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# Noncanonical roles of ATG5 and membrane atg8ylation in retromer assembly and function

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**eLife Assessment**

Masroor Ahmad Paddar and colleagues reveal noncanonical roles of ATG5 and membrane ATG8ylation in regulating retromer assembly and function. They identify ATG5's unique non-autophagic role and show that CASM partially contributes to these phenotypes. Although the mechanism by which ATG8ylation regulates the retromer remains unclear, the findings provide **important** insights with **solid** supporting evidence.

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## Abstract

ATG5 is one of the core autophagy proteins with additional functions such as noncanonical membrane atg8ylation, which among a growing number of biological outputs includes control of tuberculosis in animal models. Here we show that ATG5 associates with retromer's core components VPS26, VPS29 and VPS35 and modulates retromer function. Knockout of ATG5 blocked trafficking of a key glucose transporter sorted by the retromer, GLUT1, to the plasma membrane. Knockouts of other genes essential for membrane atg8ylation, of which ATG5 is a component, affected GLUT1 sorting, indicating that membrane atg8ylation as a process affects retromer function and endosomal sorting. The contribution of membrane atg8ylation to retromer function in GLUT1 sorting was independent of canonical autophagy. These findings expand the scope of membrane atg8ylation to specific sorting processes in the cell dependent on the retromer and its known interactors.

## Introduction

The canonical autophagy pathway, ubiquitous in eukaryotes, is manifested by the emergence of intracellular membranous organelles termed autophagosomes that capture cytoplasmic cargo destined for degradation in lysosomes [1]. Progress has been made in understanding molecular mechanisms governing canonical autophagosome biogenesis in mammalian cells [2] including ATG9A-vesicles [3–7] and membranous prophagophores [8] as precursors to LC3-positive phagophores, their reshaping as cups [9] and dynamic interactions with the omegasomes [10,11], phagophore expansion through lipid transfer [12–14], cargo recognition and sequestration [15,16], closure of the phagophore to form a double membrane phagosomes [17–20], and fusion of autophagosomes with endosomal and lysosomal organelles leading to cargo degradation [21] or secretion [22].

However, and contemporaneously with these major advances in understanding canonical autophagy, it has become evident that autophagy genes (ATGs) [23] have additional functions in mammalian cells that do not fit the canonical model [24,25]. Among such noncanonical manifestations are LAP (LC3-associated phagocytosis) [26], LANDO (LC3-associated endocytosis) [27], LC3-associated micropinocytosis (LAM) [28], conjugation of ATG8 to single membranes (CASM) [29,30], v-ATPase-ATG16L1-induced LC3 lipidation (VAIL) [31–33], ER-phagy mediated by the V-ATPase-ATG16L1-LC3C axis (EVAC) [34], LyHYP (lysosomal hypersensitivity phenotype) [35], membrane damage repair [36–39], and two distinct forms of secretory autophagy [22], SALI (secretory autophagy during lysosome inhibition) [40] and LDELS (LC3-dependent EV loading and secretion) [41]. These processes depend on or are associated with the phospholipid conjugation cascade of mammalian ATG8 proteins (mATG8s) [42] and collectively (including the canonical autophagy) represent diverse manifestations of membrane atg8ylation as a broad membrane stress, damage and remodeling response [25].

The factors governing membrane atg8ylation include two enzymatic cascades with the ATG12-ATG5 and mATG8-phosphatidylethanolamine (PE) covalent conjugates as their products. The ATG12-ATG5 conjugate [42] combines with ATG16L1 [43] or TECPR1 to form E3 ligases [38,39,44] to direct mATG8-PE conjugation and atg8ylation of target membranes. All known E3 enzymes contain the ATG12-ATG5 conjugate [38,39,42,44]. To form this conjugate, the ubiquitin like molecule ATG12 is activated by ATP, and transferred via E1 (ATG7) and E2 (ATG10) to ATG5. In preparation for the next step, an ATG12-ATG5 containing E3 ligase activates its substrate mATG8-ATG3 to transfer ATG8 and form an amide bond with aminophospholipids [42]. However, there are additional branches of these conjugation cascades, whereby ATG12 can make a noncanonical sidestep conjugate with ATG3 (ATG12-ATG3) [41,45] which is enhanced in the absence of ATG5 [35]. Apart from its role in atg8ylation, ATG5 [35,46] and potentially other ATG genes [47–49] have autophagy-independent functions. Many of the indications that such functions exist come from in vivo studies [50–52]. For example, in the case of murine models of *Mycobacterium tuberculosis* infection, atg8ylation machinery protects against tuberculosis pathogenesis but Atg5 knockout has a particularly strong phenotype exceeding other atg8ylation genes, sugestiong that ATG5 possesses atg8ylation (mATG8s lipid conjugation) independent functions [35,46,53–56]. As recently reported, one such function involves LyHYP contributing to enhanced exocytosis specifically in cells devoid of ATG5 but not of other ATG genes [35]. These developments indicate that ATG5 plays noncanonical roles in autophagy-independent vesicular transport events and homeostasis of the endolysosomal system.

Within the endosomal network, the mammalian retromer complex [57] is one of the several systems regulating vectoral transport of membranes and proteins. These systems include, among others, HOPS, CORVET, retriever, CCC/commander, and retromer [57–64] complexes that often contain and sometimes share VPS subunits. Retromer is a heterotrimer of VPS26A/B, VPS29 and

VPS35 subunits [65], first identified in yeast [66]. Retromer sorts an array of endosomal cargo in cooperation with cognate sorting nexins (SNX) [67]. Specifically, this includes SNX-BAR proteins SNX1/2-SNX5/6 [68,69], the mammalian paralogs of yeast Vps5p and Vps17p [66] which can function autonomously as an ESCPE-I complex [70], SNX-PX protein SNX3 [71], and the SNX-FERM protein SNX27 containing PX, PDZ and FERM domains [72] which sorts various transporter proteins and signaling receptors including the glucose transporter GLUT1 (SLC2A1) [73]. Retromer, together with adaptors, contributes to the complex protein sorting within the endosomal system [74–76]. Here, using unbiased proteomic approaches and follow-up mechanistic analyses, we report that retromer complex is among ATG5 interactors. We show that membrane atg8ylation, of which ATG5 is an essential component, affects retromer-dependent cargo sorting. This function is independent of the canonical autophagy pathway or a well-studied form of noncanonical membrane atg8ylation, CASM. Our findings offer a paradigm shift connecting ATG5 and membrane atg8ylation with the retromer system and its function in the endosomal cargo sorting, expanding the scope of atg8ylation machinery and its special component ATG5 beyond the current models.

# Results

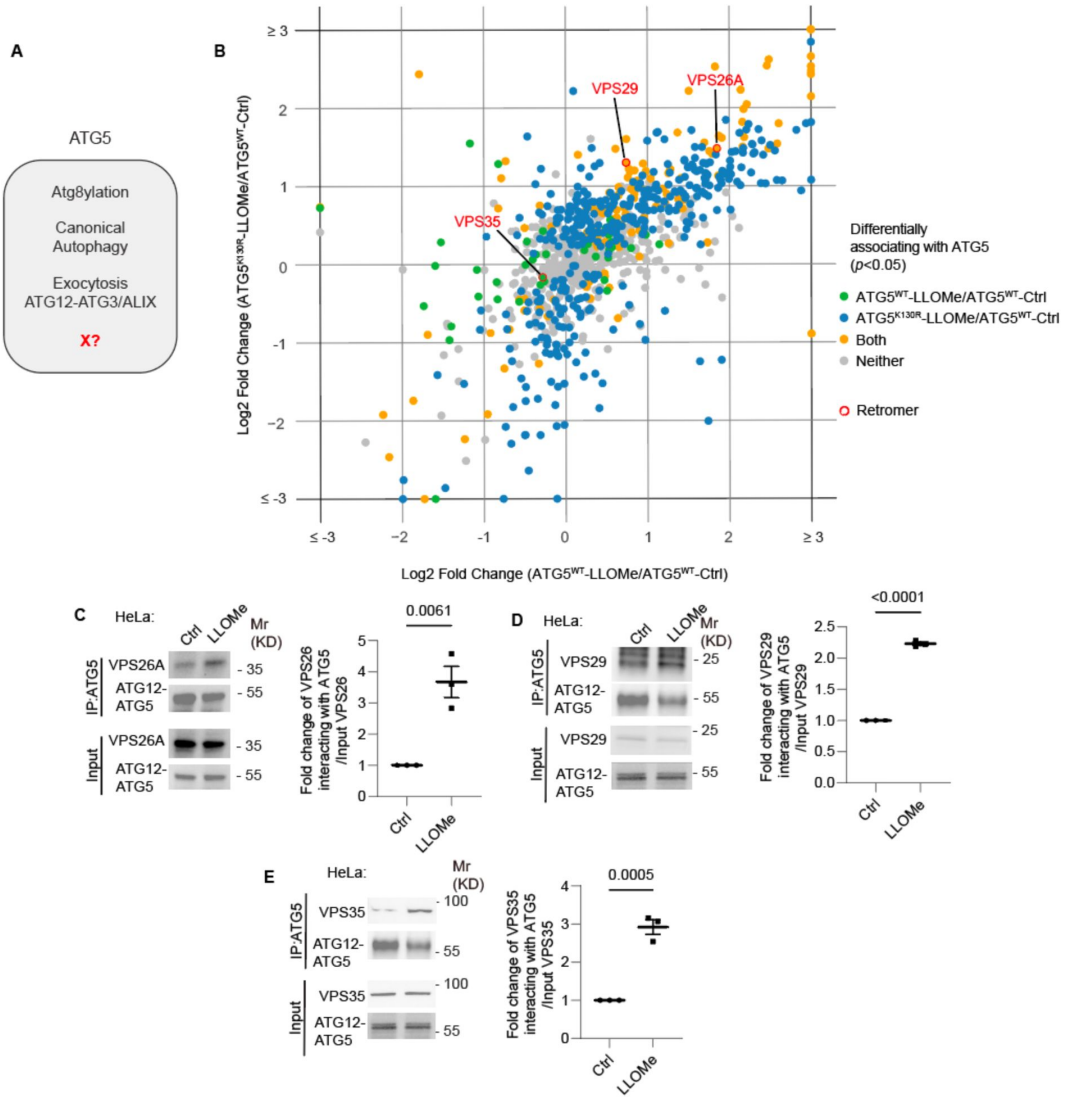
## ATG5 associates with the retromer complex

The unique aspects of Atg5 (**Fig. 1A**), outside of its role in canonical autophagy, have strong ex vivo and important in vivo phenotypes [52]. Inactivation of Atg5 in myeloid lineage renders mice excessively susceptible to acute infection with *Mycobacterium tuberculosis* attributed to excessive inflammation [35,46,53–56], which did not extend to other phases of infection as tested here in a murine model of latent tuberculosis [77–79] (**Fig. S1A-D**). In a previous study, we could only partially explain the cellular parameters associated with acute infection, which included degranulation in neutrophils from $Atg5^{fl/fl}$ LysM-$Cre^{+}$ mice [35]. Furthermore, markers of LyHYP (lysosome hypersensitivity phenotype) [35] in ATG5 knockout (KO) cells, such as galectin 3 (Gal3) response, could not be explained by the exocytic phenomena [35]. We thus sought to uncover additional processes (**Fig. 1A**) affected by ATG5 at the intracellular level. To include factors beyond the known processes, we analyzed proteomic data obtained with APEX2-ATG5 [35] and compared APEX2-$ATG5^{WT}$ with APEX2-$ATG5^{K130R}$ (an ATG5 mutant deficient in conjugation to ATG12) in cells treated with LLOMe, an agent routinely used to cause lysosomal damage [80–85] (**Figs. 1B** and **S2A**,**B**). The proximity biotinylation (APEX2)-based proteomic data indicated the presence in the vicinity of APEX2-ATG5 of several protein complexes that control important membrane trafficking pathways including retromer (**Fig. 1B**). Beside the retromer VPS subunits, there were very few other VPS proteins (**Fig. S2C**). VPS proteins were observed in ATG5 proteomic data by others [86] (**Fig. S2D**). VPS26 and VPS29 showed statistically significant increase in proximity to ATG5 in cells subjected to LLOMe treatment (**Fig. S2A-C**). Using APEX2-SGALS1 proximity biotinylation LC-MS/MS as a control, we did not observe enrichment of retromer subunits in cells treated with LLOMe (**Fig. S2E**).

Association of endogenous ATG5 with endogenous retromer subunits (VPS26A, VPS29 and VPS35) was tested in coimmunoprecipitation (co-IP) experiments (**Fig. 1C-E**). The interaction between retromer and ATG5 was detected in these experiments and showed increased association in cells subjected to lysosomal damage with LLOMe (**Fig. 1C-E**). Thus, ATG5, a key component of the known atg8ylation E3 ligases [42,87], is found in protein complexes with the retromer and this association is enhanced upon lysosomal damage.

## Retromer affects a subset of responses to lysosomal damage

During lysosomal damage a number of processes are set in motion to repair, remove and regenerate/replenish lysosomes [37,80,88–91] including membrane atg8ylation-dependent processes of repair by ESCRT machinery [92] and by lipid transfer via ATG2 [85,93]. We have




## Figure 1

### ATG5 interacts with retromer.

**A.** ATG5 functions. X, a postulated additional function. **B.** 2D scatter plot (log2 fold changes; color coded p value cutoff matrix for comparisons between samples as per the lookup table) of proximity biotinylation LC/MS/MS datasets: FlpIn-HeLa$^{APEX2\text{-}ATG5\text{-}WT}$ cells (X-axis) and FlpIn-HeLa$^{APEX2\text{-}ATG5\text{-}K130R}$ (Y-axis) treated with 2 mM LLOMe for 30 min ratioed vs. HeLa$^{APEX2\text{-}ATG5\text{-}WT}$ without LLOMe treatment (Ctrl). **C-E.** Co-IP analyses and quantification of VPS26A (C), VPS29 (D) VPS35, (E), interaction with ATG5 in HeLa cells treated with or without 2 mM LLOMe for 30 min. Data, means ± SE (n=3); unpaired *t*-test; p values indicated above the bars.

reported that ATG5 KO cells display elevated Gal3 response to lysosomal damage agents including LLOMe [35]. Gal3 is one of the well-characterized sentinel galectins alerting cellular homeostatic systems to lysosomal damage [37,80,81,88,94]. Based on the observed interactions between ATG5 and retromer, we tested whether retromer, like ATG5 [35], affected Gal3 response to lysosomal damage. We used the previously established quantitative high content microscopy (HCM) approach, which provides unbiased machine-driven image acquisition and data analysis [8,36,80,88,94]. In the experiments herein HCM was based on >500 valid primary objects/cells per well and a minimum of 5 wells per sample, with the independent biological replicates being n≥3 (≥3 separate plates) as described [35]. Upon treatment with LLOMe, HeLa$^{VPS35\text{-}KO}$ cells had increased Gal3 puncta relative to HeLa$^{WT}$ cells, comparable to HeLa$^{ATG5\text{-}KO}$ (**Fig. 2A**,**B**) [35]. The Gal3 phenotype in VPS35 knockout cells was partially complemented by expression of GFP-VPS35 (**Fig. 2C**,**D**). The observed elevated Gal3 recruitment to damaged lysosomes in the absence of VPS35 cannot be explained by increased pools of LAMP2 organelles in the cells, since the overall complement of lysosomes (quantified by LAMP2 puncta/cell) did not increase in LLOMe treated HeLa cells (**Fig. S3A**,**B**). Another marker of lysosomal damage, ubiquitination response [95], was elevated in both VPS35 and ATG5 deficient cells (**Figs. 2E**,**F**). This phenotype was confirmed in another cell line, Huh7 (**Fig. S3C**,**D**). Thus, VPS35 and ATG5 defects have a similar effect on lysosomes, previously characterized and termed lysosomal hypersensitivity phenotype/LyHYP [35].

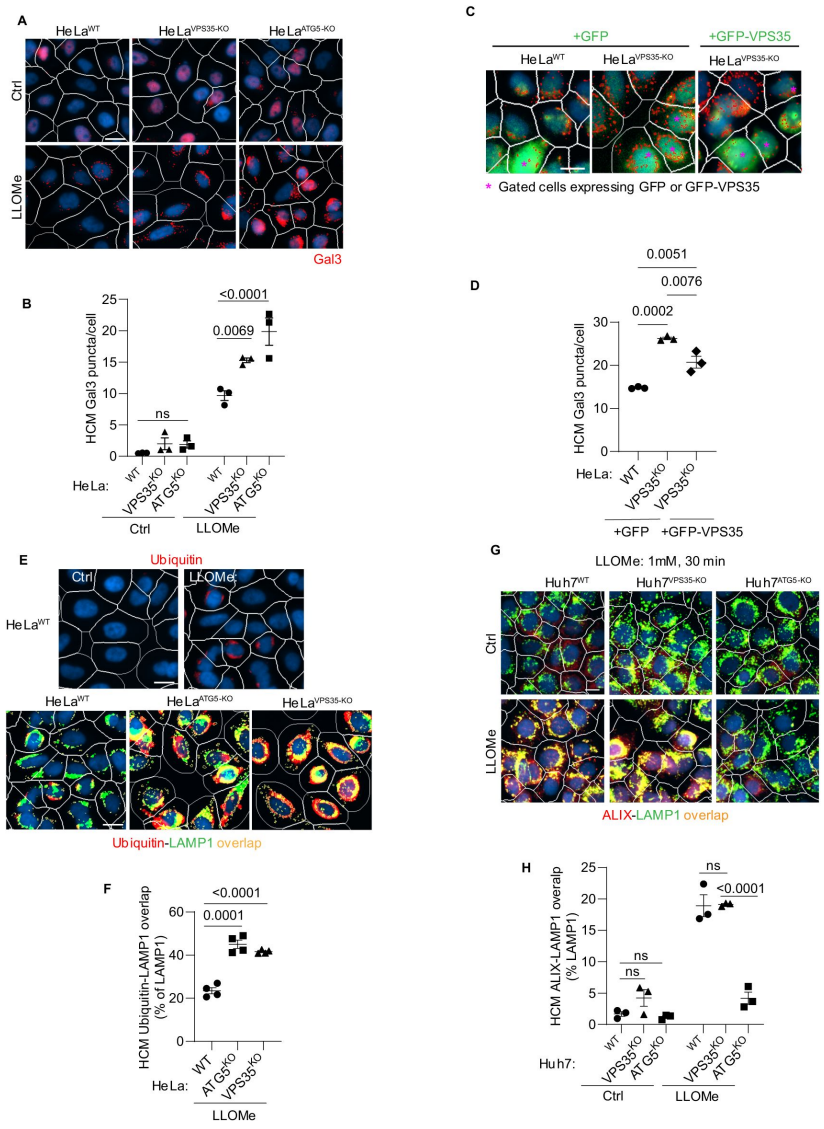
Not all aspects of LyHYP, previously observed in ATG5 knockouts [35], were seen in VPS35 KO cells. In the case of ATG5 inactivation (Huh7$^{ATG5\text{-}KO}$ and HeLa$^{ATG5\text{-}KO}$), the recruitment of ALIX, an ESCRT component involved in early stages of lysosomal repair [80,96,97] is abrogated [35], whereas in VPS35 KO cells (Huh7$^{VPS35\text{-}KO}$ and HeLa$^{VPS35\text{-}KO}$) ALIX was efficiently recruited to lysosomes upon LLOMe treatment (**Figs. 2G**,**H** and **S3E**,**F**).

The elevated Gal3 and ubiquitin markers of increased lysosomal damage in cells with inactivated VPS35 or ATG5 suggest that both may play a related role in lysosomal resilience to membrane damage, which complements the previously observed effects of aberrant VPS35/retromer on lysosomal morphology and proteolytic capacity [98,99]. The dissimilarities between ATG5 and retromer effects on the ALIX recruitment component can be explained by the previously described perturbed function of the ATG conjugation machinery specifically in ATG5 KO cells affirming the unique additional roles of ATG5 [35]. Nevertheless, the striking similarities between ATG5 KO and VPS35 KO effects on heightened Gal3 and ubiquitin responses to lysosomal membrane damage suggest that the two systems (membrane atg8ylation and retromer) closely intersect.

## ATG5 affects retromer function

To avoid the confounding, and possibly indirect, effects of lysosomal damage on endolysosomal sorting and trafficking in cells treated with LLOMe, it was necessary to test the effects of ATG5 KO on retromer function under basal conditions with unperturbed lysosomes. Our co-IP analyses with endogenous proteins (**Fig. 1C-E**) indicated that ATG5 can be found in protein complexes with retromer subunits even without the lysosomal damage. We further confirmed this in co-IPs showing that GFP-VPS35 and YFP-VPS29 were found in complexes with endogenous ATG5 in resting cells, whereas an isotype IgG control did not pulldown retromer components (**Fig. 3A**). Reverse co-IPs with GFP-VPS35 and YFP-VPS29 detected endogenous ATG5 and ATG12-ATG5 complexes in GFP/YFP immunoprecipitates (**Fig. 3B**).

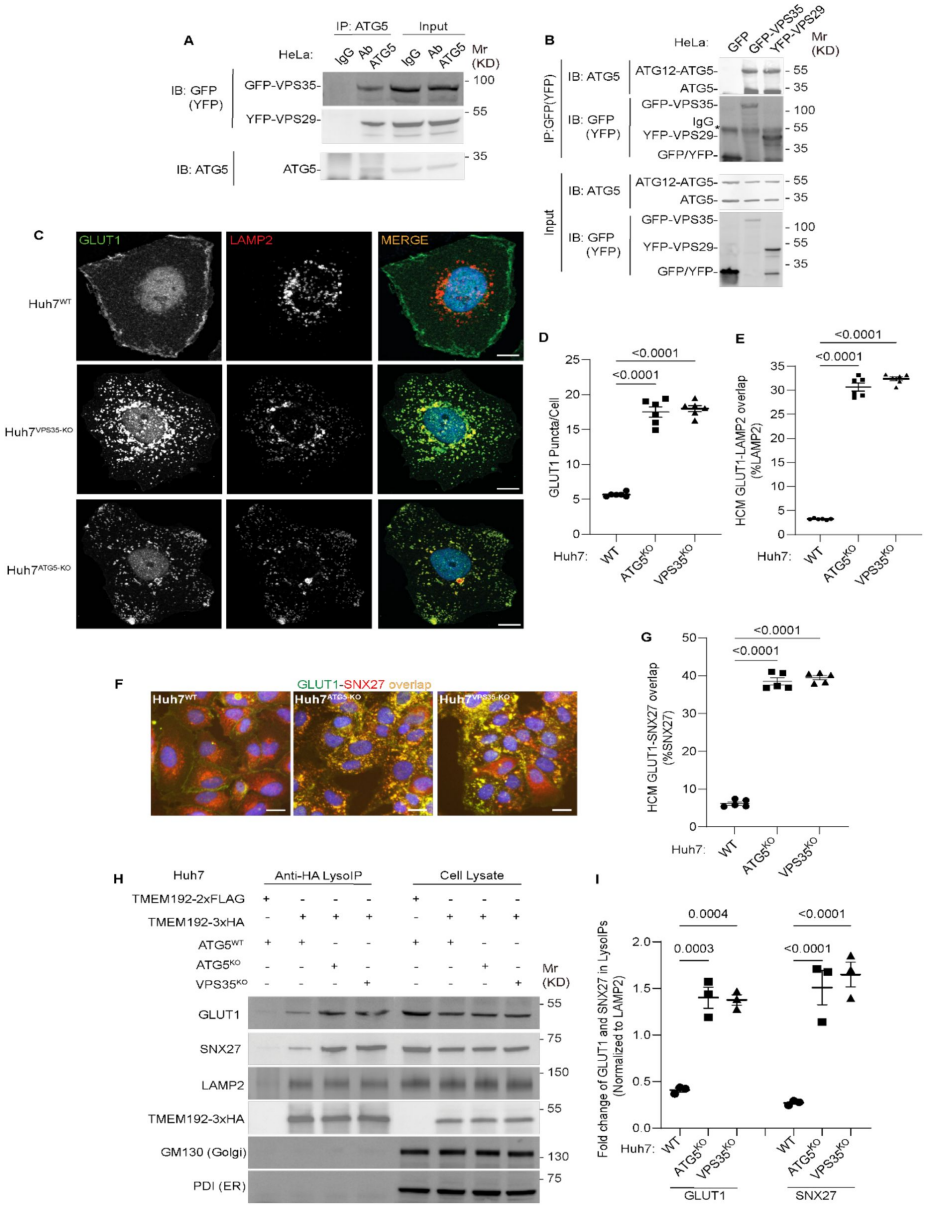
Having established that ATG5 and retromer interact under basal conditions, we tested the effects of ATG5 KO on localization of a well-established cargo for retromer-dependent sorting, GLUT1 [73,100]. We used Huh7 cells, where we generated a panel of membrane atg8ylation and VPS35 CRISPR knockouts (**Fig. S4A**), because they have a well-defined localization of GLUT1 at the plasma membrane or as intracellular profiles (**Fig. 3C**), fully amenable (and comparatively more reliable than in HeLa cells) to unbiased quantification by HCM. ATG5 KO in Huh7 cells resulted in redistribution of GLUT1 from the plasma membrane to intracellular punctate profiles




## Figure 2

### Retromer affects lysosomal sensitivity to damage.

**A,B.** High content microscopy (HCM) imaging and quantification of Gal3 response (puncta/cell of endogenous Gal3 profiles stained for immunofluorescence) in HeLa$^{WT}$, HeLa$^{ATG5}$-KO, and HeLa$^{VPS35\text{-}KO}$ cells subjected to lysosomal damage by Leu-Leu-O-Me ester hydrobromide (LLOMe; 2mM, 30 min). HCM, an unbiased machine-driven image acquisition and data analysis based on presets of >500 valid primary objects/cells per well (representative images shown; white mask, cell; red masks, Gal3 puncta), with a minimum of 5 wells per sample (sampling error), and n≥3, independent biological replicates (experimental error) in separate 96-well plates. Data, means ± SE (n=3), two-way ANOVA with Tukey's multiple comparisons. **C,D**. Complementation analysis of VPS35$^{KO}$ LyHYP (lysosome hypersensitivity) phenotype monitored by HCM quantification of Gal3 puncta in HeLa$^{VPS35\text{-}KO}$ cells transfected with GFP (control) or GFP-VPS35 expressing plasmids. Data, means ± SE (n=3); one-way ANOVA with Tukey's multiple comparisons. **E,F.** Comparative HCM analysis of ubiquitin (immunofluorescence; FK2 antibody) response to lysosomal damage (LLOMe; 2mM, 30 min) in HeLa$^{ATG5}$-KO and HeLa$^{VPS35\text{-}KO}$ cells. Yellow profiles, colocalization of ubiquitin and LAMP1. Top panels, ubiquitin immunostaining alone. Data, means ± SE (n=4), two-way ANOVA with Tukey's multiple comparisons. **G,H.** HCM quantification of ALIX localization to endolysosomal compartments (% of LAMP1 profiles positive for ALIX immunostaining) in Huh7$^{WT}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells following lysosomal damage. Yellow profiles, colocalization of ALIX and LAMP1. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line/condition.




## Figure 3

### ATG5 affects sorting of the retromer cargo GLUT1.

**A.** Co-IP analysis of endogenous ATG5 with GFP-VPS35 or YFP-VPS29 (transient transfection). **B.** Reverse Co-IP analysis GFP-VPS35 or YFP-VPS29 (transient transfection) and endogenous ATG5. Note that different antibodies were used in panels A and B: ab108327 (Abcam) recognizing preferentially the conjugated form of ATG5 and abASA-B0113 (Novateinbio), recognizing both conjugated and unconjugated ATG5. **C.** Confocal images illustrating localization of GLUT1 and LAMP2 in Huh7$^{WT}$, Huh7$^{VPS35\text{-}KO}$, and Huh7$^{ATG5\text{-}KO}$ cells. Scale bar, 10 µm. **D,E.** HCM quantification of GLUT1 (endogenous protein immunostaining) puncta/cell (D) and GLUT1 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) (E) in Huh7$^{WT}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. **F,G.** HCM quantification of GLUT1-SNX27 overlap (% of SNX7 area positive for GLUT1) in Huh7$^{WT}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **H,I.** Immunoblot analysis and quantification of proteins in lysosomes purified/enriched by LysoIP (immunoisolation with TMEM192-3xHA) from Huh7$^{WT}$, Huh7$^{ATG5\text{-}KO}$, and Huh7$^{VPS35\text{-}KO}$ cells. TMEM192-2xFLAG, negative control. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. HCM images in panel F, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.

(**Figs. 3D** and **S4B**). This phenotype was identical to the one observed in VPS35 KO (**Fig. 3D** and **S4B**). The increased intracellular GLUT1 puncta overlapped with the lysosomal marker LAMP2 in both ATG5 KO and VPS35 KO cells (**Figs. 3C**,**E** and **S4C**). A similar albeit morphologically less distinct phenotype was observed in HeLa cells (**Figs. S4D-H**). GLUT1 is sorted by retromer via VPS26's interactor SNX27, with GLUT1 (and other similar cargo) being captured by the PDZ domain of SNX27 [72,73]. In WT cells, SNX27 was found in abundant intracellular profiles (**Fig. 3F**). In both ATG5 KO and VPS35 KO cells, GLUT1 and SNX27 relocalized to the same intracellular compartment (**Fig. 3F**,**G**; **S4I**,**J**). We carried out lysosomal purification using the well-established LysoIP method [80,91,94,101], and quantified levels of GLUT1 and SNX27 in lysosomal preparations that were positive for LAMP2 and devoid of GM130 (Golgi) and PDI (ER) (**Fig. 3H**, **I**). Both GLUT1 and SNX27 were enriched in lysosomal preparations from ATG5 KO and VPS35 KO cells relative to the WT cells (**Fig. 3H**, **I**). These experiments biochemically identify the lysosomes as a compartment to which GLUT1 and SNX27 relocalize in ATG5 KO and VSP35 KO cells. The above findings show that a loss of ATG5 affects retromer-controlled trafficking events.

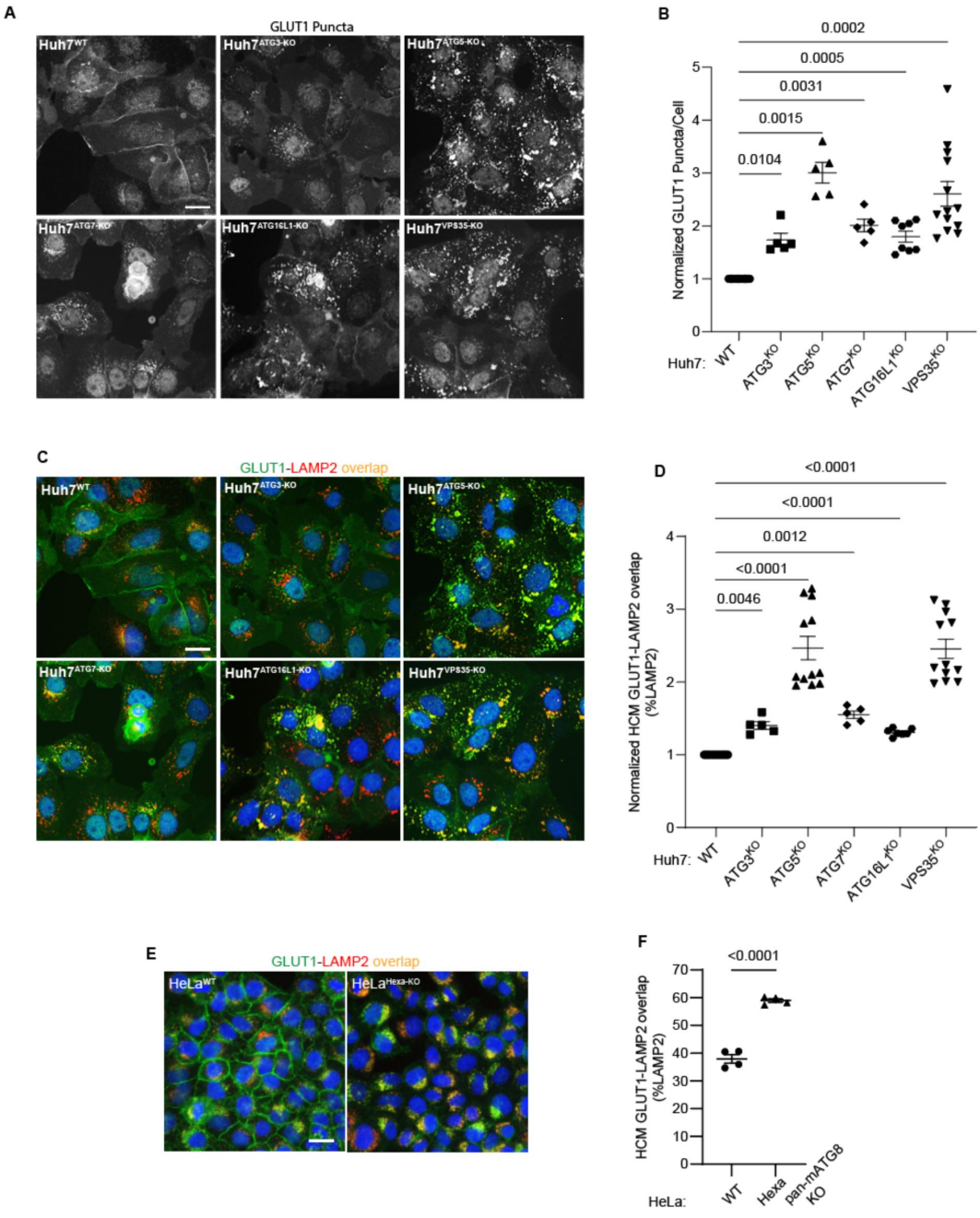
## Membrane atg8ylation apparatus contributes to retromer function in GLUT1 sorting

ATG5 is a part of the membrane atg8ylation machinery [25,42,87], also known under the term 'LC3 lipidation' [102], which functions within the canonical autophagy pathway but is also engaged in a wide array of noncanonical processes [25,38,39,44,103,104]. We tested whether other known components of the atg8ylation apparatus affect retromer. The KOs in Huh7 cells of the components of the prototypical atg8ylation E3 ligase [25,42] included: ATG5 and ATG16L1, as well as the previously characterized Huh7 lines knocked out for E1 and E2 enzymes, ATG7 [35] and ATG3 [37]. These KOs all caused entrapment of GLUT1 in intracellular punctate profiles (**Fig. 4A**, **B**) as well as an increase in GLUT1 colocalization with lysosomes ($GLUT1^+$ $LAMP2^+$ profiles; **Fig. 4C**,**D**). We tested whether retromer component levels were altered in atg8ylation mutants and found no change in total cellular levels of VPS26, VPS29 and VPS35, tested in ATG3, ATG5 and ATG7 KO Huh7 cells (**Fig. S5A**,**B**) as well as in ATG5 KO HeLa cells (**Fig. S5C**,**D**). Thus, the entire ATG16L1-dependent atg8ylation apparatus is required to maintain proper retromer-dependent sorting.

Furthermore, comparison of the previously characterized $HeLa^{Hexa\text{-}KO}$ cells [105], with all principal mATG8s inactivated, with their isogenic parental $HeLa^{WT}$ cells, showed increased GLUT1 on lysosomes in $HeLa^{Hexa\text{-}KO}$ ($GLUT1^+$ $LAMP2^+$ profiles; **Fig. 4E**,**F**). We observed a similar phenotype in separate triple knockouts (TKOs) of LC3 subfamily and GABARAP subfamily of mATG8s [105] compared to $HeLa^{Hexa\text{-}KO}$ (**Fig. S5E**). Thus, at least two or more mATG8s from two different mATG8 subclasses (LC3s and GABARAPs) and the whole membrane atg8ylation machinery were engaged in and required for proper GLUT-1 sorting.

## Canonical autophagy cannot explain effects of membrane atg8ylation on GLUT1 sorting

Membrane atg8ylation and mATGs play roles in diverse processes [25] including canonical autophagy [1]. We tested whether the participation of atg8ylation in canonical autophagy, previously reported to affect retromer-dependent cargo sorting under nutrient-limiting conditions [106,107], is the reason for reduced trafficking of GLUT1 in our experiments. We used KOs in Huh7 cells of ATG13 and FIP200/RB1CC1, two obligatory components of the canonical autophagy initiation machinery [1], to test whether canonical autophagy under the basal conditions used in our study was responsible for the effects on GLUT1 sorting. We found that in the Huh7 cells knocked out for ATG13 [35] or FIP200 [37] there was no increase in intracellular GLUT1 puncta in contrast to the increase observed with the isogenic ATG5 and VPS35 mutants (**Fig. 5A**,**B**). This was mirrored by no increase of $GLUT1^+LAMP2^+$ profiles in ATG13 and FIP200 KO cells whereas




## Figure 4

### Membrane atg8ylation apparatus affects sorting of the retromer cargo GLUT1.

**A,B.** HCM quantification of GLUT1 (endogenous protein immunostaining) puncta/cell in Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, Huh7$^{ATG7}$-KO, Huh7$^{ATG16}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **C,D.** HCM quantification of GLUT1 (endogenous protein immunostaining) colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) in Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, Huh7$^{ATG7}$-KO, Huh7$^{ATG16}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **E,F.** HCM quantification of GLUT1 (endogenous protein immunostaining) colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) in HeLa$^{WT}$, and HeLa$^{Hexa\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.

colocalization between GLUT1 and LAMP2A increased in ATG5 and VPS35 mutants (**Fig. 5C**,**D**). To further confirm that autophagy initiation mutant cells retained the ability to perturb GLUT1 trafficking due to defective atg8ylation, we knocked down ATG5 in FIP200 KO cells (**Fig. S5F**) and found that GLUT1 puncta and GLUT1$^{+}$LAMP2$^{+}$ profiles increased even in the FIP200 KO background with the effects nearing those of VPS35 knockout (**Figs. 5E-G** and **S5G**), with the difference between VPS35 KO and ATG5 KD being attributable to residual ATG5 levels in cells subjected to siRNA knockdowns. Thus, we conclude that atg8ylation but not canonical autophagy affects retromer function under basal conditions and that a functional atg8ylation apparatus is required for proper sorting of the retromer cargo GLUT1.

## ATG5 and membrane atg8ylation machinery affect Rab7 localization

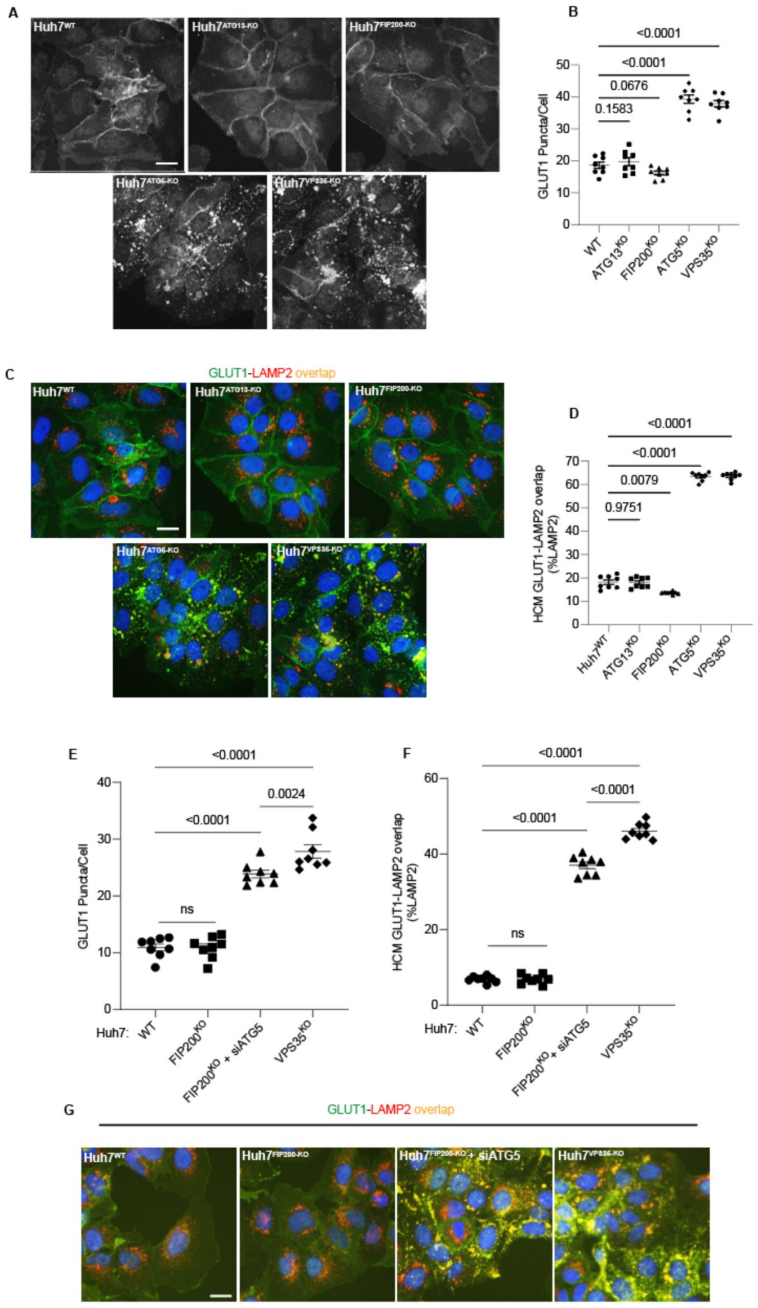
Rab7 is considered to be an interactor of the retromer [108]. Rab7 translocates in cells lacking the retromer component VPS35 to lysosomes [109] including endolysosomal domains with lysosomaly positioned mTORC1 regulatory machinery [110]. We thus tested whether absence of ATG5 and components of the atg8ylation apparatus affected Rab7 localization. ATG5, VPS35 as well as knockouts of all major components of the atg8ylation in Huh7 cells, displayed increased Rab7$^{+}$ cytoplasmic profiles (**Fig. 6A**,**B**) and overlap between Rab7 and lysosomes (LAMP2) (**Figs. 6C** and **S6A**). This was confirmed biochemically using lysosomal purification by LysoIP and immunoblotting for Rab7: there was an increase of Rab7 in lysosomal preparations from ATG5 and VPS35 KO cells (**Fig. 6D**,**E**).

This effect extended to other components of the atg8ylation machinery, as KOs in ATG3, ATG7 and ATG16L1 caused a similar effect (**Figs. 6A-C** and **S6A**). In contrast, KOs in genes specific for canonical autophagy (ATG13 and FIP200) did not alter intracellular distribution of Rab7 (**Figs. 6F-H** and **S6B**). Rab7 distribution was affected by ATG5 knockdown (**Fig. S5F**) in FIP200 KO cells (**Figs. S6C-F**). These experiments mirror the effects of ATG5 and atg8ylation on GLUT1 trafficking, showing that ATG5 and atg8ylation machinery but not canonical autophagy is required for proper localization of Rab7.

## Agonists of endolysosomal atg8ylation process CASM affect GLUT1 sorting

Membrane atg8ylation has multiple presentations that include not only canonical autophagy but also encompass processes such as CASM [103]. We thus asked whether induction of CASM [93] could affect GLUT1 trafficking. Using LLOMe as one of the inducers of CASM [93], we detect increased membrane atg8ylation (LC3 puncta formation) in response to lysosomal damage (**Fig. 7A**,**B**). When cells were treated with LLOMe [93] and another CASM inducer, monensin [29],[111],[112], GLUT1 trafficking to the plasma membrane was negatively affected and instead GLUT1 accumulated intracellularly and trafficked to lysosomes (**Figs. 7C-E** and **S7A**). Thus, lysosomal perturbation and induction of CASM may affect retromer-dependent sorting of GLUT1.

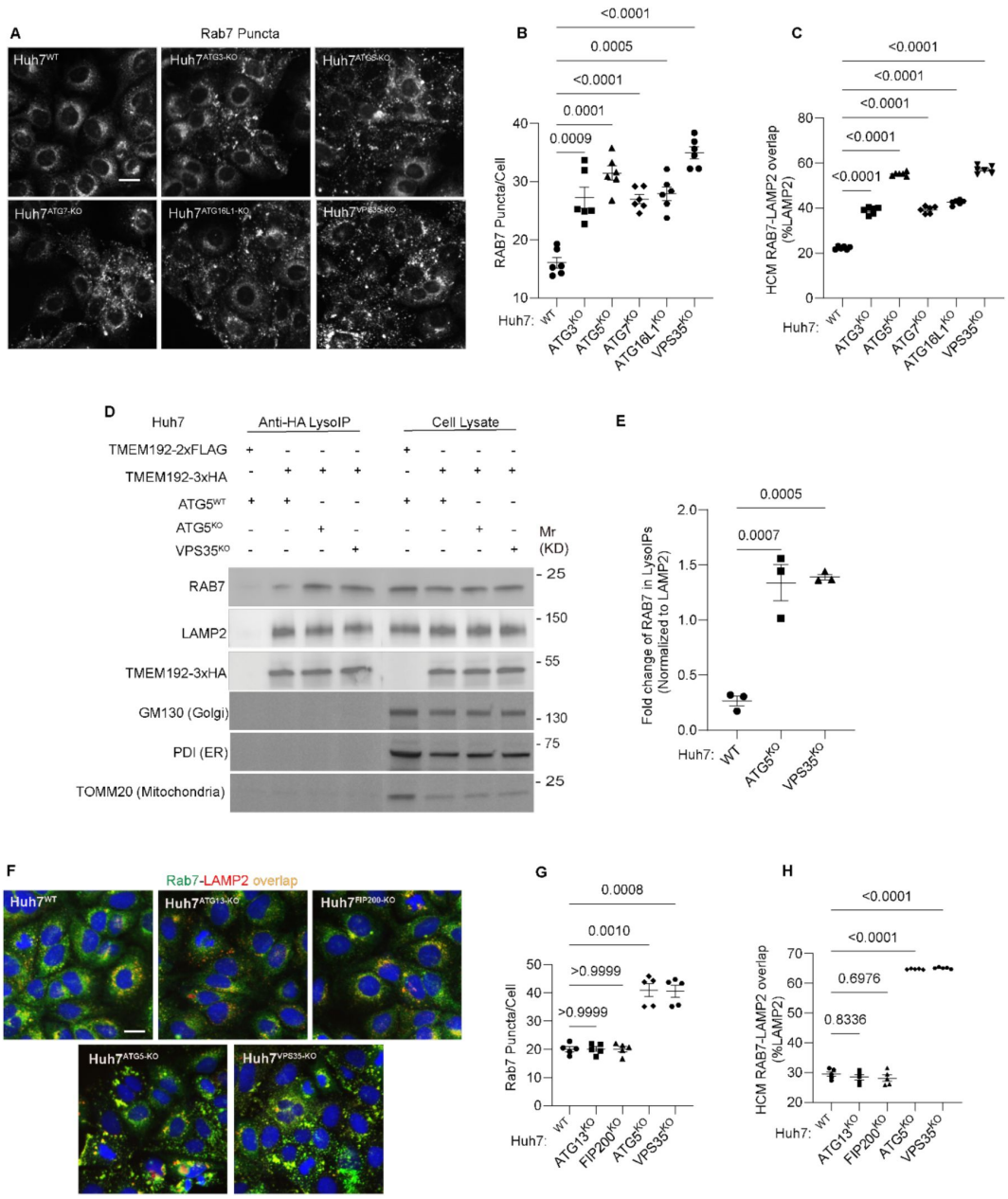
It was previously reported that under glucose starvation conditions, LC3A recruits TBC1D5, a GAP for Rab7 [113–115], away from this small GTPase [106] and a similar phenomenon could be occurring during CASM. However, under CASM-inducing conditions, no changes were detected (**Fig. S7B-D**) in interactions between TBC1D5 and LC3A or in levels of VPS35 in LC3A co-IPs, a proxy for LC3A-TBC1D5-VPS29/retromer association. This suggests that CASM-inducing treatments and additionally bafilomycin A1 do not affect the status of the TBC1D5-Rab7 system. Moreover, expression of constitutively active Rab7$^{Q67L}$ did not promote trafficking of GLUT1 to plasma membrane but rather caused accumulation of intracellular GLUT1 puncta (**Fig. 7F-H**).




## Figure 5

### Canonical autophagy does not affect sorting of the retromer cargo GLUT1.

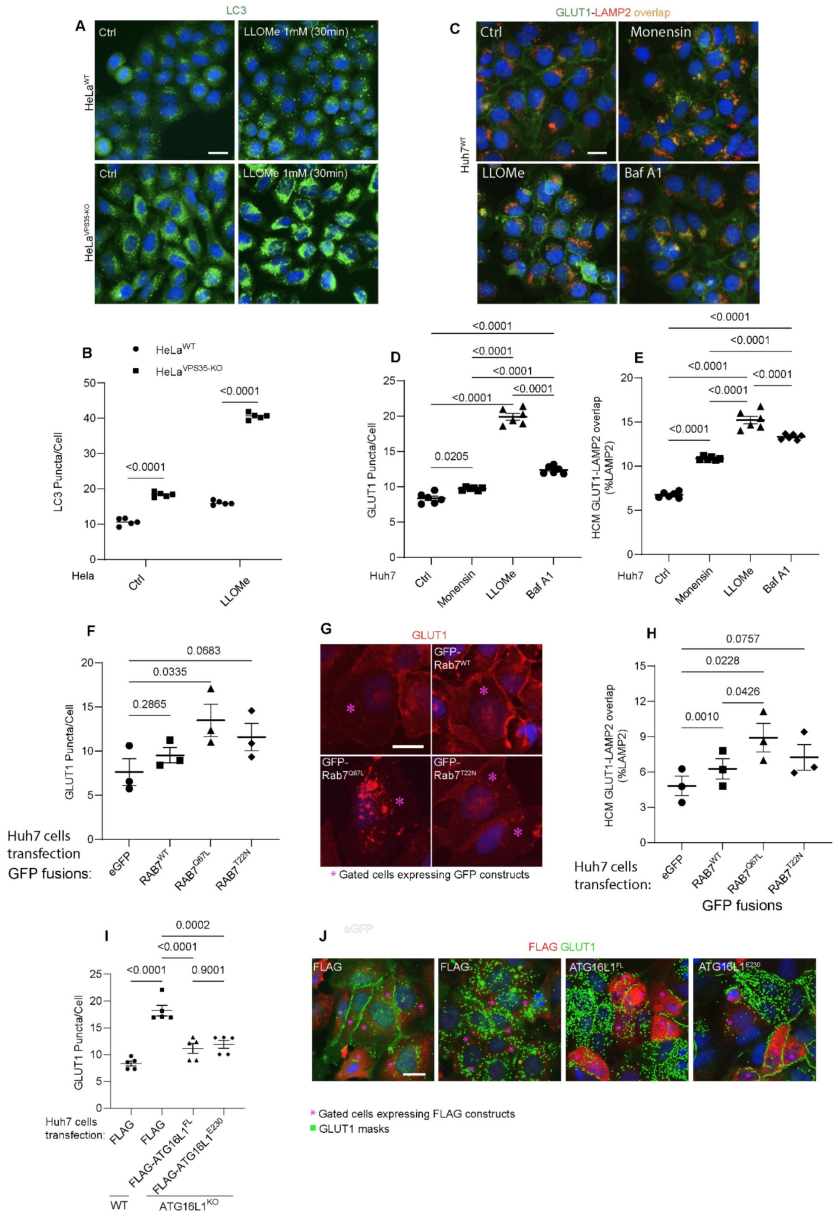
**A,B.** HCM quantification of GLUT1 (immunostaining of endogenous protein) puncta/cell in in Huh7$^{WT}$, Huh7$^{ATG13}$-KO, Huh7$^{FIP200_KO}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **C,D.** HCM quantification of GLUT1 (immunostaining of endogenous protein) colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) in in Huh7$^{WT}$, Huh7$^{ATG13}$-KO, Huh7$^{FIP200_KO}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. **E-G.** HCM quantification of GLUT1 (immunostaining of endogenous protein) puncta/cell (E) and GLUT1 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) (F) in Huh7$^{WT}$, Huh7$^{FIP200_KO}$, Huh7$^{FIP200_KO + siATG5}$, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.




## Figure 6

### Membrane atg8ylation machinery is required for proper RAB7 localization.

**A-C** HCM quantification of Rab7 (endogenous protein immunostaining) puncta/cell (B) and Rab7 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) (C) in Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, Huh7$^{ATG7}$-KO, Huh7$^{ATG16}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. **D-E.** Immunoblot analysis and quantification of proteins in lysosomes purified/enriched by LysoIP (immunoisolation with TMEM192-3xHA) from Huh7$^{WT}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. TMEM192-2xFLAG, negative control. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. **F-H.** HCM quantification of Rab7 (endogenous protein immunostaining) puncta/cell (G) and Rab7 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) (H) in Huh7$^{WT}$, Huh7$^{ATG13}$-KO, Huh7$^{FIP200_KO}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.




## Figure 7

### Lysosomal perturbations cause GLUT1 mis-sorting.

**A,B.** HCM imaging and quantification of LC3 response (puncta/cell of endogenous LC3 immunofluorescent profiles) in HeLa$^{WT}$, and HeLa$^{VPS35\text{-}KO}$ cells in response to lysosomal damage by LLOMe (1mM, 30 and 60 min). Scale bar, 20 µm. Data, means ± SE (n=5), one-way ANOVA with Tukey's multiple comparisons. **C-E.** HCM quantification of GLUT1 response (puncta/cell of endogenous GLUT1, D) and its localization to endolysosomal compartments (% of LAMP1 profiles positive for GLUT1 immunostaining, E) in Huh7$^{WT}$ treated with or without Monensin (100µM), LLOMe (100µM), and Bafilomycin A1 (100nM) for 45 minutes. Scale bar, 20 µm. Data, means ± SE (n=5), one-way ANOVA with Tukey's multiple comparisons. **F-H.** Analysis of GLUT1 puncta/cell (F,G) and its localization to endolysosomal compartments (% of LAMP1 profiles positive for GLUT1 immunostaining, (H) phenotype monitored by HCM quantification in Huh7$^{WT}$ cells transfected with GFP (control) or GFP-Rab7$^{WT}$, GFP-Rab7$^{Q67L}$, and GFP-Rab7$^{T22N}$, expressing plasmids. Scale bar, 20 µm. Data, means ± SE (n=3); one-way ANOVA with Tukey's multiple comparisons. **I-J.** Analysis of GLUT1 puncta/cell (I,J) by HCM quantification in Huh7 WT and ATG16L1-KO cells complemented with Flag (control), Flag-ATG16L1$^{FL}$, or Flag-ATG16L1$^{E230}$, expressing plasmids. Scale bar, 20 µm. Data, means ± SE (n=5); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line/condition.

## Membrane atg8ylation and endolysosomal homeostasis affect GLUT1 sorting

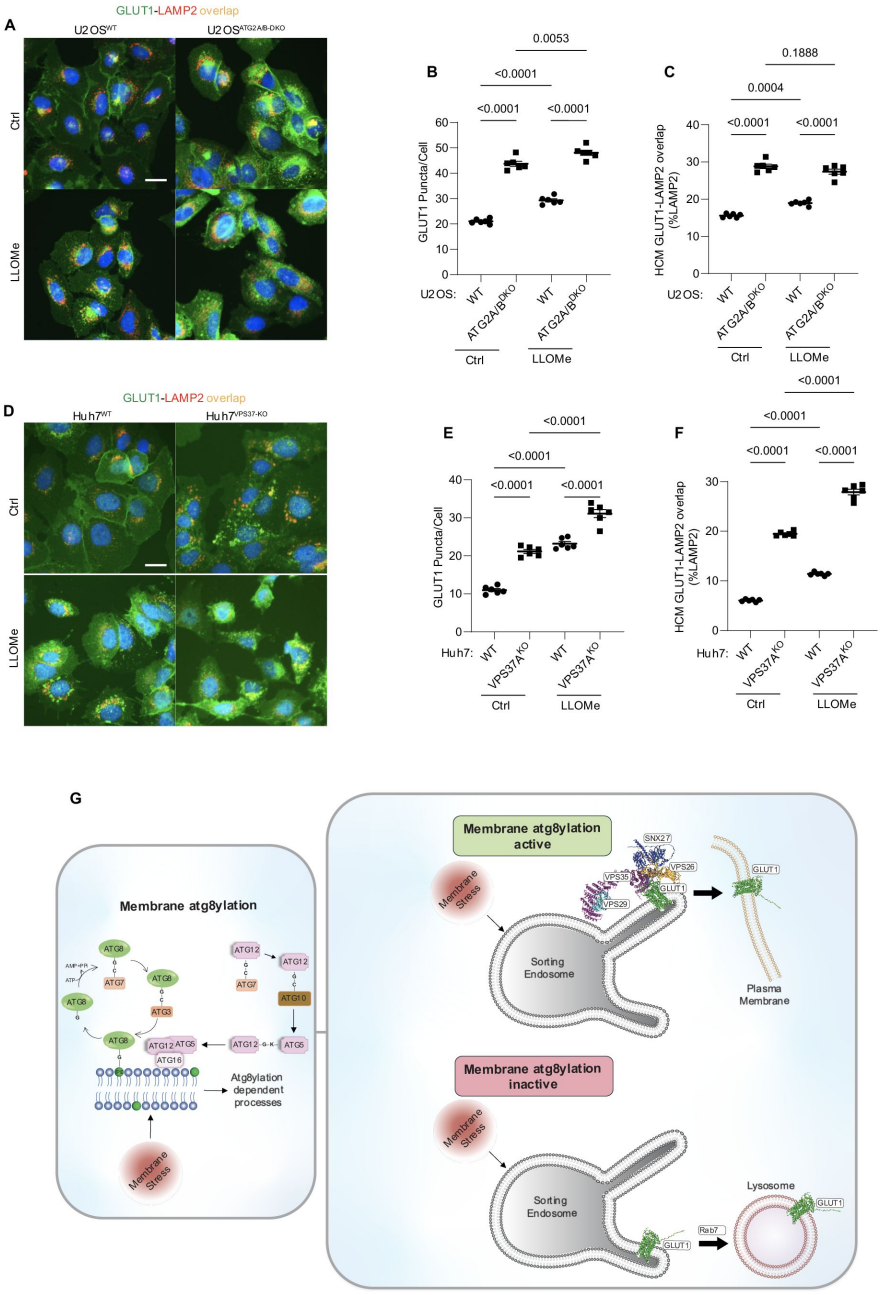
A complication of interpreting CASM as a membrane atg8ylation process responsible for effects on retromer-dependent sorting is that CASM is elicited by lysosomal perturbations using lysosomal pH modifying agents [29,103,111,112], and lysosomal stress could be an independent factor preventing proper sorting of GLUT1. Endolysosomal membrane damage (LLOMe) or perturbations of luminal pH (monensin, bafilomycin A1) negatively affected GLUT1 sorting and caused it to accumulate in lysosomes (**Fig. 7C-E**). Since bafilomycin A1 does not induce CASM [93] but disturbs luminal pH, we conclude that it is the less acidic luminal pH of the endolysosomal organelles, and not CASM, that is responsible for interference with the proper sorting of GLUT1. To additionally test this, we compared ATG16L1 full length ($ATG16L1^{FL}$) and $ATG16L1^{E230}$ [116] for complementation of the GLUT1 sorting defect in ATG16L1 KO cells (**Fig. 7I,J**). $ATG16L1^{E230}$ [116] lacks the key domain to carry out CASM via binding to V-ATPase [29,30,31–33] but retains capacity to carry out atg8ylation. Both $ATG16L1^{FL}$ and $ATG16L1^{E230}$ complemented mis-sorting of GLUT1 (**Fig. 7I,J**). Collectively, these data indicate that it is not specifically an absence of CASM/VAIL but the absence of membrane atg8ylation in general that promotes GLUT1 mis-sorting.

The above experiments suggest the role of acidification and integrity of endolysosomal compartments in GLUT1 sorting. Membrane atg8ylation is important for lysosomal repair and homeostasis with specific downstream mechanisms. These processes include: (i) ATG2A, which transfers lipids during lysosomal repair [85] and is engaged on damaged lysosomes upon membrane atg8ylation by LC3A [93], and (ii) ESCRT-based lysosomal membrane repair [80,96,97,117], with membrane atg8ylation specifically recruiting an ESCRT regulator ALG2 [92] and ESCRT-I component VPS37A [20]. Hence, we tested whether these established systems, involved in maintenance of lysosomal membrane integrity, represented by ATG2 and VPS37, can explain the mechanism underlying the observed effects of membrane atg8ylation on retromer-dependent trafficking. When U2OS ATG2A/ATG2B double KO cells were compared to parental U2OS WT cells, we observed aberrant GLUT1 trafficking, presented as accumulation of cytoplasmic GLUT1 puncta and increased colocalization of GLUT1 with lysosomes (LAMP2A) (**Figs. 8A-C** and **S8A**). A similar effect was observed when the previously characterized VPS37A knockout cells [20] were tested (**Figs. 8D-F** and **S8B**). These effects were detected with or without induced lysosomal damage (**Fig. 8A-F**).

We interpret these data as evidence that endolysosomal homeostasis and functionality, which is at all times maintained by membrane atg8ylation, even under basal conditions, contributes to proper function of retromer-dependent sorting of its cognate cargo such as GLUT1 (**Fig. 8G**).

## Effect of ATG5 knockout on MPR sorting

We tested whether ATG5 affects cation-independent mannose 6-phosphate receptor (CI-MPR). For this, we employed the previously developed methods (**Fig. S9A**) of monitoring retrograde trafficking of CI-MPR from the plasma membrane to the TGN [70,118–121]. In the majority of such studies, CI-MPR antibody is allowed to bind to the extracellular domain of CI-MPR at the plasma membrane and its localization dynamics following endocytosis serves as a proxy for trafficking of CI-MPR. We used ATG5 KOs in HeLa and Huh7 cells and quantified by HCM retrograde trafficking to TGN of antibody-labeled CI-MPR at the cell surface, after being taken up by endocytosis and allowed to undergo intracellular sorting, followed by fixation and staining with TGN46 antibody. There was a minor but statistically significant reduction in CI-MPR overlap with TGN46 in $HeLa^{ATG5\text{-}KO}$ that was comparable to the reduction in HeLa cells when VPS35 was depleted by CRISPR ($HeLa^{VPS35\text{-}KO}$) (**Fig. S9B,C**). Morphologically, endocytosed Ab-CI-MPR appeared dispersed in both $HeLa^{ATG5\text{-}KO}$ and $HeLa^{VPS35\text{-}KO}$ cells relative to $HeLa^{WT}$ cells (**Fig. S9D**). Similar HCM results were obtained with Huh7 cells (WT vs. ATG5KO; **Fig. S9E,F**). We




## Figure 8

### Membrane atg8ylation and endolysosomal homeostasis affect retromer-dependent sorting.

**A-C.** HCM quantification of GLUT1 (immunostaining of endogenous protein) puncta/cell (B) and GLUT1 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area, C) in U2OS$^{WT}$ and U2OS$^{ATG2A/B\text{-}DKO}$ cells treated with or without LLOMe (100μM) for 45 minutes. Scale bar, 20 μm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. **D-F.** HCM quantification of GLUT1 (immunostaining of endogenous protein) puncta/cell (E) and GLUT1 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area, F) in Huh7$^{WT}$ cand Huh7$^{VPS37A\text{-}KO}$ cells treated with or without LLOMe (100μM) for 45 minutes. Scale bar, 20 μm. Data, means ± SE (n =6); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line/condition. **G.** Schematic: Membrane atg8ylation maintains membrane homeostasis under basal or stress conditions and, independently of canonical autophagy, affects retromer function under. A functional atg8ylation apparatus is required for proper sorting of the retromer cargo GLUT1.

interpret these data as evidence of indirect action of ATG5 KO on CI-MPR sorting via membrane homeostasis, although we cannot exclude a direct sorting role via retromer. We favor the former interpretation based on the magnitude of the effect and the controversial nature of retromer engagement in sorting of CI-MPR [57,70,75,98,120].

### ATG5 effects on retromer function exceed those of membrane atg8ylation

Finally, we addressed the strength of effects on GLUT1 trafficking observed with ATG5 KO vs. KOs in other membrane atg8ylation genes, which were significant in all experiments but presented effects lower in magnitude than ATG5 KO (**Figs. 4** and **6**). One possibility was that since ATG3, ATG5, and ATG7 all participate in the same process of membrane atg8ylation, that our KO mutants had unequal gene inactivation levels. This was ruled out by comparing effects of ATG3 KO, ATG5 KO and ATG7 KO cells for their effects on LC3 puncta formation in a well-defined system of starvation-induced autophagy. The results of these analyses indicated that all three KOs had indistinguishable effects on LC3 puncta formation during canonical autophagy induction by starvation (**Fig. S10A**,**B**). Thus, the stronger effects of ATG5 KO on retromer vis-à-vis KOs in other atg8ylation genes reflected additional action of ATG5. In the absence of ATG5, less VPS26 and VPS35 could be pulled down in co-IPs with YFP-VPS29 (**Fig. S10C-F**). We conclude that ATG5, in addition to contributing to the retromer-dependent sorting via membrane atg8ylation and its downstream effector mechanisms which maintain healthy endolysosomal organelles, has additional effects on the retromer.

## Discussion

In this study, we have uncovered a new role of ATG5 and membrane atg8ylation machinery beyond the conventional function in canonical autophagy and the hitherto recognized noncanonical processes. ATG5 interacts with the retromer complex, whereas ATG5 and membrane atg8ylation affect retromer function in sorting of its cognate cargo GLUT1. Inactivation of ATG5 and of other membrane atg8ylation genes but not of the genes involved in canonical autophagy perturbs retromer-dependent trafficking. ATG5 and membrane atg8ylation display dual and combined effects on retromer function. The first one is through endolysosomal membrane maintenance ensuring proper cargo sorting by the retromer complex and its adaptor SNX27. The second one is via an association of ATG5 with the retromer complex. These and other activities of ATG5 underly the unique and often perplexing phenotypic manifestations of ATG5 observed in cells and in murine infection models [35,46,51,53–56], which served as the initial impetus for the present study. More generally, the finding that membrane atg8ylation influences retromer function further expands the range of this process specializing in homeostatic responses to membrane stress, damage and remodeling signals [25,104,122].

Mirroring the role of membrane atg8ylation on retromer function, a loss of VPS35 brings about LyHYP and influences cellular responses to lysosomal damage similarly to the inactivation of ATG5. The parallel between retromer and ATG5 is not perfect, as VPS35 knockout does not divert membrane repair protein ALIX from damaged lysosomes. This can be explained by sequestration of ALIX by the alternative conjugation complex ATG12-ATG3 formed in the absence of ATG5 [35]. The latter phenomenon renders ALIX unavailable for lysosomal repair [35]. VPS35 knockout has no effects on ALIX, and hence the differences between ATG5 and VPS35 in the ALIX component of LyHYP. Nevertheless, inactivation of either ATG5 or VPS35 confers similarly heightened Gal3 [80,81] and ubiquitin [95] responses as hallmarks of elevated lysosomal damage.

A further connectivity between the retromer system and membrane atg8ylation was observed at the level of LC3 puncta formation elicited by noncanonical triggers such as LLOMe treatment, which induces CASM and lysosomal repair. The effects of VPS35 on lysosomal functionality and

morphology have been previously noted [98,99], and have been ascribed to the effects of the retromer complex on the recycling of the sorting receptors for lysosomal hydrolases, such as cation-independent mannose 6-phosphate receptor (CI-MPR) [98]. We have detected significant increase in LC3 puncta in VPS35 KO cells stimulated for autophagy by starvation, consistent with the reported diminished lysosomal degradative capacity when VPS35 is absent, an effect ascribed to aberrant CI-MPR sorting [98,99]. Whether CI-MPR is sorted by retromer or independently by the ESCPE-I complex [70] remains controversial [57,70,75,98,110,120,123,124]. This controversy has been extended to the sorting of cation-dependent mannose 6-phosphate receptor (CD-MPR) [98,125], further confounded by the fact that retriever, which participates in receptor recycling, shares VPS29 subunit with the retromer [61]. Our data showing perturbances in CI-MPR sorting in ATG5 KO and VPS35 KO cells can be best explained as indirect effects via ATG5 and VPS35 roles in endolysosomal homeostasis, which in turn may affect multiple sorting complexes.

In this study, we observed a miss-localization of the small GTPase Rab7 to lysosomes in ATG5 KO cells, paralleling the effects on GLUT1. Retromer and SNX adaptors (SNX3) interact with Rab7 [108]. Rab7 affects retromer-dependent sorting [106,108,109,126]. Depletion of Rab7 reduces endosomal association of retromer [108] whereas retromer status affects Rab7, i.e., in cells with retromer subunits knocked out, Rab7 anomalously accumulates on lysosomes [109]. Consistent with this, we observed that ATG5 KO phenocopied effects of VPS35 KO and caused Rab7 re-localization to lysosomal membranes. Rab7, like other Rabs [114], is controlled by GEFs (Mon1-Cz1), GAPs (ArmusTBC1D2A, TBC1D2B, TBC1D15), and GDI [115]. It is curious that all Rab7 GAPs have LC3 interaction region (LIR) motifs and bind mATG8s: Armus/TBC12DA and TBC1D5 bind LC3A [106,113], GABARAPL1 [113] and LC3C [106]; TBCD12B binds all mATG8s [113,127]; and TBC1D15 binds LC3A [128], LC3B, LC3C, GABARAP, GABARAP L1 and GABARAPL2 [127,128]. Prior elegant studies have shown that genetic ablation of the Rab7 GAP TBCD15 results in hyperactivation of Rab7 in a wrong intracellular locale (lysosome)[109]. In our work, carried out under basal conditions or in cells subjected to lysosomal damage, we observed both translocation of Rab7 to and entrapment of GLUT1 on lysosomes. However, we did not detect any changes in TBC1D5-mATG8s interactions, suggesting that sequestration of this Rab7 GAP by mATG8s, leading to increased plasma membrane localization of GLUT1 under glucose starvation conditions [106], is not at play under basal and endolysosmal damage or luminal alkalinization conditions tested here. We did not test glucose limitation or conditions that induce canonical autophagy or engage more complex metabolic pathways. Furthermore, we found that overexpression of constitutively active Rab7 in cells grown in glucose/nutrient-rich media causes lysosomal retention of GLUT1. This is consistent with the reports by others that perturbances in the retromer-TBC1D5 complex lead to miss-localization of Rab7 to more lysosomal-like subdomains within the endosomal system [110] and that inactivation of Rab7's GAP TBC1D5 leads to entrapment within the endosomal system of the plasma membrane receptors as well as receptors that normally cycle back to the trans-Golgi network [129].

The role of ATG5 in retromer-dependent sorting of GLUT1 appears to have two components, the first one reflects ATG5 being a part of the E3 ligases for membrane atg8ylation and the second one reflects ATG5's being in protein complexes with the core retromer subunits VPS26, VPS29 and VPS35. The latter association potentially explains the stronger effects of ATG5's absence on GLUT1 sorting relative to the inactivation of other membrane atg8ylation genes. Of note, interactions between ATG5 and retromer are independent of the conjugation status of ATG5, as both ATG12—ATG5 conjugates and unconjugated ATG5 were found in protein immunoprecipitates of retromer components. This is consistent with our LC-MS/MS proteomic data indicating a conjugation status-independent increase in the proximity of ATG5 and retromer subunits during lysosomal damage. One consequence of the association between retromer and ATG5, a key component of the membrane atg8ylation apparatus, is that this could spatially direct proper membrane atg8ylation and be in part responsible for the effects of VPS35 KO on lysosomal quality and sensitivity to membrane stress or injury. In our hands, AlphaFold modeling of ATG5 with retromer components did not yield any high-confidence structures, and the best of the low-to-moderate probability

structures when subjected to mutational analysis did not result in disruption of ATG5-retromer association observed by co-IPs (data not shown). Identification of the ATG5 partners that bring it to the vicinity of the retromer is yet to be accomplished and remains one of the limitations of the present study.

We used GLUT1 as a well-defined cargo sorted by the retromer in conjunction with SNX27 [72,73]. SNX27 is a versatile adapter, linking retromer to and controlling endosome-to-plasma membrane recycling of nearly 80 proteins such as signaling receptors, ion channels, amino acid and other nutrient transporters [73]. SNX27 was also missorted in ATG5 deficient cells. This suggests that knockouts in ATG5 and in other membrane atg8ylation genes could have complex pleiotropic effects on cellular functions. Given that ATG5 has a particularly strong impact on retromer-dependent sorting, this could in part explain its unique role in the exquisite susceptibility of Atg5 mutant mice to experimental *M. tuberculosis* infections. Such effects likely contribute to the inflammatory action of neutrophils observed in $Atg5^{fl/fl}$ LysM-Cre mice [35,46,53–56]. Specifically, ATG5 KO-dependent GLUT1 miss-sorting may be one of the contributors to increased *M. tuberculosis* pathogenesis in infection sites. In the context of tuberculosis, diabetes, which includes glucose uptake dysregulation, is associated with increased incidence of active disease and adverse outcomes [130,131]. More generally, glucose uptake and metabolism are highly important for normal physiology and in various disease states including immune responses to infection, cancer, neurodegeneration, cardiovascular health, and metabolic disorders. We postulate that deficiencies or polymorphisms in membrane atg8ylation genes could have contributory roles in health and disease via GLUT1 and additional transporter proteins and signaling receptors that are dependent on retromer for their proper positioning and function in the cell.

One of the limitations of our study is that beyond the effect of membrane atg8ylation on quality of lysosomal membrane and its homeostasis, there could be more direct effects on retromer that still need to be understood. Another limitation of our study is that we have focused on basal conditions or conditions causing lysosomal damage, whereas metabolic stress including glucose excess or limitation with its multitude of metabolic effects have not been addressed. Nevertheless, we find that the membrane atg8ylation and retromer systems are intertwined, and that they affect each other's biological outputs, one being resilience of lysosomes to basal stress or induced damage (**Fig. 8G**) and the other being endosomal-plasma membrane protein sorting, both being of fundamental interest and potential therapeutic value.

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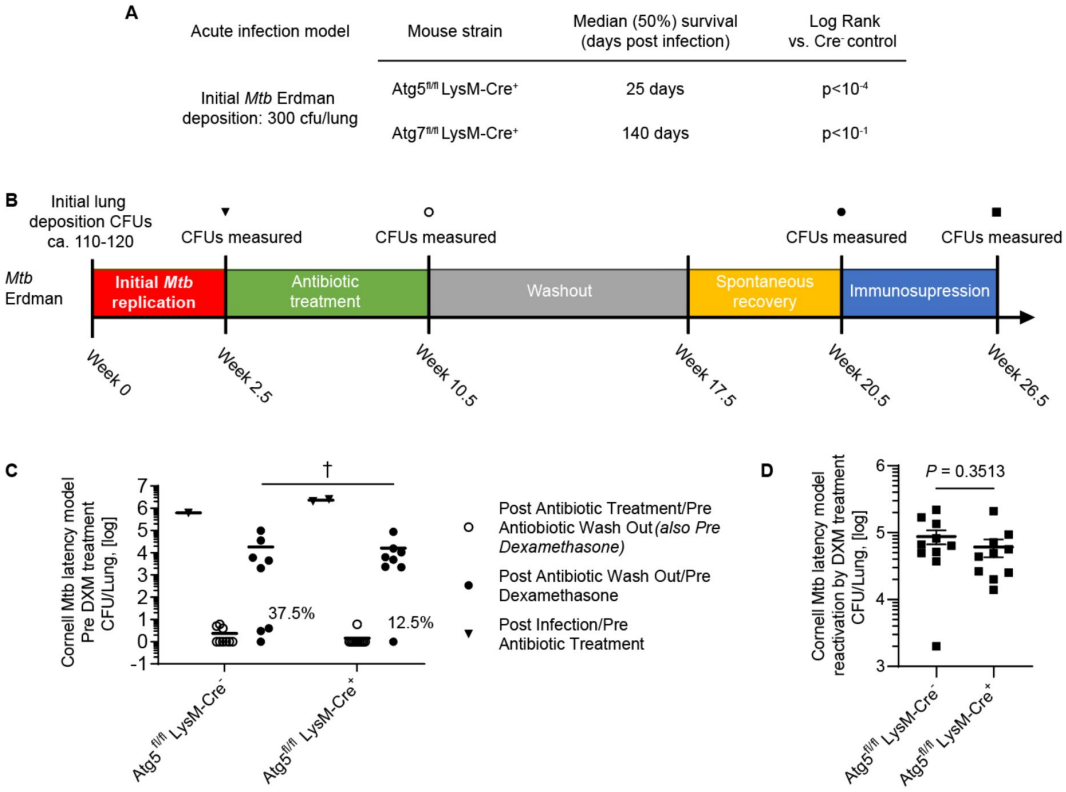
## Author contributions

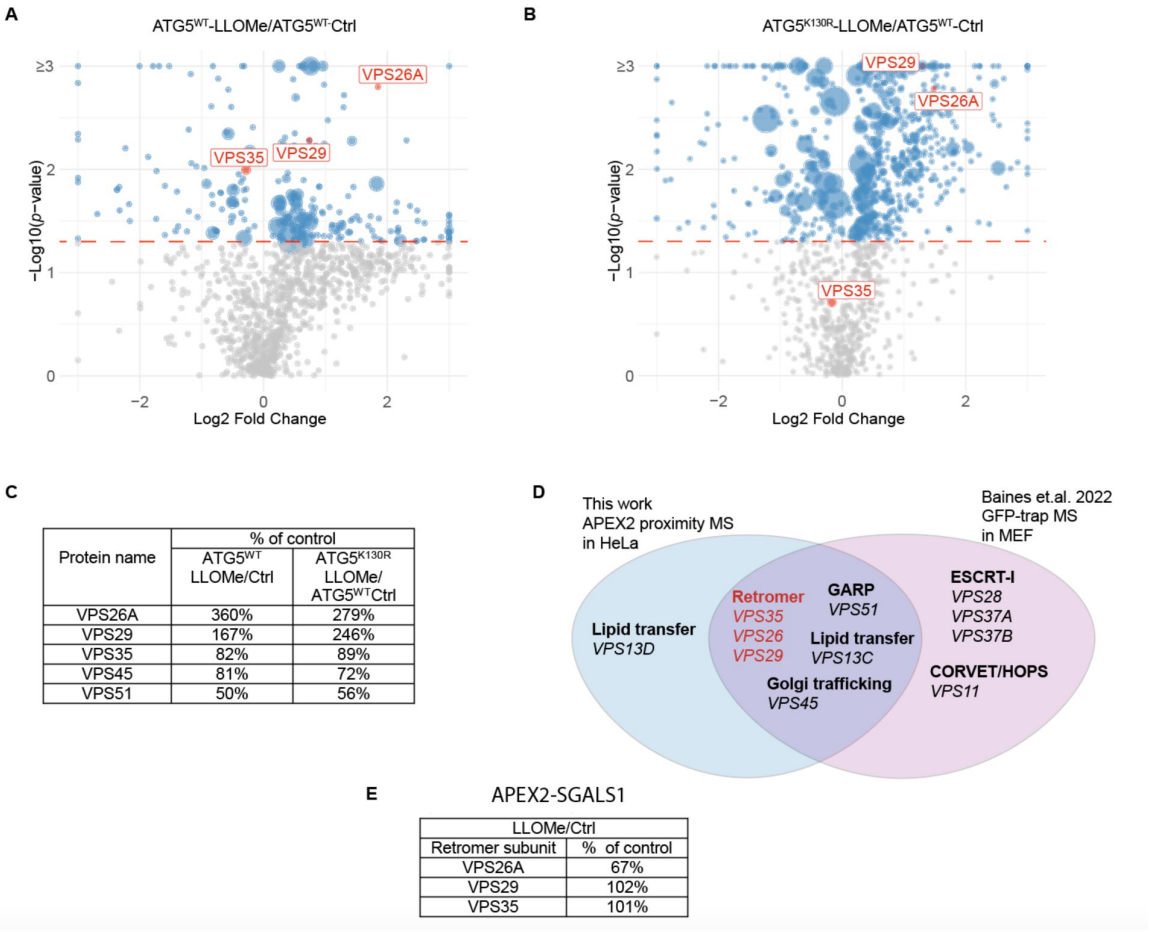
Conceptualization: M.A.P, F.W., E.S.T and V.D.; Formal Analysis: M.A.P., E.S.T., F.W., E.H., Y.H., R.J., B.P., T.L.A.D., and V.D.; Investigation and Validation: M.A.P., E.S.T., F.W., V.D.; Resources: V.D., J.J., M.H.M., Y.H.; Writing – Original Draft: V.D.; Data Curation: M.A.P., F.W., E.S.T., E.H., Y.H., M.S., B.P., and V.D.; Visualization: F.W., E.S.T., E.H., Y.H., V.D.; Supervision: V.D.; Project Administration: V.D.; Funding Acquisition: V.D.




## Supplementary Figure S1.

### Cornell model of *M. tuberculosis* latent infection in mice and effects of Atg5 loss in myeloid lineage on disease reactivation.

**A.** Summary of mice mortality data in acute infection model of *Mtb* infection (aerosol) based on survival curves in ref. 35. **B.** Details and timeline of the Cornell latency model experiments (see narrative in Methods). **C.** Effects of Atg5 loss on spontaneous reactivation of *M. tuberculosis* infection in $Atg5^{fl/fl}$ LysM-$Cre^{+}$ mice (loss of Atg5 in myeloid lineage) vs. $Atg5^{fl/fl}$ LysM-$Cre^{-}$ (control) mice. Mice were infected with an aerosol of *M. tuberculosis* (initial deposition, 100-120 CFUs per lung). After a period of 2.5 weeks, initial bacterial growth was assessed by determining lung CFUs (triangles), mice were treated PO with antibiotics (0.1g/L INH and 0.15g/L RIF in drinking water) for 8 weeks, bacterial clearance after chemotherapy assessed by determining lung CFUs (open circles), and remaining mice subjected to antibiotic washout for 7 weeks plus spontaneous reactivation period with no treatment of 3 weeks at which time the mice were sacrificed and lung CFUs determined by plating (filled circles). Data and statistics for spontaneous reactivation: means, †p≥0.05, *t* test; n=8 mice per group. **D.** Cornell murine model of *M. tuberculosis* latent infection and dexamethasone (DXM) induced reactivation and effects of Atg5 loss in myeloid lineage of $Atg5^{fl/fl}$ LysM-$Cre^{+}$ mice (vs. $Atg5^{fl/fl}$ LysM-$Cre^{-}$ control mice). Mice were infected with *M. tuberculosis* aerosols (initial lung deposition 100-120 CFUs), bacteria allowed to replicate in vivo, mice subjected to antibiotic regimen, followed by antibiotic washout period, after which immunosuppression with DXM was carried out to reactivate infection/bacterial replication (details in Methods). Data, CFU's per mouse lungs (means ± SE, *t* test, n=10 mice per group).



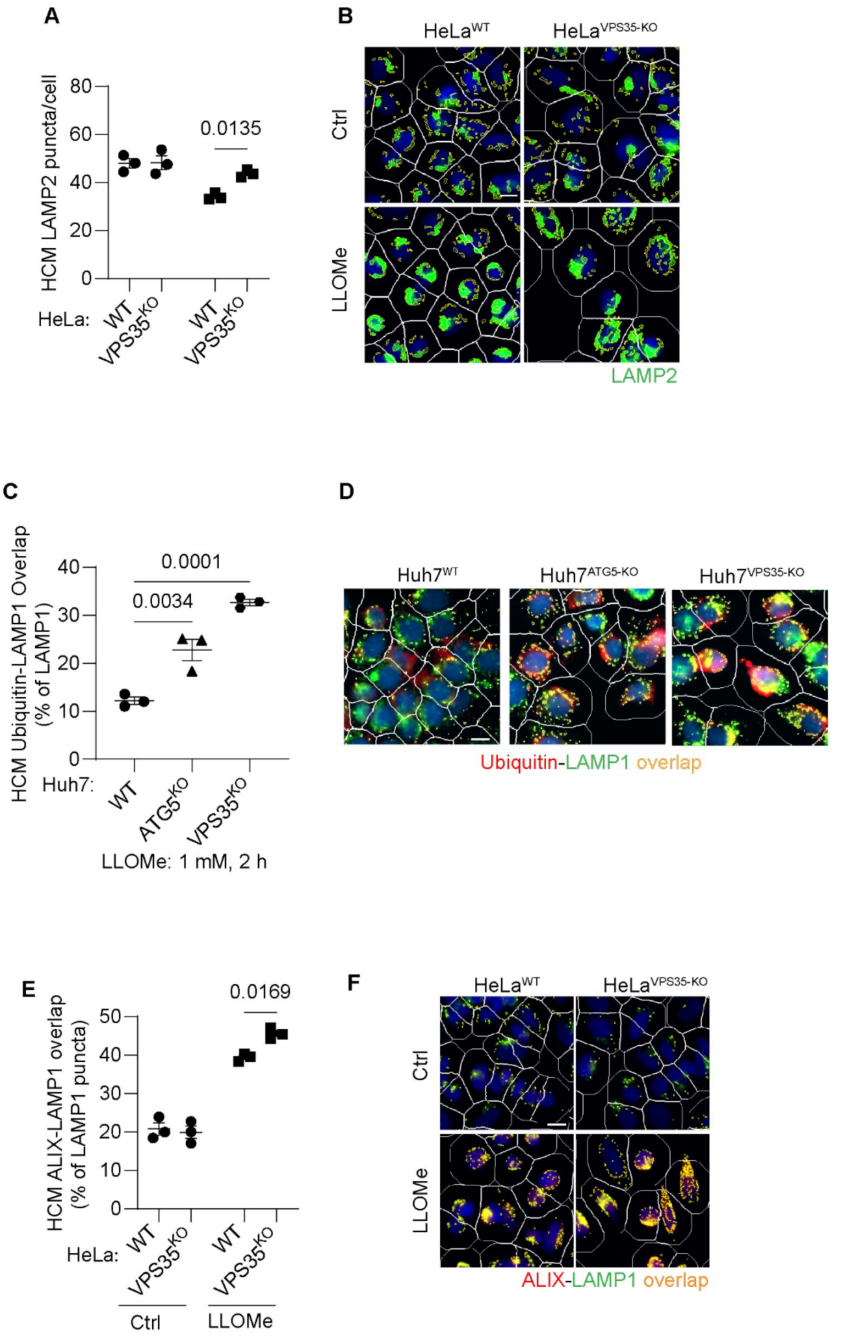

| Protein name | % of control | |
|---|---|---|
| | ATG5WT LLOMe/Ctrl | ATG5K130R LLOMe/ ATG5WTCtrl |
| VPS26A | 360% | 279% |
| VPS29 | 167% | 246% |
| VPS35 | 82% | 89% |
| VPS45 | 81% | 72% |
| VPS51 | 50% | 56% |

| LLOMe/Ctrl | |
|---|---|
| Retromer subunit | % of control |
| VPS26A | 67% |
| VPS29 | 102% |
| VPS35 | 101% |

## Supplementary Figure S2.

### ATG5 interactome analysis.

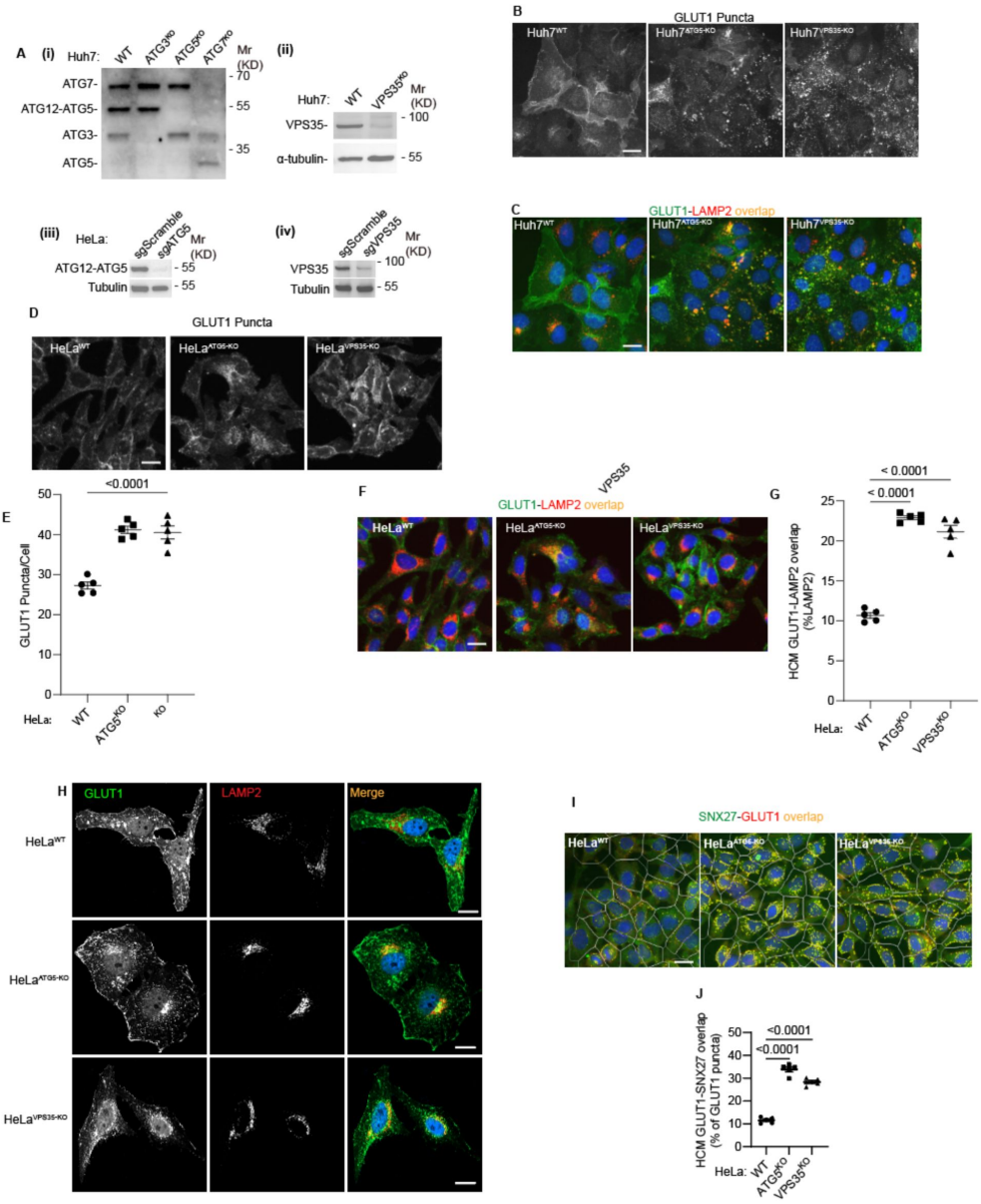
**A**. Volcano plot of proximity biotinylation LC-MS/MS interactome comparing FlpIn-HeLa$^{APEX2\text{-}ATG5\text{-}WT}$ treated with or without 2 mM LLOMe for 30 min. Diameter of symbols reflects relative number of unique peptides identified. **B**. Volcano plot of proximity biotinylation LC-MS/MS interactome comparing FlpIn-HeLa$^{APEX2\text{-}ATG5\text{-}K130R}$ treated with 2 mM LLOMe for 30 min and FlpIn-HeLa$^{APEX2\text{-}ATG5\text{-}WT}$ without LLOMe treatment. **C**. VPS proteins in the MS DIA data. **D.** VPS proteins identified here compared to VPS proteins in Ref. 86. **E.** Table: Control MS data (APEX2-SGALS1) for comparison with data in panel C; note no increase in retromer subunits with LLOMe treatment in the APEX1-SGALS1 dataset.




## Supplementary Figure S3.

### Retromer affects a subset of responses to lysosomal damage.

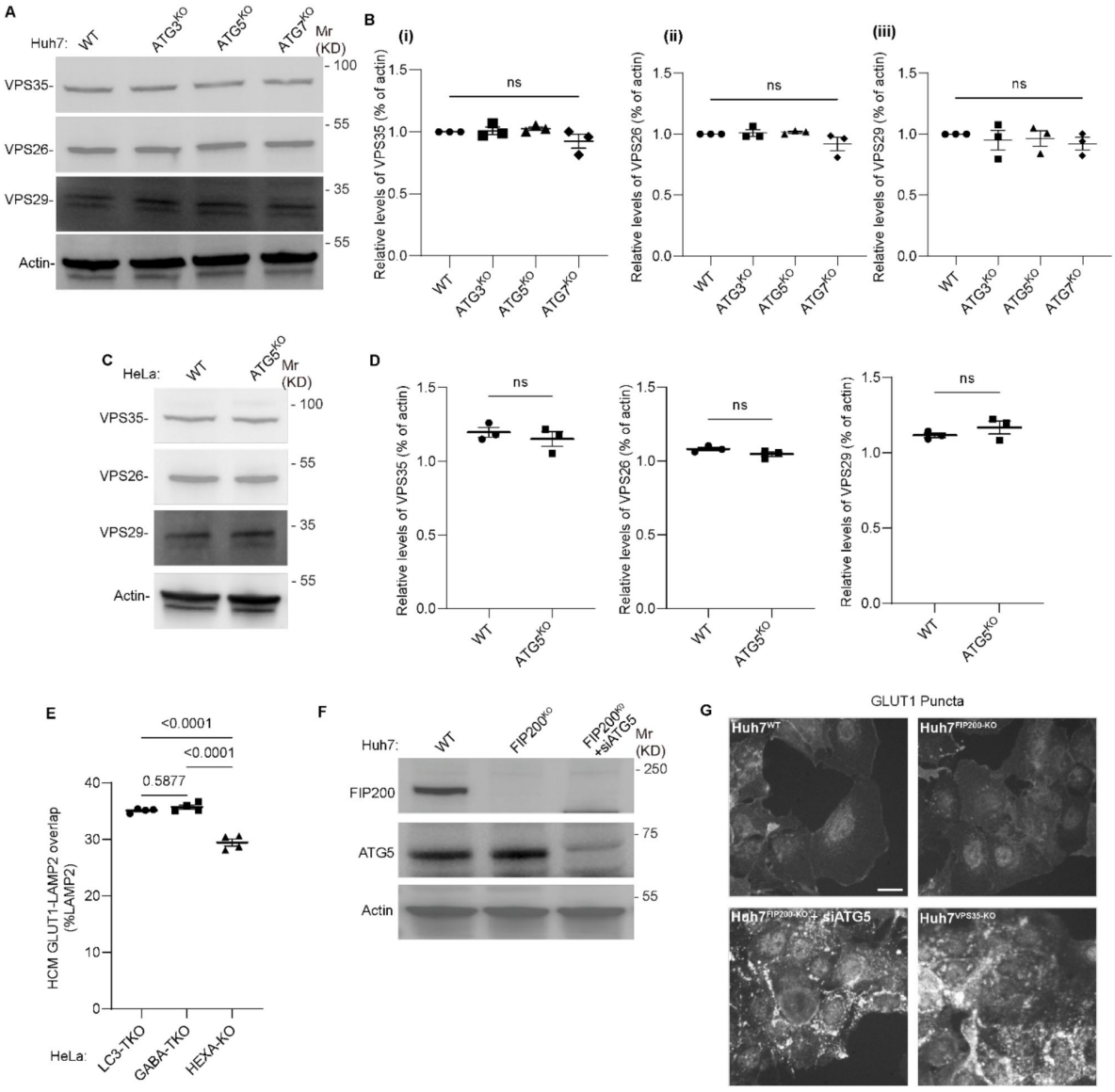
**A,B.** HCM imaging and quantification of LC3 (puncta/cell of immunofluorescently stained endogenous LC3 profiles) in HeLa$^{WT}$, and HeLa$^{VPS35-KO}$ cells in response to lysosomal damage by LLOMe. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. **C,D**. HCM analysis of ubiquitin (immunofluorescence; FK2 antibody) response to lysosomal damage (LLOMe; 1mM, 2 h) in Huh7$^{WT}$, Huh7$^{ATG5-KO}$, and Huh7$^{VPS35-KO}$ cells. Yellow profiles, colocalization of ubiquitin and LAMP1. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. **E,F.** HCM quantification of ALIX localization to endolysosomal compartments (% of LAMP1 profiles positive for ALIX immunostaining) in HeLa$^{WT}$ and HeLa$^{VPS35-KO}$ cells following lysosomal damage. Yellow profiles, colocalization of ALIX and LAMP1. Data, means ± SE (n=3); two-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line/condition.




## Supplementary Figure S4.

### Membrane atg8ylation regulates retromer function.

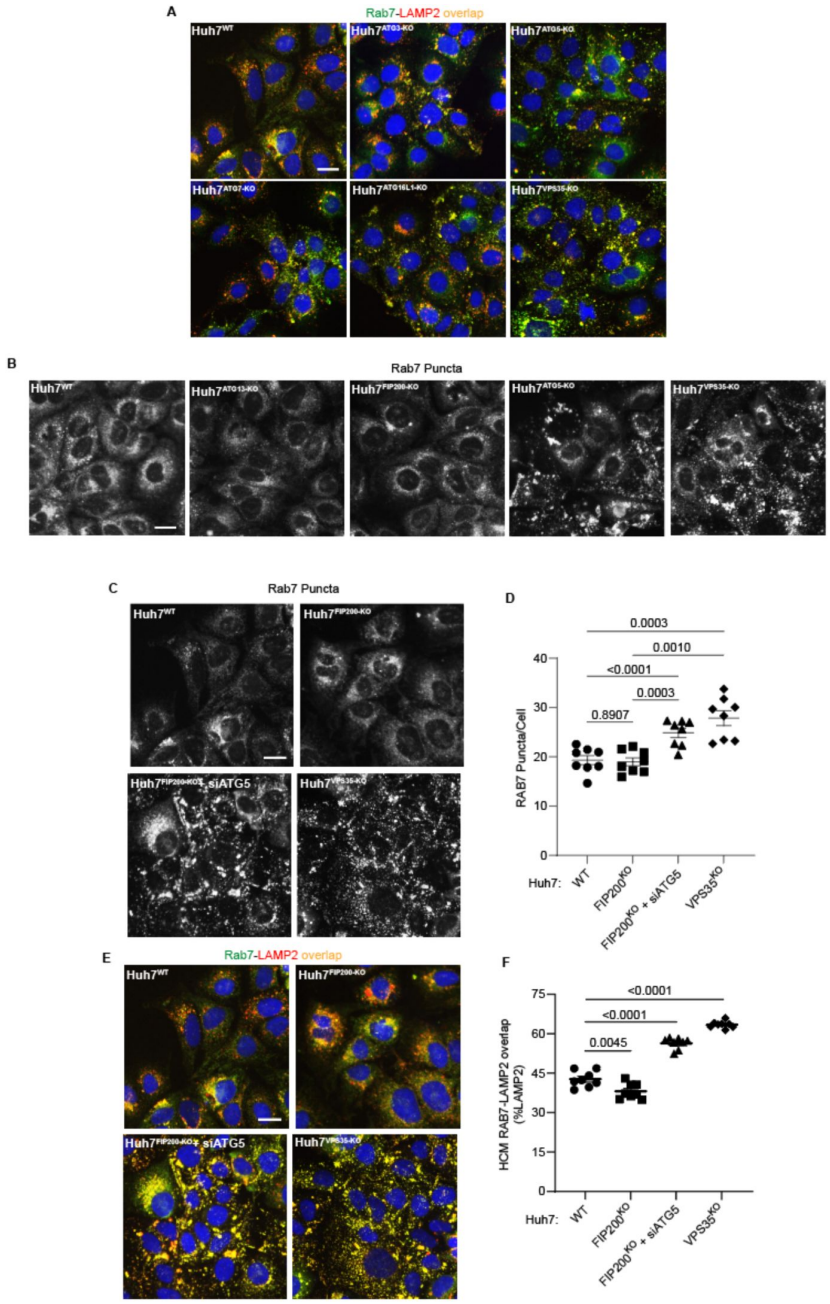
**A.** Immunoblots of CRISPR KOs: (i) ATG3, ATG5, and ATG7 in Huh7 cells; (ii) VPS35 in Huh7 cells; (iii) ATG5 in HeLa cells; and (iv) VPS35 in Hela cells. **B,C.** HCM images (example form a bank of unbiased operator-independent machine collected and processed images containing a minimum of 500 primary objects/cells) of GLUT1 (immunostaining of endogenous protein) puncta/cell (B) and GLUT1 colocalization with LAMP2 in Huh7$^{WT}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35-KO}$ cells (C). Scale bar, 20 µm. **D,E.** HCM quantification of GLUT1 (immunostaining of endogenous protein) puncta/cell in HeLa$^{WT}$, HeLa$^{ATG5}$-KO, and HeLa$^{VPS35-KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =5); one-way ANOVA with Tukey's multiple comparisons. **F,G.** HCM quantification of GLUT1 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) in HeLa$^{WT}$, HeLa$^{ATG5}$-KO, and HeLa$^{VPS35-KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =5); one-way ANOVA with Tukey's multiple comparisons. **H.** Confocal images illustrating localization of GLUT1 and LAMP2 in HeLa$^{WT}$, HeLa$^{ATG5}$-KO, and HeLa$^{VPS35-KO}$ cells. Scale bar, 10 µm. **I,J.** HCM quantification of GLUT1-SNX27 overlap (% of SNX7 area positive for GLUT1) in HeLa$^{WT}$, HeLa$^{ATG5}$-KO, and HeLa$^{VPS35-KO}$. Scale bar, 20 µm. Data, means ± SE (n=4); one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.




## Supplementary Figure S5.

### ATG5 knockout has no effect on protein levels of retromer subunits.

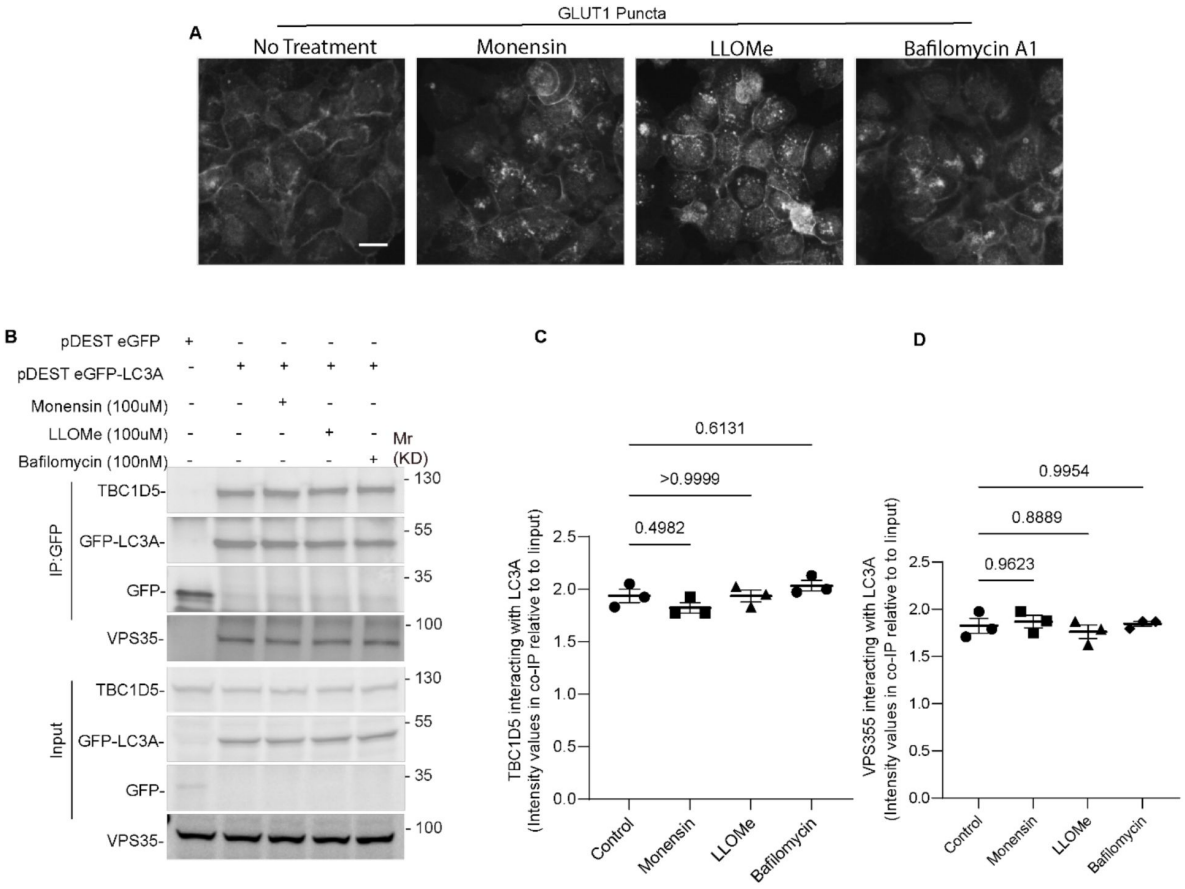
**A,B.** Immunoblot analysis (A) and quantification (B) of retromer complex proteins VPS35 (i), VPS26 (ii), and VPS29 (iii) from Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, and Huh7$^{ATG7}$-KO cells (total cell extract). **C,D.** Immunoblot analysis (C) and quantification (D) of retromer complex proteins VPS35 (i), VPS26 (ii), and VPS29 (iii) from HeLa$^{WT}$, and HeLa$^{ATG5}$-KO cells (total cell extract). **E.** HCM quantification of GLUT1-LAMP2 colocalization in HeLa$^{LC3}$-TKO, HeLa$^{GABA-TKO}$, and HeLa$^{HEXA-KO}$ cells. Data, means ± SE (n =4), one-way ANOVA with Tukey's multiple comparisons; ns (not significant), p > 0.05. **F.** Immunoblot analysis showing the siRNA mediated knockdown of ATG5 in Huh7$^{FIP200_KO}$ cells. **G.** HCM images of GLUT1 puncta/cell in Huh7$^{WT}$, Huh7$^{FIP200_KO}$, Huh7$^{FIP200_KO+ siATG5}$, and Huh7$^{VPS35-KO}$ cells. HCM images in panel F, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line/condition.




## Supplementary Figure S6.

### Loss of membrane atg8ylation but not of canonical autophagy diverts of RAB7 to lysosomal compartments.

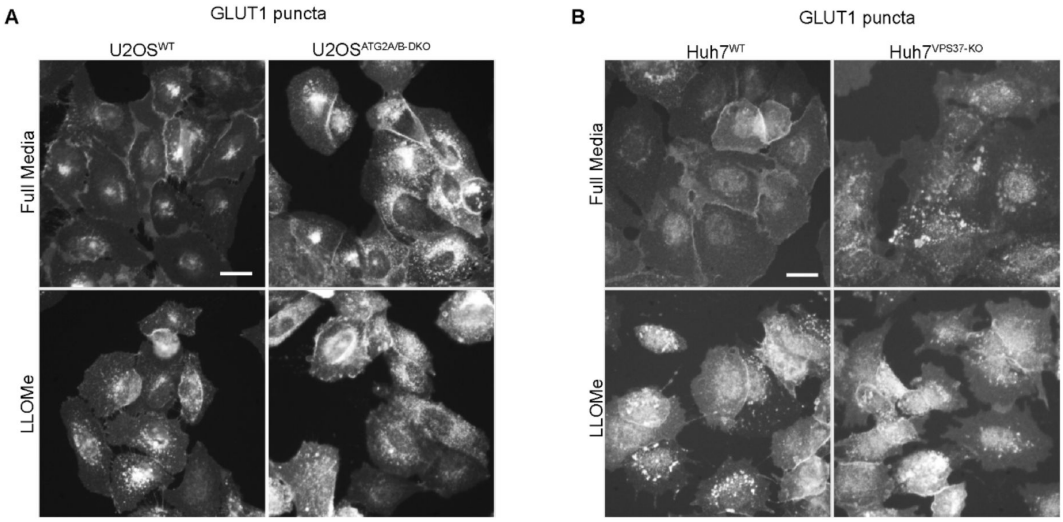
**A.** HCM images of Rab7 (immunostaining of endogenous protein) showing Rab7 colocalization with LAMP2 (% of LAMP2 profiles positive for GLUT1; overlap area) in Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, Huh7$^{ATG7}$-KO, Huh7$^{ATG16}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. **B.** HCM images of Rab7 (puncta/cell of endogenous Rab7 profiles stained for immunofluorescence) in Huh7$^{WT}$, Huh7$^{ATG13}$-KO, Huh7$^{FIP200_KO}$, Huh7$^{ATG5}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. **C,D.** HCM images (C) and quantification of RAB7 puncta (D) in Huh7$^{WT}$, Huh7$^{FIP200_KO}$, Huh7$^{FIP200_KO + siATG5}$, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =8), one-way ANOVA with Tukey's multiple comparisons. **E,F.** HCM images (E) and quantification of RAB7-LAMP2 colocalization (F) in Huh7$^{WT}$, Huh7$^{FIP200_KO}$, Huh7$^{FIP200_KO+ siATG5}$, and Huh7$^{VPS35\text{-}KO}$ cells. Scale bar, 20 µm. Data, means ± SE (n =8), one-way ANOVA with Tukey's multiple comparisons. HCM images in all relevant panels, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per cell line.




## Supplementary Figure S7.

### CASM agonists effects on retromer cargo GLUT1 in the absence of changes in TBC1D5-LC3A association.

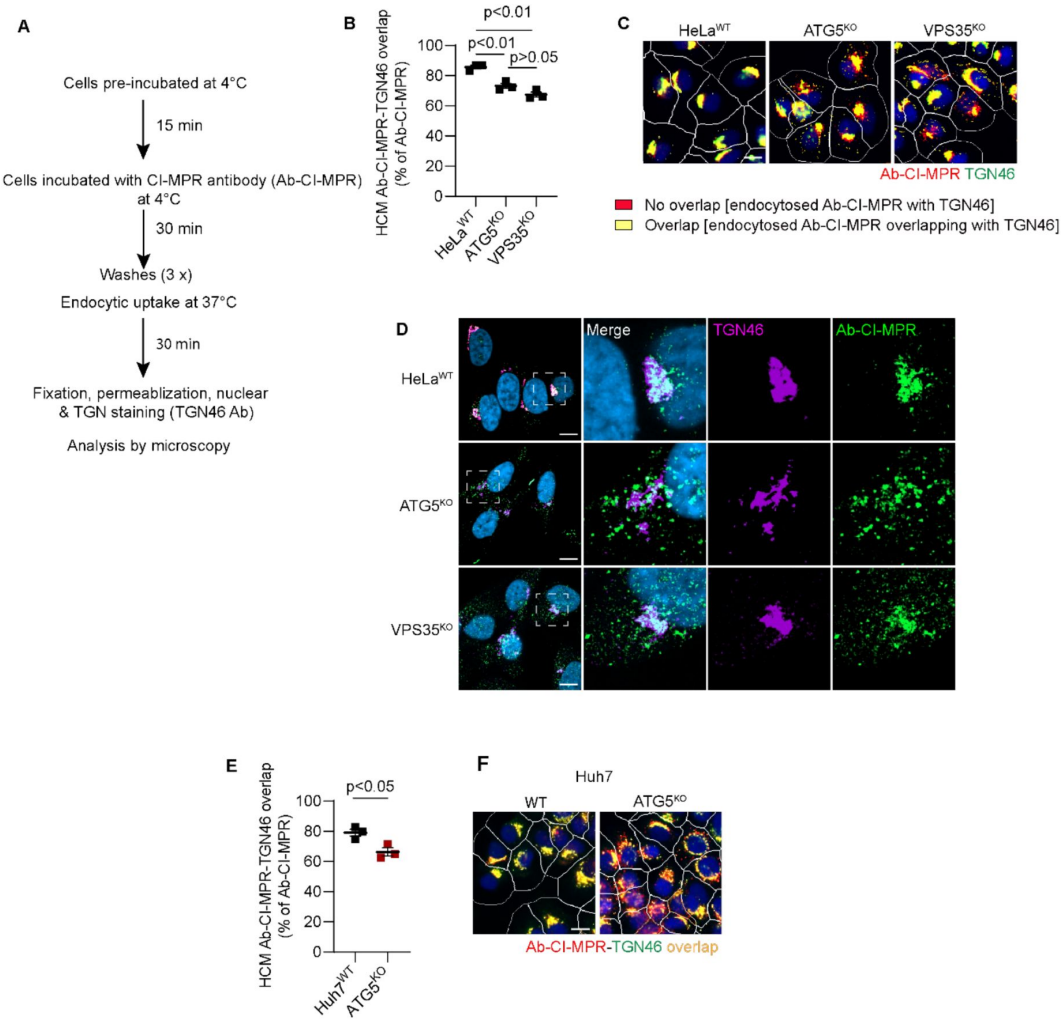
**A.** HCM images of GLUT1 puncta in Huh7$^{WT}$ cells upon treatment with Monensin (100μM), LLOMe (100μM), and Bafilomycin A1 (100nM) for 45 minutes. B-D. Co-IP analysis (B) and quantification of TBC1D5 (C) and VPS35 (D) interaction with GFP-LC3 in Huh7 cells treated with or without Monensin (100μM), LLOMe (100μM), and Bafilomycin A1 (100nM) for 45 minutes. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. HCM images in A, examples from a bank of unbiased operator-independent machine-collected and algorithm-processed fields containing a minimum of 500 primary objects/cells per well (5 wells minimum per 96-well plate; 3 plates minimum), per condition.




## Supplementary Figure S8.

### HCM images of GLUT1.

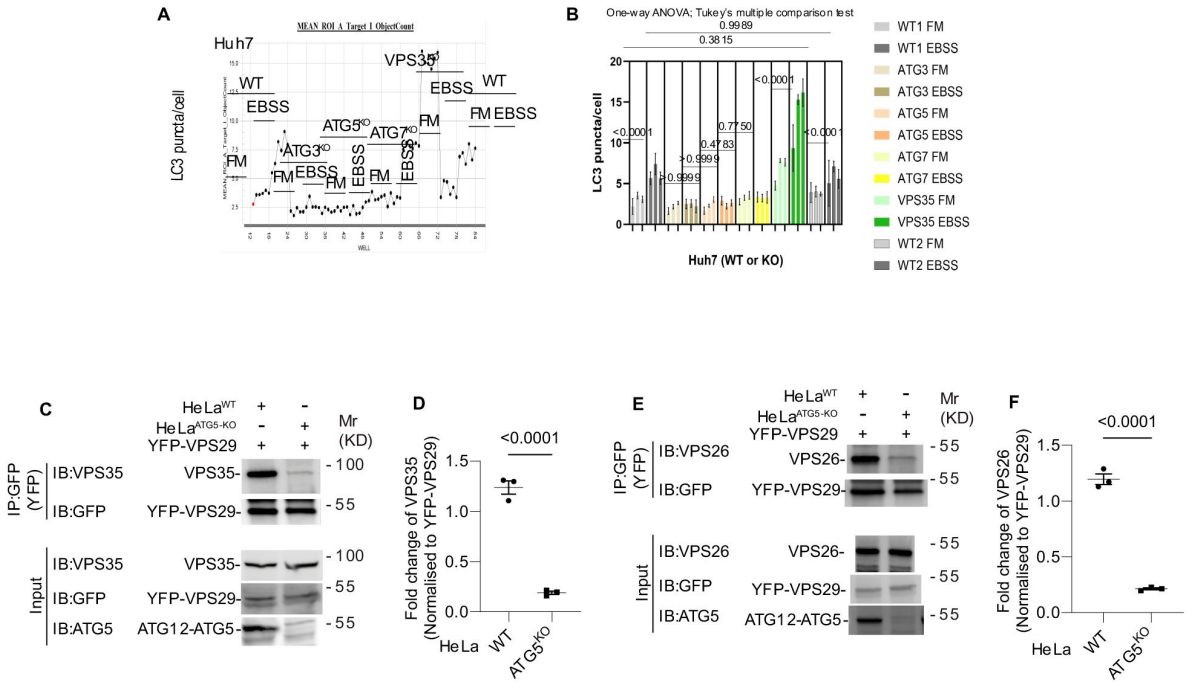
**A.** HCM representative images of GLUT1 puncta; immunostaining of endogenous protein in U2OS$^{WT}$ and U2OS$^{ATG2A/B\text{-}DKO}$ cells upon treatment with LLOMe (100μM) for 45 min. **B.** HCM representative images of GLUT1 puncta in Huh7$^{WT}$ and Huh7$^{VPS37A\text{-}KO}$ cells upon treatment with LLOMe (100μM) for 45 min.




## Supplementary Figure S9.

### Effects of ATG5 knockout on MPR sorting.

**A.** Pulse-chase procedure for monitoring CI-MPR trafficking following CI-MPR antibody uptake. **B,C.** HCM quantification and representative images of Ab-CI-MPR colocalization with TGN46 after 30 min of intracellular sorting in HeLa$^{WT}$, HeLa$^{ATG5\text{-}KO}$, and HeLa$^{VPS35\text{-}KO}$ cells. Yellow masks represent machine-assigned colocalization of Ab-CI-MPR and TGN46. Data, means ± SE (n=3); one-way ANOVA with Tukey's multiple comparisons. **D.** Confocal images of Ab-CI-MPR antibody localization relative to TGN46 in HeLa$^{WT}$, HeLa$^{ATG5\text{-}KO}$, and HeLa$^{VPS35\text{-}KO}$ cells. **E, F.** HCM quantification and representative images of Ab-CI-MPR colocalization with TGN46 after 30 min of intracellular sorting in Huh7$^{WT}$ and Huh7$^{ATG5\text{-}KO}$ cells. Data, means ± SE (n=3); unpaired *t*-test. Each experiment (independent biological repeats; n=3) consists of machine-identified 500 valid primary objects/cells per well, ≥5 wells/sample. All data collection, processing (object, ROI, and target mask assignments) and analyses were computer driven independently of human operators.




## Supplementary Figure S10.

### ATG5 affects retromer assembly.

**A,B.** HCM quantification of LC3 puncta in Huh7$^{WT}$, Huh7$^{ATG3}$-KO, Huh7$^{ATG5}$-KO, Huh7$^{ATG7}$-KO, and Huh7$^{VPS35\text{-}KO}$ cells induced for autophagy in EBSS for 90 minutes. Plot in C, an example of a whole 96-well plate HCM readout; X axis, well positions; Y axis, HCM parameter quantified. Plot in D, nested data from three different plates each one as in C. FM, full medium; EVSS, starvation medium. Data, means ± SE (n=3); one-way ANOVA with Tukey's multiple comparisons. **C,D.** Co-IP analysis (E) and quantification (F) of VPS35 and YFP-VPS29 interaction in HeLa$^{WT}$, and HeLa$^{ATG5}$-KO cells. Data, means ± SE (n=3), one-way ANOVA with Tukey's multiple comparisons. **E,F.** Co-IP analysis (G) and quantification (H) of VPS26 and YFP-VPS29 interaction in HeLa$^{WT}$, and HeLa$^{ATG5}$-KO cells. Data, means ± SE (n=3); unpaired *t*-test.

# Methods

## Cells and cell line models

HEK293T and HeLa cells were from ATCC (American Type Culture Collection). Huh7 cells were from Rocky Mountain Laboratories.

## Mice

$Atg5^{fl/fl}$ LysM-$Cre^{-}$, $Atg5^{fl/fl}$ LysM-$Cre^{+}$ mice were previously described[46,132].

## Housing and husbandry conditions of experimental animals

All mice were housed in AAALAC-accredited Animal Research Facility (ARF) of the University of New Mexico Health Sciences Center (UNM-HSC) and institutionally approved husbandry conditions and approved breeding protocols were followed. *M. tuberculosis*-infected animals were housed in a separate ABSL3 suite within the UNM HSC ARF facility and all staff followed strict ABSL3, BSL3, and animal protocols approved by the UNM HSC Biosafety committee and the Institutional Animal Care and Use Committee. The study was compliant with all relevant ethical guidelines for animal research.

## Antibodies

Antibodies from Abcam were ATG5 (1:2000 for Western blot (WB), ab108327), ATG7 (1:2000 for WB, ab52472), GFP (1:2000 for WB; 1:300 for Immunoprecipitation (IP), ab290), mCherry (1:1000 for WB, ab183628), VPS35 (1:500 for WB; 1:1000 for IF, ab10099), VPS29 (1:500 for Immunofluorescence (IF), ab10160), GLUT1 (1:3000 western blot (WB), 1:1000 for Immunofluorescence (IF), ab115730), CI-MPR(2G11) (2 µg/ml for antibody uptake assay, ab2733), RAB7 (1:2000 western blot (WB), 1:500 for Immunofluorescence (IF), ab137029).

Antibodies from Biolegend were ALIX (1:400 for Immunofluorescence (IF), #634502), Galectin-3 (1:200 for Immunofluorescence (IF), #125402).

Antibodies from Proteintech were VPS26A (1:500 for Western blot (WB), 12804-1-AP), VPS35 (1:500 for Western blot (WB), 1:1000 for Immunofluorescence (IF), 10236-1-AP), TBC1D5 (1:1000 for Western blot (WB), 17078-1-AP).

Antibodies from Sigma Aldrich were ATG3 (1:1000 for Western blot (WB), #A3231), Ubiquitin (FK2, 1:500 for immunofluorescence (IF), 04-263), mouse Anti-FLAG M2 (1:500 for immunofluorescence (IF), #F1804).

Antibodies from Cell Signaling were α-Tubulin (DM1A) (1:3000 for Western blot (WB); #3873), LAMP1 (1:3000 for immunofluorescence (IF), #9091).

Other antibodies used in this study were from the following sources: beta-Actin (1:500 for Western blot (WB), sc-47778) and GAPDH (1:500 for Western blot (WB), sc-47724) from Santa Cruz Biotechnology; LAMP2 (1:1000 for immunofluorescence (IF), H4B4) from DSHB of University of Iowa; ATG5 (1: 500 for Western blot (WB), ASA-B0113) from Novateinbio, SNX27 (1:1000 for Western blot (WB), MA5-27854) from Invitrogen.

Secondary antibodies labeled with Alexa Fluor 488, 568, 647 (1:500 for immunofluorescence (IF)) and IgG-HRP (1:10000 for Western blots (WB)) were from ThermoFisher Scientific. IgG Polyclonal Antibody Goat anti-mouse IRDye 680 (LI-COR, 925-68020), and Goat anti-rabbit IRDye 800 (LI-COR,

926-32211) secondary antibodies were from LI-COR Biosciences.

## Reagents and antibiotics

Bafilomycin A1 (BafA1, InvivoGen; 13D02-MM), Monensin (Sigma, M5273), LLOMe (Sigma, L7393), Lipofectamine 2000 (Thermo Scientific, 11668019); TritonX-100 (OmniPur, 9410-OP), saponin (Sigma, S4521-25G). DMEM (Gibco, #11995040), and Penicillin–Streptomycin (1,000 U/ml; Gibco, #15140122). OptiMEM from Life Technologies, Puromycin dihydrochloride (Sigma, P9620), Hygromycin B (Sigma, H0654)

## Plasmids and transfection

Plasmids used in this study, such as ATG5 were generated by first cloning inserts into pDONR221 (Gateway Technology cloning vector, Thermo Scientfic) using a BP cloning reaction and the expression vectors were made utilizing LR cloning reaction (Gateway, ThermoFisher) in appropriate (pDEST) destination vectors for immunoprecipitation assay. Addgene clones were: eGFP-Rab7 WT (Addgene, #12605), eGFP-Rab7$^{Q67L}$ (Addgene, #28049) and eGFP-Rab7$^{T22N}$ (Addgene, #28049). Additional plasmids were pDEST-3X flag (from Terje Jonansen), Flag-Atg16l1$^{FL}$ [8], and Flag-Atg16l1$^{E230}$ (synthetic clone prepared by Thabata Duque). Plasmid transfections were performed using the Lipofectamine 2000/3000 Transfection Reagent (ThermoFisher Scientific, #11668019).

## siRNAs

The siRNAs were from Horizon Discovery (formerly known as Dharmacon): siGENOME Non-Targeting Control siRNA (Identifier: D-001810-01-05, Target sequence: UGGUUUACAUGUCGACUAA); siGENOME human ATG5 SMARTpool siRNA (Identifier: M-004374-04-0005). ATG5 was a pool of four different siRNAs targeting a single gene with individual siRNA sequences: GGAAUAUCCUGCAGAAGAA; CAUCUGAGCUACCCGGAUA; GACAAGAAGACAUUAGUGA; and CAAUUGGUUUGCUAUUUGA.

## Cornell model of *M. tuberculosis* latent infection

For this study, a total of 68 mice were used (35 Atg5$^{fl/fl}$ LysM-Cre$^{+}$ and 33 Atg5$^{fl/fl}$ LysM-Cre$^{-}$). Early in the course of the study, one mouse died due to malocclusion and thus could not be included in the final analysis.

Inoculum was prepared by diluting *M. tuberculosis* Erdman frozen stock 1:50 in PBS/0.01% Tween for a final amount of ~7.38e6 CFU/mL. Inoculum was serially diluted 5 times at 1:10 each time in PBS/Tween. 50 µL aliquots of the 3$^{rd}$, 4$^{th}$, and 5$^{th}$ dilutions were plated on 7H11 agar plates to determine actual inoculum CFUs (Actual inoculum for this study: 6.45e6 CFU/mL). Remaining inoculum was added to Glas-Col inhalation System and mice were infected via aerosol according to the following machine settings:

Glas-Col Cycle Settings:

*M. tuberculosis* Erdman diluted 1:50, targeting 200 CFU/mouse.

1. Preheat: (15 minutes)
2. Nebulizing: (20 minutes)
3. Cloud decay: (20 minutes)
4. Decontamination: (15 minutes)-UV lights ON
5. Cool down period: (10 minutes)

Immediately following aerosol infection, 3 C57BL/6 mice, infected in parallel with the experimental cohort, were euthanized to determine lung deposition CFUs. 5 x 200uL aliquots of neat, homogenized tissue were grown for 2-3 weeks at 37°C and 5% $CO_2$. Initial deposition: ca. 100 CFUs per lung. This procedure was used for all subsequent CFU determinations in this study.

After a period of 2.5 weeks bacterial growth was assessed by determining lung CFUs (2 $Cre^+$ and 1 $Cre^-$ sacrificed)

Subsequently, mice were treated PO with antibiotics (0.1g/L INH and 0.15g/L RIF) in drinking water for 8 weeks and bacterial clearance by chemotherapy was assessed by determining lung CFUs (11 $Cre^+$ and 10 $Cre^-$ sacrificed).

The remaining mice were subjected to antibiotic washout for 7 weeks plus a spontaneous reactivation period with no treatment for 3 weeks at which time lung CFUs were determined (8 $Cre^+$ and 8 $Cre^-$ sacrificed).

After washout and reactivation periods, dexamethasone was administered by IP injection 5 times/week at 0.08mg/mouse/day to induce immunosuppression. Mice were immunosuppressed in this manner for 6 weeks after which point lung CFUs were determined (14 $Cre^+$ and 13 $Cre^-$ sacrificed).

## Generation of CRISPR mutant cells

Knockout cells ($HeLa^{ATG5}$-KO, $HeLa^{VPS35\text{-}KO}$ $Huh7^{ATG5}$-KO, $Huh7^{ATG3}$-KO, $Huh7^{ATG7}$-KO, $Huh7^{VPS35\text{-}KO}$) were generated by CRISPR/Cas9-mediated knockout system. The lentiviral vector lentiCRISPRv2 carrying both Cas9 enzyme and a gRNA targeting ATG5 (gRNA-puro: AAGAGTAAGTTATTTGACGT), ATG3 (gRNA-Hygro: GTGAAGGCATACCTACCAAC), ATG7 (gRNA-puro: CTTCCGTGACCGTACCATGC), VPS35 (gRNA-hygro: GCTCACCGTGAAGATGGACC), or Scramble (gRNA-hygro: GTGTAGTTCGACCATTCGTG) were transfected into HEK293T cells together with the packaging plasmids psPAX2 and pCMV-VSV-G at the ratio of 5:3:2. Two days after transfection, the supernatant containing lentiviruses was collected. Cells were infected by the lentiviruses with 8-10 μg/mL polybrene. 36 h after infection, the cells were selected with puromycin (1-10 μg/mL) or hygromycin (100-500 μg/ml) for one week in order to select knockout cells. All knockouts were confirmed by western blot. Selection of single clones was performed by dilution in 96-well.

## High content microscopy (HCM)

Cells in 96 well plates were fixed in 4% paraformaldehyde for 5 min. Cells were then permeabilized with 0.1% saponin in 3% Bovine serum albumin (BSA) for 30 min followed by incubation with primary antibodies for 2 h and secondary antibodies for 1h. Hoechst 33342 staining was performed for 3 min. High content microscopy with automated image acquisition and quantification was carried out using a Cellomics HCS scanner and iDEV software (ThermoFisher Scientific). Automated epifluorescence image collection was performed for a minimum of 500 cells per well. Epifluorescence images were machine analyzed using preset scanning parameters and object mask definitions. Hoechst 33342 staining was used for autofocusing and to automatically define cellular outlines based on background staining of the cytoplasm. Primary objects were cells, and regions of interest (ROI) or targets were algorithm-defined by shape/segmentation, maximum/minimum average intensity, total area and total intensity, etc., to automatically identify puncta or other profiles within valid primary objects. Each experiment (independent biological repeats; n≥3) consists of machine-identified 500 valid primary objects/cells per well, ≥5 wells/sample. All data collection, processing (object, ROI, and target mask assignments) and analyses were computer driven independently of human operators. HCM also provides a continuous variable statistic since it does nor rely on parametric reporting cells as positive or negative for a certain marker above or below a puncta number threshold.

## Antibody uptake assay

CI-MPR antibody was either incubated with cell for 1 h in 37°C incubator or pulse-chase with the following procedures: cells are preincubate at 4°C for 15 min, followed by 30 min incubation with antibody at 4°C, 3 times wash with PBS, and 30 min incubation in 37°C incubator. The cells are then fixed and subject to following procedure.

## Co-IP and immunoblotting assays

For co-IP, cells transfected with 8–10 μg of plasmids were lysed in ice-cold NP-40 buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (11697498001; Roche) and 1 mM PMSF (93482; Sigma-Aldrich) for 30 min on ice. Lysates were centrifuged for 10 min at 10,000 g at 4°C. Supernatants were incubated with (2–3 μg) antibodies overnight at 4°C. The immune complexes were captured with Dynabeads (Thermo Fisher Scientific), followed by three times washing with 1 x PBS. Proteins bound to Dynabeads were eluted with 2 x Laemmli sample buffer (Bio-Rad) and subjected to immunoblot analysis.

For immunoblotting, lysates were centrifuged for 10 min at 10,000× g at 4°C. Supernatants were then separated on 4–20% Mini-PROTEAN TGX Precast Protein Gels (Biorad) and transferred to nitrocellulose membranes. Membranes were blocked in 3% BSA for 1 h at RT and incubated overnight at 4°C with primary antibodies diluted in blocking buffer. They were then incubated with an HRP-conjugated secondary antibody, and proteins were detected using ECL and developed using ChemiDoc Imaging System (Biorad). Analysis and quantification of bands were performed using ImageJ software.

## Lysosomal purification by LysoIP

Plasmids for LysoIP were purchased from Addgene. Cells were plated in 10 cm dishes in DMEM and 10% FBS and transfected with pLJC5-TMEM192-3xHA or pLJC5-TMEM192-2XFLAG constructs at 75-85% confluency. After 24 hours of transfection, cells with or without treatment were quickly rinsed twice with PBS and then scraped in 1mL of KPBS (136 mM KCl, 10 mM $KH_2PO_4$, pH7.25 adjusted with KOH) and centrifuged at 3000 rpm for 2 min at $4^0$C. Pelleted cells were resuspended in 1000 μL KPBS and reserved 50 μL for further processing of the whole cell. lysate. The remaining cells were gently homogenized with 20 strokes of a 2 mL homogenizer. The homogenate was then centrifuged at 3000 rpm for 2 min at 4 $^0$C and the supernatant was incubated with 100 μL of KPBS prewashed anti-HA magnetic beads (ThermoFisher) on a gentle rotator shaker for 15 min. Immunoprecipitants were then gently washed three times with KPBS and eluted with 2 x Laemmli sample buffer (Bio-Rad) and subjected to immunoblot analysis.

## Immunofluorescence confocal microscopy and analysis

Cells were plated onto coverslips in 6-well plates. After treatment, cells were fixed in 4% paraformaldehyde for 5 min followed by permeabilization with 0.1% saponin in 3% BSA for 30 min. Cells were then incubated with primary antibodies for 2 h and appropriate secondary antibodies Alexa Fluor 488 or 568 (ThermoFisher Scientific) for 1 h at room temperature. Coverslips were mounted using Prolong Gold Antifade Mountant (ThermoFisher Scientific). Images were acquired using a confocal microscope (META; Carl Zeiss) equipped with a 63 3/1.4 NA oil objective, camera (LSM META; Carl Zeiss), and AIM software (Carl Zeiss).

## Sample preparation for LC-MS/MS

The previously described HeLaFp-In-APEX2-ATG5-WT and HeLaFlp-InAPEX2-ATG5-K130R cells [35] were incubated in complete medium supplemented with 500 μM biotin– phenol (AdipoGen) with or without 2 mM LLOMe for 30 min. A 1-min pulse with 1 mM $H_2O_2$ at room temperature was stopped with quenching buffer (10 mM sodium ascorbate, 10 mM sodium azide and 5 mM Trolox

in PBS). All samples were washed twice with quenching buffer, and twice with PBS for 1 min. For LC–MS/MS analysis, cell pellets were lysed in 500 µl ice-cold lysis buffer (6 M urea, 0.3 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM sodium ascorbate, 10 mM sodium azide, 5 mM Trolox, 1% glycerol and 25 mm Tris-HCl, pH 7.5) for 30 min by gentle pipetting. Lysates were clarified by centrifugation and protein concentrations were determined using Pierce 660 nm protein assay reagent.

Streptavidin-coated magnetic beads (Pierce) were washed with lysis buffer. A total of 1 mg of each sample was mixed with 100 µl of streptavidin beads. The suspensions were gently rotated at 4 °C overnight to bind biotinylated proteins. The flow-through after enrichment was removed and the beads were washed in sequence with 1 ml IP buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) twice; 1 ml 1 M KCl; 1 ml of 50 mM Na2CO3; 1 ml 2 M urea in 20 mM Tris HCl (pH 8.0); and 1 ml IP buffer. Biotinylated proteins were eluted and processed for mass spectrometry. Protein samples on magnetic beads were washed four times with 200 µl of 50 mM Triethyl ammonium bicarbonate (TEAB) with a twenty-minute shake time at 4 °C in between each wash. Roughly 2.5 µg of trypsin was added to the bead and TEAB mixture and the samples were digested over night at 800 rpm shake speed. After overnight digestion the supernatant was removed, and the beads were washed once with enough 50 mM ammonium bicarbonate to cover. After 20 min at a gentle shake the wash is removed and combined with the initial supernatant. The peptide extracts are reduced in volume by vacuum centrifugation and a small portion of the extract is used for fluorometric peptide quantification (Thermo scientific Pierce). One microgram of sample based on the fluorometric peptide assay was loaded for each LC-MS analysis.

## Liquid chromatography-tandem mass spectrometry

Peptides were desalted and trapped on a Thermo PepMap trap and separated on an Easy-spray 100 µm × 25 cm C18 column using a Dionex Ultimate 3,000 nUPLC at 200 nL/min. Solvent A = 0.1% formic acid, Solvent B = 100% Acetonitrile 0.1% formic acid. Gradient conditions = 2% B to 50% B over 60 min, followed by a 50%–99% B in 6 min and then held for 3 min than 99% B to 2% B in 2 min and total run time of 90 min using Thermo Scientific Fusion Lumos mass spectrometer. The samples were run in DIA mode; mass spectra were acquired using a collision energy of 35, resolution of 30 K, maximum inject time of 54 *ms* and an AGC target of 50K, using staggered isolation windows of 12 Da in the *m/z* range 400–1,000 *m/z*.

## DIA Quantification and Analysis

DIA data was analyzed using Spectronaut 14.10 (Biognosys Schlieren, Switzerland) using the directDIA workflow with the default settings. Briefly, protein sequences were downloaded from Uniprot (Human Proteome UP000005640), ATG5 from Uniprot and common laboratory contaminant sequences from *https://thegpm.org/crap/*. Trypsin/P specific was set for the enzyme allowing two missed cleavages. Fixed Modifications were set for Carbamidomethyl, and variable modification were set to Acetyl (Protein N-term) and Oxidation. For DIA search identification, PSM and Protein Group FDR was set at 1%. A minimum of 2 peptides per protein group were required for quantification. Proteins known to be endogenously biotinylated were excluded from consideration.

## Quantification and statistical analysis

Data in this study are presented as means ± SEM (n ≥ 3). Data were analyzed with either analysis of variance (ANOVA) with Tukey's HSD post-hoc test, or a two-tailed Student's t-test. For HCM, n ≥ 3 (independent experiments carried out on different 96-well plates). For each well in 96 well plates, ≥500 valid primary objects/cells were imaged and analyzed, with ≥ 5 wells per plate per sample. Statistical significance: p ≥ 0.05 (not significant); <0.05 (significant). For HCM, sample size was based on a historic power analysis (published studies), with large effect size (differences and

variability derived from published work), power 80%, ≥ 20%, and α 5%, assuming normal distribution and favoring type II false negative errors over type I false positive errors. Band intensity in immunoblots, n=3 (biological replicates); no power analysis was performed.

## Data availability

Raw MS DIA/DDA data have been deposited at the MassIVE proteomics repository MassIVE (MSV000090348) and Proteome Exchange (PXD036850).

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## Editors

Reviewing Editor
**Li Yu**
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Senior Editor
**David Ron**
University of Cambridge, Cambridge, United Kingdom

**Reviewer #1 (Public review):**

Summary:

In this study, Masroor Ahmad Paddar and his/her colleagues explore the noncanonical roles of ATG5 and membrane atg8ylation in regulating retromer assembly and function. They begin by examining the interactomes of ATG5 and expand the scope of these effects to include homeostatic responses to membrane stress and damage.

Strengths:

This study provides novel insights into the noncanonical function of ATG8ylation in endosomal cargo sorting process.

Weaknesses:

The direct mechanism by which ATG8ylation regulates the retromer remains unsolved.

Comments on revisions:

After revision, though the major weakness remains unsolved, other questions have been addressed experimentally or further interpreted.

https://doi.org/10.7554/eLife.100928.2.sa3

**Reviewer #2 (Public review):**

Summary:

Padder et al. demonstrates that ATG5 mediates lysosomal repair via the recruitment of the retromer components during LLOMe-induced lysosomal damage and that mAtg8-ylation contributes to retromer-dependent cargo sorting of GLUT1. Although previous studies have suggested that during glucose withdrawal, classical autophagy contributes to retromer-dependent GLUT1 surface trafficking via interactions between LC3A and TBC1D5, the experiments here demonstrate that during basal conditions or lysosomal damage, ATGs that are not involved in mATG8ylation, such as FIP200, are not functionally required for retromer-dependent sorting of GLUT1. Overall, these studies suggest a unique role for ATG5 in the control of retromer function, and that conjugation of ATG8 to single membranes (CASM) is a partial contributors to these phenotypes.

Strengths:

(1) Overall, these studies suggest a unique non-autophagic role for ATG5 in the control of retromer function. They also demonstrate that conjugation of ATG8 to single membranes (CASM) is a partial contributors to these phenotypes. Overall, these data point to a new role for ATG5 and CASM-dependent mATG8ylation in lysosomal membrane repair and trafficking.

(2) Although the studies are overall supportive of the proposed model that the retromer is controlled by CASM-dependent mATG8-ylaytion, it is noteworthy that previous studies of GLUT1 trafficking during glucose withdrawal (Roy et al. Mol Cell, PMID: 28602638) were predominantly conducted in cells lacking ATG5 or ATG7, which would not be able to discriminate between a CASM-dependent vs. canonical autophagy-dependent pathway in the control of GLUT1 sorting. Is the lack of GLUT1 mis-sorting to lysosomes observed in FIP200 and ATG13KO cells also observed during glucose withdrawal? Notably, deficiencies in glycolysis and glucose-dependent growth have been reported in FIP200 deficient fibroblasts (Wei et al. G&D, PMID: 21764854) so there may be difference in regulation dependent on the stress imposed on a cell.

Comments on revisions:

My previous comments have been addressed.

https://doi.org/10.7554/eLife.100928.2.sa2

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

In this manuscript, Padder et al. used APEX2 proximity labeling to find an interaction between ATG5 and the core components of the Retromer complex, VPS26, VPS29, and VPS35. Further studies revealed that ATG5 KO inhibited the trafficking of GLUT1 to the plasma membrane. They also found that other autophagy genes involved in membrane atg8ylation affected GLUT1 sorting. However, knocking out other essential autophagy genes such as ATG13 and FIP200 did not affect GLUT1 sorting. These findings suggest that ATG5 participates in the function of the Retromer in a noncanonical autophagy manner. Overall, the methods and techniques employed by the authors largely support their conclusions. These findings are intriguing and significant, enriching our understanding of the non-autophagic functions of autophagy proteins and the sorting of GLUT1.

Comments on revisions:

The concerns I raised have all been addressed.

https://doi.org/10.7554/eLife.100928.2.sa1

**Author response:**

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

> ***Public reviews***:
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In this study, Masroor Ahmad Paddar and his/her colleagues explore the noncanonical roles of ATG5 and membrane atg8ylation in regulating retromer assembly and function. They begin by examining the interactomes of ATG5 and expand the scope of these effects to include homeostatic responses to membrane stress and damage.*
>
> *Strengths:*
>
> *This study provides novel insights into the noncanonical function of ATG8ylation in endosomal cargo sorting process.*
>
> *Weaknesses:*
>
> *The direct mechanism by which ATG8ylation regulates the retromer remains unsolved.*

We agree with the reviewer. We do however show how at least one aspect of atg8ylation contributes to the proper retromer function, which occurs via lysosomal membrane maintenance and repair. Understanding the more direct effects on retromer will require a separate study. We now emphasize this in the revised manuscript (p. 18) and point out the limitations of the present work (p. 18): "One of the limitations of our study is that beyond effects of membrane atg8ylation on quality of lysosomal membrane and its homeostasis there could be more direct effects of membrane modification with mATG8s that still need to be understood".

> ***Reviewer #2 (Public Review):***
>
> *Summary:*

> *Padder et al. demonstrate that ATG5 mediates lysosomal repair via the recruitment of the retromer components during LLOMe-induced lysosomal damage and that mAtg8-ylation contributes to retromer-dependent cargo sorting of GLUT1. Although previous studies have suggested that during glucose withdrawal, classical autophagy contributes to retromer-dependent GLUT1 surface trafficking via interactions between LC3A and TBC1D5, the experiments here demonstrate that during basal conditions or lysosomal damage, ATGs that are not involved in mATG8ylation, such as FIP200, are not functionally required for retromer-dependent sorting of GLUT1. Overall, these studies suggest a unique role for ATG5 in the control of retromer function, and that conjugation of ATG8 to single membranes (CASM) is a partial contributor to these phenotypes.*
>
> *Strengths:*
>
> *(1) Overall, these studies suggest a unique non-autophagic role for ATG5 in the control of retromer function. They also demonstrate that conjugation of ATG8 to single membranes (CASM) is a partial contributor to these phenotypes. Overall, these data point to a new role for ATG5 and CASM-dependent mATG8ylation in lysosomal membrane repair and trafficking.*
>
> *(2) Although the studies are overall supportive of the proposed model that the retromer is controlled by CASM-dependent mATG8-ylaytion, it is noteworthy that previous studies of GLUT1 trafficking during glucose withdrawal (Roy et al. Mol Cell, PMID: 28602638) were predominantly conducted in cells lacking ATG5 or ATG7, which would not be able to discriminate between a CASM-dependent vs. canonical autophagy-dependent pathway in the control of GLUT1 sorting. Is the lack of GLUT1 mis-sorting to lysosomes observed in FIP200 and ATG13KO cells also observed during glucose withdrawal? Notably, deficiencies in glycolysis and glucose-dependent growth have been reported in FIP200 deficient fibroblasts (Wei et al. G&D, PMID: 21764854) so there may be differences in regulation dependent on the stress imposed on a cell.*

We thank the reviewer for the overall assessment of the strengths of the study. We have discussed in the manuscript the elegant study by Roy et al., PMID 28602683. To accommodate reviewer's comment, we have additionally emphasized in the text that our study is focused on basal conditions and conditions that perturb endolysosomal compartments. We agree with the reviewer that under metabolic stress conditions (such as glucose limitation) more complex pathways may be engaged and have acknowledged that in the discussion. We have now included this in the limitations of the study (p. 18): "Another limitation of our study is that we have focused on basal conditions or conditions causing lysosomal damage, whereas metabolic stress including glucose excess or limitation with its multitude of metabolic effects have not been addressed".

> *Weaknesses:*
>
> *(1) Additional controls are needed to clarify the role of CASM in the control of retromer function. Because the manuscript proposes both CASM-dependent and independent pathways in the ATG5 mediated regulation of the retromer, it is important to provide robust evidence that CASM is required for retromer-dependent GLUT1 sorting to the plasma membrane vs. lysosome. The experiments with monensin in Fig. 7C-E are consistent with but not unequivocally corroborative of a role for CASM.*

We fully agree with the reviewer. In fact, our data with bafilomycin A1 treatment causing GLUT1 miss-sorting show that it is the perturbance of lysosomes and not CASM per se that leads to mis-sorting of GLUT1 (Fig. 7D,E). Note that it has been shown (PMIDs: 28296541, 25484071 and 37796195) that although bafilomycin A1 deacidifies lysosomes it does not induce but instead inhibits CASM. This is because bafilomycin A1 causes dissociation of V1

and V0 sectors of V-ATPase, unlike other CASM-inducing agents which promote V1 V0 association. Complementing this, our data with ATG2AB DKO and ESCRT VPS37A KO (Fig. 8A-F) indicate that the repair of lysosomes is important to keep the retromer machinery functional (as illustrated in Fig. 8G). This may be one of the effector mechanisms downstream of membrane atg8ylation in general and hence also downstream of CASM. We have revised Fig. 7 title to read "Lysosomal perturbations cause GLUT1 mis-sorting" and have explained these relationships in the text (p. 12-13): "Since bafilomycin A1 does not induce CASM but disturbs luminal pH, we conclude that it is the less acidic luminal pH of the endolysosomal organelles, and not CASM, that is sufficient to interfere with the proper sorting of GLUT1."

> *Based on the results shown with ATG16KO in Fig 4A-D, rescue experiments of these 16KO cells with WT vs. C-terminal WD40 mutant versions of ATG16 will specifically assess the requirement for CASM and potentially provide more rigorous support for the conclusions drawn.*

We have carried out complementation with ATG16L1 WT and its E230 mutant (devoid of WD40 repeats but still capable of canonical autophagy) and placed these data in Fig. 7 (panels I and J) as recommended by the reviewer. This is now described on p. 13 (To additionally test this notion, we compared ATG16L1 full length (ATG16L1FL) and ATG16L1E230 (Rai et al., PMID 30403914) for complementation of the GLUT1 sorting defect in ATG16L1 KO cells (Fig. 7I,J). ATG16L1E230 [Rai, 2019, 30403914] lacks the key domain to carry out CASM via binding to VATPase 29,30 31-33 but retains capacity to carry out atg8ylation. Both ATG16L1FL and ATG16L1E230 complemented mis-sorting of GLUT1 (Fig. 7I,J). Collectively, these data indicate that it is not absence of CASM/VAIL but absence of membrane atg8ylation in general that promotes GLUT1 mis-sorting.).

> *(2) Also, the role of TBC1D5 should be further clarified. In Fig S7, are there any changes in the interactions between TBC1D5 and VPS35 in response to LLOMe or other agents utilized to induce CASM?*

We thank the reviewer for pointing this out. We do have data with VPS35 in co-IPs shown in Fig. S7. There is no change in the amounts of VPS35 or TBC1D5 in GFP-LC3A co-IPs. We now include in Fig. S7 (new panel D) a graph with quantification in the revised manuscript and emphasize this point (p. 12): "However, under CASM-inducing conditions, no changes were detected (Fig. S7B-D) in interactions between TBC1D5 and LC3A or in levels of VPS35 in LC3A co-IP, a proxy for LC3A-TBC1D5-VPS29/retromer association. This suggests that CASM-inducing treatments and additionally bafilomycin A1 do not affect the status of the TBC1D5-Rab7 system".

> *Does TBC1D5 loss-of-function modulate the numbers of GLUT1 and Gal3 puncta observed in ATG5 deficient cells in response to LLOMe?*

We agree that TBC1D5 is an interesting aspect. However, because TBC1D5 does not change its interactions in the experiments in our study, we consider this topic (i.e. whether TBC1D5 phenocopies VPS35 and ATG5 KOs in its effects on Gal3) to be beyond the scope of the present work. We underscore that LLOMe (lysosomal damage) mis-sorts GLUT1 even without any genetic intervention (e.g., in WT cells in the absence of ATG5 KO; Fig. 7). Thus, in our opinion the effects of TBC1D5 inactivation may be a moot point.

> *(3) Finally, the studies here are motivated by experiments in Fig. S1 (as well as other studies from the Deretic and Stallings labs) suggesting unique autophagy-independent functions for ATG5 in myeloid cells and neutrophils in susceptibility to Mycobacterium tuberculosis infection. However, it is curious that no attempt is made to relate the mechanistic data regarding the retromer or GLUT1 receptor mis-sorting back to the*

> *infectious models. Do myeloid cells or neutrophils lacking ATG5 have deficiencies in glucose uptake or GLUT1 cell surface levels?*

Reviewer's point is well taken. Glucose uptake, its metabolism, and diabetes underly resurgence in TB in certain populations and are important factors in a range of other diseases. This was alluded to in our discussion (lines 461-469). However, these are complex topics for future studies. We have now expanded this section of the discussion (p. 18): "In the context of tuberculosis, diabetes, which includes glucose dysregulation, is associated with increased incidence of active disease and adverse outcomes" (Dheda et al., ,PMID: 26377143; Dooley, et al., PMID:19926034).

> ***Reviewer #3 (Public Review):***
>
> *In this manuscript, Padder et al. used APEX2 proximity labeling to find an interaction between ATG5 and the core components of the Retromer complex, VPS26, VPS29, and VPS35. Further studies revealed that ATG5 KO inhibited the trafficking of GLUT1 to the plasma membrane. They also found that other autophagy genes involved in membrane atg8ylation affected GLUT1 sorting. However, knocking out other essential autophagy genes such as ATG13 and FIP200 did not affect GLUT1 sorting. These findings suggest that ATG5 participates in the function of the Retromer in a noncanonical autophagy manner. Overall, the methods and techniques employed by the authors largely support their conclusions. These findings are intriguing and significant, enriching our understanding of the non-autophagic functions of autophagy proteins and the sorting of GLUT1.*
>
> *Nevertheless, there are several issues that the authors need to address to further clarify their conclusions.*
>
> *(1) The authors confirmed the interaction between Atg5 and the Retromer complex through Co-IP experiments. Is the interaction between Atg5 and the Retromer direct? If it is direct, which Retromer complex protein regulates the interaction with Atg5? Additionally, does ATG5 K130R mutant enhance its interaction with the Retromer?*

AlphaFold modeling in the initial submission of our study to eLife (absent from the current version) suggested the possibility of a direct interaction between ATG5 and VPS35 with ATG12—ATG5 complex facing outwards, in which case K130R would not matter. However, mutational experiments in putative contact residues did not alter association in co-IPs. So either ATG5 interacts with other retromer subunits or more likely is in a larger protein complex containing retromer. It will take a separate study to dissect associations and find direct interaction partners.

> *(2) To more directly elucidate how ATG5 regulates Retromer function by interacting with the Retromer and participates in the trafficking of GLUT1 to the plasma membrane, the authors should identify which region or crucial amino acid residues of ATG5 regulate its interaction with the Retromer. Additionally, they should test whether mutations in ATG5 that disrupt its interaction with the Retromer affect Retromer function (such as participating in the trafficking of GLUT1 to the plasma membrane) and whether they affect Atg8ylation. They also need to assess whether these mutations influence canonical autophagy and lysosomal sensitivity to damage.*

Please see the response to point 1.

> ***Recommendations for the authors.***

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *While most data are solid and convincing, the following questions need to be addressed before publication:*
>
> *Major Concerns:*
>
> *(1) Examining only one cargo (GLUT1) is insufficient to reflect the retromer's function comprehensively. At least two additional cargoes should be analyzed to observe the phenotypes more accurately.*

We agree that having another retromer cargo (in addition to GLUT1) would be of interest. We point out that our data also show mis-sorting of SNX27 to lysosomes (Fig. 3H, quantifications in Fig. 3I). SNX27 in turn sorts nearly 80 ion channels, signaling receptors, and other nutrient transporters. Which of the 80 cargos to prioritize and check (the expectation is that all 80 might be missorted given that they need SNX27)? We have instead tested MPR, a SNX27-independent cargo. We now include data on effects of ATG5 knockout on CI-MPR (Fig. S9A-F). This is described in the text (p. 14; “Effect of ATG5 knockout on MPR sorting

We tested whether ATG5 affects cation-independent mannose 6-phosphate receptor (CI-MPR). For this, we employed the previously developed methods (Fig. S9A) of monitoring retrograde trafficking of CI-MPR from the plasma membrane to the TGN 70,118-121. In the majority of such studies, CI-MPR antibody is allowed to bind to the extracellular domain of CI-MPR at the plasma membrane and its localization dynamics following endocytosis serves as a proxy for trafficking of CI-MPR. We used ATG5 KOs in HeLa and Huh7 cells and quantified by HCM retrograde trafficking to TGN of antibody-labeled CI-MPR at the cell surface, after being taken up by endocytosis and allowed to undergo intracellular sorting, followed by fixation and staining with TGN46 antibody. There was a minor but statistically significant reduction in CIMPR overlap with TGN46 in HeLaATG5-KO that was comparable to the reduction in HeLa cells when

VPS35 was depleted by CRISPR (HeLaVPS35-KO) (Fig. S9B,C). Morphologically, endocytosed Ab-CI-

MPR appeared dispersed in both HeLaATG5-KO and HeLaVPS35-KO cells relative to HeLaWT cells (Fig. S9D). Similar HCM results were obtained with Huh7 cells (WT vs. ATG5KO; Fig. S9E,F). We interpret these data as evidence of indirect action of ATG5 KO on CI-MPR sorting via membrane homeostasis, although we cannot exclude a direct sorting role via retromer. We favor the former interpretation based on the strength of the effect and the controversial nature of retromer engagement in sorting of CI-MPR (57,70,75,98,120).”)

> *(2) The evidence from Alphafold predictions is weak. The direct interaction of ATG5 with retromer subunits should be tested.*

Please see the above response to Reviewer 3.

> *In addition, does retromer also interact with ATG16L1 similarly to the phenomenon in VAIL?*

We fully agree with the reviewer that finding the direct interacting partners between retromer and membrane atg8ylation machinery is an important direction as in our opinion it would expand the repertoire of E3 ligases and its adaptors. However, given the complexity and variety of possibilities, we believe that this is a topic for a future study.

> *(3) In Line 166, Figures 2C and 2D, the Gal3 phenotype does not seem to be well complemented by VPS35.*

We have adjusted the text to acknowledge incomplete complementation (p.7).

> *(4) In Figures 3 and 4, the authors show that KO of membrane atg8ylation machineries and ATG8-Hexa KO affects the localization of retromer cargo GLUT1 and SNX27. However, the mechanism by which membrane ATG8ylation affects retromer remains unresolved.*
>
> *Additionally, are other retromer subunits' locations are also affected, if so, how are they impacted? At least a speculative explanation should be provided.*

Following reviewers request, we now state on p. 19 that "one of the limitations of our study is that beyond effects of membrane atg8ylation on quality of lysosomal membrane and its homeostasis there could be more direct effects of membrane modification with mATG8s on retromer that still need to be understood".

> *(5) In Figure 3, endogenous IP results are required to examine the interaction of ATG5 with retromer if suitable retromer antibodies for IP are available.*

Endogenous IPs are given in Fig. 1. We have modified text on p. 8 to clarify this.

> *(6) In Figure 4, ATG8 Hexa KO, and triple KO of LC3s or GABARAPs all increase the localization of GLUT1 on lysosomes. It seems redundant for ATG8 family proteins here.*

Can any individual member of the ATG8 family rescue this phenotype?

If the intent of such complementation analysis is to identify a specific mATG8 responsible for the observed effects, this is already pre-empted by the fact that TKOs also have a similar effect as HEXA mutants (i.e. loss of at least two of mATG8s is enough to cause the phenotype). We now discuss this in the text (p. 10): "Thus, at least two mATG8s, each one from two different mATG8 subclasses (LC3s and GABARAPs) or the entire membrane atg8ylation machinery was engaged in and required for proper GLUT-1 sorting".

> *(7) In Figure 5, knockdown of ATG5 in FIP200 KO cells inhibited GLUT1 sorting from endosomes, leading to its trafficking to lysosomes. However, it is known that very little remnant ATG5 in ATG5 KD cells is enough to support ATG8 lipidation. Therefore, it is essential to repeat this experiment using ATG5/FIP200 double KO or ATG5 KO combined with an autophagy inhibitor.*

We point out to this limitation in the text (p. 11): "….we knocked down ATG5 in FIP200 KO cells (Fig. S5D) and found that GLUT1 puncta and GLUT1+LAMP2+ profiles increased even in the FIP200 KO background with the effects nearing those of VPS35 knockout (Figs. 5D-F and S5C), with the difference between VPS35 KO and ATG5 KD attributable to any residual ATG5 levels in cells subjected to siRNA knockdowns".

> *(8) In Figure 7, the authors show that the induction of CASM inhibited GLUT1 sorting from endosomes. However, ATG5 KO, which abolishes membrane ATG8ylation, also inhibits GLUT1 sorting. This seems paradoxical and requires a reasonable explanation or discussion.*

We understand reviewer's comment. The answer to this paradox is that it is actually the lysosomal damage that causes GLUT1 mis-sorting and not CASM. Membrane atg8ylation, such

as CASM and probably other processes given that involvement of both ATG2 and ESCRTs (Fig. 8) counteracts the damage and works in the direction of restoring/maintaining proper retromer-dependent sorting. This is now explained better in the text, and have revised the title of Fig. 7 to read “Lysosomal damage causes GLUT1 mis-sorting”. Our data with bafilomycin A1 show that it is the perturbance of lysosomes (not CASM per se) that leads to mis-sorting of GLUT1 (Fig. 7D,E), and our data with ATG2AB DKO and ESCRT (VPS37A) KO (Fig. 8A-F) indicate that repair of lysosomes is important to keep the retromer working machinery functional (as illustrated in Fig. 8G), which may be one of the effector mechanisms downstream of membrane atg8ylation in general (and hence also of CASM).

> *(9) The immuno-staining results for Figures 7F and 7G are lacking.*

We now provide the requested images.

> *(10) In Figure 8D, the quality of the image for VPS37 KO cells treated with LLOME is not sufficient to show increased colocalization between GLUT1 and LAMP2.*

We now provide a different example image. We note that these are epiflorescent HCM images

> *Minor Concerns:*
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> *(1) It would be better to distinguish the function of the membrane ATG8ylation machinery (i.e., ATG5) from the function of membrane ATG8ylation in the description. No ATG8ylation-deficient mutants were used in this study.*

We have used atg8ylation mutants (e.g. KOs in ATG3, ATG5, ATG7, and ATG16L1). We now emphasize this better in the text (p. 10).

> *(2) In Figure 2D, a green box appears there by incident.*

This has been fixed.

> *(3) In Figure 3A, the conjugate for ATG5-ATG12 is absent in the gel for IB: ATG5.*

The ATG5 antibody used in Fig. 3A recognizes primarily the conjugated form of ATG5. This is now clarified in the figure legend.

> *(4) Figure 5G is missing in the manuscript.*

Fig 5G is now mentioned in the text. Thank you.

> *(5) The gRNA sequence information for FIP200 KO is missing in the Methods section.*

Reference(s) to the already published gRNA sequence are in the manuscript.

> *(6) Suggest moving the last paragraph in Result section to Discussion section.*

We kept this single-paragraph section in Results as it contains actual data.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *(1) It is unclear why the rescue of VPS35KO cells in Fig 1C-D is so modest.*

Complementation data depend on transfection efficiency and some variability is to be expected.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Figures 2A, 2C, 2E, and 2G lack scale bars. Figure 2D has a small square above the y axis.*

Relative scale bars are now included.

> *(2) Figures S3B, S3D, and S3F lack scale bars.*

Relative scale bars are now included.

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