## Endosomal trafficking of yeast membrane proteins

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## Abstract

Various membrane trafficking pathways transport molecules through the endosomal system of eukaryotic cells, where trafficking decisions control the localisation and activity of a diverse repertoire of membrane protein cargoes. The budding yeast *Saccharomyces cerevisiae* has been used to discover and define many mechanisms that regulate conserved features of endosomal trafficking. Internalised surface membrane proteins first localise to endosomes before sorting to other compartments. Ubiquitination of endosomal membrane proteins is a signal for their degradation. Ubiquitinated cargoes are recognised by the Endosomal Sorting Complex Required for Transport (ESCRT) apparatus, which mediate sorting through the multivesicular body pathway to the lysosome for degradation. Proteins that are not destined for degradation can be recycled to other intracellular compartments, such as the Golgi and the plasma membrane. In this review we discuss recent developments elucidating the mechanisms that drive membrane protein degradation and recycling pathways in yeast.

## Membrane trafficking to the vacuole / yeast lysosome

The endosomal system is a dynamic network of membranous compartments where several trafficking pathways converge, allowing for sorting of proteins and lipids to different cellular locations. Regulation of endosomal trafficking events governs many processes in the cell, which rely on precise localisation and activity of proteins that transit the endosomal system[1–3]. In mammalian cells, integral membrane proteins that function at the plasma membrane are internalised to endosomes and can either be returned to the surface through various recycling routes or transported to other intracellular compartments, such as the Golgi apparatus or lysosomes. The budding yeast *Saccharomyces cerevisiae* has been instrumental in revealing and defining protein machinery that is responsible for various conserved endosomal trafficking mechanisms[4]. Recycling routes in yeast are not entirely clear, however a newly discovered mechanism controlled by a large group of conserved factors[5] suggests recycling may be more similar to mammalian cells than was first appreciated. The extent of internalised cargo recycling back to the cell surface in yeast is not well understood, because the majority of endocytosed proteins are thought to be instead routed through a well characterized vacuolar degradation pathway.

In this degradation pathway, the covalent attachment of ubiquitin to endosomal membrane proteins is a necessary and sufficient signal that drives delivery into the lumen of the vacuole[6,7]. Ubiquitinated cargoes are sorted to late endosomes, termed multivesicular bodies (MVBs), and packaged into luminal vesicles that are subsequently degraded in the vacuole[8]. Cargo sorting through MVBs to the vacuole is mediated by the Endosomal Sorting Complex Required for Transport (ESCRT) proteins, which are soluble cytosolic components that are recruited to endosomal membranes. The molecular basis of ubiquitin-dependent MVB sorting is achieved through multiple ESCRT functions. Firstly, various ubiquitin binding domains present in ESCRT-0, -I and -II complexes allow ESCRTs to act as receptors at the MVB for ubiquitinated cargoes that are targeted for degradation[9]. The ESCRT accessory factor Bro1, which recruits the deubiquitinating enzyme (DUb) Doa4 to the late endosome[10,11], is also a cargo specific receptor[12]. ESCRTs themselves are co-ordinated in a complex manner that is not fully understood but involves various interactions between different subunits and associations with endosomal lipids[13–15].

In addition to recognising ubiquitinated cargoes at the MVB, ESCRTs are also involved in generating luminal vesicles of the MVB (**Figure 1**). The ESCRT-III complex is comprised of subunits that are recruited to the limiting membrane of the MVB, followed by polymerisation that promotes membrane invagination and constriction. The core ESCRT-III subunits (Vps20, Snf7, Vps2 and Vps24) are involved with formation of filaments that are the minimal machinery to create luminal vesicles[16–18]. Vps20 localises to the endosomal membrane via an N-terminal myristoylation site and association with the ESCRT-II component Vps25[19,20]. Vps20 serves as a nucleation point that recruits Snf7, which in turn adopts an open conformation capable of self-polymerisation[21,22]. Spiral filaments of Snf7 are believed to be the primary driving force for invagination of endosomal membranes into the MVB lumen[23,24]. Snf7 recruits the Vps24/Vps2 sub-complex that terminates Snf7 oligomerisation through recruitment of the AAA-type ATPase Vps4[21,25]. Vps4 triggers disassembly and disassociation of ESCRT-III polymers from the endosomal membrane, thereby allowing membrane remodelling, cargo sorting into MVBs and recycling of ESCRTs[26,27]. Rapidly assembled hexamers of Vps4 are recruited to the endosome and engage ESCRT-III substrate peptides to drive destabilisation and unfolding progressively with sequential hydrolysis of ATP[25,28,29]. Vps4 has a microtubule-interacting and trafficking (MIT) domain that can bind the C-terminal MIT interacting motifs (MIMs) found in both core and accessory ESCRT-III subunits[30]. Specifically, Vps4 associates with Vps2, aided by Did2, and critically to Snf7 for its membrane remodelling function[31–33]. Other factors also control the activity of Vps4, including Vta1 that stabilises the oligomeric form of Vps4[34] and Ist1, which is structurally similar to other ESCRT-III subunits. Synthetic defects in vacuolar trafficking are observed when *ist1∆* mutations are combined with *vta1∆* or *vps60∆* deletions, however Ist1 can also bind to and inhibit Vps4; Ist1 is therefore both a positive and negative regulator of MVB sorting[35–37]. Current models predict that Vta1 and Ist1 compete for Vps4 binding and impose precise regulation. Many regulatory features of ESCRT proteins have been identified in other eukaryotic systems, in addition to species specific modulators[38]. Beyond this, ESCRTS have been shown to perform an increasing repertoire of membrane remodelling functions beyond MVB formation, such as cytokinesis, viral budding, nuclear envelope closure and repair of damaged membranes [39,40].

**Endosomal membrane protein recycling**

Not all endosomal membrane proteins are destined for packaging into MVBs. There are various recycling or retrieval mechanisms that can return proteins to earlier trafficking intermediates of the endomembrane system (**Figure 2**). A well characterised retrograde route in yeast delivers membrane proteins back to the *trans*-Golgi network (TGN). The highly conserved retromer complex was first defined as an active carrier of receptors for vacuolar hydrolases that return to the TGN for further rounds of transport[41,42]. The Vps26/Vps29/Vps35 retromer subcomplex can specifically recognise cargo and is recruited to endosomal membranes by the Vps5/Vps17 sorting nexin subcomplex, which induces tubule formation to recycle cargo back to the TGN[43]. In addition to Vps5 and Vps17, yeast express five other sorting nexins (SNX) with a Bin-Amphiphysin-Rvs domain (SNX-BAR proteins)[44], which are recruited to endosomal membranes to generate tubules that recycle specific cargo[45]. For example, the R-SNARE protein Snc1 does not rely on retromer for its recycling but instead requires a complex of Snx4/Snx42, whereas the autophagy related protein Atg27 requires a complex of Snx4/Snx41[46,47]. Membrane trafficking from the limiting membrane of the yeast vacuole have also been documented[48]. These include ESCRT mediated degradation pathways directly into the lumen[49] or via the MVB pathway[50], and a recycling route back to the TGN[51].

The trafficking of internalised cell surface proteins from endosomes to the TGN allows for their incorporation into secretory vesicles and subsequent return to the plasma membrane, which can occur through distinct trafficking mechanisms. For example, the chitin synthase Chs3 is retained at early endosomes and Golgi compartments without transiting the prevacuolar compartment[52,53]. Chs3 can be transported to the TGN from early endosomes via AP-1[53] or from late endosomes by the retromer[54]. Chs3 is then directed to the plasma membrane through physical association with the exomer complex[55–57]. The exomer has also been shown to mediate surface trafficking of Fus1 through a distinct sorting signal[58]. As mentioned, the R-SNARE Snc1 required for fusion of exocytic vesicles[59] recycles via a Snx4/Snx42 step. Additional machinery is also required for Snc1 recycling, including GTPases Ypt31/32, the GTPase-activating protein Gcs1, the flippase Drs2 with Cdc50, and Rcy1 (reviewed in[60]). More recently ubiquitination of Snc1, catalysed by Pib1 and Tul1 E3-ligase enzymes, has been shown to mediate its recycling[61,62]. K63- polyubiquination of Snc1 promotes its association with endosomally localised COPI subunits via its WD40 repeats domains[61], which interact with ubiquitin[63]. Ubiquitin conjugation is a prevalent lysosomal sorting signal, and under certain conditions targets Snc1 to the vacuole[64]. However, ubiquitination clearly has a more nuanced role in endosomal trafficking, as it is also required for Snc1 recycling.

Cargo deubiquitination removes the signal for degradation and appears to trigger recycling by default[60]. Fusion of the catalytic domain of a deubiquitinating enzyme (DUb) to various surface proteins blocks their degradation and increases steady state surface localisation[65]. Furthermore, premature removal of ubiquitin from MVB cargo can be achieved by fusing a DUb to ESCRT-0, which blocks trafficking to MVBs and induces surface recycling[66]. Similarly, cargo trapped at MVB/late endosomes of *vps4∆* cells can be recycled back to the surface following chemical dimerization with a DUb[64]. These mechanisms are in striking contrast to Snc1 recycling, which is blocked by DUb-fusion[61].

## Cell surface recycling machinery and endosomal organisation

To dissect this distinct recycling mechanism triggered by cargo deubiquitination, a synthetic recycling reporter was employed. The reporter is based on Ste3, a G-protein coupled receptor that recycles, fused to GFP and the catalytic domain of a DUb[5]. In contrast to DUb-fused Snc1, DUb-fused Ste3 recycles extremely efficiently. Furthermore, Snc1 recycling is rapidly blocked during acute inactivation of the ARF-exchange factor Sec7, which controls transit through the TGN, whereas surface recycling of DUb-fused Ste3 and the styryl dye FM4-64 were unperturbed[5]. Original interpretation of these data predicted that the latter mechanism recycled from an earlier endosome compartment distinct from the Sec7/TGN compartment traversed by Snc1. Yeast have previously been proposed to have an early endosome due to the arrival of internalised immunogold particles first at a compartment containing the Q-SNARE protein Tlg1 prior to trafficking to late endosomes[67]. Tlg1 partially colocalizes with Golgi markers but is also observed in a distinct population when assessed by immunofluorescence and biochemical fractionation[68]. Studies using endocytic tracers also propose two distinct populations of endosomes exist and *soi1* mutations disrupt trafficking between the two[69,70]. Furthermore, various mutations have been identified, which have no apparent effect on secretion kinetics but accumulate Snc1 in a compartment lacking the TGN marker Sec7[68,71,72], suggesting Snc1 recycling requires an upstream trafficking step prior to joining the secretory pathway at the TGN.

Many advances in our understanding of membrane trafficking pathways in yeast have been achieved by: 1) observing steady state localisation of trafficking cargoes / compartment markers and 2) using genetic perturbations that accumulate trafficking intermediates that cannot be observed at steady state. That said, careful interpretation is required as ensemble average approaches may not accurately reflect the dynamic nature of membrane trafficking events, and gene deletion mutants may trigger compensatory mechanisms. More recently, kinetic analyses of endosomal trafficking in living yeast cells suggest the TGN acts solely as an early and recycling endosome[73]. 3D confocal time-lapse microscopy shows that internalized proteins localize rapidly to the TGN before transit to a more stable late endosome compartment[73]. Additionally, Day and colleagues find that Sec7 is quickly recruited to TGN compartments solely marked by Tlg1. These data support a model for a streamlined endomembrane pathway in yeast and highlight the importance of understanding the kinetics of membrane trafficking cargo and reporter proteins used during imaging experiments. Presumably complex layers of regulation are required for precisely orchestrating the many protein and lipid trafficking events that occur at the TGN. How can distinct recycling mechanisms, such as those described for Snc1 and Ste3-GFP-DUb, be reconciled in this minimal model of the yeast endomembrane? It may be that recycling cargoes that exhibit a stark difference in sensitivity to *sec7ts* inactivation could be incorporated into different populations of recycling intermediates at the TGN. Another possibility is that the fluid nature of the Golgi would allow for cargo partitioning in specific TGN sub-compartments during maturation. It is also possible that residual activity of Sec7 in temperature sensitive (*sec7ts*) mutants is sufficient to recycle some cargoes but not others; indeed, cargo selective endosomal sorting in *rsp5ts* mutants has previously confused membrane trafficking models[74].

Irrespective of the precise compartments that Ste3-GFP-DUb transits *en route* to the cell surface, its exclusion from the ubiquitin / ESCRT degradation pathway, and possible exclusion from the COPI recycling mechanism used by Snc1[61], make it a robust tool to study surface recycling. The Ste3-GFP-DUb reporter was used to perform a systematic genetic screen and identified many novel and conserved candidates that may participate in surface recycling[5]. The Rag GTPases Gtr1 and Gtr2 were identified as mediators of recycling independently of their known regulation of TORC1. The recycling specific effector Ltv1 was also identified and shown to colocalize with the Rag GTPases to aberrant yeast endosomes in recycling mutants[5]. This genetic screen for recycling machinery also revealed a role for the ESCRT-III associated factor Ist1. As discussed above, Ist1 can inhibit the yeast MVB pathway[35] but may also promote the recycling of cargo back to the surface. This observation aligns with *in vitro* experiments and *in vivo* studies in animal cells, as the mammalian IST1 orthologue has been implicated in cell surface recycling[75,76]. Polymerisation of mammalian IST1 drives formation of cytosolic tubules in the opposite orientation of the luminal deformations generated by ESCRT-III, thereby driving the formation and/or scission of recycling tubules[76]. It will now be important to understand how Ist1 activity is controlled in yeast and determine if these regulatory features are conserved in mammalian systems.

**Summary**

A large number of membrane trafficking mechanisms that control cargo sorting through the endosomal system have been elucidated in yeast. Classic genetic screens identified core machinery driving the secretory, endocytic, vacuolar protein sorting and autophagy pathways, all of which impinge on the endosomal system. For example, ESCRT proteins were identified as Class E members of the vacuolar protein sorting (*vps*)pathway[77]. Other *vps* mutants comprise the retromer complex, probably the best characterised recycling machine in eukaryotic cells[43]. Recycling cargoes also rely on various components of the secretory pathway, like Sec7 at the TGN and the fusion machinery at the plasma membrane[78]. With regards to understanding different recycling mechanisms, several of the cargoes recycled through classical retrograde pathways are related to the polarization of the cell: Snc1 drives fusion of vesicles directed to the emerging bud[59], Chs3 is required for chitin ring synthesis at bud emergence[79], and Fus1 localizes to the tip of mating projections and is required for conjugation[80]. The machinery identified by screening for mutants defective in recycling Ste3-GFP-DUb may mainly be required to recycle a distinct repertoire of cargoes back to the cell surface[5]. This might explain why cargoes of this pathway are less sensitive to Sec7 inactivation and cargo deubiquitination. The precise organisation and functionality of endosomal trafficking pathways is also incompletely understood in other organisms[81] and endosomal structures seem to vary in different mammalian cell lines. Understanding the division of labour between these yeast modes of recycling will be helpful to define conserved trafficking rules. Appreciating the dynamics of endosomal reporter and marker proteins in real time will be important. New live cell imaging approaches, such as lattice light sheet and *slimfield*, which have been recently used to observe protein trafficking in living yeast cells[25,82], will likely drive insights into endosomal membrane trafficking in yeast.

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## Abbreviations

Endosomal Sorting Complex Required for Transport (ESCRT); Multivesicular bodies (MVBs); Deubiquitinating enzyme (DUb); Microtubule-interacting and trafficking (MIT); MIT interacting motif (MIM); *trans*-Golgi network (TGN); Sorting nexin with a Bin-Amphiphysin-Rvs domain (SNX-BAR); vacuolar protein sorting (*vps*).

## Keywords

Ubiquitin; ESCRTs; Lysosomes; Endosomes; Deubiquitination; Cell surface recycling; Yeast**.**

## Figure Legends

**Figure 1: Degradation of endosomal membrane proteins**

Membrane proteins are targeted for degradation by ubiquitination by E3-ubiquitin ligases. Ubiquitinated cargoes (Ub-cargo, blue) are trafficked to the late endosome where they are recognised by early Endosomal Soring Complex Required for Transport (ESCRT) machinery (complexes -0, -I and -II, yellow) that have abundant ubiquitin binding domains. The formation of luminal vesicles is driven by ESCRT-III and the AAA-ATPase Vps4 (purple). ESCRT-III polymerisation and Vps4 drives invagination of the limiting membrane of the endosome and create a vesicle into which cargo is packaged. Vps4 activity is also required to disassemble the ESCRT-III polymers..

**Figure 2: New recycling mechanisms of endosomal membrane proteins**

An array of cell surface membrane proteins in yeast (including the R-SNARE, Snc1; the G-protein coupled receptor, Ste3; and the methionine permease, Mup1) are internalised to an Early Endosome (EE) / *trans*-Golgi Network (TGN) compartment. Protein recycling of Snc1 requires its ubiquitination and subsequent interaction with the COPI coat protein, and this recycling is very sensitive to Sec7-inactivation. Other surface proteins, such as Ste3 and Mup1, are recycled more efficiently following deubiquitination.

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