







# Mammalian Atg8 proteins regulate lysosome and autolysosome biogenesis through SNAREs

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## Abstract

Mammalian homologs of yeast Atg8 protein (mAtg8s) are important in autophagy, but their exact mode of action remains ill-defined. Syntaxin 17 (Stx17), a SNARE with major roles in autophagy, was recently shown to bind mAtg8s. Here, we identified LC3-interacting regions (LIRs) in several SNAREs that broaden the landscape of the mAtg8-SNARE interactions. We found that Syntaxin 16 (Stx16) and its cognate SNARE partners all have LIR motifs and bind mAtg8s. Knockout of Stx16 caused defects in lysosome biogenesis, whereas a Stx16 and Stx17 double knockout completely blocked autophagic flux and decreased mitophagy, pexophagy, xenophagy, and ribophagy. Mechanistic analyses revealed that mAtg8s and Stx16 control several properties of lysosomal compartments including their function as platforms for active mTOR. These findings reveal a broad direct interaction of mAtg8s with SNAREs with impact on membrane remodeling in eukaryotic cells and expand the roles of mAtg8s to lysosome biogenesis.

**Keywords** autophagy; GABARAP; lysosome; SNARE; TOR

**Subject Categories** Autophagy & Cell Death; Membranes & Trafficking

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## Introduction

The autophagy pathway, controlled by a conserved set of ATG genes (Mizushima *et al.*, 2011; Levine & Kroemer, 2019), is a cytoplasmic homeostatic process at an interface between quality control and metabolism. This pathway can be activated by metabolic and stress inputs (Noda & Ohsumi, 1998; Scott *et al.*, 2004; Marino *et al.*, 2014; Garcia & Shaw, 2017; Saxton & Sabatini, 2017) or triggered by cargo recognition leading to *in situ* assembly of ATG factors via receptor regulators (Mandell *et al.*, 2014; Lazarou *et al.*, 2015; Kimura *et al.*, 2016). Autophagy can be bulk or selective (Birgisdottir *et al.*, 2013; Randow & Youle, 2014; Stolz *et al.*, 2014), whereas the multiple

biological outputs of autophagy depend on its completion and the type of termination of the autophagic pathway, e.g., degradation or secretion (Ponpuak *et al.*, 2015; Levine & Kroemer, 2019). Bulk autophagy, whereby portions of the cytoplasm of heterogeneous composition are sequestered, occurs during starvation and can be quantified by imaging (An & Harper, 2018) or biochemically (Seglen *et al.*, 2015; Engedal & Seglen, 2016) by following sequestration of the cytosolic enzyme LDH, or ultrastructurally by enumerating partially degraded electron-dense ribosomes (Tanaka *et al.*, 2000; Eskelinen, 2008). Autophagy can be selectively guided to specific cargo via a number of sequestosome 1/p62-like receptors (SLRs; Bjorkoy *et al.*, 2005; Birgisdottir *et al.*, 2013; Stolz *et al.*, 2014) or other classes of receptors besides SLRs (Kimura *et al.*, 2016; Levine & Kroemer, 2019). Autophagy machinery is often recruited to damaged or dysfunctional targets after they are marked for autophagic degradation by ubiquitin and galectin tags that in turn are recognized by cytosolic autophagic receptors such as SLRs (Randow & Youle, 2014; Stolz *et al.*, 2014). Autophagy apparatus can also be directly recruited via receptors residing on or within organelles if they become exposed following organellar membrane damage or depolarization or are modified downstream of physiological or developmental signals (Sandoval *et al.*, 2008; Wild *et al.*, 2011; Liu *et al.*, 2012; Bhujabal *et al.*, 2017; Wei *et al.*, 2017).

Degradative autophagy terminates in fusion of autophagosomes with lysosomes whereby the sequestered cargo is degraded. Examples of selective degradative autophagy include autophagy of mitochondria (mitophagy), peroxisomes (pexophagy), intracellular microbes (xenophagy), ribosomes (ribophagy), protein aggregates (aggrephagy), and specific intracellular multiprotein complexes (precision autophagy) (Birgisdottir *et al.*, 2013; Randow & Youle, 2014; Kimura *et al.*, 2016; An & Harper, 2018). Given the diversity of cargo, complexity of the protein components and membrane compartments engaged, as well as the exquisite responsiveness to a variety of cargo triggers (e.g., damaged organelles, aggregates) and stress conditions (e.g., starvation, hypoxia), autophagy is controlled by a collection of subsystems that have to come together in a modular fashion and cooperate in the initiation and execution of autophagy (Mizushima *et al.*, 2011). These include (i) formation of a

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complex between the ULK1 kinase, FIP200, ATG13, and ATG101, transducing mTOR inhibition (Ganley *et al*, 2009; Hosokawa *et al*, 2009; Jung *et al*, 2009), and AMPK activation (Kim *et al*, 2011) to induce autophagy; (ii) generation of PI3P by the ATG14-endowed class III PI3-kinase complex I (PI3KC3-CI) containing VPS34 (PI3KC3-C1) (Petiot *et al*, 2000; Baskaran *et al*, 2014; Chang *et al*, 2019) and Beclin 1 (He & Levine, 2010), which can also be modified by AMPK to specifically activate PI3KC3-C1 and not the other PI3KC3 forms (Kim *et al*, 2013)<sup>§</sup>; (iii) the ubiquitin-like conjugation system with ATG5-ATG12/ATG16L1 (Mizushima *et al*, 1998a,b) acting as an E3 ligase to lipidate mammalian homologs of yeast Atg8 (mAtg8s), some of which like LC3B have become key markers for autophagosomal membranes (Kabeya *et al*, 2000); and (iv) the only integral membrane ATG protein, ATG9, and the ATG2-WIPI protein complexes, of still unknown but essential functions (Young *et al*, 2006; Velikkakath *et al*, 2012; Bakula *et al*, 2017). These modules are for the most part interconnected, with FIP200 physically bridging via ATG16L1 the ULK1/2 complex with the mAtg8 conjugation system (Fujita *et al*, 2013; Gammoh *et al*, 2013; Nishimura *et al*, 2013), ATG16L1 and WIPI directly interacting (Dooley *et al*, 2014), ATG13 connecting the ULK1/2 complex with PI3C3-C1 via ATG13's HORMA domain binding to ATG14 of PI3KC3-C1 (Jao *et al*, 2013; Park *et al*, 2016), and PI3P, the product of VPS34 (Petiot *et al*, 2000), being detected on membranes by WIPIs (Bakula *et al*, 2017).

In yeast, a component of the above systems morphologically equated with autophagy is Atg8, with its appearance as a single punctum defining the pre-autophagosomal structure (Kirisako *et al*, 1999). In mammals, this function is spread over a set of six mAtg8s (Weidberg *et al*, 2010; Mizushima *et al*, 2011), LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2. Although it is generally accepted that mAtg8s are important for autophagy, and that mAtg8 lipidation and puncta formation herald autophagy induction and isolation membrane formation, the core function of mAtg8s and possibly a direct role in autophagosomal membrane remodeling remains to be defined. Several models, including a direct role of Atg8/mAtg8s in catalyzing membrane fusion (Nakatogawa *et al*, 2007; Weidberg *et al*, 2010, 2011), have been considered but later challenged (Nair *et al*, 2011). Nevertheless, many of the cargos, selective autophagy receptors, and regulatory components (ULK, ATG13, FIP200, ATG14, Beclin 1, VPS34, ATG4) contain LC3-interacting regions (LIRs) and can either recruit cargo (Bjorkoy *et al*, 2005; Birgisdottir *et al*, 2013) or organize core autophagy machinery (Alemu *et al*, 2012; Skytte Rasmussen *et al*, 2017; Birgisdottir *et al*, 2019). Furthermore, recent studies show that even in the complete absence of mAtg8s, autophagosome formation proceeds, albeit with a lower sequestration volume, and that mAtg8s are important for autolysosomal formation (Nguyen *et al*, 2016b). In keeping with the latter findings, depletion of components participating in mAtg8 conjugation delays autophagic flux progression (Tsuboyama *et al*, 2016). It remains to be understood precisely how mAtg8s control membrane trafficking and remodeling during phagophore formation and elongation (Xie *et al*, 2008) and during autophagosome-lysosome fusion (Weidberg *et al*, 2010; Nguyen *et al*, 2016b).

Degradative aspects of autophagy depend on autophagosome fusion with organelles of the endolysosomal system, often referred to

as autophagic flux or autophagosome-lysosome fusion (Tanaka *et al*, 2000; Klionsky *et al*, 2016). Much of the intracellular membrane trafficking and fusion processes are catalyzed by SNARE proteins, which ensure compartment/organelle specificity through pairing of cognate Qa-, Qb-, Qc-, and R-SNARE partners (Jahn & Scheller, 2006). SNAREs are often found in compartments where they function but also transit through other membranes, and thus, their fusion activities are tightly regulated by tethers, SM (Sec1/Munc18) proteins, Rabs, and additional factors that modulate their recruitment, activation, cycles of reuse, etc. (Hong & Lev, 2014). In the context of autophagy, SNAREs have been studied at different stages along the autophagy pathway (Nair *et al*, 2011; Itakura *et al*, 2012; Moreau *et al*, 2013; Kimura *et al*, 2017). At the autophagosome-lysosome fusion stage, an initially identified SNARE was the Qa-SNARE Stx17, forming complexes with Qbc-SNARE SNAP29 and R-SNARE VAMP8 (Itakura *et al*, 2012; Takats *et al*, 2013; Guo *et al*, 2014; Diao *et al*, 2015; Wang *et al*, 2016). Recent studies have indicated that additional SNAREs may be required or even be dominant in this process (Matsui *et al*, 2018; Takats *et al*, 2018). Thus, redundant, complementary or synergistic SNARE-driven fusion and regulatory events may be at work during autolysosomal fusion. We have recently shown that Stx17 interacts via its LIR motif with mAtg8s, which functions in Stx17's recruitment to autophagosomes (Kumar *et al*, 2018), and that TBK1-phosphorylated Stx17 plays a role in autophagy initiation (Kumar *et al*, 2019), in keeping with studies by others (Hamasaki *et al*, 2013; Arasaki *et al*, 2015, 2018). Thus, Stx17 contributes to the autophagy pathway at several stages from initiation to maturation.

A report of direct interaction between mAtg8s and Stx17 (Kumar *et al*, 2018) has set an unanticipated precedent, and here, we explored whether there is a broader range of interactions between mAtg8s and mammalian SNAREs. Using bioinformatics and biochemical approaches, we found additional candidate SNAREs with LIR motifs that bind mAtg8s. Among the candidates, we focused on a cognate set of SNAREs, Stx16, Vti1a, and Stx6, previously implicated in retrograde transport between endosomes and the *trans*-Golgi network (TGN). Through mutational and functional analyses, we found that Stx16 acts synergistically with Stx17 and is important for diverse types of autophagy, including mitophagy, pexophagy, ribophagy, and elimination of intracellular *Mycobacterium tuberculosis*. We have further found that Stx16 is important for cellular lysosomal content and function, and that mAtg8s modulate Stx16 localization on endolysosomal organelles. This uncovers a mechanism different from the previous views of how mAtg8s help grow autophagosomal and complete autolysosomal membranes. We conclude that mAtg8s control autophagy at least in part by directly binding to SNARE proteins engaged in the biogenesis of the endolysosomal and autolysosomal organelles.

## Results

### A subset of SNAREs including syntaxin 16 contain LC3-interacting regions

Previous studies have indicated that at least one SNARE involved in autophagy, Stx17, harbors a LIR motif that affects its distribution

<sup>§</sup>Correction added on 24 October 2019, after first online publication: the sentence has been corrected.

(Kumar *et al*, 2018). Here, we carried out a broader analysis of all SNAREs for the presence of putative LIR motifs using bioinformatics and conventional LIR motif consensus (Birgisdottir *et al*, 2013) followed up by biochemical assays (Fig 1A and Appendix Table S1). We tested a subset of SNAREs containing putative LIRs in a screen with peptide arrays for binding to GST-GABARAP as a representative mAtg8 (Fig 1A). Several SNAREs showed positive signals with GST-GABARAP in peptide arrays. These included only one R-SNARE, VAMP7, and mostly Qa-, Qb-, and Qc-SNAREs acting in different cellular compartments (Jahn & Scheller, 2006): Stx17, included as a positive control (Kumar *et al*, 2018) and already implicated in autophagy (Itakura *et al*, 2012), Stx18 acting in the ER (Hatsuzawa *et al*, 2000), GOSR1 functioning in the Golgi (Subramaniam *et al*, 1996; Mallard *et al*, 2002), Stx3 and Stx4 on plasma membrane (Low *et al*, 1996), Stx19 with no transmembrane domain and partitioning between cytosol and membranes (Wang *et al*, 2006), and Stx16, Stx6, and Vti1a, all acting in trafficking between endosomal compartments and TGN (Jahn & Scheller, 2006; Malsam & Söllner, 2011) (Figs 1B and EV1A). Thus, a number of SNAREs may be binding partners for mAtg8s indicating a previously unappreciated potential for broader intersections between mAtg8 and SNARE systems.

### Syntaxin 16 directly binds mAtg8s through its LIR motif

From the panel of SNAREs showing positive signals with GST-GABARAP in peptide arrays, we focused on a set of three cognate SNAREs, Stx16, Vti1a, and Stx6, known to form a complex (Malsam & Söllner, 2011). Co-immunoprecipitation (Co-IP) analyses of over-expressed fusion proteins confirmed that Stx16, Vti1a, and Stx6 interact with LC3B and GABARAP (Fig 1C). Co-IP analyses of endogenous proteins in HeLa and U2OS cells confirmed interactions between LC3 and Stx16 and Vti1a (Fig EV1B). In GST pull-down assays with all 6 mAtg8 proteins, Stx16 showed a positive binding signal with LC3C, GABARAP, and GABARAPL1 (Fig 1D). The peptide array binding results pointed to the <sup>219</sup>LVLV<sup>222</sup> residues in Stx16 (Fig 1B), which resembled both the LC3C-preferring LIR of NDP52 (ILVV; von Muhlinen *et al*, 2012) and the GABARAP interaction motifs with important Val residues in the second and fourth positions of the core LIR motif (Rogov *et al*, 2017). This motif was found in the linker region between the Habc and SNARE domains of Stx16 matching the requirement to be outside of known structured regions (Popelka & Klionsky, 2015). When we mutated L-219 and V-222 in the predicted (albeit atypical) LIR motif (<sup>219</sup>LVLV<sup>222</sup> mutated to <sup>219</sup>AVLA<sup>222</sup>) of Stx16, this reduced Stx16 binding to LC3C and GABARAP (Fig 1E). Additionally, we changed both Val residues within the <sup>219</sup>LVLV<sup>222</sup> motif to Ala, and the resulting Stx16 variant showed reduction in binding to LC3C and GABARAP comparable to the <sup>219</sup>AVLA<sup>222</sup> mutant in GST pull-down experiments (Fig 1F). Finally, when we mutated all 4 residues within the <sup>219</sup>LVLV<sup>222</sup> motif, the association between Stx16 and LC3C or GABARAP was completely abolished (Fig 1F).

Complementary to the above experiments with LIR mutations, we tested whether the previously defined LIR-docking site (LDS) in GABARAP (Behrends *et al*, 2010; Birgisdottir *et al*, 2013) was responsible for binding to Stx16. Two conserved hydrophobic pockets (HP1 and HP2) are known to define the LIR-docking site of GABARAP (Behrends *et al*, 2010). The Y49A mutation in the HP1

pocket of GABARAP reduced its interaction with Stx16, whereas the complete LDS mutant (double HP1 and HP2 Y49A/F104A mutation) abrogated GABARAP's binding to Stx16 in GST pull-down assays (Fig 1G). Thus, GABARAP and Stx16 interaction depends on the canonical LDS motif in GABARAP.

### Syntaxin 16 is required for efficient autophagy flux in response to starvation

Stx17 has been implicated in mammalian autophagosome-lysosome fusion (Itakura *et al*, 2012). However, additional SNAREs have now been shown to contribute to this process (Matsui *et al*, 2018), but relative contributions remain to be fully defined. We generated CRISPR/Cas9-mediated *STX17* knockout (KO) in different cell lines (HeLa and Huh7; Fig EV2A and B) and analyzed autophagy flux by monitoring LC3-II levels (Figs 2A and B, and EV2C and D). In *STX17*<sup>KO</sup> HeLa cells, the autophagic flux continued at almost the same rates as in wild-type (WT) control cells, as shown by slight increase in LC3-II levels upon starvation (Fig 2A). A similar effect was observed in hepatocellular carcinoma Huh7 *STX17*<sup>KO</sup> cells, which in general displayed faster LC3-II flux (Fig EV2C and D). Knocking out Stx17 did not cause any significant change in the levels of p62, an autophagic receptor that is degraded upon starvation (Bjorkoy *et al*, 2005), and is often used as readout of autophagic flux (Klionsky *et al*, 2016; Figs 2A and EV2C).

When we knocked out *STX16* in HeLa cells, this too had no major effects on LC3-II flux (Figs 2C and D, and EV2A and B). However, when a double *STX16/STX17* knockout was generated (Fig EV2B), the LC3 flux was completely abrogated, with LC3-II levels in starvation-induced cells accumulating at similar levels as in cells treated with bafilomycin A1 (Fig 2C and D), usually considered as a complete blocker of autophagic flux (Klionsky *et al*, 2016). The LC3-II accumulation could be partially relieved by complementation of *STX16/STX17*<sup>DKO</sup> cells transfected with FLAG-tagged WT Stx16 (Fig EV2E). Given that this indicated a block in autolysosome production, we examined *STX16/STX17*<sup>DKO</sup> cells using automated quantitative high-content microscopy (HCM) and tandem mCherry-EYFP-GABARAP, by monitoring and quantifying overlap between red (mCherry) and green (EYFP) fluorescence as another measure of autophagic flux. The overlap between mCherry and EYFP was significantly increased in *STX16/STX17*<sup>DKO</sup> cells compared to that in WT cells upon starvation in EBSS (Fig EV2F and G). At the ultra-structural level, electron microscopy quantification of initial (AVi) vs. degradative (AVd) autophagic vacuoles (Tanaka *et al*, 2000; Eskelinen, 2008) also indicated accumulation of AVi vesicles (Fig 2E and F). In conclusion, the absence of either Stx16 or Stx17 alone cannot block completion of the autophagy pathway, but when both are ablated, this prevents progression to autolysosomes.

### Syntaxin 16 and syntaxin 17 are required for completion of diverse selective autophagy processes

Mitophagy is a very well-studied process of selective autophagy and is often used as an example to study elimination of defunct organelles (Youle & Narendra, 2011). To further characterize effects of *STX16/STX17* double knockout, we employed HCM quantification of mitochondrial DNA (mtDNA) as a measure of cellular mitochondrial content often used in mitophagy studies (Lazarou *et al*, 2015;



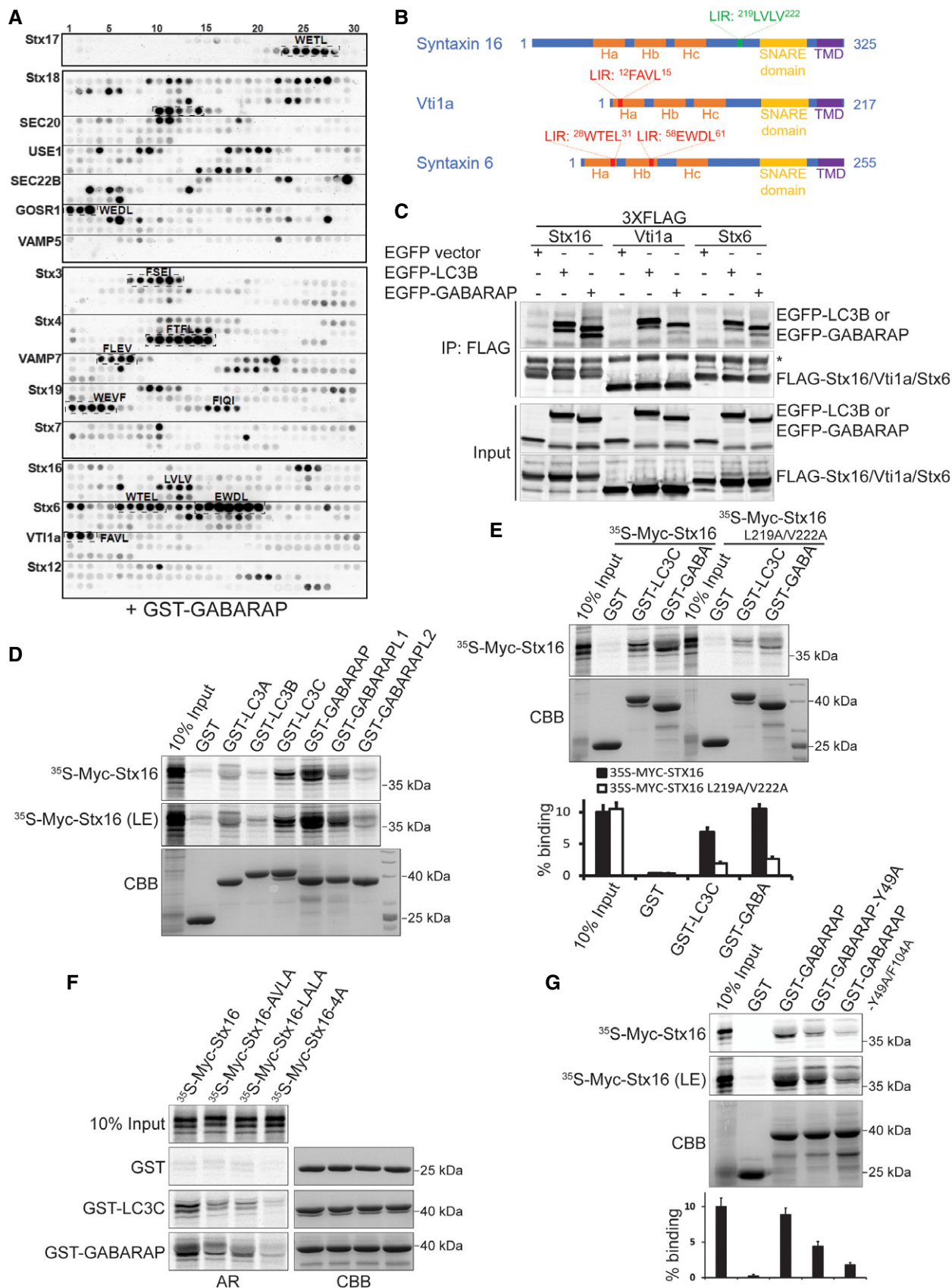


Figure 1.



**Figure 1. Many SNAREs bind mammalian Atg8 proteins.**

- A Peptide array dot blot analysis to identify LIR motifs in the indicated SNARE proteins. Amino acids for the identified LIRs from positive signals are marked on each SNARE. Amino acids are denoted as amino acid single letter codes in the blot.
- B Schematics of the functional domains of Stx16/Vti1a/Stx6 cognate SNAREs with the positions of LIR motifs. The LIR motif marked in green in syntaxin 16 indicates established LIR based on following analyses.
- C Co-immunoprecipitation (Co-IP) analysis of interactions between FLAG-tagged Stx16/Vti1a/Stx6 and EGFP-tagged LC3B or GABARAP overexpressed in HEK293T cells. \* indicates mouse IgG heavy chain of precipitated mouse anti-FLAG antibody.
- D GST pull-down analysis of interactions between radiolabeled Myc-Stx16 and GST-tagged mAtg8 proteins.
- E GST pull-down analysis of interactions between wild-type (WT) or LIR-mutant (L219A/V222A) Stx16 and GST-tagged LC3C or GABARAP. Lower panel shows percentages of WT or LIR-mutant Stx16 bound to GST-LC3C or GST-GABARAP. Data shown as means  $\pm$  SEM of precipitated Stx16,  $n = 3$ .
- F GST pull-down analysis of interactions between WT or different types of LIR-mutant Stx16 and GST-tagged LC3C or GABARAP. Lower panel shows percentages of WT or LIR-mutant Stx16 bound to GST-LC3C or GST-GABARAP.
- G GST pull-down analysis of interactions between radiolabeled Myc-Stx16 and WT or LDS-mutant GABARAP (GABARAP-Y49A and -Y49A/F104A). Lower panel shows percentages of Stx16 bound to WT or LDS-mutant GABARAP. Data shown as means  $\pm$  SEM of precipitated Stx16,  $n = 3$ .

Source data are available online for this figure.

Nguyen *et al*, 2016b). We generated *STX16/STX17* DKO in HeLa cells stably expressing YFP-Parkin (Lazarou *et al*, 2015; Fig EV3A). We then stressed mitochondria using two different depolarizing agents: CCCP or oligomycin A + antimycin A (OA), and quantified Parkin-dependent mitophagy in *STX16/STX17*<sup>DKO</sup> HeLa/YFP-Parkin cells. In wild-type cells, CCCP or OA caused a major reduction in mtDNA (Fig 3A and B). Loss of Stx16 and Stx17 caused significant reduction in mitophagy (Fig 3A and B). Clearly, additional processes were also involved as *STX16/STX17*<sup>DKO</sup> HeLa/YFP-Parkin cells still eliminated mitochondria probably through additional mechanisms (Nguyen *et al*, 2016a). Previous studies indicated that Stx17 is not important for mitophagy (Nguyen *et al*, 2016b). Nevertheless, a loss of Stx16 in the *STX17*<sup>KO</sup> background affected mitophagy significantly.

Pexophagy is another classical example of selective autophagy (Manjithaya *et al*, 2010; Gatica *et al*, 2018). We monitored pexophagy in cells treated with H<sub>2</sub>O<sub>2</sub> (Zhang *et al*, 2015) using liver cell line Huh7, which expresses wild-type LKB1 that can activate AMPK (Tan *et al*, 2018; Fig EV3B). We monitored pexophagy by quantifying levels of PEX14 and PMP70 (Zhang *et al*, 2015) proteins, and detected decrease in these peroxisomal constituents over a period of 6 hours of exposure to H<sub>2</sub>O<sub>2</sub> (Fig 3C). In cells lacking both Stx16 and Stx17, this reduction in peroxisomal protein levels was abrogated (Fig 3C and D). Thus, *STX16/STX17* DKO significantly inhibited pexophagy.

Autophagic defense against intracellular microbes, such as *Mycobacterium tuberculosis* (*Mtb*) (Gutierrez *et al*, 2004), sometimes referred to as xenophagy, is considered to be another form of selective autophagy. We tested the survival of *M. tuberculosis* in macrophage-like cell line THP1 and compared the effects of *Mtb* killing in WT, single *STX16*<sup>KO</sup> or *STX17*<sup>KO</sup> and *STX16/STX17*<sup>DKO</sup> cells (Fig EV3C). As previously reported (Gutierrez *et al*, 2004), induction of autophagy by starvation reduced survival of *Mtb* in WT THP1 cells (Fig 3E). However, this effect was lost in *STX16*<sup>KO</sup> THP1 cells (Figs 3E and EV3C). Further analyses of a CRISPR KO mutant of *STX17* alone, previously reported in THP1 cells to play a role in control of intracellular *Mtb* (Kumar *et al*, 2018), or a combination CRISPR KO mutant of both *STX16* and *STX17*, showed no further additive effects between Stx16 and Stx17 (Fig 3E).

Taken together, these data indicate that *STX16/STX17* DKO significantly reduces mitochondrial (Lazarou *et al*, 2015), peroxisomal (Manjithaya *et al*, 2010), and bacterial (*Mtb*) (Gutierrez *et al*, 2004) clearance under conditions known to induce autophagy (Fig 3F).

**Keima-based ribophagy probes indicate a role of Stx16 in autophagic maturation**

Keima fluorescent protein has become an important tool for detection of the arrival of autophagy substrates to autolysosomes (Katayama *et al*, 2011). In functional autolysosomes, despite being in a degradative compartment, Keima protein is stable but changes its spectral properties (illustrated in Fig 4A) from maximum excitation wavelength at 440 nm in neutral pH to 586 nm in acidic pH, with emission remaining constant at 620 nm (Violot *et al*, 2009). These shifts can be monitored using 440-nm and 560-nm excitation filters and emission at 620 nm in an HCM Cellomics system (see Materials and Methods). We applied Keima to monitor ribophagy (An & Harper, 2018) and generated *STX16/STX17* DKO using CRISPR/Cas9 (Fig 4B) in HEK293 cells stably expressing RPS3-Keima (fusion between 40S ribosomal protein S3 and mKeima) protein (An & Harper, 2018). RPS3-Keima was diffuse cytoplasmic in untreated cells. Under starvation conditions, we examined ribophagy as previously reported (An & Harper, 2018), by monitoring progression of RPS3-Keima into acidic autolysosomal compartments via quantification of cytoplasmic puncta at 560 nm excitation and 620 nm emission wavelengths (Fig 4C and D). The HEK293 *STX16/STX17*<sup>DKO</sup> cells showed significantly fewer autolysosomal puncta originating from RPS3-Keima than in parental WT cells (Fig 4C and D). An independent set of experiments was carried out with HCT116 cells stably expressing a fusion between 60S ribosomal protein L28 and mKeima (RPL28-Keima). We generated *STX16/STX17* DKO in these cells as well using CRISPR/Cas9 (Fig 4B). Progression of ribophagy in HCT116 RPL28-Keima *STX16/STX17*<sup>DKO</sup> cells was diminished relative to parental WT cells (Fig 4E and F). Thus, the use of previously characterized Keima ribophagy probes (An & Harper, 2018) further confirmed the importance of having both Stx16 and Stx17 for autophagic cargos to arrive into acidified compartments, including during ribophagy.

**Syntaxin 16 plays a role in lysosomal biogenesis**

When we tested autophagy response by monitoring endogenous LC3 puncta, we found an increase in LC3 dots in *STX16* knockout cells (HeLa *STX16*<sup>KO</sup>) relative to WT cells, under basal conditions and in EBSS-starved cells (Fig 5A and B). Next, we determined by HCM the overlap area between LC3 and LAMP2 profiles, and found that the overlap was strongly reduced in HeLa *STX16*<sup>KO</sup> vs WT cells

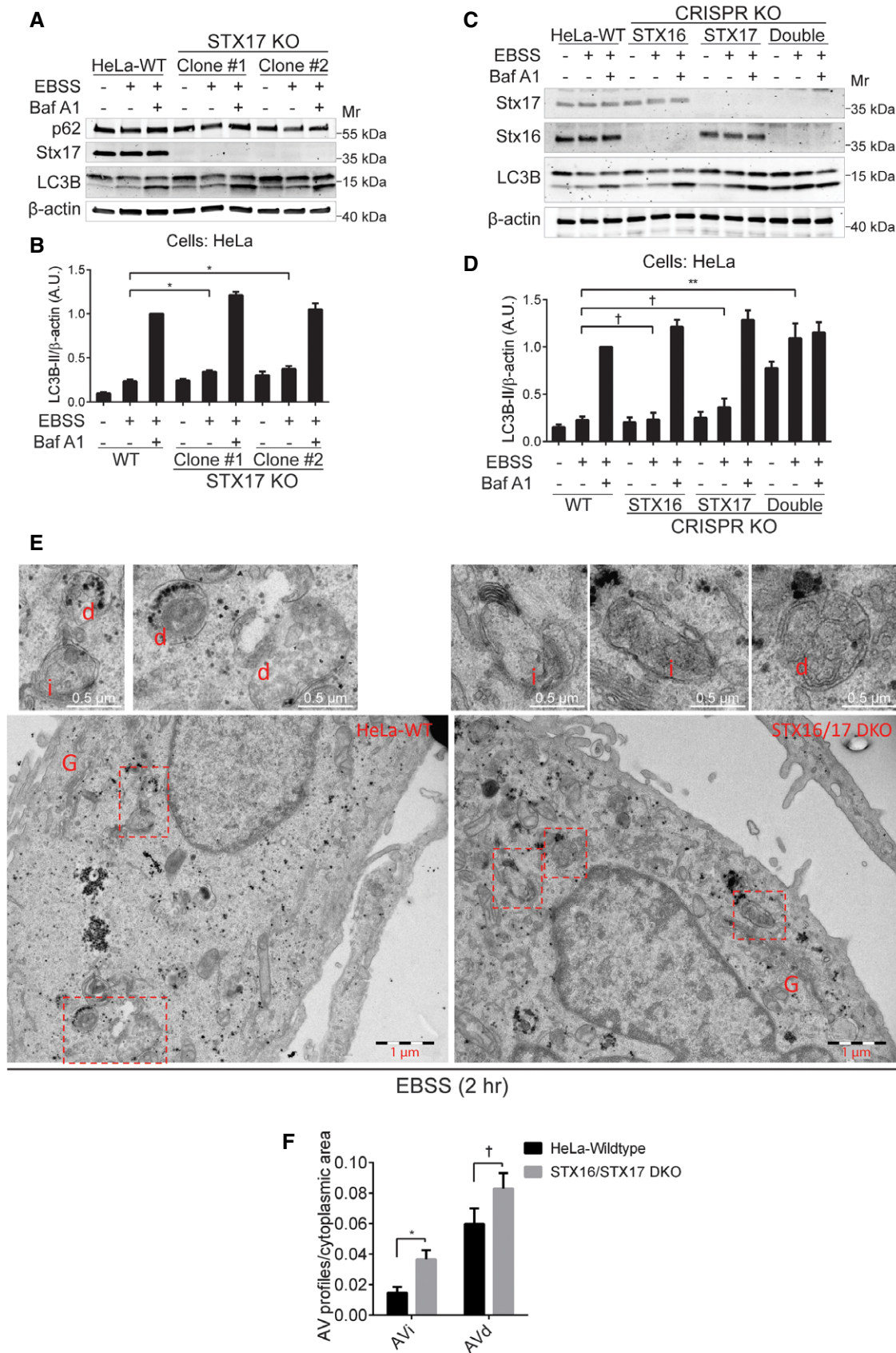


Figure 2.

**Figure 2. The mAtg8-interacting SNAREs Stx16 and Stx17 are required for efficient bulk autophagic flux.**

- A WT or *STX17* knockout (*STX17<sup>KO</sup>*) HeLa cells were starved with or without the presence of bafilomycin A1 (Baf A1, 100 nM) for 2 h, and cell lysates were subjected to Western blot analysis of LC3B and p62.
- B Quantifications of LC3B-II levels normalized to  $\beta$ -actin from cells treated as in (A); data shown as means  $\pm$  SEM of LC3B-II and  $\beta$ -actin ratios,  $n = 3$ ; \* $P < 0.05$  (one-way ANOVA).
- C WT, *STX16<sup>KO</sup>*, *STX17<sup>KO</sup>*, or *STX16/STX17* double KO (*STX16/STX17<sup>DKO</sup>*) HeLa cells were starved with or without the presence of Baf A1 (100 nM) for 2 h, and cell lysates were subjected to Western blot analysis of LC3B.
- D Quantifications of LC3B-II levels normalized to  $\beta$ -actin from (C); data shown as means  $\pm$  SEM of LC3B-II and  $\beta$ -actin ratios,  $n = 3$ ; †, not significant; \*\* $P < 0.01$  (one-way ANOVA).
- E WT or *STX16/STX17<sup>DKO</sup>* HeLa cells were starved in EBSS for 2 h and subjected to ultrastructural analysis of the autophagic vesicles (AV) with electron microscopy. AVi: initial autophagic vacuoles; AVd: degradative autophagic vacuoles; G: Golgi apparatus. Image acquisition and counting as in Methods. Scale bars, 1  $\mu$ m and 0.5  $\mu$ m (top sections).
- F Quantifications of autophagic vesicles in WT and *STX16/STX17<sup>DKO</sup>* HeLa cells treated as (E); data shown as means  $\pm$  SEM of AV profiles relative to cytoplasmic area; †, not significant; \* $P < 0.05$  (two-way ANOVA). AV profiles from 47 images of each sample were counted.

Source data are available online for this figure.

(Fig 5A and C). When we quantified LAMP2 puncta/cell, we found that LAMP2<sup>+</sup> puncta were diminished in HeLa *STX16<sup>KO</sup>* cells (Fig 5A and D). Thus, the appearance of reduced colocalization (overlap area by HCM) between LC3 and LAMP2 was not a result of fewer autolysosomes but was primarily due to the overall reduction of LAMP2<sup>+</sup> organelles in *STX16<sup>KO</sup>* cells (Fig 5A and D), a phenotype that could be complemented by reintroducing WT Stx16 but less so with a LIR mutant of Stx16 (Appendix Fig S1A and B). The reduction in cytoplasmic content of LAMP2<sup>+</sup> organelles in HeLa *STX16<sup>KO</sup>* cells was also seen by immunoblots, showing reduced total LAMP1 and LAMP2 protein levels in cells knocked out for Stx16 (Fig 5E). This was further tested by generating *STX16* knockout in different cell lines (Fig EV2A and B). As with different clones of HeLa *STX16<sup>KO</sup>* cells, different single clones of Huh7 *STX16<sup>KO</sup>* and osteosarcoma U2OS *STX16<sup>KO</sup>* cells had reduced LAMP1 and LAMP2 protein levels (Fig 5E). Thus, Stx16 is important for the maintenance of homeostatic LAMP levels. We also generated CRISPR knockouts for Stx6 and Vti1a, and found reduced levels of LAMP2 in *STX6<sup>KO</sup>* and *VTI1A<sup>KO</sup>* HeLa cells (Fig 5F). Thus, Stx16 and its cognate Qb- and Qc-SNAREs affect cellular LAMP levels.

Prior ultrastructure studies have implicated HOPS component VPS41 in LAMP1 and LAMP2 transport from the TGN to late endosomal compartments (Pols *et al*, 2013). In *STX16<sup>KO</sup>* HeLa cells, there was increased colocalization of detectable LAMP2 with the TGN marker TGN46, normalized for LAMP puncta in HCM quantifications (Fig EV3D and E). We thus tested whether Stx16 and its cognate Qb- and Qc-SNAREs, Vti1a, and Stx6 (both showing positivity in mAtg8 peptide binding arrays; Fig 1A) interact with VPS41. Using FLAG-tagged Stx6 and Stx16, we detected VPS41 in FLAG immunoprecipitates (Fig EV3F). We found VPS41 but not VPS33A (a component of the HOPS complex) in endogenous protein complexes with Stx6, and Vti1a (Appendix Fig S1C). These observations suggest a connection of Stx16 (plus Vti1a and Stx6) to VPS41 and the transport of lysosomal proteins such as LAMP1 and LAMP2, previously proposed specifically for VPS41 and not other components of HOPS (Pols *et al*, 2013).

### Starvation increases interactions of Stx16 with the lysosomal R-SNARE VAMP8

We found that Stx16 interacts with VAMP3 and VAMP4 (Fig EV3F), consistent with the known literature regarding VAMP3 and VAMP4 as cognate R-SNAREs during retrograde transport from endosomes to TGN (Mallard *et al*, 2002; Ganley *et al*, 2008). However, upon

starvation another R-SNARE, VAMP8, increased its presence in Stx16 complexes (Fig EV3F). The lysosomal SNARE VAMP8 (Jahn & Scheller, 2006) has not been previously implicated in Stx16-dependent trafficking, and our findings suggest a new relationship detected only under starvation conditions. Similarly, Stx6, a Qc-SNARE working together with Stx16, showed increased interactions with VAMP8 in starved cells (Fig EV3F). Thus, similar relationship to VAMP8 was observed with both Stx16 and Stx6. We also noticed increased Stx6-VAMP3 interactions in starved cells (Fig EV3F) and interpret this as an overall increase in trafficking between TGN and the lysosomal/endosomal pathway.

### Stx16 affects mTOR regulation

Lysosomes are not only digestive organelles but also serve as a principal cytoplasmic location for active mTOR (Betz & Hall, 2013; Saxton & Sabatini, 2017; Lawrence & Zoncu, 2019). Given that we detected defects in LAMP1/2 levels and profiles (Fig 5D and E) in *STX16<sup>KO</sup>* cells, we wondered whether mTOR would be affected and serve as another reporter of the lysosomal status in this context. We tested mTOR activity by monitoring phosphorylation of its downstream targets, such as 4E-BP1 (Gingras *et al*, 1999), and ULK1<sup>S757</sup>, with latter being a principal phospho-site for ULK1 and repression of autophagy by mTOR (Kim *et al*, 2011). Under resting conditions, no differences in mTOR activation status were observed in *STX16<sup>KO</sup>* vs WT cells (Fig 5G). Under starvation conditions, as expected, mTOR activity was reduced, but this reduction was much more pronounced in *STX16<sup>KO</sup>* cells (Fig 5G).

In resting cells, active mTOR forms cytoplasmic puncta and localizes to lysosomes (Betz & Hall, 2013; Saxton & Sabatini, 2017), but in starved cells mTOR is less punctate and translocates from lysosomes into the cytoplasm (Sancak *et al*, 2010). Under resting conditions (full medium), mTOR puncta (quantified by HCM) were not reduced in *STX16<sup>KO</sup>* vs. WT cells (Fig 5H and I). One hour of starvation reduced mTOR puncta, as quantified by HCM (Fig 5H and I), reflecting mTOR inactivation. This effect was more pronounced in *STX16<sup>KO</sup>* cells relative to WT cells (Fig 5H and I). When the cells were starved for 6 h, a time point that coincided with mTOR persistent inactivation assessed by phosphorylation of its targets (Fig 5G), we found an even stronger reduction in total mTOR puncta (Appendix Fig S2A and B). Localization of mTOR to LAMP2<sup>+</sup> profiles was reduced by starvation (examined at 6 h; Fig EV4A and B), an effect that was more pronounced in *STX16<sup>KO</sup>* relative to WT cells.



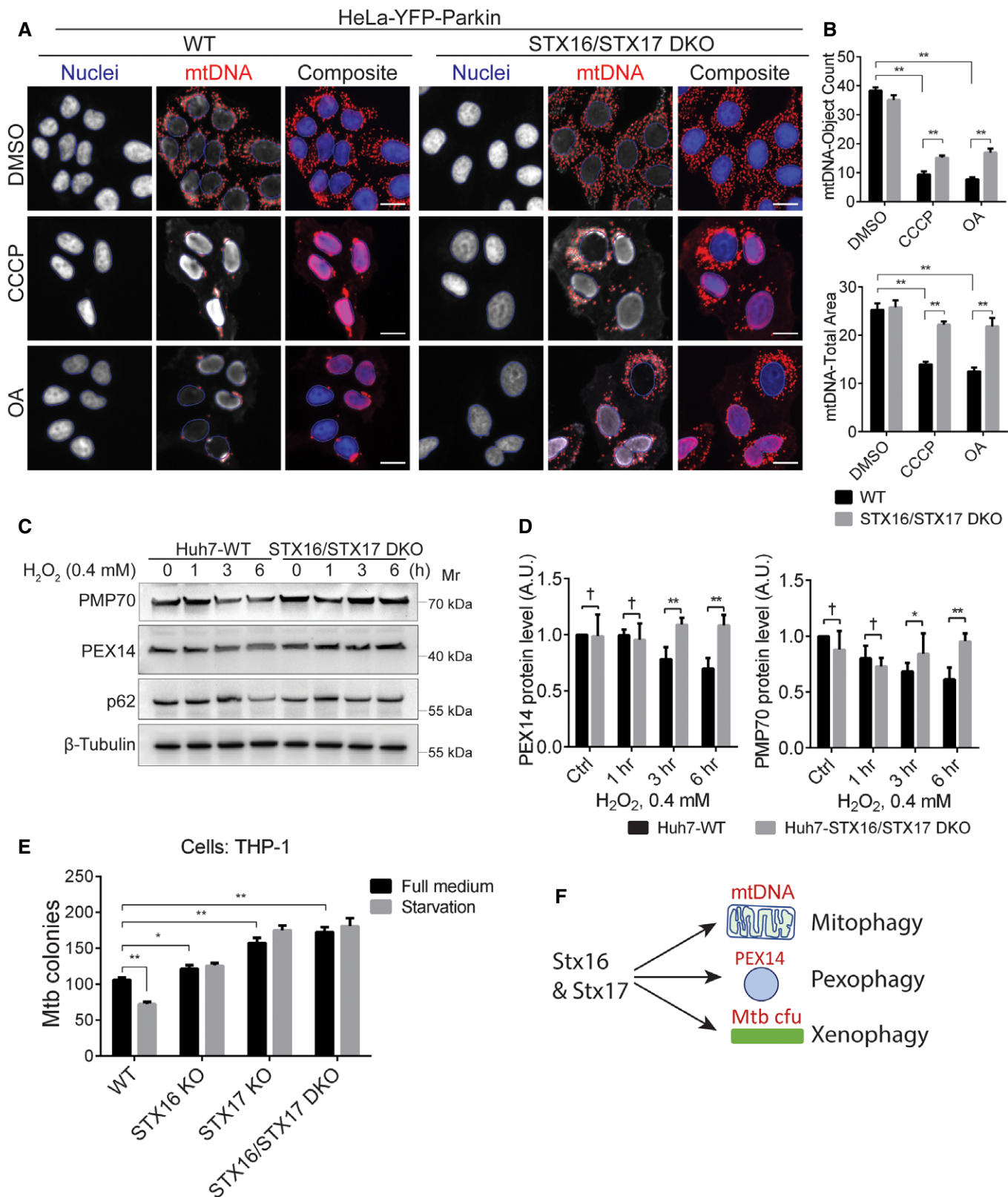


Figure 3.

**Figure 3. Stx16 and Stx17 are required for selective autophagy of mitochondria, peroxisomes, and *M. tuberculosis*.**

- A WT or *STX16/STX17*<sup>DKO</sup> HeLa-YFP-Parkin cells were treated with CCCP (10 μM) or oligomycin A (5 μM) and antimycin A (10 μM) (OA) for 16 h, and subjected to high-content microscopy (HCM) analysis of mitochondria clearance. Masks: blue, nuclei; red, mitochondria stained with mitochondrial DNA (mtDNA) antibody. Scale bar: 20 μm.
- B Quantifications of mtDNA by object count or total area per cell in WT or *STX16/STX17*<sup>DKO</sup> HeLa-YFP-Parkin cells treated as in (A); data shown as means ± SEM of mtDNA object count (upper panel) or object total area (lower panel) per cell; minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \*\**P* < 0.01 (two-way ANOVA).
- C WT or *STX16/STX17*<sup>DKO</sup> Huh7 cells were treated with H<sub>2</sub>O<sub>2</sub> (0.4 mM) for indicated time points, and autophagic clearance of peroxisomes was measured by the protein levels of PMP70, PEX14, and p62.
- D Quantifications of peroxisomal proteins PMP70 and PEX14 for WT or *STX16/STX17*<sup>DKO</sup> Huh7 cells treated as in (C); data shown as means ± SEM of PEX14 or PMP70 and β-actin ratios, *n* = 3; †, not significant; \**P* < 0.05; \*\**P* < 0.01 (two-way ANOVA).
- E WT, *STX16*<sup>KO</sup>, *STX17*<sup>KO</sup>, or *STX16/STX17*<sup>DKO</sup> THP-1 cells were infected with *Mycobacterium tuberculosis* (*Mtb*), followed by starvation to induce autophagy. Autophagic clearance of infected *Mtb* was measured by *Mtb* colonies grown on Middlebrook 7H11 agar plates; data shown as means ± SEM of *Mtb* colonies, *n* = 4; \**P* < 0.05; \*\**P* < 0.01 (two-way ANOVA).
- F Schematics for the requirement of Stx16 and Stx17 in autophagic clearance of damaged mitochondria, surplus peroxisomes, and invaded bacterial pathogens.

Thus, Stx16 affects mTOR activation state, correlating with the role of Stx16 in cellular lysosomal status.

**Stx16 is required for appropriate distribution of acidified compartments in the cell**

Since *STX16* knockout resulted in reduced cellular content of LAMP1/2, which is in lysosomal and additional acidified endosomal compartments (Lippincott-Schwartz & Fambrough, 1987) (Cheng *et al*, 2018), we anticipated that the cells might have diminished content of acidified organelles. We used LysoTracker Red DND-99 (LTR) as a standard cell biological probe for acidification of intracellular compartments (Wubbolts *et al*, 1996). Surprisingly, we found no change in total LTR fluorescence by number of profiles/cell or total area/cell (Fig 6A and B) utilizing HCM (Cellomics) and comparing WT and *STX16*<sup>KO</sup> HeLa cells under autophagy-inducing conditions (starvation). This was confirmed by total LysoTracker Green DND-26 (LTG) fluorescence and LTG puncta/cell using ImageStream flow cytometry (Amnis) in WT vs *STX16*<sup>KO</sup> cells induced for autophagy (Appendix Fig S3A–C). However, when we examined localization and morphology of LTR<sup>+</sup> profiles in *STX16*<sup>KO</sup> cells, under autophagy-inducing conditions when LTR<sup>+</sup> organelles were strongly enhanced (relative to the resting conditions), we found that the LTR labeling redistributed from its usual presentation as cytoplasmic puncta into a compartment clustering in a perinuclear region colocalizing with the TGN marker TGN46 (Fig 6C and D). To ensure that the Golgi apparatus was not perturbed, we re-examined the ultrastructural images in Fig 2E and found that the overall morphology of the Golgi stacks was similar in WT and mutant cells, but that the volume fraction of the Golgi compartment was increased (Fig 6E), consistent with an increased retention of acidified compartments in the Golgi. Furthermore, TGN46 did not show major changes in distribution relative to the Golgi marker GM130 (Fig EV4C and D) and relative to another factor in TGN-endolysosomal trafficking, mannose 6-phosphate receptor/M6PR (Fig EV4E and F). Thus, the effects of Stx16’s absence entail redistribution of acidified compartments, in keeping with a reduction in levels of the integral lysosomal membrane proteins LAMP1 and LAMP2.

**Mammalian Atg8s regulate Stx16 localization to endosomal and lysosomal compartments**

Since Stx16 is an mAtg8-binding SNARE (Fig 1), we tested whether mAtg8s influenced Stx16 localization in the cell. We used the

previously characterized triple LC3A, LC3B, LC3C knockout (Tri-LC3<sup>KO</sup>), triple GABARAP, GABARAPL1, GABARAPL2 knockout (Tri-GBRP<sup>KO</sup>), and all six mATG8 knockout (Hexa<sup>KO</sup>) HeLa cells (Nguyen *et al*, 2016b) (Fig EV5A). First, we tested LAMP1 and LAMP2 protein levels in Tri-LC3<sup>KO</sup>, Tri-GBRP<sup>KO</sup>, and Hexa<sup>KO</sup> cells. LAMP levels were not reduced under resting conditions in mAtg8-mutant cells by quantifying LAMP2<sup>+</sup> organelles (HCM, number of puncta/cell and total area/cell) (Appendix Fig S4A and B) as well as by Western blot analysis of LAMP1/2 proteins (Appendix Fig S4C). We nevertheless observed a minor LAMP reduction, possibly reflecting “exhaustion” during long-term (8 h) starvation, in Tri-GBRP<sup>KO</sup> and Hexa<sup>KO</sup> cells (Appendix Fig S4D). When we assessed localization of Stx16 to LAMP2 (lysosome) and LBPA (late endosome), we observed significantly reduced colocalizations between Stx16 and LAMP2 as well as Stx16 and LBPA by several HCM measures: (i) LAMP2-Stx16 overlap (Fig 7A and B); and (ii) LBPA-Stx16 overlap (Fig 7C; Appendix Fig S5A). Comparing *STX16* WT and its <sup>219</sup>LVLV<sup>222</sup>, <sup>219</sup>AVLA<sup>222</sup>, or LIR-4A mutants, the LIR mutants showed reduced localization to LAMP2 profiles when compared to WT (Fig EV5B and C). Thus, mAtg8s and their binding site (LIR) on Stx16 play a role in placement of Stx16 on lysosomes.

Endogenous Co-IP analyses showed reduced interactions between components of the Stx16/Vti1a/Stx6 SNARE complex in Tri-GBRP<sup>KO</sup> and Hexa<sup>KO</sup> cells (Fig EV5D), suggesting a regulatory role for mAtg8s in SNARE complex assembly or stability. A lipidation status of mAtg8s may be of significance as well, since we observed reduced interactions between Stx6 and Stx16, Vti1a or VPS41 in *ATG3*<sup>KO</sup> HeLa cells generated by CRISPR/Cas9 (Fig EV5E); *ATG3* is a key factor required for lipidation of mAtg8s (Mizushima *et al*, 2011). We next tested cellular acidified compartments by HCM and Amnis, and quantified effects of mAtg8 mutants. In Hexa<sup>KO</sup> cells, the overall LTR<sup>+</sup> profiles were reduced by HCM (Fig 7D and E) and LTG<sup>+</sup> profiles were reduced by ImageStream flow cytometry (Appendix Fig S5B–D). mAtg8s also had an effect on mTOR inactivation in response to starvation, as evidenced by a more pronounced loss of phosphorylation of mTOR targets ULK1 (phospho-Ser757) and 4E-BP1 (phospho-Thr37/46) in Hexa<sup>KO</sup> cells relative to WT HeLa cells (Fig EV5F). In summary, albeit mAtg8s do not completely parallel Stx16 phenotype, they do have an effect on Stx16 distribution and mTOR inactivation. Additionally, mAtg8s show strong effects on acidified, LTR<sup>+</sup> compartments in cells under autophagy-inducing conditions. Thus, mAtg8s and Stx16 have partially overlapping effects on the endolysosomal system.

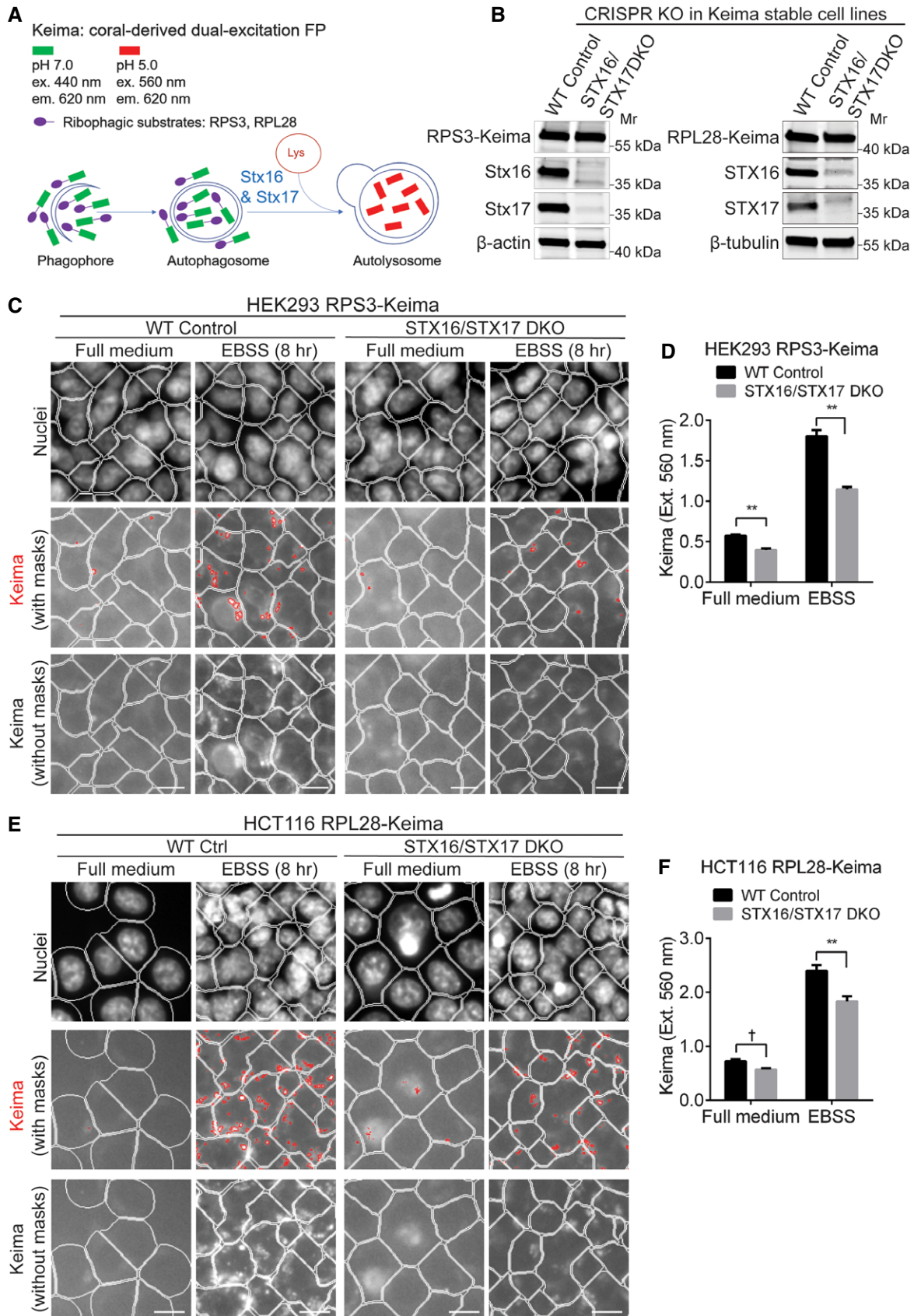


Figure 4.



**Figure 4. Stx16 and Stx17 cooperate in ribophagy.**

- A Schematic of ribophagy detection with Keima-based reporter system; FP: fluorescent protein.
- B Validation of *STX16/STX17* DKO by Western blot analysis in HEK293 cells stably expressing RPS3-Keima (left panel) and HCT116 cells stably expressing RPL28-Keima (right panel).
- C WT or *STX16/STX17*<sup>DKO</sup> HEK293 RPS3-Keima cells were cultured in full medium or starved for 8 h, and subjected to HCM analysis of Keima puncta accumulated in autolysosomes with the excitation wavelength at 560 nm. Masks: white, cells identified based on nuclei; red, Keima puncta detected with the excitation and emission wavelengths of 560 nm and 620 nm, respectively; lower panel shows Keima puncta without masks. Scale bar: 20  $\mu$ m.
- D Quantifications of autolysosomal Keima puncta in WT or *STX16/STX17*<sup>DKO</sup> HEK293 RPS3-Keima cells treated as in (C). Data shown as means  $\pm$  SEM of Keima puncta per cell, minimum 1,000 cells were counted each well from at least 12 wells, three independent experiments; \*\**P* < 0.01 (two-way ANOVA).
- E WT or *STX16/STX17*<sup>DKO</sup> HCT116 RPL28-Keima cells were cultured in full medium or starved for 8 h, and subjected to HCM analysis of Keima puncta accumulated in autolysosomes with the excitation wavelength at 560 nm. Masks: white, cells identified based on nuclei; red, Keima puncta detected with the excitation and emission wavelengths of 560 nm and 620 nm, respectively; lower panel shows Keima puncta without masks. Scale bar: 20  $\mu$ m.
- F Quantifications of autolysosomal Keima puncta in WT or *STX16/STX17*<sup>DKO</sup> HCT116 RPL28-Keima cells treated as in (E). Data shown as means  $\pm$  SEM of Keima puncta per cell, minimum 1,000 cells were counted each well from at least 12 wells, three independent experiments; †, not significant; \*\**P* < 0.01 (two-way ANOVA).

Source data are available online for this figure.

## Discussion

Here, we uncovered a broad potential for interactions between the core autophagy components, mAtg8s, and the core membrane fusion system composed of SNAREs. The bioinformatics analyses and follow-up biochemical experiments indicate that mAtg8s and several SNAREs work together in membrane trafficking and organelle biogenesis. One subset of SNAREs interacting with mAtg8s, Stx16-Vti1a-Stx6, was examined in detail, and Stx16 was fully characterized for mAtg8 binding, as has been done for Stx17 (Kumar *et al*, 2018). This leads us to propose a model departing from the conventional views of how LC3 or other mAtg8s work in autophagy, suggesting a new mechanism of control of membrane trafficking/fusion through direct action of mAtg8s on SNARE molecules (Fig 7F).

Our findings with Stx16 indicate a function for mAtg8s in the maintenance of lysosomal compartments (Fig 7F), through binding directly and regulating SNAREs of the Stx16 complex, which leads up to autolysosome biogenesis. This amends the conventional view of mAtg8s expanding the autophagosomal membranes (Carlsson & Simonsen, 2015) and complements more recent views of mAtg8s working in the recruitment of various components of the autophagic apparatus. These include (i) mAtg8 binding to autophagic receptors from the SLR (Birgisdottir *et al*, 2013; Rogov *et al*, 2014) and TRIM (Mandell *et al*, 2014; Keown *et al*, 2018) families of receptors; (ii) the ULK1-FIP200-ATG13 autophagy initiation complex (Alemu *et al*, 2012); (iii) a large number of ancillary components facilitating the autophagosome-lysosome fusion (McEwan *et al*, 2015; Olsvik *et al*, 2015; Wang *et al*, 2016; Kriegenburg *et al*, 2018); and (iv) non-autophagic roles of mAtg8s, such as LAP (Sanjuan *et al*, 2007), LAP-like processes (Florey *et al*, 2015), MVB/exosome formation (Guo *et al*, 2017), and via binding to TECPR2 in the COPII-dependent ER export (Stadel *et al*, 2015).

Human cells have 6 mAtg8s, with proposed but not fully delineated specialized functions (Shpilka *et al*, 2011). Their role has been established in the recruitment of several important ancillary factors facilitating membrane fusion at the autophagosome-lysosome fusion stage of the autophagic pathway (Nakamura & Yoshimori, 2017). An early study has shown GABARAPL2/GATE-16 interaction with NSF enhancing its ATPase activity, thus implicating it in activation of the Golgi SNARE GOS-28/GOSR1 (Sagiv *et al*, 2000), of consequence for post-mitotic Golgi reformation (Muller *et al*, 2002) and intra-Golgi transport (Sagiv *et al*, 2000). This is in keeping with

the results of our screen of mAtg8-interacting SNAREs and is compatible with the concept of mAtg8s directly acting on SNAREs.

Stx16 and its cognate SNAREs have been shown to play a role in retrograde trafficking from endosomal compartments to TGN with two distinct pairings of Qa-, Qb-, and Qc-SNAREs: (i) Stx16, Vti1a, and Stx10, required specifically for M6PR transport (we did not observe effects on M6PR relative to TGN46 in our studies), which impacts trafficking of lysosomal hydrolases (Ganley *et al*, 2008); and (ii) Stx16, Vti1a, and Stx6, which is independent of the Stx10/M6PR pathway and controls trafficking to TGN of endocytosed incoming toxins bound to membrane glycoconjugates (Mallard *et al*, 2002). There is also a Stx16-independent retrograde pathway that instead depends on GOS-28/GOSR1 (Tai *et al*, 2004), another SNARE that incidentally binds mAtg8s (see Fig 1A, 6<sup>th</sup> peptide array from the top). Of note, GOS-28/GOSR1 has been first described as a GATE-16-binding SNARE (Sagiv *et al*, 2000) with Golgi functions. Most of the prior studies with Stx16 complexes have been focused on retrograde trafficking to the TGN, and thus, our observations could be due to perturbed retrograde membrane and cargo flow. However, our findings that Stx16 is in complexes with VPS41, previously implicated in anterograde transport of LAMP1/2 (Pols *et al*, 2013), and reduced abundance of LAMP1/2<sup>+</sup> organelles in *STX16*<sup>KO</sup> cells suggest that Stx16 defect blocks the prograde delivery of LAMP1/2 during lysosomal biogenesis.

Trafficking of various components that make up functional lysosomes is only partially understood (Luzio *et al*, 2000; Saftig & Klumperman, 2009; Bright *et al*, 2016) especially regarding anterograde pathways to lysosomes of integral membrane proteins such as LAMP1/2 and trafficking to and assembly at the lysosome of the V<sub>1</sub>V<sub>0</sub> holoenzyme vacuolar H<sup>+</sup>-ATPase (Saftig & Klumperman, 2009). Our findings suggest that Stx16 is responsible for LAMP1/2 trafficking to lysosomes with secondary consequences on the overall levels of LAMPs when this delivery is disrupted. It is likely that Stx16 does not affect vacuolar H<sup>+</sup>-ATPase's ability to function but these acidified compartments may be displaced due to a lack of clearly defined lysosomal destination. Some of *STX16*<sup>KO</sup> phenotypes (perturbation of acidified compartments) are echoed in mAtg8s null Hexa<sup>KO</sup> cells, which show diminished overall content of acidified organelles. These phenomena can in part be explained by mAtg8s affecting Stx16 localization. Since Hexa<sup>KO</sup> effects on acidification are more pronounced than the effects of *STX16*<sup>KO</sup>, this indicates additional functions for mAtg8s, specifically in H<sup>+</sup>-ATPase function. For example, it has recently been shown that LC3 affects ATP6V1E1

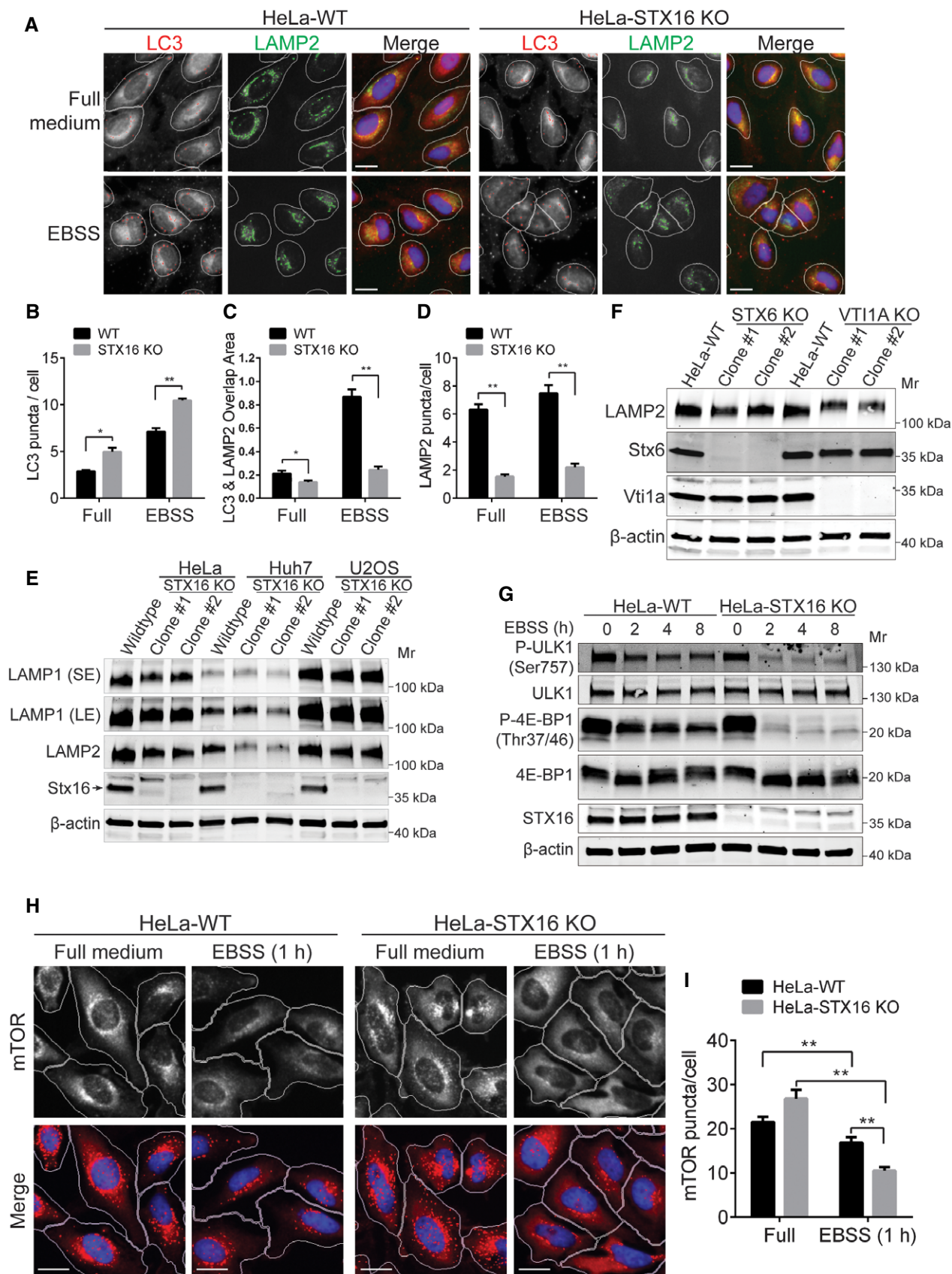


Figure 5.

**Figure 5. Stx16 is required for the maintenance of lysosomal homeostasis and mTOR activity.**

- A WT or *STX16*<sup>KO</sup> HeLa cells were starved in EBSS for 2 h and subjected to HCM analysis of LAMP2 and LC3 puncta. Masks: white, cells identified based on nuclei; red, LC3 puncta; green, LAMP2 puncta; yellow, overlap of LC3 and LAMP2. Scale bar: 20  $\mu$ m.
- B–D Quantifications of LC3 puncta per cell (B), LC3, and LAMP2 overlap area per cell (C), and LAMP2 puncta per cell (D) in WT or *STX16*<sup>KO</sup> cells treated as in (A). Data shown as means  $\pm$  SEM of puncta or overlap area per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \**P* < 0.05; \*\**P* < 0.01 (two-way ANOVA).
- E Western blot analysis of LAMP1 and LAMP2 protein levels in WT and separate clones of *STX16*<sup>KO</sup> HeLa, Huh7, and U2OS cells.
- F Western blot analysis of LAMP2 protein levels in WT and separate clones of *STX6*<sup>KO</sup> or *VTI1A*<sup>KO</sup> HeLa cells.
- G Time course of mTOR inactivation during EBSS starvation in WT vs. *STX16*<sup>KO</sup> HeLa cells analyzed by Western blotting for mTOR substrates ULK1 and 4E-BP1.
- H WT or *STX16*<sup>KO</sup> HeLa cells were starved in EBSS for 1 h and subjected to HCM analysis of mTOR puncta. Masks: white, cells identified based on nuclei; red, mTOR puncta. Scale bar: 20  $\mu$ m.
- I Quantifications of mTOR puncta in WT or *STX16*<sup>KO</sup> cells treated as in (H). Data shown as means  $\pm$  SEM of mTOR puncta per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \*\**P* < 0.01 (two-way ANOVA).

Source data are available online for this figure.

subunit of the  $V_1$  domain of vacuolar  $H^+$ -ATPase (Guo *et al.*, 2017). Although mAtg8s do not phenocopy in full the Stx16 phenotype, they do affect Stx16 distribution and mTOR activity. Thus, mAtg8s and Stx16 have only partially overlapping effects on the endolysosomal system most likely due to mAtg8 action exceeding the reach of the Stx16 function.

Since lysosomes are the location of active mTOR (Betz & Hall, 2013; Saxton & Sabatini, 2017), we used mTOR activity as another readout of Stx16 effects on the lysosomal function. Somewhat surprisingly, given the strong reduction of LAMP1/2 levels and diminished number of LAMP<sup>+</sup> organelles, *STX16*<sup>KO</sup> did not show effects on basal mTOR activity. However, during starvation, which inactivates mTOR and triggers its translocation from the lysosomes, mTOR was inhibited far more prominently in *STX16*<sup>KO</sup> cells and this was also reflected in fewer mTOR<sup>+</sup> cytoplasmic puncta. One interpretation is that the reduced LAMP1/2 content differentially affects subpopulations of lysosomes (Bright *et al.*, 2016) and that the ones that are platforms for active mTOR are preferentially preserved/maintained. Nevertheless, upon stress and perturbation, inactivation of mTOR is more effective and more durable in *STX16*<sup>KO</sup> cells. This could be a reflection of altered trigger thresholds dependent on diminished lysosomal sources of nutrients and/or difficulties in re-establishing nutrient equilibrium and mTOR activity. Alternatively, Stx16 may be important in transport to lysosomes of additional mTOR regulatory components important for its reactivation.

The Qa-SNARE Stx17 was first appreciated as a key SNARE in autophagosome–lysosome fusion (Itakura *et al.*, 2012; Takats *et al.*, 2013; Guo *et al.*, 2014; Diao *et al.*, 2015; Wang *et al.*, 2016). Recent studies have uncovered the yeast SNARE, Ykt6, as important in

autophagosome–vacuole/lysosome fusion in yeast (Bas *et al.*, 2018; Gao *et al.*, 2018), and that mammalian Ykt6 ortholog (Matsui *et al.*, 2018; Takats *et al.*, 2018) plays a potentially dominant (over Stx17) role in mammalian autophagosome–lysosome fusion. Whereas the exact mechanism of Ykt6 in maturation has not been agreed upon, with differences in proposed models (Matsui *et al.*, 2018; Takats *et al.*, 2018), it is worth noting that Ykt6 is also part of one of the retrograde trafficking routes from endosomes to TGN that includes GOS-28/GOSR1 (Tai *et al.*, 2004), an mAtg8-binding SNARE as discussed above. Nevertheless, our data here suggest that Stx17 still plays a very important role in autophagosomal flux, revealed in the requirement for a *STX16/STX17* double KO to block autophagic flux using the conventional and well-accepted LC3 flux assay (Klionsky *et al.*, 2016), and reflected in autophagic degradation of a diverse panel of substrates: mitochondria, peroxisomes, *M. tuberculosis*, and ribosomes.

Stx17 interacts via its LIR motif with mAtg8s, and this may play a role in its recruitment to autophagosomes as a prelude to fusion with the lysosomes (Kumar *et al.*, 2018). However, Stx17's role in autophagy begins much earlier, during initiation (Hamasaki *et al.*, 2013; Arasaki *et al.*, 2015, 2018; Kumar *et al.*, 2019) ensuring that once autophagosomes are formed they are enabled for fusion with endolysosomal compartments. The present screen for other SNAREs that bind mAtg8s yielded a number of candidates in addition to Stx16, such as Vti1a and Stx6, which partner with Stx16, and Stx3 and Stx4, SNAREs that are engaged in secretory autophagy (Kimura *et al.*, 2017). This prompts us to suggest that mAtg8s' function in autophagy should be reconsidered and some of its mechanistic principles revisited. We propose that mAtg8s act by reshaping overall membrane trafficking in the cell through direct interactions and

**Figure 6. Stx16 regulates proper distribution of acidified compartments in the cell.**

- A WT or *STX16*<sup>KO</sup> HeLa cells were starved in EBSS for 1 h, followed by starvation with the presence of LysoTracker Red DND-99 (LTR) (100 nM) for additional 30 min, and subjected to HCM analysis of LTR. Scale bar: 20  $\mu$ m.
- B Quantifications of LTR count per cell or total area per cell in WT and *STX16*<sup>KO</sup> HeLa cells treated as in (A). Data shown as means  $\pm$  SEM of LTR puncta per cell or object total area per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; †, not significant (two-way ANOVA).
- C WT or *STX16*<sup>KO</sup> HeLa cells were starved in EBSS for 1 h, followed by starvation with the presence of LTR (100 nM) for additional 30 min, and subjected to HCM analysis of the colocalization between LTR and TGN46. Masks: white, cells identified based on nuclei; red, LTR puncta; green, TGN46 puncta; yellow, overlap between LTR and TGN46. Scale bar: 20  $\mu$ m.
- D Quantifications of overlaps between LysoTracker Red and TGN46 in WT and *STX16*<sup>KO</sup> cells treated as in (C). Data shown as means  $\pm$  SEM of LTR and TGN46 overlap area per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \*\**P* < 0.01 (two-way ANOVA).
- E WT or *STX16/STX17*<sup>DKO</sup> HeLa cells were starved in EBSS for 2 h and subjected to ultrastructural analysis of the Golgi apparatus with electron microscopy. VF of Golgi: volume fraction of Golgi relative to total cytoplasmic area. Scale bar, 2  $\mu$ m. Red arrows indicate Golgi apparatus.



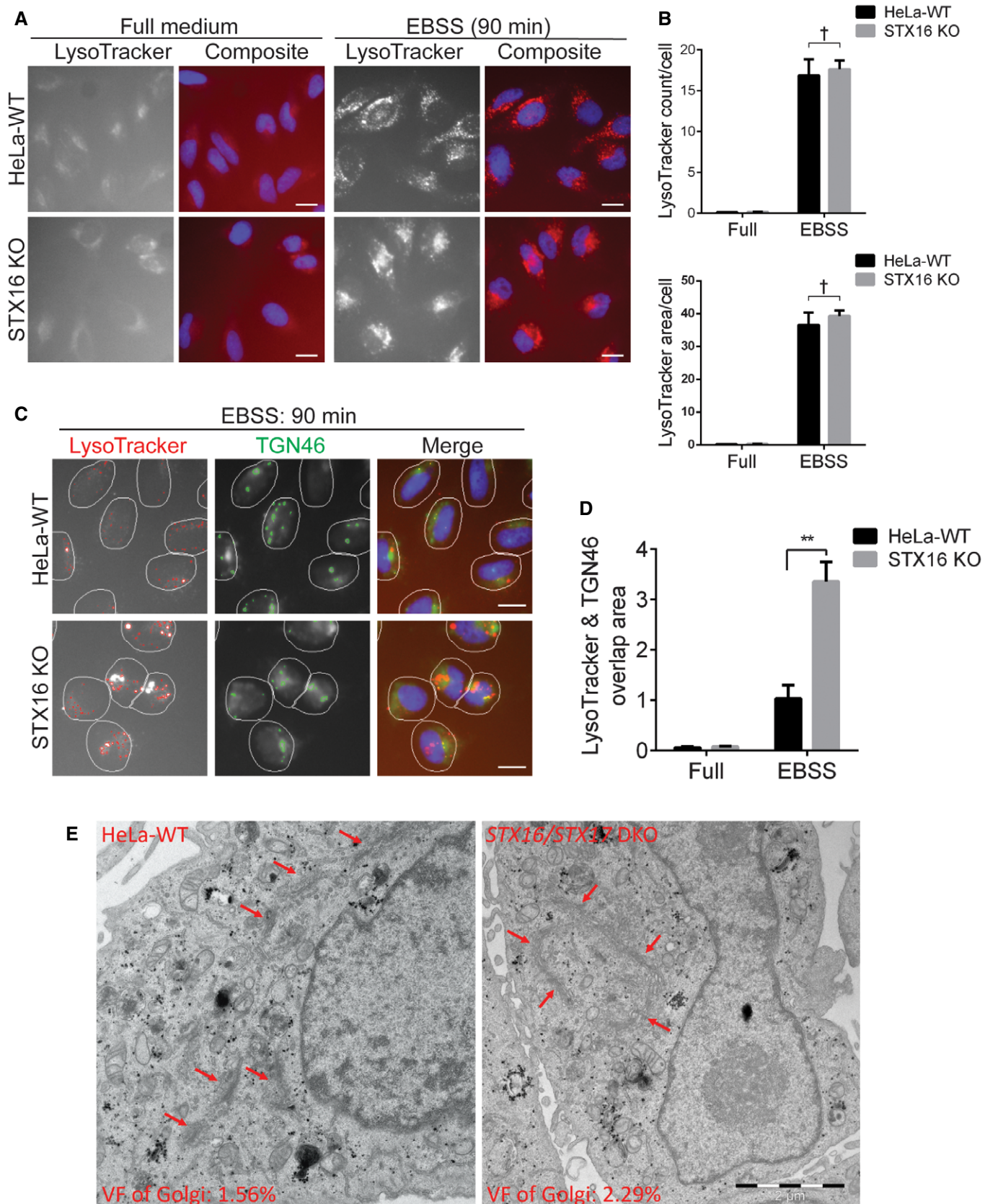


Figure 6.

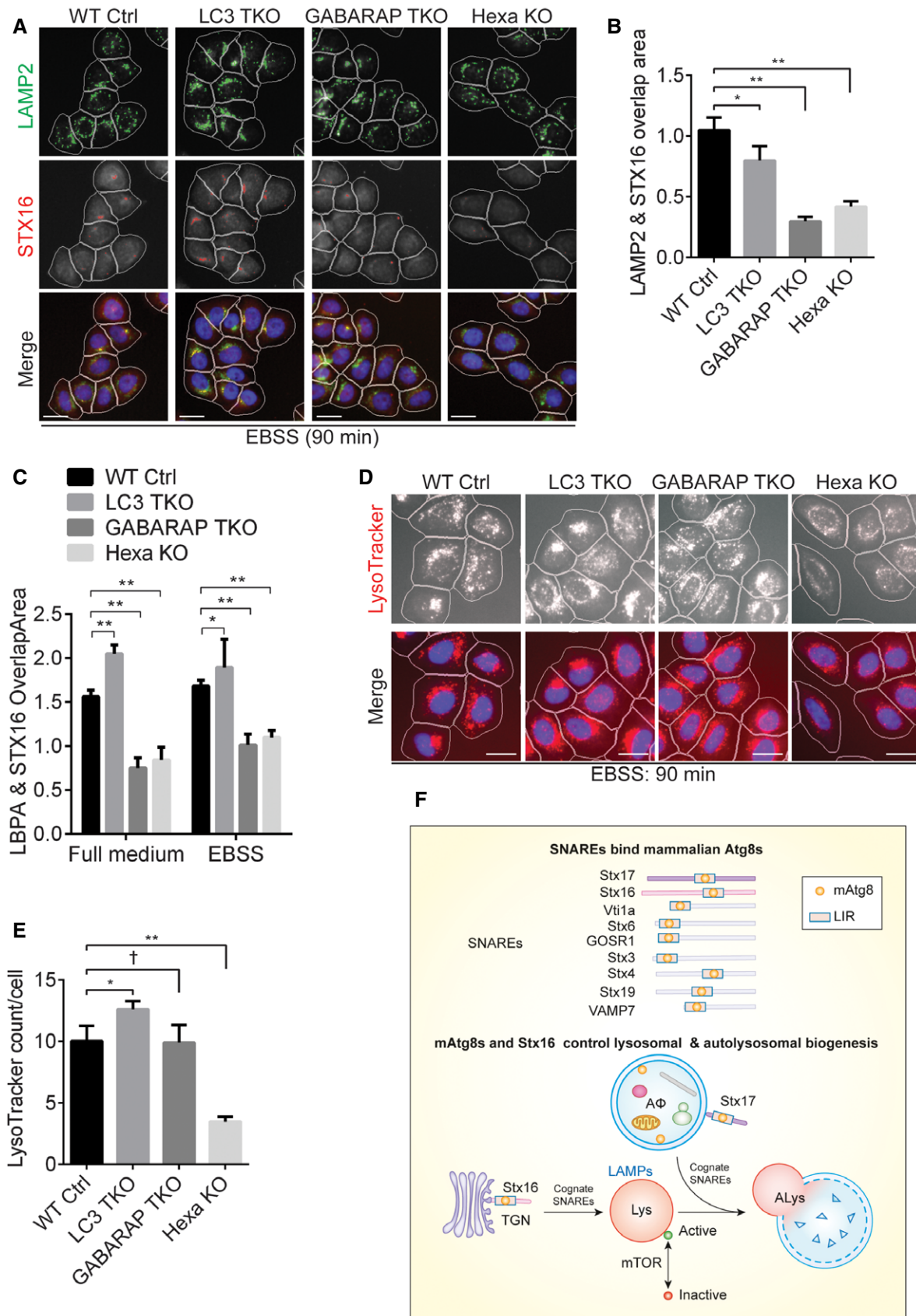


Figure 7.



**Figure 7. Mammalian Atg8s regulate Stx16 localization and acidification of endolysosomal organelles.**

- A WT, LC3<sup>TKO</sup>, GABARAP<sup>TKO</sup>, or Hexa<sup>KO</sup> HeLa cells were starved in EBSS for 90 min and subjected to HCM analysis of overlaps between LAMP2 and Stx16. Masks: white, cells identified based on nuclei; green, LAMP2 puncta; red, Stx16 puncta; yellow, overlap of LAMP2 and Stx16. Scale bar: 20  $\mu$ m.
- B Quantifications of overlaps between LAMP2 and Stx16 in WT, LC3<sup>TKO</sup>, GABARAP<sup>TKO</sup>, or Hexa<sup>KO</sup> HeLa cells treated as in (C). Data shown as means  $\pm$  SEM of LAMP2 and Stx16 overlap area per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \* $P$  < 0.05; \*\* $P$  < 0.01 (one-way ANOVA).
- C Quantifications of overlaps between LBPA and Stx16 in WT, LC3<sup>TKO</sup>, GABARAP<sup>TKO</sup>, or Hexa<sup>KO</sup> HeLa cells grown in full medium or treated as in (A). Data shown as means  $\pm$  SEM of LBPA and Stx16 overlap area per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; \* $P$  < 0.05; \*\* $P$  < 0.01 (one-way ANOVA). Representative images are shown in Appendix Fig S5A.
- D WT, LC3<sup>TKO</sup>, GABARAP<sup>TKO</sup>, or Hexa<sup>KO</sup> HeLa cells were starved in EBSS for 1 h, followed by starvation with the presence of LTR (100 nM) for additional 30 min, and subjected to HCM analysis of LTR. Scale bar: 20  $\mu$ m.
- E Quantifications of LTR puncta in WT, LC3<sup>TKO</sup>, GABARAP<sup>TKO</sup>, or Hexa<sup>KO</sup> HeLa cells treated as in (D). Data shown as means  $\pm$  SEM of LTR puncta per cell, minimum 500 cells were counted each well from at least 12 wells, three independent experiments; †, not significant; \* $P$  < 0.05; \*\* $P$  < 0.01 (one-way ANOVA).
- F Schematic model of this study. Mammalian Atg8 proteins directly bind Stx16 through its LIR motif, which controls the proper localization of the Stx16 SNARE complex at the endolysosomal compartments. The Stx16 SNARE complex functions in the maintenance of lysosomal homeostasis upon autophagy induction, thus controlling mTOR activity.

effects on specific SNAREs and redirect membrane flow toward autophagy at its initiation and termination stages.

## Materials and Methods

### Antibodies and reagents

Antibodies: Rabbit anti-LC3B (L7543, for WB), rabbit anti-STX17 (HPA001204), and mouse anti-FLAG (F1804, for WB and IP) were purchased from Sigma-Aldrich; mouse anti-LC3 (M152-3, for WB and IP) and rabbit anti-LC3 (PM036, for IF) were purchased from MBL International; mouse anti-p62 (#610833) and mouse anti-GM130 (#610823) were purchased from BD Biosciences; rabbit anti-STX16 (NBP1-92467), rabbit anti-VAMP3 (NB300-510), rabbit anti-VAMP4 (NBP2-13512), rabbit anti-VPS33A (NBP2-20872), and rabbit anti-TGN46 (NBP1-49643) were purchased from Novus Biologicals; mouse anti-STX6 (H00010228-M01, for WB and IP) was purchased from Abnova; mouse anti-Vti1a (sc-136117), mouse anti-VAMP8 (sc-166820), mouse anti-ATG3 (sc-393660), and mouse anti- $\beta$ -actin (sc-47778) were purchased from Santa Cruz Biotechnology; rabbit anti-VPS41 (ab181078), rabbit anti-PEX14 (ab183885), rabbit anti-PMP70 (ab85550), rabbit anti-GFP (ab290), and rabbit anti- $\beta$ -tubulin (ab18251) were purchased from Abcam; rabbit anti-LAMP1 (D2D11) (#9091), Phospho-AMPK $\alpha$  (Thr172) (#2531), AMPK $\alpha$  (#2532), Phospho-ULK1 (Ser757) (#6888), ULK1 (D8H5) (#8054), Phospho-4EBP1 (Thr37/46) (#2855), 4E-BP1 (#9452), Phospho-mTOR (Ser2448) (#2971), rabbit anti-mTOR (7C10) (#2981), rabbit anti-LKB1 (27D10) (#3050), and Autophagy Atg8 Family Antibody Sampler Kit (#64459) were purchased from Cell Signaling Technology; mouse anti-LBPA (C64) (MABT837) was purchased from EMD Millipore; mouse anti-DNA antibody (#61014) was purchased from Progen; and mouse anti-LAMP2 (H4B4) and mouse anti-M6PR (22d4) were purchased from Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa.

Earle's Balanced Salt Solution (EBSS) (E3024) and hydrogen peroxide solution (H1009) were purchased from Sigma-Aldrich. Bafilomycin A1 was purchased from InvivoGen (Cat. Code: tlrl-baf1). CCCP (C2759), oligomycin A (#75351), and antimycin A (A8674) were purchased from Sigma-Aldrich. LysoTracker<sup>TM</sup>Red DND-99 (L7528) and LysoTracker<sup>TM</sup> Green DND-26 (L7526) were purchased from Thermo Fisher Scientific and used at 100 nM final concentration for 30 min.

### Cell culture and transfection

HeLa, HEK293T, U2OS, and THP-1 cells were purchased from the American Type Culture Collection (ATCC). HeLa, HEK293T, and U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, and 1 $\times$  penicillin–streptomycin (Thermo Fisher Scientific) at 37°C in a 5% CO<sub>2</sub> atmosphere. THP-1 cells were grown in RPMI 1640 with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM  $\beta$ -mercaptoethanol (M6250, Sigma-Aldrich) and 10% FBS. The Huh7 cell line was purchased from Rocky Mountain Laboratory. Keima reporter cell lines HEK293 RPS3-Keima and HCT116 RPL28-Keima were obtained from J. Wade Harper, Harvard University (An & Harper, 2018).

For Co-IP analysis of the interactions between overexpressed Stx16/Vti1a/Stx6 and mAtg8s, HEK293T cells in 10-cm petri dishes were transfected with corresponding plasmids via the ProFection Mammalian Transfection System (E1200, Promega). For other experiments, cells were transfected with Lipofectamine 2000 Transfection Reagent (# 11668-019, Thermo Fisher Scientific) according to the manufacturer's manual.

### Plasmid constructs

STX16 plasmid was obtained from DNASU (#HsCD00396980); STX6 was obtained from Addgene (#31581); VTI1A DNA was PCR amplified from cDNA reverse transcribed from total RNAs isolated from HeLa cells. All constructs were transferred into pDEST-EGFP or pDEST-3XFLAG vectors using Gateway cloning system (#12535-019, Thermo Fisher Scientific) and verified by sequencing at GENEWIZ. EGFP-tagged LC3B and GABARAP constructed in pDEST vector were described before (Alemu *et al.*, 2012). LIR mutants of STX16 were generated with QuikChange Lightning Site-Directed Mutagenesis Kit (#210519, Agilent).

### GST pull-down assay and peptide array analysis

Recombinant GST and GST-fusion proteins were expressed in competent *Escherichia coli* SoluBL21 (Genlantis, #C700200) by inducing overnight bacterial cultures with 50–75  $\mu$ g/ml isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). Expressed proteins were purified



by immobilization on Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, #17-5132-01). For GST pull-down assays, myc-tagged proteins were *in vitro* translated in the presence of radioactive methionine (<sup>35</sup>S-methionine) using the TNT T7 Reticulocyte Lysate System (Promega, #14610). 10 µl of *in vitro* translated proteins was first precleared to remove unspecific binding with 10 µl of empty Glutathione Sepharose beads in 100 µl of NETN buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, #1183617001) for 30 min at 4°C. This was followed by incubation of the precleared mixture with purified GST or GST-fusion proteins for 1–2 h at 4°C. The mixture was washed five times with NETN buffer by centrifugation at 2,500 g for 2 min followed by addition of 2XSDS gel-loading buffer (100 mM Tris pH 7.4, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 200 mM dithiothreitol (DTT) (Sigma, #D0632) and heating for 10 min. The reaction was then resolved by SDS-PAGE and the gel stained with Coomassie Brilliant Blue R-250 Dye (Thermo Fisher Scientific, #20278) to visualize the GST and GST-fusion proteins. The gel was then vacuum-dried and radioactive signal detected by Bioimaging analyzer BAS-5000 (Fujifilm).

Peptide arrays of 20-mer amino acids of each SNARE protein were synthesized and immobilized on a membrane with the MultiPrep peptide synthesizer (Intavis Bioanalytical Instruments AG). The membrane was first blocked with 5% non-fat dry milk and then incubated with GST-GABARAP for 24 h. The membrane was washed three times and visualized by immunoblotting with anti-GST antibody as described (Rasmussen *et al*, 2019).

### Generation of knockout cells with CRISPR/Cas9 gRNA

Sequences of gRNAs targeting *STX16* and *STX17* are shown in Fig EV2A. *ATG3* gRNA target sequence: TAGTCCACCACTGTCC AACAA; *STX6* gRNA target sequence: ACATGTCCCAGCGCATCGGA; *VTI1A* gRNA target sequence: AGATACCACCCCAAAGTCTGA. All the single KO and *STX16/STX17* DKO HeLa, Huh7, U2OS, THP1, and HeLa-YFP-Parkin cells were generated by infecting target cells with single-guide RNA (sgRNA) lentiviral vectors, lentiCRISPRv2, as described in Sanjana *et al* (2014). Briefly, HEK293T cells were transfected with lentiCRISPRv2 sgRNA vectors together with psPAX2 and pMD2.G at the ratio of 4 µg, 2.5 µg, and 1.5 µg/6-cm dish. Sixty hours later, the supernatants containing lentiviruses were collected and spun down at 300 g for 5 min to clear cell debris. Lentiviruses were diluted with DMEM full medium at 1:2 ratio and used to infect target cells overnight with the presence of 8 µg/ml of polybrene (Hexadimethrine bromide, H9268, Sigma-Aldrich) in 12-well plates. Then, the medium with lentivirus was removed and changed to fresh medium for another 24 h. Cells were selected with puromycin (1 µg/ml) for 5 days before validation of the knockout. Single clones of HeLa, Huh7, and U2OS KO cells were isolated by seeding single cells in 96-well plates after serial dilutions. For THP1 KO cells, it is impossible to isolate single clones due to their suspension nature, and thus, depletion of Stx16 and Stx17 was not complete. Data are shown in Fig EV3C. Generation of LC3 TKO, GABARAP TKO, and Hexa KO HeLa cells was described as before (Nguyen *et al*, 2016b).

### Western blotting and co-immunoprecipitation (Co-IP)

Cell lysates were prepared with a standard procedure using RIPA buffer (Pierce, #89900) supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, #1183617001). Protein concentrations were determined using Pierce™ BCA Protein Assay Kit (#23225). Protein electrophoresis was carried out using TGX™ SDS Gels (Bio-Rad, #4561091), followed by blotting to a nitrocellulose membrane (Bio-Rad, #1620112). Membranes were incubated with primary antibodies diluted in blocking buffer (3% BSA diluted in 1XTBS buffer plus 0.05% Tween-20) overnight at 4°C. After incubation with fluorescently labeled secondary antibodies goat-anti-mouse IRDye 680LT or goat-anti-rabbit IRDye 800CW (LI-COR Biosciences) diluted 1:10,000 in blocking buffer, membranes were scanned using a fluorescence scanner Odyssey (LI-COR Biosciences).

For Co-IP of overexpressed proteins, HEK293T cells cultured in 10-cm dishes were lysed with 1 ml NP-40 cell lysis buffer (Invitrogen, FNN0021). Cell lysates were spun down at top speed for 10 min at 4°C to clear cell debris. The supernatants were collected and incubated with 2.5 µl anti-FLAG antibody (M2) for 4 h, followed by incubation with Dynabeads® Protein G for 1 h, and precipitated proteins were subjected to Western blot analysis with rabbit anti-GFP antibody. For endogenous Co-IP analysis, cell lysates were prepared from cells cultured in 15-cm dishes and incubated with 2.5 µg mouse anti-Stx6 or mouse anti-LC3 antibody overnight at 4°C, followed by Dynabeads® Protein G (Thermo Fisher Scientific, #10004D) binding and Western blot analysis of bound proteins.

### Immunostaining and staining of acidified compartments for HCM and ImageStream flow cytometry

For HCM, cells were seeded into 96-well plates. After treatments, cells were washed once with 1× PBS, followed by fixation with 4% paraformaldehyde (PFA) for 5 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After incubation with blocking buffer (3% goat serum in 1× PBS), cells were stained with primary antibodies in blocking buffer for 1 h at room temperature or overnight at 4°C, followed by staining with secondary antibodies (Alexa Fluor conjugates, Thermo Fisher Scientific) diluted 1:1,000 in blocking buffer for 1 h at room temperature. For LysoTracker Red DND-99, cells in 96-well plates were cultured in full medium or starved for 1 h in EBSS, followed by adding LTR for another 30 min at the final concentration of 100 nM. For ImageStream flow cytometry analysis of LysoTracker Green DND-26, cells in 6-cm dishes were starved in EBSS for 1 h, followed by adding LTG for another 30 min at the final concentration of 100 nM. Cells were collected by trypsinization and resuspended in 200 µl 1× PBS for Amnis analysis.

### High-content microscopy (HCM) computer-based image acquisition and quantification

High-content microscopy with automated image acquisition and quantification was carried out using a Cellomics ArrayScan VTI platform (Thermo Fisher Scientific). A minimum of 500 primary objects

(valid cells) per well were imaged for the quantifications of intracellular targets (regions of interest, ROI). Scanning parameters and object masks were preset with HCS Studio Scan software for algorithm-defined automated image acquisition and analysis. The images were viewed, and ROI quantified with HCS View software iDEV provided with the Cellomics (Mandell *et al*, 2014).

### ImageStream flow cytometry analysis

ImageStreamX Mark II flow cytometer Amnis® (EMD Millipore) was used to analyze LysoTracker Green DND-26 (LTG) staining after autophagy induction by starvation. The 488-nm laser was used for excitation. Debris and doublets were gated out. Bright field (430–480 nm, Channel 01) and LTG (505–560 nm, Channel 02) channels were measured, and at least 8,000 events of single cells per sample were collected. For analysis, the IDEAS version 6.0 software was used. Gating was applied to focused single cells using gradient root mean square of the bright field image then bright field area and aspect ratio, respectively. Fifty cells with low/no fluorescent intensity and 50 cells with high fluorescent intensity and verified punctate LTG staining by image collection from WT cells were selected to create a template. The template was then applied to all other samples in the same experiment to generate quantifications (counts of LTG/cell) for each sample.

### Electron microscopy

Wild-type or *STX16/STX17<sup>DKO</sup>* HeLa cells were grown in 6-cm dishes until they became semi-confluent. The cells were starved for 2 h in EBSS, followed by fixation with 2% glutaraldehyde (EM grade) in 0.2 M HEPES, pH 7.4. After 30-min initial incubation, cells were scraped under a small volume of fixative and transferred to Eppendorf tubes. The tubes were spun at full speed for 10 min at room temperature to get a firm pellet. The pellets were continued being fixed until total fixation time is 2 h. Thin sections were cut using an ultramicrotome, collected onto electron microscopy grids, and stained with uranyl acetate and lead citrate. In order to count autophagic compartments or Golgi apparatus, 47 images for each sample were taken at primary magnification of 5,000×, using the principles of uniform random sampling. The images were zoomed on computer screen. Autophagic compartments and the Golgi apparatus were counted, and the cytoplasmic area was estimated by point counting (Eskelinen, 2008).

### *Mycobacterium tuberculosis* killing assay

*Mycobacterium tuberculosis* killing assay was carried out according to previously described (Kumar *et al*, 2018). Briefly, *Mycobacterium tuberculosis* Erdman (Erdman) culture was prepared by thawing frozen stock aliquot and grown in 7H9 Middlebrook liquid medium supplemented with oleic acid, albumin, dextrose, and catalase (OADC, Becton Dickinson, Inc., Sparks, MD, USA), 0.5% glycerol, and 0.05% Tween 80. Cultures were grown at 37°C. WT, *STX16<sup>KO</sup>*, *STX17<sup>KO</sup>*, and *STX16/STX17<sup>DKO</sup>* THP1 cells (Fig EV3C) were infected with Erdman at MOI 10 and incubated with full medium for 18 h or 16 h followed by starvation in EBSS for 2 h to induce autophagy, lysed, and plated on 7H11 agar plates. CFU was enumerated 3 weeks later.

### Quantifications and statistical analysis

Data, means ± SEM ( $n \geq 3$ ), were analyzed with paired and unpaired two-tailed Student's *t*-test, or by one-way or two-way ANOVA (analysis of variance) followed by post hoc Tukey's test. Statistical significance was defined as: † $P \geq 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Expanded View** for this article is available online.

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### Author contributions

Conceptualization, YG, TJ, SK, and VD; Investigation and Validation, YG, VD, and TJ; Methodology, YG, YPA, SK, BB, SWC, JJ, and E-LE; Formal Analysis, YG, SK, VD, and TJ; Resources, ML; Writing—Original Draft, YG and VD; Writing—Review and Editing, YG, VD, ML, and TJ; Supervision, VD and TJ; Funding Acquisition, VD and TJ.

### Conflict of interest

The authors declare that they have no conflict of interest.

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