Cargo ubiquitination is essential for multivesicular body intraluminal vesicle formation

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The efficient formation of a variety of transport vesicles is influenced by the presence of cargo, suggesting that cargo itself might have a defining role in vesicle biogenesis. However, definitive *in vivo* experiments supporting this concept are lacking, as it is difficult to eliminate endogenous cargo. The Endosomal Sorting Complexes Required for Transport (ESCRT) apparatus sorts ubiquitinated membrane proteins into endosomal intraluminal vesicles (ILVs) that accumulate within multivesicular bodies. Here we show that cargo ubiquitination is required for effective recruitment of the ESCRT machinery onto endosomal membranes and for the subsequent formation of ILVs.

Keywords: ubiquitin; lysosome; multivesicular body; endosome; vacuole

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INTRODUCTION

Lysosomal degradation of membrane proteins is mediated by their attachment to ubiquitin (Ub), which serves as a sorting tag for entry into intraluminal vesicles (ILVs) that bud from the limiting membrane of endosomes [1–3]. The ILVs accumulate in late endosomes/multivesicular bodies (MVBs), which fuse to lysosomes and thereby deliver ubiquitinated, vesicle-incorporated membrane proteins to the lysosome lumen for degradation. The ESCRTs (Endosomal Sorting Complexes Required for Transport) encompass a set of interacting protein complexes that recognize ubiquitinated membrane proteins (Ub-cargo) and facilitate their sorting into ILVs. ESCRT-0 and ESCRT-I capture cargo and initiate ILV formation, after which ESCRT-II and, ultimately, ESCRT-III are recruited to the ESCRT apparatus to complete vesicle formation and fission from the endosomal limiting membrane. Multiple Ub-binding domains (UBDs) are found within ESCRT-0, -I and -II. Inactivation of individual UBDs causes specific defects in the sorting of Ub-cargo

into MVBs without compromising other ESCRT functions such as maintenance of endosomal morphology and recycling of proteins between endosomes and the trans-Golgi network (TGN). However, when multiple UBDs are lost, or UBD loss occurs in the context of mutations that weaken the ability of ESCRTs to bind one another, the ESCRT pathway is more broadly compromised [4–6]. This suggests that UBDs have an intimate role in controlling ESCRT activity. One possible explanation is that UBDs might not simply serve as a means for ESCRTs to gather cargo, but also enable Ub-cargo to communicate with and regulate ESCRTs. Thus, rather than being passively incorporated into constitutively made vesicles, Ub-cargo could itself actively stimulate the function of ESCRTs by promoting or coordinating their assembly.

RESULTS AND DISCUSSION

To determine whether Ub-cargo has a role in ESCRT-mediated ILV formation, we examined the consequences of eliminating endosomal Ub-cargo. To this end, we expressed a dominant-acting protein in which the ESCRT-0 subunit Hse1 (the homologue of mammalian STAM) is fused to the catalytic domain of the deubiquitinating peptidase domain of Herpes Virus UL36, resulting in the formation of an Hse1-DUB chimeric protein (Fig 1A). Our previous studies showed that when Hse1-DUB is expressed in wild-type cells, either as the sole copy of Hse1 or in addition to endogenous Hse1, it effectively removes Ub from cargo during the MVB-sorting process, and blocks the sorting of a variety of cargoes that would normally undergo Ub-dependent sorting into MVB ILVs [7]. As a further measure of the effectiveness of Hse1-DUB to block MVB sorting of ubiquitinated cargo, we followed green fluorescent protein (GFP)-tagged Ub (GFP-Ub; [8]). Earlier studies indicated that GFP-Ub could be used as a proxy to monitor the delivery of a wide variety of cargoes into the MVB pathway. For instance, wild-type cells accumulate GFP-Ub in the vacuole and loss of the late-acting Doa4 DUB, which is required for removing much of the Ub from MVB cargo before its entry into ILVs, causes hyper-accumulation of GFP-Ub within the vacuole [8]. As expected, we find that GFP-Ub effectively enters into the general pool of Ub as it is found readily conjugated to a variety of proteins (Fig 1B), and it also accumulates in a processed form within the vacuole lumen in *Pep+* cells (Supplementary Fig S1 online). Fig 1C shows that, although wild-type cells accumulate GFP-Ub in the vacuole, GFP-Ub is excluded from the

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Fig 1 | Hse1 fused to a deubiquitinating enzyme blocks MVB sorting of ubiquitinated proteins. (A) Schematic of the Hse1-DUb fusion protein showing key functional domains of the Hse1 subunit of ESCRT-0 fused to the catalytic domain of the deubiquitinating enzyme UL36. VHS (Vps27, Hrs, STAM), UIM (Ub-interacting motif) and SH3 (Src-homology 3). (B) *pep4Δ* cells expressing either empty vector (–) or GFP-Ub (+) were grown to log phase and radiolabelled with three 5-min pulses of ^{35}S -methionine before spheroplasting and lysis; lysates were then subjected to immunoprecipitation using anti-GFP antibodies. (C) Localization of GFP-Ub in WT cells or WT cells carrying integrated *CUP1-Hse1-DUb* (upper panels). Cells were grown with CuCl_2 for 7 h before microscopy. Lower panels show localization of GFP-Ub in *vps4Δ* with or without expression of Hse1-DUb. Cells were counter-labelled with the lipid-binding endocytic tracer FM4-64 (at 30 °C for 30 min), and then chased in rich media for 15 min. Accumulation of FM4-64 within enlarged endosomal 'class E compartments' (arrowheads) was clearly observed in *vps4Δ* mutants. Scale bar, 5 μm. (D) Class E compartment endosomes (identified by accumulation of FM4-64) from 275 mutant *vps4Δ* cells or 101 *vps4Δ* cells expressing Hse1-DUb were scored for colocalization with GFP-Ub. Bars represent the mean and error bars s.d. ESCRT, Endosomal Sorting Complexes Required for Transport; GFP, green fluorescent protein; MVB, multivesicular body; Ub, ubiquitin; WT, wild type.

vacuole in cells expressing Hse1-Dub, indicating that a wide variety of Ub-cargoes are blocked from MVB sorting. Importantly, vacuolar morphology was normal in Hse1-Dub-expressing cells, which is in contrast to the formation of enlarged endosomes, known as class E compartments, observed upon deletion of any of the ESCRT genes from yeast. Previous experiments also showed that in contrast to ESCRT-null strains, Hse1-DUB expression does not cause secretion of the vacuolar protease carboxypeptidase Y (CPY), further indicating that the endocytic system is generally functional [7]. In addition, Hse1-DUB does not block MVB sorting of cargo fused in frame to the amino terminus of Ub; in this orientation, Ub is resistant to removal by the carboxy-terminal-specific hydrolase activity of UL36 [7]. To address whether Hse1-DUB could deubiquitinate proteins within endosomes, we analysed the distribution of GFP-Ub in *vps4Δ* mutants. Loss of the AAA-ATPase Vps4 blocks ESCRT function, causing the accumulation of ESCRTs and other endosomal proteins on enlarged 'class E compartment' endosomes [9]. GFP-Ub also accumulates on class E endosomal compartments in *vps4Δ* cells, consistent with previous studies in yeast and mammalian cells demonstrating the accumulation of ubiquitinated proteins upon loss of ESCRT function [8]. Expressing Hse1-DUB within *vps4Δ* cells reversed the accumulation of GFP-Ub within endosomes (Fig 1C,D). Notably, Hse1-DUB expression did not suppress the formation of class E endosomes identified by the endocytic tracer FM4-64. These data, together with published studies using Hse1-DUB, demonstrate that Hse1-DUB is an effective tool to deplete ubiquitinated cargo on endosomes.

We next used electron microscopy to assess the production of MVB ILVs in *pep4Δ atg8Δ* mutant yeast cells (Fig 2). Mutant *pep4Δ* cells lack the soluble vacuolar hydrolase Pep4 and fail to degrade ILVs, allowing them to accumulate within the vacuolar lumen [10]. Lack of Atg8, the LC3 homologue required for autophagy, ensured that any intraluminal structures observed were from the ESCRT-dependent MVB pathway rather than by autophagy [11]. The *pep4Δ atg8Δ* cells correctly sorted the cargo Mup1-GFP into the lumen of the vacuole (Fig 2B). Mup1 is a polytopic methionine transporter that undergoes Ub-dependent sorting into MVB ILVs in response to methionine in the growth media [12]. Cells expressing Mup1-GFP were spheroplasted, energy depleted to allow for electron-dense phosphates and metals to dissipate from the vacuole interior and imbedded for transmission electron microscopy. As expected, these cells contained abundant intravacuolar vesicles (Fig 2C). In contrast, expression of Hse1-DUB in *pep4Δ atg8Δ* cells blocked the accumulation of Mup1-GFP in the vacuole lumen and markedly reduced the number of ILVs found in the vacuole. Quantification of the number of ILVs showed that these structures were virtually absent when Hse1-DUB was expressed (Fig 3A). In addition, cryo-transmission electron microscopy analysis confirmed that ILVs were abundant within *pep4Δ atg8Δ* cells, but not within *pep4Δ atg8Δ* cells expressing Hse1-DUB (Fig 1D). As expected, expressing a catalytically inactive version of Hse1-DUB had no effect on ILV accumulation in the vacuole (Fig 3C). Finally, we found that Hse1-DUB also depleted vacuolar ILVs from *vma4Δ* mutant cells as analysed in cells that were fixed before spheroplasting (Fig 3B). Mutant *vma4Δ* cells lack the V-ATPase and do not accumulate intravacuolar metabolites that obscure visualization of ILVs within vacuoles [10]. These results, obtained

Fig 2 | Loss of ubiquitinated cargo abolishes the generation of intraluminal vesicles. (A) Schematic illustration showing forms of cargo (Mup1-GFP) with or without fusion to Ub, and forms of the ESCRT-0 subunit Hse1 with or without fusion to the UL36 DUB enzyme, which were used in each experimental group. (B) Localization of Mup1-GFP in *pep4Δ atg8Δ* cells without or with the expression of Hse1-DUB. Localization of Mup1-GFP-Ub in *pep4Δ atg8Δ* Hse1-DUB cells is also shown (right panels). Cells were grown in rich media (YPD replete with methionine) for 12 h in the presence of CuCl_2 . Scale bar, 5 μm . (C) TEM-based analysis of the cells shown in B. Cells were spheroplasted, fixed, stained and analysed by TEM. V, M and N are labelled. Scale bar, 1 μm . (D) Cryo-electron microscope-based analysis of cells in B. Scale bar, 1 μm . (E) Magnification of cryo-electron microscope images of MVB-localized ILVs in D. Scale bar, 50 nm. DUB, deubiquitinating; ESCRT, Endosomal Sorting Complexes Required for Transport; GFP, green fluorescent protein; ILV, intraluminal vesicle; M, mitochondria; MVB, multivesicular body; N, nuclei; TEM, transmission electron microscope; Ub, ubiquitin; V, vacuoles.

with a combination of different electron microscopic methods, support the idea that loss of Ub-cargo blocks ILV formation rather than simply depleting the ILVs of protein cargo.

If Hse1-DUB blocks ILV formation by eliminating Ub-cargo as opposed to interfering with other aspects of ESCRT function, then expressing cargo that is resistant to deubiquitination by Hse1-DUB should restore ILV formation. Fig 2 shows that Mup1-GFP-Ub, in which Mup1-GFP was fused to the N terminus of Ub, is sorted normally into the vacuole lumen of cells expressing Hse1-DUB. Because the DUB domain (UL36) is a Ub C-terminal hydrolase, the orientation of Ub within Mup1-GFP-Ub is immune to Hse1-DUB cleavage. Both transmission electron microscopy and cryo-electron microscopy showed that the expression of

Mup1-GFP-Ub restored the accumulation of ILVs within Hse1-DUB-expressing cells. Quantification revealed that introducing DUB-resistant cargo largely restored the number of ILVs, and that the average diameter of the ILVs was the same as those found in wild-type cells (Fig 3A). ILVs were also restored to Hse1-DUB-expressing cells by expressing other DUB-resistant Ub-cargo including Ste3-GFP-Ub and Ub-GFP-Cps1, suggesting that the effect of Ub-cargo was not specific to only Mup1-based fusion proteins (Fig 3C). Together, these results demonstrate that Ub-cargo has an essential role in ILV formation. Moreover, they imply that flux through the MVB pathway can be regulated in large measure simply by supplying more Ub-cargo.

Fig 3 | Additional ubiquitinated cargo replenishes accumulation of ILVs. (A) Morphometric analysis of TEM experiment described in Fig 2C. Average number of ILVs (per cell per section) as calculated by counting ILVs within the vacuoles of 24 wild-type cells, 52 wild-type cells expressing Hse1-DUB and 36 wild-type cells coexpressing Hse1-DUB and Mup1-GFP-Ub. Error bars = s.d. Average vesicle size was calculated from measurements of 329 ILVs from wild-type cells, and 333 ILVs from cells coexpressing Hse1-DUB and Mup1-GFP-Ub. Error bars = s.d. (B) TEM images of *vma4Δ* cells with and without expression of Hse1-DUB. Cells were fixed before spheroplasting, imbedding and electron microscopy analysis. (C) Fluorescence localization and TEM analysis of WT cells expressing Mup1-GFP-Ub alone or expressing Mup1-GFP and Hse1-dub* in which the DUB carries an inactivating mutation, and cells expressing Hse1-DUB (active) that coexpress either Ste3-GFP-Ub or Ub-GFP-Cps1. DUB, deubiquitinating; ESCRT, Endosomal Sorting Complexes Required for Transport; GFP, green fluorescent protein; ILV, intraluminal vesicle; TEM, transmission electron microscopy; Ub, ubiquitin; V, vacuoles.

The dependence of ILV formation on the presence of Ub-cargo indicates that there might be interactions between cargo and the UBDs within the ESCRT apparatus that help organize the sorting machinery into a productive pathway. The role of such interactions might be to stabilize ESCRTs on endosomes until they have initiated later stages of vesicle formation. To examine this possibility, we assessed whether eliminating Ub-cargo changed the extent of ESCRT recruitment to endosomal membranes.

In wild-type cells, GFP-tagged ESCRT-0, ESCRT-I and ESCRT-II subunits localized to punctate structures, consistent with their well-established endosomal function (Fig 4A). The GFP-tagged ESCRT subunits were all functional, as they mediated MVB sorting and proper CPY sorting when present as the sole copy of their respective subunit (Fig 5A,B). Hse1-DUB expression shifted the distribution of both ESCRT-I (visualized with Vps23-GFP and Vps28-GFP) and ESCRT-II (visualized with Vps22-GFP and

Fig 4 | Effect of Ub-cargo on ESCRT localization to endosomes. (A) Localization of GFP-tagged subunits of ESCRT-0 (Vps27), ESCRT-I (Vps23 and Vps28) and ESCRT-II (Vps22 and Vps36) in wild-type cells in the absence or presence of Hse1-DUb. Cells were grown in minimal media in the presence of CuCl₂ for 7 h before microscopy. Localization of GFP-tagged ESCRTs in Hse1-Dub-expressing cells that were coexpressing Mup1-RFP-Ub is also depicted (right hand columns). Scale bar, 5 μm. (B) Localization of GFP-tagged ESCRT subunits in *vps4Δ* cells in the absence or presence of Hse1-DUb expression. Scale bar, 5 μm. DUB, deubiquitinating; ESCRT, Endosomal Sorting Complexes Required for Transport; GFP, green fluorescent protein; Ub, ubiquitin; WT, wild type.

Vps36-GFP) to the cytosol. Hse1-DUb did not, however, alter the distribution of ESCRT-0, reinforcing the idea that the activity of Hse1-DUb is concentrated on endosomes. Remarkably, expression of Mup1-RFP-Ub (a non-deubiquitinatable cargo) restored the endosomal localization of ESCRT-I and ESCRT-II (Fig 4A).

The ability of ESCRTs to cycle on and off membranes depends of Vps4, an AAA-ATPase that can dismantle membrane-bound polymers that form from ESCRT-III subunits. Loss of Vps4 essentially locks ESCRTs onto membranes where they accumulate. We next assessed whether a block in Vps4 activity could still cause accumulation of ESCRTs onto endosomes of cells expressing Hse1-DUb. Fig 4 shows that *vps4Δ* mutants accumulated GFP-tagged ESCRT-I and ESCRT-II subunits on endosomes as expected. In addition, the ESCRTs still accumulated on *vps4Δ* endosomes even upon expressing Hse1-DUb (Fig 4B), even though Ub-cargo was found not to accumulate on these endosomes (Fig 1C).

The elimination of Ub-cargo by Hse1-DUb and the resulting loss of ILV accumulation, combined with the restoration of ILVs upon expression of DUB-resistant Ub-cargo, strongly argue that Ub-cargo is required for ILV formation. Our assays detected the presence of ILVs within the vacuole lumen, where they accumulate normally in wild-type cells that lack active vacuolar proteases. Importantly, Hse1-DUb does not perturb other aspects of the endocytic pathway including vacuolar and endosomal morphology or sorting of soluble vacuolar hydrolases. Thus, the loss of ILVs from the vacuole indicates a defect in ILV formation within endosomes, rather than failure of endosomes to deliver their contents to the vacuole. Loss of any of the ESCRTs in yeast results in a panoply of phenotypes that include not only the loss of ILV formation and MVB sorting of Ub-cargo but also the accumulation of large aberrant endosomes that accumulate

recycling proteins (for example, Kex2 and Vps10), as well as vacuolar membrane proteins such as the V-ATPase complex. It remains unclear how these phenotypes relate to one another; however, the profound defect that Hse1-DUb expression causes in MVB sorting and generation of ILVs without the other concomitant defects observed in ESCRT null mutants demonstrates that these phenotypes can be separated and suggests that they represent distinct functions of the ESCRTs. We also found that Hse1-DUb did not suppress the 'class E *vps*' phenotype when expressed in *vps4Δ* cells even though Hse1-DUb was effective at removing ubiquitinated proteins that otherwise accumulate within the enlarged endosomal class E compartment. Thus, the abnormal endosome function of ESCRT mutants does not seem to be driven by the accumulation of ubiquitinated proteins that might be sensed by factors that regulate endosome maturation.

Ub-cargo clearly promotes the association of ESCRTs with membranes. One possibility is that in the absence of cargo ESCRTs never associate with endosomes. Alternatively, ESCRTs might simply recycle off membranes rapidly without cargo, unable to associate stably enough to lead to productive ILV formation. We favour the latter model (Fig 5C), as ESCRTs in Hse1-DUb-expressing cells could still be captured onto endosomes when their release was blocked by loss of Vps4. Our finding that Ub-cargo is required for recruitment of ESCRTs to endosomes implies that interactions between the ESCRTs themselves and interactions between ESCRTs and cargo work cooperatively to create enriched endosomal subdomains that subsequently form ILVs. Nevertheless, they do not rule out the possibility that Ub-cargo might have a more active role in configuring and/or activating the ESCRT apparatus to trigger vesicle formation. Moreover, the disappearance of ILVs in the absence of Ub-cargo suggests that few, if any,

Fig 5 | Function and Functional modelling of GFP-tagged ESCRTs. (A) WT cells or cells expressing the indicated GFP-tagged ESCRT subunit as their sole source of that protein were analysed for proper MVB sorting of Sna3-RFP to the vacuole interior. Also shown is the same analysis of Vps28-GFP and Vps36-GFP cells lacking *VPS4*. (B) CPY was immunoprecipitated from intracellular and secreted extracellular fractions from cells pulse-labelled with ^{35}S -methionine for 10 min and chased for 50 min at 30 °C. (C) Model for how Ub-cargo could drive ESCRT recruitment or retention and subsequent ILV formation. In the absence of cargo, ESCRTs rapidly recycle off endosomes and are unable to form a productive complex. In the presence of cargo, ESCRTs are retained better, allowing them to organize properly and complete the formation of ILVs while concomitantly sorting Ub-cargo into those vesicles. CPY, carboxypeptidase Y; E, extracellular; ESCRT, Endosomal Sorting Complexes Required for Transport; GFP, green fluorescent protein; I, intracellular; ILV, intraluminal vesicle; MVB, multivesicular body; RFP, red fluorescent protein; Ub, ubiquitin; WT, wild type.

Ub-independent cargoes are sorted into ILVs. Our results also have implications for the biogenesis of transport vesicles other than ILVs. A number of *in vivo* and *in vitro* studies indicate that cargo abundance influences the dynamics of assembly for COPI, COPII and clathrin-coated vesicles [13–15]. It is conceptually axiomatic that the mechanisms underlying the biogenesis of all transport vesicles would have in common a cargo-instigated feedback mechanism to prevent the formation and accumulation of empty vesicles. Thus, the strict requirement for Ub-cargo in the biogenesis of ILVs revealed here, and the need for cargo and the vesicle coat machinery to collaborate, might be fundamental to a variety of vesicle transport steps.

METHODS

Yeast strains and plasmids are described (Tables 1 and 2). Cells for electron microscope analysis were grown in rich yeast peptone

dextrose (YPD) media with 100 μM CuCl_2 , spheroplasted in the presence of 2% NaN_3 and fixed in 2% glutaraldehyde and 100 mM Na cacodylate [4]. Cells were either stained with ruthenium red and osmium tetroxide, embedded, sectioned and stained with lead citrate/uranyl acetate or frozen in 2.3 M sucrose, sectioned and stained with uranyl acetate. Electron microscope analysis was also performed in Hse1-Dub-expressing *vma4* Δ cells using electron microscope procedures previously described [10,18]. Localization of ESCRTs in the presence and absence of Hse1-DUB (expression induced from the *CUP1* promoter) was assessed in wild-type SEY6210 cells carrying a *VPS27-GFP* low-copy expression plasmid, or in cells in which GFP was stably integrated at the 3'-end of the *VPS22*, *VPS23*, *VPS28* or *VPS36* open-reading frame according to described methods [19]. The Vps27-GFP protein was made so that the last 25 residues of Vps27 were C-terminal to the GFP to allow for the association to clathrin.

Table 1 | Yeast strains used in the study

Strain	Genotype	Use	Reference
SEY6210 wild type	<i>Mat a, leu1-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-D9</i>	Figs 1C,4	Robinson <i>et al</i> [16]
PLY2463	SEY6210; <i>pep4-ΔH3</i>	Fig 1C	This study
PLY3987	SEY6210; <i>pep4-ΔH3 ura3-52::CUP1-Hse1-UL36-HA::URA3</i>	Fig 1C	This study
PLY4099	SEY6210; <i>pep4-ΔH3 vps4Δ::KanR</i>	Fig 1C	This study
PLY4100	SEY6210; <i>pep4-ΔH3 ura3-52::CUP1-Hse1-UL36-HA::URA3 vps4Δ::KanR</i>	Fig 1C	This study
PLY3986	SEY6210; <i>pep4-ΔH3 atg8Δ::KanR</i>	Figs 2B–E, 3C	This study
PLY3988	SEY6210; <i>pep4-ΔH3 atg8Δ::KanR CUP1-Hse1-UL36-HA::URA3</i>	Figs 2B–E, 3C	This study
GOY25	SEY6210; <i>vma4Δ::URA3</i>	Fig 3B	Odorizzi <i>et al</i> [10]
SJY028	SEY6210; <i>vps22::GFP-HIS5MX</i>	Figs 4, 5 (A,B)	This study
SJY034	SEY6210; <i>vps23::GFP-HIS5MX</i>	Figs 4, 5 (A,B)	This study
SJY040	SEY6210; <i>vps28::GFP-HIS5MX</i>	Figs 4, 5 (A,B)	This study
SJY02	SEY6210; <i>vps36::GFP-HIS5MX</i>	Figs 4, 5 (A,B)	This study
PLY4064	SEY6210; <i>vps22::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4065	SEY6210; <i>vps23::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4066	SEY6210; <i>vps28::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4067	SEY6210; <i>vps36::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4103	SEY6210; <i>vps4Δ::KanR vps22::GFP-HIS5MX</i>	Fig 4	This study
PLY4105	SEY6210; <i>vps4Δ::KanR vps23::GFP-HIS5MX</i>	Fig 4	This study
PLY4107	SEY6210; <i>vps4Δ::KanR vps28::GFP-HIS5MX</i>	Fig 4	This study
PLY4109	SEY6210; <i>vps4Δ::KanR vps36::GFP-HIS5MX</i>	Fig 4	This study
PLY4104	SEY6210; <i>vps4Δ::KanR vps22::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4106	SEY6210; <i>vps4Δ::KanR vps23::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Fig 4	This study
PLY4108	SEY6210; <i>vps4Δ::KanR vps28::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Figs 4, 5 (A,B)	This study
PLY4110	SEY6210; <i>vps4Δ::KanR vps36::GFP-HIS5MX CUP1-Hse1-UL36-HA::URA3</i>	Figs 4, 5 (A,B)	This study

Table 2 | Plasmids used in the study

Plasmid	Description	Use	Reference
pPL4586	Yeast integrating plasmid-encoding <i>CUP1-HSE1-UL36-HA</i> in a <i>URA3</i> -based integrating plasmid (pRS303)	Throughout	This study
pPL3878	pRS315-expressing GFP-Ub from <i>PRC1</i> promoter	Fig 1B,C	Ren <i>et al</i> [8]
pPL4146	pRS315-expressing Mup1-GFP from <i>CUP1</i> promoter	Fig 2B	Stringer & Piper [7]
pPL4147	pRS315-expressing Mup1-GFP-Ub from <i>CUP1</i> promoter	Fig 2B	Stringer & Piper [7]
pPL4012	pRS316-expressing HSE1-UL36-HA from <i>CUP1</i> promoter	Fig 3B	Stringer & Piper [7]
pPL4131	pRS316-expressing HSE1- <i>ul36^{C>A}</i> -HA from <i>CUP1</i> promoter	Fig 3C	Stringer & Piper [7]
pPL3484	pRS315-expressing Ste3-GFP-Ub from <i>STE3</i> promoter	Fig 3C	Bilodeau <i>et al</i> [17]
pPL3267	pRS315-expressing Ub-GFP-Cps1 from <i>PRC1</i> promoter	Fig 3C	Ren <i>et al</i> [8]
pPL4656	pRS313-expressing Vps27-GFP from <i>VPS27</i> promoter	Figs 4A, 5A,B	This study
pPL4064	pRS315-expressing Sna3-RFP from <i>SNA3</i> promoter	Fig 5A	This study

To visualize ESCRT-GFP, cells were grown in minimal media, coverslipped on glass slides and photographed during the first 3 s of exposure of excitation light to avoid photobleaching. Measuring secretion of newly synthesized CPY by pulse/chase immunoprecipitation was performed as described [4].

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: CM and NJB built reagents, conducted the experiments and analysed data. RCP and SDE helped design experiments and analyse data. CM wrote the manuscript. DKS provided initial observations and methodology underpinning the study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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