The intralumenal fragment pathway mediates ESCRT-independent surface transporter down-regulation

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Surface receptor and transporter protein down-regulation is assumed to be exclusively mediated by the canonical multivesicular body (MVB) pathway and ESCRTs (Endosomal Sorting Complexes Required for Transport). However, few surface proteins are known to require ESCRTs for down-regulation, and reports of ESCRT-independent degradation are emerging, suggesting that alternative pathways exist. Here, using *Saccharomyces cerevisiae* as a model, we show that the hexose transporter Hxt3 does not require ESCRTs for down-regulation conferring resistance to 2-deoxyglucose. This is consistent with GFP-tagged Hxt3 bypassing ESCRT-mediated entry into intralumenal vesicles at endosomes. Instead, Hxt3-GFP accumulates on vacuolar lysosome membranes and is sorted into an area that, upon fusion, is internalized as an intralumenal fragment (ILF) and degraded. Moreover, heat stress or cycloheximide trigger degradation of Hxt3-GFP and other surface transporter proteins (Itr1, Aqr1) by this ESCRT-independent process. How this ILF pathway compares to the MVB pathway and potentially contributes to physiology is discussed.
Surface polytopic proteins including receptors, transporters, and channels are internalized and sent to lysosomes for degradation. Precise control of their surface levels underlies diverse physiology, including endocrine function, wound healing, tissue development, nutrient absorption, and synaptic plasticity. Damaged surface proteins are also cleared by this mechanism to prevent proteotoxicity. To trigger this process, surface proteins are labeled with ubiquitin—in response to changing substrate levels, heat stress to induce protein misfolding or cellular signaling for example—and then selectively internalized by the process of endocytosis.

The within-cell, they are sent to endosomes where they encounter ESCRTs (endosomal sorting complexes required for transport). These five protein complexes (ESCRT-0, -I, -II, -III, and the Vps4 complex) sort and package these internalized surface proteins into IntraLuminal Vesicles (ILVs). After many rounds, ILVs accumulate creating a mature multivesicular body (MVB). The MVB then fuses with lysosomes to expose protein laden ILVs to lumenal hydrolases for catabolism.

Although many examples of ESCRT-mediated protein degradation have been published, reports of ESCRT-independent degradation of surface proteins are emerging. These protein complexes (ESCRT-0, -I, -II, -III, and the Vps4 complex) sort and package these internalized surface proteins into IntraLuminal Vesicles (ILVs). After many rounds, ILVs accumulate creating a mature multivesicular body (MVB).

The MVB then fuses with lysosomes to expose protein laden ILVs to lumenal hydrolases for catabolism.

Upon reexamination of micrographs presented in earlier reports on receptor and transporter down-regulation, we found that some internalized surface polytopic proteins appear on vacuole membranes on route to the lumen for degradation, e.g., the high-affinity tryptophan permease Tat238, peptide transporter Ptr239, myo-inositol transporter Itr140, and glucose transporters Hxt1 and Hxt341. We also noticed that most of these published studies do not directly assess whether ESCRTs are required for protein degradation. Given that internalized surface transporters and receptors can appear on vacuole membranes, we decided to test the hypothesis that the ILF pathway represents an alternative, ESCRT-independent mechanism for degradation of surface polytopic proteins (Fig. 1a).

**Results**

ESCRTs are not required for 2-deoxyglucose resistance. ESCRTs have been implicated in the down-regulation of surface transporters required for *Saccharomyces cerevisiae* cell survival and proliferation in the presence of toxic substrates. For example, to prevent entry of the toxic arginine analog canavanine, the surface arginine permease Can1 is endocytosed and sorted for degradation by ESCRTs. Similarly, the surface glucose transporter Hxt3 is internalized and degraded in the presence of 2-deoxyglucose, a toxic glucose analog.

We tested this hypothesis by treating yeast cultures with increasing concentrations of either toxin and then assessed effects on cell viability by imaging and counting dead yeast cells stained with methylene blue. As expected, deleting components of ESCRT-0 (VPS27) or ESCRT-II (VPS36)—to disrupt different early- or late-acting complexes, respectively, necessary for protein sorting into ILVs—enhanced sensitivity to the toxin canavanine. However, deleting these ESCRT genes had no effect on cell viability in the presence of 2-deoxyglucose. As controls, we examined cells lacking CAN1 or HXT3 and found that they were resistant to canavanine or 2-deoxyglucose, respectively, as predicted. Thus, these data suggest that ESCRTs mediate degradation of Can1 but may not be required for Hxt3 degradation triggered by 2-deoxyglucose.

**Hxt3 protein degradation is ESCRT-independent.** Based on micrographs presented in previous reports showing Hxt3 on vacuole membranes, we first assessed the possibility that internalized Hxt3 bypassed ESCRTs altogether at the endosome and instead were delivered to vacuole membranes where they may be sorted for degradation. To test this hypothesis, we used fluorescence microscopy to monitor the distribution of GFP-tagged Hxt3 in live *S. cerevisiae* cells over time after addition of 2-deoxyglucose. As controls, we examined cells lacking CAN1 or HXT3 and found that they were resistant to canavanine or 2-deoxyglucose, respectively, as predicted. Thus, these data suggest that ESCRTs mediate degradation of Can1 but may not be required for Hxt3 degradation triggered by 2-deoxyglucose.
internalized Can1-GFP, an ESCRT-client, never appears on vacuole membranes on route to the vacuole lumen for degradation when cells were treated with canavanine (Fig. 1c–e), as predicted for the canonical MVB pathway. To confirm that proteolysis occurs, we conducted western blot analysis to detect cleavage of GFP from Hxt3 or Can1 in whole-cell lysates regardless of route taken to the vacuole lumen.
In all, these findings suggest that internalized Hxt3-GFP bypasses ESCRT function at endosomes, and instead is delivered to vacuole membranes on route to the lumen where it is degraded in response to 2-deoxyglucose. If true, then deleting ESCRT genes should have no effect on this process. To test this hypothesis, we tracked the subcellular distribution of Hxt3-GFP after 2-deoxyglucose treatment in cells lacking the ESCRT gene VPS36 (Fig. 1c–e). Although Hxt3-GFP continues to be delivered to the vacuole lumen over time, we noticed that on route, Hxt3-GFP abnormally accumulated on puncta and vacuole membranes, even prior to treatment, in mutant cells. This is consistent with previous reports showing that deleting ESCRT genes impairs, but does not entirely block, endocytosis and delivery of vacuole cargo proteins in addition to completely abolishing protein sorting into ILVs. Importantly, GFP continued to be cleaved from Hxt3 (Fig. 1f) confirming that it continues to be degraded in absence of VPS36. Thus, delivery of internalized Hxt3-GFP to the vacuole lumen for degradation in response to 2-deoxyglucose does not require ESCRTs.

2-deoxyglucose triggers Hxt3 degradation by ILF pathway. What mediates ESCRT-independent Hxt3-GFP degradation? Because this surface polytopic proteins appear on the vacuole membrane, we reasoned that this is where it is sorted and packaged into intralumenal vesicles or fragments for degradation. Although microautophagy may be responsible, no intermediate structures resembling elongated invaginations extending into the vacuole lumen were observed and there are no reports of selective protein sorting associated with this process (rather these structures seem to be devoid of membrane proteins). Instead, we considered two processes reported to mediate selective degradation of vacuole membrane proteins: the ESCRT-dependent vReD (vacuole membrane Recycling and Degradation) pathway and ESCRT-independent ILF pathway. Given that ESCRTs were not required for Hxt3-GFP down-regulation, we eliminated the possibility that the vReD pathway was responsible and focused on the ILF pathway.

Upon further examination of micrographs showing Hxt3-GFP distributed on vacuole membranes after toxin addition, we found that it was present within boundary membranes between docked vacuoles, the area of membrane that is internalized as an ILF and degraded upon fusion (Fig. 2a, b). To confirm, we measured the GFP fluorescence within the vacuole boundary membranes in the cell population (Fig. 2c). To determine if Hxt3-GFP was getting sorted into the boundary, we compared its distribution to three GFP-tagged ILF-client proteins that are residents of the vacuole membrane: Fet5 (a copper oxidase) which is known to be excluded from boundaries, Vph1 (the stalk domain of the V-type H^+ATPase) which is distributed uniformly on vacuole membranes, and Fth1 (an iron transporter) which is enriched in boundaries (Fig. 2b–d; see ref. 37). From this analysis, it was clear that the Hxt3-GFP boundary profile was most similar to Fth1-GFP (Fig. 2b, c), suggesting that it was enriched and thus possibly sorted into the ILF pathway.

We also found that the distribution of these three GFP-tagged resident vacuole proteins was unaffected by 2-deoxyglucose (Fig. 2d), suggesting that their turnover was not stimulated by this toxin. Similarly, 2-deoxyglucose does not trigger down-regulation of GFP-tagged Can1 or the surface myo-inositol transporter Itrl (Fig. 2d). Moreover, Hxt3-GFP was not down-regulated when cells were treated with canavanine (Fig. 2a). We assessed all cells imaged, quantified these observations and confirmed that only Hxt3-GFP was downregulated by 2-deoxyglucose but it was unaffected by canavanine (Fig. 2e). These important findings imply that Hxt3-GFP was selectively sorted into the ILF pathway in response to only its cognate toxic substrate 2-deoxyglucose.

Finally, to demonstrate that Hxt3-GFP was internalized into the lumen during fusion we recorded homotypic vacuole membrane fusion events in live cells treated with 2-deoxyglucose, and confirmed that Hxt3-GFP decorated ILFs created by fusion events (Fig. 2f; Supplementary Movie 1). Homotypic vacuole fusion was not stimulated by 2-deoxyglucose, as we counted the same number of fusion events within cells in the absence (CTL) and presence of the toxin (Fig. 2g). Thus, vacuole fusion persists under these conditions and seems to mediate the selective degradation of Hxt3 after endocytosis.

Together, these observations led us to conclude that Hxt3 avoids ESCRTs and is instead selectively sorted for degradation by the ILF pathway. Two questions immediately arose after making this exciting discovery: Is surface protein degradation by the ILF pathway only triggered by toxic substrates, or does it contribute to other triggers of surface transporter downregulation? Is the ILF pathway responsible for degradation of other surface polytopic proteins? In other words, how pervasive is the contribution of the ESCRT-independent ILF pathway to surface transporter and receptor protein downregulation?

ILF pathway degrades Hxt3 in response to diverse stimuli. When yeast cells are challenged with acute heat stress, surface
**Fig. 2** Hxt3-GFP is selectively degraded by the ILF pathway in response to 2-deoxyglucose. **a** Micrographs of live wild type cells expressing GFP-tagged Hxt3 after treatment with 2-deoxyglucose for 30 min, or canavanine for 30 min or 6 h. Vacuole membranes were stained with FM4–64. Arrowhead indicates GFP on the vacuole membrane. **b** Line plots of GFP or FM4–64 fluorescence intensity for lines shown in **a** and **d**, to indicate vacuole membrane localization. GFP values greater than the FM4–64 signal at boundaries (near 2, for two membranes) indicate enrichment. **c** Using micrographic data presented in **a** and **d**, we generated cumulative probability plots of GFP-tagged Hxt3, Fet5, Vph1 or Fth1 fluorescence measured within the boundary membrane of docked vacuoles within live wild-type cells in the absence or presence of 2-deoxyglucose (2DG). Averages ± S.E.M. are shown in insets. *P > 0.05, as compared to Vph1 by t-test. n = 3 experiments whereby 72 Vph1-GFP, 77 Fet5-GFP, 107 Fth1-GFP or 82 Hxt3-GFP + 2DG boundaries within cells were analyzed.**

**d** Micrographs of live wild type cells expressing GFP-tagged resident vacuole transporters Fet5, Vph1 or Fth1, or surface transporters Can1 or Itr1, before (control) after treatment with 2-deoxyglucose for 30 min. Vacuole membranes were stained with FM4–64. **e** Using micrographs shown in **a** and **d**, we calculated the proportion of wild type cells showing GFP fluorescence on the plasma membrane (PM), intracellular puncta, vacuole membrane (Vac Mem) or vacuole lumen (Vac Lumen) after treatment with 2-deoxyglucose or canavanine. For control conditions, n = 218 Hxt3-GFP, 187 Fet5-GFP, 265 Vph1-GFP, 267 Fth1-GFP, 233 Can1-GFP, and 189 Itr1-GFP cells. After 2-deoxyglucose treatment, n = 200 Hxt3-GFP, 143 Fet5-GFP, 161 Vph1-GFP, 158 Fth1-GFP, 150 Can1-GFP, and 178 Itr1-GFP cells. For canavanine treatment, n = 171 or 166 Hxt3-GFP cells after 0.5 or 6 h, respectively. **f** Images from time-lapse video showing a homotypic vacuole fusion event within a live wild-type cell expressing Hxt3-GFP stained with FM4–64 to label vacuole membranes and treated with 2-deoxyglucose. Arrowhead indicates newly formed ILF. See Supplementary Movie 1. **g** Analysis of micrographic data shown in **f** showing the proportion of cells that displayed a vacuole fusion event within 5 min in the absence (CTL) or presence of 2-deoxyglucose (2DG). Averages ± S.E.M. are shown. *P > 0.05, when 2DG was compared to CTL by t-test. n = 5 experiments whereby a total of 121 (CTL) or 174 (2DG) Hxt3-GFP cells were analyzed. Scale bars, 1 µm
polytopic proteins are thought to misfold, which disrupts their function and contributes to membrane permeabilization preceding cell death. If they cannot be refolded (through the activity of chaperones), they are internalized and sent to the vacuole lumen for degradation—presumably by the MVB pathway—to mediate surface polytopic protein quality control20.

Thus, to answer the first question, we tested whether treating cells with heat stress also triggers Hxt3-GFP degradation by the ILF pathway using fluorescence microscopy to monitor its distribution within living cells. As with 2-deoxyglucose, we found that Hxt3-GFP is internalized from the surface after heat stress and accumulates on vacuole membranes (Fig. 3a, b) within boundaries between docked organelles (Fig. 3a, c) and in the vacuole lumen (Fig. 3d). We confirmed that Hxt3-GFP decorated ILFs formed during homotypic vacuole fusion events in live cells (Fig. 3e; Supplementary Movie 2), which correlated with enhanced Hxt3-GFP degradation as assessed by western blot analysis of whole-cell lysates expressing Hxt3-GFP after heat stress (HS). Western blot analysis of whole-cell lysates prepared from wild-type or vps36Δ cells expressing GFP-tagged Fet5 (excluded), Vph1 (ubiquitous) and Fth1 (enriched) are shown for reference (see Fig. 2D). Averages ± S.E.M. are shown in insets. *P < 0.05, as compared to Vph1 by t-test. n = 3 experiments whereby a total of 81 WT or 102 vps36Δ cells were analyzed under control conditions, and 75 WT or 88 vps36Δ cells were analyzed after heat stress. e Images from time-lapse videos showing homotypic vacuole fusion events within live wild type or vps36Δ cells expressing Hxt3-GFP treated with heat stress. Vacuole membranes were stained with FM4-64. Arrowheads indicate newly formed ILFs. See Supplementary Movies 2 and 4. Example shown is a representation of n = 4 experiments. f Western blot analysis of whole-cell lysates prepared from wild-type or vps36Δ cells expressing Hxt3-GFP after heat stress (HS) stained with anti-GFP antibody. Estimated molecular weights and cleaved GFP band densities relative to CTL normalized to load controls (G6PDH) are shown. Blots shown are representatives of n = 3 experiments. Scale bars, 1 μm.

Fig. 3 Quality control of Hxt3-GFP is mediated by the ILF pathway. a Fluorescence and DIC micrographs of live wild type or vps36Δ cells expressing GFP-tagged Hxt3 before (control) or after heat stress (37 °C for 15 min). Vacuole membranes were stained with FM4-64. Arrowsheads indicate GFP on the vacuole membrane. 3-dimensional GFP fluorescence intensity (FI) plots and line plots of Hxt3-GFP or FM4-64 fluorescence intensity for lines shown in a, to indicate vacuole membrane localization after heat stress. b Micrographic data shown in a was used to calculate the proportion of wild type or vps36Δ cells that show Hxt3-GFP fluorescence on the plasma membrane (PM), intracellular puncta, vacuole membrane (Vac Mem) or vacuole lumen (Vac Lumen) before or after treatment with heat stress. Under control (CTL) conditions, n = 218 WT cells or 243 vps36Δ cells under control conditions (CTL); n = 163 WT cells or 196 vps36Δ cells after heat stress. c, d Micrographic data shown in a was used to generate cumulative probability plots of Hxt3-GFP fluorescence measured within the boundary membrane (c) or lumen (d) of vacuoles within live wild type or vps36Δ cells before or after heat stress (HS). GFP-tagged Fet5 (excluded), Vph1 (ubiquitous) and Fth1 (enriched) are shown for reference (see Fig. 2D). Averages ± S.E.M. are shown in insets. *P < 0.05, as compared to Vph1 by t-test. n = 3 experiments whereby a total of 81 WT or 102 vps36Δ cells were analyzed under control conditions, and 75 WT or 88 vps36Δ cells were analyzed after heat stress. e Images from time-lapse videos showing homotypic vacuole fusion events within live wild type or vps36Δ cells expressing Hxt3-GFP treated with heat stress. Vacuole membranes were stained with FM4-64. Arrowheads indicate newly formed ILFs. See Supplementary Movies 2 and 4. Example shown is a representation of n = 4 experiments. f Western blot analysis of whole-cell lysates prepared from wild-type or vps36Δ cells expressing Hxt3-GFP after heat stress (HS) stained with anti-GFP antibody. Estimated molecular weights and cleaved GFP band densities relative to CTL normalized to load controls (G6PDH) are shown. Blots shown are representatives of n = 3 experiments. Scale bars, 1 μm.
quality control and degrades this transporter protein in response to cycloheximide.

ILF pathway machinery mediates Hxt3-GFP degradation in vitro. To verify that the observed degradation of internalized Hxt3-GFP was conducted by the ILF pathway machinery, we repeated experiments using in vitro fusion reactions containing isolated vacuoles based on the following reasoning [see 37]: (1) all molecular machinery underlying the ILF pathway copurifies with vacuoles, in a preparation that excludes possible cytoplasmic contributors to Hxt3-GFP degradation (e.g., the proteasome) as well as the protein translation machinery to eliminate interference by biosynthesis. (2) activation of the Rab GTPase Ypt7 is critical for sorting proteins into the ILF pathway. However, deleting YPT7 chronically blocks all vacuole fusion events including MVB-vacuole fusion preventing study of the ILF and MVB pathways in live cells. Instead, purifying protein inhibitors of Ypt7–rGdi1 (a Rab-GTPase chaperone protein that extracts Ypt7 from membranes) and Gyp1-46 (the catalytic domain of the Rab GTPase activating protein Gyp1 that inactivates Ypt7 [45]) can be added to in vitro vacuole fusion reactions containing healthy organelles to acutely block Ypt7 permitting study of protein sorting into ILFs. Thus, if the ILF machinery is responsible for Hxt3-GFP degradation, then it should continue to occur in vitro and be sensitive to Ypt7 inhibitors.
To test this hypothesis, we first isolated vacuoles from untreated wild-type cells, imaged them and found that Hxt3-GFP decorated their membranes (Fig. 5a). Further investigation revealed that surface Hxt3-GFP is down-regulated in live yeast cells during the organelle isolation procedure (e.g., during spheroplasting when their cell walls are enzymatically removed; Fig. 5a). We made a similar observation when live cells were withdrawn from glucose for 5 min in growth medium (Fig. 5a), a
novel trigger of Hxt3-GFP degradation. All solutions for the organelle isolation procedure are devoid of glucose. Thus, we reasoned that glucose depletion during vacuole purification triggers Hxt3-GFP downregulation, accounting for its presence on vacuole membranes and within the lumen in vitro.

Next, we added ATP to isolated vacuoles to initiate homotypic fusion in vitro and imaged reactions 30 min afterwards (Fig. 5b). As expected, we found that Hxt3-GFP was present in boundary membranes (Fig. 5c) and within the vacuole lumen (Fig. 5d). Consistent with these findings, we found that more Hxt3-GFP was degraded after fusion by western blot analysis (Fig. 5e). Thus, heat stress or CHX further enhances clearance of Hxt3-GFP in vivo (see Figs. 3 and 4). We reasoned that this is because Hxt3-GFP found on isolated vacuole membranes is already marked for degradation, as its presence is the product of downregulation presumably triggered by glucose withdrawal during the organelle isolation procedure (Fig. 5a) and it is degraded during fusion in vitro under control (unstimulated) conditions (Fig. 5b–e). This step of the procedure requires 60 min, which may explain why band patterns look similar to those observed at 60 min after 2-deoxyglucose treatment in vivo (Fig. 1f). This also explains why relatively high levels of lumenal GFP fluorescence (~60% of total) are observed in vacuole preparations prior to fusion (Fig. 6b). Thus, we are confident that the observed changes in GFP-cleavage reflect lumenal Hxt3-GFP degradation that correlates with internalization during vacuole fusion, suggesting that the ILF pathway mediates Hxt3-GFP degradation in vitro.

Importantly, both heat stress and CHX significantly increased the amount of Hxt3-GFP present in the boundary (Fig. 5b, c), internalized (Fig. 5d) and degraded (Fig. 5e) by the ILF pathway in vitro. We made similar observations when experiments were repeated with vacuoles isolated from vps36Δ cells (Fig. 5b–e), confirming that ESCRTs are not required for protein sorting. However, these responses were not as robust as those observed in vivo (see Figs. 3 and 4). We reasoned that this is because Hxt3-GFP found on isolated vacuole membranes is already marked for degradation, as its presence is the product of downregulation presumably triggered by glucose withdrawal during the organelle isolation procedure (Fig. 5a) and it is degraded during fusion in vitro under control (unstimulated) conditions (Fig. 5b–e). Thus, heat stress or CHX further enhances clearance of Hxt3-GFP that is already destined for degradation. Because these responses are additive, we reasoned that heat stress and CHX likely target distinct mechanisms, at least in part, from those that respond to glucose withdrawal. Because heat stress and CHX trigger Hxt3-GFP degradation in vitro, the underlying machinery must be present on vacuole membranes, whereas the machinery that senses glucose withdrawal must be, in part, present on the plasma membrane to accommodate endocytosis, and neither likely include ESCRTs.

Next, we pretreated in vitro vacuole fusion reactions with Ypt7 inhibitors and found that they blocked Hxt3-GFP sorting, internalization and degradation (Fig. 5b–e), implicating Ypt7 and its effectors in Hxt3-GFP sorting into boundary membranes.
However, closer examination of western blots revealed that some residual Hxt3-GFP cleavage occurred in the presence of inhibitors under all conditions tested (Fig. 5e; compare 0–120 min with fusion inhibitors). One explanation is that perhaps some vacuoles were docked, and contained Hxt3-GFP in their boundary membranes, upon isolation (i.e., they were engaged in the fusion process in live cells before lysis). If so, then Ypt7 inhibitors would be unable to block subsequent Ypt7-independent stages of fusion responsible for Hxt3-GFP internalization and degradation, accounting for the observed residual GFP-cleavage. This also accounts for the small fraction of the vacuole population that contains relatively high levels of Hxt3-GFP fluorescence in the boundary and lumen in the presence of Ypt7 inhibitors (Fig. 5c, d). Thus, all things considered, we are confident that Ypt7 and the ILF pathway machinery located on the vacuole membrane is likely responsible for Hxt3-GFP degradation triggered by diverse stimuli.

Clearance of ESCRT-client proteins from endosome membranes is thought to require multiple rounds of ILV formation3, raising the question: How efficient is protein clearance from vacuole membranes by the ILF pathway? To answer it, we initiated homotypic vacuole fusion in vitro with ATP and imaged reactions every 30 min over 2 h (Fig. 6a). Some GFP fluorescence was observed in the vacuole lumen prior to fusion (0 min) and this signal increased over the course of the fusion reaction (Fig. 6b). Moreover, the amount of Hxt3-GFP on vacuole membranes decreased over time (Fig. 6c), confirming that this transporter was getting internalized into the lumen during the fusion reaction. Similar observations were made after isolated vacuole were subjected to heat stress prior to initiation of fusion (Fig. 6a–c), except the effect was more pronounced whereby nearly all of Hxt3-GFP on the membrane was internalized into the lumen after 120 min (also see Fig. 6d), consistent with complete degradation observed by western blot analysis (Fig. 5c). Previously, we showed that heat stress has no effect on the rate of vacuole fusion in vitro37 and it is known that the population of isolated vacuoles undergoes 1.5 fusion events after 120 min under these conditions46. Thus, we estimate that Hxt3-GFP can be completely cleared from vacuole membranes within two fusion events.

ILF pathway mediates quality control of many surface proteins. Is the ILF pathway responsible for downregulation of other surface transporter proteins? To answer this (second) question, we used fluorescence microscopy to screen for other ILF-client proteins by examining the intracellular membrane distribution of GFP-tagged surface transporter or receptor proteins after live cells were challenged with heat stress, which should trigger degradation of all surface polytopic proteins. Five surface proteins showed clear phenotypes: Unlike Can1, Ste3 (a G-protein coupled receptor) or Mup1 (a methionine permease [see 47]), internalized GFP-tagged Itr1 (a myo-inositol transporter)40 and Aqr1 (a major facilitator superfamily-type transporter that excretes amino acids)48 appeared on vacuole membranes on route to the vacuole lumen for degradation (Fig. 7a, c). Both transporters were present within boundary membranes between organelles (Fig. 7b) and accumulated in the vacuole lumen of live wild type cells after heat stress (Fig. 7d). This correlated with enhanced Itr1-GFP and Aqr1-GFP degradation after heat stress as assessed by western blot analysis of whole-cell lysates (Fig. 7e). Importantly, deleting VPS27, a component of ESCRT-0, had no effect on the, internalization, sorting or degradation of Itr1-GFP or Aqr1-GFP after heat stress (Fig. 7a–e), confirming that the ESCRT-independent ILF pathway is critical for quality control of these transporter proteins. Thus, the ILF pathway mediated degradation of three of six proteins examined, suggesting that it may play a considerable role in surface transporter protein downregulation.

Discussion

Here we demonstrate that whereas some internalized surface polytopic proteins (Can1) rely on the canonical ESCRT-dependent MVB pathway for downregulation, other presumed ESCRT-client proteins (Hxt3, Itr1, Aqr1) bypass ESCRT function at endosomes and, upon MVB-vacuole fusion, are delivered to vacuole membranes where they are selectively sorted for degradation by the ILF pathway (see Fig. 1a). Both pathways are triggered by the presence of toxic substrates, substrate withdrawal or cycloheximide and are critical for protein quality control. Thus, the assumption that all surface proteins are degraded by the MVB pathway is no longer reasonable, and we speculate that the ILF pathway may play an equally important role in surface polytopic protein downregulation. This conclusion raises many new questions concerning this important area of fundamental cell biology: What evidence supports ESCRT-mediated protein down-regulation? What distinguishes one pathway from the other? What determines protein entry into each pathway? What is the potential impact of this discovery on eukaryotic physiology?

Herein, we reveal that internalized surface polytopic proteins may take two distinct pathways to the vacuole lumen for degradation. Paramount to this discovery was visualizing internalized surface receptors within live cells over time to track their routes to the vacuole lumen for degradation (e.g., Figure 1c). This revealed that some internalized proteins accumulate on the vacuole membrane: a trafficking intermediate that does not occur in the MVB pathway, but is a requirement for the ILF pathway. Unfortunately, the majority of previous reports on surface protein degradation do not provide such detailed kinetic analyses and instead present only light micrographs of cells before and after treatment, indicating surface and vacuole lumen protein distributions respectively35,13,14,41,49–52. Many important papers originally describing degradation of surface receptors or transporters do not include any micrographs53. Thus, the pathway responsible for their degradation now seems enigmatic. Moreover, when comprehensive datasets are provided, many internalized surface transporters appear on vacuole membranes58, suggesting that ILF-dependent degradation of surface proteins may be widespread.

Further confounding this issue is that protein sorting and ILV formation by ESCRTs cannot be accurately visualized in biological systems using light microscopy due to limitations of spatial resolution. Rather, samples must be fixed and imaged using electron microscopy coupled with immune-gold labeling. Due to associated technical challenges, very few reports provide electron micrographs to definitively demonstrate protein sorting or internalization by ESCRTs54–56. However, for the ILF pathway, here we show surface transporters being delivered to the lumen during homotypic vacuole fusion in real time within live cells using HILO microscopy (Figs. 2f, 3e, and 4e; Supplementary Movies 1–5). In addition to providing definitive proof that the ILF pathway contributes to degradation of these transporters, this approach (including FRAP37) offers the advantage of allowing us to study the fundamental properties of protein sorting for transporter and receptor downregulation.

Finally, although we present data from complimentary experimental approaches to support our conclusions, we recognize that both pathways rely on the same membrane trafficking route within cells, i.e. endocytosis culminating with MVB-vacuole fusion (see Fig. 1a). As such, we expect that genetic manipulations affecting both pathways will cause pleiotropic phenotypes. For example, in addition to blocking ILV formation, deleting ESCRT
genes partially inhibits MVB-vacuole fusion, which is required to deliver internalized proteins to the ILF pathway, and prevents efficient delivery of some biosynthetic cargoes to the vacuole lumen (e.g., carboxypeptidase S and other vacuole hydrolases). This perhaps explains why Hxt3-GFP accumulates on puncta and the vacuole membrane in vps36Δ cells (e.g., Fig. 1c–e) and its degradation profile is different than in wild-type cells (Figs. 1f, 3f, 4f, 5e, and 7e). If true, then appearance of internalized proteins on these aberrant structures in ESCRT knockout cells is not a phenotype exclusive to ESCRT-client proteins, warranting reevaluation of published data that relies on this phenotype to infer protein degradation by the MVB pathway. All things considered, we provide a logical framework to determine which pathway mediates surface protein degradation and are currently conducting screens to comprehensively understand the contributions of each to surface receptor and transporter down-regulation in S. cerevisiae.

Fig. 7 Quality control of surface transporters Itr1 and Aqr1 is mediated by the ILF pathway. a Fluorescence and DIC micrographs of live wild type (top) and vps27Δ (bottom) cells expressing GFP-tagged Hxt3, Itr1, Aqr1, Ste3, or Mup1 before (control) and after heat stress (37 °C for 15 min). Vacuole membranes were stained with FM4–64. Arrowheads indicate GFP on the vacuole membrane. Scale bar, 1 μm. b Three-dimensional GFP fluorescence intensity (FI) plots and line plots (left) of GFP or FM4–64 fluorescence intensity for lines shown in a to indicate boundary membrane localization after heat stress. c, Using micrographic data shown in a, we measured the proportion of wild type or vps27Δ cells with GFP fluorescence observed on the vacuole membrane (e) or within the lumen (d) before (CTL) or after heat stress. Averages ± S.E.M. are shown. *P < 0.05, as compared to CTL by t-test. n = 4 experiments whereby a total of 238 Hxt3-GFP cells, 180 Itr1-GFP cells, 180 Aqr1-GFP cells were analyzed under CTL conditions, and 222 Hxt3-GFP cells, 161 Itr1-GFP, 167 Aqr1-GFP cells were analyzed after HS. e Western blot analysis of whole-cell lysates prepared from wild type or vps27Δ cells expressing Itr1-GFP or Aqr1-GFP before (CTL) or after heat stress (HS) stained with anti-GFP antibody. Estimated molecular weights and cleaved GFP band densities (Dens.) relative to CTL normalized to load controls (G6PDH) are shown. Blots are representatives of n = 3 experiments.
shared with the MVB pathway, which employs protein ubiquityla-
tion as a label for selective degradation. This is based on the fol-
lowing reasoning: (1) prior to this work, Hxt3, Itr1 and Aqr1 were re-
ported to be ubiquitylated by specific E3-ligase and E4-
adapter proteins in response to the same stimuli that drive their
downregulation by the ILF pathway (e.g., Rsp5 and Art5, respec-
tively, for Itr1). 2 The same ubiquitylation machinery labels polytopic proteins at the plasma membrane, endosome membranes, and vacuole membranes (e.g., E3 ligases Rsp5 and Pib1, and E4
adapter Shd4). This possibly explains why heat stress triggers
degradation of Hxt3-GFP on isolated vacuole membranes in vitro
(Figs. 5 and 6), as it does for Hxt3-GFP on the plasma membrane in
live cells (Figs. 3 and 7). 3 When ESCRT genes are deleted, ESCRT-
client proteins Can1, Ste3, and Mup1 continue to be degraded,
whereas the function of ESCRT clients, such as Can1, may be
therefore, each ILV generated by ESCRTs has an estimated membrane area of 0.03μm² (assuming it is spherical with a radius of 0.05 μm) whereas each ILV generated by a
vacuole fusion event has an area up to 2.26 μm² (assuming it is a
double boundary membrane disk with a radius of 0.6 μm, average vacuole radius of 1.0 μm, and all of the boundary membrane is
internalized). Thus, membrane internalized by a single ILF is
assumed to be ubiquitylated by specific E3-ligase and E4-
adapter proteins in response to the same stimuli that drive their
downregulation by the ILF pathway (e.g., Rsp5 and Art5, respec-
tively, for Itr1). This possibly explains why heat stress triggers
degradation of Hxt3-GFP on isolated vacuole membranes in vitro
(Figs. 5 and 6), as it does for Hxt3-GFP on the plasma membrane in
live cells (Figs. 3 and 7). When ESCRT genes are deleted, ESCRT-
client proteins Can1, Ste3, and Mup1 continue to be degraded,
whereas the function of ESCRT clients, such as Can1, may be
degradation within eukaryotic cells. Through these different mechan-
isms, we speculate that the ILF pathway plays a distinct but
equally important role as the MVB pathway in surface polytopic
protein degradation.

Surface receptor and transporter protein downregulation is critical for diverse physiology in all eukaryotes. ESCRT-
dependence has been demonstrated for only a handful of human
receptors and transporters. These represent a minuscule fraction of an estimated ~5500 polytopic proteins encoded by
the human genome, all of which presumably have finite lifetimes and must be selectively degraded. Thus, for the subset
that represents surface polytopic proteins, it is reasonable to
propose that this burden is split by at least two pathways. For
example, here we discovered that the ILF pathway mediates
downregulation of the yeast hexose transporter (Hxt3) when
is withdrawn from the growth medium (Fig. 5a). Downregulation of orthologous glucose transporters (e.g., GLUT1) triggered by glucose depletion is observed in mamar-
alian epithelial cells lining the ileum or proximal tubule of the
nephron but it is not clear what mediates this process, which
is needed for sugar (re)absorption. Genes encoding the
proposed machinery underlying the ILF pathway are conserved
in all eukaryotes including human, like genes encoding
ESCR Ts. Thus, it is tempting to speculate that the ILF pathway
also contributes to the down-regulation of these sugar trans-
porters, as well as other receptors and transporters, in mamar-
ian cells.

Why two pathways? Evolutionary theory predicts that each pathway must perform a distinct, fundamental function
necessary for eukaryotic physiology to be conserved. The concept of two pathways for surface polytopic protein
downregulation is obviously still in its infancy, but in support, we
offer three speculative explanations for their coexistence: (1)
whereas, the function of ESCRT clients, such as Can1, may be
unnecessary or not tolerated on vacuole membranes, ILF-client
proteins may function on vacuoles (or lysosomes) as well as on
the plasma membrane. However, this is unlikely for Hxt3
because after it is endocytosed, it concentrates in boundary
membranes (Figs. 2c, 3c, 4c, and 5c) and is efficiently cleared
from vacuole membranes (see Fig. 6). (2) Unlike ILF clients,
ESCRT-client proteins may require immediate sequestration
from the cytoplasm after endocytosis. For example, activated
hormone receptors must be sequestered into ILVs for accurate
signal termination. (3) ESCRT clients are can be reused either
by ILV back fusion, i.e., membrane fusion between ILV and
MVB perimenter membranes (although this is contentious) or by
MVB-plasma membrane fusion which releases protein-laden
ILVs as exosomes. Whereas degradation is certain for ILF-
client proteins.

In addition to their role in the MVB pathway, ESCRTs are
recruited to other compartmental membranes where they make
important contributions to cytokinesis, reformation of the
nuclear envelope, and exosome release—process that terminate
with membrane fission. Orthologous components of the ILF
machinery (e.g., CORVET) are present on other endosomal
compartments where it drives fusion events needed for mem-
brane trafficking as well as organelle biogenesis and
homeostasis. Thus, from a broader perspective, it seems that
ESCRTs drive membrane remodeling prior to fission, whereas
the components of the ILF pathway remodel membranes prior to
fusion within eukaryotic cells. Through these different mechan-
isms, we speculate that the ILF pathway plays a distinct but
equally important role as the MVB pathway in surface transporter and receptor downregulation.
Table 1 Yeast strains used in this study

| Strain      | Genotype                  | Source                  |
|-------------|---------------------------|-------------------------|
| BY4741      | MATa his3-D1 leu2-3,112 met15-40 ura3-1          |           |
| Can1-GFP    | BY4741, Can1-GFP:His3MX   |            |
| Ste3-GFP    | SEY6210, Ste3-GFP:KanMX   |            |
| Mup1-GFP    | SEY6210, Mup1-GFP:KanMX   |            |
| Hxt3-GFP    | BY4741, Hxt3-GFP:His3MX   |            |
| Hxt3-GFP:vps36Δ| BY4741, Hxt3-GFP:His3MX, vps36:KanMX | This study |
| Hxt3-GFP:vps27Δ| BY4741, Hxt3-GFP:His3MX, vps27:KanMX | This study |
| Itr1-GFP    | BY4741, Itr1-GFP:His3MX   |            |
| Aqr1-GFP    | BY4741, Aqr1-GFP:His3MX   |            |
| Fet5-GFP    | BY4741, Fet5-GFP:His3MX   |            |
| Fh1-GFP     | BY4741, Fh1-GFP:His3MX    |            |
| Vph1-GFP    | BY4741, Vph1-GFP:His3MX   |            |

Methods

Yeast strains and reagents. All *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. All unique yeast strains generated for this study are available from the authors. To knock out VPS27 by homologous recombination we used the Longtime method36 and the following primers: (forward; CBO2002) 5'-GCTAAGGGTAGAATGAGTACGTTTAAAGCAATAGACGTTGGAGTC-3' and (reverse; CBO2003) 5'-TAGAGTTCCCTTTTAAATTTATTATTTTATTTTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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**Author contributions**

C.L.B and E.K.M. conceived the project. E.K.M. performed experiments and prepared all data for publication. C.L.B. guided experimental design and analysis. E.K.M. and C.L.B. wrote the paper.

**Additional information**

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**Competing interests:** The authors declare no competing interests.

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