Functional Studies of Vesicular Transport in Yeast

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Abstract

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A basic feature of eukaryotic cells is their compartmental organisation. To maintain this cellular order, a strictly regulated intracellular transport is essential. The compartments are defined by their lipid membranes and the transport between different compartments is therefore mediated by lipid vesicles.
The yeast *Saccharomyces cerevisiae* is a very suitable model organism for studies of intracellular transport. In order to find new yeast genes involved in intracellular transport, a screen was performed using a knockout yeast strain collection and two drugs known to interfere with intracellular transport, monensin and brefeldin A. In an initial screen, seven new genes were found (*MON1-2* and *BRE1-5*) that caused an increased sensitivity to either drug when knocked out. When the monensin screen was extended to the entire yeast genome, a total of 63 sensitive strains were found. Most of the encoded proteins are known to be involved in post-Golgi transport.
Some of the 63 monensin-sensitive knockout mutants showed synthetic interactions with a *vma1* knockout mutant, ranging from complete synthetic lethality to reduced growth. These interactions were specific for genes involved in retrograde transport.
One of the new genes found in the screen, *MON1*, is involved in transport to the vacuole. A *mon1* deletion strain is impaired in maturation of proaminopeptidase both during starvation and during nutrient-rich conditions, indicating that Mon1p is involved in both the autophagic and the cytoplasm-to-vacuole targeting pathways. A GFP-Mon1p fusion protein localizes to the cytosol, and to punctate structures in the cytosol. Furthermore, the autophagosomal marker protein GFP-Aut7p accumulates in *mon1* deleted cells at punctate structures near the vacuole.
The two syntaxins, Sso1p and Sso2p, are involved in the fusion of vesicles to the plasma membrane, where they can functionally replace each other. However, the *SSO1* gene also has an essential function in meiosis, which *SSO2* cannot provide. Gene fusions between *SSO1* and *SSO2* were used to map this meiosis-specific function to two short elements within the 3'-untranslated region of the *SSO1* gene.

Keywords: Brefeldin A, monensin, *Saccharomyces cerevisiae*, syntaxin, vacuole, vesicular transport

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This Thesis Is Dedicated To My Father

Att vara stark är inte
Att aldrig falla
Att alltid veta
Att alltid kunna

Att vara stark är inte
Att alltid orka skratta
Att hoppa högst
eller vilja mest

Att vara stark är inte
Att lyfta tyngst
Att komma längst
eller att alltid lyckas

Att vara stark är
Att acceptera livet som det är
Att acceptera dess kraft
och ta del av den
Att falla till botten
Slå sig hårt
och alltid komma igen

Att vara stark är
Att våga hoppas
när ens tro är som svagast

Att vara stark är
Att se ljus i mörkret
och alltid kämpa
för att nå dit.

Contents

Appendix

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Murén E., Öyen M., Barmark G., Ronne H., (2001) Identification of yeast deletion strains that are hypersensitive to brefeldin A or monensin, two drugs that affect intracellular transport. *Yeast* 18:163-172.

II. Meiling-Wesse K., Barth H., Voss C., Barmark G., Murén E., Ronne H., Thumm M., (2002) Yeast Mon1p/Aut12p functions in vacuolar fusion of autophagosomes and cvt-vesicles. FEBS Letters 530: 174-180.

III. Barmark G., Gustavsson M., Larsson J., Murén E., Ronne H., Functional genomics of monensin sensitivity in yeast: implications for post-Golgi traffic. Manuscript.

IV. Barmark G., Murén E., Harnemark H., Gustavsson M., Ronne H. Mapping of sequences in the 3'-untranslated region of the yeast plasma membrane syntaxin gene *SSO1* that are required for sporulation. Manuscript.

Abbreviations

List of abbreviations used in the text:

ALP	alkaline phosphatase
API	aminopeptidase I
BFA	brefeldin A
COG	conserved oligomeric Golgi complex
CPS	carboxypeptidase S
CPY	carboxypeptidase Y
Cvt	cytoplasm to vacuole transport
ER	endoplasmatic reticulum
GARP	Golgi-Associated Retrograde Protein complex
GDF	GDI-Displacement Factor
GDI	Rab GDP Dissociation Inhibitor
GEF	GDP-GTP Exchange Factor
HOPS	HOmotypic fusion and vacuole Protein Sorting complex
LE	late endosome (same as PVC)
LEP	leading edge protein coat
MVB	multivesicular bodies (same as PVC)
NSF	*N*-ethylmaleimide sensitive factor
PM	plasma membrane
PrA	proteinase A
PrB	proteinase B
PVC	prevacuolar compartment (same as MVB)
SNARE	*s*oluble *N*SF *a*daptor protein *re*ceptor
SPB	spindle pole body
TGN	*trans*-Golgi network
TOR	target of rapamycin
TRAPP	TRAnsport Protein Particle complex
t-SNARE	target membrane SNARE
UTR	untranslated region
VAM	vacuolar morphology
v-ATPase	vacuolar ATPase
VFT	Vps fifty three complex
VPS	vacuolar protein sorting
v-SNARE	vesicle SNARE

Introduction

The yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a species of budding yeast, and it is perhaps the most thoroughly studied eukaryotic model organism. The name *Saccharomyces cerevisiae* is derived from its ability to metabolise a number of carbohydrates, commonly glucose (greek *saccharon* = sugar and *myketes* = fungus). Egypt was the location where yeast was first used to bake bread, approximately 4000 B.C. In 1859, Louis Pasteur discovered how yeast worked and explained the fermentation that takes place when you brew beer and wine. The industrial utilisation of yeast is, however, not limited only to traditional processes like baking of bread, and fermentation of wine and beer. Yeast is also a rich source for industrially important enzymes like invertase. Furthermore, yeast extract has important uses as a source for vitamin B and D, in enhancing the taste of food and as a common ingredient in microbiological growth media (Grainger J, 2001). The natural habitats of the yeast *S. cerevisiae* are fruits and fruit juices. The commercial yeast that is used now is somewhat different from the original wild type yeast since it has been modified by geneticists to be more suitable for baking and brewing (Madigan, Martinko & Parker, 1997).

Yeast as a model organism

There are many advantages to using the yeast *Saccharomyces cerevisiae* as a model system. It was the first eukaryotic organism whose genome was completely sequenced. The complete genome sequence was published as a supplement to *Nature*, Vol 387, on the 29th of May 1997 (Grainger J, 2001). Properties like stable haploid and diploid phases and the fact that it is viable even if burdened with several different auxotrophic selection markers makes yeast more suitable for molecular studies than many other microorganisms. A haploid yeast cell has 16 chromosomes, containing approximately 6000 genes. Out of these 6000 genes about 20 percent are essential for growth (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). In the haploid phase the yeast *S. cerevisiae* exists in two different mating types, MATa and MATα. They can be crossed to each other to form a diploid cell. Upon nutrient starvation these diploid cells sporulate during which they undergo meiosis and form four new haploid spores, two of each mating type.

The yeast *S. cerevisiae* is easy to work with and is not pathogenic. It is easy to transform *S. cerevisiae*, to use methods like targeted gene knockouts by homologous recombination, and to perform different screens using plasmid libraries. The rapid growth and the budding pattern that result in dispersed cells is also valuable when working with yeast (Guthrie & Fink, 1991). Much of our current knowledge in biochemistry, cell biology, and molecular genetics is based on work performed in yeast.

Organelles of the yeast cell

Yeast is, as all other eukaryotes, divided into compartments and organelles like the nucleus, the endoplasmic reticulum (ER), mitochondria, the Golgi complex, the vacuole, endosomes, and peroxisomes. The different compartments are separated by lipid membranes and contain enzymes and other proteins that are specific for that particular compartment. It is of vital importance that these proteins are kept separate from each other in order for the cell to function properly. Correct sorting of newly synthesized proteins to different intracellular compartments is therefore an essential and highly conserved process in all eukaryotes.

The vacuole

The yeast vacuole is the equivalent to the mammalian lysosome and the vacuole of plant cells. The vacuole plays an essential role in adaptation of the cell to environmental changes and during stress conditions like nutrient depriviation. It also plays an important role in the maintenance of cellular pH and ion-homeostasis, and functions as a storage compartment for ions (Jones, Webb & Hiller, 1997) and amino acids. It is the main site of protein degradation and contains a variety of degradative enzymes, such as proteinase A (PrA), proteinase B (PrB), carboxypeptidase Y (CPY), carboxypeptidase S (CPS), aminopeptidase I (API), alkaline phosphatase (ALP), trehalase, RNase and ∝-mannosidase (Klionsky, Herman & Emr, 1990, and references therein). The vacuole is the terminal destination for most membrane-mediated transport in the cell. In wild type yeast cells, the vacuole takes up as much as 25% of the cellular volume, making it the single largest organelle.

Storage

The vacuole serves as a storage site for amino acids, purines, polyamines, S-adenosylmethionine and polyphosphates (Kucharczyk & Rytka, 2001). The vacuolar lumen is acidic, and the resulting pH gradient across the vacuolar membrane is important for the transport of arginine, asparagine, glutamine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan and tyrosine into the vacuole (Jones, Webb & Hiller, 1997).

Degradation

There are two main routes for protein degradation in the yeast cell. One is via the proteasome and the other via the vacuole. The vacuolar system is more suitable for degradation of larger compounds like protein complexes and organelles (Teter & Klionsky, 2000). Degradation of cellular macromolecules is important during nutrient stress, when the synthesis of essential macromolecules needs to be maintained at the expense of non-essential ones. Some proteins, like cell surface receptors, are downregulated by being transported to the vacuole, where they are degraded. There are several different hydrolases in the vacuole that are responsible for the degradation of proteins (Jones, Webb & Hiller, 1997).

Ion homeostasis

The vacuole functions as a reservoir for mono- and divalent cations (Kucharczyk & Rytka, 2001). Ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Li^{+}, phosphate, K^{+}, Cl^{-} and Cd^{2+} are stored in the vacuole. In the case of Ca^{2+}, it has been shown that the vacuole regulates the ion homeostasis by releasing two protons for every Ca^{2+} ion that it sequesters. The vacuole also has a detoxifying function, since storage of for example Cd^{2+} or Sr^{2+} ions in the vacuole means that the cytosolic levels of them are kept low (Jones, Webb & Hiller, 1997, and references therein).

Acidic pH

Another feature common to both the yeast vacuole and the mammalian lysosome is that they are acidic organelles. It is the vacuolar ATPase (v-ATPase) that is responsible for establishing and maintaining the low vacuolar pH. The v-ATPase utilizes the energy generated by the hydrolysis of ATP to pump protons into the vacuolar lumen (Jones, Webb & Hiller, 1997). In addition to the difference in pH between the cytoplasm and the vacuolar lumen, there is also a pH gradient between different non-cytoplasmic compartments in the cell, from the endoplasmic reticulum to the Golgi complex, and then on to the late endosome, and finally the vacuole, with a lower pH closer to the vacuole (Demaurex, 2002).

Intracellular transport

Intracellular transport is vital for maintenance of the intracellular organization of the eukaryotic cell. The transport is hence strictly regulated. Specificity in transport is mediated by several different protein complexes that are involved in the transport, docking and fusion of a transport vesicle to the right target membrane. The specific transport pathways involved, see Figure 1, and some of the most important proteins and complexes that function in intracellular transport, are further discussed below.

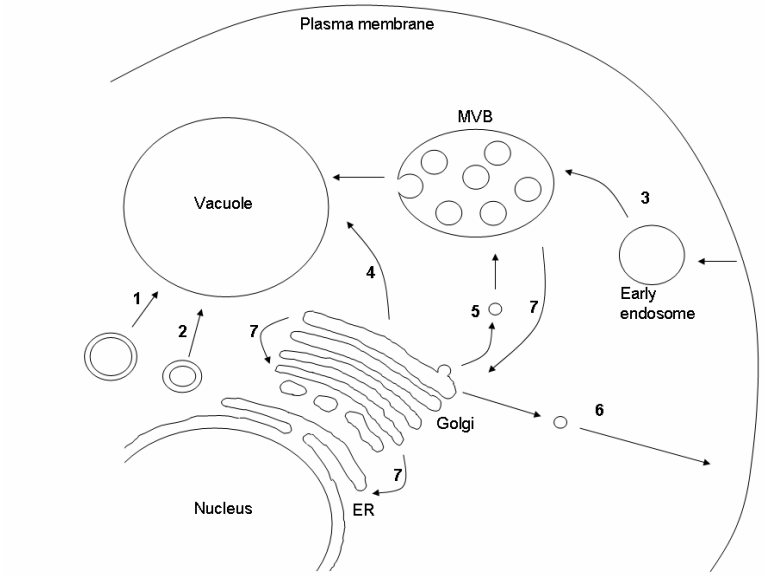

Fig. 1. The intracellular transport pathways of the yeast cell.

1. Autophagic pathway
2. Cvt-pathway (Cytoplasm-to-vacuole targeting)
3. Endocytic pathway
4. ALP-pathway (Alkaline phosphatase)
5. CPY-pathway (Carboxypeptidase Y)
6. Exocytic pathway
7. Retrograde transport

MVB, multivesicular body; ER, endoplasmic reticulum.

Transport pathways

ER to Golgi transport

Newly synthesized proteins that are destined for the secretory pathway carry a signal peptide which targets them for co-translational translocation across the membrane of the endoplasmic reticulum, ER. The secretory proteins are modified by glycosylations within the ER. Upon arrival to the Golgi complex the proteins are further modified. Transport between the ER, and the Golgi comprises both anterograde transport, where newly synthesised proteins are transported from the ER to the Golgi, and retrograde transport, where ER resident proteins are recycled to the ER from the Golgi complex.

There are two complexes that have been implicated in ER to Golgi transport, TRAPP and the COG complex. TRAPP (transport protein particle) is a Golgi-associated complex, which exists in two forms in the yeast cell, either as TRAPP II, containing ten subunits, or as TRAPP I, containing only seven subunits.

TRAPP I and TRAPP II may act at different steps in the secretory pathway, since the TRAPP I complex binds to ER-derived COPII vesicles, but TRAPP II does not. This indicates that the TRAPP II specific subunits (Trs120p, Trs130p and Kre11p) somehow interfere, either directly or indirectly, with the binding of TRAPP to the COP II vesicle. TRAPP I binding may be linked to COPII vesicle uncoating, raising the possibility that TRAPP I may participate in the targeting and/or attachment of ER-derived vesicles to the Golgi. A strain mutated in the TRAPP II specific TRS130 is deficient in intra-Golgi transport (Oka & Krieger, 2005).

The COG complex comprises eight proteins, COG1-8. It has been proposed to be involved in three different activities: protein sorting on exit from the ER, attachment and/or targeting of ER-derived vesicles to the Golgi, and retention and/or retrieval of Golgi-localized proteins. There are genetic and/or physical interactions between the COG complex and several components of the secretory pathway, including the COPI coat, and several SNAREs and small GTPases. The precise role of the COG complex in Golgi function and membrane trafficking remains to be established. (Oka & Krieger, 2005, and references therein).

Exocytosis

Proteins destined for secretion (exocytosis) are translated at the rough ER and translocated co-translationally into the lumen of the ER. They are then transported inside lipid vesicles to the Golgi complex. At the late Golgi, they are further transported inside new lipid vesicles to the plasma membrane. There are at least two different routes from the Golgi to the plasma membrane, as evidenced by the fact that there exists two different populations of late secretory vesicles that accumulate in secretory mutants. One vesicle population contains Bgl2p and the major plasma membrane ATPase, Pma1p, as well as Snc1p, while the other population contains the enzymes invertase and acid phosphatase (Harsay & Bretscher, 1995).

A number of SEC genes that are involved in the secretory pathway were originally discovered as temperature-sensitive mutations (Novick, Field & Schekman, 1980). The late-acting SEC genes SEC1, 2, 3, 4, 5, 6, 8, 9, 10 and 15, as well as SSO1, SSO2, SNC1 and SNC2 are involved in the fusion of Golgi derived vesicles to the plasma membrane (Neiman, 1998). Each vesicle that is transported from the Golgi complex to the plasma membrane carries the v-SNAREs Snc1p and Snc2p on its surface, which can interact with the t-SNAREs Sec9p, Sso1p and Sso2p on the inner surface of the plasma membrane. This interaction is regulated by the Rab GTPase Sec4p. The exocyst is composed of the eight proteins Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p and is involved in the tethering and docking of transport vesicles to the plasma membrane. The Mso1p protein (Aalto et al., 1997) is also part of the exocyst (Knop et al., 2005). In yeast cells deficient for one of the exocyst subunits, secretory vesicles accumulate in the cell, presumably because the vesicles are unable to dock with and fuse to the plasma membrane. During cytokinesis, the exocyst is localized to the mother-daughter cell connection, while at the beginning

of the cell cycle it is localized to the bud tip (Lipschutz & Mostov, 2002). The localizations reflect the fact that these are the major sites at which new membrane is added during cytokinesis and mitotic growth. The exocyst is known to interact both genetically and physically with the translocon, which is positioned at the endoplasmic reticulum (ER) and is responsible for the translocation of proteins across the membrane into the ER lumen (Guo & Novick, 2004). Overexpression of the β-subunit of the translocon, Sbh1p, can partially suppress growth defects in all exocyst sec mutants (Toikkanen et al., 2003). It has also been shown in mammalian cells that the β-subunit of the translocon associates with the exocyst (Lipschutz, Lingappa & Mostov, 2003). These interactions may provide a way to maintain the balance between protein synthesis and secretion of proteins.

Transport to the vacuole

There are several different transport pathways that converge on the vacuole in yeast. Newly synthesised vacuolar proteins are transported through the common secretory pathway from the ER membrane or lumen, through the Golgi complex, but are then diverted from the secretory pathway and instead transported via specific vesicles to the vacuole. This pathway is called the CPY (carboxypeptidase Y) pathway. CPY is synthesized as a precursor protein, Prc1p, which is translocated into the ER lumen concomitant with cleavage of the ER signal peptide. The protein is glycosylated in the ER and further modified in the Golgi. Upon arrival to the vacuole, CPY is processed to its mature form by vacuolar hydrolases (Jones, Webb & Hiller, 1997) Material from the cell surface is also delivered to the vacuole, by endocytosis. The CPY and endocytic pathways converge at the MVBs (multivesicular bodies). There is a third pathway, the ALP (alkaline phosphatase) pathway, that diverges from the CPY and secretory pathway at the late Golgi, and bypasses the MVBs. ALP is a vacuolar membrane protein, which is synthesized as a precursor (Pho8p), just like CPY, and cleaved to its mature form upon arrival to the vacuole (Jones, Webb & Hiller, 1997). The ALP pathway does not require clathrin. Furthermore, proteins can be delivered to the vacuole from the cytosol, either by Cvt (cytoplasm-to-vacuole targeting) pathway or by autophagy (see below). Upon budding, about 50% of the vacuolar material is transferred to the daughter cell in a process called vacuolar inheritance (Kucharczyk & Rytka, 2001).

Many mutants have been isolated whose gene products are required for Golgi to vacuole transport. They either missort soluble vacuolar proteins (vps), lack vacuolar protease activity (pep) or have aberrant vacuolar morphology (vam) (Rothman & Stevens, 1986; Rothman, Howald & Stevens, 1989; Bankaitis, Johnson & Emr, 1986; Robinson et al., 1988; Jones, 1977; Wada, Ohsumi & Anraku, 1992). There is a significant overlap between these three sets of genes. Over 70 vacuolar protein sorting (VPS) genes have been identified in different screens (Bryant and Stevens, 1998, Conibear and Stevens, 1998, Bonangelino, Chavez & Bonifacino, 2002). These VPS genes have been classified into six different classes (A-F) according to the vacuolar morphology of the mutant yeast strain (Banta, 1988; Raymond et al., 1992).

The HOPS complex (HOmotypic fusion and vacuole Protein Sorting) contains Pep5p (Vps11p), Vps16p, Pep3p (Vps18p), Vps33p, which are class C Vps proteins, and Vam6p (Vps39p) and Vps41p, two class B proteins. The HOPS complex, together with Ypt7p, is required for docking of vacuoles to each other in homotypic vacuole fusion (Seals et al., 2000). The four class C Vps proteins in the HOPS complex are also found together in the smaller class C Vps complex. This complex is involved in docking and fusion of cargo vesicles from the biosynthetic pathway to the vacuole. The class C Vps complex regulates the assembly of the trans-SNARE complexes formed at the vacuolar membrane (Sato et al., 2000).

The Ccz1p-Mon1p complex is required at the tethering/docking stage (Wang et al., 2002). Ccz1p can function either in association with Mon1p and Ypt7p in the fusion at the vacuole, but can also interact with Arl1p at an earlier step in transport to the vacuole (Hoffman-Sommer et al., 2005).

Retrograde transport

In most transport pathways, there is a process for retrieving certain components so that they can be reused. The GARP (Golgi-Associated Retrograde Protein)/VFT (Vps Fifty Three) complex consists of four proteins, Vps51p-Vps54p. It is required for retrograde transport of Golgi-localized proteins from the late endosome to the Golgi complex. Vps52p binds directly to Ypt6p, which is an essential protein for the retrograde traffic to the Golgi. Vps51p interacts with the N-terminal region of Tlg1p and Vps53p with the Arl1p protein. The GARP complex is highly conserved, and has also been found in worms, flies, mammals and plants (Oka & Krieger, 2005 and references therein).

The retromer complex is involved in transport from the endosome to the Golgi. The retromer comprises the five proteins Vps35p, Vps29p, Vps26p, Vps17p and Vps5p, and is also conserved in mammalian cells (Seaman et al., 1997, Seaman, McCaffery & Emr, 1998; Seaman, 2004). The retromer is important for the retrieval of the Vps10p protein to the Golgi complex. Vps10p functions as a receptor for vacuolar hydrolases such as carboxypeptidase Y (CPY). It binds to the target proteins in the late Golgi, where Vps10p and its bound target are packed into a vesicle and transported to the vacuole (Seaman, 2004 and references therein). Vps35p is the protein that is responsible for selecting the cargo proteins to be transported (Nothwehr, Ha & Bruinsma, 2000).

Endocytosis

Endocytosis is an important process in all eukaryotic cells. It is involved in different cellular functions such as nutrient uptake, and in the regulation of the expression of cell surface receptors, transporters and ion channels (Dupré, Urban-Grimal & Haguenauer-Tsapis, 2004). During endocytosis, macromolecules are taken up by the cell through invagination of the plasma membrane. The endocytosed substances first arrive to early endosomes, then move on to late endosomes, and finally to the vacuole, where they are degraded by different hydrolases.

Studies with the fluorescent dye FM4-64 (Vida & Emr, 1995) revealed that there are separate compartments corresponding to early endosomes and late endosomes in the yeast cell. The early endosomes tend to be tubular and are located towards the cell periphery, while the late endosomes are more spherical and often located closer to the nucleus. The late endosomes are capable of both homotypic fusion with other late endosomes and heterotypic fusion with the vacuole (Piper & Luzio, 2001). A subset of late endosomes has internal vesicles and are hence called multivesicular bodies, MVBs (Katzmann, Odorizzi & Emr, 2002). These vesicles are derived from invaginations of the endosomal membrane. The ESCRT complexes (see below) are involved in the formation of the MVB. Mutations in the class E VPS genes cause accumulation of an aberrant endosome (Xu et al., 2004). Proteins in the MVB membrane and inside the lumen of the MVB are eventually transported to the vacuole (Katzmann et al., 2002). The MVB is therefore also referred to as the PVC, the prevacuolar compartment. Late endosomes have a lower lumenal pH than early endosomes, and this pH gradient has been implicated in controlling the direction of intracellular transport.

Ubiquitin is required for sorting of cargo both at the plasma membrane, at the late endosome, and at the TGN, trans-Golgi network (Hicke, 2001). At the plasma membrane, ubiquitination of membrane proteins is a sorting signal for endocytosis. Proteins destined for endocytosis are thus covalently tagged with monoubiquitin (Hicke, 2001; Hicke & Dunn, 2003). Ubiquitin is used as a signal for endocytosis both in Saccharomyces cerevisiae, and in higher eukaryotes (Hicke, 2001). It should be noted that ubiquitination is also a signal for degradation via the proteasome (Hershko, Ciechanover & Varshavsky, 2000). The difference, however, is that when destined for proteasomal degradation, the protein is tagged with a polyubiquitin chain instead (Hicke, 2001, and references therein). There are several proteins that are involved in catalysing the ubiquitination steps. E1 is a ubiquitin-activating enzyme, E2 is a ubiquitin-conjugating enzyme and E3 a ubiquitin ligase (Hicke, 1999). ATP is needed both for the ubiquitin-protein ligation and the action of the 26S proteasome complex in protein degradation (Hershko, Ciechanover & Varshavsky, 2000).

There are three different ESCRT complexes (Endosomal Sorting Complex Required for Transport), the subunits of which are encoded by 10 of the class E VPS genes. The ESCRT complexes are involved in selecting and sorting of cargo proteins into the interior of the multivesicular body (MVB), for delivery to the vacuole (Babst et al., 2002b). The ESCRT-III complex is recruited to the endosomal membrane by the ESCRT-II complex, which in turn is recruited by the ESCRT-I complex. The ESCRT-I complex contains a single copy of Vps23p and multiple copies of Vps28p and Vps37p. The ESCRT-II complex consists of Vps22p, Vps25p and Vps36p, while the ESCRT-III complex contains two functionally distinct subcomplexes, the Vps20p-Snf7p subcomplex which binds to the endosomal membrane and the Vps2p-Vps24p subcomplex which binds to the Vps20p-Snf7p subcomplex and serves to recruit additional cofactors (Babst et al., 2002a).

There is a link between actin and endocytosis, since actin patches are formed at pre-existing sites containing components of the endocytic machinery. The actin polymerization that occurs at these sites has been proposed to be the force that drives invagination of the endocytic vesicles into the cell (Kaksonen, Sun & Drubin, 2003).

Autophagy and the Cvt pathway

Autophagy is a pathway utilized for transporting proteins and organelles to the vacuole for degradation during nutrient starvation, so that the resulting amino acids can be made available for de novo synthesis of essential proteins. There is also some recycling of proteins by vacuolar degradation under normal growth conditions, for which the related but distinct cytoplasm-to-vacuole-targeting pathway (cvt) is utilized. Most of the genes involved in the autophagic and cvt-pathways are common to both pathways, but there are also genes specific for each pathway (Harding et al., 1996; Scott et al., 1996) The autophagic pathway is universal to all eukaryotic cells, while the cvt-pathway so far has been described only in Saccharomyces cerevisiae (Farré & Subramani, 2004). There is a huge capacity in the cell to degrade proteins. Half of the cellular proteins can thus be degraded in 24 hours (Teichert et al., 1989).

There are three forms of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy (Klionsky, 2005). Autophagy in yeast most often occur by macroautophagy. A vesicle with double membranes, called an autophagosome, fuses to the vacuole, leaving a single-membrane autophagic body inside the vacuole. In microautophagy cytosolic compounds are either invaginated into the vacuole or taken up via an enwrapping mechanism, in which the vacuolar membrane surrounds the cytosolic material. Chaperone-mediated autophagy, finally, is a secondary response to starvation, and involves direct translocation of the targeted proteins across the vacuolar membrane (Massey, Kiffin & Cuervo, 2004).

Cvt vesicles also have double membranes but are smaller than autophagosomes and do not include bulk cytosol. The cvt pathway is specific with respect to the targeted proteins, while the autophagic pathway is non-specific and also not easily saturated (Teter & Klionsky, 2000). Upon fusion of the cvt vesicles to the vacuole, cvt bodies are released into the vacuolar lumen, see Figure 2. These are single bilayer vesicles that are subsequently lysed in the vacuole. The lysis of the vesicles is dependent on the acidic pH of the vacuolar lumen and on proteinase B (Nakamura et al., 1997; Takeshige et al., 1992). Both autophagosomes and cvt-vesicles are thought to originate from the same pre-autophagosomal structure, PAS (Suzuki et al., 2001; Kim et al., 2001), but the exact mechanism of vesicle formation is not known so far.

When cells are starved for either nitrogen or carbon, the switch from the cvt pathway to the autophagic pathway is dependent on a Tor (target of rapamycin) kinase signalling cascade (Teter & Klionsky, 2000). Autophagy is inhibited by the Tor kinase under basal i.e. nutrient-rich conditions (Klionsky, 2005). API,

aminopeptidase I, is a protein that is specifically transported by the autophagic pathway during starvation and by the cvt pathway otherwise. It is transported as a precursor and cleaved to its mature form upon arrival to the vacuole. Studies of whether pro-API accumulates within a cell can therefore be used to see if there is a block in the transport to the vacuole. Several mutants have been isolated in genes encoding proteins necessary for the autophagic or cvt pathways. The apg mutants, isolated by Tsukada and Ohsumi (1993) were shown to be essential for autophagy, while the cvt mutants, isolated by Klionsky's group were shown by Harding et al (1995) to be defective in API maturation. Complementation studies (Scott et al., 1996; Harding et al., 1996) have shown that the cvt mutants largely overlap with the apg mutants and also with the aut mutants isolated by Thumm et al. (1994), that also are defective in autophagy.

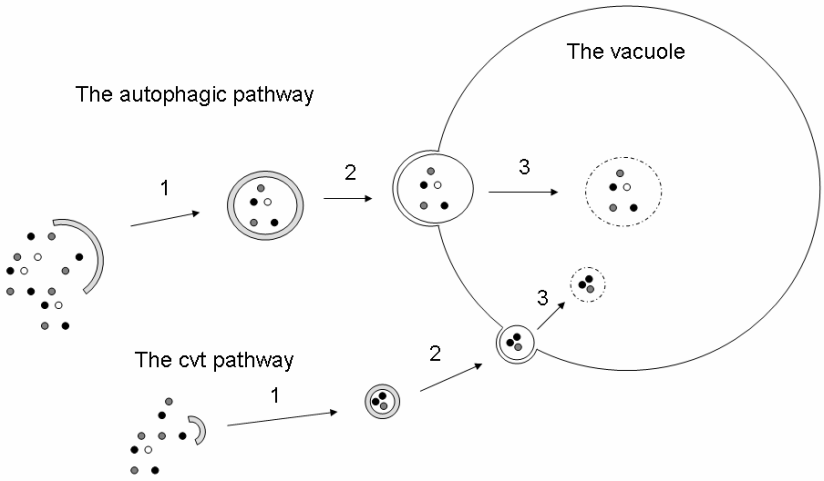

Fig. 2. Formation of an autophagic double membrane vesicle (autophagosome) and a cvt vesicle, and their subsequent uptake into the vacuole.

1. Formation of a double bilayer membrane vesicle
2. Fusion of the autophagosome/cvt vesicle with the vacuole
3. Lysis of the autophagic body/cvt body inside the vacuole, and subsequent release of the cargo into the vacuolar lumen
Cvt, cytoplasm-to-vacuole targeting

Pexophagy

Pexophagy is the specific turnover of peroxisomes. The peroxisomes are organelles without DNA, unlike the mitochondria. They have a single membrane and enclose enzymes that produce and degrade hydrogen peroxide. Redundant, damaged or non-functional peroxisomes are degraded in the vacuole by

autophagy-related processes (Farré & Subramani, 2004). There are two independent pathways for peroxisome degradation, one similar to macroautophagy and the other similar to microautophagy. Pexophagy has been best characterized in the yeasts Hansenula polymorpha and Pichia pastoris, which have more peroxisomes than Saccharomyces cerevisiae (Thumm, 2000).

Proteins involved in intracellular transport

The transport between different organelles within the cell is mediated by lipid vesicles. These vesicles have proteins attached to them that direct the vesicles to the proper target membrane through protein-protein interactions. This is a strictly regulated process, since it is of high importance that the right transport vesicle is targeted to the right membrane in order to maintain the intracellular order. SNARE proteins, SM (Sec1p/ Munc18) proteins, small GTPases of the rab family, and membrane tethering factors are necessary for vesicle targeting and fusion (Bowers & Stevens, 2005).

Coat complexes

Coating of the transport vesicles with proteins takes place during the formation of the vesicles by budding from the donor membrane. Before the fusion of the transport vesicle to the target membrane the coat is released. The vesicle loses its coat in a multi-step process which includes inactivation of the small GTPase, phosphoinositide hydrolysis, and the action of uncoating enzymes.

The first vesicle coat to be described was the clathrin coat, discovered by Roth and Porter (1964) and Pearse (1975). It was later found that clathrin coats are restricted to post-Golgi transport steps, including transport from the Golgi to the plasma membrane, transport within the trans-Golgi network (TGN), transport from the late Golgi to the endosomes (Lewis et al., 2000; Sipos et al., 2004; Ha et al., 2003; Bonifacino & Glick, 2004), and transport from the plasma membrane to the early endosome (Kirchhausen, 2000). Vesicles transported between the ER and the Golgi are instead coated with COPI or COPII complexes. The COPI complex is involved in intra-Golgi transport and retrograde transport from the Golgi to the ER (Letourneur et al., 1994), while COPII is involved in anterograde transport from the ER to the Golgi complex (Barlowe et al., 1994), see Figure 3.

COPII

Vesicular transport between the ER and the Golgi can be minimally reconstituted using only five proteins: the Sec23p-Sec24p complex, the Sec13p-Sec31p complex and the small GTPase Sar1p, which belongs to the Arf family (Barlowe et al., 1994). These five proteins acting together are capable of capturing the cargo, deforming the budding membrane, inducing the scission that detaches the vesicle from the donor membrane, and also inducing the coat release. The COPII-coat assembly is activated by the recruitment of a Sar1p-GTP complex to the membrane. This allows the binding of the Sec23p-Sec24p complex to occur and stimulates the recruitment of the cargo. The next step is membrane deformation,

which is caused by the binding of the Sec13p-Sec31p complex. The vesicle buds off when the coat is complete. The GTPase activity of Sar1p is stimulated by Sec23p, which act as a timer, leading to inactivation of Sar1p and subsequent uncoating (Kirchhausen, 2000; Mancias & Goldberg, 2005). The disassembly of the coat complex makes it possible for the tethering factors to bind to the naked transport vesicle and target it to the correct membrane.

COPI

The COPI coatomer is a complex of seven proteins, α, β, β', γ, δ, ε and ζ. The initial event in the COPI pathway is the association of the small GTPase Arf1p in its active GTP-bound form to the membrane, which leads to the subsequent recruitment of the COPI coatomer (Kirchhausen, 2000). COPI-dependent transport is not unidirectional like COPII-dependent transport (ER to Golgi), but can occur in both the anterograde and retrograde direction. The recruitment of Arf1p-GTP to the membrane also stimulates the recruitment of cargo proteins to the budding site. GTP hydrolysis is slow when Arf1p is bound to its preferred cargo, allowing kinetic regulation of coat recruitment. Thus, when a non-preferred cargo is bound, GTP hydrolysis is fast, and the Arf1 protein will not be retained long enough to complete the coat assembly. Membrane deformation occurs at the same time as coat recruitment. The vesicle buds when the coat is complete. The GTPase activity of Arf1p is stimulated by ARF1GAP, which act as a timer, leading to inactivation of Arf1p and uncoating (Kirchhausen, 2000). This resembles the action of Sec23p on Sar1p in the case of COPII vesicles.

Clathrin

The clathrin heavy and light chains are encoded by the CHC1 and CLC1 genes, respectively. Chc1p polymerizes into a triskelion (Kirchhausen, 2000). These cytosolic triskelions assemble into a coat that drives membrane deformation. Clathrin-coated vesicles have a large number of associated proteins, in contrast to the COPI- and COPII-coated vesicles. At the trans-Golgi, clathrin coat assembly is activated by the recruitment of Arf1p to the membrane. It is not clear how clathrin coat assembly is activated for vesicles that are formed at the plasma membrane. Arf1p in turn recruits the adaptor complex AP-1 to the Golgi membranes. (Vesicles formed at the plasma membrane instead carry the related AP-2 complex.) One end of the adaptor proteins binds to the cargo proteins and the other end to clathrin. Clathrin triskelions polymerize into hexagons and pentagons, forming a cage, which leads to membrane deformation. When the coat is almost complete, dynamin, together with accessory proteins, pinches off the vesicle (Kirchhausen, 2000).

Adaptor proteins

Adaptor protein complexes act as vesicle coat components in different membrane transport pathways. The defining characteristic of an adaptor is that it binds to the cargo proteins and serves as a link between the substrates for internalization and coat components (Rappoport, Benmerah & Simon, 2005). The four different AP complexes and the GGA proteins all have distinct distributions in the cell, and most likely help to sort cargo proteins into distinct types of vesicles. Arf1p is

required for the recruitment of all adaptors onto intracellular membranes (Robinson, 2004).

Multimeric adaptors

There are four known AP complexes, AP-1, AP-2, AP-3 and AP-4. They are all heterotetrameric complexes consisting of two heavy chains, one medium chain and one small chain (Dell'Angelica, Mullins & Bonifacino, 1999). The AP-1 complex interacts both physically and genetically with clathrin (Panek et al., 1997; Yeung, Phan & Payne, 1999; Rad, 1995). It functions in late Golgi to endosome trafficking (Bonifacino & Traub, 2003; Bowers & Stevens, 2005). The AP-2 complex is carried by clathrin-coated vesicles formed at the plasma membrane (Bowers & Stevens, 2005). Transport of cargo via the ALP pathway requires the AP-3 adaptor complex. Clathrin is not needed in this case (Bowers & Stevens, 2005), but AP-3 nevertheless contains clathrin-binding sequences (Barois & Bakke, 2005). Deletion of any of the AP-3 subunits results in a selective mislocalization of the vacuolar membrane protein ALP and the vacuolar t-SNARE Vam3p without affecting carboxypeptidase Y sorting to the vacuole (Cowles et al., 1997; Stepp, Huang & Lemmon, 1997; Vowels & Payne, 1998). The AP-4 complex, finally, was discovered by Dell'Angelica et al. (1999) and Hirst et al. (1999). It has been proposed to be associated with nonclathrin-coated vesicles (Hirst et al., 1999).

The cycling of the AP-1, AP-3 and the non-clathrin COPI coat is regulated by the same small GTP-binding protein, Arf1p. The active, GTP-bound form of Arf1p promotes coat association to membranes.
The drug brefeldin A inhibits the exchange of GDP for GTP on Arf1p. This causes redistribution of the coats to the cytosol due to the block in coat-membrane association (Dell'Angelica, Mullins & Bonifacino, 1999).

Monomeric adaptors

The three monomeric clathrin-adaptors are called α-arrestin, β-arrestin1 and β-arrestin2 (Kirchhausen, 1999). The arrestins act together with the AP-2 complex at the plasma membrane to bring different types of cargo into the same population of coated vesicles (Robinson, 2004). Arrestins act as adaptors linking cargo recruitment to a coated pit, and thus have the potential to indirectly influence the nucleation or regulation of coated pit formation (Kirchhausen, 1999).

GGA proteins

The GGA (Golgi-localized, gamma-ear-containing, Arf-binding) proteins Gga1p, Gga2p and Gga3p are adaptor proteins, that bind to ubiquitin and which are involved in sorting of the ubiquitinated cargo at the late Golgi. They are also thought to play a role in the endocytic pathway, perhaps in the sorting of ubiquitinated proteins destined for the vacuole during formation of the multivesicular body (MVB) (Scott et al., 2004; Pelham, 2004; Babst, 2004; Puertollano & Bonifacino, 2004). Consistent with this, deletions of the GGA proteins cause delayed transport of endocytosed proteins to the vacuolar lumen (Scott et al., 2004).

As discussed above, both MVB vesicle formation and sorting of ubiquitinated cargo into MVB vesicles depend on the function of the ESCRT complexes, ESCRT-I – III (Katzmann, Odorizzi & Emr, 2002). Puertollano et al. (2004) have identified a two-hybrid interaction between one of the ESCRT-I subunits, Vps23p, and Gga3p, indicating that Gga3p might act in concert with the ESCRT machinery in the sorting of ubiquitinated endosomal cargo proteins (Babst, 2004).

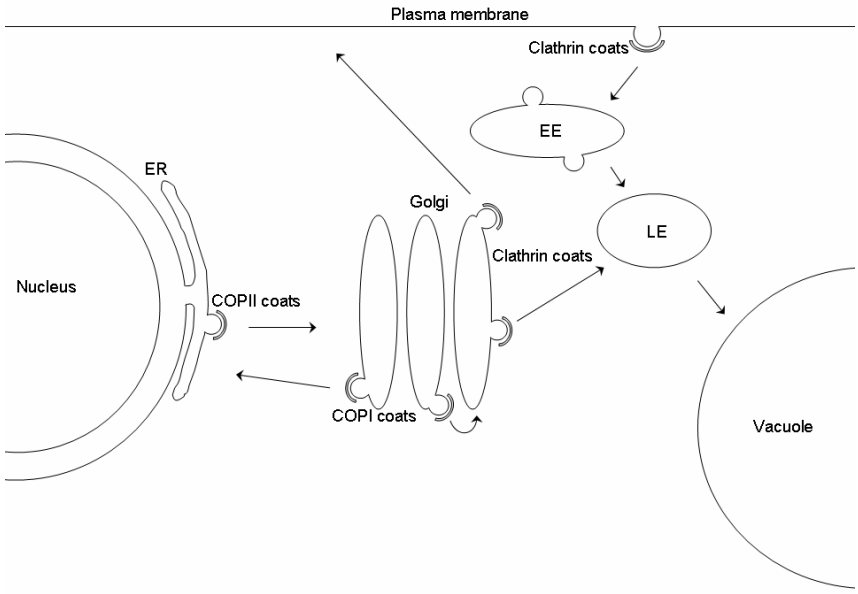

Fig. 3. The three different kind of coated vesicles that are found in the membrane traffic pathways. COPI coated vesicles are involved in intra-Golgi transport and retrograde traffic from the Golgi complex to the endoplasmic reticulum. COPII coated vesicles are mainly involved in anterograde transport from the ER to the Golgi. Clathrin coated vesicles, finally, are involved in endocytosis and exocytosis as well as transport from the Golgi complex to the late endosome.
ER, endoplasmic reticulum; EE, early endosome; LE, late endosome.

Rab GTPases

Rab GTPases are important regulators of all vesicular transport steps. They function mainly in recruiting tethering and docking proteins prior to the fusion of lipid transport vesicles to the target membrane.
The GTP hydrolysis that is catalysed by the Rab protein occurs subsequent to membrane fusion. After GTP hydrolysis, the GDP-bound Rab is bound by the cytosolic protein GDI (Rab GDP Dissociation Inhibitor) and transported in this complex to the correct compartment. The GDI-Displacement Factor (GDF) then releases the Rab protein from the GDI protein, so that the former can be reinserted into a membrane, where a GDP-GTP Exchange Factor (GEF) activates the Rab protein. The activated GTP-bound Rab protein is then again able to interact with

the Rab effector (Pfeffer, 2001). Binding of the Rab protein to the Rab effector tethers the vesicle to the target membrane and allows the SNAREs to interact with each other. There are different Rab effectors depending on which target membrane that is involved. The HOPS complex, for example, is involved in the docking with and fusion of transport vesicles to the vacuole.

SNAREs

NSF, N-ethylmaleimide sensitive factor, was the first protein identified that could stimulate an in vitro vesicular transport reaction (Block et al 1988). NSF requires SNAPs, Soluble NSF Attachment Protein, to bind to membranes. Soon thereafter another set of proteins, the SNAREs (SNAP receptors) was discovered. In 1994, the SNARE hypothesis was proposed according to which every transport vesicle carries a specific v-SNARE protein that binds to a cognate t-SNARE protein on the target membrane (Rothman, 1994). The interaction between these v- and t-SNAREs was proposed to mediate specificity in the fusion of transport vesicles to target membranes. To this SNARE complex, ∝-SNAP would bind and recruit NSF, which in turn would lead to ATP hydrolysis and fusion of the vesicles to the target membrane.

It is now clear that the original SNARE hypothesis was wrong in the sense that target specificity is not mediated by interactions between v-SNARE and t-SNARE proteins. In fact, the SNAREs are quite promiscuous in their interactions and can form many different complexes with other SNAREs (Pelham, 2001). Instead, it has been found that different tethering complexes are responsible for the initial recognition of vesicle and target membrane prior to the SNARE interactions, while the t- and v-SNAREs now are thought to be involved in the actual fusion step, see Figure 4 (Pfeffer, 1999). Thus, the t- and v-SNAREs assemble into a four-helix bundle upon docking and this complex promotes the fusion of the vesicle to the target membrane.

The most important t-SNAREs are the syntaxins, which form a distinct family of related and highly conserved proteins. There are seven proteins in yeast that belong to the syntaxin family, while the human genome encodes 15 members of the syntaxin family (Teng, Wang & Tang, 2001). The syntaxins have three N-terminal helices, one core helix (also known as the SNARE motif) and a C-terminal transmembrane domain. Sometimes the classification into v- and t-SNAREs is not meaningful, for example in homotypic fusion of vesicles. The SNAREs can alternatively be classified into R- or Q-SNAREs, reflecting the presence of an arginine or a glutamine at a characteristic position within the SNARE motif (Fasshauer et al., 1998). Although these classifications have different grounds, there is a rough correspondence of R-SNAREs with v-SNAREs and of Q-SNAREs with t-SNAREs.

Specific SNAREs are thought to mediate fusion at each trafficking step, including traffic from ER to Golgi and from Golgi to other compartments (Söllner et al., 1993; Sögaard et al., 1994; Rothman, 1994). The yeast SNAREs involved in Golgi complex to plasma membrane transport are Sso1p and Sso2p, which are

syntaxin 1 homologues (Aalto, Ronne & Keränen, 1993), Sec9p, which is a SNAP-25 homologue (Brennwald et al., 1994), and Snc1p and Snc2p, which are homologues of synaptobrevin (Protopopov et al., 1993). In non-meiotic cells, Sso1p and Sso2p function only in Golgi to plasma membrane transport, but other syntaxins such as Tlg2p can function in more than one intracellular transport step. The same syntaxin may then interact with different other SNARE proteins in different transport steps (Pelham, 2001).

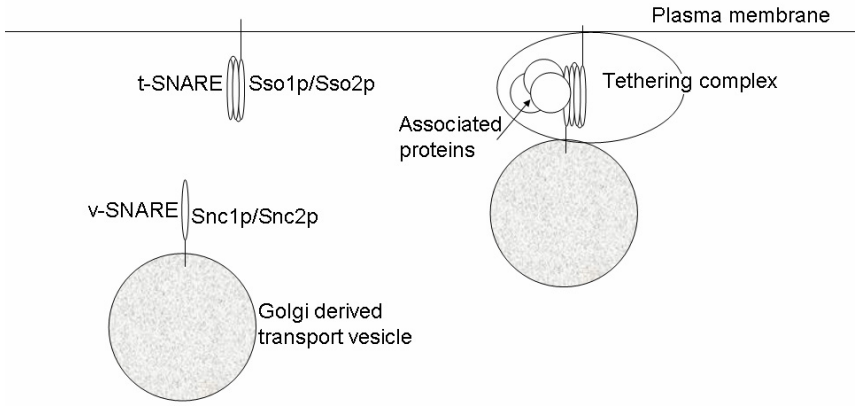

Fig. 4. A schematic picture of the interaction between a v-SNARE and a t-SNARE, resulting in a formation of a four-helix bundle, which enables the subsequent fusion of a tethered Golgi derived vesicle to the plasma membrane.

Sporulation

The life cycle of the yeast *Saccharomyces cerevisiae* switches between a haploid and a diploid phase, see Figure 5. Haploid cells exist in two different mating types, Mat a and Mat α. These cells are analogous to the gametes of higher eukaryotes, but unlike the latter, haploid yeast cells can divide to produce new haploids. When one type of haploid cell is mated to the other type, a diploid cell is formed. This diploid cell can undergo mitotic division and form new diploid cells. Both haploid and diploid yeast cells divide by budding, which is a process in which a new cell is formed as a small outgrowth of the old cell. The bud is enlarged until nuclear division takes place, after which the bud separates from the mother cell. The diploid cell can also undergo meiotic division and in this way generate four new

haploid cells, also called a tetrad. The difference between a mitotic division and a meiotic division is the existence of the meiosis I in the latter. In a mitotic division there is a single round of DNA replication followed by a single round of DNA segregation, while in the meiotic division DNA replication is followed by two rounds of DNA segregation. The first of these, meiosis I, differs from a normal mitosis in that it is the two parental chromosomes that segregate from each other. In the subsequent meiosis II divison, chromatids within each chromosome segregate from each other, just as in a mitotic division.

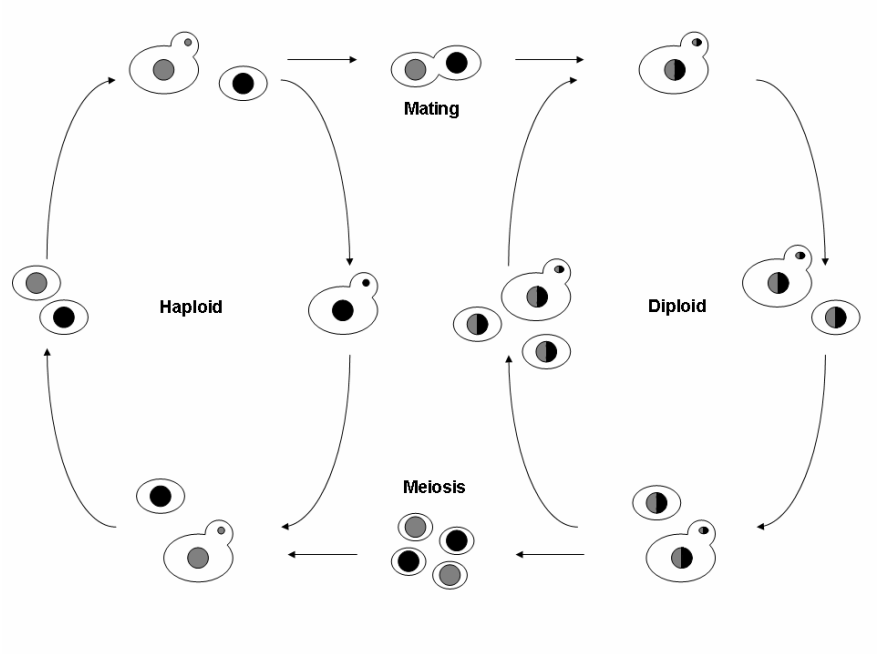

Fig. 5. The life cycle of the yeast *Saccharomyces cerevisiae.* There is both vegetative reproduction by budding of haploid or diploid cells, and sexual reproduction, where two haploid cells of different mating types fuse to form a diploid cell. The diploid cell can in turn sporulate, *i. e.* go through meiosis and form four spores with haploid nuclei.

Upon starvation for certain nutrients, typically nitrogen and glucose starvation in the presence of a non-fermentable carbon source, diploid yeast cells undergo meiosis and sporulate, see Figure 6. This is an important way for yeast cells to survive in the nature, since they can persist as dormant spores for a very long time when the nutrient supply is low. The remnant of the diploid cell within which sporulation takes place is called the ascus, and the new haploid cells that are formed inside the ascus are called ascospores (Madigan, Martinko & Parker, 1997). Each spore is surrounded by a spore wall, which is formed *de novo* in the sporulating cell (Kupiec *et al.,* 1997).

When the four spores are formed, each one is at first surrounded by a prospore membrane, which will eventually become the plasma membrane of the new

haploid cell. However, the prospore membrane differs from a normal plasma membrane in that it is a double membrane, similar to the two membranes that enclose the autophagosomes (see above). This structure originally forms as a small flat membrane sack at the meiotic plaque, near the spindle pole body (SPB). During meiosis II, the sack extends itself and wraps around the new haploid nucleus like a hand in a glove that grabs a ball. At the end of meiosis II, the prospore membrane closes by fusion to itself on the other side of the nucleus, and each haploid nucleus is thus surrounded by a double prospore membrane, in addition to (and outside) the nuclear membrane. Organelles that are inherited by the spores, such as mitochondria, are also enclosed by the prospore membrane, and are thus found between it and the nuclear membrane.

The spindle pole body is the functional equivalent to the centrosome in higher eukaryotes and serves as a microtubule-organizing centre. During sporulation, the SPB is duplicated twice. At the cytoplasmic side of the SPBs there are so called meiotic plaques, where the prospore membrane attaches. These enlarged plaques are meiosis-specific and it is composed of Mpc54p and Mpc70p together with Spo74p (Moreno-Borchart & Knop, 2003; Knop & Strasser, 2000, and references therein). Interestingly, the formation of the prospore membrane is dependent on several late *SEC* genes that are involved in transport from the Golgi to the plasma membrane in non-meiotic cells (Knop & Strasser, 2000, and references therein). A possible explanation for this is that the prospore membrane is formed from fused vesicles that are derived from the late Golgi (Neiman, 1998). A complex called the leading edge protein coat, LEP, is also important in the formation of prospore membrane. The LEP consists of at least three different proteins, Ssp1p, Ady3p and Don1p, which are expressed only during meiosis. It covers the leading edge of the prospore membrane as it grows and wraps around the haploid nucleus (Moreno-Borchart *et al*., 2001).

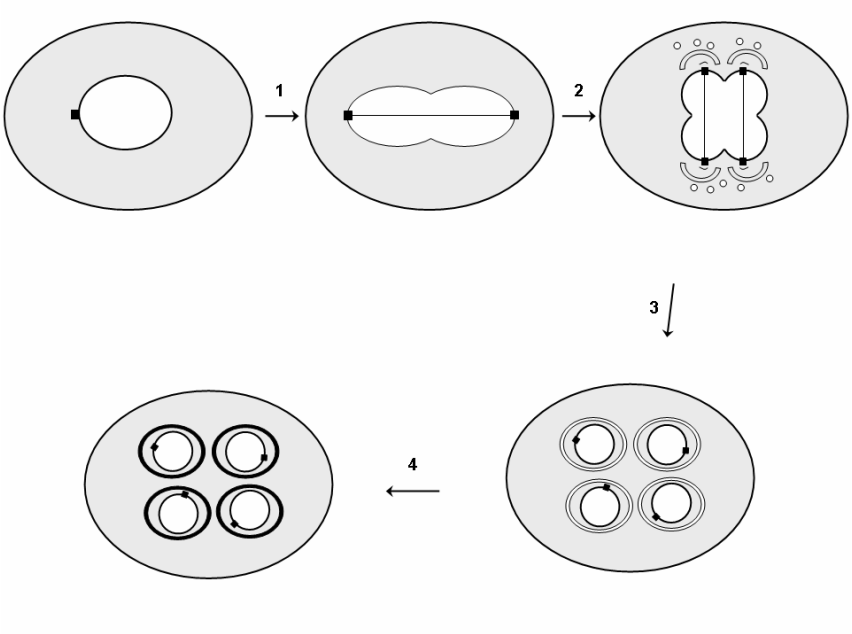

Fig. 6. The sporulation process in *Saccharomyces cerevisiae*.

1. Duplication of the spindle pole body, SPB, and meiosis I.
2. Meiosis II, duplication of the SPB once more and formation of the prospore membrane at the meiotic plaque by fusion of Golgi-derived vesicles to each other.
3. Enclosure of the newly formed haploid nuclei by prospore membranes.
4. Spore walls are formed around the haploid spores, by deposition of material in between the two layers of the prospore membrane.

The double prospore membrane serves as a scaffold for the formation of the spore wall, which is constructed in the lumen in between the two prospore membranes (Neiman, 1998, and references therein). The spore wall protects the spore from environmental damage and consists of four distinct layers, two inner polysaccharide layers composed of β-glucan and mannan, a central chitosan layer and an outermost layer containing dityrosine.

A number of sporulation specific genes (*spo* genes) have been identified in classical genetic screens (Bresch, Muller & Egel, 1968; Esposito & Esposito, 1969). In addition, it was found that the plasma membrane syntaxin Sso1p is required for sporulation, and that this function cannot be provided by Sso2p, which functions redundantly with Sso1p in secretion (Jäntti *et al*., 2002) Either Sso1p, Sso2p or both localize to the growing prospore membrane (Neiman, Katz & Brennwald, 2000). Interestingly, there is another case where yeast has two duplicated t-SNAREs, where at least one of them is required for sporulation. Thus, the mammalian SNAP-25 protein has two homologues in yeast: Sec9p which functions in secretion, and Spo20p which functions in sporulation (Neiman, 1998; Neiman, Katz & Brennwald, 2000). It should be noted, however, that Spo20p has

no apparent role in secretion, unlike Sso1p, which functions in both processes. Moreover, Sec9p and Spo20p seems to have partially overlapping functions during meiosis, since prospore membrane formation is completely inhibited when *SPO20* is deleted in a *sec9* temperature sensitive mutant, while there is still some prospore membrane formation in a *spo20* deletion mutant with a wild type *SEC9* gene (Neiman, 1998).

The present investigation: Results and Discussion

Paper I: Identification of yeast deletion strains that are hypersensitive to brefeldin A or monensin, two drugs that affect intracellular transport.

In order to find new genes involved in intracellular transport in yeast, 620 knockout mutants (a subset of the Eurofan deletion strain collection) were screened for increased sensitivity to the drugs monensin and brefeldin A.

Both drugs are known to interfere with intracellular transport. Brefeldin A is a fungal metabolite isolated from *Penicillium brefeldianum* (Dinter & Berger, 1998, and references therein). The main targets of brefeldin in yeast are the Sec7 domain family of ARF exchange factors: Gea1p, Gea2p and Sec7p (Peyroche *et al.*, 1999). The function of ARF proteins is to recruit COPI and AP-1/clathrin coat protein complexes to Golgi membranes (Peyroche *et al.,* 1999) By interfering with GTP-GDP exchange on the ARF proteins, brefeldin A inhibits COPI binding to the Golgi membranes and thereby blocks the transport.

The other drug that we used, monensin, is incorporated into membranes and act as a H^+/Na^+ exchanger. Treating cells with monensin leads to swelling of the *trans*-Golgi, but the precise mode of action of monensin is still unclear (Dinter & Berger, 1998). It has been suggested, however, that a major mechanism of monensin action is that its H^+/Na^+ exchange activity reduces the acidity of compartments such as the late Golgi, the endosome and the vacuole, which is important for proper transport.

Secretion is an essential process in yeast, since growth of the daughter cell (bud) depends on it. Studies of genes involved in secretion have therefore relied on the isolation of conditional lethal, temperature-sensitive mutations in these genes (Novick & Schekman, 1979; Novick, Field & Schekman, 1980). We reasoned that other genes might exist that are important, though not essential, for secretion, and that the corresponding knockout mutants would then be viable, but with a partial block in secretion. To search for such genes, we screened the knockout strain collection for increased sensitivity to brefeldin A and monensin. The rationale for this screen was our expectation that knockout mutants with a partial block in secretion would be particularly sensitive to drugs that interfere with secretion.

Accordingly, we expected such mutants to be unable to grow in the presence of reduced drug concentrations that would fail to completely inhibit growth of the wild type strain.

Using this approach, we were able to identify 18 genes whose knockout mutants showed increased sensitivity to either monensin or brefeldin A. Seven previously unknown genes were found and named *BRE1-BRE5* for sensitivity to brefeldin A and *MON1-MON2* for sensitivity to both monensin and brefeldin A (none of them was sensitive only to monensin). The genes found in the screen could be classified into four distinct categories.

Five of the genes, *VAC7*, *VAM6*, *TLG2*, *SYS1* and *RCY1,* were already known to be involved in intracellular transport. This revealed a high level of relevance in the screen. *VAC7* encodes an integral vacuolar membrane protein (Bonangelino, Catlett & Weisman, 1997). Cells with mutations in *VAC7* have enlarged unlobed vacuoles and are blocked in retrograde transport from the vacuole (Bryant *et al*., 1998). Vam6p is part of the HOPS complex (Seals *et al.,* 2000), mediating fusion at the vacuolar membrane. *TLG2* encodes a syntaxin (t-SNARE) implicated in endocytosis and in maintenance of Golgi resident proteins. It binds to the Sec1 homologue Vps45p (Nichols, Holthuis & Pelham, 1998; Seron *et al.,* 1998). Tlg2p is also required for the cytoplasm-to-vacuole trafficking of API (aminopeptidase I) and for retrieval of the v-SNARE Snc1p from early endosomes (Abeliovich, Darsow & Emr, 1999; Lewis *et al*., 2000). Sys1p was identified as a high copy number suppressor of the deletion of *YPT6* (Tsukada & Gallwitz, 1996), which encodes the homologue of the mammalian Rab6p protein. Rab6p has been proposed to be involved in both anterograde and retrograde transport between the late Golgi and the late endosome (Lazar, Gotte & Gallwitz, 1997). Cells disrupted for both Sys1p and Ypt6p have fragmented vacuoles. Rcy1p is a F-box protein that interacts with Skp1p and is thereby implicated in ubiquitinylation (Patton, Willems & Tyers, 1998). Rcy1p has been shown to be involved in endocytosis (Wiederkehr *et al*., 2000). It is required for recycling of plasma membrane proteins, like the v-SNARE Snc1p, which are internalized by endocytosis (Galan *et al*., 2001).

Three of the genes found in our screen, *ERG4*, *ALG9* and *ALG12*, are known to be involved in membrane or cell wall biogenesis. Eight genes, *HOS2*, *LSM1*, *BRE1*, *BRE2*, *MON1*, *BRE3*, *BRE4* and *BRE5*, had no obvious links to intracellular transport or secretion at that time. Several of these new genes discovered in paper I have subsequently been shown to be involved in either intracellular transport or gene expression. Thus, cells that lack Mon1p are deficient in both the autophagic pathway and the cvt-pathway, suggesting a role for Mon1p in transport to the vacuole (Meiling-Wesse *et al.*, 2002; paper II). Mon1p interacts with Ccz1p, forming a complex at the vacuole (Wang *et al*., 2002). Bre1p is one of several E3 ubiquitin ligase partners for the E2 ubiquitin conjugating enzyme Rad6p, which mediates mono- and polyubiquitylation of target proteins. Bre1p is the specific E3 partner of Rad6p that is required for monoubiquitylation of histone H2B (Yamashita, Shinohara & Shinohara, 2004). Bre1p contains a RING finger domain, and is necessary for transcription of Notch

target genes. The Notch signalling pathway controls numerous cell fate decisions during development in higher eukaryotes and has also been implicated in various human diseases including cancer (Bray, Musisi & Bienz, 2005, and references therein). Interestingly, Bre1p also contains a myosine-like domain that is weakly similar to the Uso1p protein, which is required for the initial docking or tethering of ER-derived transport vesicles at the *cis*-Golgi (Cao, Ballew & Barlowe, 1998).
Bre2p is a subunit of the Set1 complex (COMPASS complex), a multiprotein complex involved in methylation of histone H3 which is required for transcriptional silencing near the telomere. Methylation of histones is an important way to regulate transcription, since it affects the accessibility of chromatin. Bre2p is homologous to the *Drosophila* trithorax protein Ash2p. The trithorax (Trx) family of proteins is required for maintaining a specific pattern of gene expression in *Drosophila* (Krogan *et al.,* 2002; Roguev *et al.,* 2001; Nagy, 2002).
Bre3p (Lem3p) is a membrane glycoprotein which is important for translocation of phospholipids and alkylphosphocholine drugs across the plasma membrane (Kato *et al.,* 2002; Hanson *et al.,* 2003). Bre4p is a zinc finger protein containing five transmembrane domains. The *bre4* deletion mutant exhibits strongly fragmented vacuoles (Wiederkehr, Meier & Riezman, 2001). Bre5p, finally, acts together with Ubp3p in a complex which de-ubiquitinate the COPII protein Sec23p (Cohen *et al.,* 2003).

The two genes *ENT4* and *MON2*, finally, shared homology with other genes known to be involved in intracellular transport. Mon2p is a member of the Sec7 family of Arf GEFs (guanine nucleotide exchange factors) and subsequently been shown to have a role in endocytosis and in maintenance of the vacuole structure (Jochum *et al.,* 2002). Ent4p contains an ENTH (epsin *N*-terminal homology) domain. Ent4p is a protein of unknown function, but with sequence similarity to Ent1p and Ent2p, which are required for endocytosis (Wendland, Steece & Emr, 1999), and to Ent3p, which is involved in clathrin recruitment and traffic between the Golgi and endosomes. Ent3p associates with the clathrin adaptor Gga2p (Duncan, Costaguta & Payne, 2003). All four *ENT* genes interacts with the EH-domain containing protein Pan1p. The *ENT* genes are homologous to the mammalian protein epsin, which is an EH-domain-binding protein that has been implicated in clathrin binding during endocytosis (Wendland *et al*., 1999).

Paper II: Yeast Mon1p/Aut12p functions in vacuolar fusion of autophagosomes and cvt-vesicles.

*MON1*encodes a cytosolic protein of 645 aa initially identified in our screen for mutants sensitive for the drug monensin (Paper I). *MON1* also appeared in a screen for mutants that secrete CPY (Bonangelino, Chavez & Bonifacino, 2002) In paper II, a collaboration with Michael Thumm and coworkers, we investigated the role of Mon1p in the autophagic and the cytoplasm-to-vacuole targeting pathways. Mon1p was found to be involved in autophagy when Thumm and coworkers recovered it in a screen for strains with defective proaminopeptidase I maturation, and it is therefore also named Aut12p. Mon1p was found to be necessary for the fusion of both cvt-vesicles and autophagosomes to the vacuole,

as evidenced from the fact that proaminopeptidase I maturation is blocked in both starved and non-starved *mon1*Δ cells. Moreover, we found that the autophagosomal marker protein GFP-Aut7p accumulates at punctate structures outside the vacuole in *mon1*Δ cells. GFP-Mon1p was similarly shown to localize to the cytosol and to cytosolic punctate structures in the cell. These results suggest that Mon1p is located at the vacuolar membrane, since that is the place where the CPY pathway, the cvt-pathway and the autophagic pathway converge. It was later shown by Wang *et al.* (2002) that Mon1p interacts with Ccz1p, forming a complex at the vacuolar membrane.

Paper III: Functional genomics of monensin sensitivity in yeast: implications for post-Golgi traffic.

The aim of this study was to do a screen similar to the previous screen of the Eurofan collection, but with the complete collection of viable haploid knockout mutants from Euroscarf, and only using monensin this time. Out of the 4709 strains tested, 63 strains were found to be sensitive to monensin. Many of the genes identified in the screen encode proteins already known to be involved in intracellular transport, in particular transport to the vacuole.

We found one uncharacterised ORF, *YNL080c* (which we named *MON3),* that may represent a new gene involved in intracellular transport. Other genes found were genes encoding proteins involved in vacuolar fusion and endosomal fusion, all the proteins of the HOPS complex except Vps11p, all the proteins of the GARP/Vft complex except Vps53p, all the proteins of the ESCRT-II complex and one of the proteins of the ESCRT-III complex. We also found several genes involved in endosome to Golgi transport, various genes encoding Golgi proteins and genes involved in early endocytosis.

By comparing our study to a *VPS* screen, where secretion of CPY was investigated (Bonangelino, Chavez & Bonifacino, 2002) and to a *VAM* screen where vacuolar morphology was studied (Seeley *et al.*, 2002) we could see that there is a considerable overlap between the genes found in the three screens. Interestingly, however, none of the vacuolar ATPase subunit deletions were sensitive to monensin, even though all of them appeared in either or both of the *VPS* and *VAM* screens. We also compared these three screens to a screen for increased sensitivity and resistance to salt (Warringer *et al.*, 2003) These salt-responsive mutants have been classified into three categories: efficiency-of-growth mutants, rate-of-growth mutants and time-of-adaptation mutants (the same mutant may appear in more than one of these groups). There was more correlation between the mutants that appeared in our monensin sensitivity screen and those that appeared in the *VPS* and *VAM* screens, than with the salt-sensitive mutants. On the other hand, the different types of salt-sensitive mutants were more strongly correlated with each other. Of the salt-sensitive mutants, the rate-of-growth mutants were most strongly correlated with the mutants that appeared in the *MON*, *VPS* and *VAM* screens.

In order to further investigate why the vacuolar ATPase subunits failed to appear in our screen, we made double mutants between our 63 mutants and a deletion of one of the genes encoding a vacuolar ATPase subunit, *VMA1*. The latter deletion eliminates all vacuolar H^+-ATPase activity. The v-ATPase in the vacuolar membrane and the v-ATPase in the Golgi membrane differ by one subunit, Vph1p in the vacuolar type and Stv1p in the Golgi type. The other subunits of the v-ATPases are the same in both enzymes. The Golgi v-ATPase is functions in maintaining acidification of the Golgi complex, while the vacuolar ATPase functions in the vacuole. Interestingly, we found that several of the 63 monensin-sensitive knockout mutants showed synthetic interactions with the *vma1* knockout mutant, ranging from complete synthetic lethality to reduced growth. Furthermore, this interaction appears to specific for genes involved in retrograde transport, and was not seen with genes involved in transport to the vacuole or vacuolar biogenensis.

Paper IV: Mapping of sequences in the 3'-untranslated region of the yeast plasma membrane syntaxin gene *SSO1* which are required for sporulation.

SSO1 and *SSO2* encode two proteins that are closely related, being 74% identical and 86% similar at amino acid level (Aalto, Ronne & Keränen, 1993). Together, Sso1p and Sso2p provide an essential function in vegetative cells, where they are involved in mediating fusion of transport vesicles derived from the Golgi complex to the plasma membrane. Although the two proteins are redundant in secretion, Sso1p is specifically needed during sporulation, while Sso2p is not (Jäntti *et al.*, 2002). The N-terminal Ha and Hb helices in Sso1p were found to be important but not essential for sporulation (Öyen *et al.*, 2004). In contrast, sporulation was completely dependent on the 3'-untranslated region (3'-UTR) of *SSO1*. Thus, sporulation was 10-fold reduced when the region containing the Ha and Hb helices was switched to the corresponding *SSO2* sequence, but completely inhibited when the 3'-UTR of *SSO1* was replaced by that of *SSO2*.

In paper IV, we have further mapped the ability of the *SSO1* 3'-UTR to sustain sporulation to two short elements within the 3'-UTR. These elements span the regions between 20 and 50 nucleotides and between 150 and 200 nucleotides downstream of the *SSO1* stop codon.

The *SSO1* sequence is more AT-rich and the *SSO2* sequence more GC-rich in the first 30 nuclotides downstream of the stop codon. This is followed by a short region with a high degree of similarity (between positions 30 and 50), where the sequences of *SSO1* and *SSO2* differ in only 6 positions. Beyond position 50 of the 3'UTR, there is little similarity between the *SSO1* and *SSO2* sequences. Interestingly, there is a predicted Puf protein binding site at position 45, and another predicted Puf site at position 150 in the *SSO1* 3'UTR. The fact that these two motifs coincide with the two elements that are important for sporulation suggests that Puf proteins may be involved in mediating the sporulation-specific function of *SSO1*.

There are six different Puf proteins in yeast. Three of them have been proposed to bind to UGUA followed by UA two to four nucleotides further downstream (Gerber, Herschlag & Brown, 2004). The two predicted Puf sites in the *SSO1* mRNA that are described above are the only sites that have an UA dinucleotide close to the UGUA motif. In the first element, there are two UAs, one directly after and the other 6 bases after the UGUA. In the second element, the UGUA is followed by an UA with 2 nucleotides in between. The functions of the Puf1p and Puf2p proteins are unknown. Puf3p is known to bind *COX17* mRNA and promote its degradation (Olivas and Parker, 2000), while Puf6p is known to bind *ASH1* mRNA and inhibit its translation, as well as having an effect, still poorly understood, on localization of the *ASH1* mRNA to the bud (Gu *et al*., 2004). This raises the intriguing possibility that the two predicted Puf-binding sites in *SSO1* might be involved in mRNA transport. In this context, it should be noted that meiotic cells differ from normal mitotic cells in that a new membrane, the prospore membrane, is formed inside the cell, from vesicles that would normally be transported to the plasma membrane (Neiman, 1998). Finally, it should be noted that Puf2p was reported to bind the *SSO2* mRNA, but not the *SSO1* mRNA in *in vitro* experiments with extracts from mitotic cells (Gerber et al., 2004). This is surprising since the two predicted Puf-binding sites which coincide with the meiosis-specific elements in *SSO1* are absent from *SSO2*. It is not known, however, to what sequence Puf2p binds, so it remains a possibility that there are Puf2p-binding sequences in the *SSO2* mRNA which prevent its proper function during meiosis. Nor can it be ruled out that one or several Puf proteins bind the *SSO1* mRNA in meiotic cells, where other proteins may modulate mRNA-Puf protein interactions, even if they fail to do so when using extracts from mitotic cells.

Conclusions and future perspectives

We have identified several new proteins involved in intracellular transport. Mon1p is known to form a complex with Ccz1p, and this complex is important for the vesicle docking with and fusion to the vacuolar membrane. The exact function of Mon1p remains to be elucidated. One way to further characterize Mon1p would be to look for interaction partners other than Ccz1p and Ypt7p by, for example, a two hybrid screen using Mon1p as a bait.

Mon3p is another interesting protein found in the screen for monensin sensitivity, which is possibly involved in intracellular transport. The function of Mon3p is still unknown but there are a number of experiments that could be done to elucidate its function. One strategy would be to perform a yeast two hybrid screen using Mon3p. Another strategy would be to do a multicopy suppressor screen using a *mon3* deletion strain.

There seems to be two elements in the 3'-untranslated region (3'-UTR) of the *SSO1* mRNA that are important for sporulation. The two elements, located between 20 and 50 nucleotides and between 150 and 200 nucleotides downstream of the stop codon seem to be redundant, as sporulation occur with either one of them present. The second element, however, seems to be more important than the first element, as it alone can support sporulation to the same level as the wild type *SSO1* gene. The fact that the two elements coincide with two predicted binding sites for Puf proteins suggests that the latter may be important for the meiosis-specific function of *SSO1*. This hypothesis can be tested by introducing point mutations in the two Puf consensus motifs. Another experiment that could be done is to check whether knockout strains that lack one or several Puf proteins still can sporulate.

The question whether the essential function of the 3'-UTR of the *SSO1* gene has to do with a sporulation-specific localization of the *SSO1* mRNA also remains unsolved. This could be tested by tagging the *SSO1* mRNA and studying its intracellular localization in both mitotic and meiotic cells.

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