

# Syntaxin 17 recruitment to mature autophagosomes is temporally regulated by PI4P accumulation

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## Abstract

During macroautophagy, cytoplasmic constituents are engulfed by autophagosomes. Lysosomes fuse with closed autophagosomes but not with unclosed intermediate structures. This is achieved in part by the late recruitment of the autophagosomal SNARE syntaxin 17 (STX17) to mature autophagosomes. However, how STX17 recognizes autophagosome maturation is not known. Here, we show that this temporally regulated recruitment of STX17 depends on the positively charged C-terminal region of STX17. Consistent with this finding, mature autophagosomes are more negatively charged compared with unclosed intermediate structures. This electrostatic maturation of autophagosomes is likely driven by the accumulation of phosphatidylinositol 4-phosphate (PI4P) in the autophagosomal membrane. Accordingly, dephosphorylation of autophagosomal PI4P prevents the association of STX17 to autophagosomes. Furthermore, molecular dynamics simulations support PI4P-dependent membrane insertion of the transmembrane helices of STX17. Based on these findings, we propose a model in which STX17 recruitment to mature autophagosomes is temporally regulated by a PI4P-driven change in the surface charge of autophagosomes.

### eLife assessment

This paper addresses a **fundamental** issue in the field of autophagy: how is a protein responsible for autophagosome-lysosome fusion recruited to mature autophagosomes but not immature ones? The work succeeds in its ambition to provide a new conceptual advance. The evidence supporting the conclusions is **convincing**, with fluorescence microscopy, biochemical assays, and molecular dynamics simulations. This work will be of broad interest to cell biologists and biochemists studying autophagy, and also those focusing on lipid/membrane biology.

## Introduction

Macroautophagy (hereafter, autophagy) is a highly conserved process of intracellular degradation ([Mizushima and Levine, 2020](#); [Soreng et al., 2018](#)). Membrane cisternae (called isolation membranes or phagophores) elongate, bend, and engulf cytoplasmic components. Closure of the rim of the cup-shaped structures results in the formation of autophagosomes. Subsequently, the autophagosomes fuse with lysosomes to degrade their enclosed contents. Autophagosome–lysosome fusion is strictly regulated; lysosomes fuse with only fully closed autophagosomes. If lysosomes were to fuse with intermediate unclosed structures and their inner membrane is degraded, then harmful lysosomal enzymes would leak out into the cytosol. This temporal regulation of autophagosome–lysosome fusion is achieved in mammals by two mechanisms. One is the regulated translocation of the autophagosomal SNARE syntaxin 17 (STX17), which is recruited immediately before or after the closure of autophagosomes ([Itakura et al., 2012](#); [Takáts et al., 2013](#); [Tsuboyama et al., 2016](#)). The other is the activation of YKT6, a conserved autophagosomal SNARE ([Matsui et al., 2018](#)), which is kept inactivated by ULK1 (a homolog of yeast Atg1)-mediated phosphorylation until the completion of autophagosome formation (ULK1/Atg1 is dissociated at this time) ([Barz et al., 2020](#); [Gao et al., 2020](#); [Sanchez-Martin et al., 2023](#)). Then, STX17 interacts with SNAP29 and the lysosomal SNARE protein VAMP7 or VAMP8, and YKT6 interacts with SNAP29 and the lysosomal STX7 to mediate the fusion between autophagosomes and lysosomes.

However, the molecular mechanism underlying the late recruitment of STX17 is not known. A previous study reported that LC3/GABARAP family proteins and immunity-related GTPase M (IRGM) are involved in autophagosomal recruitment of STX17 ([Kumar et al., 2018a](#)), whereas other studies have shown that LC3/GABARAP family proteins are not required ([Nguyen et al., 2016](#); [Tsuboyama et al., 2016](#)). It has also been recently reported that phosphorylation of STX17 and binding to filamin A are important for STX17 recruitment ([Wang et al., 2023](#)). Although these factors may help STX17 recruitment, these mechanisms do not explain the strict temporal regulation of STX17 recruitment.

Accordingly, we hypothesized that there exists a yet unknown change in some autophagosomal properties during maturation that is recognized by STX17. In this study, we reveal that STX17 recruitment to autophagosomes requires positively charged amino acids in the C-terminal region of STX17. Consistently, the membrane of autophagosomes becomes more negatively charged when autophagosomes acquire STX17. Furthermore, we show that phosphatidylinositol 4-phosphate (PI4P), a negatively charged phospholipid, accumulates during autophagosome maturation and is required for STX17 recruitment. Based on these findings, we propose a model in which STX17 recruitment to mature autophagosomes is temporally regulated by the electrostatic change of autophagosomes.

## Results

### Autophagosomal localization of STX17 requires a positively charged C-terminal region

STX17 has an N-terminal Habc domain, followed by a SNARE domain, two tandem transmembrane helices, and a short C-terminal region, with both N- and C-terminal ends facing the cytosol (**Figure 1A**) ([Itakura et al., 2012](#)). A short construct containing only the transmembrane helices and the C-terminal region (STX17TM), which behaves similarly to full-length STX17 ([Itakura et al., 2012](#)), colocalized with ring-shaped autophagosomes labeled with the general autophagic

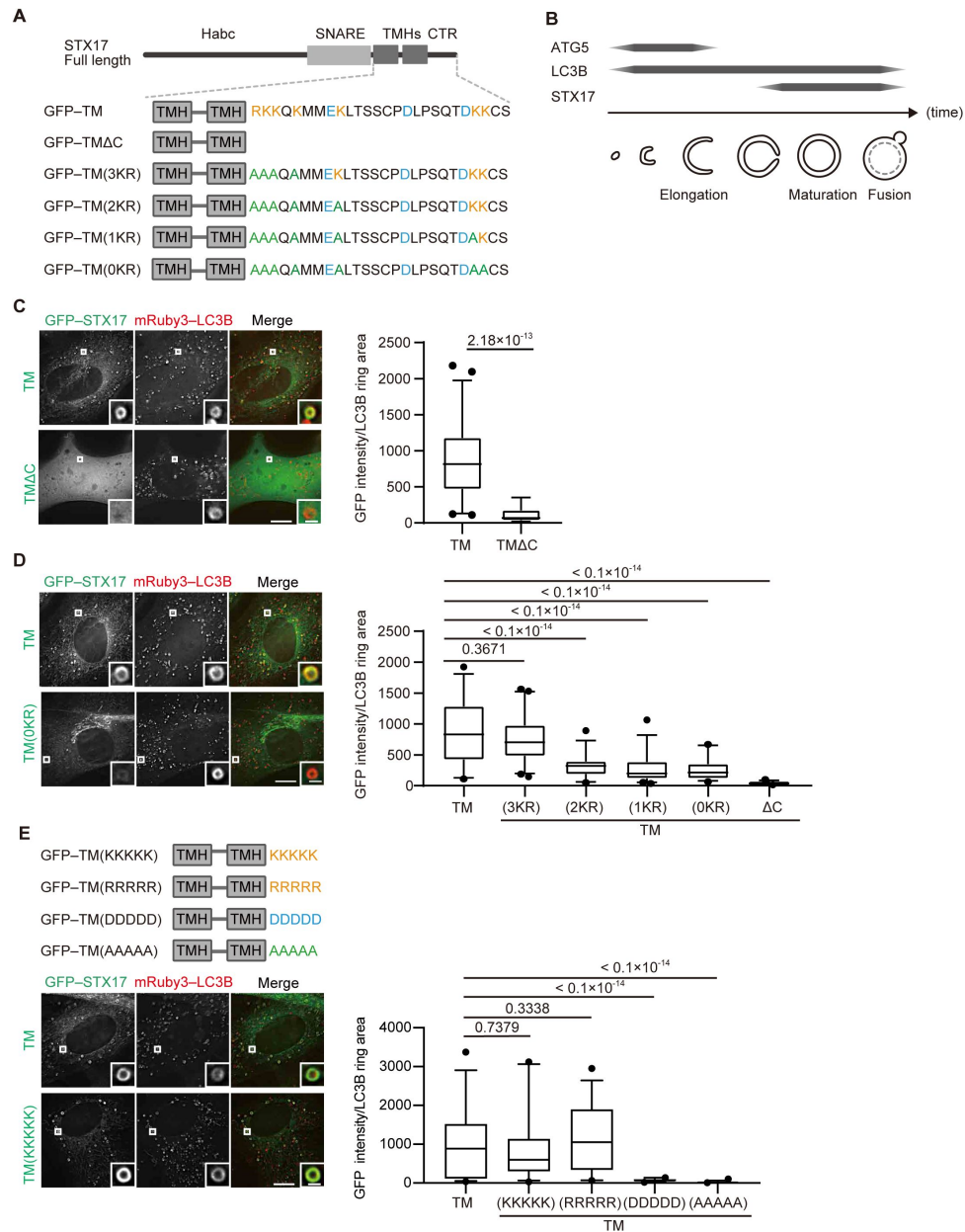
membrane marker microtubule-associated protein light chain 3B (LC3B) (**Figure 1B** [↗](#) and **C** [↗](#)). STX17 is a tail-anchored protein, and the C-terminal region of tail-anchored proteins is generally important for specific membrane targeting (*Rao et al., 2016* [↗](#); *Yagita et al., 2013* [↗](#)). Therefore, we determined the role of the C-terminal region of STX17. In the following experiments, we used the STX17TM construct in order to avoid detecting an indirect effect of SNARE domain-mediated translocation. Deletion of the C-terminal cytosolic region from STX17TM (TMΔC) resulted in a diffuse cytosolic pattern (**Figure 1C** [↗](#)), indicating that not only the transmembrane helices but also the C-terminal region of STX17 are required for its proper autophagosomal localization. Although the role of STX17 in autophagosome–lysosome fusion is conserved in both *Drosophila melanogaster* (Dm) (*Takáts et al., 2013* [↗](#)) and *Caenorhabditis elegans* (Ce) (*Guo et al., 2014* [↗](#)), the amino acid sequence of the C-terminal region is not conserved in these organisms (**Figure 1—figure supplement 1A** [↗](#)). Nevertheless, when expressed in mammalian cells, DmSTX17TM and CeSTX17TM were recruited to autophagosomes, although less efficiently (**Figure 1—figure supplement 1B** [↗](#)), suggesting that recruitment of STX17 does not depend on the specific amino acid sequence in the C-terminal region.

In general, cationic amino acids in the C-terminal region of tail-anchored proteins are important for targeting membranes (*Borgese et al., 2001* [↗](#); *Horie et al., 2002* [↗](#); *Yagita et al., 2013* [↗](#)). STX17 has cationic amino acids in its C-terminal region, and the replacement of lysine and arginine with alanine impaired autophagosomal localization in a dose-dependent manner (**Figure 1A** [↗](#) and **D** [↗](#), **Figure 1—figure supplement 1C** [↗](#)).

Moreover, the replacement of the C-terminal region with artificial sequences consisting of five residues of positively charged lysine or arginine, but not negatively charged aspartic acid or uncharged alanine, restored the localization of STX17 to autophagosomes (**Figure 1E** [↗](#), **Figure 1—figure supplement 1D** [↗](#)). These data suggest that autophagosomal localization of STX17 requires positively charged residues, but not sequence-specific structures, in its C-terminal region.

## The membrane of autophagosomes becomes negatively charged during maturation

Given the importance of positively charged residues in the C-terminal region, we hypothesized that STX17 favors negatively charged membranes. To evaluate this hypothesis, we first conducted an *in vitro* membrane binding assay using liposomes with different compositions of phospholipids. STX17TM was efficiently recruited to liposomes containing anionic phospholipids such as phosphatidylserine (PS), phosphatidylinositol (PI) 3-phosphate (PI3P) or PI4P (**Figure 2A** [↗](#)). The association of STX17TM with PI4P-containing membranes was abolished in the presence of 1 M NaCl (**Figure 2B** [↗](#)). These data suggest that STX17 can be recruited to negatively charged membranes via electrostatic interaction independent of the specific lipid species.



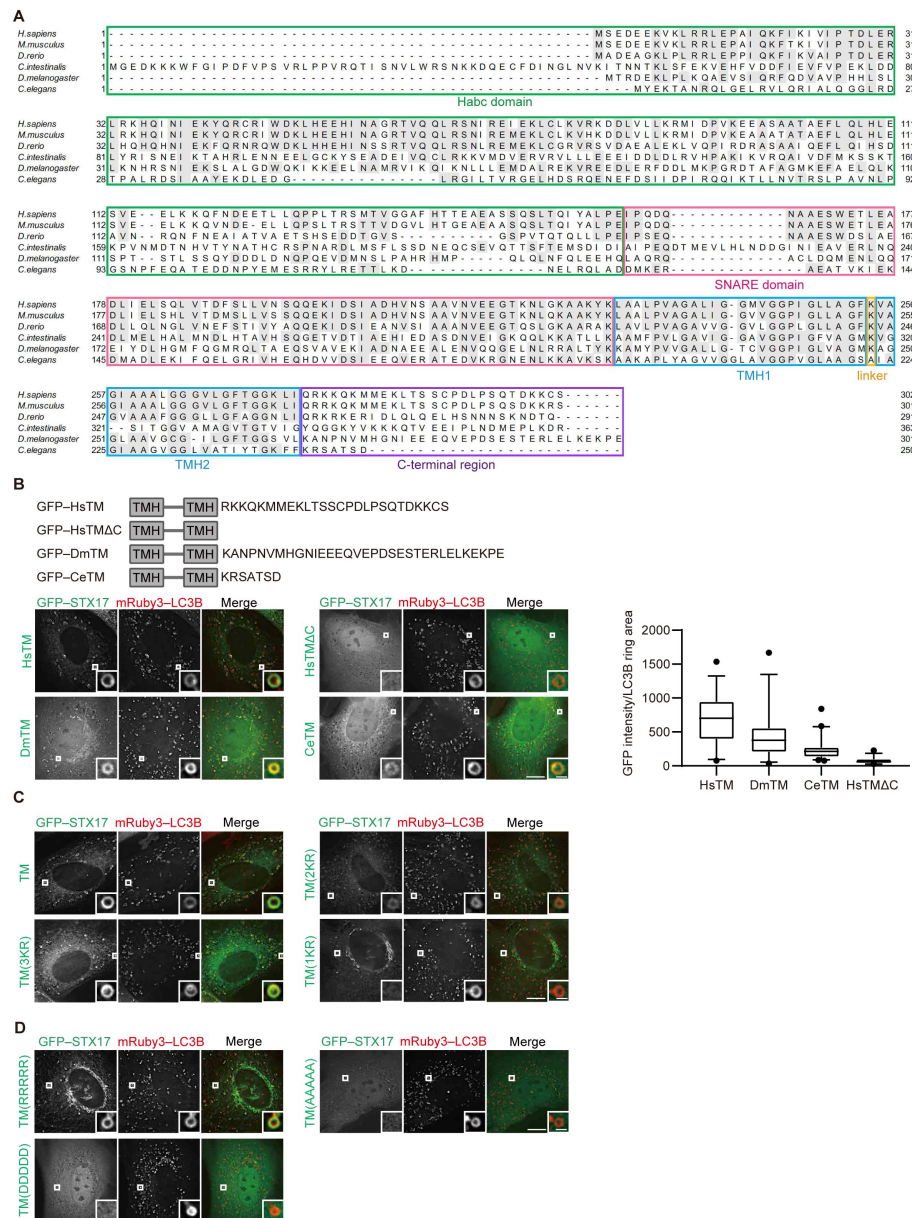
**Figure 1.**

### The recruitment of STX17 to autophagosomes is dependent on its positively charged C-terminal region.

**(A)** Schematic representation of the structures of STX17 and its C-terminal variants. The positively (orange) and negatively (blue) charged residues are shown. Alanine substitutions are shown in green. TMH, transmembrane helix; CTR, C-terminal region.

**(B)** Schematic representation of the localization of ATG5, LC3B, and STX17 during autophagosome formation and maturation.

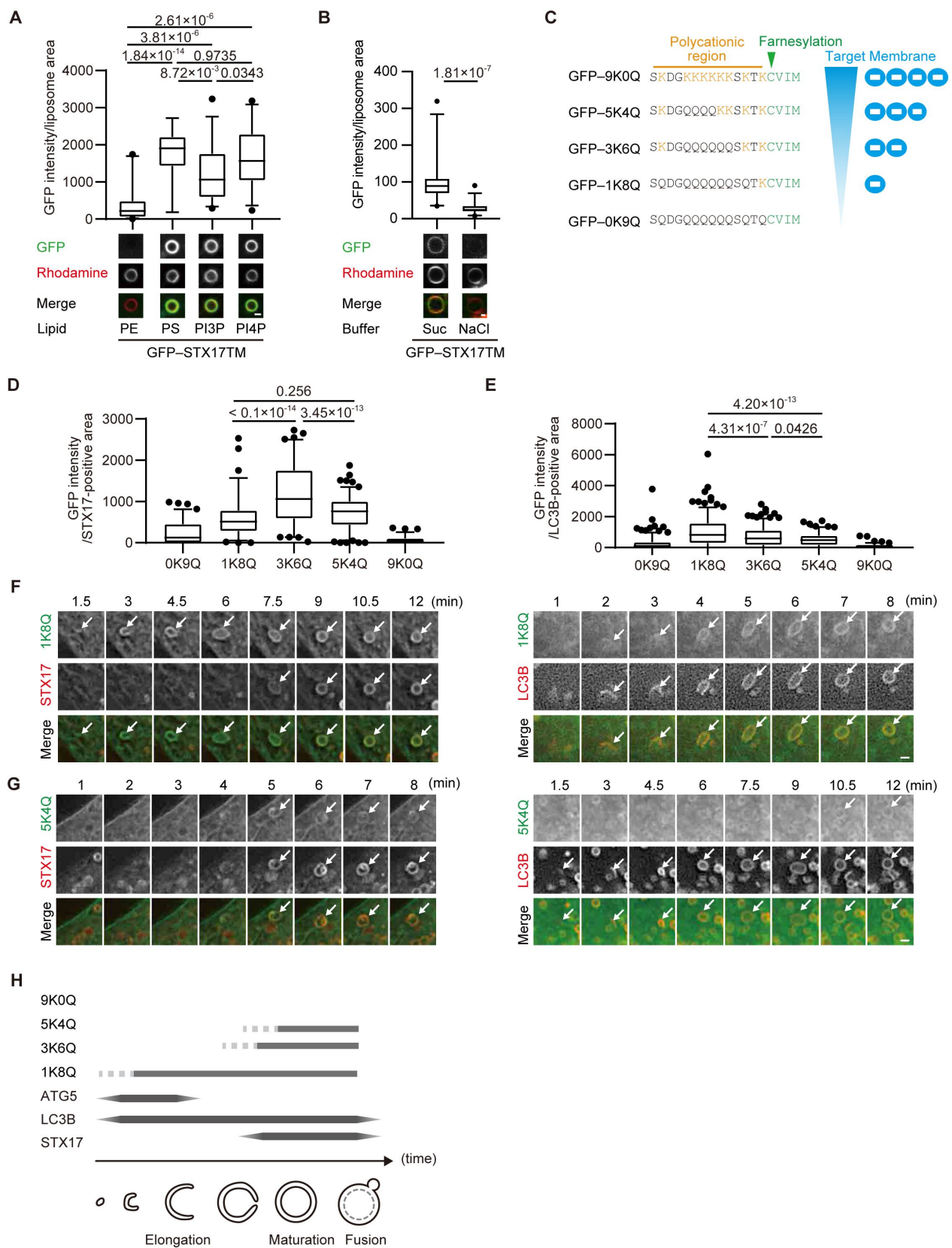
**(C–E)** Mouse embryonic fibroblasts (MEFs) stably expressing mRuby3-LC3B and GFP-STX17TM (containing the two transmembrane helices and the C-terminal region) or its mutants were cultured in starvation medium for 1 h. Quantification of GFP-STX17TM intensity of mRuby3-LC3B-positive ring-like structures ( $n > 30$ ) are shown in the graphs. In box plots, solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. Differences were statistically analyzed by Welch's *t*-test (C) or one-way ANOVA followed by Dunnett's multiple comparison test (D and E). Experiments were performed three times independently. Scale bars, 10  $\mu$ m (main), 1  $\mu$ m (inset) (C, D, and E).



**Figure 1—figure supplement 1.**

## Recruitment of STX17 depends on the abundance of cationic amino acids in the C-terminal region but not on its specific amino acid sequence.

**(A)** Multiple sequence alignment of STX17 proteins from *Homo sapiens* (Hs), *Mus musculus*, *Danio rerio*, *Ciona intestinalis*, *Drosophila melanogaster* (Dm), and *Caenorhabditis elegans* (Ce). Identical residues between more than two species are indicated with gray boxes. Domains of STX17 are indicated with different colors: green, the Habc domain; magenta, the SNARE domain; blue, transmembrane helix (TMH); orange, a linker between the TMDs; and purple, the C-terminal region. **(B)** Mouse embryonic fibroblasts (MEFs) stably expressing mRuby3-LC3B and either GFP-tagged HsSTX17TM, DsSTX17TM, or CeSTX17TM were cultured in starvation medium for 1 h. GFP-STX17TM intensities of mRuby3-LC3B-positive ring-like structures were quantified ( $n > 30$ ). In box plots, solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. **(C)** MEFs stably expressing one of the GFP-tagged alanine replacement mutants (shown in **Figure 1A**) and mRuby3-LC3B were cultured in starvation medium for 1 h. Quantification results are shown in **Figure 1D**. **(D)** MEFs stably expressing one of the GFP-tagged charge replacement mutants (shown in **Figure 1E**) and mRuby3-LC3B were cultured in starvation medium for 1 h. Quantification results are shown in **Figure 1E**. Experiments were performed three times independently. Scale bars, 10  $\mu$ m (main), 1  $\mu$ m (inset) (B, C, and D).



**Figure 2.**

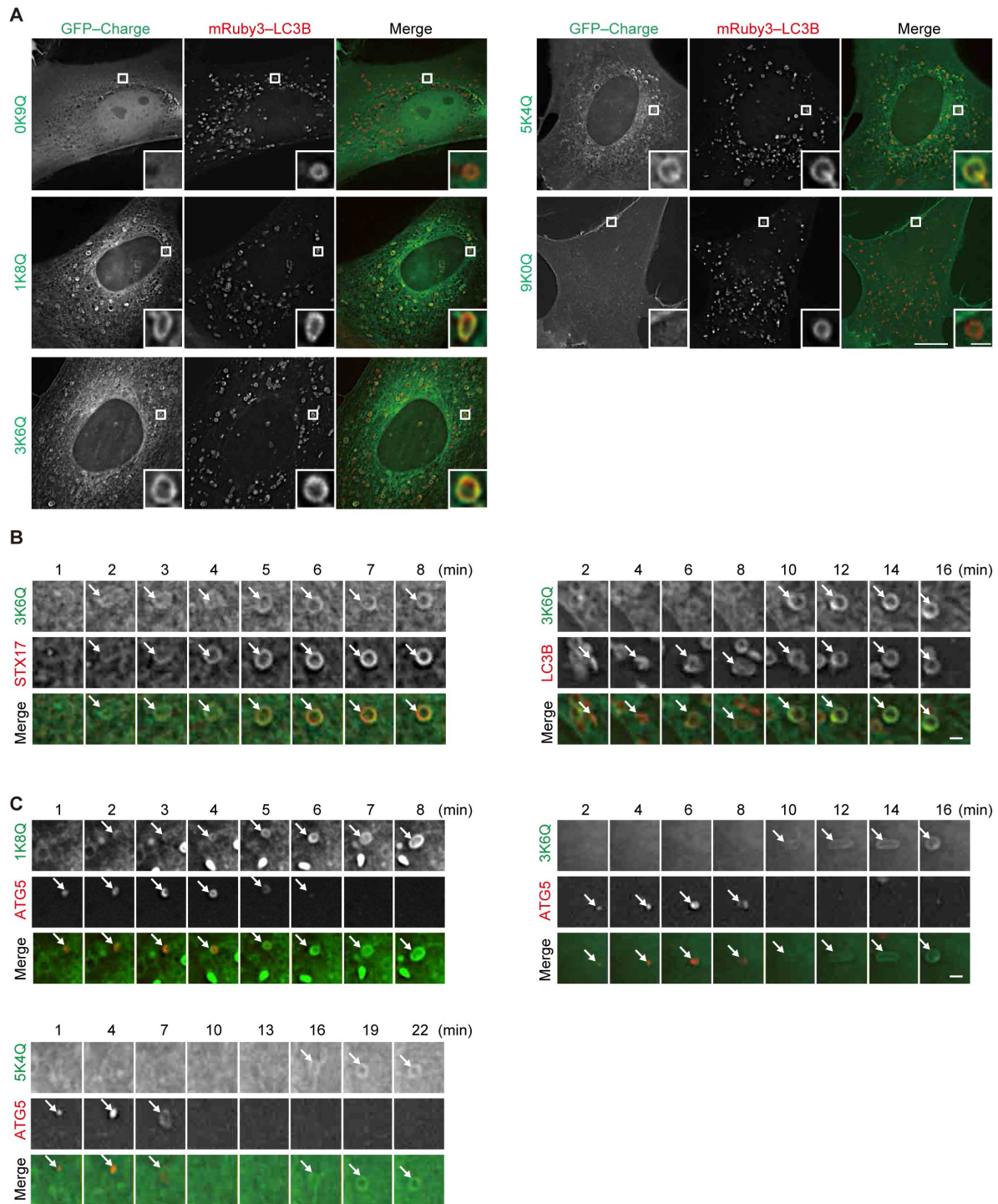
### The membrane of autophagosomes becomes negatively charged during maturation.

(A) GFP-STX17TM translated *in vitro* was incubated with rhodamine-labeled liposomes containing the indicated concentrations of phospholipids: 70% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), and 10% of either PE, phosphatidylserine (PS), phosphatidylinositol 3-phosphate (PI3P), or phosphatidylinositol 4-phosphate (PI4P). GFP intensities of liposomes are quantified and shown as in **Figure 1C** ( $n > 30$ ). (B) GFP-STX17TM translated *in vitro* was incubated with rhodamine-labeled liposomes containing 70% PC, 20% PE and 10% PI4P in the presence of 1 M NaCl or 1.2 M sucrose. GFP intensities of liposomes were quantified and shown as in **Figure 1C** ( $n > 30$ ). (C) Amino acid sequences of GFP-tagged membrane surface charge probes. The positively charged residues are shown in orange. The farnesylation motif is indicated in green. (D and E) Mouse embryonic fibroblasts (MEFs) stably expressing one of the GFP-tagged charge probes and mRuby3-STX17TM (C) or mRuby3-LC3B (D) were cultured in starvation medium for 1 h. GFP intensities of mRuby3-STX17TM-positive (C) or mRuby3-LC3B-positive (D) ring-like structures were quantified ( $n > 70$ ). (F and G) Time-lapse analysis of MEFs stably expressing the GFP-tagged 1K8Q (E) or 5K4Q (F) charge probes and mRuby3-STX17TM or mRuby3-LC3B cultured in starvation medium. Autophagosomes are indicated by arrows. (H) Summary of electrostatic dynamics of autophagosome formation. In box plots, solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. Differences were statistically analyzed by Welch's *t*-test (B) or one-way ANOVA followed by Sidak's multiple comparison test (A, C, and D). Experiments were performed three times independently. Scale bars, 1  $\mu$ m (A, B, F, and G).

Next, we tested whether autophagosomal membranes are indeed negatively charged. To monitor membrane charge *in vivo*, we used membrane surface charge biosensors (**Figure 2C**) (Simon *et al.*, 2016; Yeung *et al.*, 2006). These surface charge probes have a diverse range of positively charged peptides and a farnesylated anchor at their C terminus, with GFP fused to their N terminus. The name of each probe is indicated by the number of lysine (positively charged) followed by that of glutamine. The probe for the most negatively charged membrane (9K0Q) labeled the plasma membrane, which is known to have a highly negative charge (Li *et al.*, 2014), but not autophagosomes (**Figure 2D** and **E**, **Figure 2—figure supplement 1A**). However, the probes for intermediate (5K4Q and 3K6Q) and weakly (1K8Q) negative charges labeled not only the plasma membrane but also LC3B- and STX17-positive autophagosomes (**Figure 2D** and **E**, **Figure 2—figure supplement 1A**). The recruitment kinetics differed between the intermediate and weakly negative charge probes. The 1K8Q probe was recruited to not only STX17-positive spherical autophagosomes but also STX17-negative, LC3B-positive elliptic structures that should correspond to unclosed autophagosomes (Tsuboyama *et al.*, 2016) (**Figure 2F**). In contrast, the intermediate charge probes (3K6Q and 5K4Q) were recruited to spherical LC3B-positive autophagosomes at almost the same time as STX17 (**Figure 2G**, **Figure 2—figure supplement 1B**). Structures positive for ATG5, a marker for unclosed autophagosomes, were labeled with 1K8Q but not with 3K6Q or 5K4Q (**Figure 2—figure supplement 1C**). These results, which are summarized in **Figure 2H**, suggest that mature autophagosomes are more negatively charged compared with unclosed intermediate structures.

### The accumulation of PI4P in mature autophagosomes coincides with STX17 recruitment

We next explored the mechanism that generates the negative charges of autophagosomal membranes. As the change in the membrane charge was rapid and robust, we reasoned that it could be caused by a change in lipid composition or modification. We screened negatively charged lipids that are enriched in autophagosomal membranes using various phospholipid probes (Platre and Jaillais, 2016). Among them, we found that the probes for PI4P (GFP-fused PH domain of CERT; GFP-CERT(PHD) and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P<sub>2</sub>) (GFP-TRPML1(PHD))



**Figure 2—figure supplement 1.**

### The membrane of autophagosomes becomes negatively charged during maturation.

**(A)** Mouse embryonic fibroblasts stably expressing one of the GFP-tagged charge probes and mRuby3-LC3B were cultured in starvation medium for 1 h. **(B)** Time-lapse analysis of MEFs stably expressing GFP-3K6Q and mRuby3-STX17TM or mRuby3-LC3B cultured in starvation medium. Autophagosomes are indicated by arrows. **(C)** Time-lapse analysis of MEFs stably expressing one of the GFP-tagged charge probes and mRuby3-ATG5 cultured in starvation medium. Autophagosomes are indicated by arrows. Experiments were performed three times independently. Scale bars, 10  $\mu$ m (A [main]), 1  $\mu$ m (A [inset], B, and C).

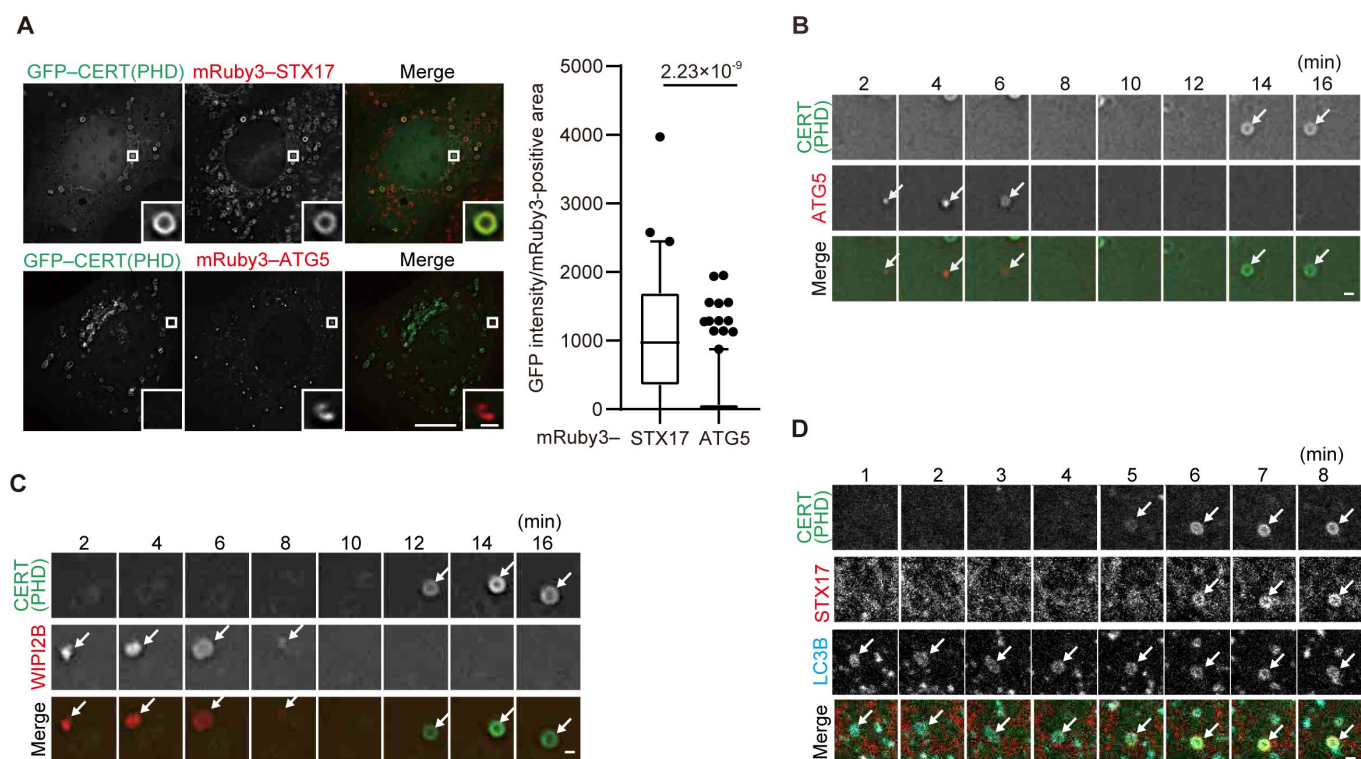
colocalized with STX17-positive ring-shaped structures (**Figure 3—figure supplement 1A** [↗](#)). Since PI(3,5)P<sub>2</sub> was primarily present in lysosomes (**Figure 3—figure supplement 1B** [↗](#)) (*Dong et al., 2010* [↗](#)), PI(3,5)P<sub>2</sub> enrichment likely occurs after fusion with lysosomes. The CERT(PHD) (W33A) mutant, lacking PI4P-binding activity, was not co-localized with STX17TM (**Figure 3—figure supplement 2A** [↗](#)) (*Sugiki et al., 2012* [↗](#)). Consistently, other PI4P probes, including FAPP(PHD), OSBP(PHD), and P4M-SidMx2, colocalized with STX17TM (**Figure 3—figure supplement 2B** [↗](#) and **C** [↗](#)) (*Hammond et al., 2014* [↗](#); *Platre and Jaillais, 2016* [↗](#)).

PI4P is an important lipid component of the Golgi complex and the plasma membrane as well as a major regulator of conserved eukaryotic cellular processes (*Balla, 2013* [↗](#); *Platre et al., 2018* [↗](#); *Schink et al., 2016* [↗](#)). In the context of autophagy, it was previously reported that GABARAP-dependent PI4P production by PI4K2A (PI4KIIα) is required for autophagosome–lysosome fusion (*Wang et al., 2015* [↗](#)), and that PI4KB (PI4KIIβ) is detected in ATG9A vesicles and early autophagic structures and is furthermore required for autophagosome formation (*Judith et al., 2019* [↗](#)). Consistently, we detected faint signals of PI4K2A and PI4KB on LC3B-positive structures (**Figure 3—figure supplement 1C** [↗](#)). However, the dynamics of PI4P production during autophagosome maturation has not been systematically determined.

The PI4P probes (CERT(PHD), FAPP(PHD), OSBP(PHD), and P4M-SidMx2) colocalized with STX17 but not with ATG5 (**Figure 3A** [↗](#), **Figure 3—figure supplement 2B** [↗](#) and **C** [↗](#)), suggesting that PI4P accumulates in mature autophagosomes. We then investigated the dynamics of PI4P enrichment by time-lapse microscopy. The signal of the CERT(PHD) probe appeared on LC3B-positive membranes only after the disappearance of the unclosed autophagosome markers ATG5 and WIPI2B (**Figure 3B** [↗](#) and **C** [↗](#)) and almost at the same time as STX17 localized (**Figure 3D** [↗](#)). This was followed by the appearance of LysoTracker signals (note that LysoTracker signals have been previously observed to appear ring-shaped until inner membrane degradation (*Tsuboyama et al., 2016* [↗](#))) (**Figure 3—figure supplement 2D** [↗](#)). To confirm that PI4P accumulation was independent of autophagosome–lysosome fusion, we evaluated the colocalization among the PI4P probe, LC3B, and LAMP1 in STX17- and YKT6-double-knockdown cells, in which unfused autophagosomes accumulated (*Matsui et al., 2018* [↗](#)). The punctate structures of CERT(PHD) were still colocalized with LC3B-positive and LAMP1-negative structures in these double-knockdown cells (**Figure 3—figure supplement 2E** [↗](#)). In addition, STX17 recruitment and PI4P enrichment occurred normally in cells lacking all ATG8 family proteins (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2) (**Figure 3—figure supplement 2F** [↗](#)). These results suggest that PI4P accumulates in mature autophagosomal membranes independent of lysosome fusion and ATG8 proteins, and that the kinetics of the accumulation of STX17, the PI4P probes, and the negative charge probes are correlated.

## STX17 recruitment to autophagosomes depends on PI4P

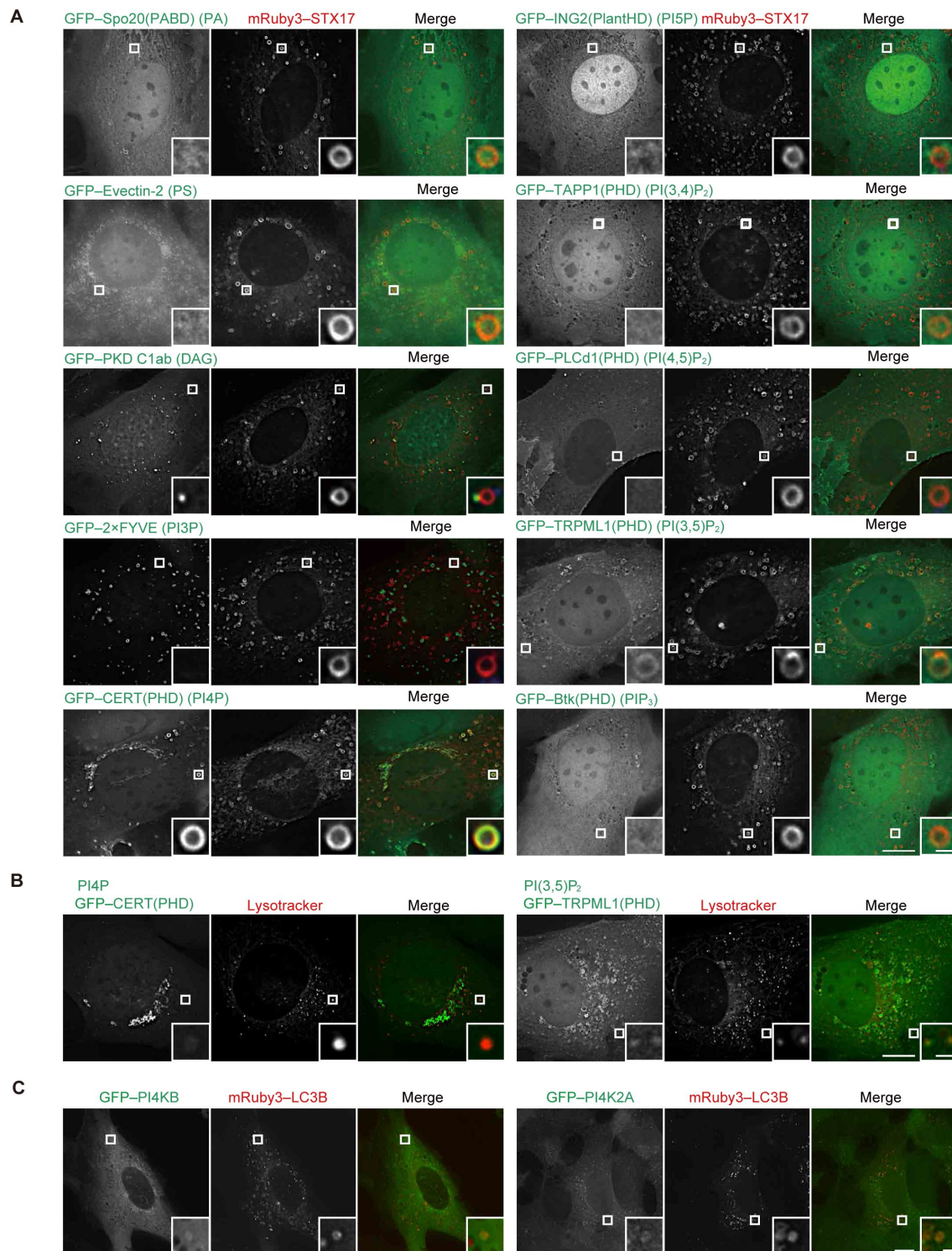
To determine whether PI4P is required for the recruitment of STX17 to autophagosomes, we first tried to dephosphorylate PI4P by ectopic expression of SAC1, a PI4P-phosphatase, on autophagosomes (*Manford et al., 2010* [↗](#)). To this end, we fused the phosphatase domain of yeast Sac1 (Sac1PD) to the N terminus of LC3B and expressed them in different cell lines, using several methods, including lipofection and retrovirus- and adenovirus-mediated transfection. Although Sac1PD–LC3B localized to autophagosomes, it did not reduce either the level of autophagosomal PI4P or STX17 recruitment (unpublished observation). High expression of Sac1PD–LC3B inhibited autophagosome formation (unpublished observation). Mammals possess four PI 4-kinases. We next tried to deplete each or combinations of these PI 4-kinases by using siRNA or an auxin-inducible degron system (*Yesbolatova et al., 2020* [↗](#)) or inhibit them using PI 4-kinase inhibitors, including PI-273 (*Li et al., 2017* [↗](#)), BF738735 (unpublished observation) (*van der Schaar et al., 2013* [↗](#)), and NC03 (**Figure 4—figure supplement 1** [↗](#)) (*Sengupta et al., 2019* [↗](#)), but ultimately failed to reduce the autophagosomal PI4P levels and STX17 recruitment. Although the precise reason for this failure was not known, these approaches were insufficient to deplete autophagosomal PI4P *in vivo*.



**Figure 3.**

### Phosphatidylinositol 4-phosphate (PI4P) is enriched in the autophagosomal membrane during maturation.

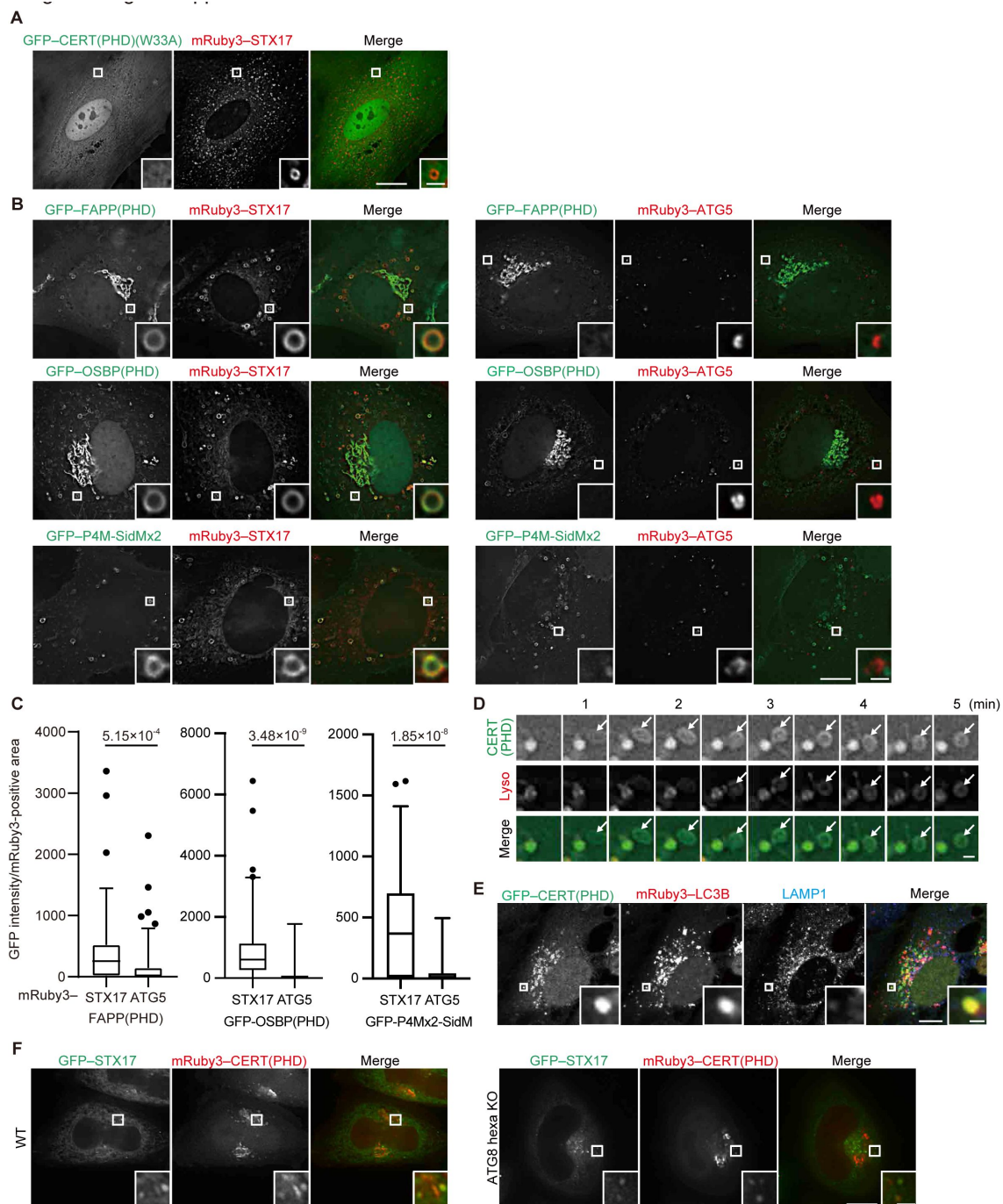
(A) Mouse embryonic fibroblasts (MEFs) stably expressing GFP-CERT(PHD) and mRuby3-STX17TM or mRuby3-ATG5 were cultured in starvation medium for 1 h. GFP intensities of mRuby3-positive structures ( $n > 60$ ) were quantified. In box plots, solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. Differences were statistically analyzed by Welch's  $t$ -test. (B–D) Time-lapse analysis of MEFs stably expressing GFP-CERT(PHD) and mRuby3-ATG5 (B), WIP12B-mRuby3 (C), or mRuby3-STX17TM and HaloTag-LC3B (visualized with SaraFluor 650T HaloTag ligand) (D) cultured in starvation medium. Autophagosomes are indicated by arrows. Experiments were performed three times independently. Scale bars, 10  $\mu$ m (A [main]), 1  $\mu$ m (A [inset], B–D).



**Figure 3—figure supplement 1.**

### Localization of phospholipids in mature autophagosomes.

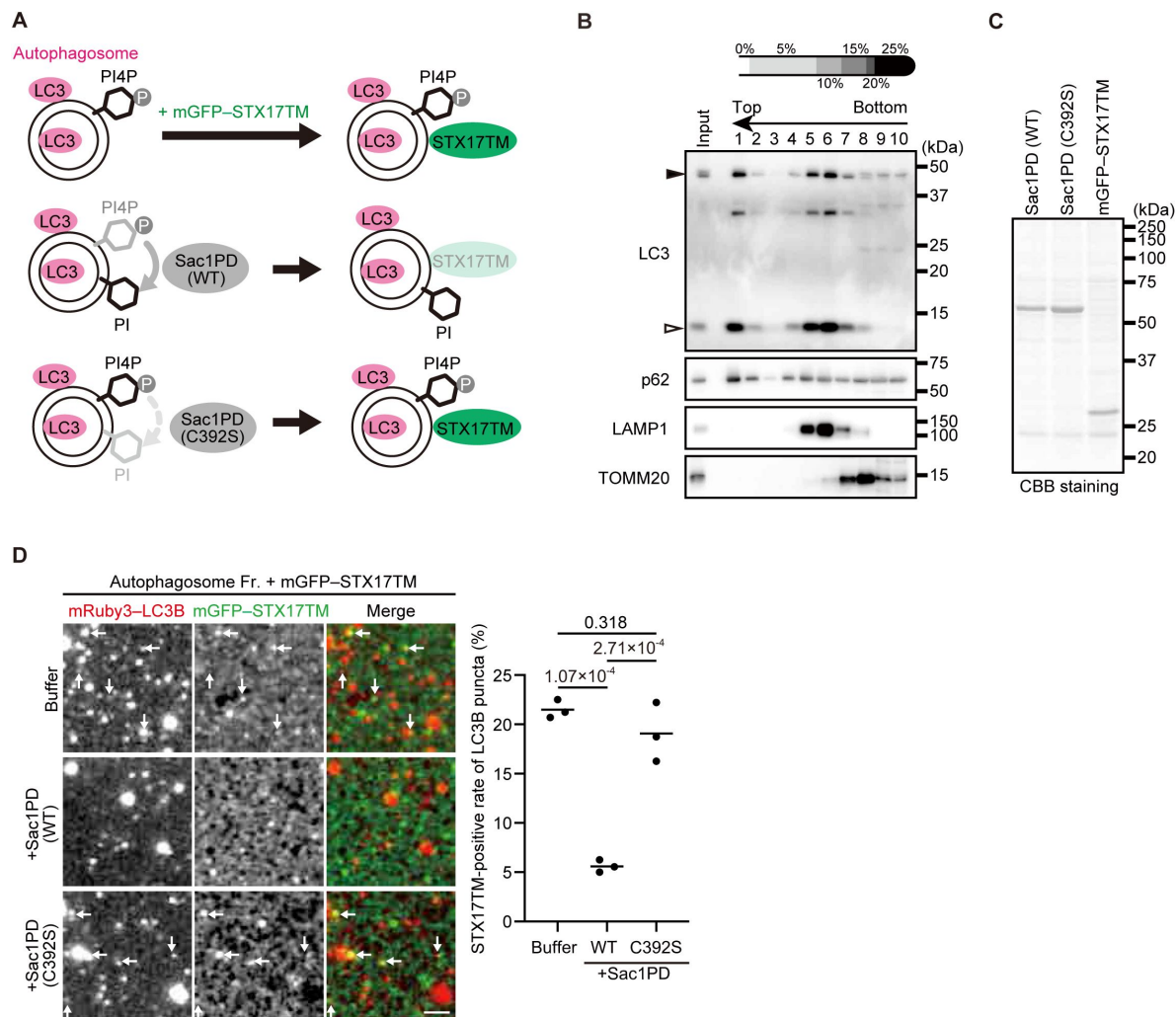
**(A)** Mouse embryonic fibroblasts (MEFs) stably expressing the indicated GFP-tagged phospholipid probe and mRuby3-STX17TM were cultured in starvation medium for 1 h. The following phospholipid probes were used: phosphatidic acid (PA), Spo20(PABD); PS, Evectin-2; diacylglycerol (DAG), PKD C1ab; PI3P, 2xFYVE; PI4P, CERT(PHD); PI5P, ING2(PlantHD); PI(3,4)P<sub>2</sub>, TAPP1(PHD); PI(4,5)P<sub>2</sub>, PLCd1(PHD); PI(3,5)P<sub>2</sub>, TRPML1(PHD); and PIP<sub>3</sub>, Btk(PHD). **(B)** MEFs stably expressing GFP- CERT(PHD) or TRPML1(PHD) were cultured in starvation medium containing LysoTracker Deep Red for 1 h. **(C)** MEFs stably expressing GFP-PI4KB or GFP- PI4K2A and mRuby3-LC3B were cultured in starvation medium for 1 h. Experiments were performed three times independently. Scale bars, 10  $\mu$ m (main), 1  $\mu$ m (inset).



**Figure 3—figure supplement 2.**

### Phosphatidylinositol 4-phosphate (PI4P) is enriched in mature autophagosomes before fusion with lysosomes.

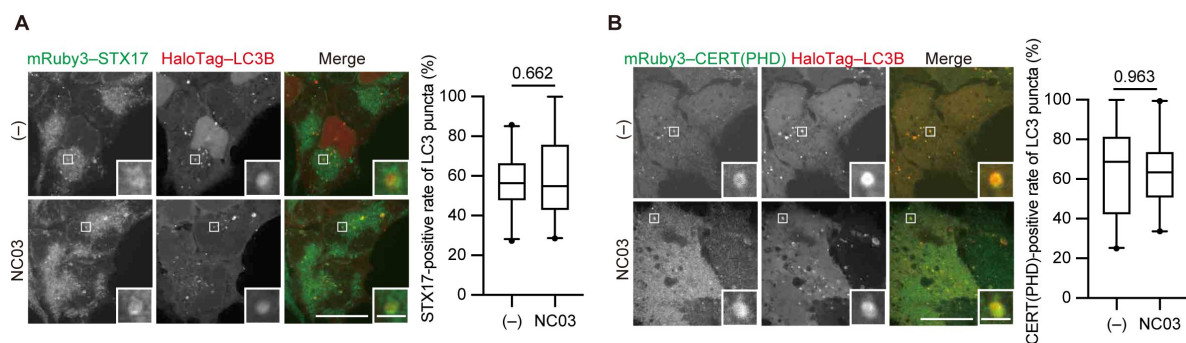
(A and B) Mouse embryonic fibroblasts (MEFs) stably expressing the indicated GFP-tagged PI4P probe, CERT(PHD)(W33A), FAPP(PHD), OSBP(PHD) or P4M-SidMx2, and mRuby3-STX17TM or mRuby3-ATG5 were cultured in starvation medium for 1 h. (C) GFP intensities of mRuby3-positive structures ( $n > 50$ ) in (B) were quantified. In box plots, solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. Differences were statistically analyzed by Welch's *t*-test. (D) Time-lapse analysis of MEFs stably expressing GFP-CERT(PHD) cultured in starvation medium containing LysoTracker Deep Red. (E) U2OS cells stably expressing GFP-CERT(PHD) and mRuby3-LC3B were transfected with siSTX17 and siYKT6. After 3 days, cells were cultured in starvation medium for 1 h, and immunostained with anti-LAMP1 antibodies. (F) WT and ATG8 hexa KO HeLa cells stably expressing GFP-STX17TM and transiently expressing mRuby3-CERT(PHD) were cultured in starvation medium for 1 h. Experiments were performed three times independently. Scale bars, 10  $\mu$ m (A, B, and E [main]), 1  $\mu$ m (A, B, E [inset], and D).



**Figure 4.**

### STX17 recruitment to autophagosomes depends on phosphatidylinositol 4-phosphate (PI4P) *in vitro*.

**(A)** Schematic representation of the *in vitro* autophagosome recruitment assay. Isolated autophagosomes were mixed with mGFP-STX17TM and either recombinant Sac1-phosphatase domain (Sac1PD) or its phosphatase-dead mutant (C392S). **(B)** Isolation of mature autophagosomes prior to their fusion with lysosomes. Homogenates of STX17 knockout HeLa cells stably expressing mRuby3-LC3B cultured in starvation medium at 1 h were separated by the OptiPrep membrane flotation method. The autophagosome-containing fraction (#1: LC3-positive and LAMP1-negative) was collected. The positions of mRuby3-LC3B (black arrowhead) and endogenous LC3B (white arrowhead) are indicated. **(C)** Purification of recombinant yeast Sac1 (phosphatase domain, PD) and its phosphatase-dead (C392S) mutant and mGFP-STX17TM from High Five cells. **(D)** *In vitro* autophagosome association assay. Isolated autophagosomes were mixed with recombinant Sac1 (WT or C392S) for 30 min and then with mGFP-STX17TM for another 30 min. Representative images are shown. STX17-positivity rates were determined across three independent experiments (two of the three experiments were performed in a blind manner, and 80 autophagosomes were counted in each experiment). Solid horizontal lines indicate means. Differences were statistically analyzed by one-way ANOVA followed by Tukey's test. The scale bar, 2.5  $\mu$ m.



**Figure 4—figure supplement 1.**

### The PI 4-kinase inhibitor NC03 failed to suppress autophagosomal PI4P accumulation and STX17 recruitment.

HEK293T cells stably expressing mRuby3-STX17TM (A) or mRuby3-CERT(PHD) (B) and HaloTag-LC3B were cultured in starvation medium for 1 h and then treated with and without 10  $\mu$ M NC03 for 10 min. Representative confocal images are shown. STX17TM-or CERT(PHD)-positive rates of LC3B structures per cell ( $n > 30$  cells) are shown in the graphs. Solid horizontal lines indicate medians, boxes indicate the interquartile ranges (25th to 75th percentiles), whiskers indicate the 5th to 95th percentiles, and dots represent outliers. Differences were statistically analyzed by Welch's *t*-test. Scale bars, 10  $\mu$ m (main), 1  $\mu$ m (inset).

Therefore, we instead used an *in vitro* system to determine whether PI4P is important for STX17 recruitment to autophagosomes (**Figure 4A** [↗](#)). Mature autophagosomes prior to fusion with lysosomes were isolated from *STX17* knockout (KO) cells (**Figure 4B** [↗](#), Fraction #1) (*Matsui et al., 2018* [↗](#)). Recombinant Sac1PD, its phosphatase-dead mutant (C392S), and mGFP-STX17TM were generated using insect cells (**Figure 4C** [↗](#)). Autophagosomes were first incubated with or without Sac1PD or Sac1PD (C392S) for 30 min and then further incubated with mGFP-STX17TM for another 30 min. Recombinant mGFP-STX17TM was associated with autophagosomes, but it was significantly impaired by treatment with Sac1PD. Phosphatase-dead Sac1PD (C392S) showed no effect (**Figure 4D** [↗](#)). These data suggest that PI4P is important for autophagosomal recruitment of STX17.

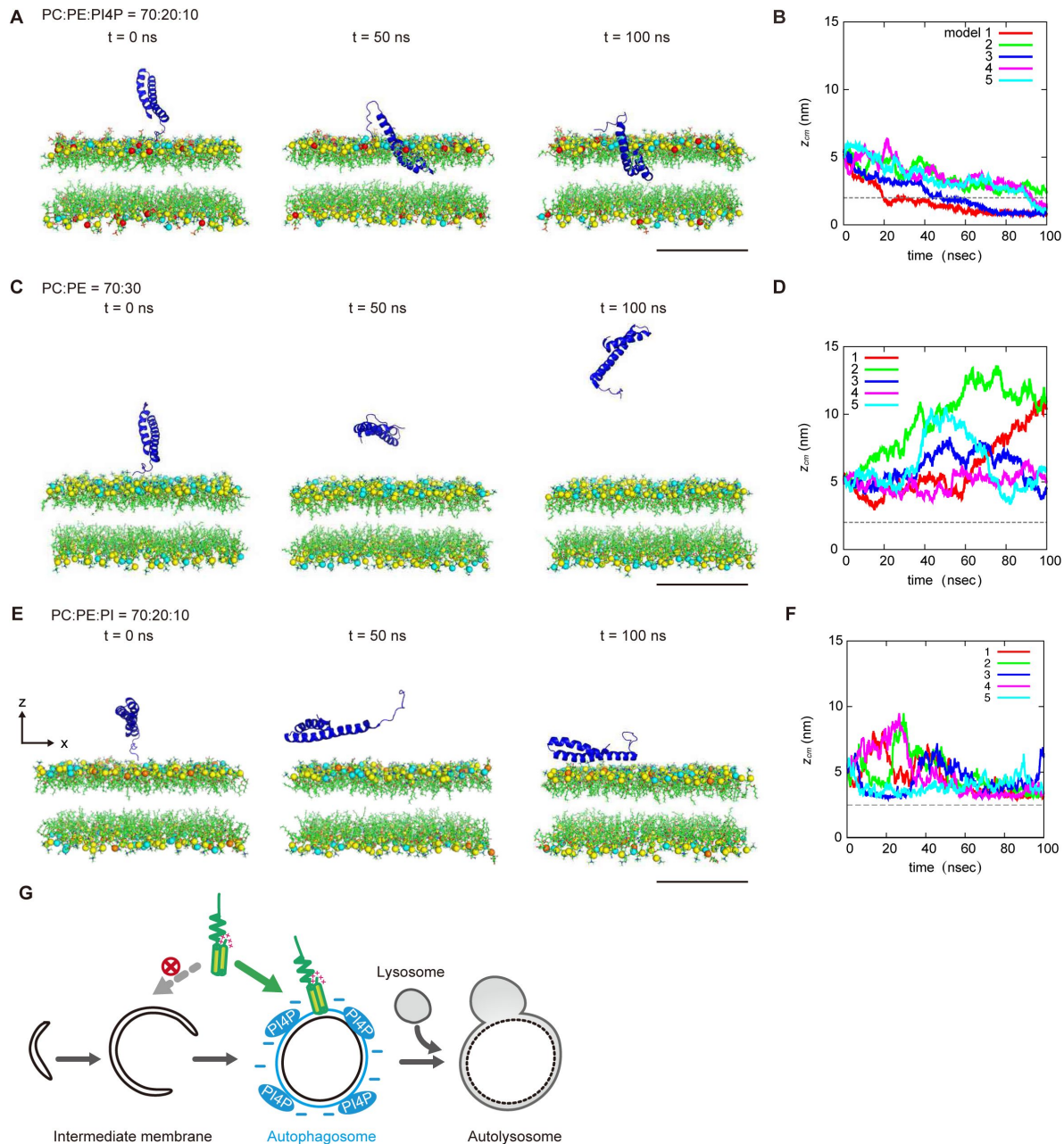
We further determined the effect of PI4P on the dynamics of STX17TM on a membrane by performing a molecular dynamics simulation. We used all-atom models for STX17TM and the highly mobile membrane-mimetic (HMMM) model for the lipid bilayer (*Ohkubo et al., 2012* [↗](#)). The structure of STX17TM was predicted by trRosetta (*Du et al., 2021* [↗](#)), yielding five different models, all of which were used in the simulation. The initial configuration was prepared such that the center of mass of STX17TM was located 3 nm above the membrane surface, after which the movement of STX17TM was tracked. In independent simulations using four out of the five predicted STX17TM structures, STX17TM was inserted into the membrane with PI4P (PC:PE:PI4P = 70:20:10) within a short time scale of 100 ns (**Figure 5A, B** [↗](#), Video 1).

The two transmembrane helices were inserted into the membrane, while the charged C-terminal region remained bound to the membrane surface (Video 1). In contrast, STX17 diffused freely in the solution and was not inserted into the membrane without PI4P (PC:PE = 70:30) in simulations of all five structures (**Figure 5C, D** [↗](#), Video 2). Moreover, if the membrane contained PI instead of PI4P, STX17TM approached the PI-containing membrane but was not inserted into the membrane (**Figure 5E, F** [↗](#), Video 3). These results suggest that STX17TM can be readily inserted into PI4P-containing membranes.

## Discussion

Based on the results of this study, we propose a model in which temporally regulated autophagosome-lysosome fusion involves a dynamic change in electrostatic status during autophagosome maturation; the recruitment of STX17 to mature autophagosomes is primarily mediated by the electrostatic interaction between the positively charged C-terminal region of STX17 and negatively charged autophagosomal membranes likely due to accumulation of PI4P (**Figure 5G** [↗](#)). During revising this manuscript, Juhász's group also reached a consistent model that PI4P is required for autophagosomal localization of STX17 (*Laczko-Dobos et al., 2024* [↗](#)). Because STX17 does not localize to all negatively charged membranes (e.g., the plasma membrane), the transmembrane helices appear to also regulate the specificity of target membranes. This is consistent with the “coincidence detector” mechanism in which both a cationic motif and a nearby membrane-anchored moiety are important for membrane targeting (*Carlton and Cullen, 2005* [↗](#); *Yeung and Grinstein, 2007* [↗](#)).

A striking finding of this study is that the membrane of autophagosomes become more negatively charged during maturation, most likely through an increase in the PI4P content of their membranes, although we do not exclude the possible involvement of other negatively charged molecules. Given the possibility that fluorescence lipid probes may give false-negative results, a more comprehensive biochemical analysis, such as lipidomics analysis of mature autophagosomes, would be imperative to elucidate the potential involvement of other negatively charged lipids.



**Figure 5.**

### Molecular dynamics simulations of phosphatidylinositol 4-phosphate (PI4P)-dependent STX17TM insertion into membranes.

(A, C and E) An example of a time series of simulated results of STX17TM insertion into a membrane consisting of 70% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), and 10% PI4P (POPI14) (A), 70% PC and 30% PE (C) or 70% PC, 20% PE and 10% phosphatidylinositol (PI) (E). STX17TM is shown in blue. Phosphorus in PC, PE, PI4P and PI are indicated by yellow, cyan, red and orange, respectively. Short-tailed lipids are represented as green sticks. The time evolution series of (A), (C) and (E) are shown in Videos 1, 2 and 3. (B, D and F) Time evolution of the  $z$ -coordinate of the center of mass ( $z_{cm}$ ) of the transmembrane helices of STX17TM in the case of membranes with PI4P (B) or PI (F) and without PI4P or PI (D). Five independent simulation results are represented by solid lines of different colors. The gray dashed lines indicate the locations of the lipid heads. Scale bars, 5 nm (A, C, and E). (G) Model of the PI4P-driven electrostatic maturation of the autophagosome and STX17 recruitment.

While PI3P is important for autophagosome formation, we do not think that PI3P contributes to the increase in negative charge. It was reported that PI3P is dephosphorylated to PI during autophagosome formation or maturation ([Allen et al., 2020](#); [Cebollero et al., 2012](#); [Taguchi-Atarashi et al., 2010](#)), and that the PI3P effectors DFCP1 and WIPI family proteins are not detected on mature autophagosomes ([Koyama-Honda et al., 2013](#)). In fact, we did not detect WIPI2B and the PI3P reporter GFP-2×FYVE on PI4P-enriched structures (**Figure 3C**) and STX17-positive structures (**Figure 3—figure supplement 1A**), respectively. Considering the change in autophagosomal membrane charge, the amount of PI4P produced during autophagosome maturation is likely to be higher than that of PI3P in the early stages. Our data of the 1K8Q probe suggest that immature autophagosomal membranes may also have slight negative charges (**Figure 2F**). Although the source of the negative charge of immature autophagosomes is currently unknown, it may be derived from low levels of PI4P, which is undetectable by the PI4P probes and/or other negatively charged lipids such as PI and PS ([Schmitt et al., 2022](#)).

PI4P is involved in many cellular processes, including autophagosome formation and maturation. It was reported that PI4KB regulates the early stage of autophagosome formation through interaction with ATG9A ([Judith et al., 2019](#)). It was also reported that PI4K2A is recruited to autophagosomes by binding to the ATG8 family proteins GABARAP and GABARAPL1 and produces PI4P in the autophagosomal membranes ([Wang et al., 2015](#)). Furthermore, the knockdown of PI4K2A resulted in the accumulation of abnormally enlarged autophagosomes, presumably owing to a defect in autophagosome-lysosome fusion ([Wang et al., 2015](#)). Indeed, a freeze-fracture replica labeling study showed that autophagosomal membranes contain PI4P in the cytoplasmic leaflets of the outer and inner membranes ([Kurokawa et al., 2019](#)). Very recently, it was reported that the knockdown of PI4KIIa in *Drosophila* blocked autophagic flux at a late step of autophagy in the fat body ([Liu et al., 2023](#)).

Although these studies are consistent with our finding that PI4P production induces STX17 recruitment for fusion with lysosomes, our repeated attempts to deplete PI4P genetically (i.e., by depletion of PI 4-kinases or attachment of Sac1 to autophagosomes) or pharmacologically did not deplete autophagosomal PI4P or inhibit STX17 recruitment. Liu et al. also showed that the knockdown of PI4KIIa in *Drosophila* did not inhibit STX17 recruitment. This may be because PI 4-kinases function redundantly. In any case, it would be important to elucidate how PI 4-kinase activity or PI4P synthesis is upregulated during autophagosome maturation.

Surface charge, which is now recognized as one of the key physical properties of cellular membranes, regulates various biological processes ([Yeung and Grinstein, 2007](#)). For example, the negatively charged plasma membrane, where anionic phospholipids such as PI(4,5)P<sub>2</sub> and PS are enriched, recruits positively charged proteins, including K-Ras and Rac1 ([Bigay and Antonny, 2012](#); [Heo et al., 2006](#); [Li et al., 2014](#); [Platre et al., 2018](#); [Yeung et al., 2008](#); [Yeung et al., 2006](#)), some of which are released from the membrane when the negative charge of the surface is reduced during phagocytosis ([Yeung et al., 2006](#)). However, little has been reported on the role of electrostatic properties of other organelles, including autophagosomes.

Generally, PI4P can be structurally recognized by a series of PI4P-binding domain-containing proteins ([Lemmon, 2008](#); [Moravcevic et al., 2012](#)). However, recent studies suggest that PI4P can also generate a negatively charged electrostatic field and recruit positively charged proteins independent of specific amino acid motifs ([Hammond et al., 2012](#); [Simon et al., 2016](#)). Examples of the role of PI4P in electrostatic signature include the recruitment of the polar auxin transport regulator PINOID and other signaling molecules to the cell plate in plants ([Simon et al., 2016](#)); the recruitment of D6 protein kinase ([Barbosa et al., 2016](#)), NPH3 ([Reuter et al., 2021](#)), and the exocyst complex ([Synek et al., 2021](#)) to the plasma membrane in plants; the recruitment of Lgl and Dlg proteins, which function in cell polarity and tumorigenesis, to the plasma membrane in *Drosophila* ([Dong et al., 2015](#); [Lu et al., 2021](#)); and the recruitment of NLRP3 to the disassembled trans-Golgi network during inflammasome activation in mammals ([Chen and Chen,](#)

2018). In the present study, we showed that autophagosomal PI4P also serves as a regulator of surface charge. Given that the function of elongating unclosed autophagosomes (i.e., sequestration) and mature closed autophagosomes (i.e., fusion with lysosomes) differs despite using seemingly the same membrane, electrostatic maturation would be an efficient way for the function to switch.

## Materials and Methods

### Plasmids and antibodies

First, cDNAs encoding human STX17 (Itakura *et al.*, 2012), *D. melanogaster* STX17 (NM\_079202), *C. elegans* STX17 (NM\_059941), rat LC3B (Hara *et al.*, 2008), and mouse ATG5 (NM\_053069) were inserted into pMRXIP (harboring a puromycin-resistant marker) (Kitamura *et al.*, 2003; Saitoh *et al.*, 2002), pMRXIZ (harboring a zeocin-resistant marker) (Morita *et al.*, 2018), and pMRXIB (harboring a blasticidin-resistant marker) (Morita *et al.*, 2018) together with enhanced GFP or mRuby3 (codon-optimized, Addgene #74252). STX17 fragments and their point mutations were generated by a standard PCR method or PCR-mediated site-directed mutagenesis.

Membrane surface charge probes were constructed by annealing with the following oligonucleotides (purchased from Thermo Fisher Scientific), after which they were inserted into pMRXIP plasmid with enhanced GFP:

GFP-9K0Q (GGCTCGGGATCCGGAATTCATCCAAAGATGGAAAAAAAAAGAAGAAGAA AAGTAAAACCAAATGCGTGATTATGTAACGAGAGCGGCCGCT, AGCGGCCGCTCTCGAGTTACATAATCACGATTTGGTTTACTTTTCTTCTTCT TTTTTTTCCATCTTTGGATGAATTCGCGGATCCCGAGCC); GFP-5K4Q (GGCTCGGGATCCGGAATTCAGCAAAGACGGCCAGCAGCAACAAAAGAA GTCTAAGACCAAGTGTGAATCATGTAACGAGAGCGGCCGCT, AGCGGCCGCTCTCGAGTTACATGATTACACACTTGGTCTTAGACTTCTTTTGT TGCTGCTGGCCGCTTTGCTTGAATTCGCGGATCCCGAGCC); GFP-3K6Q (GGCTCGGGATCCGGAATTCATCCAAGGACGGACAACAGCAGCAACAACA GAGTAAACTAAATGCGTGATAATGTAACGAGAGCGGCCGCT, AGCGGCCGCTCTCGAGTTACATTATCACGATTTAGTTTACTCTGTTGTTGC TGCTGTTGTCCGTCCTTGGATGAATTCGCGGATCCCGAGCC); GFP-1K8Q (GGCTCGGGATCCGGAATTCATCCAGGACGGTCAGCAGCAACAACAGCA ATCACAATAAATGTGAATAATGTAACGAGAGCGGCCGCT, AGCGGCCGCTCTCGAGTTACATTATTACACATTTAGTTTGTGATTGCTGTTGTT GCTGCTGACCGTCTGGGATGAATTCGCGGATCCCGAGCC). GFP-0K9Q was generated by PCR-mediated site-directed mutagenesis.

To generate lipid probes, cDNAs encoding human 2×Spo20(PABD) (NM\_005633; amino acids 422–551) (Kassas *et al.*, 2017), CERT(PHD) (NM\_001130105; amino acids 1–116) (Hanada, 2006), FAPP (NM\_001807; amino acids 1–101) (Balla *et al.*, 2005), OSBP (NM\_002556; amino acids 87–185) (Levine and Munro, 1998), 2×ING2(PlantHD) (NM\_001564; amino acids 190–280) (Gozani *et al.*, 2003), 2×TAPP1(PHD) (NM\_001001974; amino acids 184–304) (Dowler *et al.*, 2000), 2×TRPML1(PHD) (NM\_020533; amino acids 1–69) (Dong *et al.*, 2010), Btk (NM\_000061; amino acids 1–177) (Baraldi *et al.*, 1999), and rat PLCd1 (NM\_017035; amino acids 1–175) (Varnai and Balla, 1998) were inserted into pMRXIP. GFP-Evectin-2 was provided by Hiroyuki Arai; GFP-PKD C1ab was provided by Tamas Balla; and mRFP-2×FYVE was provided by Harald Stenmark. GFP-P4M-SidMx2 (51472; Addgene) was amplified by PCR and subcloned into pMRXIP.

For the *in vitro* autophagosome recruitment assay, DNA fragments encoding GST–HRV3C-tagged yeast Sac1 (phosphatase domain; 2–517 amino acids) (NM\_001179777) or its C392S mutant and mGFP (monomeric enhanced GFP with A206K mutation)–STX17TM were inserted downstream of the polyhedrin promoter of the pFastBac Dual Expression vector (10712024; Thermo Fisher Scientific).

For immunoblotting, mouse monoclonal anti-TOMM20 (sc-11415; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-LAMP1 (ab24170; Abcam), anti-p62 (PM045; MBL), and anti-LC3B ([Kabeya et al., 2000](#) [DOI](#)) antibodies were used as primary antibodies. HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG (111-035-003, 111-035-144; Jackson ImmunoResearch Laboratories) antibodies were used as secondary antibodies.

For immunostaining, rabbit polyclonal anti-LAMP1 (ab24170; Abcam) was used as a primary antibody, while Alexa Fluor 660-anti-rabbit IgG (A-21074; Molecular Probes) was used as secondary antibodies.

## Cell culture

Mouse embryonic fibroblasts (MEFs), U2OS cells, HeLa cells, and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (D6546; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (S1820500; Biowest) and 2 mM L-glutamine (25030081; Gibco) in a 5% CO<sub>2</sub> incubator. For the starvation treatment, cells were washed twice with phosphate-buffered saline (PBS) and incubated in amino acid-free DMEM (04833575; Fujifilm Wako Pure Chemical Corp.) without FBS. The STX17 KO HeLa cells used in the present study were as previously described ([Matsui et al., 2018](#) [DOI](#)). ATG8 hexa KO HeLa cells (lacking LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2) were kindly provided by Michael Lazarou ([Nguyen et al., 2016](#) [DOI](#)).

For transient expression of mRuby3–CERT(PHD), HeLa cells were transiently transfected using FuGENE HD (VPE2311; Promega) with pMRXIB-mRuby3–CERT(PHD) for 24 h in Opti-MEM (31985-070; Gibco).

## Retroviral infections and generation of stable cell lines

HEK293T cells were transiently transfected using Lipofectamine 2000 (11668019; Thermo Fisher Scientific) or FuGENE HD (VPE2311; Promega) with retrovirus vectors, pCG-gag-pol, and pCG-VSV-G (provided by Teruhiko Yasui). After cells were cultured for 2–3 days, the supernatant was collected and filtered through a 0.45-μm syringe filter unit (SLHV033RB; EMD Millipore). The obtained cells were cultured with retrovirus and 8 μg/ml polybrene (H9268; Sigma-Aldrich). Uninfected cells were eliminated by puromycin (P8833; Sigma-Aldrich), blasticidin (02218713; Fujifilm Wako Pure Chemical Corp.), geneticin (10131; Thermo Fisher Scientific), and zeocin (R25005; Thermo Fisher Scientific).

## Fluorescence microscopy

Live-cell fluorescence imaging was performed using a Delta Vision Elite widefield fluorescence microscope (GE Healthcare Life Science) equipped with a 100× PlanAPO oil-immersion objective lens (Olympus, NA1.40) and a cooled-CCD camera (Photometrics, CoolSNAP HQ2). Cells stably expressing GFP and mRuby3 were grown on a glass-bottom dish (617870; Greiner bio-one, or 11004006; IWAKI). To observe lysosomes, 50 nM LysoTracker Red DND99 (L7528; Thermo Fisher Scientific) or LysoTracker Deep Red (L12492; Thermo Fisher Scientific) was added to the medium.

To observe HaloTag–LC3B, cells were observed in the presence of 200 nM SaraFluor 650T HaloTag ligand (GoryoChemical, A308-02). During live-cell imaging, the culture dish was mounted in a chamber (INUB-ONI-F2; TOKAI HIT) to maintain the culture conditions (37°C, 5% CO<sub>2</sub>). Images

were acquired at 30-s intervals. Time series of 16-bit images were converted into RGB Tiff images using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, <http://imagej.nih.gov/ij/>, 1997–2018). GFP intensity of autophagosomal structures was determined using ImageJ. Additionally, mRuby3–LC3 or Atg5-positive structures, including punctate and cup-shape structures (premature autophagosomes) and ring-like structures (mature autophagosomes) were extracted by the Analyze Particle function after binarization using the Auto Threshold v1.17 plugin (Method, Max Entropy). The GFP intensity of each particle was measured by the Measure ImageJ plugin, and the background signal (i.e., average GFP intensity of the entire cell) was subtracted.

The GFP–STX17TM intensity of mature autophagosomes or liposomes was determined using ImageJ. The center of ring-like structures in the red channel (LC3B or liposome signals) was first selected using the multi-point tool. The region of interest (ROI) was then defined by drawing a 10- $\mu$ m-diameter circle around the previously determined center and capturing the red channel (LC3B or liposome signals). The GFP–STX17TM intensity was measured by the Radial Profile Plot ImageJ plugin, and the background signal of the surrounding cytosol area was subtracted.

## Preparation of recombinant proteins

*In vitro* transcription and translation in the rabbit reticulocyte lysate system were performed according to a published method with minor modifications (Sharma *et al.*, 2010). For *in vitro* mRNA synthesis, DNA templates containing the SP6 RNA polymerase promoter site upstream of the sequence to be transcribed were generated by a standard PCR method and then incubated for 1 h at 37°C using the mMACHINE SP6 Transcription Kit (AM1340; Thermo Fisher Scientific). For *in vitro* protein synthesis, transcripts were incubated with rabbit reticulocyte lysates (L4960; Promega) for 30 min at 37°C.

Recombinant yeast Sac1 (amino acids 2–517) and mGFP–STX17TM (amino acids 229–302) proteins were prepared using the Bac-to-Bac baculovirus expression system (10359016; Thermo Fisher Scientific). Bacmid DNAs were prepared using DH10Bac *E. coli* cells (10361012; Thermo Fisher Scientific), after which High Five insect cells (BTI-TN-5B1-4; B85502; Thermo Fisher Scientific) were transfected with the bacmid DNAs using Cellfectin II (10362100; Thermo Fisher Scientific).

Baculoviruses were generated and amplified in the High Five cells. After a 96-h viral infection, the cells were suspended in 2 ml of ice-cold homogenization buffer (20 mM HEPES-KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA, and protease inhibitor cocktail). Cells were then disrupted by ultrasonication using a UD-201 ultrasonic disruptor (TOMY). After centrifugation at 16,500  $\times g$  for 10 min, the supernatants were incubated with Glutathione Sepharose 4B (17075601; GE Healthcare) for 3 h at 4°C with gentle rotation. The sepharose resins were washed three times with homogenization buffer and then treated with HRV3C protease (206-18151; Fujifilm Wako Pure Chemical Corp.) for 12 h at 16°C. The eluted proteins were stored at –80°C. To confirm recombinant expression, purified samples were separated by SDS-PAGE and detected by CBB staining using CBB Stain One Super (11642-31; Nacalai Tesque).

## Preparation of phospholipid vesicles

Liposomes were prepared as follows. First, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (850375 C; Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (850725 C; Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) (840035P; Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (18:1 PI) (850149P; Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate) (18:1 PI3P) (850150P; Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4'-phosphate) (18:1 PI4P) (850151P; Avanti Polar Lipids), and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4'-bisphosphate) (18:1 PI(3,4)P<sub>2</sub>) (850153P; Avanti Polar Lipids) dissolved in chloroform to 1  $\mu$ M final concentrations were mixed in a glass tube at the indicated ratios in the figure legends. Then, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000) Biotin)

(880129C; Avanti Polar Lipids) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (18:1 Liss Phod PE) (810150C; Avanti Polar Lipids) were added to the mixtures to label the liposomes. The chloroform was evaporated under argon gas and then completely dried in a vacuum desiccator overnight. The lipid film was hydrated in KHM buffer (20 mM HEPES-NaOH [pH 7.4], 110 mM potassium acetate, 2 mM MgCl<sub>2</sub>) at a concentration of 1 mM and incubated for 16 h at 30°C.

## Purification of mature autophagosomes

STX17 KO HeLa cells stably expressing mRuby3-LC3B were harvested from two 10-cm dishes and washed twice with ice-cold PBS. The cell pellets were collected after centrifugation at  $700 \times g$  for 5 min and resuspended in 1 ml of ice-cold homogenization buffer (20 mM HEPES-KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA, and protease inhibitor cocktail). Cells were then disrupted by passage through a 25-gauge needle. The homogenized cells were centrifuged at  $3,000 \times g$  for 10 min to remove cell debris and undrupted cells. The supernatant was diluted with an equal volume of 50% OptiPrep (1893; Cosmo Bio) with complete EDTA-free protease inhibitor (11873580001; Roche). Discontinuous OptiPrep gradients were generated in MLS-50 tubes (344057; Beckman Coulter) by overlaying each of the following OptiPrep solutions in homogenization buffer (20 mM HEPES-KOH [pH 7.4], 250 mM sucrose, 1 mM EDTA): 1.25 ml of the diluted supernatant in 25% OptiPrep, 0.25 ml in 20%, 0.75 ml in 15%, 0.75 ml in 10%, 2.0 ml in 5%, and 0.25 ml in 0%. The gradients were centrifuged at  $150,000 \times g$  in MLS-50 rotors (Beckman Instruments) for 3 h, and subsequently, 10 fractions (0.5 ml each) were collected from the top. Proteins in each fraction were isolated by TCA precipitation. The samples were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (IPVH00010; EMD Millipore). Immunoblotting analysis was performed with the indicated antibodies. Immobilon Western Chemiluminescent HRP Substrate (P90715; EMD Millipore) was used to visualize the signals, which were detected on an IQ800 biomolecular imager (Cytiva). Contrast and brightness adjustments were performed using Photoshop 2022 (Adobe).

## *In vitro* recruitment assay

For the liposome binding assay, the prepared liposomes were mixed with recombinant proteins produced by rabbit reticulocyte lysate for 15 min at 30°C. A 0.2-mm-thick chamber was created by attaching two glass cover slips (Matunami glass) together with double-sided tape applied along the long edges. NeutrAvidin Protein (31000; Thermo Fisher Scientific) was added to the chamber for 15 min at room temperature before being washed out with KHM buffer. The liposome–recombinant protein mixture was then transferred to the NeutrAvidin Protein-coated chamber. Images were captured immediately after using a confocal microscope (FV3000; Olympus) equipped with a 60× PlanAPO oil-immersion objective lens (Olympus, NA1.42).

For the autophagosome recruitment assay, the LC3-positive, LAMP1-negative autophagosomal fraction was incubated with 0.5 µg of Sac1 proteins from insect cells for 30 min at 37°C. Then, mGFP-STX17TM was added to the mixture, which was then incubated again for 30 min at 37°C. The mixtures were transferred to a glass-bottom dish (617870; Greiner bio-one), and images were captured using a fluorescence microscope (BZ-810; Keyence) equipped with a 60× oil-immersion objective lens (Nikon, NA1.40).

## RNA interference

Stealth RNAi oligonucleotides were purchased from Thermo Fisher Scientific. The following sequences were used: human YKT6 siRNA antisense, 5'-GGTGTGGTCATTGCTGACAATGAAT -3'; human YKT6 siRNA antisense sense, 5'-ATTCATTGTCAGCAATGACCACACC -3' (*Matsui et al.*, 2018 [DOI](#)). The siRNA oligonucleotides for human STX17 (HSS123732 antisense, 5'-AATTAAGTCCGCTTCTAAGGTTTCC -3'; HSS123732 sense, 5'-GGAAACCTTAGAAGCGGACTTAATT -3') were previously reported (*Itakura et al.*, 2012 [DOI](#)). The stealth RNAi oligonucleotides were

transfected into cells using Lipofectamine RNAiMAX (13778150; Thermo Fisher Scientific) according to the manufacturer's instructions. Three days after transfection, cells were used for analysis.

## Immunostaining

Cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde phosphate buffer solution (0915485; Nacalai Tesque) for 10 min at room temperature. Fixed cells were permeabilized with 50 µg/ml digitonin (D141; Sigma-Aldrich) in PBS for 5 min, blocked with 3% BSA in PBS for 10 min, and then incubated with primary antibodies for 16 h at 4°C. After being washed three times with PBS, cells were incubated with secondary antibodies for 1 h at room temperature. The coverslips were observed using a confocal laser microscope (FV3000; Olympus) equipped with a 60× PlanAPO oil-immersion objective lens (Olympus, NA1.42).

## Sequence alignment

Amino acid sequences of STX17 from the following species were obtained from the NCBI protein database: *Homo sapiens* (NP\_060389.2), *Mus musculus* (NP\_080619.2), *Danio rerio* (NP\_001007450.1), *Ciona intestinalis* (NP\_492342.1), *Drosophila melanogaster* (NP\_523926.1), and *Caenorhabditis elegans* (NP\_492342.1). The sequences were aligned using clustal W (<https://www.genome.jp/tools-bin/clustalw>) as implemented in Molecular Evolutionary Genetics Analysis X (Kumar et al., 2018b).

## Molecular dynamics simulation

We used the all-atom model for SXT17TM utilizing initial structures predicted by trRosetta, a method based on deep learning (Du et al., 2021). Because five different three-dimensional structural models were predicted (TM score > 0.7), each was used in the simulations. The lipid bilayer was modeled using the HMMM model, which was developed to study protein-membrane interactions more efficiently by replacing the membrane lipids with short-chain lipid and organic solvents to facilitate lateral diffusion (Ohkubo et al., 2012). The lipid headgroups in the HMMM model were identical to those in the all-atom model and faithfully represented the membrane surface. The lipid membrane consisted of 150 lipid molecules in each leaflet. The membranes with and without PI4P were examined for lipid compositions of POPC:POPE:POPI4P (POPI14) = 70:20:10, 70:30:0 or POPC:POPE:POPI = 70:20:10 respectively. The initial configuration was prepared so that the center of mass of SXT17TM was located 3 nm above the membrane surface and the first principal axis of the atomic configuration was tilted 45 degrees from the z axis. The protein and lipids were solvated in a 10 nm × 10 nm × 30 nm box with the TIP3P water model and 0.15 M KCl ions. The initial configurations were built by the Membrane Builder module in the CHARMM-GUI server (Jo et al., 2008). All molecular dynamics simulations were performed using GENESIS (Jung et al., 2015). The CHARMM36 force-field was used to describe the interactions in the system (Huang et al., 2017). Energy minimization was performed for 1000 steps by the steepest descent algorithm and then by the conjugate gradient algorithm. Then, a 250 ps NVT simulation was performed at 303.15 K for solvent equilibration, followed by a 1.6 ns NPT equilibration to 1 atm using the Langevin thermostat/barostat (Quigley and Probert, 2004). The production simulations were performed for 100 ns with a time-step of 2.5 fs and the Langevin thermostat/barostat. Long-range electrostatic interactions were simulated using the particle-mesh Ewald method (Darden et al., 1993; Essmann et al., 1995). The short-range electrostatic and van der Waals interactions both used a cutoff of 12 Å. All bonds were constrained by the SHAKE/RATTLE algorithm (Andersen, 1983; Ryckaert et al., 1977).

## Statistical analysis

Comparisons between data from two groups were evaluated using Welch's *t*-test, and comparisons of data among multiple groups were performed using one-way analysis of variance (ANOVA) followed by the Dunnett's, Sidak's, or Tukey's multiple comparison tests as implemented in

GraphPad Prism 8 and 9 (GraphPad Software). Data distributions were assumed to be normal, but this was not formally tested.

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S.S., H.Y., and N.M. designed the project and analyzed the data. S.S. performed most experimental work and analyzed data. Y.S. designed, performed, and analyzed the molecular dynamics simulation. S.S., T.M., M.U., and H.Y. performed *in vitro* reconstitution experiments. S.S., I.K.H., J.S., and H.Y. performed *in vivo* PI4P depletion analysis. S.S., Y.S., H.Y., and N.M. wrote the manuscript. H.Y. and N.M. supervised the project. All authors commented on the manuscript.

## Competing Interest

Noboru Mizushima: a Reviewing editor, *eLife*. The other authors declare that no competing interests exist.

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**David Ron**

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### Reviewer #1 (Public Review):

In this manuscript, the authors report a molecular mechanism for recruiting syntaxin 17 (Syn17) to the closed autophagosomes through the charge interaction between enriched PI4P and the C-terminal region of Syn17. How to precisely control the location and conformation of proteins is critical for maintaining autophagic flux. Particularly, the recruitment of Syn17 to autophagosomes remains unclear. In this paper, the author describes a simple lipid-protein interaction model beyond previous studies focusing on protein-protein interactions. This represents conceptual advances.

<https://doi.org/10.7554/eLife.92189.2.sa2>

### Reviewer #2 (Public Review):

Summary:

Syntaxin17 (STX17) is a SNARE protein that is recruited to mature (i.e., closed) autophagosomes, but not to immature (i.e., unclosed) ones, and mediates the autophagosome-lysosome fusion. How STX17 recognizes the mature autophagosome is an unresolved interesting question in the autophagy field. Shinoda and colleagues set out to answer this question by focusing on the C-terminal domain of STX17 and found that PI4P is a strong candidate that causes the STX17 recruitment to the autophagosome.

Strengths:

The main findings are: 1) Rich positive charges in the C-terminal domain of STX17 are sufficient for the recruitment to the mature autophagosome; 2) Fluorescence charge sensors of different strengths suggest that autophagic membranes have negative charges and the charge increases as they mature; 3) Among a battery of fluorescence biosensors, only PI4P-binding biosensors distribute to the mature autophagosome; 4) STX17 bound to isolated autophagosomes is released by treatment with Sac1 phosphatase; 5) By dynamic molecular simulation, STX17 TM is shown to be inserted to a membrane containing PI4P but not to a membrane without it. These results indicate that PI4P is a strong candidate that STX17 binds to in the autophagosome.

Weaknesses:

- It was not answered whether PI4P is crucial for the STX17 recruitment in cells because manipulation of the PI4P content in autophagic membranes was not successful for unknown reasons.
- The question that the authors posed in the beginning, i.e., why is STX17 recruited to the mature (closed) autophagosome but not to immature autophagic membranes, was not answered. The authors speculate that the seemingly gradual increase of negative charges in autophagic membranes is caused by an increase in PI4P. However, this was not supported by

the PI4P fluorescence biosensor experiment that showed their distribution to the mature autophagosome only. Here, there are at least two possibilities: 1) The increase of negative charges in immature autophagic membranes is derived from PI4P. However the fluorescence biosensors do not bind there for some reason; for example, they are not sensitive enough to recognize PI4P until it reaches a certain level, or simply, their binding does not occur in a quantitative manner. 2) The negative charge in immature membranes is not derived from PI4P, and PI4P is generated abundantly only after autophagosomes are closed. In either case, it is not easy to explain why STX17 is recruited to the mature autophagosome only. For the first scenario, it is not clear how the PI4P synthesis is regulated so that it reaches a sufficient level only after the membrane closure. In the second case, the mechanism that produces PI4P only after the autophagosome closure needs to be elucidated (so, in this case, the question of the temporal regulation issue remains the same).

<https://doi.org/10.7554/eLife.92189.2.sa1>

### Reviewer #3 (Public Review):

#### Summary:

In this study, the authors set out to address the question of how the SNARE protein Syntaxin 17 senses autophagosome maturation by being recruited to autophagosomal membranes only once autophagosome formation and sealing is complete. The authors discover that the C-terminal region of Syntaxin 17 is essential for its sensing mechanism that involves two transmembrane domains and a positively charged region. The authors discover that the lipid PI4P is highly enriched in mature autophagosomes and that electrostatic interaction with Syntaxin 17's positively charged region with PI4P drives recruitment specifically to mature autophagosomes. The temporal basis for PI4P enrichment and Syntaxin 17 recruitment to ensure that unsealed autophagosomes do not fuse with lysosomes is a very interesting and important discovery. Overall, the data are clear and convincing, with the study providing important mechanistic insights that will be of broad interest to the autophagy field, and also to cell biologists interested in phosphoinositide lipid biology. The author's discovery also provides an opportunity for future research in which Syntaxin 17's c-terminal region could be used to target factors of interest to mature autophagosomes.

#### Strengths:

The study combines clear and convincing cell biology data with in vitro approaches to show how Syntaxin 17 is recruited to mature autophagosomes. The authors take a methodical approach to narrow down the critical regions within Syntaxin 17 required for recruitment and use a variety of biosensors to show that PI4P is enriched on mature autophagosomes.

#### Weaknesses:

There are no major weaknesses, overall the work is highly convincing. It would have been beneficial if the authors could have shown whether altering PI4P levels would affect Syntaxin 17 recruitment. However, this is understandably a challenging experiment to undertake and the authors outlined their various attempts to tackle this question.

<https://doi.org/10.7554/eLife.92189.2.sa0>

### Author Response

The following is the authors' response to the original reviews.

### Reviewer #1 (Public Review):

*In this manuscript, the authors report a molecular mechanism for recruiting syntaxin 17 (Syn17) to the closed autophagosomes through the charge interaction between enriched PI4P and the C-terminal region of Syn17. How to precisely control the location and conformation of proteins is critical for maintaining autophagic flux. Particularly, the recruitment of Syn17 to autophagosomes remains unclear. In this paper, the author describes a simple lipid-protein interaction model beyond previous studies focusing on protein-protein interactions. This represents conceptual advances.*

We would like to thank Reviewer #1 for the positive evaluation of our study.

### Reviewer #2 (Public Review):

#### Summary:

*Syntaxin17 (STX17) is a SNARE protein that is recruited to mature (i.e., closed) autophagosomes, but not to immature (i.e., unclosed) ones, and mediates the autophagosome-lysosome fusion. How STX17 recognizes the mature autophagosome is an unresolved interesting question in the autophagy field. Shinoda and colleagues set out to answer this question by focusing on the C-terminal domain of STX17 and found that PI4P is a strong candidate that causes the STX17 recruitment to the autophagosome.*

#### Strengths:

*The main findings are: 1) Rich positive charges in the C-terminal domain of STX17 are sufficient for the recruitment to the mature autophagosome; 2) Fluorescence charge sensors of different strengths suggest that autophagic membranes have negative charges and the charge increases as they mature; 3) Among a battery of fluorescence biosensors, only PI4P-binding biosensors distribute to the mature autophagosome; 4) STX17 bound to isolated autophagosomes is released by treatment with Sac1 phosphatase; 5) By dynamic molecular simulation, STX17 TM is shown to be inserted to a membrane containing PI4P but not to a membrane without it. These results indicate that PI4P is a strong candidate that STX17 binds to in the autophagosome.*

We would like to thank Reviewer #2 for pointing out these strengths.

#### Weaknesses:

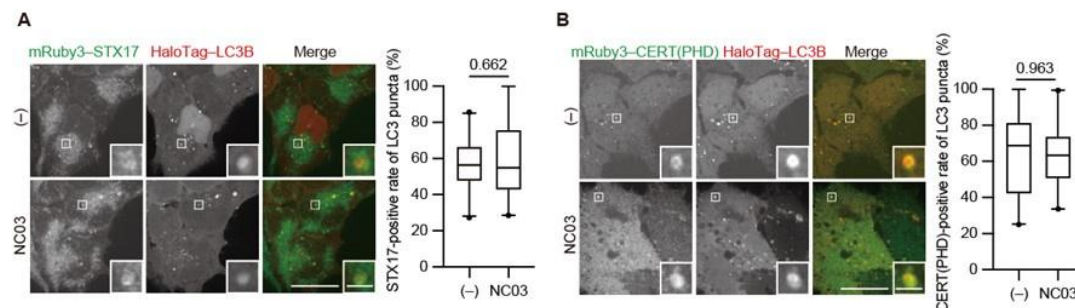
- It was not answered whether PI4P is crucial for the STX17 recruitment in cells because manipulation of the PI4P content in autophagic membranes was not successful for unknown reasons.*

As we explained in the initial submission, we tried to deplete PI4P in autophagosomes by multiple methods but did not succeed. In this revised manuscript, we added the result of an experiment using the PI 4-kinase inhibitor NC03 (Figure 4—figure supplement 1), which shows no significant effect on the autophagosomal PI4P level and STX17 recruitment.

### Author response image 1.

The PI 4-kinase inhibitor NC03 failed to suppress autophagosomal PI4P accumulation and STX17 recruitment. HEK293T cells stably expressing mRuby3-STX17TM (A) or mRuby3-CERT(PHD) (B) and Halotag-LC3 were cultured in starvation medium for 1 h and then treated with and without 10  $\mu$ M NC03 for 10 min. Representative confocal images are shown. STX17TM- or CERT(PHD)-positive rates of LC3 structures per cell ( $n > 30$  cells) are shown in the graphs. Solid horizontal lines indicate medians, boxes indicate the interquartile ranges

(25th to 75th percentiles), and whiskers indicate the 5th to 95th percentiles. Differences were statistically analyzed by Welch's t-test. Scale bars, 10  $\mu\text{m}$  (main), 1  $\mu\text{m}$  (inset).

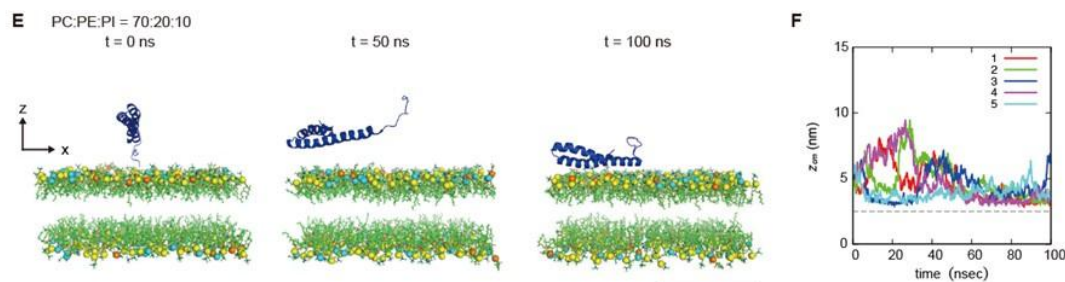


- The molecular simulation study did not show whether PI4P is necessary for the STX17 TM insertion or whether other negatively charged lipids can play a similar role.

As the reviewer suggested, we performed the molecular dynamics simulation using membranes with phosphatidylinositol, a negatively charged lipid. STX17 TM approached the PI-containing membrane but was not inserted into the membrane within a time scale of 100 ns in simulations of all five structures. This data suggests that PI4P, which is more negatively charged than PI, is required for STX17 insertion. Thus, we have included these data in Figure 5E and F and added the following text to Lines 242–244. “Moreover, if the membrane contained phosphatidylinositol (PI) instead of PI4P, STX17 approached the PI-containing membrane but was not inserted into the membrane (Figure 5E, F, Video 3).”

## Author response image 2.

(E) An example of a time series of simulated results of STX17TM insertion into a membrane consisting of 70% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), and 10% phosphatidylinositol (PI). STX17TM is shown in blue. Phosphorus in PC, PE and PI are indicated by yellow, cyan, and orange, respectively. Short-tailed lipids are represented as green sticks. The time evolution series are shown in Video 3. (F) Time evolution of the z-coordinate of the center of mass ( $z_{\text{cm}}$ ) of the transmembrane helices of STX17TM in the case of membranes with PI. Five independent simulation results are represented by solid lines of different colors. The gray dashed lines indicate the locations of the lipid heads. A scale bar indicates 5 nm.



- The question that the authors posed in the beginning, i.e., why is STX17 recruited to the mature (closed) autophagosome but not to immature autophagic membranes, was not answered. The authors speculate that the seemingly gradual increase of negative charges in autophagic membranes is caused by an increase in PI4P. However, this was not supported by the PI4P fluorescence biosensor experiment that showed their

*distribution to the mature autophagosome only. Here, there are at least two possibilities: 1) The increase of negative charges in immature autophagic membranes is derived from PI4P. However the fluorescence biosensors do not bind there for some reason; for example, they are not sensitive enough to recognize PI4P until it reaches a certain level, or simply, their binding does not occur in a quantitative manner. 2) The negative charge in immature membranes is not derived from PI4P, and PI4P is generated abundantly only after autophagosomes are closed. In either case, it is not easy to explain why STX17 is recruited to the mature autophagosome only. For the first scenario, it is not clear how the PI4P synthesis is regulated so that it reaches a sufficient level only after the membrane closure. In the second case, the mechanism that produces PI4P only after the autophagosome closure needs to be elucidated (so, in this case, the question of the temporal regulation issue remains the same).*

We thank the reviewers for pointing this out. While the probe for weakly negative charges (1K8Q) labeled both immature and mature autophagosomes, the probes for intermediate charges (5K4Q and 3K6Q) and PI4P labeled only mature autophagosomes (Figure 2F, Figure 2-figure supplement 1B). Thus, we think that the autophagosomal membrane rapidly and drastically becomes negatively charged, and at the same time, PI4P is enriched. Although immature membranes may have weak negative charges, we did not examine which lipids contribute to the negative charges. Thus, we have added the following sentences to the Discussion part.

“Our data of the 1K8Q probe suggest that immature autophagosomal membranes may also have slight negative charges (Figure 2E). Although the source of the negative charge of immature autophagosomes is currently unknown, it may be derived from low levels of PI4P, which is undetectable by the PI4P probes and/or other negatively charged lipids such as PI and PS (Schmitt et al., EMBO Rep, 2022).” (Lines 279–283) “In any case, it would be important to elucidate how PI 4-kinase activity or PI4P synthesis is upregulated during autophagosome maturation.” (Lines 302–303)

### **Reviewer #3 (Public Review):**

#### *Summary:*

*In this study, the authors set out to address the question of how the SNARE protein Syntaxin 17 senses autophagosome maturation by being recruited to autophagosomal membranes only once autophagosome formation and sealing is complete. The authors discover that the C-terminal region of Syntaxin 17 is essential for its sensing mechanism that involves two transmembrane domains and a positively charged region. The authors discover that the lipid PI4P is highly enriched in mature autophagosomes and that electrostatic interaction with Syntaxin 17's positively charged region with PI4P drives recruitment specifically to mature autophagosomes. The temporal basis for PI4P enrichment and Syntaxin 17 recruitment to ensure that unsealed autophagosomes do not fuse with lysosomes is a very interesting and important discovery. Overall, the data are clear and convincing, with the study providing important mechanistic insights that will be of broad interest to the autophagy field, and also to cell biologists interested in phosphoinositide lipid biology. The author's discovery also provides an opportunity for future research in which Syntaxin 17's c-terminal region could be used to target factors of interest to mature autophagosomes.*

#### *Strengths:*

*The study combines clear and convincing cell biology data with in vitro approaches to show how Syntaxin 17 is recruited to mature autophagosomes. The authors take a methodical approach to narrow down the critical regions within Syntaxin 17 required for*

*recruitment and use a variety of biosensors to show that PI4P is enriched on mature autophagosomes.*

We would like to thank Reviewer #3 for the positive comments.

*Weaknesses:*

*There are no major weaknesses, overall the work is highly convincing. It would have been beneficial if the authors could have shown whether altering PI4P levels would affect Syntaxin 17 recruitment. However, this is understandably a challenging experiment to undertake and the authors outlined their various attempts to tackle this question.*

We thank Reviewer #3 for pointing this out. Please see our above response to Reviewer #2 (Public Review).

*In addition, clear statements within the figure legends on the number of independent experimental repeats that were conducted for experiments that were quantitated are not currently present in the manuscript.*

As pointed out by Reviewer #3, we have added the number of independent experimental repeats in the figure legends.

**Reviewer #1 (Recommendations For The Authors):**

*This paper is well written and all experiments were conducted with a high standard. Several minor issues should be addressed before final publication.*

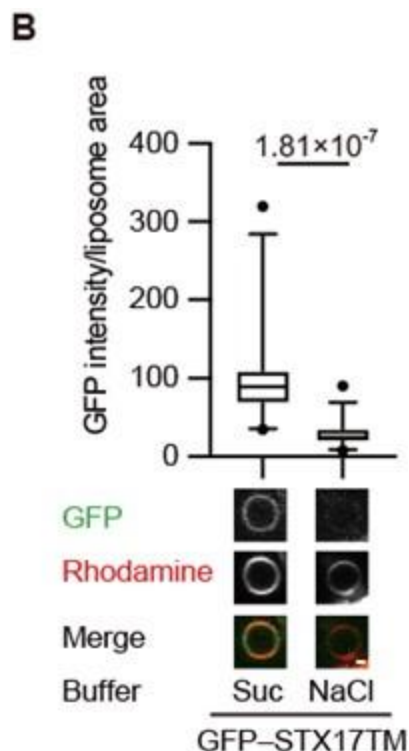
*(1) To further confirm the charge interaction, a charge screening experiment should be performed for Fig. 2A.*

We have asked Reviewer #1 through the editor what this experiment meant and understood that it was to see the effects of high salt concentrations. We monitored the association of GFP-STX17TM with liposomes in the presence or absence of 1 M NaCl and found that it was blocked in a high ionic buffer. This data supports the electrostatic interaction of STX17 with membranes. We have included this data in Figure 2B and added the following sentences to Lines 124–126.

“The association of STX17TM with PI4P-containing membranes was abolished in the presence of 1 M NaCl (Figure 2B). These data suggest that STX17 can be recruited to negatively charged membranes via electrostatic interaction independent of the specific lipid species.”

### **Author response image 3.**

GFP-STX17TM translated in vitro was incubated with rhodamine-labeled liposomes containing 70% PC, 20% PE and 10% PI4P in the presence of 1 M NaCl or 1.2 M sucrose. GFP intensities of liposomes were quantified and shown as in Figure 1C (n > 30).



(2) The authors claim that "Autophagosomes become negatively charged during maturation", based on experiments using membrane charge probes. Since it's mainly about the membrane, it's better to refine the claim to "The membrane of autophagosomes becomes...", which would be more precise and close to the topic of this paper.

We would like to thank the reviewer for pointing this out. This point is valid. As recommended, we have collected the phrases "Autophagosomes become negatively charged during maturation" to "The membrane of autophagosomes becomes negatively charged during maturation" (Line 72, 118, 262, 969 (title of Figure2), 1068 (title of Figure2-figure supplement1)).

(3) The authors should add more discussion regarding the "specificity" for recruiting Syn17 through the charge interaction. Particularly, how Syn17 could be maintained before the closure of autophagosomes? For the MD simulations in Fig. 5, the current results don't add much to the manuscript. The cell biology experiments have demonstrated the conclusion. The authors could try to find more details about the insertion by analyzing the simulation movies. Do membrane packing defects play a role during the insertion process? A similar analysis was conducted for alpha-synuclein (<https://pubmed.ncbi.nlm.nih.gov/33437978/>).

Regarding the mechanism of STX17 maintenance in the cytosol, we do not think that other molecules, such as chaperones, are essential because purified recombinant mGFP-STX17TM used in this study is soluble. However, it does not rule out such a mechanism, which would be a future study.

In the paper by Liu et al. (PMID: 33437978), small liposomes with diameters of 25–50 nm are used. Therefore, there are packing defects in the highly curved membranes, to which alpha-synuclein helices are inserted in a curvature-dependent manner. On the other hand,

autophagosomes are much larger (~1  $\mu\text{m}$  in diameter) and almost flat for STX17 molecules, so we think it is unlikely that STX17 recognizes the packing defect.

**Reviewer #2 (Recommendations For The Authors):**

- *The two (and other) possibilities with regards to the interpretation of the negative charge/PI4P result in autophagic membranes are hoped to be discussed.*

As mentioned above, we have added the following sentences to the Discussion section. “Our data of the 1K8Q probe suggest that immature autophagosomal membranes may also have slight negative charges (Figure 2E). Although the source of the negative charge of immature autophagosomes is currently unknown, it may be derived from low levels of PI4P, which is undetectable by the PI4P probes and/or other negatively charged lipids such as PI and PS (Schmitt et al., EMBO Rep, 2022).” (Lines 279–283)

“In any case, it would be important to elucidate how PI 4-kinase activity or PI4P synthesis is upregulated during autophagosome maturation.” (Lines 302–303)

- *Fluorescence biosensors are convenient to give an overview of the intracellular distribution of various lipids, but some of them show false-negative results. For example, evectin-2-PH for PS binds to endosomes but not to the plasma membrane, even though the latter contains abundant PS. With regards to PI4P, some biosensors illuminate both the Golgi and autophagosome, while others do not appear to bind the Golgi. Moreover, fluorescence biosensors for PI(3,5)P2 and PI(3,4)P2, which are also candidates for the STX17 insertion issue, are less reliable than others (e.g., those for PI3P and PI(4,5)P2). These problems need to be considered.*

We agree with Reviewer #2 that fluorescence biosensors are not perfect for detecting specific lipids. Based on the Reviewer’s suggestion, we have included a comment on this in the Discussion section as follows (Lines 265–268).

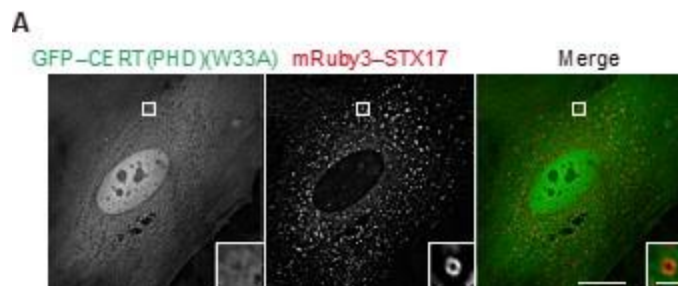
“Given the possibility that fluorescence lipid probes may give false-negative results, a more comprehensive biochemical analysis, such as lipidomics analysis of mature autophagosomes, would be imperative to elucidate the potential involvement of other negatively charged lipids.”

- *A negative control for the PI4P biosensor, i.e., a mutant lacking the PI4P binding ability, is better to be tested to confirm the presence of PI4P in autophagosomes.*

We would like to thank the Reviewer for this comment. We conducted the suggested experiment and confirmed that the CERT(PHD)(W33A) mutant, which is deficient for PI4P binding (Sugiki et al., JBC. 2012), was diffusely present in the cytosol and did not localize to STX17-positive autophagosomes. This data supports our conclusion that PI4P is indeed present in autophagosomes. We have included this data in Figure 3–figure supplement 2A and explained it in the text (Lines 164–166).

**Author response image 4.**

Mouse embryonic fibroblasts (MEFs) stably expressing GFP–CERT(PHD)(W33A) and mRuby3–STX17TM were cultured in starvation medium for 1 h. Bars indicate 10  $\mu\text{m}$  (main images) and 1  $\mu\text{m}$  (insets).



• As a control to the molecular dynamic simulation study, STX17 TM insertion into a membrane containing other negative charge lipids, especially PI, needs to be tested. PI is a negative charge lipid that is likely to exist in autophagic membranes (as suggested by the authors' past study).

We thank the reviewers for this suggestion. As mentioned above (Reviewer #2, Public Review), we performed the molecular dynamics simulation using membranes containing PI and added the results in Figure 5E and F and Video 3.

• If the putative role of PI4P could be shown in the cellular context, the authors' conclusion would be much strengthened. I wonder if overexpression of PI4P fluorescence biosensors, especially those that appear to bind to the autophagosome almost exclusively, may suppress the recruitment of STX17 there.

We would like to thank the Reviewer for asking this question. In MEFs stably overexpressing PI4P probes driven by the CMV promoter, STX17 recruitment was not affected. Thus, simple overexpression of PI4P probes does not appear to be effective in masking PI4P in autophagosomes.

Another idea is to use an appropriate molecule (e.g., WIPI2, ATG5) and to recruit Sac1 to autophagic membranes by using the FRB-FKBP system or the like. I hope these and other possibilities will be tested to confirm the importance of PI4P in the temporal regulation of STX17 recruitment.

We tried the FRB-FKBP system using the phosphatase domain of yeast Sac1 fused to FKBP and LC3 fused to FRB, but unfortunately, this system failed to deplete PI4P from the autophagosomal membrane.

#### **Reviewer #3 (Recommendations For The Authors):**

A few areas for suggested improvement are:

(1) It would be helpful if the authors could clarify for all figures how many independent experiments were conducted for all experiments, particularly those that have quantitation and statistical analyses.

As pointed out by Reviewer #3, we have added the number of independent experimental repeats in the figure legends.

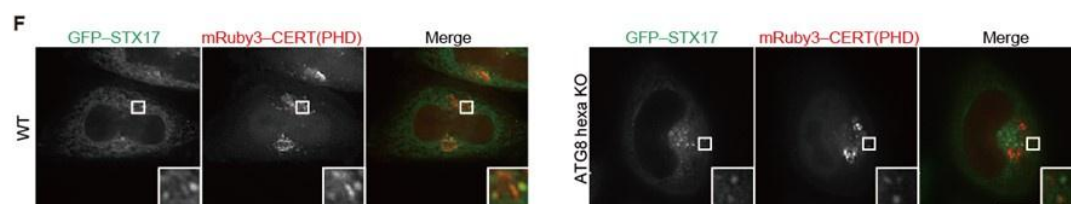
The authors made several attempts to modulate PI4P levels on autophagosomes although understandably this proved to be challenging. A couple of suggestions are provided to address this area:

(2) Given the reported role of GABARAPs in PI4K2a recruitment and PI4P production on autophagosomes, as well as autophagosome-lysosome fusion (Nguyen et al (2016) J Cell Biol) it would be worthwhile to assess whether GABARAP TKO cells have reduced PI4P and reduced Stx17 recruitment

According to the Reviewer's suggestion, we examined the localization of STX17 TM and the PI4P probe CERT(PHD) in ATG8 family (LC3/GABARAP) hexa KO HeLa cells that were established by the Lazarou lab (Nguyen et al., JCB 2016). As in WT cells, STX17 TM and CERT(PHD) were still colocalized with each other in hexa KO cells, suggesting that neither STX17 recruitment nor PI4P enrichment depends on ATG8 family proteins (note: the size of autophagosomes in HeLa cells is smaller than in MEFs, making it difficult to observe autophagosomes as ring-shaped structures). We have included this result in Figure 3—figure supplement 2(F) and explained it in the text (Lines 194–196, 198).

#### Author response image 5.

(F) WT and ATG8 hexa KO HeLa cells stably expressing GFP-STX17TM and transiently expressing mRuby3-CERT(PHD) were cultured in starvation medium. Bars indicate 10  $\mu$ m (main images) and 1  $\mu$ m (insets).



(3) Can the authors try fusing Sac1 to one of the PI4P probes (CERT(PHD)) that were used, or alternatively to the c-terminus of Syntaxin 17? This approach would help to recruit Sac1 only to mature autophagosomes and could therefore prevent the autophagosome formation defect observed when fused to LC3B that targeted Sac1 to autophagosomes as they were forming. Understandably, this approach might seem a bit counterintuitive since the phosphatase is removing PI4P which is what is recruiting it but it could be a viable approach to keep PI4P levels low enough on mature autophagosomes so that Syntaxin 17 is no longer recruited. A Sac1 phosphatase mutant might be needed as a control.

We would like to thank the Reviewer for these suggestions. We tried the phosphatase domain of yeast Sac1 or human SAC1 fused with STX17TM, but unfortunately, these fusion proteins did not deplete PI4P from autophagosomes.