPKI γ splicing variants. We employed a clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 strategy to precisely delete exon 17 using two single guide RNAs (sgRNAs) that were located at each end of exon 17 and a ssDNA donor that directly linked intron 16 and intron 17 (Fig. S2). The genotypes of the *PIPKI γ 5^{-/-}* mice were confirmed by PCR genotyping and sequence analysis. The *PIPKI γ 5^{-/-}* mice survived to adulthood, and they were fertile and had normal litter sizes. We established MEFs derived from both *PIPKI γ 5^{+/+}* (WT) and *PIPKI γ 5^{-/-}* (KO) mouse embryos, and the absence of PIPKI γ 5 in the knockout cells was verified by Western blotting (Fig. 1H). Similar to PIPKI γ 5 knockdown human cells, *PIPKI γ 5^{-/-}* MEF cells show enhanced levels of active Rab7a (Fig. 1H and 1I) compared with those in *PIPKI γ 5^{+/+}* MEFs. This finding indicates that these phenotypes, which are caused by the loss of PIPKI γ 5, are common in both humans and mice. Re-expressing of PIPKI γ 5 in *PIPKI γ 5^{-/-}* MEF cells can decrease active Rab7a levels (Fig. 1H and 1I). This further supports the function of PIPKI γ 5 in modulating Rab7a activation levels.

PIPKI γ 5 is required for Rab7a recruitment to early endosomes.

Although Rab7a is a marker of late endosomes, it can also be recruited to early endosomes, which is a key step in the maturation of early endosomes into late endosomes (29). Endocytic membrane proteins are first accumulated in early endosomes, and then the early endosomes mature into late endosomes. Finally, the late endosomes fuse with lysosomes, and the endocytic membrane proteins can be degraded by the lysosomes. Ras-related protein Rab-5a (Rab5a) is a small GTPase mainly located in early endosomes that plays a critical role in the organization of early endosomes (30, 31). During the early to late endosome maturation process, Rab5a must be replaced by Rab7a via a highly coordinated process (14, 29, 32). The recruitment of Rab7a to Rab5a-positive early endosomes indicates the start of Rab5a to Rab7a conversion. The molecular mechanisms mediating Rab7a recruitment to early endosomes are still not clear. The colocalization of PIPKI γ 5 with the early endosome marker Early Endosome Antigen 1 (EEA1) indicates that PIPKI γ 5 can translocate to early endosomes (Fig. 2A). This indication suggests that PIPKI γ 5 may play a role in Rab7a recruitment to early endosomes by interacting with Rab7a. Therefore, the effects of PIPKI γ 5 knockdown on Rab7a translocation to early endosomes were examined by IF staining. The loss of PIPKI γ 5 significantly decreased the colocalization of Rab7a with EEA1 (Fig. 2B and 2C). Consistently, PIPKI γ 5 knockdown also reduced the colocalization of Rab7a with another early endosome marker Rab5a (Fig. 2D and 2E). These results suggest that PIPKI γ 5 is required for Rab7a recruitment to early endosomes. In contrast, the loss of PIPKI γ 5 did not affect the colocalization levels of Rab5a with EEA1 (Fig. 2F and 2G), suggesting that PIPKI γ 5 is not required for Rab5a to recruit its effector EEA1 to early

endosomes. Loss of PIPKI γ 5 causes morphological change of early endosomes. EEA1-positive early endosomes in the PIPKI γ 5-knockdown cells are more aggregated and enlarged (Fig. 2B and 2F). This suggests that PIPKI γ 5 may play an important role in modulating endosome function.

PIPKI γ 5 and Rab7a are required for early to late endosome maturation.

To further determine whether PIPKI γ 5 controls early to late endosome maturation, the sorting of endocytic membrane proteins from early to late endosomes was tracked. EGFR is one of the most investigated membrane receptors for endocytosis and endosomal trafficking. Alexa Fluor 555-EGF was employed to stimulate the internalization and subsequent endosomal trafficking of EGFR. The trafficking of EGFR to early or late endosomes could be monitored by tracking the colocalization of Alexa Fluor 555-EGF with different endosome markers. As shown in Fig. 3A and 3B, after EGF treatment for 15 minutes, there was no significant difference in EGF colocalization with EEA1 in control or PIPKI γ 5 knockdown cells. This finding indicates that PIPKI γ 5 does affect the internalization and accumulation of EGFR in early endosomes. Upon treatment with EGF for a longer time, EEA1 disassociated from EGF-positive endosomes, indicating the maturation of early endosomes into late endosomes. Therefore, EGF-EEA1 colocalization was decreased in control cells. The disassociation of EEA1 from EGF-positive endosomes was significantly inhibited in PIPKI γ 5 knockdown cells (Fig. 3A and 3B). These results indicate a deficiency in early to late endosome maturation. The loss of Rab7a has effects on the inhibition of EEA1 disassociation from EGFR-containing early endosomes similar to those of the loss of PIPKI γ 5 (Fig. 3A and 3B). This phenomenon confirms that Rab7a is required for the maturation of EGFR-containing early endosomes into late endosomes. The knockdown of PIPKI γ 5 or Rab7a protein levels was confirmed by Western blot (Fig. 3C).

Consistently, the colocalization of EGF with Rab7a was dramatically decreased in PIPKI γ 5 knockdown cells (Fig. 3D and 3E). This further indicates a deficiency of EGFR-containing early endosomes maturation into Rab7a-positive late endosomes caused by the loss of PIPKI γ 5.

The blockage of EGFR-containing early endosomes maturation into late endosomes should decrease EGFR final degradation by lysosomes. To confirm this, the down-regulation of EGFR induced by EGF stimulation was examined by Western blot. As shown in Fig. S3, loss of PIPKI γ 5 or Rab7a blocked the EGF-stimulated EGFR down-regulation. This suggests that both PIPKI γ 5 and Rab7a are required for EGFR down-degradation.

PIPKI γ 5 controls autophagy flux.

Rab7a not only controls the endosomal sorting of internalized membrane proteins but also controls other critical vesicular trafficking events, such as the process of autophagic degradation (16, 17, 33). Autophagy is a catabolic degradation process whereby cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled to maintain cellular metabolism (34). Because PIPKI γ 5 interacts with Rab7a and controls Rab7a subcellular localization (Fig. 1 and 2), the possible effects of PIPKI γ 5 on the autophagic pathway were examined. The production of LC3-II, a well-established

marker of autophagy and autophagy-related processes (35), was examined in control and PIPKI γ 5 knockdown cells. As shown in Fig. 4A and 4B, the knockdown of PIPKI γ 5 dramatically enhanced the formation of LC3-II puncta. Consistently, the loss of PIPKI γ 5 increased LC3-II protein levels (Fig. 4C and 4D). Increased LC3-II levels can be associated with either enhanced autophagy initiation or reduced autophagosome turnover (36). Therefore, the levels of p62/SQSTM1, an autophagy substrate, were examined. Active autophagic degradation leads to a decrease in p62 levels (37). The loss of PIPKI γ 5 dramatically increased the p62 levels, suggesting that it blocked autophagic degradation (Fig. 4C). The blocking of autophagy initiation by Spautin-1 or wortmannin treatment in PIPKI γ 5 knockdown cells was not able to fully downregulate the levels of LC3-II or p62 so that they were equivalent to the levels in control cells (Fig. 4C and 4D). This phenomenon further supports a decreased autophagic degradation caused by loss of PIPKI γ 5.

To further trace the effect of PIPKI γ 5 loss on autophagic trafficking, IF staining with LC3-II antibodies was performed. The costaining of LC3-II and the late endosome/lysosome marker LAMP1 or LysoTracker was significantly enhanced in PIPKI γ 5 knockdown cells (Fig. 4E, 4F, and 4G). This indicates that LC3-II-containing autophagosomes can still fuse with lysosomes to form autolysosomes in PIPKI γ 5 knockdown cells. This result also suggests that autolysosome function is disturbed in PIPKI γ 5 knockdown cells so that LC3-II cannot be efficiently degraded, which causes an increase in the accumulation of LC3-II in autolysosomes. Consistently, the costaining of p62 with LysoTracker or LAMP1 was also significantly increased (Fig. 4H, 4I, and 4J). This further suggests a dysfunction of autophagy substrate degradation at autolysosomes.

Similar to the results observed in PIPKI γ 5 knockdown, LC3-II levels were significantly increased in PIPKI γ 5^{-/-} MEFs compared with those in PIPKI γ 5^{+/+} MEFs (Fig. 4K and 4L). The reintroduction of PIPKI γ 5 expression in PIPKI γ 5^{-/-} MEFs significantly decreased the LC3-II levels, indicating the presence of a rescue effect (Fig. 4K and 4L). These results further support the function of PIPKI γ 5 in modulating autophagic flux. The loss of other PIPKI γ splicing variants such as PIPKI γ 2 did not have a similar effect on autophagy as the loss of PIPKI γ 5 (Fig. S4A and S4B). This finding indicates that the effect on the modulation of autophagy is specific to PIPKI γ 5.

PIPKI γ 5 regulates the sorting of hydrolases to lysosomes and controls lysosome function.

The effects of PIPKI γ 5 on the modulation of autophagic degradation (Fig. 4) suggest that PIPKI γ 5 may be critical for lysosome function. PIPKI γ 5 and Rab7a play important roles in the endosomal system (Figs. 2 and 3). Endosomes not only control the fate of internalized membrane receptors but also control the sorting of cargos, such as lysosomal hydrolases, from the TGN to lysosomes, which is critical for normal lysosome function (38). The digestive function of lysosomes in the breakdown of biological macromolecules is mediated by lysosomal hydrolases. Over 50 soluble hydrolases, including cathepsins, have been found in lysosomes (39, 40). These hydrolases are synthesized in the rough endoplasmic reticulum and travel along the secretory pathway until they reach the TGN, where they are sorted and delivered to late endosomes/lysosomes (5, 41). The inhibition of lysosomal hydrolases, such

as cathepsins, can induce lysosome dysfunction and prevent autophagic degradation (42, 43). To determine whether PIPKI γ 5 and/or Rab7a control the sorting of hydrolases to lysosomes, the subcellular distribution of the lysosomal hydrolase cathepsin D (CTSD) was monitored. The loss of PIPKI γ 5 significantly decreased the colocalization of CTSD with the late endosome/lysosome marker LAMP1 (Fig. 5A and 5B), suggesting there was a decrease in sorting to late endosomes/lysosomes. The colocalization of cathepsin B (CTSB), another lysosomal hydrolase, with LAMP1 was also significantly decreased in PIPKI γ 5 knockdown cells (Fig. S5A and S5B). This further suggests that the sorting of hydrolases to lysosomes is disrupted by the loss of PIPKI γ 5. Cathepsin hydrolases are proteolytically processed in the acidic environment of lysosomes during maturation. Therefore, the blockade of late endosome/lysosome sorting decreases the levels of mature cathepsin hydrolases in cells (44). To confirm the changes in the levels of CTSB and CTSD hydrolases in lysosomes, the lysosome components were enriched using a lysosome isolation kit (Sigma). Mature CTSB and CTSD levels were significantly decreased in PIPKI γ 5 knockdown cells (Fig. 5C and 5D). To rule out siRNA-induced off-target effects, two different PIPKI γ 5 siRNAs were used, and both resulted in the knockdown of PIPKI γ 5 expression, enhanced LC3II levels, and a decrease in mature CTSD levels (Fig. S5C).

The morphological changes in lysosomes in PIPKI γ 5 knockdown cells were detected with an electron microscopy (EM) approach. As shown in Fig. 5E and 5F, the lysosomes were significantly enlarged in PIPKI γ 5 knockdown cells compared with those in control cells. Enlarged lysosomes always indicate impaired lysosomal degradative capacity and lysosome dysfunction (45, 46). This is consistent with the reduction in hydrolase sorting to lysosomes (Fig. 5C). To examine whether loss of PIPKI γ 5 affects lysosome pH, LysoSensor Green DND-189 was used. The LysoSensor Green DND-189 (pK_a 5.2) is brightly fluorescent at acidic pH and is almost nonfluorescent at neutral or basic pH. As shown in Fig. S5D and S5E, treatment of Bafilomycin A1, the V-ATPase inhibitor that increases lysosome pH, significantly decreased LysoSensor Green DND-189 staining fluorescence intensity. While, loss of PIPKI γ 5 did not decrease LysoSensor Green DND-189 staining fluorescence intensity compared with control (Fig. S5D and S5E). This indicates that loss of PIPKI γ 5 does not disturb the acidic environment of lysosome.

The loss of Rab7a significantly decreased the mature CTSD levels (Fig. S6A and S6B), similar to that observed for the loss of PIPKI γ 5. The loss of Rab7a also robustly increased the levels of LC3-II and increased the costaining of LC3-II with LAMP1 (Fig. S6A, S6C, and S6D), which was also similar to that observed for the loss of PIPKI γ 5. These results indicate that Rab7a and PIPKI γ 5 are both required for the sorting of hydrolases to lysosomes and the modulation of autophagy flux.

PIPKI γ 5 controls retromer-Rab7a connection and regulates CI-MPR subcellular localization.

The sorting of lysosomal hydrolases such as cathepsins from the TGN to late endosomes/lysosomes is dependent on the sorting receptor CI-MPR (5, 47). This receptor is transported from the TGN to endosomes to deliver newly synthesized lysosomal hydrolases (47). To maintain the iterative cycling of hydrolase delivery, CI-MPR must be recycled from late

endosomes to the TGN (48). As shown in Fig. 6A, the knockdown of CI-MPR dramatically decreased the levels of mature CTSD and CTSB. Moreover, the loss of CI-MPR led to a robust increase in the LC3-II levels, suggesting the inhibition of autophagic degradation. These results confirm that CI-MPR is required for lysosomal hydrolase sorting to lysosomes and is critical for normal lysosome function. The loss of the cation-dependent mannose 6-phosphate receptor (CD-MPR), which is another hydrolase sorting receptor, showed less effect on the CTSD/CTSB and LC3-II levels (Fig. 6A).

To determine whether PIPKI γ 5 or Rab7a can control CI-MPR, the subcellular distribution of CI-MPR was monitored. The loss of Rab7a significantly enhanced the colocalization of CI-MPR with LAMP1 (Fig. 6B and 6C), indicating the increased distribution of CI-MPR in late endosome/lysosomes. Although the loss of PIPKI γ 5 partially decreased the CI-MPR expression levels (Fig. 6A), the loss of PIPKI γ 5 dramatically promoted the translocation of CI-MPR in late endosome/lysosomes (Fig. 6B and 6C). These results indicate that PIPKI γ 5 and Rab7a are required for the retrograde sorting of CI-MPR from late endosomes/lysosomes. Therefore, the loss of PIPKI γ 5 or Rab7a leads to a dramatic accumulation of CI-MPR at late endosome/lysosomes. As a consequence, this can decrease the retrograde sorting of CI-MPR to TGN and decrease the efficiency for hydrolase delivery to lysosomes. The knockdown of PIPKI γ 5 or Rab7a protein levels was confirmed by Western blot (Fig. 6D).

The retromer complex is responsible for the retrograde sorting of CI-MPR from late endosomes to the TGN (49, 50). In mammalian cells, the retromer complex is composed of a cargo-selective VPS trimer (VPS35, VPS26 or VPS29) and SNX components (SNX1 or SNX2 with SNX5 or SNX6) (51). Our previous work indicated that PIPKI γ 5 directly interacts with SNX5 and SNX6 (21). This suggests that PIPKI γ 5 may regulate CI-MPR trafficking between endosomes and the TGN by modulating the components of the retromer complex. As shown in Fig. 6E and 6F, the loss of PIPKI γ 5 significantly decreased the distribution of the retromer component VPS26 in Rab7a-positive late endosomes/lysosomes. Similarly, the subcellular location of another retromer component, SNX6, in Rab7a-positive late endosomes/lysosomes was also dramatically decreased in PIPKI γ 5 knockdown cells (Fig. S7A and S7B). These results indicate that the loss of PIPKI γ 5 blocks the recruitment of the retromer complex to late endosome/lysosomes. This indicates a mechanism by which PIPKI γ 5 controls retromer complex-mediated CI-MPR retrograde sorting from late endosomes/lysosomes.

Rab7a interacts with the retromer VPS subcomplex and controls the endosomal localization of the VPS subcomplex (52). Therefore, the effects of PIPKI γ 5 loss on retromer-Rab7a interaction were examined. Retromer prefers to interact with active Rab7a (Rab7a-GTP) (52). Purified His₆-Rab7a was preloaded with GMP-PNP (a non-hydrolyzable analog of GTP), and then used for the pulldown of retromer VPS subcomplex from control or PIPKI γ 5-knockdown cells. As shown in Fig. 6G–6I, loss of PIPKI γ 5 significantly decreased Rab7a interaction with retromer components VPS35 and VPS26, which indicates a declined connection between Rab7a and retromer. This result suggests a mechanism by which PIPKI γ 5 modulates retromer recruitment to Rab7a-positive late endosomes. Consistently, exogenous expression of wild type PIPKI γ 5 significantly promotes Rab7a

interaction with retromer components VPS29 and VPC35 (Fig. S7C–S7F). The expression of PIPKI γ 5 kinase dead mutant cannot increase Rab7a-retromer interaction (Fig. S7C–S7F). This suggests that the kinase activity of PIPKI γ 5 to produce PI4,5P₂ is required for modulating Rab7a-retromer interaction.

PIPKI γ 5 controls TBC1D5 subcellular localization to modulate active Rab7a levels.

The retromer complex can regulate Rab7a activity (53). Deletion of the retromer leads to Rab7a hyperactivation and enhanced Rab7a localization in late endosomes (53), similar to the effects of PIPKI γ 5 knockdown (Fig. 1). The retromer component VPS29 directly binds to TBC1D5, a Rab7a-specific GTPase-activating protein (GAP), to recruit TBC1D5 to late endosomes (54). This process is required for the conversion of active Rab7a (GTP-bound) to inactive Rab7a (GDP-bound) (53). The knockdown of TBC1D5 significantly enhances the translocation of Rab7a to late endosomes/lysosomes, indicating the enhancement of Rab7a activation (Fig. 7A and 7B). This effect is similar to that caused by the loss of PIPKI γ 5. Consistently, the expression of TBC1D5 was shown to reverse the increase in active Rab7a levels caused by the loss of PIPKI γ 5 (Fig. 7C).

As PIPKI γ 5 controls the subcellular localization of the retromer complex (Fig. 6), the effects of PIPKI γ 5 on TBC1D5 subcellular distribution were examined. As shown in Fig. 7D and 7E, the loss of PIPKI γ 5 significantly decreased the subcellular localization of TBC1D5 in Rab7a-positive late endosomes. The loss of TBC1D5 recruitment to late endosomes can lead to accumulation of active Rab7a at late endosomes. To exclude the possibility that the decrease of TBC1D5 recruitment is an indirect consequence of active Rab7a recruitment to late endosomes, a constitutively active Rab7a mutant Rab7a-Q67L was expressed in control or PIPKI γ 5-knockdown cells. Rab7a-Q67L is consistently localized at late endosomes. As shown in Fig. S7G and S7H, TBC1D5 can colocalize with constitutively active Rab7a-Q67L at endosomes, which suggests that active Rab7a recruitment to late endosomes does not lead to TBC1D5 loss from late endosomes. Loss of PIPKI γ 5 still caused decreased colocalization of TBC1D5 with Rab7a-Q67L (Fig. S7G and S7H). This supports that PIPKI γ 5 is required for TBC1D5 recruitment to late endosomes. In PIPKI γ 5 knockdown cells, TBC1D5 still localized to retromer-positive endosomes (Fig. 7F and 7G), while the loss of the retromer component VPS35 led to a decrease in TBC1D5 localization in endosomes (Fig. 7F). This finding indicates that the endosomal distribution of TBC1D5 is dependent on the retromer. In contrast, the loss of TBC1D5 did not cause the disassociation of VPS35 from endosome vesicles (Fig. 7F). This indicates that the endosomal distribution of VPS35 is independent on TBC1D5. These results indicate the mechanism by which PIPKI γ 5 controls the level of Rab7a activation and the subcellular distribution. The loss of PIPKI γ 5 blocks the recruitment of the retromer to Rab7a-positive late endosomes/lysosomes (Fig. 6). As a consequence, the recruitment of TBC1D5 to Rab7a-positive late endosomes/lysosomes is disrupted, which leads to increased levels of active Rab7a and increased Rab7a translocation in late endosomes.

Overall, this research indicates a model in which PIPKI γ 5 and Rab7a form a novel molecular nexus that controls endosome maturation and lysosome function (Fig. 8). PIPKI γ 5 directly interacts with Rab7a and is required for Rab7a recruitment to early

endosomes, which is a key step in the maturation of early endosomes into late endosomes. PIPKI γ 5 also controls the subcellular localization of the retromer complex. The loss of PIPKI γ 5 decreases the recruitment of retromer complex to Rab7a-positive late endosomes/lysosomes, which blocks the retrograde sorting of CI-MPR from late endosomes/lysosomes to the TGN. As a consequence, this causes decreased hydrolase sorting to late endosomes/lysosomes and leads to lysosome dysfunction. Furthermore, the loss of PIPKI γ 5 blocks retromer-dependent TBC1D5 recruitment to late endosome/lysosomes, which increases the levels of active Rab7a at late endosome/lysosomes.

Discussion

This study reveals the important role of the PI4,5P₂-producing kinase PIPKI γ 5 in the control of endosome maturation and lysosome function. Different phosphoinositide species play distinct roles in regulating membrane dynamics and vesicular transport. Previous research on the role of phosphoinositides in the endosomal system has mostly focused on phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 3,5-bisphosphate (PI3,5P₂) because they are abundant in early endosomes and late endosomes. Compared to PI3P and PI3,5P₂, PI4,5P₂ is most abundant at the plasma membrane (55). Therefore, previous research on the function of PI4,5P₂ in vesicle trafficking has mainly focused on membrane endocytosis. The function of PI4,5P₂ in endosomal sorting is less clear. However, recent research has revealed that PI4,5P₂ also plays critical roles in the endosomal-lysosomal system. For instance, decreases in PI4,5P₂ levels impair endosomal recycling of internalized transferrin to the plasma membrane (56). PI4,5P₂ signaling also controls the polarized trafficking of integrins and E-cadherin from endosomes to the plasma membrane (57–59). Furthermore, PI4,5P₂ can regulate autophagic membrane trafficking by modulating autophagic lysosome formation (60). Our previous work revealed that PI4,5P₂ production is required for EGFR sorting from endosomes to lysosomes for degradation (21, 61). All of these results, together with those of the current study, support a role for PI4,5P₂ in the endosomal-lysosomal system, which was previously underestimated.

The local production of phosphoinositides by specific kinases is critical for the control of vesicle trafficking. PI3P is produced by the kinase Vps34 and is concentrated in early endosomes (62, 63), where it plays a central role in recruiting effectors that are involved in the endosomal sorting of targeting proteins. The maturation of early endosomes into late endosomes requires the conversion of PI3P to PI3,5P₂ in the limiting membranes of endosomes. This process depends on PIKfyve, a PI3P 5-kinase that binds to PI3P via its FYVE domain and uses PI3P as a substrate to produce PI3,5P₂ (64). Among the kinases responsible for PI4,5P₂ production in the endosomal system, we identified PIPKI γ 5 as an important kinase (21). As a PI4,5P₂-producing kinase specifically localized in endosomes, PIPKI γ 5 controls the downregulation of EGFR signaling by promoting EGFR lysosomal sorting (21). The results of the current study suggest that the function of PIPKI γ 5 is not just limited to modulating EGFR and that PIPKI γ 5 plays a broad role in the regulation of both endosome and lysosome function by interacting with Rab7a.

The maturation of early endosomes into late endosomes requires the conversion of early endosome-specific Rab5a and PI3P to late endosome-specific Rab7a and PI3,5P₂ (65). The

recruitment of Rab7a and its effector WDR91 inhibit PI3K activity, which allows for the turnover of endosomal PI3P and facilitates the conversion of PI3P to PI3,5P₂ (66). The current study found that PIPKIγi5 is required for Rab7a recruitment to early endosomes (Fig. 2). Therefore, PIPKIγi5 may also be required for the conversion of PI3P into PI3,5P₂. This idea is supported by results that show that the loss of PIPKIγi5 inhibited the disassociation of EEA1 from endosomes (Fig. 3A). The recruitment of EEA1 to endosomes depends on its binding to PI3P (67). The prolonged translocation of EEA1 in endosomes indicates a defect in endosomal PI3P down-regulation.

Rab7a interacts with the retromer complex, and their cooperation is required for normal endosomal/lysosomal function (52, 53). PIPKIγi5 directly interacts with the retromer components SNX5 and SNX6 to control their modulation of EGFR sorting to intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) (21). The current study found that PIPKIγi5 controls the cooperation between Rab7a and the retromer complex. The loss of PIPKIγi5 blocks retromer recruitment to Rab7a-positive late endosomes (Fig. 6), which leads to accumulation of CI-MPR in late endosomes. As a consequence, this decreases CI-MPR-mediated hydrolase delivery to late endosomes/lysosomes (Fig. 6).

A recent study reported that Rab7a-positive endosomes possess high levels of PI4P, which is primarily produced by PI4K2A kinase (68). PI4P is the substrate of PIPKIγi5, which produces PI4,5P₂. The acute conversion of endosomal PI4P to PI4,5P₂ can lead to the release of Rab7a from late endosomes (68). Our results may indicate a molecular mechanism that explains this phenomenon. PIPKIγi5 interacts with Rab7a and is responsible for the conversion of PI4P to PI4,5P₂ in late endosomes. Consistently, the loss of PIPKIγi5 enhances Rab7a translocation in late endosomes (Fig. 1). Furthermore, PIPKIγi5 is required to trigger Rab7a inactivation in late endosomes. The loss of PIPKIγi5 blocks retromer recruitment to late endosomes, which decreases retromer-dependent TBC1D5 translocation to Rab7a-positive late endosomes (Fig. 7). As a result, TBC1D5 cannot convert Rab7a-GTP to Rab7a-GDP, and the levels of active Rab7a are increased.

By modulating the sorting of hydrolases to lysosomes, PIPKIγi5 controls autophagic degradation (Figs. 4 and 5). PIPKIγi5 has also been reported to interact with ATG14 to control autophagy initiation (69). However, the loss of PIPKIγi5 did not block autophagy initiation in our cell line system (Fig. S4C). It is possible that the dependence of autophagy initiation on ATG14 is cell context specific. The knockdown of ATG14 did not block autophagy initiation in our cell line system (Fig. S4D).

Although PIPKIγi5 plays important roles in controlling Rab7a, *PIPKIγi5*^{-/-} mice still can survive to adulthood. Loss of PIPKIγi5 leads to elevated Rab7a activation levels, which may not prevent the *PIPKIγi5*^{-/-} mice survival. Rab7a constitutively active mutants have been found in humans and are related to Charcot-Marie-Tooth type 2 (CMT2) neuropathies (70). But the patients can still survive to adulthood. This suggests that the elevated Rab7a activation is not lethal. There is no any CMT2 like symptoms found in *PIPKIγi5*^{-/-} mice. It is possible that the effect of PIPKIγi5 loss on Rab7a activation is not as strong as caused by Rab7a constitutively active mutants.

Altogether, this study revealed a novel Rab7a-PIPKI γ i5 molecular nexus that controls endosome maturation, the sorting of hydrolases to lysosomes, and autophagic degradation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations:

Rab7a	Ras-related protein Rab-7a
Rab7b	Ras-related protein Rab-7b
Rab5a	Ras-related protein Rab-5a
PIPKIγi5	type Igamma phosphatidylinositol phosphate 5-kinase i5
PI4,5P₂	phosphatidylinositol 4,5-bisphosphate
CI-MPR	cation-independent Mannose 6-Phosphate Receptor
GAP	GTPase-activating protein
TGN	trans-Golgi network
EGFR	epidermal growth factor receptor
IF	Immunofluorescence
CRISPR	clustered regularly interspaced short palindromic repeats
sgRNA	single guide RNA
MEF	mouse embryonic fibroblasts
EEA1	Early Endosome Antigen 1
TBC1D5	TBC1 domain family member 5
SNX5	Sorting Nexin 5
SNX6	Sorting Nexin 6
VPS26	vacuolar protein sorting-associated protein 26
VPS29	vacuolar protein sorting-associated protein 29
VPS35	vacuolar protein sorting-associated protein 35
PI4P	phosphatidylinositol-4-phosphate

PI3P	phosphatidylinositol-3-phosphate
PI3,5P₂	phosphatidylinositol 3,5-bisphosphate
LAPTM4B	Lysosomal-associated transmembrane protein 4B
RILP	Rab-interacting lysosomal protein
LAMP1	lysosome-associated membrane glycoprotein 1
CTSD	cathepsin D
CTSB	cathepsin B

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Figure 1. PIPKIγi5 interacts with Rab7a and controls Rab7a activation levels.

(A) UMSCC1 cells were subjected to immunoprecipitation with Rab7a antibody and then immunoblotted with antibodies as indicated. (B) Recombinant His₆-Rab7a and GST-PIPKIγi5 were purified from *E. coli* and subjected to His₆-pulldown assays. His₆-Rab7a was preloaded with GDP or GMP-PNP. (C) IF staining of HA-PIPKIγi5 (green) and endogenous Rab7a (red). Bar, 20 μm. (D) IF staining of endogenous LAMP1 (green) and Rab7a (red) in control or PIPKIγi5 knockdown UMSCC1 cells. Bar, 20 μm. (E) Quantification of LAMP1-Rab7a colocalization (n=50 cells from three independent

experiments). (F) HA-RALP was used to pull down Rab7a-GTP in control or PIPKI γ 5 knockdown UMSCC1 cells. (G) Quantification of active Rab7a (Rab7a-GTP) levels in control or PIPKI γ 5 knockdown UMSCC1 cells. (H) Myc-PIPKI γ 5 expression was introduced in *PIPKI γ 5^{+/+}* (WT) or *PIPKI γ 5^{-/-}* (KO) MEFs via lentivirus. An empty lentivirus vector was used as a control. Levels of PIPKI γ 5, total Rab7a (in whole cell lysate), and active Rab7a (pulled down by HA-RILP) in *PIPKI γ 5^{+/+}* (WT) and *PIPKI γ 5^{-/-}* (KO) MEFs were detected via Western blot. (I) Quantification of active Rab7a levels in WT or KO MEFs transfected with or without PIPKI γ 5. The error bars indicate the mean $\pm$ SEM from three independent experiments. An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$.

Figure 2. PIPKI γ i5 is required for Rab7a recruitment to early endosomes.

(A) IF staining of HA-PIPKI γ i5 (green) and EEA1 (red) in UMSCC1 cells. (B) Costaining of EEA1 and Rab7a in control or PIPKI γ i5 knockdown UMSCC1 cells. (C) Quantification of Rab7a-EEA1 colocalization. (D) IF staining of Rab7a in mCherry-Rab5a-expressing control or PIPKI γ i5 knockdown UMSCC1 cells. (E) Quantification of mCherry-Rab5a-Rab7a colocalization. (F) IF staining of EEA1 and Rab5a in control or PIPKI γ i5 knockdown UMSCC1 cells. (G) Quantification of EEA1-Rab5a colocalization. The error

bars indicate the mean $\pm$ SEM. (n=50 cells from three independent experiments). An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$. Bar in IF images, 20 μm .

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Figure 3. PIPK1γi5 and Rab7a are required for early to late endosome maturation.

(A) Costaining of EEA1 (green) and Alexa Fluor 555-EGF (red) in control, PIPK1γi5 knockdown, or Rab7a knockdown UMSCC1 cells. (B) Quantification of Alexa Fluor 555-EGF and EEA1 (n=50 cells from three independent experiments). One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed. ***, $P < 0.0001$. (C) PIPK1γi5 and Rab7a protein levels in control, PIPK1γi5 knockdown, or Rab7a knockdown UMSCC1 cells were detected by Western blot. (D) Costaining of Rab7a (green) and Alexa Fluor 555-EGF (red) in control or PIPK1γi5 knockdown UMSCC1 cells. (E) Quantification of Alexa Fluor 555-EGF and Rab7a. The error bars indicate the mean $\pm$ SEM (n=50 cells).

from three independent experiments). An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$. Bar in IF images, 20 μm .

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Figure 4. PIPKIγ5 controls autophagic flux.

(A) IF staining of LC3-II in control or PIPKIγ5 knockdown UMSCC1 cells. (B) Quantification of LC3-II puncta (n=50 cells from three independent experiments). (C) UMSCC1 cells were transfected with control or PIPKIγ5 siRNA and then treated with or without spautin-1 or wortmannin. The indicated protein levels were detected by Western blotting. (D) Quantification of LC3-II protein levels. (E) Costaining of LC3 and LAMP1 in control or PIPKIγ5 knockdown UMSCC1 cells. (F) Costaining of LC3 and LysoTracker. (G) Quantification of LC3 colocalization with LAMP1 or LysoTracker (n=50 cells from three independent experiments). (H) Costaining of p62 and LysoTracker in control or PIPKIγ5 knockdown UMSCC1 cells. (I) Costaining of HA-p62 and LAMP1 in control or PIPKIγ5 knockdown UMSCC1 cells. (J) Quantification of p62 colocalization with LAMP1 (n=50 cells from three independent experiments). (K) Western blotting of PIPKIγ5, HA, tubulin, and LC3 in WT and KO cells transfected with Vector or HA-PIPKIγ5. (L) Quantification of LC3-II levels in WT and KO cells transfected with Vector or HA-PIPKIγ5.

three independent experiments). (H) Costaining of endogenous p62 and LysoTracker. (I) Costaining of HA-p62 and LAMP1. (J) Quantification of p62 colocalization with LysoTracker (n=50 cells from three independent experiments). (K) HA-PIPKI γ 5 expression was introduced in *PIPKI γ 5^{+/+}* (WT) or *PIPKI γ 5^{-/-}* (KO) MEFs via lentivirus. An empty lentivirus vector was used as a control. Then, the PIPKI γ 5 and LC3-II levels were detected by Western blot. (L) Quantification of LC3-II levels in MEFs. The error bars indicate the mean $\pm$ SEM from three independent experiments. An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$. Bar in IF images, 20 μ m.

Figure 5. PIPK1 γ i5 regulates hydrolase sorting to lysosomes.

(A) Costaining of CTSD (red) and LAMP1 (green) in control or PIPK1 γ i5 knockdown UMSCC1 cells. Bar, 20 μ m. (B) Quantification of CTSD-LAMP1 colocalization (n=50 cells from three independent experiments). (C) The lysosome components were enriched using a lysosome isolation kit, and then mature CTSD and CTSB levels were detected by Western blotting. (D) Quantification of mature CTSD and CTSB levels in control or PIPK1 γ i5 knockdown UMSCC1 cells. (E) The morphology of lysosomes (indicated in EM images by arrows) was detected by electron microscopy (EM). (F) The lysosome diameters in control

or PIPKI γ 5 knockdown UMSCC1 cells was quantified (n=50 cells from three independent experiments). The error bars indicate the mean $\pm$ SEM from three independent experiments. An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$.

Figure 6. PIPKI γ 5 controls the colocalization of the retromer complex with Rab7a and regulates CI-MPR subcellular localization.

(A) The indicated siRNAs were transfected into UMSCC1 cells, and then the levels of the indicated proteins were detected by Western blotting. (B) IF staining of CI-MPR (red) and LAMP1 (green) in control, PIPKI γ 5 knockdown, or Rab7a knockdown UMSCC1 cells. (C) Quantification of CI-MPR-LAMP1 colocalization (n=50 cells from three independent experiments). (D) PIPKI γ 5 and Rab7a protein levels in control, PIPKI γ 5 knockdown, or Rab7a knockdown UMSCC1 cells were detected by Western blot. (E) IF staining of Rab7a (green) and VPS26 (red) in control or PIPKI γ 5 knockdown UMSCC1 cells. (F)

Quantification of Rab7a-VPS26 colocalization (n=50 cells from three independent experiments). The error bars indicate the mean $\pm$ SEM. An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$. Bar in IF images, 20 μ m. (G) Same amount (200 ng) of His₆-Rab7a loaded with GMP-PNP was used to pulldown retromer components in cell lysate from control or PIPKI γ i5-knockdown UMSCC1 cells. The indicated proteins in cell lysate or pulled down by His₆-Rab7a-GMP-PNP were detected by Western blot. (H) Quantification of VPS35 associated with His₆-Rab7a-GMP-PNP in control or PIPKI γ i5-knockdown UMSCC1 cells. (I) Quantification of VPS26 associated with His₆-Rab7a-GMP-PNP in control or PIPKI γ i5-knockdown UMSCC1 cells. The error bars indicate the mean $\pm$ SEM from three independent experiments. An unpaired Student's two-tailed t-test was used. **, $P < 0.001$.

Figure 7. PIPKIγi5 is required for TBC1D5 recruitment to late endosomes.

(A) IF staining of Rab7a (green) and LAMP1 (red) in control or TBC1D5 knockdown UMSCC1 cells. (B) Quantification of Rab7a-LAMP1 colocalization (n=50 cells from three independent experiments). (C) Flag-TBC1D5 was transfected into control or PIPKIγi5 knockdown UMSCC1 cells, and then the levels of active Rab7a were measured. (D) IF staining of TBC1D5 (green) and Rab7a (red) in control or PIPKIγi5 knockdown UMSCC1 cells. (E) Quantification of TBC1D5-Rab7a colocalization (n=50 cells from three independent experiments). (F) IF staining of TBC1D5 (green) and VPS35 (red) in control,

PIPKI γ i5 knockdown, VPS35 knockdown, or TBC1D5 knockdown UMSCC1 cells. (G) Quantification of TBC1D5-VPS35 colocalization (n=50 cells from three independent experiments). The error bars indicate the mean $\pm$ SEM. An unpaired Student's two-tailed t-test was used. ***, $P < 0.0001$. Bar in IF images, 20 μ m.

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Figure 8. Model of the modulation of endosome maturation and lysosome function by PIPKIγi5 and Rab7a.

PIPKIγi5 interacts with Rab7a and is required for Rab7a recruitment to early endosomes, which is a critical step in the initiation of the maturation of early endosomes into late endosomes. PIPKIγi5 is also required for retromer translocation to Rab7a-positive late endosomes. The retromer recruits TBC1D5 to convert Rab7a-GTP into Rab7a-GDP, which leads to the release of Rab7a from late endosomes. The retromer also mediates the retrograde sorting of CI-MPR from late endosomes to the TGN, which facilitates CI-MPR-dependent hydrolase delivery to late endosome/lysosomes.