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*Review*

# *Saccharomyces cerevisiae* — a model organism for the studies on vacuolar transport✪

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**The role of the yeast vacuole, a functional analogue of the mammalian lysosome, in the turnover of proteins and organelles has been well documented. This review provides an overview of the current knowledge of vesicle mediated vacuolar transport in the yeast *Saccharomyces cerevisiae* cells. Due to the conservation of the molecular transport machinery *S. cerevisiae* has become an important model system of vacuolar trafficking because of the facile application of genetics, molecular biology and biochemistry.**

A basic feature of the eukaryotic cell is compartmental organisation which requires mechanisms precisely sorting and distributing newly synthesised proteins and metabolites to their appropriate target organelles. Although a lot has been learned concerning the general principles of intracellular communication, the detailed mechanism of integration of these processes into the network of cellular interactions remains an open and essential question. Due to an impressive conservation of the molecular transport machinery across phyla (Schekman & Orci, 1996; Rothman, 1996), a great deal of the current knowledge

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**Abbreviations:** ALP, alkaline phosphatase; COP, coatomer protein; CPY, soluble serine carboxypeptidase; Cvt, cytoplasm-to-vacuole targeting; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; MVB, multivesicular body; PM, plasma membrane; t-SNAREs, plasma membrane syntaxin related receptors on the target membrane; v-SNAREs, synaptobrevin-related receptors on the vesicle membrane.

on the functional interactions has been acquired by the study of the yeast *Saccharomyces cerevisiae.* This unicellular eukaryote is organised into the same major membrane-bounded compartments as all other eukaryotic cells and shares the metabolic processes of higher eukaryotes. Apart from being easy to manipulate, *Saccharomyces cerevisiae*, like other micro-organisms represents an excellent system for the study of functional networks among genes. This approach combines methods of classical and molecular yeast genetics with the tools deriving from complete sequencing of the yeast genome and from genome databases from other species.

Due to the complexity of the problem the present article is limited to the transport pathways in yeast cells converging upon the vacuole.

## THE YEAST VACUOLE

The yeast vacuole is an equivalent of the mammalian lysosome and the vacuole of plant cells. In wild type cells it takes up as much as 25% of the cellular volume. The multiprotein vacuolar proton pumping ATP-ase (V-ATPase) generates and maintains the acidic pH of this compartment. As the main degradative site in the cell, the vacuole contains a variety of degradative enzymes required for intracellular digestion, including endo- and exoproteases, ribonucleases, polyphosphatases, $\alpha$-mannosidase, trehalase, and alkaline phosphatase (ALP). The vacuole also serves as a storage site for certain cellular nutrients, such as amino acids, purines, polyamines, *S*-adenosylmethionine and polyphosphates, which can be mobilised by the cell. The yeast vacuole, similarly to the plant one, functions as a reservoir for mono- and divalent cations. Due to its ability to sequester high concentrations of ions it fulfils a detoxification function.

The defects in vacuolar function lead to protein mislocalisation, disturbances in ion homeostasis, and affect such complicated processes as osmoregulation and sporulation (Jones *et al.,* 1997; Conibear & Stevens, 1998; Klionsky, 1998, and references therein). Thus, it is not surprising that in *S. cerevisiae,* genetic and biochemical studies along with the data obtained by computational analysis of the genome led to the identification of more than 500 ORFs whose products are, or could be, directly or indirectly involved in the biogenesis and functioning of the vacuole.

## A VARIETY OF PROTEIN TRANSPORT PATHWAYS TO THE YEAST VACUOLE

In yeast cells several different transport pathways converge upon the vacuole (Fig. 1). Newly synthesised proteins, such as vacuole resident proteases, are transported through the secretory pathway to the endoplasmic reliculum (ER) lumen or membrane, pass from the ER through the Golgi apparatus and in late Golgi are diverted from the secretory pathway. The vacuole is also a recipient of material from the cell surface delivered by the process of endocytosis. These two pathways overlap at the stage of the multivesicular body, MVB (known also as the prevacuolar compartment – PVC or late endosome). Another pathway which diverts at the late Golgi, referred to as the ALP pathway, bypasses the MVB when delivering alkaline phosphatase to the vacuole. Hydrolase aminopeptidase I (API) is supplied to the vacuole from the cytoplasm by cytoplasm-to-vacuole targeting (Cvt) overlaping with autophagy, which nonselectively delivers cytosolic proteins and organelles to the vacuole for degradation and recycling. Recent data show that fructose-1,6-bisphosphatase (FBPase) is delivered to the vacuole for degradation in a novel type of vesicles called vacuole import and degradation vesicles (Vid pathway). Ions and small molecules reach the vacuole *via* fluid-phase endocytosis. About 50% of vacuolar material is transferred from mother to the daughter

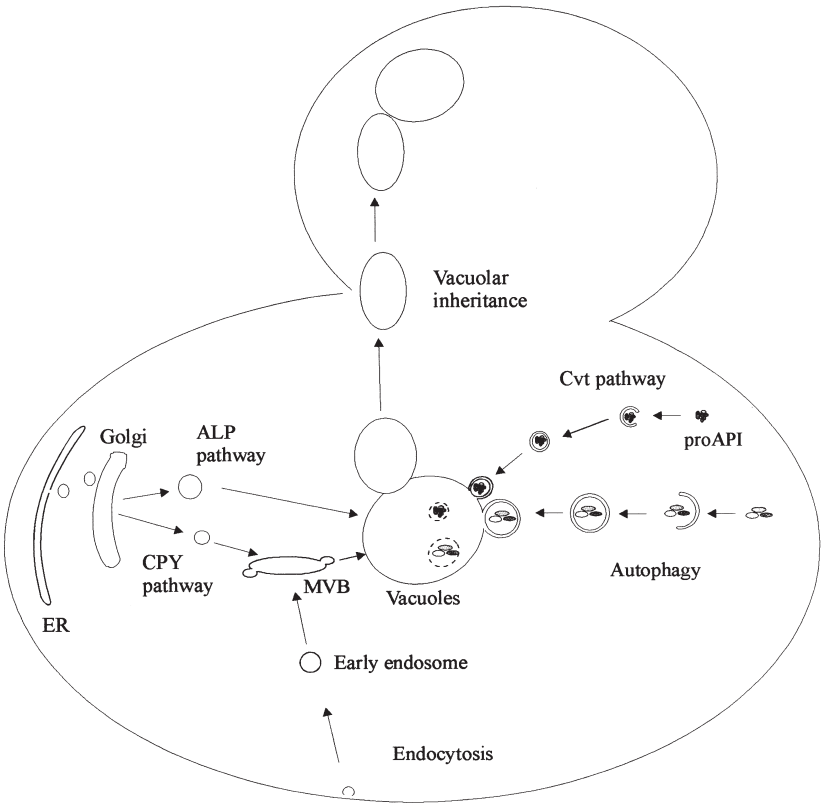


**Figure 1. Multiple routes of transport to the yeast vacuole.**

Vacuolar proteins are sorted in the vesicles targeted to the vacuole at the level of the late Golgi. The soluble hydrolase CPY is delivered to the vacuole *via* the MVB, but alkaline phosphatase (ALP) bypasses it. Proteins endocytosed from the cell surface are also transported via the MVB. The other pathways shown are: the biosynthetic cytoplasm-to-vacuole pathway (cvt) taken by aminopeptidase I (API), autophagy, and transmission of vacuoles from the mother to the daughter cell.

cell in the process of vacuolar inheritance. Beside the anterograde transport to the vacuole the retrograde transport of proteins exists between vacuole and earlier compartments: the MVB, Golgi or ER (Gotte & Lazar, 1999). Genetic analysis allowed the identification of a substantial number of genes required for vacuole function and protein sorting in yeast. The main classes of these genes include *VPS* (*v*acuolar *p*rotein *s*orting ), *VAC* (*vac*uole partitioning), APG (*a*uto*p*ha*g*ocytosis), *END* (*end*ocytosis), and *VID (v*acuolar *i*mport and *d*egradation) (for a review see: Catlett & Weisman, 2000; Kim & Klionsky, 2000; Sorkin, 2000; Shieh *et al*., 2001). The vacuolar protein sorting mutants (*vps)* are arranged in six groups depending on the phenotype (Raymond *et al.*, 1992). The characteristics of *vps* mutants is presented in Table 1.

## VESICULAR TRANSPORT

The transport pathways to the yeast vacuole are vesicle mediated. The inter-compartment small vesicles bud from the donor organelle membrane and carry molecules from the site of vesiculation to a specific recipient organelle. The assembly of protein coat on vesicles, a set of proteins known as coatomer protein (COP), together with adapter proteins provides for selective partitioning of cargoes into the vesicles and for precisely directing them to the site of destination. A coat protein complex, COP I, mediates retrograde transport from the Golgi to the ER and through the Golgi. The COP II proteins form a coat on anterograde vesicles budding from the ER to the early Golgi. The clathrin coat is necessary for budding of vesicles destined to MVB from

the late Golgi. The delivery of cargo to the recipient organelle is accomplished by membrane recognition known as tethering, followed by docking and fusion. Tethering factors are peripherally membrane-associated protein complexes consisting of up to 10 different subunits. The docking stage involves specific sets of membrane-anchored proteins, so-called SNARE proteins. There are two families of integral membrane proteins essential for the final interaction of two compartments: v-SNAREs (synaptobrevin-related receptors on the vesicle membrane) and t-SNAREs (plasma membrane syntaxin related receptors on the target membrane). According to the SNARE model (Sollner *et al.,* 1993; Weber *et al.,* 1998) in both the docking and membrane fusion stages, the assembly of a membrane fusion complex starts with the pairing of a v-SNARE with its cognate t-SNARE (Ungermann *et al.,* 1998). The formation of a stable four-helix bundle may generate enough energy to promote mixing of the lipid bilayers of the fusing membranes (Sutton *et al.,* 1998). The v-SNARE and t-SNARE complex formation is regulated by Sec18p and Sec17p which are required at all transport steps; the dissociation of SNARE components is coupled to ATP hydrolysis by Sec18p (Griff *et al.,* 1992; Wilson *et al.,* 1989). Recent studies of SNARE function in yeast indicate that both v- and t-SNAREs can interact with more than one partner what questioned generality of the model. It appears that, important in specific vesicle targeting, SNAREs act in multiprotein complexes which are beginning to be identified (Fig. 2) (Darsow *et al.,* 1997; Nichols & Pelham, 1998).

**Table 1. Morphological classification of *vps* mutants***

| Class | Phenotypes of mutants |
|---|---|
| A | wild-type or slightly perturbed vacuoles |
| B | fragmented vacuoles, mislocalisation of vacuolar proteins |
| C | lack of organised vacuole, dispersed intracellular localisation of vacuolar membrane proteins, defective CPY sorting |
| D | defects in vacuole inheritance and acidification, ALP dispersed in membranes of multiple vesicular bodies |
| E | vacuoles similar to wild-type, exaggerated and acidified prevacuolar compartment, ALP localised to vacuole |
| F | large central vacuoles surrounded by small vacuole-like compartments, severe CPY sorting defects |

*Compiled from the data of Raymond *et al.* (1992) and from MIPS (http://mips.gsf.de/).

## THE Rab/Ypt GTPases

The small monomeric GTPases (200–230 amino acids) named Rab in mammals and Ypt in yeast play a key regulatory role at different stages of vesicles traffic. The family of Ypt GTPases belongs to the superfamily of Ras-related proteins. There are 29 members of this superfamily in *S. cerevisiae.* Apart from eleven Ypt GTPases it comprises four other families playing a regulatory roles: Ras in the cell cycle, Ran in nuclear import, Arf/Sar in vesicle budding and Rho in cytoskeletal organisation and cell wall biosynthesis (reviewed in: Lazar *et al.*, 1997; Gotte *et al.,* 2000). The proteins of the Ras superfamily from different species are characterised by a highly conserved guanine nucleotide binding regions and a posttranslationally added isoprenoid tail, which anchors Ras proteins to their target membranes. The alignment of the conserved G regions is presented in Table 2.

The first Rab proteins found in yeast were Sec4p and Ypt1p (Salminen & Novick, 1987; Gallwitz *et al*., 1983), the remaining nine proteins are Ypt31p, Ypt32p, Ypt51p, Ypt52p, Ypt53p, Ypt6p, Ypt7p, Ypt10p and Ypt11p. The last two were identified by screening the entire genome by virtue of common sequence motifs (Lazar *et al.,* 1997).

Transport GTPases cycle between donor/vesicular and acceptor membranes, between active GTP-bound and inactive GDP-bound state. They are maintained in the GDP-bound

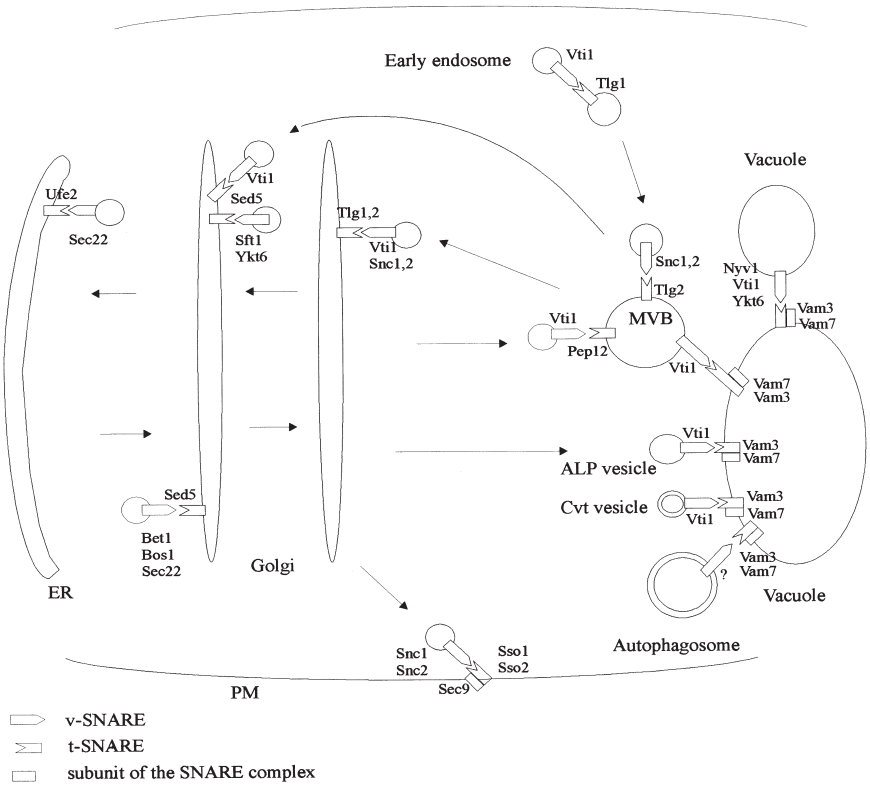


**Figure 2. Interaction of v- and t-SNAREs in yeast.**

ER, endoplasmic reticulum; PM, plasma membrane; MVB, multivesicular body.

inactive conformation by GDP-dissociation inhibitor (GDI), which is able to release the Rab-GDP form from target membranes and direct it to the proper donor membrane, where it is recognised by a specific receptor. One or more so called GDI displacement factors (GDF) disrupts the complex, releasing GDI into cytosol, and enable membrane binding of Rab-GDP (Ullrich *et al.,* 1994; Soldati *et al.,* 1994), then the guanine nucleotide exchange factor (GEF) catalyses GDP/GTP exchange. The active Rab-GTP protein is resistant to removal from the membrane by Rab GDI. The new cycle starts after GTP hydrolysis accelerated by a GTPase-activating protein, GAP (Strom *et al.,* 1993). In contrast to the conserved Rab proteins the factors that function at particular steps of the cycle appear to share no sequence similarity and generally are specific for individual Rab family members (Ullrich *et al.,* 1994; Soldati *et al.,* 1994; Fukui *et al.,* 1997). Activated, vesicle-bound GTP-bound Rab/Ypt proteins influence the function of the SNARE complex.

**Table 2. Conserved sequence motifs in Ras proteins from different species**

| | G1 | G2 | G3 | G4 | G5 |
|---|---|---|---|---|---|
| Ypt7 yeast | GDSGVGKT | YKATI | WDTAGQE | GNKID | FL-TSAK |
| p21/ras-rat | GAGGVGKS | YDPTI | LDTAGQE | GNKCD | FIETSAK |
| RhoH human | GDGGCGKT | YTPTV | WDTAGQD | GCKTD | YHRGQEM |
| Ran-mouse | GDGGTGKT | YVATL | WDTAGQE | GNKVD | YYDISAR |

G1, binds $\alpha$ and $\beta$ phosphates of GTP/GDP; G2, effector domain essential for GTP hydrolysis; G3, binds $\gamma$ phosphate of GTP; G4, binds the guanine ring; G5, stabilises the guanine–G4 interaction.

## VESICULAR TRANSPORT FROM THE ENDOPLASMIC RETICULUM TO THE GOLGI

The Golgi compartment in *S. cerevisiae* cells does not consist of a series of parallel stacks as in mammalian cells. Although immunoelectron microscopy revealed the presence of single cisternae (Preuss *et al.,* 1992), different compartments could be defined by the sequential carbohydrate modification of proteins occurring in distinct Golgi compartments (Graham & Emr, 1991). Most of the soluble vacuolar hydrolases, as well as membrane-associated proteins, passage through the Golgi apparatus proceeding to the vacuole along the early stages of the secretory pathway. In the last compartment of the Golgi, vacuolar and secreted proteins are sorted and follow separate routes.

In *S. cerevisiae* cells the endoplasmic reticulum (ER) to Golgi transport begins with the formation of vesicles at the ER surface followed by docking and fusion with the Golgi membrane. These processes are carried out by proteins classified as the Sec group (from secretory mutants). The vesicles budding from the ER are coated with the cytosolic proteins COPII forming heterodimeric complexes Sec23p/Sec24p and Sec13p/Sec31p in the presence of the small GTPase Sar1p, which recruits the cytosolic coat components (Barlowe, 1997; Klumperman, 2000). The mechanism of cargo selection to the budding vesicles remains obscure. However, the data obtained by the Gallwitz group indicate that the COPII subunit Sec24p plays a role in this process (Peng *et al.* 1999).

Another set of proteins participate in the step of vesicle docking and fusion with the early Golgi membrane. The multiprotein complex TRAPP (transport protein particle) containing ten subunits (Bet1p, Bet3p and Bet5p and seven proteins encoded by uncharacterised open reading frames) together with Uso1p/115p and Sec34p/Sec35p are implicated in the ER-to-Golgi tethering. The TRAPP complex is a highly conservative element of vesicular traffic and orthologs of most of the TRAPP proteins have been identified in mammals. The docking is mediated by the GTP-ase Ypt1p (Barrowman *et al.,* 2000; Singer-Kruger *et al.,* 1998; Wang *et al.,* 2000). The SNAREs Sec22p, Bos1p, Bet1p and Sed5p are necessary for membrane fusion and are distributed between the vesicle and the Golgi. The Bet1, Bos1 and Sec22 proteins act on the vesicles, whereas Sed5p is required on the acceptor membrane (Stone *et al.,* 1997).

Inter-Golgi protein transport occurs through the COP I coated vesicles, however, the molecular mechanism of this process has not been solved yet. Two Ypt proteins, Ypt31p and Ypt32p, function in the inter-Golgi transport and in the budding of vesicles from the Golgi complex (Benli *et al.,* 1996).

The crucial stage in the vacuolar transport occurs at the late Golgi (the yeast equivalent of the mammalian *trans*-Golgi network, TGN). At that stage at least five routes branch out: two parallel secretory pathways to the cell surface (Harsay & Bretscher, 1995), the retrograde pathway to the early Golgi and ER (Harris & Waters, 1996), and two that carry proteins to the vacuole, i.e., one transversing the MVB (the CPY pathway) and the alternative ALP pathway (Bryant & Stevens, 1998, and references therein). This complexity demands a high level of regulation and the factors involved in the formation of vacuolar-destined vesicles from the late Golgi are beginning to be uncovered.

The Vps1p GTPase, is involved in the formation of both classes of vesicles transporting proteins from the late Golgi to the vacuole (Finken-Eigen *et al.,* 1997). Also Arf1 and Arf2, small GTP-binding proteins, are present on the cytoplasmic surface of Golgi cisternae and vesicles that bud from the Golgi. They bind to the membrane in the GTP form and recruit the coatomer COP I complex (MIPS Search Reference Database). Clathrin, the Vps34p phosphatidylinositol 3-kinase, and the

Vps15 protein kinase have been clearly implicated in the formation of Golgi vesicles, named "clathrin coated vesicles", carrying cargo to the MVB (Conibear & Stevens, 1995). It has been proposed that Vps15p and Vps34p function together as components of a membrane-associated signal transduction complex that regulates intracellular protein trafficking decisions through protein and lipid phosphorylation (Stack *et al.,* 1993). Recently it has been shown that Gga1 and Gga2 proteins representing an evolutionarily conserved protein family with homology to the clathrin adaptor AP-1 $\gamma$ subunit play an important role in cargo-selective clathrin-mediated protein traffic from the *trans*-Golgi network to endosomes (Costaguta *et al.,* 2001). The transport from the Golgi to the MVB relies upon a number of Vps proteins. Vps45p, one of the four Sec1-like proteins identified in yeast (functioning as negative regulators of v-SNAREs docking interaction with t-SNAREs), is required for transport from the Golgi to the MVB. t-SNAREs which function in late Golgi or endosomal compartments are Tlg1p and Tlg2p forming a trimeric complex with Vps45p (Nichols *et al.*,1998).

## CPY PATHWAY

The soluble serine carboxypeptidase (CPY) is encoded by the *PRC1* gene. Its trafficking to the vacuole has been very well characterised (for a review see: Jones *et al.,* 1997; Johnson *et al.,* 1987; Bryant & Stevens, 1998). CPY is synthesised as an inactive precursor, preproCPY containing a signal sequence that directs it into the lumen of ER. In the ER the signal sequence is cleaved and proCPY undergoes N-linked glycosylation resulting in the intermediate ER form, p1CPY 67-kDa. Like secretory proteins, glycosylated p1CPY is then packed into vesicles that bud from the ER followed by subsequent fusion of the vesicle with Golgi membranes. In the Golgi compartment p1CPY undergoes sequential oligosaccharide modifications to produce the Golgi modified 69-kDa, p2CPY form. Transport within the Golgi occurs also through vesicles. In the late Golgi compartment the zymogen p2CPY is diverted away from the secretory pathway. The vacuolar sorting sequence (QRPL – residues 24 to 27) in the propeptide region of p2CPY directs the protein into the vacuolar pathway. The current model of the receptor-mediated pathway taken by p2CPY is as follows: in the late Golgi p2CPY binds to Vps10p, the CPY receptor (Marcusson *et al.,* 1994); the complex travels to the MVB in Golgi derived transport vesicles in the *VPS45*-dependent way; p2CPY dissociates from its receptor in the MVB and is transported to the vacuole, where it is cleaved by vacuolar proteases into its active, mature 61-kDa form (mCPY). Vps10p is recycled back to the Golgi. Genes encoding homologs of Vps10p have been identified within the *S. cerevisiae* genome, and it was shown that protein products of two genes *VTH1* and *VTH2* can act as functional receptors for CPY. These data suggest that *S. cerevisiae* contains a family of receptors that participate to various degrees in the sorting of soluble hydrolases to the vacuole (Cooper & Stevens, 1996; Westphal *et al.,* 1996).

## ANTEROGRADE AND RETROGRADE TRAFFICKING BETWEEN LATE GOLGI AND MVB

Evidence for the existence of the MVB in yeast came from a study of class E *vps* mutants (Table 1), which contain an exaggerated membrane structure adjacent to the vacuole. Proteins transported from late Golgi to the vacuole and endocytosed proteins accumulate in the MVB of E *vps* mutants (Bryant & Stevens, 1997; Horazdovsky *et al.,* 1995). On the other hand, the members of class D *VPS* genes appear to be involved in the transport of proteins from the Golgi to the MVB. The phenotypes of class D *vps* mutants include a block in the transport of soluble CPY to the vacuole,

altered vacuolar morphology, and a phenotype unique to this class, the accumulation of 40- to 60-nm vesicles (Raymond *et al.,* 1992).

The proteins encoded by the class D genes such as the t-SNARE Vps6p (Pep12p) and its partner v-SNARE Vti1p have for a long time been known to act in vesicle transport. Vps45p (homolog of Sec1p), the Ypt GTPase Vps21p (Ypt51p) and its guanine nucleotide exchange factor (GEF), Vps9p, and three other proteins, Vac1p (Pep7p or Vps19p), Vps8p, and Vps3p are found in a complex with the SNAREs (for a reference see: Tall *et al.,* 1999; Gotte & Lazar, 2000).

Also the components of the retrograde trafficking route MVB-to-late Golgi have been identified recently. The latest data indicate that Vps52p, Vps53p and Vps54p form a stable complex peripherally associated with the late Golgi, which functions at a step in protein transport between late Golgi and MVB and in the retrograde pathway recycling Golgi membrane proteins from the MVB back to late Golgi, probably as a regulator of docking or fusion of retrograde vesicles. The proteins Vps35, Vps29, Vps26, Vps5 and Vps17 cooperate in the so called retromer complex retrieving Golgi proteins from the MVB (Conibear & Stevens, 2000).

## ENDOCYTOSIS OF CARGO FROM THE PLASMA MEMBRANE TO THE MVB COMPARTMENT

As is shown in Fig. 1, the MVB serves as an intermediate compartment where traffick through the endocytic system meets the receptor-mediated pathway from the late Golgi. Endocytosis is an important process of membrane trafficking from the plasma membrane (PM) to the vacuole compartment. A number of plasma membrane proteins and extracellular fluid are taken up by endocytosis. Such proteins as the transporter of a-factor Ste6p, the multidrug transporter Pdr5p, and the mating pheromone receptors Ste2p and Ste3p are endocytosed constitutively. However, these proteins may be internalised rapidly in response to external stimuli in the process of receptor-mediated (or ligand stimulated) endocytosis. Amino acid (Gap1p) and uracil (Fur4p) permeases, the inositol permease (Itr1p) and maltose transporter (Mal61p) undergo constitutive and signal-regulated endocytosis (for a review see: Bryant & Stevens, 1998; D'Hondt *et al.,* 2000; Hicke, 2001; Katzmann *et al.,* 2001; Wendland *et al.,* 1998). The endocytosis starts with internalisation from the PM. In mammalian cells, short amino-acid sequences have been identified as signals directing PM proteins for endocytosis. Only in two yeast PM proteins, Kex2p and Ste3p, the NPFXD signal motif has been found (Tan *et al.,* 1996). The well documented internalisation signal in yeast is ubiquitination. Multiubiquitination of substrates is generally required for the recognition and degradation of cytosolic, ER and nuclear proteins by the proteasome (for a review see Hershko *et al.,* 2000), whereas the presence of a single or a few ubiquitins (with a different ubiquitin linkage) per plasma membrane protein leads to the recognition by the endocytic machinery and targeting toward the vacuole for degradation. The ubiquitin ligase Rsp5p (Mdp1p) mediates ubiquitination of proteins that is a signal for endocytosis. The mechanism by which the ubiquitin moiety is recognised by the endocytic machinery is not clear (for a review see: D'Hondt *et al.*, 2000; Rotin *et al.*, 2000; Sorkin, 2000). It has been proposed that Pan1p (Eps15p) functions as a multivalent adaptor that associates with Rsp5p, End3p, Inp51p and many other proteins into a complex involved in the selection of ubiquitinated substrates for endocytic vesicles (Tang *et al.,* 1997; Gagny *et al.,* 2000). The first step of endocytosis consists in the formation of vesicles from the PM. In mammalian cells, the cargo is endocytosed via clathrin-coated pits. The internalisation step requires an interaction of clathrin, dynamin (a GTPase), amphiphysin (an SH3 domain pro-

tein that has been implicated in the endocytosis of synaptic vesicle) and Eps15p – a tyrosine kinase substrate (McMahon *et al.*, 1997). In yeast cells the internalisation step is different. Deletion of the *CHC1* gene encoding clathrin heavy chain caused only a 50% reduction of pheromone receptors internalisation (Payne *et al.,* 1998). Isolation of endocytosis mutants (*end, ren, dim*) led to the identification of proteins involved in the internalisation step. It turned out that the internalisation in yeast depends upon actin and actin associated proteins and lipids. The evidence for separate compartments corresponding to early endosomes and the MVB came from studies on the kinetics of appearance of radiolabelled, internalised pheromone in cell fractions (Singer-Krüger *et al.,* 1993), and from following the fate of the fluorescent vital dye FM4-64 (Vida & Emr, 1995) or visualisation of the $\alpha$-factor receptor by immunofluorescence (Hicke *et al.,* 1997). Prescianotto- Baschong and Riezman (1998) developed the use of positively charged Nanogold to follow the endocytic pathway in yeast by electron microscopy. The authors have shown that positively charged Nanogold fulfills all the criteria for an endocytic marker. This method allowed them to visualise and confirm the existence of distinct organelles along the endocytic pathway, including primary endocytic vesicles, and a vesicular/tubular structure equivalent to mammalian early endosome, the MVB similar to the late endosome, and vacuoles. In animal cells the small GTPase Rab5 functions as a regulatory factor in the early steps of the endocytic pathway and is involved in homotypic fusion of early endosomes. Also some of its regulatory factors have been identified such as the guanine exchange factor Rabex5 or EEA1, early endosomal antigen. Vps21p (Ypt51p) was identified as the yeast homologue of Rab5p, but the Ypt51p-dependent step in the endocytic pathway remained unclear since its function in the Golgi-to-MVB step had been suggested (Tall *et al.,* 1999). The latest data from the Stevens laboratory (Gerrard *et al.,* 2000) suggest that Vps21p controls both the endocytic and the biosynthetic pathways of protein transport into the MVB. The finding that in *vps21* mutant two markers: endocytic – Ste3p, and late Golgi – Vph1p accumulated in different transport intermediates indicates that Vps21p controls different trafficking steps before their convergence at the MVB. Other proteins proposed as components of the endocytic machinery were two t-SNARE family members, Tlg1p and Tlg2p (Abeliovich *et al.,* 1998; Holthius *et al.,* 1998). Tlg2p is a t-SNARE involved in a stage of transport between primary endocytic vesicles and early endosomes (Seron *et al.,* 1998). However, according to Abeliovich *et al.* (1999), the primary function of Tlg2p is regulation of aminopeptidase I (API) transport to the vacuole *via* the Cvt pathway.

Lately model for ubiquitin-dependent sorting into the MVB has been proposed (Katzman *et al.,* 2001). Ubiquitinated cargo is recognised by a protein complex called ESCRT-I (endosomal sorting complex required for transport). The 350 kDa ESCRT-I, composed of multiple copies of Vps28p and Vps37p and one copy of Vps23p, associates with the endosomal membrane. ESCRT-I initiates the entry of cargo into the MVB. It acts in concert with a number of class E Vps proteins recruiting the deubiquitinating enzyme Doa4. The ubiquitin is removed from cargo prior to its entry into MVB. Free ubiquitin is recycled into the cytoplasm.

There is a growing body of evidence that ubiquitination is also required as a signal for sorting at the late Golgi of the amino-acid permeases Gap1 and Tat2, that undergo regulated trafficking. In this case monoubiquitination of the proteins promotes their transport to the cell surface, whereas polyubiquitination is a signal that targets the proteins to the vacuole (Hicke, 2001, and references therein).

## MVB-TO-VACUOLE TRANSPORT

Genetic and biochemical analysis of class B and C *vps* mutants (Table 1) enabled the identification of some of the proteins that are part of the very large vacuole-associated complexes required in MVB-vacuolar docking and fusion and also in the process of vacuole-vacuole homotypic fusion. Vam3p plays a central role in vacuolar protein transport as this vacuole-localised t-SNARE is required for the fusion of multiple vesicular intermediates with the vacuole (Darsow *et al.,* 1997). The docking event in the vacuole fusion reaction is dependent on the Rab GTPase Ypt7p (Mayer & Wickner, 1997; Ungermann *et al,* 1998; Kucharczyk *et al.,* 2000; Kucharczyk *et al.,* 2001). The multiple reactions at the stage of docking and fusion of MVB with the vacuole are directed by the class C-Vps protein complex associated with the vacuolar membrane and composed of Vps11p, Vps16p, Vps18p, Vps33p, Vps39p and Vps41p (Rieder & Emr, 1997). The class C-Vps complex interacts with unpaired Vam3p. It has been postulated that this complex associated with unpaired Vam3p mediates the assembly of t-SNARE complexes during MVB-vacuole, ALP and API transporting vesicles-vacuole docking/fusion and vacuole-to-vacuole fusion. The class C-Vps complex interacts with the active, GTP-bound form of Ypt7 (Price *et al.,* 2000; Sato *et al.,* 2000; Wurmser *et al.,* 2000).

## THE ALP PATHWAY

Alkaline phosphatase (ALP) encoded by the gene *PHO8* is an integral vacuolar membrane protein. Similarly to CPY, alkaline phosphatase is synthesised as a zymogen and undergoes proteolytic cleavages. ALP starts its vacuolar traffic at the ER and is transported through the Golgi where it is glycosylated. However, from the late Golgi compartment ALP takes its own way to the vacuole known as the "ALP pathway" that bypasses the MVB (Fig.1). The MVB-independent pathway was recognised by proper vacuolar localisation of ALP in mutants defective in the trafficking into or out of the MVB (Raymond *et al.,* 1992; Bryant & Stevens, 1998). In a separate study, the vacuolar localisation of mature ALP was found in mutants blocked in maturation of the vacuolar membrane protein carboxypeptidase S (CPS) or additionally blocked in the late secretory pathway (Piper *et al.,* 1997). The observation that ALP is not carried from the late Golgi by the *VPS45*-dependent route to the MVB, implied the existence of a second class of vesicles that carry vacuolar cargo. Transport of a subset of membrane proteins to the yeast vacuole along the ALP pathway requires the function of AP-3, an adaptor protein complex consisting of four subunits: Apl6p, Apl5p, Apm3p and Aps3p (homologs of the mammalian adaptins $\beta$3, $\mu$3 and $\sigma$, respectively). The AP-3 complex does not require clathrin to mediate ALP sorting. It has been proposed that the AP-3 complex recognises, at the late Golgi, a sorting signal in the cytosolic N-tail of ALP (Piper *et al.* 1997). The membrane association of AP-3 is regulated by Vps41p (Vam2p) and Arf GTP-ase. Although the ALP pathway depends on the product of the *VPS41* gene the pleiotropic defects of *vps41*$\Delta$ mutant indicated that Vps41p functions in several sorting pathways (Lemmon & Traub, 2000; Odorizzi *et al.,* 1998; Rehling *et al.,* 1999).

## AUTOPHAGY

Autophagy is generally a non-specific process that takes place in all eukaryotic cells. It sequesters the cytoplasm and organelles into large double-membrane vesicles (300–900 nm) known as autophagosomes, which deliver cargo to the vacuole where it is degraded and recycled. The outer autophagosomal membrane fuses with the vacuole to release a single-membrane-bounded autophagic body into the vacuolar lumen (Fig. 1). Autophagy is

highly regulated through the action of various kinases, phosphatases and GTPases. It is inhibited under nutrient-rich conditions and induced by nitrogen or carbon starvation or by treatment with rapamycin, an inhibitor of the Tor kinase (reviewed in: Kim & Klionsky, 2000; Klionsky & Emr, 2000).

An insight into the mechanism of autophagocytosis resulted from a study of a variety of *aut* and *apg* mutants defective in degradation of marker proteins or starvation-sensitive (Thumm *et al.,* 1994; Matsuura *et al.,* 1997). Four steps of autophagocytosis can be distinguished: Tor kinase dependent induction, formation of the autophagosomes, docking and fusion with the vacuole, and autophagic body breakdown

In yeast, as in mammalian cells, the delivery of autophagosomes to the vacuole depends on microtubules. *AUT2* and *AUT7,* two newly isolated *S. cerevisiae* genes essential for autophagocytosis encode microtubule-associated proteins. Aut7p is attached to microtubules *via* Aut2p, which interacts with the tubulins Tub1p and Tub2p. Aut2p- and Aut7p-depleted cells accumulate autophagosomes in the cytoplasm. This phenotype indicates that microtubules with the attached Aut2p–Aut7p complex are involved in the delivery of autophagic vesicles to the vacuole (Lang *et al.,* 1998).

The components of the vacuolar SNARE complex are required for autophagosome docking and fusion. They include proteins known from other pathways, i.e., the vacuolar t-SNAREs Vam3p and Vam7p, the GTP-binding protein Ypt7p, and the Vps11, Vps16, Vps18, Vps33, Vps39, Vps41 protein complex (for a review see: Klionsky & Emr, 2000).

A unique feature of autophagy is the process of autophagic body breakdown. Cytosolic proteins appear inside the vacuole enclosed in autophagic vesicles, which are broken down in the vacuole together with their cytosolic content. The autophagic bodies are then degraded by active vacuolar hydrolases (Baba *et al.,* 1994; 1997). Recently Aut4p, a putative integral vacuolar membrane protein, was identified as a component essential for the desintegration of autophagic vesicles inside the vacuole of *S. cerevisiae* cells (Suriapranata *et al.,* 2000).

## CYTOPLASM-TO-VACUOLE TARGETING

Aminopeptidase I and $\alpha$-mannosidase are vacuolar-resident hydrolases. In growing yeast cells they are imported into the vacuole by a constitutive non-classical vesicular transport mechanism, the cytoplasm-to-vacuole targeting (Cvt) pathway. Targeting of these enzymes to the vacuole may occur by an alternative transport route, the autophagy pathways, which operates under starvation conditions (Fig. 1) (Baba *et al.,* 1997; Scott *et al.,* 1997; Klionsky, 1998; Hutchins & Klionsky, 2001).

The marker protein of the Cvt pathway, API, is synthesised in the cytoplasm on free ribosomes as an inactive zymogen. The precursor form pro-API contains an amino-terminal region necessary for correct vacuolar sorting. This region is predicted to form two $\alpha$-helices. The first amphipathic helix seems to be critical for the correct sorting of the enzyme. In the cytosol, the newly synthesised API oligomerises forming a homododecamer of 732 kDa (Martinez *et al.,* 1997; Oda *et al.,* 1996). The oligomer is sequestered into double-membraned Cvt vesicles (130–150 nm). The Cvt vesicles fuse with the vacuole and release a single bilayer vesicle, the Cvt body, into the vacuolar lumen. API is released into the vacuolar lumen upon lysis of Cvt bodies, and the propeptide is cleaved resulting in the mature enzyme.

A significant degree of overlap has been uncovered between mutants isolated as defective in the Cvt pathway and mutants that were isolated as defective in autophagy. However, these are distinct processes since *cvt* mutants have been isolated that are not defective in autophagy, as well as aut mutants correctly

processing API (Harding *et al.,* 1996; Scott *et al.,* 1997).

According to the model proposed by Abeliovich *et al.* (1999), the Tlg2 and Vps45 proteins are required at an early step of assembly of Cvt vesicles that precedes the sequestration of API into the vesicle. Once formed, the Cvt vesicle undergoes docking and fusion with the vacuolar membrane where the Cvt pathway converges with the CPY and ALP pathways. The final docking and fusion of Cvt vesicles with the vacuolar membrane depends on the Vps proteins Vam3p and Vps18p. In the conditions of autophagocytosis induction API is sequestered into autophagosomes in a Tlg2p- and Vps45p-independent fashion.

Interestingly, some *cvt* mutants are allelic to the *vps* mutants *vps39* to *cvt4* and *vps41* to *cvt8* (Harding *et al.* 1995). A systematic screen of *vps* strains showed that *vps1, vps8, vps15, vps16, vps17, vps18* and *vps26* mutants are also defective in API and $\alpha$-mannosidase targeting. These data indicate that the same set of proteins is utilised by the CPY and Cvt pathways (Bryant & Stevens, 1998; Fischer von Mollard & Stevens,1999).

## HOMOTYPIC VACUOLE FUSION

Homotypic vacuole fusion depends on a t-SNARE–v-SNARE interaction as in the heterotypic vesicle-vacuole fusion. In the process of vacuole-vacuole fusion the two t-SNAREs Vam3p and Vam7p interact with the v-SNAREs Nyv1p, Vti1p and Ykt6p. These proteins form a *cis*-complex as it resides on the same membrane (Ungermann *et al.,* 1999). In resting vacuoles a tetrameric complex composed of Vam3p, Nyv1p, Sec17p and Vam7p resides on the vacuolar membrane. Transformation of resting vacuoles into fusion-competent ones requires the step known as "priming" when new, complementary SNARE complexes are formed on different membranes. At that step takes place the disintegration of the tetrameric complex, catalysed by the Sec18p ATPase, and Sec17p is released into the cytosol. The fusion-competent vacuoles have only one type of SNARE on their membrane and they dock to each other when the SNAREs are complementary. Docking is mediated by the active, GTP-bound form of Ypt7p, which regulates also the MVB-to-vacuole docking. The fusion of vacuoles requires the phosphoprotein phosphatase PPI and the $Ca^{2+}$-binding protein calmodulin (for a review see: Gotte & Lazar, 1999).

## VACUOLAR INHERITANCE

Yeast cells grow asymmetrically by budding, thus portions of all their organelles, including the nucleus, must be delivered to the bud. Vacuolar inheritance initiates early in the cell cycle. After the actin cytoskeleton is polarised, the vacuole aligns along the mother-bud axis towards the incipient bud site. Next, a region of the vacuolar membrane extends into the emerging bud *via* tubular and vesicular segregation structures. The vesicles deposited in the growing bud by the segregation machinery use homotypic fusion to form one or several larger vacuoles within the daughter cell. Mutants defective in vacuolar inheritance (*vac*) have been isolated by microscopic examination of cells that secrete CPY to identify those that lack the segregation structures. The *vac* mutants are divided into three classes on the basis of their vacuole morphology: Class I, that have normal vacuole morphology, but lack the vacuole in the bud; Class II, that have less vacuole lobes and often a node is detected on the vacuole membrane that is oriented towards the bud; Class III, that have a greatly enlarged, single vacuole and in one fourth of the cell population a single vacuole is shared between the mother and the bud. The lack of vacuolar inheritance seen in the *vac* mutants is not due to some gross perturbation of cell division, since many of the *vac* mutants are normal for the inheritance of other

organelles such as the nucleus and mitochondria, and their buds grow to a normal size. The actin cytoskeleton is critical for vacuole inheritance. It has been proposed that myosin serves as a motor for vacuole transport along actin filaments from the mother cell into the bud (for a review see Catlett & Weisman, 2000).

## ROLE OF LIPIDS IN THE REGULATION OF VESICULAR TRAFFICKING

To conclude the review it is worth to mention a new trend in the studies on vesicular traffic. The major lipid constituents of the plasma membrane, phosphatidylinositols (PIs), sphingolipids and sterols, have emerged as important transport regulators indicating that not only proteins, but also lipids play a role in this process. PIs are substrates for PI kinases and PI phosphatases, which modify their inositol head group to generate different PIs, phosphorylated at one or a combination of positions (D'Hondt *et al.,* 2000, and references therein).

PI-4,5-$P_2$ plays a role in the internalisation step of clathrin-mediated endocytosis and PI-3-P functions at a postinternalisation step of endocytosis and in the endosomal/vacuolar traffic. Sphingolipids, together with ergosterol, form sterol-sphingolipid-rich domains in the plasma membrane (rafts) that may define the membrane spatial specificity by recruiting the endocytic machinery to a distinct membrane site. Moreover, rafts are involved in the biosynthetic delivery of some proteins to the plasma membrane (Bagnat *et al.,* 2000; Simonsen *et al.*, 2001).

## CONCLUDING REMARKS

Molecular genetic studies of vacuolar transport using *S. cerevisiae* have led to the identification of many cellular components required for this process. The importance of studies on this very complex and highly regulated process is highlighted by numerous inherited human diseases, viral infections and cancers that are linked to disorders in protein transport.

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