Functional Studies of Plasma Membrane Syntaxins in Yeast

Mattias Öyen
Department of Plant Biology and Forest Genetics
Uppsala

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Abstract

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Syntaxins are required for fusion of membranes in eukaryotic cells and belong to a group of proteins known as t-SNAREs. This thesis primarily focuses on the role of the plasma membrane syntaxins Sso1p and Sso2p in the yeast *Saccharomyces cerevisiae*. The plasma membrane syntaxins are required for viability in yeast, but in the vegetatively growing cell, the Sso proteins have seemingly reduntant functions. We generated a mutant allele of *SSO2, sso2-1*, that has a conditional lethal phenotype in the absence of *SSO1*. Overexpression of genes coding for other SNARE proteins; Sec9p, Snc1p and Snc2p, suppressed the lethal phenotype. The corresponding mutant allele of *SSO1*, *sso1-1*, is also temperature-sensitive and interacts synthetically with a disruption of *MSO1*, which codes for a Sec1p interacting protein.

Most notably, both *SSO1* and *MSO1*, but not *SSO2,* were shown to be necessary for spore formation during meiosis. Mapping of functions within the Sso1p protein showed that a region in the N-terminus of Sso1p is needed for efficient sporulation. Unexpectedly, the 3'-untranslated region of *SSO1* is absolutely required for sporulation and also sufficient to enable some spore formation when fused to the *SSO2* open reading frame.

Inspection of the *sso1/sso1* phenotype during sporulation using transmission electron microscopy showed that prospore membrane assembly at the meiotic plaque of the spindle pole body is completely blocked in the mutant.

A second part of this thesis deals with screening for uncharacterized genes involved in intracellular transport by exposing deletion mutants for drugs known to inhibit intracellular transport. The screen identified two new genes whose deletions made the cell sensitive to monensin, and those were given the names *MON1* and *MON2*. Five new genes caused sensitivity to Brefeldin A when deleted, and were named *BRE1-BRE5*.

Key words: Brefeldin A, meiosis, monensin, prospore membrane, SNARE, sporulation, *SSO1, SSO2,* syntaxin, 3'-UTR, vesicular transport.

Authors address: Mattias Öyen, Department of Plant Biology and Forest Genetics, Uppsala Genetic Centre, Swedish University of Agricultural Sciences, Box 7080, S-750 07 Uppsala, Sweden. Electronic mail: Mattias.Oyen@vbsg.slu.se

‘But I should like to know – ’ Pippin began.
‘Mercy!’ cried Gandalf. ‘If the giving of information is to be the cure of your inquisitiveness, I shall spend all the rest of my days in answering you. What more do you want to know?’
‘The names of all the stars, and of all living things, and the whole history of Middle-earth and Over-heaven and of the Sundering Seas,’ laughed Pippin. ‘Of course! What less?’

Excerpt from “The two towers” by JRR Tolkien

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IV. Öyen M., Jäntti J., Larsson J., Ronne H. Characterization of the role of the yeast syntaxin Sso1p in sporulation. Manuscript.

Abbreviations

List of abbreviations used in the text:

ALP	alkaline phosphatase
AP (-1, -2, -3)	adaptor protein complex -1, -2, -3
API	amminopeptidase I
ATP	adenosine-tri-phosphate
BFA	brefeldin A
cDNA	complementary DNA
COG	conserved oligomeric golgi
COP (I, II)	coat protein complex (-I and -II)
CPS	carboxypeptidase S
CPY	carboxypeptidase Y
Cvt	cytoplasm to vacuole transport
DAPI	4',6-diamidino-2-phenyindole
ER	endoplasmatic reticulum
GARP	golgi associated retrograde protein
GDI	GDP dissociation inhibitor
GDP	guanosine-di-phosphate
GEF	GDP-GTP exchange factor
GFP	green fluorescent protein
GTP	guanosine-tri-phosphate
LE	late endosome (same as PVC)
MP	meiotic plaque
MVB	multivesicular bodies (same as PVC)
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
NSF	N-ethylmaleimide sensitive factor
ORF	open reading frame
PM	plasma membrane
PrA	Proteinase A
PrB	Proteinase B
PVC	prevacuolar compartment
SC	synaptonemal complex
SDS	sodium-dodecyl sulphate
SM-proteins	Sec1p/Munc18 proteins
SNAP	Soluble NSF attachment protein
t-SNARE	target membrane SNAP receptor
v-SNARE	vesicular SNAP receptor
SPB	spindle pole body
TEM	transmission electron microscopy
TM, TMD	transmembrane, transmembrane domain
TRAPP (I, II)	transport protein particle (-I, -II)
UTR	untranslated region
VFT	Vps fifty three complex
VPS	vacoular protein sorting
VSVG	G-protein from the vesicular stomatitis virus

Introduction

Much progress in science is about the available tools and how to use these tools in new ways. Robert Hookes construction of the compound microscope allowed him to observe mundane things at a previously unseen magnification. He saw and reported that cork and leaves were composed of cells (Hooke, 1664), and he was the first to use the word "cell" in a biological context. Today, microscopy has developed further and it is in a light microscope possible to get a thousand-fold magnification with a detail resolution of 0.1 μm. With an electron beam instead of light much greater magnifications can be achieved.

The yeast *Saccharomyces cerevisiae* is perhaps best known as a tool in brewery and baking, where one takes advantage of its conversion of sugar to ethanol or its ability to produce carbon dioxide respectively. This alone is a good reason to do research on yeast. For example, how can yeast be selected to be more efficient in baking? Research and development has resulted in dried yeast, in pre-made dough, ready to be baked at the local shop, and in more efficient production of baking yeast. In beer and wine brewing, the yeast strain used affects the fermentation process and has a large influence of the final product. Any organism with such commercial value would have attracted scientific interest.

Yeast is also used as a model system to study eukaryotic cell functions. Although it bears little obvious semblance to animals and plants it shares all the basic cell functions with them. It has a nucleus and organises its DNA in several chromosome pairs. It has mitochondria, peroxisomes, a cytoskeleton and both internal membrane structures, such as endoplasmatic retitulum and Golgi apparatus, as well as a plasma membrane surrounding the cell. In common with other fungi and plants it also has a cell wall and a vacuole. Compared to cells from humans and most other multicellular organisms, yeast cells are quite easy to grow and they have a comparatively short generation time, which makes individual experiments quicker. There are a few properties of *Saccharomyces cerevisiae* that make it an outstanding model when using molecular genetics. The genome is small and the genes contain few introns, making it possible to work easily with whole genomic fragments instead of cDNA clones. Yeast can grow both as a diploid and as a haploid cell, with the consequence that the phenotype of recessive mutations can be readily inspected. Furthermore, synthetically lethal mutant combinations can be investigated by crossing the haploid strains to generate a heterozygous diploid strain. This is followed by sporulation and inspection of the haploid progeny. Most importantly, homologous recombination is very efficient in yeast, which means that it is very easy to use gene targeting to delete genes or introduce specific mutations into the yeast genome.

Saccharomyces cerevisiae was the first eukaryotic organism whose whole genome was completely sequenced (Goffeau *et al.*, 1996), something which opened up many new possibilities in research. For example, knowing the complete sequence made it possible to systematically delete all genes and study the effects of the deletions. Such work showed that the majority of the genes were non-essential for cell viability (Winzeler *et al.*, 1999). It also made it possible to study the expression pattern of all the genes during different growth conditions (Horak & Snyder, 2002). Another important development is the cloning and utilization of the

green fluorescent protein (GFP) and color variants of it, which have simplified studies of the subcellular localization of proteins and also made it possible to study protein-protein interactions and dynamics in living cells (for a review, (Matz, Lukyanov & Lukyanov, 2002). In conclusion, *Saccharomyces cerevisiae* is a model organism where the laboratory methods are well established and defined, and where a large number of mutants have been described and are readily available. It has therefore served as a front organism in the "new biology".
The present study focuses on a family of membrane bound proteins called syntaxins, and their role in vesicular cell transport. In the overview, I will discuss rather briefly the main steps in vesicular transport within a eukaryotic cell. I will then discuss the proteins that bring membranes together in the cell, the SNARE proteins. Before presenting my own work, I will also cover the syntaxin family in some detail. Much of the research that I will discuss in the introduction was carried out in yeast. This does not mean that research on intracellular transport has been made only in yeast, but rather that I have to restrict the scope of the introduction.

Yeast as a tool for investigating intracellular protein transport.

The yeast *Saccharomyces cerevisiae* is a useful model for eukaryotic cells as it shares many properties with cells in multicellular eukaryotes while it is just as easy to work with as bacteria. Yeast is particularly well suited for studies of intracellular transport since a large number of genes, cloned by complementing a lethal mutation, have had their gene product function assigned to different transport steps within the cell (Kaiser, Gimeno & Shaywitz, 1997). The yeast cell uses vesicular transport to the plasma membrane for secretion of proteins, and to expand the cell surface, including bud formation that eventually leads to the formation of new daughter cells. Since transport to the plasma membrane is crucial for cell growth, many genes involved in transport are essential. Vesicular transport is also used in earlier transport steps within the cell, *e. g.* from ER to Golgi and within the Golgi (Cleves & Bankaitis, 1992).
Saccharomyces cerevisiae is able to grow at different temperatures, which makes it possible to use temperature sensitive mutants – mutations whose gene products are functional at a permissive temperature but non-functional at an elevated temperature. These conditional mutants have been of paramount importance in identifying the proteins involved in the secretory pathway. In particular, the isolation of 23 *sec* mutants that accumulated invertase at a restrictive temperature by Novick et al. (Novick & Schekman, 1979; Novick, Field & Schekman, 1980) was of key importance for further research into intracellular transport. These *sec* mutants fell into several distinct categories that were impaired in ER to Golgi transport, transport within the Golgi, and transport from *trans*-Golgi to the plasma membrane.
The pathway for transport from *trans*-Golgi to the vacuole has also been characterized using several systematic mutant screens (Jones, Webb & Hiller, 1997) and the results obtained have suggested that there are several distinct pathways for transport between *trans*-Golgi and the vacuole. Notably, these pathways have almost no overlap with the main secretory pathway leading from trans-Golgi to the plasma membrane.

Background

Overview of transport steps in the secretory pathway

ER translocation

The perhaps most significant obstacle for a protein destined for transport is the need to pass trough the ER membrane and fold correctly at the same time. The general mechanism for translocation of proteins over the ER membrane is well characterized. Responsible for ER import and ER membrane insertion is a heterotrimeric complex that is conserved in both eukaryotes and prokaryotes. In prokaryotes it is called SecYEG and in eukaryotes the Sec61-complex (Matlack, Mothes & Rapoport, 1998). The translocation complex in yeast consists of Sec61p, Sss1p and Sbh1p, where four Sec61p molecules form the membrane spanning channel while Sss1p may stabilize the channel (Matlack, Mothes & Rapoport, 1998; Romisch, 1999). The Sec61-complex provides a way through the membrane, but not the driving force for the translocation. There are two ways of translocation, co-translational and post-translational. The Sec61-complex can associate with the ribosome and then a direct path for translated proteins into the ER lumen is formed. Alternatively, already translated proteins are translocated post-translationally through the same channel. The Sec61-complex associates with the Sec62/63p, which is needed for gating the translated protein into the ER lumen (Kaiser, Gimeno & Shaywitz, 1997; Matlack, Mothes & Rapoport, 1998). The post-translational translocation is thought to be done mainly through a second translocon: the Ssh1p complex, with Ssh1p being a Sec61p homologue. This complex is not necessary for cell viability, but seems to provide translocation capacity needed for fast growth (Corsi & Schekman, 1996; Robb & Brown, 2001). Finally, it should be noted that the Sec61p complex has been shown to interact with the plasma membrane exocyst complex, which may indicate a regulatory link between protein translocation and exocytosis (Toikkanen *et al.*, 2003).

Vesicular transport from ER to Golgi

Already while proteins are translocated into the ER, their processing and maturation is started. Useful markers for this step are the removal of the signal peptide by signal peptidase and the addition of core oligosaccharides (Chen *et al.*, 2001). The removal of the signal peptide is essential: Sec11p that is a component of the yeast signal peptidase was therefore identified as a conditional secretory mutant (Novick, Field & Schekman, 1980; YaDeau, Klein & Blobel, 1991). The ER and the Golgi are separate organelles with no direct contact. Transport from ER to Golgi is therefore conducted via transport vesicles that are formed at the ER membrane. The vesicles are initially covered with a coat that is later shed, after which the transport vesicles start to fuse with each other, thus forming tubular clusters (Bannykh, Rowe & Balch, 1996). By observation of early yeast secretory mutants using electron microscopy, two different phenotypes were distinguished; class I mutants (*sec 12, sec13, sec16* and *sec23*) had the same vesicle amounts as the wild type, whereas class II mutants (*sec18, sec17* and *sec22*) had increased amounts of vesicles. Furthermore, genetic analysis revealed that class I mutants

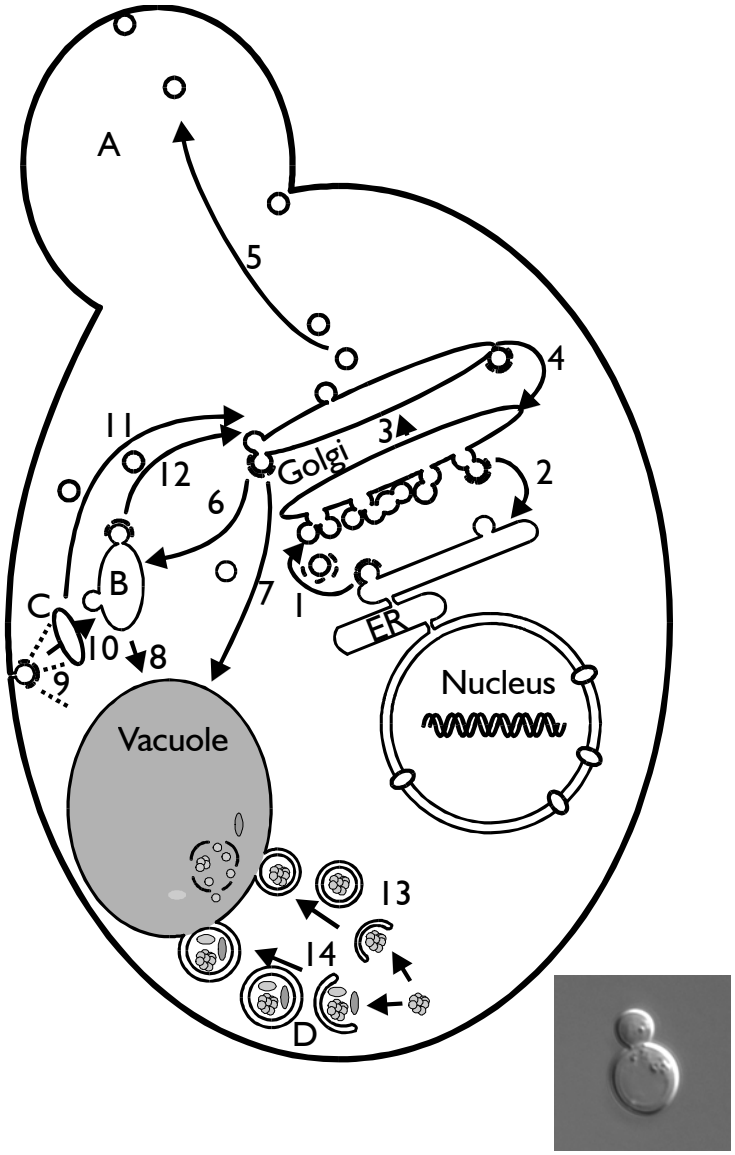

Figure 1 Compartments and transport pathways within the yeast cell.

Compartments. A: Budding daughter cell, B: Prevacuolar compartment, C: Early endosome, D: Autophagosomes.
Transport pathways. 1: Anterograde ER to Golgi, 2: Retrograde Golgi to ER, 3: Cisternal maturation within Golgi, 4: Retrograde transport within Golgi, 5: Transport from late Golgi to the growing daughter cell, 6: CPY transport from late Golgi to the PVC, 7 ALP transport from late Golgi to the vacuole, 8: PVC to vacuole transport, 9: Endocytosis (dependent on actin), 10: Early endosome to PVC transport, 11: Early endosome to late Golgi transport, 12: Recycling from PVC to late Golgi, 13: Cvt pathway, 14: Autophagocytic pathway.
The small picture shows a yeast cell seen with Normarski optics at x1000 magnification.

were epistatic to class II mutants. The interpretation of these findings was that the class I mutants are needed for vesicle formation, and the class II mutants for vesicle docking (Kaiser & Schekman, 1990).
Subsequent work has confirmed that the class I genes code for proteins that are required for vesicle formation. These proteins form the COPII class vesicle coat, which is the coat that is found on vesicles that are budded from ER. Among these proteins is Sec12p, a guanidine exchange factor (GEF) that interacts with the ARF GTPase Sar1p. Sar1p-GTP then forms a complex with Sec23p-Sec24p that can bind different cargo proteins. This complex is bound to Sec13p-Sec31p, which forms a polymer that deforms the membrane and thus drives the formation of the vesicle (Barlowe, 2002). It has further been demonstrated that Sec24p has binding sites that mediate cargo selection (Miller *et al.*, 2003). These binding sites were identified in a study of the structure of Sec23p-Sec24p-Sar1p complex (Bi, Corpina & Goldberg, 2002).
It has also been confirmed that the class II genes encode proteins that are involved in membrane fusion. Thus, Sec22p is a t-SNARE that is located in both ER and Golgi, and Sec17p and Sec18p are both needed for disassembly of the SNARE complex prior vesicle fusion, as discussed further below.

The Golgi apparatus

The Golgi apparatus is not a very prominent structure in *Saccharomyces cerevisiae*. Electron microscopy images do not show the stacked membrane structures typically seen in mammalian and plant cells, except in *sec7* or *sec14* secretory mutants (Morin-Ganet *et al.*, 1998). The Golgi apparatus appears to be rather quickly and transiently formed from vesicular clusters that coalesce into larger tubular structures. Finer and then larger nodular networks subsequently appear, which are replaced by secretory granulae (Morin-Ganet *et al.*, 2000). Secretory pathway proteins in yeast are nevertheless modified in the same sequential manner as in cells with stacked Golgi membranes, indicating that the biochemical compartments within the organelle are similar to those in other eukaryotes (Cleves & Bankaitis, 1992; Kaiser, Gimeno & Shaywitz, 1997). Other yeasts, such as *Pichia pastoris* have prominent Golgi structures (Mogelsvang *et al.*, 2003).
As in higher eukaryotes, the yeast Golgi apparatus is a junction of several transport pathways: transport from the ER, transport back to the ER, anterograde and retrograde transport between different Golgi compartments, and finally transport to the plasma membrane and transport to the vacuole.

Retrograde transport and COPI vesicles

Retrograde transport is required for recycling of proteins that are functional components of the transport pathway itself. Golgi to ER transport in yeast is selective, but some protein cycling between ER and Golgi occurs. There are two major motifs that have been identified as being important for ER retention of proteins in yeast: HDEL and KKXX. HDEL is found at the C-terminal end of soluble ER resident proteins, and has been shown to be sufficient for ER retrieval when added to normally secreted proteins, such as pro-α-factor (Dean & Pelham, 1990). It is also needed for ER retention, since when the HDEL motif is deleted

from Kar2p, the latter is secreted into the culture medium (Hardwick *et al.*, 1990) In mammalian cells, the ER retention motif is slightly different, KDEL, but seems to have the same function (Harter & Wieland, 1996). The ER retrieval motif KKXX, which is located at the C-terminus of integral membrane proteins, confers similar properties to proteins as HDEL. Addition of KKXX localizes proteins to the ER and removal of it leads to either vacuolar degradation or secretion (Kaiser, Gimeno & Shaywitz, 1997). Both HDEL-proteins and KKXX-proteins have been shown to undergo Golgi-specific carbohydrate modifications, indicating that the ER-retrieval motifs rather allow efficient recycling of the proteins than completely block their transport from the ER.

The COPI vesicles are responsible for this motif selective retrograde Golgi to ER transport. The COPI complex consists of seven proteins; in yeast they are known as Ret1p, Sec26p, Sec27p, Sec21p, Ret2p, Sec28p and Ret3p. As with the COPII complex, the components are conserved in all eukaryotic cells (Wieland & Harter, 1999; Kirchhausen, 2000). COPI assembly is essential for recycling of KKXX-proteins to the ER, and it has been shown that the coat component Sec21p interacts directly with the KKXX motif. (Letourneur *et al.*, 1994; Harter *et al.*, 1996). The HDEL motif is found in soluble ER proteins, and it binds to a HDEL receptor protein that interacts with coat proteins (Kirchhausen, 2000).

Retrograde transport is the most established role for COPI-vesicles. It has however been proposed that they could also have other roles, such as anterograde transport between ER and Golgi, and transport within the Golgi complex (Kaiser, Gimeno & Shaywitz, 1997).

Transport within the Golgi

The yeast *Saccharomyces cerevisiae* has not been extensively used for studies of transport within the Golgi since the Golgi is not a very prominent structure in yeast. Mutational analyses have, however, established that the yeast Golgi complex is involved in step-wise processing of proteins similar to its role in higher eukaryotes (Kaiser, Gimeno & Shaywitz, 1997). Biochemical studies of Golgi transport have been made primarily with cell free systems derived from mammalian cell lines.

Different models have been proposed for how anterograde transport occurs between the Golgi cisternae. The Golgi cisternae could be reasonably static membrane structures, with budding vesicles shuttling material between the different Golgi compartments. Another way for transport from *cis*- to *trans*-Golgi would be gradual maturation of entire Golgi cistarnae. Combinations of these models have also been discussed, see (Beznoussenko & Mironov, 2002) for a review. Recent results support a cisternal maturation model, as both the small G-protein from vesicular stomatitis virus (VSVG) and large aggregates of procollagen traverse the Golgi with the same speed and without leaving the cisternae they enter when they first arrive from the ER (Mironov *et al.*, 2001). Consistent with this, it has been shown by immuno-EM that vesicles in the vicinity of the Golgi complex contain proteins either residing in the Golgi or ER, and to a lesser degree VSVG proteins, that are transported through the Golgi (Martinez-Menarguez *et al.*, 2001), commented in (Pelham, 2001). Recent TEM studies of

the *Pichia pastoris* Golgi complex support the cisternael maturation model (Mogelsvang *et al.*, 2003).

Transport from the late Golgi to the vacuole: the CPY and ALP pathways

In the yeast late Golgi compartment, equivalent to the mammalian *trans*-Golgi network, proteins destined for the vacuole are sorted from protein destined to the plasma membrane or for secretion. Soluble proteins require a recognition sequence in order to be transported to the vacuole, while membrane bound proteins in contrast seem to be transported to the vacuole by default, in the absence of other transport signals (Bryant & Stevens, 1998).

Transport to the vacuole is easy to study as many vacuolar proteins are processed, first during transport from ER to late Golgi, and then subsequently within the vacuole. This has made it possible to study the effect that different yeast mutants that are defective in transport have on the processing of vacuolar proteins. Several mutant screens have identified a large number of mutants that are deficient in vacuolar function. Mutants deficient in the maturation of carboxypeptidase Y (CPY) (*pep1-pep15)* were isolated in a screen not specifically aimed at studying the vacuole, but it was subsequently shown that several of these genes encode proteins involved in vacuolar biogenesis and/or function. Vacuolar protein sorting, *vps,* mutants were isolated using a CPY-invertase fusion. If the CPY precursor was not sorted to the vacuole, the fusion protein resulted in a positive invertase assay. Other genetic screens aimed at studying vacuolar morphology (*vam* mutants) and endocytosis (*end* mutants) have also provided insights into the mechanisms of vacuolar transport (Jones, Webb & Hiller, 1997).

The route that CPY takes from the late Golgi to the vacuole is commonly termed as the CPY pathway. In short, the CPY precursor Prc1p is imported in the ER, at which time the N-terminal signal sequence is removed. Glycosylation of the protein occurs during its transport through the ER and Golgi. Once it arrives to the vacuole, the precursor is processed twice, first by proteinase A (PrA) and then by proteinase B (PrB) (Jones, Webb & Hiller, 1997). Pro-CPY has a recognition sequence that binds to a receptor, Pep1p/Vps10p, in the late Golgi. In absence of either Pep10p or the recognition sequence, most CPY will not end up in the vacuole, but is instead secreted from the cell (Bryant & Stevens, 1998; Kucharczyk & Rytka, 2001). Subcellular fractionation studies have further shown that CPY enters a separate membrane structure termed the prevacuolar compartment (PVC) before being processed in the vacuole. The CPY receptor Pep1p does not enter the vacuole, but is instead recycled back to the late Golgi (Horazdovsky, DeWald & Emr, 1995). Several other proteins are sorted in the same manner as CPY and are therefore affected by the same mutants in the vps-dependent pathway. This group of proteins includes both soluble proteins, such as PrA and PrB and membrane bound proteins, such as the vacuolar ATPase subunit Vph1p.

Transport of the transmembrane vacuolar protein alkaline phosphatase (ALP), which is coded by *PHO8* in yeast, is not affected by the same mutants as transport of CPY. For example, it reaches the vacuole in *pep12* mutants, which CPY does not. The transport route that ALP uses is referred to as the ALP pathway. A signal sequence at the N-terminal end of ALP was both necessary and sufficient for

transport within this pathway, as determined by domain swapping with the *PEP12* sorting dependent transmembrane protein carboxypeptidase S, CPS. Overexpression of ALP shifts some of the protein into *PEP12* dependent sorting, indicating that the ALP pathway is easily saturated (Cowles *et al.*, 1997b). Sorting of ALP depends on the adaptor complex AP-3 and deletion of any of the four genes that code for AP-3 components results in mislocalization of ALP (Cowles *et al.*, 1997a). Unlike the other adaptor protein complexes in yeast, AP-3 does not seem to associate with clathrin, although the mammalian AP-3 complex does (Liu *et al.*, 2001). Instead the protein Vps41p has been shown to directly interact with AP-3 through a motif in its N-terminus, while the C-terminus has a heavy clathrin chain motif that promotes homo-oligomerization. Both motifs are required for correct sorting of ALP (Darsow *et al.*, 2001).

Vesicle coat formation during vacuolar transport

Vesicle formation is required for transport within both the CPY and ALP pathways. In the CPY pathway, soluble proteins destined for the vacuole bind to transmembrane cargo proteins. These receptors are not produced in the same amount as their cargo, indicating that they are recycled and reused for transport in the same way as the components involved in the COPII and COPI transport machinery.

The best studied vesicle coat in mammalian cells is the clathrin vesicle coat, which is involved in vesicle formation at the plasma membrane during endocytosis and in transport from the *trans*-Golgi, see (Alberts *et al.*, 2002). Clathrin heavy and light chains form triskelion unit spontaneously in solution. When the triskelion units are assembled onto a membrane, recruited by adaptor protein complexes, they form a lattice that deforms the membrane and results in a clathrin coated pit. The vesicle is severed from the membrane by the action of dynamin in a GTP-dependent action. The adaptor protein complex AP-1 is located in vesicles derived from the *trans*-Golgi and the adaptor complex AP-2 complex in vesicles derived from the plasma membrane. (Kirchhausen, 2000) This rather straightforward biochemical picture is complicated by the fact that deletion of all genes coding for known adaptor proteins in yeast cells does not result in the same phenotype as a lack of clathrin does. Moreover, clathrin coated vesicles are still formed in such cells, showing that clathrin coat formation does not depend on adaptor protein function (Huang *et al.*, 1999).

In yeast, clathrin is required for transport between the late Golgi and the PVC, and also for receptor-mediated endocytosis. Deletion of the genes encoding the clathrin heavy and light chains, *CHC1* and *CLC1*, results in a viable cell that has several distinct phenotypes: slow growth, temperature sensitivity, slow endocytosis, inability to retain Golgi-resident proteins and defects in mating and sporulation. Vacuolar transport is, however, not affected in these cells (Kaiser, Gimeno & Shaywitz, 1997).

The Cvt and autophagocytic pathways

Proteins can also be directly imported into the vacuole from the cytoplasm; a process named the Cvt (cytoplasm-to-vacuole) pathway. The vacuolar hydrolase aminopeptidase I (API) which is transported by this pathway, reaches the vacuole

independently of any Sec or Vps proteins, as it is never translocated into the ER lumen (Klionsky, Cueva & Yaver, 1992). Vam3p is however required for delivery of API to the vacuole, showing that the transport is mediated by membrane fusion (Darsow, Rieder & Emr, 1997). The pro-API oligomerizes into clusters in the cytosol and is packed into double membrane vesicles that fuse directly with the vacuolar membrane. When the Cvt vesicle first fuses with the vacuole, a single membrane layered Cvt body is released into the vacuole lumen. The Cvt body is subsequently degraded and the pro-API peptides cleaved, thus producing mature API (Kim, Scott & Klionsky, 2000; Kucharczyk & Rytka, 2001).

Morphologically, the Cvt pathway resembles autophagocytosis, which is a bulk transport of cytoplasm and organelles to the vacuole. It is induced as a response to nutrient starvation or, in mammalian cells. also by hormonal stimuli (Klionsky & Emr, 2000). As with the Cvt vesicle, the autophagosome consists of a double membrane that encloses part of the cytosol and delivers its contents to the vacuole in a Vam3p dependent manner (Darsow, Rieder & Emr, 1997). There is a significant overlap between genes involved in Cvt pathway and autophagocytosis and recently a unified nomenclature for the involved genes was proposed (Klionsky *et al.*, 2003). The synthesis of the double membrane bodies is essentially the same in both pathways. The difference lies in regulation, as the Cvt is active under normal growth conditions and autophagocytosis during starvation (Huang & Klionsky, 2002). Although the formation of Cvt-vesicles and autophagosomes use essentially the same machinery, there seems to be different requirements for their synthesis. Thus, Cvt-vesicle formation requires Tlg2p and Vps45p which autophagosome formation does not, whereas the reverse is true for the COPII components Sec12p, Sec16p, Sec23p and Sec24p (Abeliovich, Darsow & Emr, 1999; Ishihara *et al.*, 2001).

Endocytosis and recycling in yeast

Enodocytosis, uptake and internalization of plasma membrane components, is in eukaryotic cells used to regulate the plasma membrane size and to control the amount of surface proteins. In higher eukaryotes, there is a number of signaling receptors that are internalized upon ligand binding, such as receptor tyrosine kinases, cytokine receptors and G-protein coupled receptors. Endocytotic processes seem to be mediated by two different pathways, one which is clathrin dependent and one which is clathrin independent (Mousavi *et al.*, 2003).

Yeast has only one form of cell-cell communication, which is by now well understood: the pheromone response. Haploid *MAT*α cells secrete α-factor, which binds to the Ste2p receptor on the surface of the *MAT*a cell. The internalization of Ste2p is more rapid in the presence of α-factor and is partially dependent on clathrin, but uptake still occurs at 50% in a *chc1* mutant, indicating that proteins are endocytosed also in the absence of clathrin (Kaiser, Gimeno & Shaywitz, 1997; D'Hondt, Heese-Peck & Riezman, 2000). The AP-2 complex, which is required for endocytosis in mammalian cells seems not to be required at all in yeast under normal growth conditions (Boehm & Bonifacino, 2002). Endocytoic defects are easily detected in yeast using the uptake of either radiolabeled α-factor or of fluorescent compounds, such as lucifer yellow or FM4-64 (Vida & Emr, 1995; D'Hondt, Heese-Peck & Riezman, 2000). In yeast, there is an absolute

requirement for actin in endocytosis while in mammalian cells this does not seem to be the case (Geli & Riezman, 1998). The direct involvement of actin in endocytosis could be through the actin patches as discussed in (Schott, Huffaker & Bretscher, 2002). In this context, it should also be noted that membrane lipid compositions and direct lipid protein interactions are important for endocytosis as reviewed in (D'Hondt, Heese-Peck & Riezman, 2000; Bankaitis & Morris, 2003; Gruenberg, 2003).

In yeast, the main signal for endocytosis is monoubiquitination of the membrane protein. Generally, ubiquitination requires action of two or three proteins. The ubiquitin is activated by an activating enzyme (E1), then transferred to a conjugating enzyme (E2) and finally covalently bound to the target molecule, often with assistance of a ubiquitin-ligase (E3) (Hicke, 1999). Ubiquitination was first discovered as a mechanism for targeting proteins for degradation in the proteasome, and in that case several ubiquitin molecules are attached to the protein. Monoubiquitinated proteins at the plasma membrane are instead targeted to the PVC compartment and subsequently degraded in the vacuole, as demonstrated with the amino acid permeases Tat2p and Gap1p (Hicke, 2001).

When an endocytotic vesicle has separated from the plasma membrane, it fuses with the early endosome, a weakly acidic compartment that allows the separation of receptors and ligands. The content of the early endosome migrates to the vacuole through a late endosomal compartment. At some point in the transport to the vacuole there is an intersection between the endocytotic and biosynthetic pathways. The class E vacuolar protein sorting (*vps*) mutants fail to correctly localize both endocytosed proteins and proteins transported within the CPY pathway (Bryant & Stevens, 1998; D'Hondt, Heese-Peck & Riezman, 2000).

Some endocytosed material is not transported to the vacuole but recycled to the late Golgi using the same recyling pathway as Vps10p. A specific vesicle coat has been identified as being involved in this retrograde traffic, the retromer coat (Seaman, McCaffery & Emr, 1998). The Vps35p protein, which is part of this coat complex, has been shown to interact with retrieval sequences and facilitate the retainment of Golgi proteins (Nothwehr, Ha & Bruinsma, 2000). The picture of transport from the plasma membrane to the late Golgi is, however, not uniform: a study of the v-SNARE Snc1p cycling indicated that it was sorted directly from the early endosome to the late Golgi. This cycling did not require a functional retromer coat (Lewis *et al.*, 2000).

The adaptive power of the yeast cell is demonstrated in the study of Vps10p trafficing in *chc1Δ* deletion mutants. Thus, in the absence of clathrin, Vps10p is localized to the plasma membrane and then internalized by endocytosis. It is then detached from its ligand, the pre-CPY, within the PVC, after which it is returned to the late Golgi (Deloche & Schekman, 2002).

Transport to the plasma membrane

Soluble proteins that are translocated into the ER lumen, folded properly, and lack a specific sorting signal are transported through the Golgi and then secreted out of the cell. In yeast, transport to the plasma membrane is almost exclusively directed to the growing bud. Consequently one important phenotype associated with deficient secretion is a general block in cell growth and an internal accumulation of secretory vesicles. In yeast, secretion is generally constitutive in the sense that

there are no major internal storage compartments for secretory proteins, although recently a regulated secretion of chitin synthase was described (Valdivia & Schekman, 2003). Typical cases of regulated secretion from other organisms are release of substances as an answer to external stimuli. Examples of this are the release of neurotransmitter substance from nerve cells and the release of histamine from mast cells. In cells with both constitutive and regulated secretion, the two modes of secretion involve different vesicles, which are separated at the *trans*-Golgi stage. The content in vesicles subject to regulated secretion are concentrated using a clathrin dependent mechanism, and these more dense vesicles can be clearly distinguished by electron microscopy (Alberts *et al.*, 2002). In contrast to other defined vesicular transport steps in yeast, transport from the late Golgi to the plasma membrane seems not to depend on coated vesicles. That is, in strains where the clathrin genes *CHC1* and *CLC1* have been deleted, transport to the plasma membrane is generally unaffected. There is however a subtle effect of *CHC1* deletion on the late Golgi to PM transport. Thus, the secretory vesicles that accumulate in *sec6* and *snc* strains were shown to be of two categories: one of lower density that contains the H^+-transporter Pma1p and one of higher density that contains invertase and acid phosphatase (Harsay & Bretscher, 1995; David, Sundarababu & Gerst, 1998). When either *CHC1* or *VPS1*, which is a dynamin homologue, is deleted in the *sec6* background, no high-density vesicles are seen, and the invertase activity localizes to the lower density fractions when the cells are separated on a density gradient. The high-density vesicles also disappear in a *sec6 pep12Δ* strain, implying that the formation of high-density vesicles may involve trafficking via the PVC (Gurunathan, David & Gerst, 2002). This is possibly a similar mechanism to that which concentrates vesicles involved in regulated secretion in mammalian cells.

Of the 23 secretion mutants isolated in Novicks and Schekmans original screen for temperature sensitive mutants in *Saccharomyces cerevisiae* (Novick, Field & Schekman, 1980) 10 mutants are directly involved in late secretion. None of these late secretory mutants are involved in vacuolar transport, emphasizing that transport to the vacuole and to the plasma membrane are two very distinct processes.

Sec4p, Rab GTPases and their roles in vesicular transport

Sec4p is one of the proteins that are needed for transport to the plasma membrane. It is a Rab GTPase, *i.e.* it belongs to the Rab GTPase protein family, which in turn belongs to the large Ras GTPase protein super-family. Other members of this protein super-family are the Ras-proteins, involved in signal transduction from cell surface receptors, and Rho-proteins, which are involved in signalling from the cell surface to the cytoskeleton. The Ras super-family members have a few properties in common: they cycle between an inactive state bound to GDP and an active state bound to GTP, and they are associated with membranes through covalently attached fatty acid chains. Their activity is regulated by guanidine exchange factors, GEFs, which facilitate the exchange of bound GDP for GTP, thereby activating the GTPase. The GTPase activity in Ras proteins, which converts them into the inactive form, may be stimulated by specific GTPase activating proteins, or GAPs (Stenmark & Olkkonen, 2001).

Sec4p performs an essential function in the cell that is required for vesicle transport from the late Golgi to the plasma membrane. Sec4p localizes to the bud tip, to vesicles derived from the Golgi, and is also present in a cytoplasmic pool (Kaiser, Gimeno & Shaywitz, 1997). There are 10 other Rab GTPases that have been identified in yeast, but none of them overlaps functionally with Sec4p. Generally, each Rab GTPase is associated with a distinct transport step within the cell (Lazar, Gotte & Gallwitz, 1997). Several proteins are involved in the GTP-GDP cycle of Sec4p. The binding of GTP to Sec4p causes a major conformational change in the protein which enables it to interact with its downstream effector, the exocyst component Sec15p (Novick & Guo, 2002); (Stroupe & Brunger, 2000). The Sec4p GEF Sec2p facilitates the GDP to GTP nucleotide exchange. Sec2p must be recruited to vesicles where Sec4p-GDP already resides for successful exchange (Ortiz *et al.*, 2002). The interaction between GTP-bound Sec4p and Sec15p eventually leads to *trans*-SNARE pairing and subsequent vesicle fusion with plasma membrane. After membrane fusion, Sec4p-GDP localizes to the plasma membrane, from where it is extracted by the protein Gdi1p. Loss of *GDI1*, allelic to *SEC19*, is lethal and will lead to depletion of vesicle bound Sec4p (Garrett *et al.*, 1994).

Tethering of the transport vesicle to the target membrane

The Rab GTPases are reasonably well characterized, while their effectors, the proteins they interact with in their active GTP-bound states, are less well understood. The Sec4p effector Sec15p is a part of large protein complex called the exocyst. The other proteins in this complex are Sec3p, Sec5p, Sec6p, Sec8p, Sec10, Exo70p and Exo84p. They perform an essential function and the absence of any of these proteins results in a blocked secretion and is therefore lethal. The exocyst components localize to the bud tip in yeast, which is the main secretion site in growing yeast cells (Novick & Guo, 2002). Exocyst function precedes membrane fusion and it is thought to involve docking of the vesicle which makes it available for the subsequent fusion, a process termed “tethering”. (Pfeffer, 1999; Grote, Carr & Novick, 2000). Tethering is defined as a transient state where a transport vesicle is attached to the target membrane, but has not yet fused with it. Another example of a protein involved in tethering is Uso1p, a downstream effector of the Rab GTPase Ypt1p, which functions in ER to Golgi transport (Cao, Ballew & Barlowe, 1998). Uso1p is a large rod-shaped coiled-coil protein, that possibly could perform its function in tethering by bridging the gap between the vesicle and the target membrane (Yamakawa *et al.*, 1996).

The exocyst complex and Uso1p may represent different ways of tethering a vesicle; they are both conserved between yeast and animals. The human exocyst complex is referred to as the Sec6/Sec8 complex and the human homologue of Uso1p is known as p115 (Whyte & Munro, 2002). In yeast, there are two complexes that resemble the exocyst: the COG (conserved oligomeric Golgi) complex and the VFT (Vps fifty three) complex, They share a conserved domain at the N-terminal end of most of their subunits and are termed “quatrefoil” complexes. The COG complex has been implicated in Golgi tethering and the VFT complex in recycling from PVC to the late Golgi (Short & Barr, 2002; Whyte & Munro, 2002). There are also three complexs that show no homology with each

other but are all targets for Rab GTPases: TRAPP I & II (transport protein particle) that functions in ER to Golgi traffic, the Class C Vps complex that appears to be involved in both late Golgi to PVC and PVC to vacuole transport, and the Dsl1 complex that is hypothesised to be involved in Golgi to ER retrograde traffic (Whyte & Munro, 2002). In contrast to the Rab GTPases and the SNARE proteins, it seems that the homology between different tethering complexes is low or absent, indicating that the mechanics involved may differ for different transport steps (Guo *et al.*, 2000; Whyte & Munro, 2002). In addition to the interaction of Sec4p with Sec15p, there are other interactions that are known to occur between GTPases and exocyst components. Sec3p, which localizes to the bud independently of vesicluar traffic and is regarded as the spatial mark for exocyst localization (Finger, Hughes & Novick, 1998), has thus been shown to interact with the Rho1 GTPase, and to be dependent on it for correct localization (Guo, Tamanoi & Novick, 2001). Rho1p has several roles, among them organization of polarized growth (Orlean, 1997). The Rho1 GTPase binds a region of Sec3p that also is a binding site for another GTPase, Cdc42p. However, Rho1p and Cdc42p do not seem to compete for Sec3p binding, but rather act at different times in the cell cycle. Cdc42p is also a Rho GTPase, and it is important for initiation of polarized growth (Novick & Guo, 2002). As the exocyst mislocalizes in *rho1* or *cdc42* mutant strains, it is evident that Sec3p and its interactions with Rho1p and Cdc42p are important for proper localization of secretion to the growing bud.

Membrane fusion in eukaryotes

A proteinaceous lipid bilayer vesicle which is brought close to another similar membrane in a hydrophilic milieu is not able to overcome the forces separating the membranes by itself (Blumenthal *et al.*, 2003). For successful membrane fusion, the vesicle needs to be brought to the correct target membrane and actively fused with it.

The membrane fusion leads to mixing of the vesicle lumen contents with the content behind the target membrane, whether that is an organelle lumen or the extracellular space. The membrane fusion event is a central part of cell dynamics and both the specificity and the fusion process itself have therefore been the subject of much recent research.

Evolutionary conserved proteins involved in membrane fusion

The release of neurotransmitters from a nerve cell axon into the synaptic cleft is a highly regulated process. The release of transmitter is triggered by a Ca^{2+} influx that in turn is the consequence of a depolarization of the plasma membrane. After release, the transmittor substance is taken up again into the cell, transported to the endosome and recycled back to the plasma membrane. The molecular components involved in the vesicle recycling were isolated and characterized mostly by biochemical methods (Jahn & Sudhof, 1993). An ATP-binding protein, NSF (N-ethylmaleimide Sensitive Factor) was shown to be required for membrane fusions in all eukaryotic cells. NSF binds to several SNAPs (Soluble NSF Attachment Proteins) and forms a complex with them. The NSF-SNAP complex interacts with

three synaptic proteins, SNAP-25, synaptobrevin and syntaxin. (Sollner *et al.*, 1993b). It was also shown that these interacting proteins are absolutely required for release of neurotransmittor substance, as they are the target for different bacterial neurotoxins, reviewed in (Schiavo, Matteoli & Montecucco, 2000). The synapse is a specialized cell structure, but the proteins that are crucial for successful vesicle fusion are present in all animal cells and are also conserved in yeast. Specifically, the plasma membrane syntaxins that are involved in synapse function are homologous to the products of the duplicated yeast genes *SSO1* and *SSO2* (Aalto, Ronne & Keränen, 1993). Synaptobrevin is homologous to the products of the duplicated genes *SNC1* and *SNC2* (Protopopov *et al.*, 1993), and SNAP-25 is homologous to the *SEC9* gene product (Brennwald *et al.*, 1994).
NSF is homologous to Sec18p, and one of the SNAP-proteins to Sec17p. NSF is known to be involved in almost every membrane fusion event in the cell. It assembles to a hexamer that associates to the membrane by interaction with a SNAP protein (Sec17p in yeast). It then separates some of the complexed membrane fusion proteins from each other in an ATP consuming reaction that is not yet fully understood (Whiteheart, Schraw & Matveeva, 2001).

The SNARE hypothesis

The observation that NSF-SNAP binding proteins, termed SNARE proteins for SNAP receptor, resided on different membranes led to a classification of synaptobrevin as a v-SNARE, as it is present on transport vesicles, and of SNAP-25 and syntaxin as t-SNAREs, as they were isolated from the target-membranes (Sollner *et al.*, 1993a). This was suggested to be a general mechanism for membrane fusion in all eukaryotic cells. The SNARE hypothesis states that the v-SNARE is the address tag on the vesicle that binds specifically to the corresponding t-SNARE on the target membrane. The complex that the v-SNARE form with the t-SNARE is stable in absence of NSF-SNAP and vesicle fusion will not occur if any of the participating SNARE proteins are not present. Membrane fusion was supposed to involve the ATPase activity of SNAP-NSF (Rothman & Warren, 1994; Sudhof, 1995).
The suggestions put forward in the SNARE hypothesis have subsequently been disproven: SNAREs are not required for vesicle docking (Pfeffer, 1999) and NSF is not required for the membrane fusion (Mayer, Wickner & Haas, 1996). Instead, NSF disassembles *cis*-SNARE complexes after vesicle fusion (Whiteheart, Schraw & Matveeva, 2001). Nevertheless, the classification of these proteins as v-SNAREs and t-SNAREs has been maintained. It has helped to identify complexes involved in membrane fusion in different organisms and the SNARE hypothesis has stimulated the development in the field.

The SNARE core complex

Interactions between different SNARE proteins involves the formation of a SNARE complex which is composed of four α-helixes (Katz *et al.*, 1998; Sutton *et al.*, 1998; Antonin *et al.*, 2000), aligned parallel to each other. These helices are 60-70 amino acids long (Weimbs *et al.*, 1997) and are referred to as SNARE motifs. The SNARE complex is very stable. For example, treatment with tetanus

or botulinum neurotoxins, which cause proteolysis of free SNARE proteins, shows that the core complex is insensitive to these toxins once it has been formed. The complex is also resistant to SDS treatment at this stage (Pellegrini *et al.*, 1995).

Once the three-dimensional structure of the neuronal SNARE complex was resolved (Sutton *et al.*, 1998) it became possible to model also other related SNARE complexes using this structure. This modeling showed that the residues of SNARE proteins that are located in the middle of the 4-helix bundle are most conserved. In particular, in one position, a glutamine residue was found to be conserved in most t-SNARE proteins and a arginine in most v-SNARE proteins (Fasshauer *et al.*, 1998). The authors therefore argued for a reclassification of v-SNAREs and t-SNAREs into Q-SNAREs and R-SNAREs and also noted that most known SNARE complexes contain three Q-SNARE helixes and one R-SNARE helix. It has subsequently been shown that the SNARE complex is sensitive to changes in the 3Q/1R balance, although a 4Q complex remains functional (Katz & Brennwald, 2000; Ossig *et al.*, 2000). A neuronal t-SNARE complex composed of two syntaxin molecules and one SNAP-25 molecule, which would form a 4Q bundle, was shown to be more flexible than the previously characterized neuronal SNARE complex (Xiao *et al.*, 2001). The proposed reclassification of SNARE proteins from v- and t-SNAREs to R- and Q-SNAREs has not been adopted widely, and in most cases v-SNAREs equals R-SNAREs and t-SNAREs equals Q-SNAREs (Jahn & Sudhof, 1999).

The formation of the SNARE core complex is spontaneous and is readily reproduced in a test tube. Furthermore, the SNARE pairing is not selective when tested *in vitro*. Instead, SNARE proteins from different compartments of the cell can form complexes with each other. These “incorrect” SNARE complexes may also be as resistant to heat and SDS as SNARE complexes known to be formed *in vivo* (Fasshauer *et al.*, 1999; Yang *et al.*, 1999). However, when Golgi SNARE complex formation was tested using reconstituted vesicles, a situation more resembling the *in vivo* situation was observed, in that the complex formation was more restrictive (Parlati *et al.*, 2002). With the same system it was further shown that efficient fusion only occurred when Golgi t-SNAREs and v-SNAREs were present on different membranes (Parlati *et al.*, 2000). SNARE complexes exert their biological role when formed between membranes, and those complexes are called *trans*-SNARE complexes. After membrane fusion the components are on the same membrane, in a *cis*-conformation, and it is these complexes that Sec18p subseqeuntly breaks up after membrane fusion (Whiteheart, Schraw & Matveeva, 2001).

The role of the SNARE proteins has been reassessed. Instead of being address tags involved in vesicle docking, they are now thought to be directly involved in membrane fusion. Membrane spanning SNARE proteins have their SNARE motifs close to their transmembrane regions. The formation of a SNARE complex where the participating proteins are on different membranes will therefore bring the two membranes very close to each other. As mentioned above, the SNARE complex is a very stable state, and cannot be broken up without the ATP-dependent action of SNAP-NSF (Sollner *et al.*, 1993a). Whether the SNARE complex is sufficient for membrane fusion or if there are additional steps involved after SNARE complex formation has not yet been established (Jahn & Grubmuller, 2002). Recently, it was, however, shown that SNARE proteins, which were modified to expose the

SNARE motif on the cell surface, could induce cell fusion, something which lends further support to a direct role for these proteins in membrane fusion (Hu *et al.*, 2003). In their large review about membrane fusion and exocytosis (Jahn & Sudhof, 1999) discuss the similarities between the SNARE complex and viral fusion proteins, and note that although these genes are unrelated, the encoded proteins have similar mechanical properties. Virus infected cells are also known be able to fuse with each other (Blumenthal *et al.*, 2003), similar to the cells expressing the engineered SNARE-proteins.

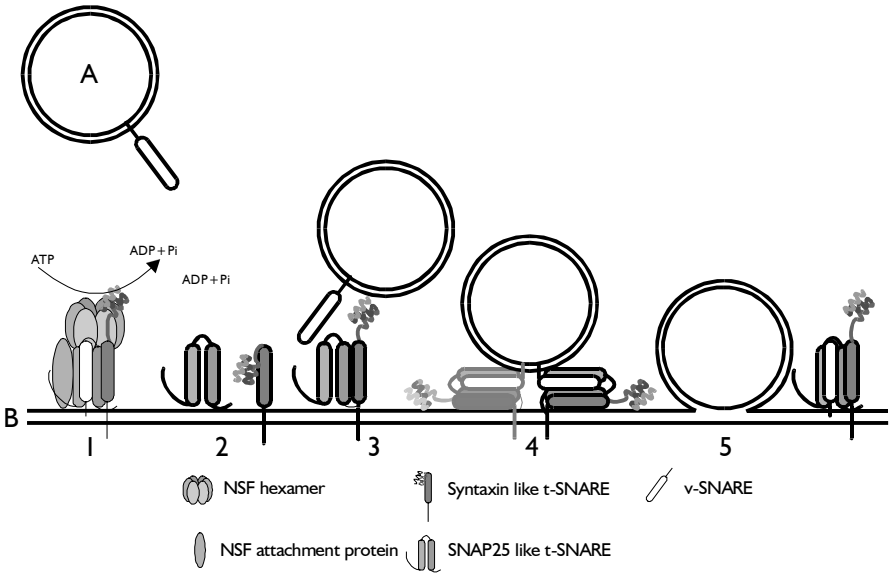

Figure 2: Heterotypic membrane fusion at the plasma membrane
A: Transport vesicle, B: Plasma membrane.
1: The NSF-SNAP complex disassembles *cis*-SNARE complexes in an ATP consuming process. *Cis*-SNARE complexes remain at the plasma membrane after vesicle fusion. 2: The syntaxin adopts a ”closed conformation” when it is not bound to other SNARE components, which is followed by association with SNAP-25 (Nicholson *et al.*, 1998) 3: The t-SNAREs associate before binding the v-SNARE. 4: Several *trans*-SNARE complexes are formed at vesicle fusion, and they initiate direct membrane contact. 5: The SNARE complex remains on the plasma mebrane until it is broken up by NSF-SNAP and then the v-SNAREs can recycle (Nicholson *et al.*, 1998; Jahn & Sudhof, 1999).

Plasma membrane SNAREs in yeast

The best characterized SNARE pairing event, in yeast as well as in higher eukaryotes, is the complex that is formed at the plasma membrane. In yeast as well as in higher eukaryotes, the core components are Sec9p, Sso1p or Sso2p and Snc1p or Snc2p. The Snc proteins are 79% identical to each other and are, as far as we know, functionally redundant. Interestingly, a *snc1 snc2* strain is viable, which shows that the synaptobrevins are not essential for secretion. These cells are however, impaired in transport from Golgi to the plasma membrane, accumulate transport vesicles, and are unable to grow under certain conditions (Protopopov *et al.*, 1993). Thus, the double *snc* mutant can grow on synthetic medium only at temperatures equal to or less than 30°C indicating that some vesicle fusion may occur by Sso1/2p – Sec9p SNARE pairing alone (Marash & Gerst, 2001). The Snc proteins are about 40% homologous to the human plasma membrane synaptobrevin, but expression of human synaptobrevin in yeast does not complement the *snc* mutants (Gerst, 1997).

The Snc proteins cycle between the plasma membrane and intracellular compartments. Recycling is dependent on a methionine residue in the SNARE helix, Snc1p-Met43 and Snc2p-Met42 respectively, and changing the methionine to an alanine results in a temperature sensitive cell. GFP tagged mutant *snc1-M43A* localizes similar to GFP tagged *SNC1* in wild type cells. In *sec6* mutant cells at the restrictive temperature, fluorescence from the *GFP-snc1-M43A* construct is retained at the plasma membrane, while the wild type *GFP-SNC1* localizes in intracellular structures. Analysis of this recycling deficient *snc1* mutant has shown that all endocytosis is impaired: vacuole staining with the fluorescent stain FM4-64 and vacuole degradation of Ste2p α-receptor are both abolished in the *snc1 M43A* strain (Gurunathan *et al.*, 2000). A dual role for the Snc proteins in both exocytosis and endocytosis is plausible as no specific endocytotic v-SNARE has been identified in yeast (Grote *et al.*, 2000; Gurunathan *et al.*, 2000). Sec9p has two SNARE helices and no transmembrane domains. Its human homologue SNAP-25 has been shown to localize to the plasma membrane independently of syntaxin 1a, the mammalian homologue of the Sso proteins. Association of SNAP-25 with the plasma membrane is mediated by palmitylated cystein residues that are located between the two SNARE helices (Loranger & Linder, 2002). Sec9p has also been shown to localize to the plasma membrane, as have the two Sso proteins (Brennwald *et al.*, 1994). Investigations using two different temperature sensitive mutants of *SEC9*: *sec9-4,* which was isolated in the original *sec* mutant screen (Novick, Field & Schekman, 1980) and *sec9-7* (Rossi *et al.*, 1997), demonstrated how important the residue matching is in SNARE formation. Thus, protein lysates from *sec9-4* cells were unable to form SNARE complexes *in vitro*, which was possible with *sec9-7* cell lysates. Still, neither mutant strain is viable at the restrictive temperature (Rossi *et al.*, 1997). The *sec9-7* protein proved to be unable to form stable *in vivo* SNARE complexes, a defect which could be rescued by a second mutation in the Snc2 protein, *snc2-1*, which can be interpreted as a symmetry in the SNARE complex being restored. Analysis of these mutants led to the suggestion that the SNARE helices in the yeast plasma

membrane SNARE complex are arranged in parallel (Katz *et al.*, 1998), similar to the human neuronal SNARE complex.

The syntaxin family

The syntaxins are strongly conserved in all eukaryotes and they seem to have to the same overall structure: three N-terminal helices, one core helix and a C-terminal transmembrane domain. Most of the protein faces the cytosol. In *Saccharomyces cerevisiae* there are seven syntaxins, while there are 15 in man (Teng, Wang & Tang, 2001). In *Arabidopsis thaliana* there are even more: sequence analysis have identified 24 syntaxins that can be ordered into ten subfamilies (Sanderfoot, Assaad & Raikhel, 2000). The function of all *Arabidopsis* syntaxins have not yet been addressed. The wider diversity of membrane fusion complexes is exemplified by the fact that no less than five distinct SNARE complexes have been identified in the *trans*-Golgi and prevacuole compartments of *Arabidopsis* (Sanderfoot *et al.*, 2001).

Syntaxin structure and function

The third SNARE protein in the plasma membrane SNARE complex is a member of the strongly conserved syntaxin family. In yeast, there are two plasma membrane syntaxins, encoded by the *SSO1* and *SSO2* genes. They were cloned as multicopy suppressors of the *sec1-1* mutant and were initially regarded as functionally redundant (Aalto, Ronne & Keränen, 1993). The Sso proteins are 73% identical at the protein level and seemingly redundant during vegetative growth. Unlike the synaptobrevins, however, they have an essential function: a deletion of both *SSO* genes is lethal (Aalto, Ronne & Keränen, 1993). Experiments with promotor shutoff and temperature-sensitive mutations have shown that cells depleted of Sso proteins accumulate vesicles in the same manner as is seen for late secretory pathway *sec* mutations at restrictive temperatures.

The structure of the Sso1 protein has been determined both by x-ray analysis of the crystallized protein (Munson *et al.*, 2000) and by nuclear magnetic resonance (NMR) spectroscopy (Fiebig *et al.*, 1999). Sso1p is a 290 amino acid protein with a 22 amino acid transmembrane (TM) region at the C-terminal end. Adjacent to the TM is the SNARE motif that forms an α-helix, the Hcore helix. In solution, the SNARE motif is partially unstructured. The N-terminal half of the protein forms three shorter helices both in solution and when the protein is complexed with other SNARE proteins. These helices are named Ha, Hb and Hc respectively. The region N-terminal to Ha is unstructured, while the region between Hc and the Hcore forms two short linker helices, HL1 and HL2.

The human plasma membrane syntaxin 1a has a similar structure (Lerman *et al.*, 2000), as have the more distantly related yeast vacuolar syntaxin Vam3p (Dulubova *et al.*, 2001). This suggests that the three N-terminal helices is a common feature of all syntaxins.

In Sso1p, the N-terminal helices have been shown to be important for regulating SNARE complex assembly. Thus, removal of the N-terminal part of Sso1p increases the rate of SNARE complex assembly with Sec9p and Snc2p by a magnitude of three (Nicholson *et al.*, 1998). Structurally, the N-terminal helices

fold back and interact with the Hcore helix, which may explain the reduced assembly rate for the full-length protein. Consistent with this, mutations that disturb the Ha, Hb and Hc structures in Sso1p result in faster formation of the SNARE complex (Munson *et al.*, 2000).
Studies of the Sso proteins further showed that they could suppress mutations in some of the exocyst genes: *sec3-2, sec5-24* and *sec15-1* when overexpressed. In addition, they could also suppress *sec9-4* (Aalto, Ronne & Keränen, 1993).
Gerst and coworkers have explored how phosphorylation regulates syntaxin function in both Sso1p and in the endocytic t-SNAREs Tlg1p and Tlg2p. It seems that dephosphorylation activates the syntaxins. Interestingly, mutant proteins where the proposed PKA phosphorylation sites have been changed: Sso1p-S79A, Tlg1p-S31A, Tlg2-S90A could suppress different effects of the *snc1snc2* double knockout. Thus, the *tlg* mutations restored endocytosis and the *sso1* mutation restored exocytosis. *sso1*-S79A in combination with any of the *tlg* mutations could also restore full growth on rich medium of *snc1snc2* cells (Gurunathan *et al.*, 2002).

Subcellular localization of syntaxins

Because syntaxins are localised to different compartments there must clearly be a sorting mechanism for them. They do not have an N-terminal signal sequence and have only their C-terminal transmembrane region inserted into the membrane, while the rest of the protein faces the cytoplasm. An investigation of Sso2p insertion and transport when expressed in mammalian cells showed that the protein was inserted into the ER membrane and transported in a normal way along the secretory pathway (Jäntti *et al.*, 1994). A comparative study in mammalian cells of syntaxins 3 and 4 which are localized to the plasma membrane, and syntaxin 5, which localizes to the *cis*-Golgi, suggested that the length of the transmembrane region is a determinant for the protein either being retained in the Golgi or transported to the plasma membrane. Thus, by fusing the cytoplasmatic region of syntaxin 5 to the TMD of syntaxin 3, the latter was transported to the plasma membrane, and the reverse experiment, as well as a truncation of the longer syntaxin 3 TMD, resulted in *cis*-Golgi retainment of the protein (Watson & Pessin, 2001). The TMD effect of syntaxin 5 localisation was confirmed in another study that also showed that the correct intracellular localisation of syntaxin 7 and 8 depends on a dileucine motif. For syntaxin 7 this motif was necessary for correct internalisation from the plasma membrane, while syntaxin 8 required this motif for cycling between the *trans*-Golgi network and the endosome (Kasai & Akagawa, 2001). The mechanism for membrane insertion and the dependence of TMD length for subcellular localization is not restricted to syntaxins, but is also true for other tail-anchored proteins (Kutay *et al.*, 1995; Bulbarelli *et al.*, 2002).
Syntaxins are known to be involved in cytokinesis in plants. Thus, in *Arabidopsis thaliana* there is a syntaxin, KNOLLE, which interacts with the Sec1p like protein KEULE (Assaad *et al.*, 2001) and has its expression tightly regulated during the cell cycle. KNOLLE is required for targeting of transport vesicles to the phragmoplast, the foundation for the cell plate that will divide the plant cell at completion of cell division (Batoko & Moore, 2001). The transcriptional

regulation of KNOLLE is important for its function during cytokinesis. Expression of KNOLLE from a strong constitutive promotor, 35S, did not rescue *knolle* mutant embryos, although the protein was present in the cell. *In situ* hybridisation revealed that *KNOLLE* mRNA accumulated in the cell during the M-phase and that the 35S-promotor controlled *KNOLLE* produced less transcript than the endogenous *KNOLLE* promotor. The inability of 35S-*KNOLLE* to complement *knolle*-deficient embryos was therefore attributed to a difference in gene expression (Volker, Stierhof & Jurgens, 2001).

The two yeast syntaxins Pep12p and Vam3p are both involved in transport from ER to Golgi. Pep12p is required for transport within the CPY pathway. Thus, in a *pep12* null mutant ALP is transported normally. In contrast, Vam3p is required for transport both within the CPY and the ALP pathway. When overexpressed, *VAM3* can substitute for *pep12* and the reverse is also true (Darsow, Rieder & Emr, 1997; Gotte & Gallwitz, 1997). In a mutant screen for *vam3* alleles that could suppress *pep12* null mutants even if not overexpressed, several mutations in the linker region were recovered. Vam3p itself is transported by the ALP pathway and a deletion of the AP-3 components results in mislocalisation of the syntaxin (Cowles *et al.*, 1997a). Vam3p contains a dileucine motif within the linker region that is required for the AP-3 association. Mutations in this motif led to incorrect sorting of the protein, which may explain why the mutants protein could functionally replace Pep12p (Darsow, Burd & Emr, 1998).

The Sec1 protein family

SEC1, whose allele *sec1-1* was the first isolated secretory mutant (Novick and Schekman, 1979), is essential for viability (Aalto *et al.*, 1991). Yeast strains with the mutant allele *sec1-1* or *sec1-11* accumulate vesicles and are impaired in secretion at the restrictive temperature. These mutants have been further characterized at a molecular level (Brummer *et al.*, 2001). Localization studies of GFP tagged Sec1p have shown that it is concentrated at the sites of secretion, reminiscent of the localization of the exocyst complex, and furthermore that this localization is abolished in a temperature-restricted *sec4* strain, but remains in a temperature-restricted *sec18* strain (Carr *et al.*, 1999). The same study used *in vitro* binding assays to show that Sec1p binds to assembled SNARE complexes and not to any specific SNARE protein. This illustrates an enigma of the Sec1p/Munc18-proteins (SM-proteins): they are conserved in all eukaryotes, there are also conserved Sec1p homologues involved in different transport steps in the cell, they all interact with SNARE proteins that also are conserved, but yet the specific interactions differ from case to case.

In yeast there are four SM-proteins: Sec1p, Vps45p, Sly1p and Vps33p (Toonen & Verhage, 2003) that are involved in different steps of intracellular transport. Interactions with specific SNARE proteins have, besides for Sec1p also been demonstrated for Vps45p and Sly1p. Interestingly, they both interact with the N-terminal ends of the syntaxins. Thus, Vps45p interacts with the endocytic syntaxin Tlg2p and also with Pep12p (Dulubova *et al.*, 2002), while Sly1p interacts with the ER syntaxin Ufe1p and with the Golgi syntaxin Sed5p (Yamaguchi *et al.*, 2002; Dulubova *et al.*, 2003). Vps45p is also involved in vesicle traffic to the vacuole, as is Vps33p. Their interactions with the vacuolar syntaxins Pep12p and

Vam3p respectively have not yet been mapped, but do not seem to be mediated by the syntaxin N-termini (Toonen & Verhage, 2003). In overexpression studies of *VAM3* and *PEP12* it was found that Vam3p required the presence of Vps45p to substitute for Pep12p, though it is known to interact with Vps33p in vacuolar fusion. This indicates that the action of Vps45p is site-specific, but not syntaxin specific (Darsow, Rieder & Emr, 1997).
Illustrating further the diversity in the SM – syntaxin interactions, it was found that the human homologue of Sec1p, Munc18, does not interact with the assembled SNARE complex, but rather with the N-terminal helices of syntaxin 1a (Dulubova *et al.*, 1999; Yang *et al.*, 2000). The importance of SM-proteins in membrane fusion is clearly established, but their diverse interactions with the SNARE proteins are puzzling (Gallwitz & Jahn, 2003; Toonen & Verhage, 2003). Finally, in addition to the interaction with the exocytic SNARE complex, Sec1p also interacts with the small, hydrophilic protein Mso1p. This protein, which has no apparent homologue in higher eukaryotes, is involved in vesicle fusion, and its absence causes an accumulation of vesicles in the bud, indicating that secretion is partially compromised, but cell growth is not strongly affected. Deletion of *MSO1* is however synthetically lethal in combination with *sec1, sec2* or *sec4* mutations at the permissive temperature and has strong synthetic effects also with mutant alleles of several other exocyst genes (Aalto *et al.*, 1997).

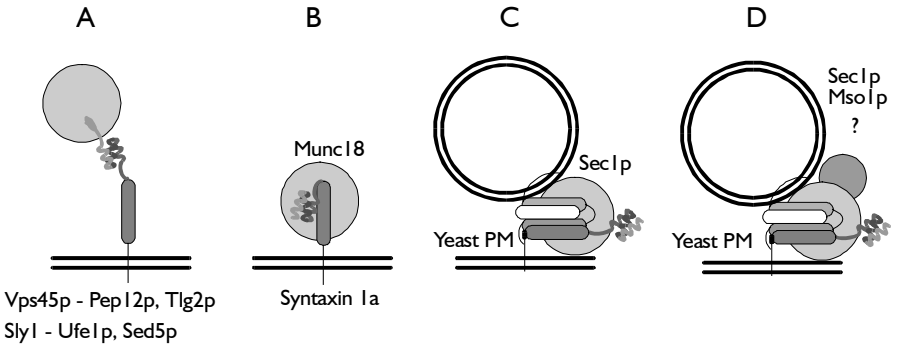

Figure 3 Different interactions between syntaxins and SM proteins

A: Vps45p interacts with the N-terminal end of Pep12p and Tlg2p as does Sly1p with Ufe1p and Sed5p. B: Munc18 binds to the closed conformation of syntaxin 1a. C: Sec1p binds to the assembled yeast plasma membrane SNARE complex. D: It is possible that theinteraction between Mso1p and Sec1p is specifically needed for the binding of Sec1p to Sso1p containing, but not Sso2p containing SNARE complexes.

Table 1: Syntaxins in yeast and their interacting SM proteins

Syntaxin	Site of action	Interacting SM protein	Reference
Sso1p	Exocytosis, prospore membrane	Sec1p	(Carr *et al.*, 1999)
Sso2p	Exocytosis	Sec1p	(Carr *et al.*, 1999)
Tlg2p	Endocytosis, PVC to Golgi	Vps45p	(Dulubova *et al.*, 2002)
Pep12p	Golgi to PVC	Vps45p	(Bryant & James, 2001; Dulubova *et al.*, 2002)
Vam3p	PVC to vacuole	Vps33p	(Dulubova *et al.*, 2001)
Ufe1p	ERto Golgi	Sly1p	(Yamaguchi *et al.*, 2002)
Sed5p	Intra Golgi	Sly1p	(Yamaguchi *et al.*, 2002)

Meiosis and spore formation in yeast

Diploid yeast cells, which is the normal ploidity for yeast, use meiosis and sporulation as a way to survive nutrient starvation. Sporulation requires the *MAT* locus to be heterozygous, *MAT*a/*MAT*α, but is also dependent on growth on a non-fermentable carbon source and a lack of nitrogen. Yeast proceeds through meiosis in the same manner as higher eukaryotes with the exception that the nuclear envelope does not break down but instead remains continuous until the spores have been formed. The transcriptional cascade that is initiated at the beginning of meiosis is extensive, and the initiator of it, *IME1* is elaborately regulated (Vershon & Pierce, 2000). Activation of *IME1* leads to transcription of early meiotic genes that in turn activate the middle meiotic genes, which then activate the late meiotic genes (Kupeic *et al.*, 1997; Clancy, 1998).

The morphological changes that yeast undergoes during meiosis have been characterised by electron microscopy. During meiosis, the DNA is replicated after which homologous chromosomes are paired in a structure called the synaptonemal complex (SC). At the same time, meiotic recombination takes place, but SC formation and recombination are not necessarily linked (Kupeic *et al.*, 1997). At the beginning of DNA replication the spindle pole bodies, SPBs, are duplicated and at the disassembly of the SC the SPBs have migrated along the nuclear envelope to opposite ends of the cell, and a spindle structure has been developed. At this stage, the SPBs are duplicated once more. At the end of anaphase II, when the chromosomes have separated, there are four SPBs and four distinct lobes inside the original cell (Kupeic *et al.*, 1997). At that stage the SPBs acquire a meiosis-specific structure, a thicker outer plaque. From this meiotic plaque, MP, the prospore membrane is formed, which extends from the SBP and forms a lobe that encloses the nucleoplasm and part of the cytoplasm before it closes (Moens, 1971; Moens & Rapport, 1971). Formation of the MP and proper progression of prospore membrane formation is crucial for spore development (Moreno-Borchart & Knop, 2003). The lipid vesicles that participate in the formation of the prospore membrane come from the late Golgi and formation of prospore membrane is thus a branch of the exocytic pathway. Consistent with this, some exocytosis mutants, *sec1-1* and *sec4-8*, are deficient in spore formation (Neiman, 1998). The t-SNARE Sec9p is, however, not required during sporulation as it is functionally replaced by the meiosis specific t-SNARE Spo20p (Neiman, 1998; Neiman, Katz & Brennwald, 2000). After the prospore membrane has been formed, the formation of the spore cell wall starts with the deposition of material between the two

membranes. When completed, the spore cell wall consists of a large, electron transparent layer mainly made up of polysaccharides, and outside it two distinct layers, an inner layer made up of chitosan and an outer layer made up of dityrosine (Kupeic *et al.*, 1997). As a consequence of having the spore wall, yeast spores are much more resistant to draught, heat and organic solvents than are vegetatively growing cells.

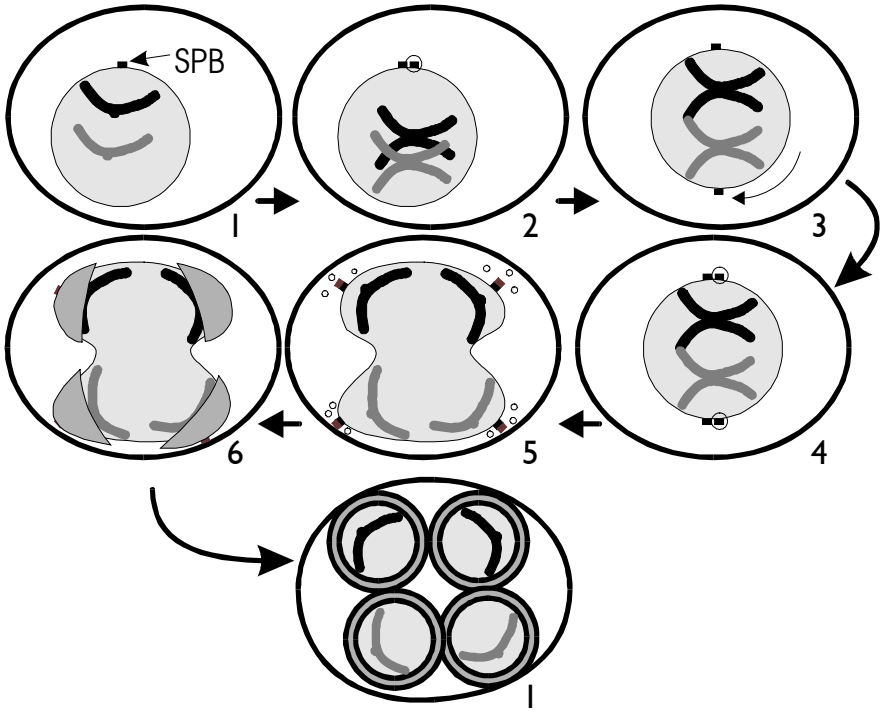

Figure 4 Spore formation in *Saccharomyces cerevisae*

1: Initiation of sporulation 2: At the onset of meiosis the genome is replicated and the SPBs are duplicated. 3: The SPBs separate from each other. 4: The SPBs are duplicated again. 5: The nuclear envelope deforms and the nuclei separate from each other. The SPB develop the meiosis specific outer plaque where vesicles fuse and form the prospore membrane. 6: The prospore membranes embrace a part of the lobular nuclear envelope including some cytoplasm. 7: The spores mature and acquire a thick cell wall. (Moens & Rapport, 1971; 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.

Understanding organised intracellular transport is ultimately a key to understanding the eukaryotic cell. The cell harbours many enzymatic reactions, such as those within lyzosomes and vacuoles, which would be deleterious unless confined by lipid barriers. Over the years, the yeast *Saccharomyces cerevisiae* has been an important tool for elucidating the mechanisms of intracellular transport. One of the most important abilities of yeast is the haploid growth phase, which allows recessive mutations to have a phenotype that can be directly observed. The secretory pathway has been dissected using several mutant screens, as outlined in the introduction. The completion of the yeast genome sequence made it possible to generate deletion mutants for all 6000 yeast genes and subject them to different phenotypic tests. One set of deletion mutants was generated as part of the Eurofan II gene deletion project, where 631 uncharacterised ORF were deleted. We set out to test if drugs, known to disturb intracellular transport, could be used to identify new components of the transport machinery. We choose to test the strains for sensitivity of the fungal metabolite brefeldin A (BFA) which blocks transport from ER to Golgi, and to the carboxylic ionophore monensin, which incorporates in membranes and acts as a Na^+/H^+ exchanger. Monensin treatment of mammalian cells results in block of secretion and an enlargement of *trans*-Golgi can be observed (Dinter & Berger, 1998). The mechanism of action of BFA has recently been elucidated. The targets of BFA are the ARF guanine exchange factors Sec7p, Gea1p and Gea2p. ARF proteins belong, as the rab GTPases do, to the ras super-family of GTPases, and Arf1p-GTP is involved in both COPI complex and clathrin coat formation (Kirchhausen, 2000). BFA binds to its target when it is complexed with ARF-GDP in a non-competitive manner and depletes the cell of active ARF-GTP complexes (Peyroche *et al.*, 1999).

A number of genes were identified as being sensitive for either drug in the screen, several of which were already known to be involved in vesicular transport. Two of these genes were *TLG2*, which codes for a syntaxin involved in endocytosis and recycling (Seron *et al.*, 1998), and *RCY1* which codes for an F-box proteins that has been shown to be involved in the recycling of Snc1p from the early endosome (Galan *et al.*, 2001).

Seven previously uncharacterised genes were named *BRE1-BRE5* and *MON1-MON2*. These genes have subsequently been further characterised mainly by other investigators.

A deletion of *MON1* impairs import into the vacuole of aminopeptidase I, ApeI, a marker protein for the cvt-pathway during rich growth conditions, and for the autophagocytic pathway during restricted growth conditions (Meiling-Wesse *et*

al., 2002). Mon1p interacts with Ccz1p; moreover a *ccz1* deletion mutant has a similar phenotype to a *mon1* deletion, and the complex seems to be required for delivery of proteins to the vacuole by several distinct pathways (Wang *et al.*, 2002). *MON2* was also identified in another screen that was aimed at identifying mutants defective in vacuolar protein sorting (Avaro *et al.*, 2002). *BRE1* has been shown to encode a ubiquitine ligase, which is required for ubiquitination of histone H2B (Hwang *et al.*, 2003; Wood *et al.*, 2003). *BRE2* encodes a subunit of the Set1 complex, Set1C, proposed to be a H4 lysine-4 methyltransferese (Roguev *et al.*, 2001). *BRE3* is identical to *LEM3*, and Lem3p was shown to be involved in the uptake of alkylphophocholine drugs, which may explain why it appeared in our drug sensitivity screen (Hanson *et al.*, 2003). A *BRE5* deletion, finally, was recently shown to be defective in ER to Golgi transport, and also had a delayed processing of CPY and of the plasma membrane protein Gas1p. Bre5p was shown to be required for proper function of Ubp3p, which is involved de-ubiquititnation. In particular, Ubp3p de-ubiquitinates Sec23p, a COPII component, thus providing a link between Bre5p function and intracellular transport (Cohen *et al.*, 2003).

Paper II: Characterization of temperature-sensitive mutations in the yeast syntaxin 1 homologues Sso1p and Sso2p and evidence for a distinct function for Sso1p in sporulation

The *SSO1* and *SSO2* genes were identified as multicopy suppressors of the *sec1-1* mutation, and are homologues of the human plasma membrane syntaxin 1a. Their interaction with genes in the late secretory pathway was investigated by overexpression in secretory mutants (Aalto, Ronne & Keränen, 1993). A third multicopy suppressor of *sec1-1* cloned with the same approach, *MSO1*, showed no homology to the *SSO* genes or to any other gene in the yeast genome, but interacted directly with Sec1p (Aalto *et al.*, 1997). We generated a temperature sensitive mutant allele of *SSO2* by shuttle mutagenesis. The mutant allele has a base substitution that cause an exchange of an arginine at position 200 to lysine. This arginine residue is conserved in almost all syntaxins and would be located in a –8 layer in centre of the SNARE bundle, if one extends the modelling described in (Fasshauer *et al.*, 1998) beyond layer –7. The temperature-sensitive *sso2-1* phenotype is seen only when *SSO1* is deleted, in which case growth is restricted at 30° and higher temperatures. However, already at the permissive temperature the cells have wider bud necks, accumulate vesicles and frequently fail to form a septum between mother and daughter cell. The corresponding mutation in Sso1p, R196K, was introduced by site-directed mutagenesis and resulted in the allele *sso1-1*. An *sso1-1 sso2Δ* strain exhibits the same phenotypes as a *sso1Δ sso2-1* strain, but to a lesser degree. The restrictive temperature is 36°C, and *sso1-1* cells grown at the restrictive temperature exhibit the same morphological phenotypes as *sso2-1* cells at permissive temperature. In contrast, *sso1-1* cells have almost no phenotype at permissive temperatures. The same pattern is seen with secretion of Hsp150p, where the effect of the *sso1-1* mutation is less severe than that of *sso2-1*. There is also a synthetic effect in *SSO1 sso2 mso1* cells that is not seen in *sso1 SSO2 mso1* cells. It thus appears that *SSO1* cannot fully support cell growth in absence of *MSO1*, while *SSO2* is not dependent on *MSO1*.

The most striking difference between the *SSO* genes is, however, our finding that *SSO1,* like *MSO1*, is required for sporulation while *SSO2* is dispensable for sporulation. We established that this difference was not due to differences in transcription, as *SSO1* expressed from the *SSO2* promotor was able to complement the sporulation deficiency, while *SSO2* did not suppress the sporulation deficiency when expressed from the *SSO1* promotor. Overexpression of *SSO2*, but of no other plasma membrane SNARE component, from a 2μ-plasmid did however result in a slightly restored sporulation.

Paper III: Mapping of sporulation-specific functions in the yeast syntaxin *SSO1* gene

The aim of this study was to characterise the nature of *sso1/sso1* sporulation defect and to determine which amino acids in Sso1p are specifically required for successful sporulation.

We could establish both by staining with DAPI, a fluorescent compound that binds to DNA, and by FACS analysis, that *sso1/sso1* cells proceed through both meiotic divisions and also separate the DNA into four distinct nuclei. However, progress beyond this point is blocked in the mutant strain, as there is no ascus formation at all.

The specific requirement for Sso1p during sporulation is striking since Sso1p and Sso2p are 74% identical at the amino acid level. Most of the differences are found in the N-terminal region. In contrast, there is only one non-conserved and two conserved differences between the two proteins within the SNARE motif. We used a PCR-based approach to make hybrids between the two genes where one part of the gene was replaced by the corresponding part from the other gene. The hybrid genes were cloned into *CEN*-plasmids and expressed from the *SSO1* promoter in diploid cells where both copies of *SSO1* had been deleted. All hybrids produced could complement the temperature-sensitive phenotypes of both *sso1-1* and *sso2-1* mutants during mitotic growth. The successive N-terminal replacement of Sso1p encoding sequences by Sso2p encoding sequences resulted in hybrids with successively decreased sporulation frequencies until Sso2p-123/120-Sso1p, which sustained 4% sporulation. Exchanges beyond that point did not further decrease the sporulation frequency. We could conclude that the N-terminal Ha and Hb helixes provide a function that is important for efficient sporulation but not completely necessary, nor could the N-terminus of Sso1p alone enable sporulation when transferred to Sso2p.

Surprisingly, further experiments revealed an absolute requirement for the 3' untranslated region of the *SSO1* gene in sporulation. One possible explanation of this unexpected finding would be if the 3' untranslated region of *SSO1* confers increased expression of the gene during meiosis. We therefore proceeded to investigate if the Sso1p and Sso2p proteins are differently expressed during sporulation. These experiments were performed in the SK1 genetic background, which proceeds through sporulation rapidly and synchronously, usually within 12h (Padmore, Cao & Kleckner, 1991). However, we found no evidence that Sso1p is preferentially expressed during sporulation. In contrast, the protein levels of both Sso1p and Sso2p remained relatively constant during sporulation.

This dependency on the 3' UTR for function has not been described previously for any secretory pathway protein, but it is not unknown that the 3'UTR can have various effects both on translation and localization of the gene product. In (Kuersten & Goodwin, 2003), the authors discuss 3' UTR dependent translational and spatial localisation of mRNA in *Drosophila* and *C. elegans*. In yeast, the localisation of Ash1p to daughter cells is mediated by the interaction between the *ASH1* 3' UTR and the myosin V She1p (Takizawa *et al.*, 1997; Chartrand *et al.*, 1999; Gonzalez *et al.*, 1999). The mechanisms of mRNA transport and advantages of transporting mRNA instead of the protein have been discussed by (Jansen, 2001). Asymmetric distribution of mRNA may be achieved through active and directed transport, as in the case of the *ASH1* mRNA, through local stabilisation of mRNA or through local immobilisation of diffusing mRNA. In the case of *SSO1,* we do not know if mRNA localization is involved, but immunoblots of lysate from cells in the sporulating culture showed that the Sso1-Sso2 hybrid proteins, expressed with the *SSO2* 3' UTR, were as abundant after 60h of sporulation as Sso1p expressed with the *SSO1* 3' UTR. This at least indicates that the *SSO1* 3' UTR does not affect protein stability.

Paper IV: Characterisation of the role of yeast plasma membrane syntaxin Sso1p in sporulation

The other gene coding for a SNARE protein known to be required for sporulation, Spo20p, has a null mutant phenotype that has been characterised by electron microscopy (Neiman, 1998). Mutant *spo20* cells initiate formation of prospore membranes, but instead of enclosing the haploid nuclei, the membranes extend into narrow tubes that exclude the cytoplasm and also may exclude the nuclei. A similar phenotype is also seen in an *ady4/ady4* deletion mutant strain, as reported in (Nickas ME, 2003), that identified Ady4p as a component of the meiotic SPB.
Electron microscopy of *sso1/sso1* cells during sporulation showed that formation of prospore membranes was not initiated at all. Vesicles accumulated at the meiotic plaque, but they were not able to fuse into a continuos membrane, indicating that initiation of the membrane fusion process had failed. Interestingly, this phenotype has also been observed in a *mso1/mso1* strain (Jäntti et al*., unpublished results*) suggesting that the two proteins may interact directly during prospore membrane formation.
We proceeded to use overexpression of *SEC1, SSO1, SSO2* and *SPO20* from high copy number plasmids to study their ability to suppress or complement the sporulation deficiency of *sso1* and *spo20* diploid mutants. We found that *SEC1* suppresses the *spo20* mutation slightly, but not the *sso1* mutation, while *SSO2* suppresses *sso1* slightly but not *spo20*. As expected, *SPO20* complements *spo20* but results in no sporulation in *sso1* mutant. Overexpression of *SSO1* resulted in a slight suppression of *spo20*, but also complemented *sso1* poorly. The reason for this is unclear. It was not a dominant negative effect, since *SSO1* strains transformed with the same overexpression construct sporulated well, and immunoblot analysis showed that the protein was present at higher than normal levels. We also noted that expression of *SSO1* from the 2μ construct did not complement the *sso2-1* phenotype completely, which the same insert expressed from a *CEN*-plasmid did. It is conceivable that this reflects a situation similar to

that observed in the *Arabidopsis knolle* mutant, where overexpression of the cDNA from a strong promoter was not sufficient to complement the mutant

The intracellular distribution of the Sso proteins was examined using N-terminal fusions to a green fluorescent protein (GFP), expressed from an inducible promotor. The constructs complemented the *sso1-1* and *sso2-1* phenotypes, but the GFP-Sso1p construct failed to complement the *sso1* sporulation phenotype. The latter was to be expected, since these constructs did not use the *SSO1* 3'UTR, which we found to be necessary for sporulation, but the *CYC1*-terminator. We found that both GFP-Sso1p and GFP-Sso2p localize to the membranes in mitotic as well as meiotic cells, though a significant accumulation of GFP-SSo1p in the cytoplasm also was observed. Significantly, GFP-Sso2p localised to the periphery of the spores during sporulation, indicating that Sso2p is not excluded from the prospore membrane. This rules out one possible explanation why Sso1p is specifically needed during sporulation.

Conclusions and future perspectives

In Paper I we set out to identify new proteins involved in intracellular transport by treating the cells with drugs that are known to disturb protein transport. The method was successful, and of the new genes identified, *MON1*, *MON2* and *BRE5* have subsequently been confirmed to be involved at different stages in the secretory pathway.

The major part of this thesis work was about functional characterisation of the yeast plasma membrane syntaxins Sso1p and Sso2p. The initial work on the *SSO1* and *SSO2* genes indicated that they are functionally redundant, but together their function in Golgi to plasma membrane transport is essential. Mapping of *SSO* genetic interactions using conditional mutants showed the *SSO1* function is partially dependent on *MSO1*. Thus, in the presence of *MSO1,* the phenotype of the *sso1-1* mutation seems to be milder than that of the *sso2-1* mutation, which is defective in cytokinesis at the permissive temperature. The most interesting observation in this context was that both *MSO1* and *SSO1* are essential for sporulation, while *SSO2* has no role in sporulation. It has previously been reported that Sec1p is required for the spore formation (Neiman, 1998) which opens an interesting possibility. As Sso1p requires Mso1p, which is known to interact with Sec1p, for growth at elevated temperatures and they are both also needed for sporulation, it could be that Mso1p interacts with Sec1p in a manner that gives Sec1p a preference for Sso1p rather than Sso2p.

The domain swap experiments with Sso1p and Sso2p showed that the two N-terminal regulatory helices of Sso1p are required for efficient sporulation. However, they are not essential for sporulation and they do not alone confer an ability to support sporulation when transferred to Sso2p. Unexpectedly, we instead found that the *SSO1* 3'UTR is both necessary and sufficient for Sso protein function during sporulation.

Electron microscopy on *sso1/sso1* cells showed that without *SSO1,* prospore membrane synthesis at the meiotic plaque is not initiated. A comparison with the phenotype seen in a *spo20/spo20* strain, where prospore membrane synthesis does initiate, suggests that Sso1p and Spo20p may function at somewhat different stages in the process.

In conclusion, the specific function of Sso1p in sporulation underscores the importance of membrane fusions in eukaryotic cell development. Spore formation may serve as a model for other forms of cytokinesis that is initiated inside the cell, such as plant cytokinesis and spermatogenesis.

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