Protein sorting and cell surface polarity in yeast

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1. Abstract

The studies presented here were focused on the understanding of the principles for protein sorting from the Golgi to the cell surface. As a marker protein we used Fus1p, a type I plasma membrane protein that is O-glycosylated on the extracellular domain and plays a role in cell fusion during yeast mating. Additionally, we analyzed mechanisms responsible for asymmetric distribution of Fus1p in mating cells.

We demonstrated that the glycans attached to the protein act as a sorting determinant for protein transport to the cell surface. In cells lacking *PMT4*, encoding a mannosyltransferase involved in the initial step of O-glycosylation, Fus1p was not glycosylated and accumulated in late Golgi structures. A similar defect in exocytosis was observed when a Fus1p mutant lacking the O-glycosylated domain was expressed in wild-type cells, however, the cell surface delivery could be rescued if the 33 amino acid portion of the Fus1p ectodomain, containing 15 potentially glycosylated sites was added to the protein.

It was previously well documented in epithelial cells that different types of protein glycosylation and association with lipid rafts play a role of determinants for protein delivery to the apical plasma membrane. However, otherwise the machinery responsible for cargo sorting to the apical membrane is poorly understood. Our finding that also in yeast, protein glycosylation can function as a sorting determinant provides a new possibility to investigate underlying mechanisms.

Despite a number of screens done in yeast for the identification of genes involved in secretion (SEC genes), very little is known about the machinery for cargo sorting to Golgi-derived exocytic vesicles. It was demonstrated that yeast cells, similarly to mammalian cells have two pathways from the Golgi to the cell surface. In mutants that block one pathway, cargo is delivered to the cell surface via the remaining transport route. Existence of parallel and redundant pathways to the cell surface complicates the identification of the machinery responsible for cargo sorting. We developed a new, genome-wide screening strategy that allows for the

identification of mild transport defects expected when only one of the bifurcated exocytic pathways is affected. We introduced a plasmid expressing the chimeric Fus-Mid, our model cargo protein fused to the green fluorescent protein (GFP), to the yeast mutant array, comprising 4848 deletion strains covering in total 3/4 of the yeast genome. We used microscopy analysis to search for mutants with abnormal localization of Fus-Mid. This screen revealed a requirement of several enzymes regulating the synthesis of raft lipids (sphingolipids and ergosterol) for correct and efficient delivery of the marker protein to the cell surface. Additionally, we identified mutants of the cytoskeleton, several unknown genes and known membrane traffic regulators, Kes1p, Rvs161p and Chs5p. Because our marker protein was partially transported to the vacuole we were able to identify genes involved in Golgi to vacuole transport. Interestingly, we found that vacuolar sorting is abolished in several mutants that block N-glycosylation. These observations point to the possibility that N-glycosylation plays an important role in organization of the transport from the Golgi to the vacuole. The exact mechanism, however, remains to be investigated.
The approach presented here provides a new tool that can now be used for further screens using different cargoes of secretory vesicles, with the goal for the systematic genome-wide search for regulators of post-Golgi trafficking.

Yeast mating cells provide a useful paradigm to understand mechanisms for cell surface polarization. Upon pheromone stimulation, cells become polarized and localize a specific subset of proteins involved in signal trunsduction, cell adhesion and fusion to the mating projection in order to facilitate cell fusion. Two different models were proposed for Fus1p localization to the tip of the mating projection. One model is based on clustering of raft components, another model that was recently put forward, employs an ongoing cycle of endocytosis and polarized delivery of membrane components. We recapitulated the mechanism by which Fus1p is polarized on the cell surface and demonstrated that Fus1p, unlike some other proteins, localizes to the mating projection independently of endocytic recycling. Instead, Fus1p was retained at the mating projection through its cytoplasmic domain that interacted with multi-protein caffolding machinery. Our studies clearly demonstrated that different

mechanisms operate independently to polarize membrane components on the surface of yeast cells during mating.

2. Introduction

2.1. Cell polarity and asymmetry of cell surface

Most eukaryotic cells exhibit some kind of cell polarization that often is necessary for proper function of the cell in its tissue context. Neurons collect signals from neighboring cells on the dendrites and cell body and transfer the impulse to the receiving cells through the long axonal process. Cells that migrate like fibroblasts, activated neutrophils or metastatic cancer cells have usually distinctly organized leading and rear edges of the cells. Specific clustering of membrane components occurs during the immunological synapse formation in activated lymphocytes. Also yeast cells are polarized during cell replication and mating.

One of the most interesting issues in cell polarity is the polarization of the cell surface. Epithelial cells provide a great paradigm to study segregation of plasma membrane components. These specialized cells form monolayers that cover the lumen of many organs including lung, kidney, intestine and pancreas and function as a barrier between the lumen of the organ and underlying tissues (Lubarsky and Krasnow, 2003). To fulfill these functions epithelial cells polarize their surface to an apical membrane facing the lumen and a basolateral domain contacting neighboring cells or the extracellular matrix. These two membrane domains are separated by the tight junction that provides a diffusion barrier for apical and basolateral membrane components (Rodriguez-Boulan et al., 2005).

The apical membrane works as a protective shield for the cell exposed to digestive enzymes and osmotic pressure present in the lumen of organs (Lubarsky and Krasnow, 2003). The apical membrane is believed to be especially robust due to its enrichment in sphingolipids (Simons and van Meer, 1988) that together with cholesterol form tightly packed microdomaines - lipid rafts (Simons and Ikonen, 1997). The apical membrane is also specialized for secretion of many proteins needed for the organ function. The basolateral membrane facing neighboring cells is a place of cell-cell contacts and secretion of molecules absorbed from the lumen of organs (Rodriguez-Boulan et al., 2005).

2.2. Organization of epithelial cell polarity – cargo sorting and polarized exocytosis

The organization of these functionally different domains on the plasma membrane of epithelial cells is achieved by specialized sorting of lipids and proteins and polarized exocytosis to the apical and basolateral domains (Fig. 1). In some other cell types cell surface polarization requires polarized surface delivery but is often independent of cargo sorting (Fig. 1).

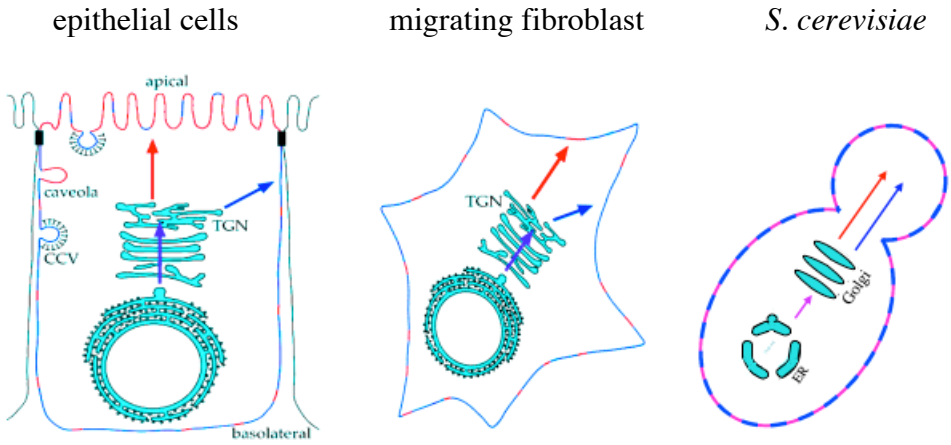


Figure 1. Polarization of the epithelial cells requires cargo sorting to the apical (red) and basolateral (blue) pathways. In other eukaryotic cells also two different exocytic routes were found. Although polarization of the cell often requires polarized exocytosis (like in yeast, picture on the right) it does not depend on cargo sorting into different pathways. In yeast, both exocytic pathways are targeted to the site of polarized growth (discussed later in the text).

2.3. Biosynthetic cargo is sorted in the TGN and in endosomes

The trans Golgi network (TGN) is recognized as a major hub for sorting of biosynthetic cargo (Keller and Simons, 1997; Traub and Kornfeld, 1997). Recently, it was demonstrated that sorting can also take place in endosomal compartments (Traub and Apodaca, 2003). Using video microscopy, immunoelectron microscopy and cell fractionation, Ang at al. showed that the basolateral membrane cargo vesicular stomatitis virus glycoprotein G (VSV-G) was passing through the recycling endosome on its way from the Golgi to the plasma membrane (Ang et al., 2004). In addition, enzymatic inactivation of recycling endosomes lead to intracellular accumulation of VSV-G (Ang et al., 2004). Similar observations were obtained when an apically

sorted mutant of VSV-G was used. These results suggest that the biosynthetic cargoes that have to reach different destinations might use recycling endosomes as an intermediate compartment on their way to the cell surface, and that sorting to different pathways occurs not only in the TGN but also in endosomes.

2.4. Determinants for protein sorting to the apical and basolateral pathways

Different determinants on proteins can mediate their sorting to the apical and basolateral plasma membrane. With some exceptions, basolateral sorting signals are present on the cytoplasmic tail of the protein, while apical transport is mediated by determinants in the extracellular or transmembrane domain of the protein (see Table 1).

Table 1. Determinants for protein sorting to the apical and basolateral membranes.

Site	Determinant	Polarity	Examples*
Cytoplasmic domain	Tyrosine-based motif	Basolateral	LDL receptor, transferrin receptor, vesicular stomatitis virus glycoprotein
	Dileucine motif	Basolateral	IgG Fc receptor, E-cadherin
	PDZ-binding motif	Apical/basolateral	CFTR (a), BGT-1 GABA transporter (bl), ERbB-2 receptor tyrosine kinase (bl)
	Others	Apical/basolateral	H,K-ATPase α-subunit (a), megalin (a), GAT-2 GABA transporter (bl)
Membrane anchor	Transmembrane domain	Apical	Influenza virus hemagglutinin and neuraminidase
	GPI-anchor	Apical	Placental alkaline phosphatase, decay-accelerating factor, Thy-1
Extracellular domain	*N*-glycosylation	Apical	Occludin (truncated version), FcLR (Fc receptor-LDL receptor chimera), GLYT2 glycine transporter
	O-glycosylation	Apical	Neurotrophin receptor, sucrase isomaltase
	Oligomerization domain?	Apical?	Kv1 potassium channel (neurons)

*a, apical; bl, basolateral.

Adopted from Schuck and Simons 2004

2.4.1. Sorting to the basolateral membrane

Proteins sorted basolaterally usually contain a tyrosine-based motif or a di-leucine motif, which are recognized by the adaptor protein complex AP-1B (Sugimoto et al., 2002). The family of heterotetrameric adaptor protein (AP) complexes is involved in cargo sorting and vesicle formation at different sites in the cell. AP-1B, the closely related AP-1A, AP-3 and AP-4 mediate sorting events at the level of the TGN and endosomes, whereas AP-2 functions in endocytic clathrin coated vesicle formation at the plasma membrane (Simmen et al., 2002). The AP-1B complex is specifically expressed in some epithelial cells (Ohno et al., 1999) and was localized to the Golgi and recycling endosomes, supporting the role of endosomes in basolateral delivery (Ang et al., 2004). Each complex consists of two large subunits (β and γ), a medium

subunit (μ) and a small subunit (σ). The β subunit is responsible for the interaction with clathrin, whereas the μ subunit binds to selected cargo molecules (Traub and Apodaca, 2003). Interestingly, the LLC-PK1 kidney cells which do not express μ1B (component of AP-1B), missort several basolateral proteins to the apical membrane (Folsch et al., 1999). It was demonstrated that also AP-4 binds to basolateral cargo via its μ4 subunit and plays a role in protein sorting to the basolateral membrane (Simmen et al., 2002).

2.4.2. Sorting to the apical membrane

Much less is known about mechanisms that regulate apical exocytosis. It was postulated that association with lipid rafts and different types of protein glycosylation determine sorting to the apical plasma membrane.

2.4.2.1. Lipid rafts in apical sorting

It was proposed that liquid ordered microdomains, rafts, floating within the membrane plane, serve as transport platforms for apical sorting (Simons and Ikonen, 1997). This hypothesis was based on the observation that the apical plasma membrane is enriched in glycosphingolipids (Simons and van Meer, 1988). Further support for this postulation comes from the observation that apical sorting is particularly sensitive to depletion of cholesterol and sphingolipids (Hansen et al., 2000; Keller and Simons, 1998; Lipardi et al., 2000; Mays et al., 1995). Thus, proteins and lipids that associate with rafts at the TGN would be separated from the rest of the membrane and transported apically.

2.4.2.1.2. Detergent resistant membranes and lipid rafts

The most used method to analyze raft association is isolation of detergent resistant membranes (DRMs). Sphingolipids and cholesterol in rafts are more densely packed than lipids in a non-raft environment and are resistant to extraction with non-ionic detergents like Triton X-100 or Chaps at 4°C (Schuck et al., 2003). DRMs can be separated from the solubilized membrane components in a gradient centrifugation

(Schuck et al., 2003). However, the solubilization of membranes with detergent might trigger clustering of the insoluble membrane components. Therefore, DRMs do not reflect exactly the molecular composition of lipid rafts in live cells, but only demonstrate a tendency for proteins and lipids to partition into rafts (Munro, 2003; Schuck et al., 2003).

2.4.2.1.3. Determinants for proteins to associate with lipid raft

Several determinants were described to facilitate protein association with rafts and apical transport.

2.4.2.1.3.1. Extracellular leaflet.

GPI-anchored proteins generally associate with rafts on the basis of the favorable packing of the GPI anchor into the more ordered raft phase (Brown and Rose, 1992). However, GPI anchors are very diverse and raft association might be different for various GPI anchored proteins (Benting et al., 1999; Mayor and Riezman, 2004). It was shown that glycosylation of GPI-anchored proteins might determine apical sorting (Benting et al., 1999). Raft association is often mediated by the transmembrane domain (TMD). It is not clear which properties of the TMD mediate partitioning of proteins into lipid rafts. The DRM association of influenza virus hemagglutinin (HA) was shown to depend critically on amino acids in the transmembrane domain facing the outer leaflet of the bilayer (Scheiffele et al., 1997). The TMD can also be responsible for lipid-protein interactions that could lead to protein partitioning into rafts, possibly by exerting some conformational changes in the protein (Simons and Vaz, 2004).

2.4.2.1.3.2. Length of TMD, thickness of membranes and rafts

It was postulated that the length of the TMD could determine the partitioning of proteins into lipid rafts, as well as their intracellular localization (Munro, 1991; Munro, 1995). ER/Golgi resident proteins have a TMD that is about five amino acids shorter than that of plasma membrane proteins (Bretscher and Munro, 1993). Munro

has demonstrated that artificial lengthening of the TMD can lead to missorting of proteins from the Golgi to the cell surface (Munro, 1995). This finding was in agreement with observations from electron microscopy studies, indicating that the thickness of the lipid bilayer increases along the secretory pathway in non-polarized plant cells (Sandelius et al., 1986). Interestingly, the thickness of the bilayer containing raft lipids enriched at the plasma membrane is higher than the non-raft environment. It was proposed that proteins are sorted to different compartments according to "hydrophobic matching" and localized to the compartments where exposure of lipophilic amino acids out of the membrane is minimal (Mouritsen and Bloom, 1984; Munro, 1991; Munro, 1995). Recent analysis of membrane purified from polarized rat hepatocytes, using a very sensitive solution x-ray scattering (SXS) technique, revealed that the thickness of the lipid bilayer indeed increases from the ER to the Golgi, 37.5 +/- 0.4 A and 39.5 +/- 0.4 A, respectively. However, there is a significant difference in thickness between the apical and basolateral plasmamembrane, 35.6 +/- 0.6 A and 42.5 +/- 0.3 A, respectively (Mitra et al., 2004). The fact that the apical membrane is the thickest is in agreement with the observation that this domain is very enriched in raft lipids. It is unexpected however, that the basolateral membrane is even thinner than the ER membrane. This could encourage to assumption that proteins with longer TMDs associate with lipid rafts and are sorted apically while proteins with shorter TMDs localize to the basolateral side. Such an assumption is however not valid because no difference in the length of TMDs for apical and basolateral proteins has been observed. It is still not clear, how basolateral proteins with relatively long TMDs are incorporated into the thin ER membrane (37,5 ± 0,4 A)**,** then are transported to the Golgi where the membrane is thicker than in the ER (39,5 ± 0,4 A), and finally are placed in the thinnest the basolateral membrane (35,6 ± 0,6 A) (Mitra et al., 2004). It is possible that proteins (or protein complexes) localy increase the membrane thickness. Alternatively, a protein with a long TMD could tilt in the membrane or oligomerize to reduce the hydrophobic surface exposed on the outside of the membrane. Interestingly, protein oligomerization was reported to influence association with lipid rafts and intracellular sorting (Bagnat et al., 2001; Lee et al., 2002). It must be added that the thickness of the ER, Golgi and basolateral membrane but not of the apical membrane was dependent on proteins but not on cholesterol concentration. When purified membranes were treated with proteases, the

differences in thickness of lipid bilayers were significantly smaller (Mitra et al., 2004). This observation suggests that proteins influence bilayer thickness of ER, Golgi and basolateral membranes but the thickness of the apical plasma membrane is solely based on its lipid composition.

2.4.2.1.3.3. Cytoplasmic leaflet

Some cytoplasmic proteins are found in the DRM fraction and are thus thought to be associated with raft domains via the cytoplasmic leaflet of the lipid bilayer. These include several signaling molecules such as G subunits of heterotrimeric G proteins or the src-like protein tyrosine kinases lck, fyn, and lyn that depend on multiple acylation for DRM association (Mumby, 1997; Rodgers et al., 1994; Shenoy-Scaria et al., 1994; Wolven et al., 1997).

2.4.2.2. Protein glycosylation in apical delivery

There are many reports that different types of protein glycosylation, O-glycosylation (Remacle-Bonnet et al., 1995; Spodsberg et al., 2001; Yeaman et al., 1997) and N-glycosylation (Fiedler and Simons, 1995; Scheiffele et al., 1995), could function as sorting determinants for apical delivery. This was well illustrated for the rat growth hormone. This soluble protein was secreted from both, apical and basolateral, surfaces when unglycosylated. However, when a signal for N-glycosylation was added to the protein, it was secreted predominantly from the apical side (Scheiffele et al., 1995).

2.4.3. Hierarchy of sorting signals

Obviously, glycosylated proteins and rafts, i.e. caveolae, are also transported to the basolateral membrane. So how does this fit with their role in apical sorting? Many proteins contain more than one sorting signal. Signals for basolateral delivery are usually dominant over the apical determinants and proteins are sorted apically when the basolateral determinant is missing. This was well illustrated in studies on the sorting of rat growth hormone. As described above, the secretion pattern of this soluble protein is changed from random to apical when the protein is glycosylated (Scheiffele et al., 1995). However, when the hormone was fused to the

transmembrane and cytoplasmic domain of the low-density lipoprotein (LDL) receptor, which has a strong basolateral determinant, the chimeric protein was transported to the basolateral membrane regardless of its glycosylation status. When the basolateral sorting signal on the cytoplasmic tail was mutated, the glycosylated protein was sorted apically again. When all determinants for surface delivery were removed, the growth hormone-LDL construct was accumulated in the TGN (Benting et al., 1999).

2.4.4. Clustering of lipid rafts as a sorting principle

The observation that the apical plasma membrane is enriched in rafts and that protein glycosylation is important for apical sorting, led to the hypothesis that glycosylated proteins can be bound by lectins that are able to oligomerize and cluster rafts with associated lipids and proteins into apical sorting platforms (Fig. 2) (Simons and Ikonen, 1997).

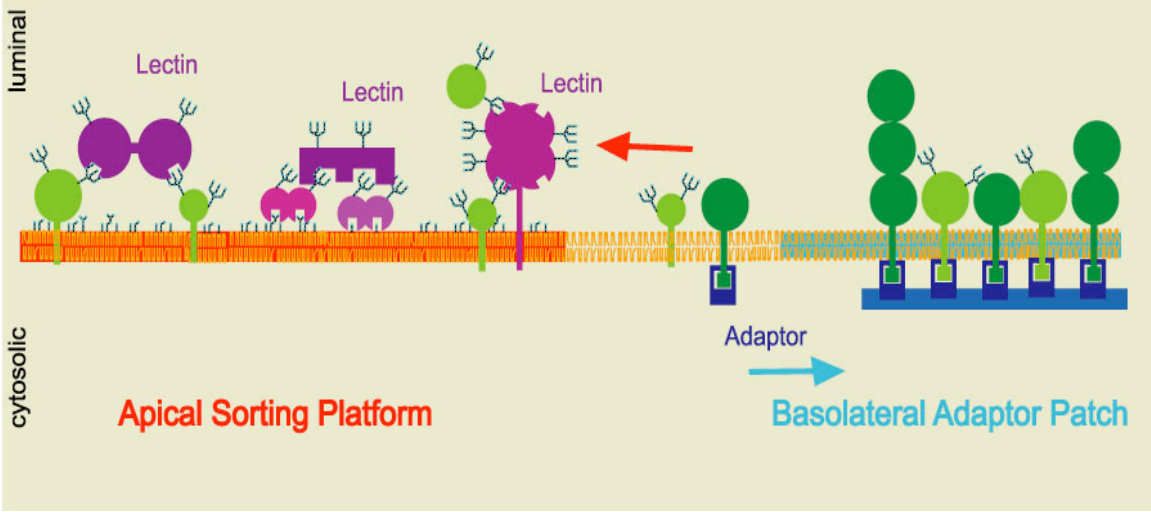


Figure 2. Lectins cluster glycoproteins for apical sorting. It was postulated that there is a family of lectins that would be crucial for establishing an apical sorting platform. Multivalent lectins would crosslink different apical cargo proteins and glycolipids. This clustering would stabilize weak individual interactions between proteins and lipid microdomains. The lectins are likely glycoproteins, and by binding also to each other they would create a lattice of glycoproteins and lectins. Another prediction is that the lectins would have an affinity for raft microdomains. Glycoproteins containing a basolateral sorting determinant would be sequestered away from the apical lectin raft platform with the help of adaptor proteins (adopted from Fullekrug and Simons 2004).

Despite numerous attempts, a lectin responsible for sorting at the TGN could not be identified. Two additional models for the sorting of glycosylated proteins were proposed. Rodriguez-Boulan and Gonzalez (Rodriguez-Boulan and Gonzalez, 1999) have suggested that glycans change the biophysical properties of an apical protein such that the presentation of a proteinaceous sorting signal to a hypothetical sorting receptor is facilitated. Alternatively, the glycans contribute to a transport-permissive conformation of the apical protein that facilitates its incorporation into lipid rafts and thus into the apical pathway. It is interesting that still, 10 years after first reports that glycans serve as a sorting determinants, the underlying molecular mechanism remains unknown. It should be added that clustering of rafts can be triggered by many different protein-protein interactions and are not only governed by interactions with carbohydrates.

2.4.5. Clustering of lipid rafts as a mechanism for vesicle formation

Clustering of lipid rafts could also provide a mechanism for vesicle formation.

When a liquid ordered (raft) phase coexists with a liquid disordered phase, line tension is generated on the boundary between these two phases (Schuck and Simons, 2004). Line tension is the two-dimensional equivalent of surface tension and arises from the immiscibility of membrane components that prefer different phases. Importantly, the line tension increases with the size of the domains and the length of the boundaries between domains in the membrane. It was initially postulated on theoretical grounds and recently proven in studies on model membranes that when the line tension increases, the minority phase present as isolated domains buds out and eventually pinches off from the rest of the membrane (Baumgart et al., 2003). Therefore, clustering of lipid rafts could provide the energy necessary to form a transport vesicle. Interestingly, it was demonstrated that membranes containing different sterols form different types of membrane curvature and the budding vesicles have different shapes and sizes (Bacia et al., 2005). Roux et al., showed recently that the lipid composition is significantly different in tubes and vesicles budding from giant unilamellar vesicles (GUVs) made of either purely phospholipidic membrane or of biological membranes (Roux et al., 2005). Tubes emerging from the Golgi were also reported to exist in living cells (Trucco et al., 2004).

2.5. MDCK is a difficult model to study – limited tools

Although epithelial cells serve as a very interesting model to study cell polarization and sorting and transport of cargo to distinct domains on the cell surface, this system has several limitations. Polarized MDCK cells are difficult to transfect with standard methods. One efficient method to introduce genetic material into these cells is infection with recombinant viruses, the preparation of which is time consuming and side effects from virus infections could complicate the interpretation of the experiments. The recent progress in RNAi technology allowed the inactivation of genes but this effect is transient, does not work equally well for all genes, and often optimization of conditions is required (Schuck et al., 2004). Studies of gene function in mammalian cells is additionally complicated by the fact that many genes are duplicated. Mammalian cells have about 30, 000 genes, of which many are expressed in different forms like splice-variants, products of alternative transcription or translation. These modifications significantly complicate studies on gene function in epithelial cells.

2.6. Yeast as an easy experimental model

The budding yeast *Saccharomyces cerevisiae* is a simple and powerful model to study various aspects of cell biology. This unicellular organism is easy to culture and has a relatively small genome comprising of about 6000 genes, 4500 of which are non-essential for cell viability. The ability of yeast cells to perform homologous recombination allows various genetic manipulations including precise gene deletion and the introduction of fusion constructs into the genome. The commercially available library of deletion mutants covering all non-essential genes greatly contributes to the studies of gene function. These features all together have allowed for a number of genetic screens that have identified the function of many genes involved in different cellular processes.

Budding yeast is especially suitable for studying membrane trafficking. The isolation of temperature sensitive mutant alleles of genes essential in membrane transport allowed for conditional blocking of intracellular traffic at different stages in the exocytic and endocytic pathways. In particular, temperature sensitive mutants of the exocyst components allowed for intracellular accumulation of Golgi-derived exocytic

vesicles – a great prerequisite for the purification and characterization of the secretory vesicles. (Potenza et al., 1992).

2.7. Organization of exocytic and endocytic compartments in yeast.

The organization of the exocytic and endocytic pathways in yeast is of particular interest. Several pathways connecting different compartments have been proposed but their existence and organization have not been well documented. Knowledge about transport routes from the Golgi to the endosome is also important for understanding how the secretory pathways are organized, since one branch of the exocytic pathways was proposed to overlap with endosomal transport (Gurunathan et al., 2002; Harsay and Schekman, 2002).
In the next chapters I will summarize existing interpretations of the organization of intracellular trafficking in yeast.

2.7.1. Organelle identity and transport specificity

The correct localization of proteins and lipids to different cellular compartments is important for the proper function of the cell. The unique distribution of molecules requires maintenance of organelle identity, cargo sorting to the correct transport pathway, and finally specific vesicular fusion with the correct target compartment.

The organell identity is maintained on different levels. First, a concert of specific kinases and phosphatases facilitates a unique distribution of phosphatidylinositol-phosphates (PIPs). These lipids are present on the cytoplasmic leaflet of membranes on specific compartments, with PI(4)P marking the Golgi, PI(4,5)P2 the plasma membrane, PI(3)P the endosomes and PI(3,5)P2 the vacuole (Gary et al., 2002; Kihara et al., 2001; Stefan et al., 2002; Walch-Solimena and Novick, 1999). Additionally, transport specificity is regulated by Rab proteins and SNARE complexes (soluble N-ethylmaleimide-sensitive factor attachment protein receptor). Rab proteins are small GTPases that orchestrate interactions with effector proteins on specific membranes and facilitate the formation of vesicles or the fusion of vesicles with the target membrane (Zerial and McBride, 2001). In vesicle fusion with the

acceptor membrane, Rab proteins together with their efectors facilitate vesicle thethering, and the assemble of the SNARE complexes between vesicular v-SNAREs and partners present on the target membranes, t-SNAREs, provide the energy necessary for membrane fusion (Jahn et al., 2003). Originally, it was proposed that the SNARE complex consists simply of a t-SNARE and a partner v-SNARE (Rothman, 2002). This hypothesis was oversimplified. Currently it is known that four SNARE helices are involved, and that specific amino acids, glutamines (Q) and arginine (R), conserved in the helices are critical for complex formation. In the SNARE complex three helices contain glutamines (Qa, Qb and Qc) and one v-SNARE helix provides the arginine (R) (Jahn et al., 2003). The yeast genome contains eight t-SNAREs and their specific intracellular localization was determined. ER membranes contain Ufe1p, early Golgi is marked with Sed5p, late Golgi and early endosomes share Tlg1p and Tlg2p, the fusion of secretory vesicles with the plasma membrane is specified by Sso1p and Sso2p, late endosomes contain Pep12p, and the fusion with the vacuole is mediated by Vam3p.

The drawing of a general road map for intracellular transport is additionally complicated by the fact that target compartments can change their identity.

2.7.2. Dynamic nature of endocytic and exocytic compartments

As discussed above, organelle identity is specified by the presence of marker proteins and lipids. The localization of resident proteins is facilitated by targeted delivery to, retention in particular compartments or retrieval of molecules that were mislocalized to other compartments. It is well known that Golgi resident proteins must be constantly retrieved from the plasma membrane and endosomes in order to be enriched in Golgi structures. The late Golgi in yeast was defined as a compartment containing Kex2p, a protease involved in processing of the secreted protein alpha factor and other proteins (Redding et al., 1991). However, Kex2p to a certain degree leaks from the Golgi with vesicular transport to endosomes and the plasma membrane and must be retrieved back to the TGN (Wilsbach and Payne, 1993a; Wilsbach and Payne, 1993b). The block of retrieval pathways from endosomes to Golgi leads to mislocalization of Kex2p to the endosomes, and plasma membrane (Wilsbach and Payne, 1993a). The studies on Kex2p localization revealed that, at steady state, Kex2p

is also found on early endosomes (Brickner and Fuller, 1997) . Additionally, late Golgi and early endosomes are sharing the same t-SNARE, indicating that the same transport carriers directed to the Golgi could potentially also fuse with early endosomes (Holthuis et al., 1998). These altogether suggest that both organelles are very similar. It is believed that in *Saccharomyces cerevisiae* the early endosome is an intermediate state between the late Golgi and the *sensu stricto* endosome (late endosome, defined as a Pep12p positive compartment). Early endosomes, depleted of Golgi proteins, can mature to late endosomes, which can fuse with the vacuole (Pelham, 2002). Taking all these factors into account, it seems that the compartments in the secretory and endocytic pathways are dynamic and can mature into one another.

Also particular Golgi compartments appear to be able to change their identity from early to late Golgi elements. It is a subject of intensive debate whether the cargo traffic through the Golgi apparatus is organized by vesicular transport or by maturation of cisternae (in mammalian cells) or Golgi compartments in budding yeast (Golgi apparatus in *S. cerevisiae* is not organized in stacks but, instead, early, middle and late Golgi compartments are scattered throughout the cytoplasm). Evidence for both models of cargo traffic through the Golgi exists. According to the cisternal maturation model, the Golgi can disappear when transport is blocked and reappear *de novo* from the ER membrane (Bevis et al., 2002; Glick, 2002).
In *Pichia pastoris,* Golgi can be made de novo from ER membrane (Bevis et al., 2002; Glick, 2002) (Fig. 3). Francois Kepes and colleagues used electron microscopy to demonstrate that upon block of protein synthesis, Golgi compartments disappear in an ordered manner in *S. cerevisiae*. When the traffic from the ER was blocked, first early, then medial and finally late Golgi vanished and reappeared in the same order after release from the traffic block (Morin-Ganet et al., 2000). These observations fit well with the compartment maturation model shown in *Pichia pastoris* (Fig 3).

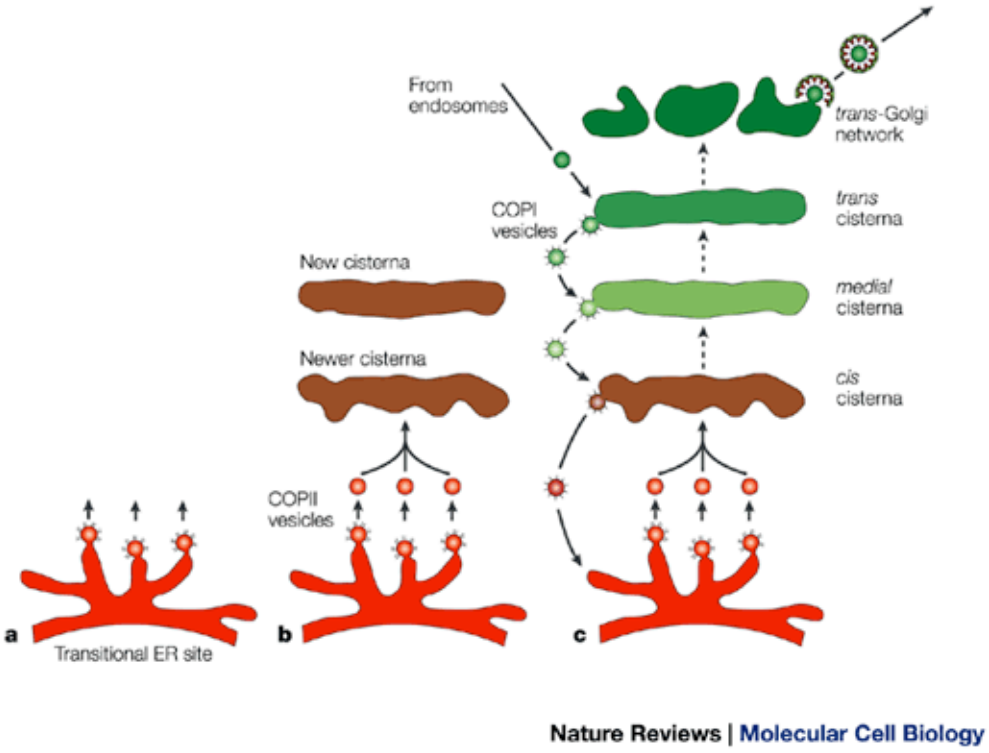


Figure 3. A hypothetical mechanism for de novo Golgi formation (adopted from B. Glick, 2002). (a) The process begins when a cell contains a transitional endoplasmic reticulum (tER) site that is not functionally connected to a Golgi stack. Such a situation might arise in several ways: a tER site itself could form de novo, as seems to happen in the yeast *Pichia pastoris* (B. J. Bevis and B. S. G., unpublished observations). (b) This tER site produces coatomer protein complex (COP)II vesicles that contain Golgi membrane proteins. The vesicles fuse homotypically to form a new cisterna. Once this cisterna is complete, a second cisterna is assembled. (c) Further rounds of COPII-vesicle budding produce more cisternae. These cisternae become polarized as a result of COPI-mediated retrograde transport between the cisternae and from the cisternae back to the ER. Meanwhile, peripheral proteins bind to the cisternae from the cytosol (not shown), and further membrane components are delivered by vesicles that are derived from later compartments, such as endosomes.

In contrast, fluorescence microscopy studies in *S. cerevisiae* showed that when ER export is blocked, only a subset of the Golgi proteins is redistributed to the ER, plasma membrane and endosomes, while leaving other Golgi proteins in punctate structures (Todorow et al., 2000; Wooding and Pelham, 1998). These experiments contradict the Morin-Ganet's observations and suggest that a complete and reversible disassembly of the Golgi is not possible in *S. cerevisiae*.

In support of the cisternal maturation model, mammalian cells were shown to produce secreted molecules like collagen, which are too big to be incorporated into vesicles

(Stephens and Pepperkok, 2002). It was demonstrated, however, that the Golgi stacks can be connected via a system of tubes, and that the formation of these tubes was driven by the production of secretory material (Trucco et al., 2004). This observation gives another explanation for the paradigm of transport of big molecules through the Golgi apparatus. It is likely that transport through the Golgi compartments is partially based on vesicular traffic, partially on tubes connecting cisternae (in mammalian cells), and partially on the maturation of cisternae. In conclusion, these results suggest that Golgi compartments can be dynamic and at least in some organisms can change their identity from early to late Golgi structures.

2.7.3. Endocytic compartments in yeast and mammalian cells

Knowledge about the organization of the mammalian transport pathways between different compartments has been useful for the identification of similar pathways in yeast. However, in many aspects, the organization of the yeast endocytic and exocytic systems is different from the mammalian one.

In yeast there is no evidence for the existence of recycling endosomes or homologues of the mammalian Rab4 protein (Pelham, 2002). Internalized cargo is transported to early endosomes and from there either further to the late endosomes and vacuole for degradation, or back to the Golgi for sorting to the plasma membrane (Lewis et al., 2000; Valdivia et al., 2002). Therefore, it seems that there is no direct transport from early endosomes to the cell surface (it was proposed that one branch of the exocytic pathway is going through late endosomes (Gurunathan et al., 2002; Harsay and Schekman, 2002), this will be discussed later).

It was also speculated that the yeast late endosome is more similar to the mammalian early endosome and the yeast early endosome is more similar to a Golgi compartment (Pelham, 2002). This controversial hypothesis was based on several observations. Syntaxin16, a mammalian equivalent of the Tlg2p, a t-SNARE present on both, early endosomes and late Golgi in yeast, is restricted to the TGN in mammalian cells. Moreover, the t-SNAREs that were found on mammalian early endosomes, syntaxin7 and the related synaxin13, appear to be equivalent of the yeast late endosome SNARE Pep12p (Mallard et al., 2002; Pelham, 2002). Interestingly, the mammalian homologues of yeast proteins associated with functions of the late endosome, such as

Hrs (Vps27p), Snx1 (Vps5p), Rab5 (Ypt51p) and rabenosyn5 (Vac1p), have been localized to early endosomes (Chin et al., 2001; de Renzis et al., 2002; Pelham, 2002; Raiborg et al., 2001; Teasdale et al., 2001).

Furthermore, in mammalian cells the formation of multivesicular bodies occurs in early endosomes while in yeast it happens in the late compartments (Pelham, 2002). All these findings together indicate that yeast late endosomes are more like an early endosome in mammalian cells. Some similarities between the yeast vacuole and mammalian late endosomes were also observed. In mammals PI(3,5)P2 is present on the late endosomes and in yeast it is localized only to the vacuole (Pelham, 2002).

It is clear that the expression of proteins in a heterologous system can lead to mislocalization, and such experiments can easily generate artifacts. However, as discussed above, one has to be very careful when comparing organization of vesicular transport between yeast and mammalian cells. Especially the function of the adaptor protein complexes seems to differ between mammalian cells and yeast.

2.8. Endosomal and vacuolar sorting

From the Golgi there are at least three routes for protein transport to the vacuole: a direct, AP-3 mediated transport route, a pathway through the intermediate endosomal compartments, and endocytosis of cargo that was first delivered to the cell surface.

2.8.1. Direct transport to the vacuole

The vacuolar alkaline phosphatase ALP and the vacuolar t-SNARE Vam3p are transported directly from the Golgi to the vacuole (Piper et al., 1997; Rehling et al., 1999). Vesicles on this pathway are not coated with clathrin and vacuolar delivery of transported cargo is not affected in mutants in which endosome function is blocked, like *vps4, vps27, pep12* (Piper et al., 1997). It was demonstrated that the formation of vesicles on this pathway is dependent on the AP-3 complex (Rehling et al., 1999) and the interacting Vps41 protein (Darsow et al., 2001). Cargo is sorted to this route through interactions mediated by the acidic di-leucine motif present on the cytoplasmic domain of ALP and Vam3p. (Darsow et al., 1998).

2.8.2. Transport to the endosomal compartments

The organization of transport from the Golgi to endosomes is still a matter of a debate. Formation of transport carriers in this pathway involves clathrin coat assembly and requires either the AP-1 complex or GGA proteins. It is not clear, however, whether these clathrin adaptors function on the same or different pathways and what the target compartments for these routes are.

When transport from the Golgi to endosomes is blocked, the cargo is often rerouted to the cell surface, and soluble vacuolar enzymes like carboxypeptidase Y (CPY) are often secreted to the medium and can be detected by western-blot analysis (Klionsky and Emr, 1989; Rothman et al., 1989). This easy assay allowed for the identification of genes that affect vacuolar proteins sorting (*VPS*) (Rothman et al., 1989), which were categorized into six different classes based on observed changes in vacuolar morphology (Raymond et al., 1992). It was demonstrated that CPY was secreted in the absence of its membrane receptor Vps10p, which constantly cycles between the Golgi and endosomes (Cooper and Stevens, 1996). CPY was also secreted in several mutants that blocked traffic through endosomal compartments including *vps4*, *vps27, vps34* and the late endosomal t-SNARE, Pep12p (Deloche and Schekman, 2002; Gurunathan et al., 2002; Harsay and Schekman, 2002). These experiments demonstrated that CPY is delivered to endosomes before it reaches the vacuole.

In 1984 Mueller and Branton identified clathrin coated vesicles in yeast. They predicted that “the presence of coated vesicles in yeast cells suggests that this organism will be useful for studying the function of clathrin-coated vesicles” (Mueller and Branton, 1984). However, one year later, Payne and Schekman published that yeast cells survive without clathrin (Payne and Schekman, 1985). This observation started an interesting debate how “Life without clathrin” can be possible (Rothman, 1986). For many years the only defect observed in clathrin deficient cells was alteration in processing of alpha factor, missorting of Kex2p protease to the cell surface and slow cell growth (Payne and Schekman, 1989). Only the small defect in the kinetics of CPY delivery to the vacuole observed in clathrin knockout cells suggested a role of clathrin in transport to endosomes, as was known to be the case in mammalian cells (Seeger and Payne, 1992). Phan et al. (Phan et al., 1994) identified

the *APS1* gene encoding a yeast homolog of the small subunit of the mammalian AP-1 complex known to operate with clathrin in cargo delivery from the TGN to endosomes. However, in yeast there was no clear phenotype for mutants in AP-1 components.

In the year 2000 Hirst and collaborators identified and characterized yeast GGA (Golgi-associated, γ-adaptin homologues, Arf-binding) proteins, homologues of the mammalian clathrin-associated γ-adaptins that were involved in Golgi to endosome transport (Hirst et al., 2000). Importantly, according to the Hirst observations in mammalian cells the GGAs, unlike γ-adaptin, were not associated with clathrin-coated vesicles or with any of the components of the AP-1 complex (Hirst et al., 2000). In yeast, the double deletion of *GGA1* and *GGA2* resulted in missorting of CPY to the cell surface while single mutants exhibited only mild phenotypes (Hirst et al., 2000). As mentioned above, at that time there was no evidence for a block of CPY pathway in clathrin mutants. The same year Black and Pelham proposed that GGA, "possibly in association with clathrin", function in the pathway from the Golgi to late endosomes, which, as they proposed, is distinct from the AP-1 mediated pathway (as postulated) transporting cargo to the early endosomes (Black and Pelham, 2000). It is clear, that Pep12p is delivered from the Golgi to late endosomes but it is still a matter of debate whether GGA1/2p and AP-1 operate on the different transport routes.

Black and Pelham hypothesis was based on several indications coming from the following experiments. First, vacuolar mislocalization of the overexpressed late endosomal t-SNARE Pep12p was prevented when the FSD motif on the protein was mutated, leading to the accumulation of protein in early endosomes, a similar defect was observed in *gga2Δ* mutant cells, but not in mutants of AP-1 complex. It was postulated that the FSD is a sorting signal for GGA-mediated transport to late endosomes, and that the Pep12 mutant was missorted to another pathway that leads to the early endosomal compartment (assuming it must be AP-1 mediated). As discussed above, the distinction between early endosomes and late Golgi is very difficult and *de facto* the protein could be retained in the Golgi. Second, the localization of the Pep12p mutant to the vacuole was restored when the coiled-coil domain of pep12p (residues 193-261) was deleted. This was interpreted as a deactivation of an unknown signal for retention in the early endosomal compartment. The readout in this experiment, vacuolar localization, is unfortunately not specific because proteins can

be also transported to the vacuole for degradation when misfolded and/or marked with ubiquitin. Third, it was shown that plasma membrane proteins carrying the NH2 terminal portion of Pep12p without the coiled-coil domain are missorted to the vacuole and that surface delivery can be rescued when the FSD motif on the chimeric protein was mutated. Similarly, "rescue" of surface delivery was observed when the chimeric protein was expressed in *gga1/2* or clathrin mutant cells, but not in mutants of AP-1 components. Based on these observations it was postulated that GGA function in correct targeting of Pep12p from the Golgi to late endosomes, and that this pathway is distinct from AP-1 mediated. Recently, it was found that GGA proteins bind to ubiquitin, interact with clathrin and function as receptors for sorting of ubiquitinated proteins for degradation (Babst, 2004; Mattera et al., 2004; Puertollano and Bonifacino, 2004; Scott et al., 2004). The block of vacuolar delivery observed by Black and Pelham in *GGA2* deficient cells could be due to a defect in transport of proteins that were misfolded, ubiquitinated and should be degraded.

Further support for the Black and Pelham hypothesis came from observations that mutations in clathrin or AP-1 components alone does not lead to CPY secretion (Costaguta et al., 2001; Pelham, 2002; Seeger and Payne, 1992). In contrast, the double mutants of *gga* and clathrin or *gga* and AP-1 components do missort CPY (Costaguta et al., 2001). It suggested that AP-1 and GGA might have redundant functions as adaptors in clathrin vesicle formation but did not discriminate the possibility that both adaptors operate on the same type of vesicles. In 2002 (two years after Black and Pelham's report), Deloche and Schekman discovered that in clathrin mutant cells Vps10p and CPY are indeed missorted to the cell surface as both proteins were found on secretory vesicles in chlathrin/sec6 double mutant (Deloche and Schekman, 2002; Gurunathan et al., 2002; Harsay and Schekman, 2002) but after arrival to the cell surface CPY did not dissociate from its receptor and both were endocytosed and finally delivered to the vacuole (Deloche and Schekman, 2002). It is not clear, why in some mutants that blocked endosomal sorting including *vps1, vps34* and *pep12*, CPY is secreted to the medium whereas in others, like clathrin, CPY is not released to the medium (Bonangelino et al., 2002; Seeger and Payne, 1992). These observations suggest that some genes that affect endosomal transport might escape from the identification in this simple CPY secretion assay (recently a genome-wide screen identified over a hundred mutants that secret CPY using this assay; clathrin

was not identified (Bonangelino et al., 2002)). Vps10p was also found together with the AP-1 complex on immunopurified clathrin coated vesicles (Deloche et al., 2001). Similarly, GGA proteins were present on clathrin coated vesicles and in addition were found to interact physically with both, clathrin and Apl2p, the β1 subunit of the AP-1 complex (Costaguta et al., 2001). These observations suggest that both adaptors may function on the same vesicles.

In summary, it is very likely that vesicular transport from the late Golgi to the early endosomes exists, although it was never documented properly in yeast. Alternatively, the late Golgi compartment can undergo maturation into the early endosomal structures.

The distinction between transport routes to the early and late endosomes is complicated because of the dynamic nature of compartment identity, cargo molecules could be delivered via alternative routes and the involvement of the same molecular machinery on different transport pathways. The formation of Golgi-to-endosomes transport carriers involves clathrin, which is recruited to the membrane via its adaptors AP-1 and GGA. These adaptors interact with transported cargo, with GGA functioning also as a receptor for ubiquitinated proteins. Both adaptors seem to be redundant for interaction with the clathrin coat but are likely specific for the transported cargo. It is surprising that the AP-1 interacting cargo molecules were not identified in yeast and the role of AP-1 in protein sorting is still not clear. In cells carrying mutations in AP-1 components only a defect in the retrieval of a subset of Golgi membrane proteins was reported (Valdivia et al., 2002). Similarly, in *gga2Δ,* there is a defect in early endosome to Golgi transport (Black and Pelham, 2000) suggesting that either, AP-1 and GGAs also have a function in retrograde transport, or mutations in these adaptor proteins could lead to indirect effects. To date there is no clear evidence that yeast GGA and AP-1 are present on different populations of vesicles. Very likely, clathrin coated vesicles might contain both adaptors which contribute to vesicle formation. The target compartment for these vesicles might be specified by different principles, like the presence of the specific combinations of SNAREs. Therefore, the modified Pep12p may not be an informative cargo molecule to study pathways that are specific for late endosomal delivery.

2.9. Secretory pathways in yeast.

2.9.1. Identification of first *SEC* genes

Invertase Suc2p is a soluble secreted enzyme that catalyzes the extracellular hydrolysis of sucrose. Goldstein and Lampen developed an easy assay to measure the enzymatic activity of invertase present in the medium (Goldstein and Lampen, 1975). Four years later, this assay was used in the first screen for genes involved in secretion (*SEC*). Cells were subjected to mutagenesis and analyzed for thermo-sensitive defects in invertase secretion and cell growth. These studies identified the first two genes involved in secretion, named *SEC1* and *SEC2* (Novick and Schekman, 1979). The electron microscope inspection revealed that, at the restrictive temperature, these mutant cells accumulate organelles of the secretory apparatus that turned out to be post Golgi secretory vesicles. Such a massive accumulation of internal membranes leads to a change in the density of the mutant cells. This observation was used by Novick, Field and Schekman to design a next screen for the identification of new SEC genes. They mutagenized yeast cells and screened them at the restrictive temperature for: (1) defects in growth, (2) internal accumulation of membrane – the mutant cells became denser and could be enriched in gradient centrifugation, (3) internal accumulation of invertase (Novick et al., 1980). This approach revealed several essential (mutations caused a block of growth and secretion) *SEC* genes that were involved at different stages in the secretory pathway. Most of the identified genes are required for fusion of the exocytic vesicles with the plasma membrane and in the ER to the Golgi transport. Surprisingly, still very little is known about cargo sorting and formation of the secretory vesicles at the TGN.

2.9.2. Machinery for vesicle fusion with the plasma membrane

In yeast, there are two types of exocytic vesicles (discussed later) and both populations employ the same essential machinery for fusion with the cell surface. The Rab GTPase Sec4p is recruited to the vesicles (Guo *et al.,* 1999) and when converted to its active (GTP bound) form by a guaninenucleotide exchange factor (GEF) Sec2p, it interacts with its effector, the exocyst, a protein complex containing eight subunits (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p) (TerBush *et al.,*

1996). These interactions tether vesicles to the plasma membrane at the sites of exocytosis and the SNARE complexes of Snc1/2p, Sso1/2p and Sec9p facilitate membrane fusion (Fig. 4) (Aalto *et al.*, 1993; Protopopov *et al.*, 1993; Jahn *et al.*, 2003).

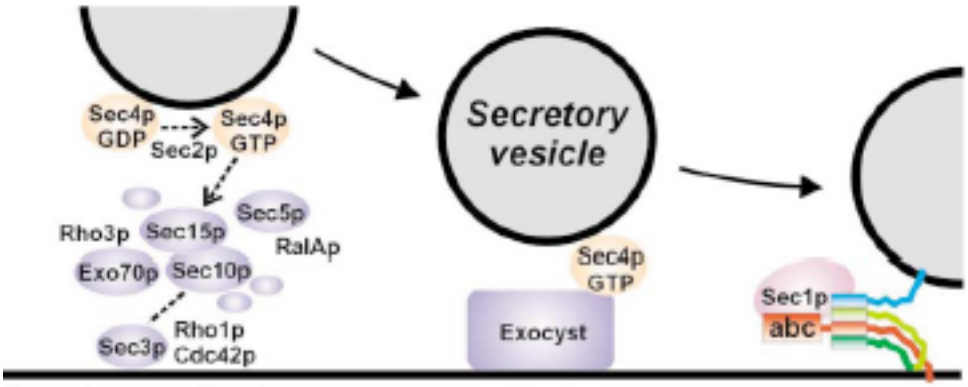


Figure 4. Fusion of the secretory vesicles with the plasma membrane. Adopted from Jahn et al., 2003.

2.9.3. ER to Golgi transport

There are two vesicular pathways from the ER to the Golgi that utilize the same essential machinery for vesicle formation. First, it was demonstrated that the Golgi delivery of GPI-anchored proteins but not other cargo molecules strongly depends on the production of ceramides (Sutterlin et al., 1997). Later, Muniz et al. demonstrated that GPI-anchored proteins are transported to the Golgi in specialized vesicles (Muniz and Riezman, 2000). It is still not clear how the cargo segregation to those two types of carriers is regulated.

The budding of the ER-derived vesicles is driven by the assembly of the COPII coat that includes the Sec13p-Sec31p and the Sec23p-Sec24p protein complexes and the small Ras-like GTPase Sar1p (Fig. 5) (Bonifacino and Glick, 2004).

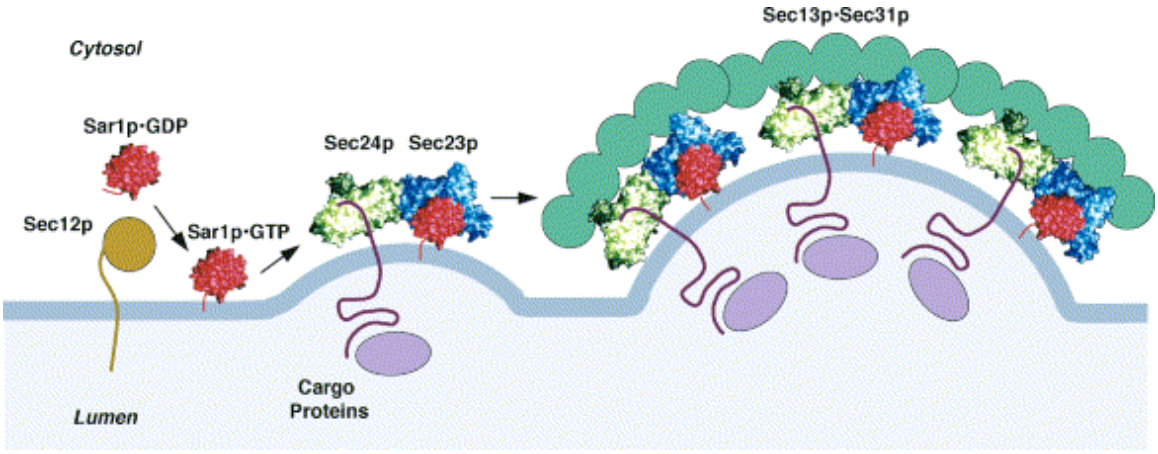


Figure 5. Assembly of COPII coat (Adopted from Bonifacino and Glick, 2004). Cytosolic Sar1p•GDP is converted to membrane bound Sar1p•GTP by the transmembrane protein Sec12p. Sar1p•GTP recruits the Sec23p•Sec24p subcomplex by binding to Sec23p, forming the "pre-budding complex". Transmembrane cargo proteins gather at the assembling coat by binding to Sec24p. The Sec13p•Sec31p subcomplex polymerizes onto Sec23p•Sec24p and crosslinks the pre-budding complexes. Cargo proteins are further concentrated.

Most of the transmembrane proteins that leave the ER bind directly to COPII components (Aridor et al., 1998; Kuehn et al., 1998; Votsmeier and Gallwitz, 2001), but some transmembrane and soluble cargo proteins bind indirectly to COPII through the transmembrane export receptors including Erv29p, Erv25p, Emp24p, Emp46p, Emp47p. (Appenzeller et al., 1999; Muniz and Riezman, 2000; Powers and Barlowe, 2002). Export receptors leave the ER together with their ligands, unload their cargo into the early Golgi, and recycle back to the ER on vesicles coated with COPI proteins (Bonifacino and Glick, 2004).

2.9.4. Organization of post-Golgi secretory pathways in yeast

Budding yeast like polarized epithelial cells and other mammalian cells, have two routes from the Golgi to the cell surface. Two populations of secretory vesicles were isolated based on their differences in density and transported cargo. The light population of vesicles contains Pma1p, the major plasma membrane ATPase, and β-glucanase Bgl2p, while the much less abundant dense population of vesicles is enriched in the soluble secreted enzymes invertase Suc2p and acidic phosphatase

Pho5p (Gurunathan et al., 2002; Harsay and Bretscher, 1995; Harsay and Schekman, 2002).

It was recently discovered that in mutants that block transport from the Golgi to and through endosomes, including *vps1, vps4,* clathrin (*chc1Δ*), and the late endosomal t-SNARE *pep12,* the dense vesicles were not formed and transported cargo was missorted to the low density, Pma1p containing vesicles (Gurunathan et al., 2002; Harsay and Schekman, 2002). Additionally, in these mutants, vacuolar CPY and its receptor Vps10p, normally transported to late endosomes (see "Endosomal and vacuolar sorting"), were rerouted to the cell surface and found on Pma1p containing vesicles (Gurunathan et al., 2002; Harsay and Schekman, 2002). Based on these observations it was postulated that one branch of the exocytic pathway overlaps with the route by which CPY is transported to late endosomes. Similarly, in mammalian epithelial cells, some basolateral proteins were found to travel to the cell surface via endosomes (see "basolateral sorting"). The proposed model implicates the sorting of exocytic material in endosomes and the existence of direct transport from endosomes to the cell surface. In contradiction to this hypothesis, previous studies demonstrated that a functional retrograde endosome-to-Golgi pathway was necessary for surface recycling of previously endocytosed cargo. This suggests that direct transport from endosomes to the plasma membrane is not possible in yeast (Lewis et al., 2000; Valdivia et al., 2002). Additionally, in cells that are mutated in the PI-3P kinase *vps34*, and have therefore low levels of PI3P and impaired endosome function, CPY was missorted to the cell surface in low density vesicles, however, high density vesicles containing invertase were formed normally (Gurunathan et al., 2002). This experiment clearly demonstrated that the pathway for CPY transport to endosomes could be separated from the invertase route to the cell surface. Therefore, it seems possible that mutations that block traffic from the Golgi to endosomes, could have indirect effects on the exocytic pathways. For instance, the function of the invertase pathway, found to be the minor route (Harsay and Bretscher, 1995), could be dependent on the recycling of the molecular machinery from the plasma membrane. In support for this hypothesis it was found that endocytosis deficient cells (*end4*) accumulated high density vesicles but not the other type (Gall et al., 2002; Harsay and Bretscher, 1995). It is known that a block in transport from Golgi to endosomes (like

in *vps1* mutant cells) leads to a defect in recycling from endosomes to the Golgi (Wilsbach and Payne, 1993a; Wilsbach and Payne, 1993b).
Although it was never tested properly (vesicle purification from the mutant cells) there are some indications that the AP-1 complex rather than GGA proteins is important for the formation of invertase containing vesicles (Gall et al., 2002).

In conclusion, the simplest explanation of the results obtained by Harsay and Schekman and Gurunathan et al. would be that the invertase transport route involves an intermediate endosomal compartment but other possibilities should still be considered.

2.9.5. Determinants for cell surface delivery

Surprisingly little is known about what determines protein localization to the plasma membrane in yeast. Similarly to the apical sorting in epithelial cells, protein association with lipid rafts and the length of the TMD were reported to facilitate protein exocytosis in yeast. An equivalent to the cytoplasmic sorting signals of mammalian proteins targeted to the basolateral route has not been defined in yeast. However, as discussed above, sorting of some cargo, e.g. invertase, requires genes involved in TGN-to-endosome/vacuole delivery (Gurunathan et al., 2002; Harsay and Schekman, 2002), a pathway that involves the formation of clathrin-coated vesicles and cargo selection through cytoplasmic tails.

2.9.5.1. Lipid rafts in yeast

The work done by Bagnat has demonstrated that the major lipid components of rafts in yeast are sphingolipids and ergosterol (yeast do not produce cholesterol) (Bagnat et al., 2000). In yeast, DRMs were detected already in the ER where ergosterol and ceramides are produced (Bagnat et al., 2000). In the Golgi, ceramides are converted into the more complex sphingolipids: inositol phosphorylceramide (IPC), mannosyl-inositol phosphorylceramide IPC (MIPC) and mannosyldi-IPC ($M(IP)_2C$), the most abundant sphingolipid in yeast (van Meer and Holthuis, 2000). The concentration of sphingolipids increases along the secretory pathway (Bagnat et al., 2001; Bagnat et

al., 2000). The endosomal and especially vacuolar membranes are depleted from raft lipids and DRMs. Mostly plasma membrane proteins were found to be associated with lipid rafts, including Fus1p, Gas1p, Hxt2p and Pma1p (Bagnat et al., 2001; Bagnat et al., 2000; Bagnat and Simons, 2002b). It seems that GPI-anchored proteins like Gas1p are associated with rafts already in the ER while other proteins, like Pma1p, translocate to rafts later in the secretory pathway as judged by DRM extraction (Bagnat et al., 2000; Lee et al., 2002). Up to date there are only two non-raft integral plasma membrane proteins identified, the general amino acid permease Gap1p and the poorly characterized Ypl176c (Bagnat and Simons, 2002a).

2.9.5.2. Association with lipid rafts in plasma membrane delivery

Pma1p is the most abundant protein that travels trough the yeast secretory pathway and it is the marker protein for the light population of post-Golgi exocytic vesicles (Gurunathan et al., 2002; Harsay and Bretscher, 1995; Harsay and Schekman, 2002). Detailed studies on trafficking of Pma1p revealed that the surface delivery of this protein requires oligomerization and association with lipid rafts (Bagnat et al., 2001; Lee et al., 2002). When sphingolipid (and ceramide) production is blocked in the *lcb1-100* mutant, Pma1p is missorted to the vacuole and rapidly degraded. There is also a temperature-sensitive allele *pma1-7* that loses its raft association and is missorted to the vacuole when cells are incubated at the restrictive temperature. Because *PMA1* is essential for cell viability, it was possible to isolate a high copy suppressor of *pma1-7*, Ast1p, which restores cell surface delivery of the pma1-7p mutant (Chang and Fink, 1995). Ast1p directly interacts with *pma1-7* and facilitates its oligomerization and raft association, leading to exocytosis of the mutant protein (Bagnat et al., 2001; Lee et al., 2002). Similarly, it was demonstrated for the tryptophan permease Tat2p that depletion of ergosterol in *erg6Δ* affected protein exocytosis (Umebayashi and Nakano, 2003). These studies demonstrated that also in yeast, raft lipids are involved in sorting to the plasma membrane.

2.9.5.3. Length of the TMD

As was reported in mammalian cells, also in yeast the length of the TMD can determine protein localization. Yeast plasma membrane proteins have much longer

TMDs then the ER or vacuolar resident proteins. Lengthening of the TMD of the ER t-SNARE Ufe1p, allows for transport of the protein along the secretory pathway to the cell surface or the vacuole (Rayner and Pelham, 1997). The choice between these destinations was determined by the length and amino acid composition of the TMD. A longer TMD was required to reach the plasma membrane and shorter TMD directed proteins to the vacuole (Rayner and Pelham, 1997). These results are in agreement with the observation that the yeast plasma membrane enriched in raft lipids is thicker than the lipid bilayers of intracellular organells. However, as discussed earlier for epithelial cells, protein sorting cannot be explained with a simple "matching" between the length of the TMD and the thickness of the lipid bilayer.

2.9.5.4. Protein glycosylation and sorting in yeast

Studies on epithelial cells clearly demonstrated that glycans attached to proteins are involved in sorting to the apical plasma membrane. However, in yeast, the role of protein glyosylation in sorting has not been defined. Sanders et al. observed that unglycosylated Axl2p, a protein involved in bud side selection, was degraded in the Golgi instead of being transported to the cell surface (Sanders et al., 1999). In this case, it is not clear whether the defect in exocytosis of Axl2p was due to the lack of a sorting signal or incorrect folding leading to rapid degradation of the protein (Sanders et al., 1999).

It was also demonstrated that the vacuolar CPY, an N-glycosylated protein, is missorted to the cell surface in several mutants that block N-glycosylation (Bonangelino et al., 2002).

2.9.6. Sorting from the Golgi to the cell surface

As described above, we have a good understanding of mechanisms that regulate vesicle formation and cargo sorting from the ER to the Golgi, and of the machinery required for fusion of the Golgi-derived vesicles with the plasma membrane. Surprisingly, little is known about the machinery involved in the formation of secretory vesicles at the Golgi. It was demonstrated that phosphatidylinositol-4-psosphate (PI4P), generated at the TGN by the PI4-OH kinase Pik1p, is crucial for

cell viability. The *pik1-100* mutant cells showed abnormal morphology of the Golgi apparatus and reduction in secretion of invertase (Walch-Solimena and Novick, 1999). The second known protein involved in the regulation of the Golgi exit is Sec14p, the yeast phosphatidylinositol-transfer protein. Mutant cells carrying the thermosensitive allele of this essential protein block exocytosis when incubated at non-permissive temperature (Bankaitis et al., 1989). Two rab proteins, Ypt31p and Ypt32p, which have 90% similarity, were also implicated in secretion. These proteins seem to have redundant function, yeast cells can tolerate deletion of either the *YPT31* or the *YPT32* gene, but not both (Benli et al., 1996). Cells deleted for the *YPT31* gene and carrying a conditional mutation in *YPT32* exhibit abnormal Golgi structures and a block in secretion (Benli et al., 1996). The role of these two Ypts in exocytosis is still not clear since these proteins are implicated in Golgi-endosome transport (Chen et al., 2005) and could have an indirect function in transport to the cell surface.

So far, only one protein, Chs5p, was identified that directly regulates Golgi exit for some plasma membrane proteins. This non-essential protein was shown to localize to the TGN and regulate surface delivery of Chs3p and Fus1p through the direct binding to their cytoplasmic domain (Santos et al., 1997; Santos and Snyder, 1997; Santos and Snyder, 2003; Valdivia et al., 2002). Interestingly, it has been shown that the GPI-anchored cell wall protein Crh2p, which has no domains exposed to the cytoplasm, also requires cytosolic Chs5p for plasma membrane delivery (Rodriguez-Pena et al., 2002). The molecular mechanism for Chs5p function remains to be investigated.

2.9.7. Why did the machinery responsible for cargo sorting at the TGN escape from identification?

Why, despite several screens and detailed studies on the machinery involved in secretion, is there so little known about cargo sorting from the TGN to the cell surface? A potential explanation is that blocking only one route in the bifurcated pathway does not lead to a strong defect in secretion. Gurunathan et al., and Harsay and Schekman demonstrated that, when the invertase pathway was blocked, the dense vesicle cargo was missorted to the Pma1p pathway and still delivered to the cell surface (Gurunathan et al., 2002; Harsay and Schekman, 2002). Taking this into account, mutations that block only one secretory pathway (1) would not be lethal, (2)

would not accumulate internal membranes (3) and would not block completely the transport of cargo to the cell surface. Therefore, these mutants could easily escape identification in previous studies (see chapter "Secretory pathways in yeast"). Although there are also two pathways from the ER to the Golgi, both routes use the same essential machinery for vesicle formation. Similarly, two types of post-Golgi exocytic vesicles use the same essential proteins for the fusion with plasma membrane identified in previous screens (except for the SNAREs, Sso1/2p and Snc1/2 that are duplicated in the yeast genome). Most likely, different machinery for vesicle formation is used in these two exocytic routes and therefore corresponding genes are expected to be non-essential for cell viability.

Additionally, studies on mutants that affect exocytosis were mostly focused on analyzing defects in invertase secretion, as this can be easily addressed with an enzymatic assay (see chapter "secretory pathways in yeast"), but the defect is observed only when both transport routes are blocked.

The biggest obstacle in screening for the mutants that affect sorting of exocytic cargo at the TGN is the lack of an assay that allow for the detection of proteins missorted from one branch of the secretory pathway to the other.

2.10. Cell surface polarization in yeast

Yeast cells exhibit cell polarity during cell replication, invasive growth and mating (Chant, 1999; Madden and Snyder, 1998). During vegetative growth, a new cell is formed at specific sites. Haploid cells bud axially – the mother cell forms a bud adjacent to the previous budding site, diploid cells bud in a bipolar pattern – the daughter cell emerges distally from the previous birth site (Chant, 1999; Madden and Snyder, 1998). A second type of polarized growth occurs when access to specific nutrients, such as nitrogen, is decreased. Under this conditions yeast cells initiate invasive growth, in which cells elongate, bud from the one pole of the cells and form connected chains of cells that can spread across the substrate and invade neighboring areas rich in nutrients. The third type of cell polarity occurs during mating, when two haploid cells make elongated projections (at this stage the elongated cells are called shmoos) towards each other and fuse to make a diploid cell (Fig. 6).

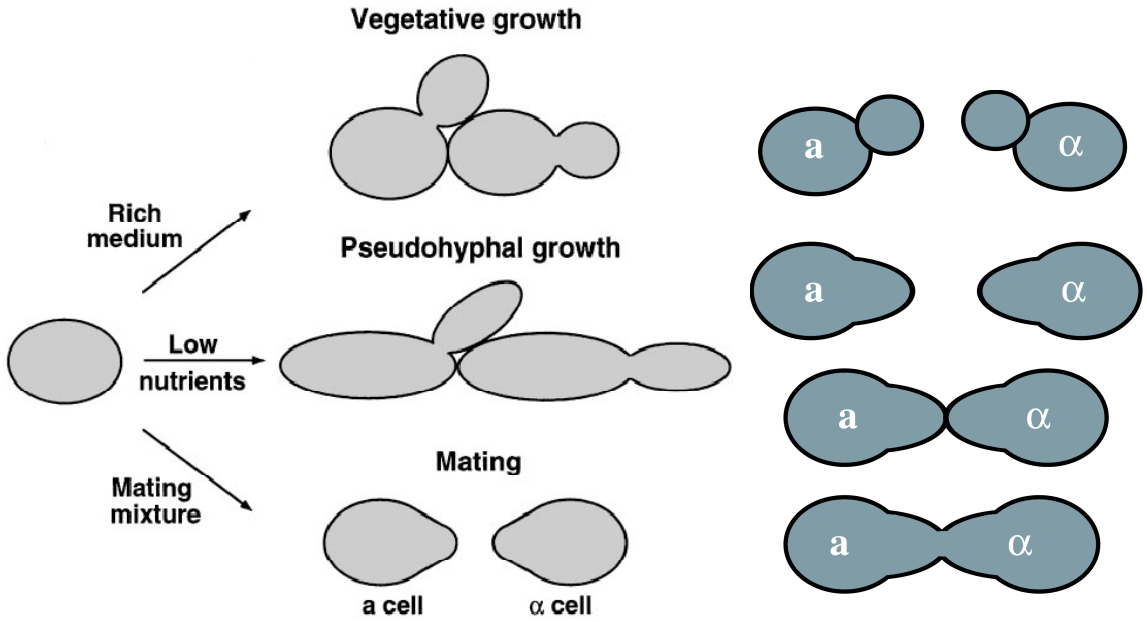


Figure 6. (Left) Yeast cells exhibit three types of cell polarity during cell replication in rich medium, pseudohyphal growth and mating. (Right) During mating, haploid cells exposed to pheromone from cells of the opposite mating type (called "a" and "α") make a projection towards each other in order to minimize the distance between them and subsequently fuse to make a diploid cell. Adopted from Madden and Snyder 1998 and from Bagnat and Simons 2004.

The polarization process in budding and mating is characterized by a hierarchy of steps. First, the site on the cell surface is selected by intrinsic and extrinsic cues. This site is marked by the deposition of landmark proteins. Second, cell polarity is established by the activation of small GTPases with Cdc42p as the major player. Last, a multiprotein machine is assembled that spools out actin cables to direct post-Golgi traffic to the site of polarized cell growth (Chang and Peter, 2003; Drubin and Nelson, 1996; Madden and Snyder, 1998; Pruyne and Bretscher, 2000).

In budding, membrane traffic is directed by actin cables to the bud and the septin ring at the mother-daughter cell neck region provides a physical barrier, preventing diffusion of membrane components from the bud to the mother cell. During mating, the biosynthetic transport is directed to the shmoo tip (Pruyne and Bretscher, 2000). In the mating projection, there is no diffusion barrier like the septin ring, and most proteins diffuse laterally across the entire cell surface. Only a specific subset of proteins required for mating is clustered at the tip of the mating projection.

The mating yeast cells provide a simple paradigm for analyzing mechanisms underlying the generation of cell surface polarity. Two models have been proposed for formation and maintenance of cell surface asymmetry in shmoos: clustering of lipid rafts and endocytic recycling.

2.10.1. Clustering of lipid rafts

One model is based on clustering of lipid rafts containing proteins involved in cell-cell adhesion and the fusion machinery responsible for mating (Bagnat and Simons, 2002a). It was demonstrated that the raft associated protein Fus1p, which is required for efficient mating, loses its polarized localization to the tip of the mating projection when the production of sphingolipids and ergosterol is blocked in *lcb1-100*/*lcb3Δ* or *erg6Δ* mutants, respectively. In agreement with the role of lipid rafts in mating, both mutants were not able to form diploid cells. (Bagnat and Simons, 2002a)

2.10.2. Endocytic recycling

Another model was recently put forward, employing an ongoing cycles of endocytosis and polarized delivery of membrane components to the tip of shmoos. This model was based on the observation that the polarized distribution of Snc1p, a v-SNARE involved in vesicle fusion with plasma membrane, is dependent on endocytosis (Valdez-Taubas and Pelham, 2003). Also Sso1p, a plasma membrane t-SNARE that is equally distributed over the entire cell surface, was polarized to the mating projection when a signal for endocytosis was added to the protein. Valdez-Taubas and Pelham proposed that the same mechanism, endocytic recycling and not clustering on the tip of the mating projection is involved in Fus1p polarization (Valdez-Taubas and Pelham, 2003).

2.11. Goals of these studies

1. The Role of O-glycosylation in cell surface delivery in yeast.
2. The development of a new method that would allow for the identification of genes involved in Golgi to plasma membrane transport.
3. The identification of the machinery involved in cargo sorting to the plasma membrane.
4. The analysis of mechanisms that regulate cell surface polarization during mating.

3. Results

3.1. Role of Fus1p O-glycosylation

3.1.1. Protein O-glycosylation in yeast

In baker's yeast the O-glycans attached to the proteins are unbranched chains of up to 5 mannoses linked to serine or threonine on the protein (Strahl-Bolsinger et al., 1999). O-glycosylation is initiated in the ER through the action of 6 protein O-mannosyl transferases (Pmt1-6p) that transfer first mannose from the dolichol-6-phosphate mannose to the acceptor site on the protein (Fig. 7) (Strahl-Bolsinger et al., 1999). The PMT family was classified into *PMT1*, *PMT2* and *PMT4* subfamilies, which differ in their protein substrate specificity (Gentzsch and Tanner, 1997; Girrbach and Strahl, 2003). The PMT family exhibit certain degree of redundancy and only the simultaneous deletion of *PMT1*/*PMT2* and *PMT4* subfamily members is lethal (Gentzsch and Tanner, 1996; Girrbach *et al.*, 2000). After the first mannose is attached to the acceptor sites protein is transported to the Golgi apparatus where mannose chains are elongated by a concert of enzymes including Ktr3p, Kre2p, Ktr1p, Mnt1p that are highly redundant in function. For the elongation of mannose chains in the Golgi the GDP-mannose serves as a donor of sugars (Fig. 7) (Strahl-Bolsinger et al., 1999).

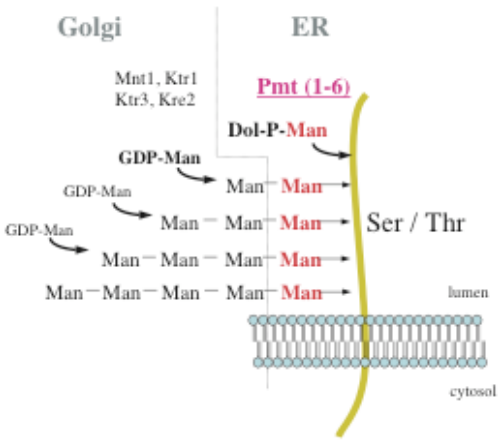


Figure 7. Schematic illustration of protein O-glycosylation in yeast, See text for details.

3.1.2. Unglycosylated Fus1p accumulates intracellulary in *pmt4Δ* mutant cells.

Fus1p is an O-glycosylated type I integral membrane protein, which is raft associated and is required for cell fusion during yeast mating (Bagnat and Simons, 2002a; Trueheart et al., 1987; Trueheart and Fink, 1989). The function of the O-glycosylation of Fus1p is not known. To gain insight into the role of O-glycosylation we first determined which particular member of the *PMT* gene family is responsible for the glycosylation of Fus1p. We expressed an epitope-tagged version of the protein in all *pmt* mutants under the control of the inducible GAL-S promoter. In western blot analysis of Fus1p expressed in wild-type cells we detected four specific bands (Fig. 8A). These different forms are due to the processing of the protein (Bagnat and Simons, 2002a; Trueheart and Fink, 1989) (also see below). A similar pattern was observed when the protein was expressed in *pmt1Δ*, *pmt2Δ*, *pmt3Δ*, *pmt5Δ* and *pmt6Δ* mutants, indicating that in these mutants the protein was processed normally (Fig. 8A). In contrast, in *pmt4Δ* cells Fus1 was detected as a single band, suggesting that in this mutant Fus1p was not properly processed. We observed variation in the relative amount of the different forms of the protein produced in different *pmt* mutants; however, similar variations were observed for the protein produced in wild-type cells in different experiments.

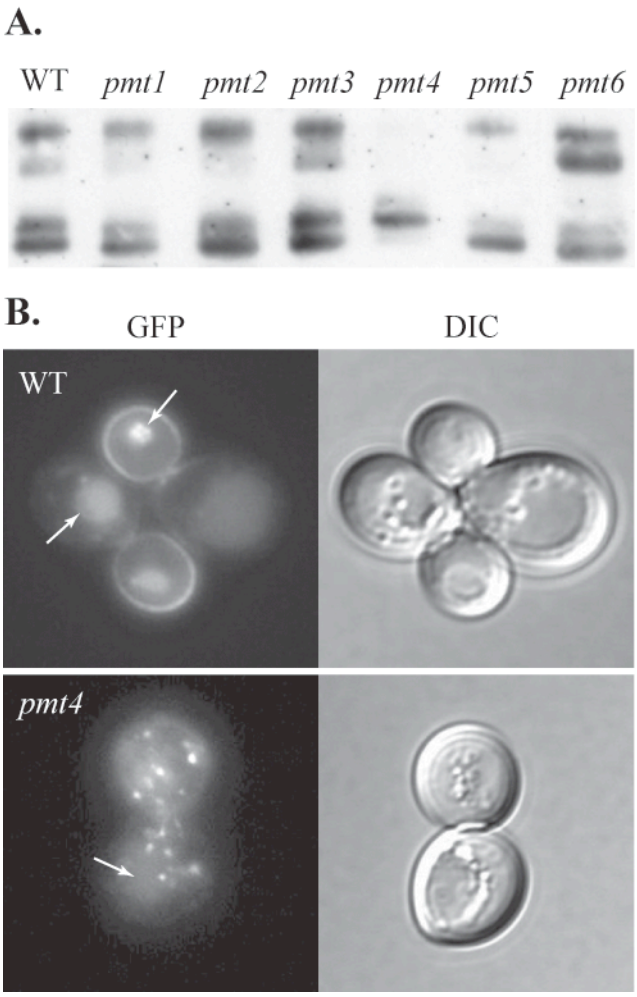


Figure 8. Fus1 processing and its surface delivery is blocked in *pmt4Δ* cells. (A) Western blot analysis of Fus1-TAP expressed in different *pmt* mutants. (B) Cellular localization of Fus1-GFP in wild-type and *pmt4Δ* cells. In wild-type cells the protein is localized to the plasma membrane and the vacuole (arrow). In *pmt4Δ* cells protein accumulates in dot-like structures inside the cell and in the vacuole (arrow). Quantitative analysis of fluorescence from plasma membrane and inside of the cell (see Materials and Methods) demonstrated that Fus1-GFP is much more efficiently delivered to the cell surface in wild-type cells than in the *pmt4Δ* cells (see Fig. 16).

Next we tested whether altered processing of Fus1p in *pmt4Δ* mutant cells affected its cellular localization. A green fluorescent protein fusion (Fus1-GFP) was expressed from the GAL-S promoter (Bagnat and Simons, 2002a) in wild-type and *pmt4Δ* cells. In wild-type cells, 3h after induction of expression, Fus1-GFP was localized to the plasma membrane of the bud and to vacuoles (Fig. 8B). Fus1p has a very fast turn-over (half life less than 1h) and after delivery to the cell surface it is rapidly

endocytosed and transported to the vacuole for degradation. In *pmt4Δ* cells transport of Fus1-GFP to the cell surface was inhibited (only 3.6% of cells had surface staining; N°=300) instead, the protein accumulated intracellularly in dot-like structures and in vacuoles (Fig. 8B). These structures resembled Golgi or endosomal elements.

To examine which form of Fus1p is produced in *pmt4Δ* cells we expressed the protein in thermo-sensitive mutants that block biosynthetic traffic along the secretory pathway at different stages. *SEC53* encodes a phospho-mannomutase, necessary for the production of dolichol-P-mannose and GDP-mannose, donors of sugars for both N- and O-glycosylation (Babczinski and Tanner, 1973; Kepes and Schekman, 1988; Ruohola and Ferro-Novick, 1987; Sharma et al., 1974). When *sec53* cells were incubated at 37°C (the restrictive temperature) both N- and O-glycosylation were blocked. Fus1p expressed in *sec53* cells at the restrictive temperature migrated as a single band with the same mobility as the band generated in *pmt4Δ* cells (Fig. 9), suggesting that Fus1 was not glycosylated in the *pmt4Δ* mutant. In *sec18* cells, when the protein accumulated in the ER, Fus1 migration on SDS-PAGE was shifted compared to the *sec53* form (Fig. 9), due to partial glycosylation of the protein. This precursor (p) form was transported to the Golgi where elongation of the mannose chains takes place. The fully glycosylated form of Fus1p, accumulating in the Golgi in the *sec14* mutant cells, migrated as the slowest band - m1 (mature1). A second mature form of Fus1p – m2 (mature-2 that migrates as the fastest band) was also generated in the Golgi complex (Fig. 9). The big shift in migration between the m1 and m2 forms suggested that the protein was proteolytically cleaved. In *sec14* cells both mature forms were generated, indicating that the cleavage occurred in the Golgi complex.

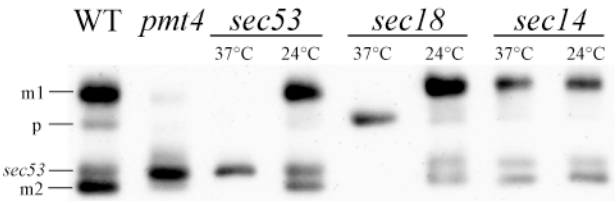


Figure 9. Unglycosylated Fus1 accumulates in *pmt4Δ* cells. Western blot analysis of Fus1-TAP expressed in wild-type, *pmt4Δ* and different secretory mutant cells. The form of Fus1 that is produced

in the *pmt4Δ* mutant migrates at the level of the unglycosylated protein produced in *sec53* cells at 37°C (sec53 form). In *sec18* cells at 37°C the protein accumulated in the ER and migrated as a partially glycosylated precursor (p) form. In *sec14* cells at 37°C the protein accumulated in the Golgi as a fully glycosylated mature form (m1). In the Golgi complex a second mature form (m2) was generated. The m2 form migrated more rapidly than all the other forms. In *pmt4Δ* cells minute amounts of m1, p and m2 forms were also detected.

We also followed the maturation of Fus1-GFP in wild-type and *pmt4Δ* mutant cells in a pulse-chase experiment. Cells were grown in medium containing raffinose as carbon source and expression of Fus1-GFP was induced for 15 minutes by addition of galactose. Then cells were pulse-labeled with [^{35}S] methionine for 5 minutes and chased for various times. In wild-type cells at the beginning of the chase the unglycosylated (*sec53* form) form and the ER precursor form (p) of the protein were detected (Fig. 10). After 5 minutes of chase the mature form (m1) appeared and after 30 min of chase the m2 form was generated. As the protein matured the amount of the precursor form was reduced. In contrast, in the *pmt4Δ* mutant Fus1-GFP migrated as the unglycosylated form throughout the whole chase period. Only faint bands that represent other forms of Fus1p were detected. Thus in the *pmt4Δ* mutant Fus1p was not degraded and remained unglycosylated throughout the chase period.

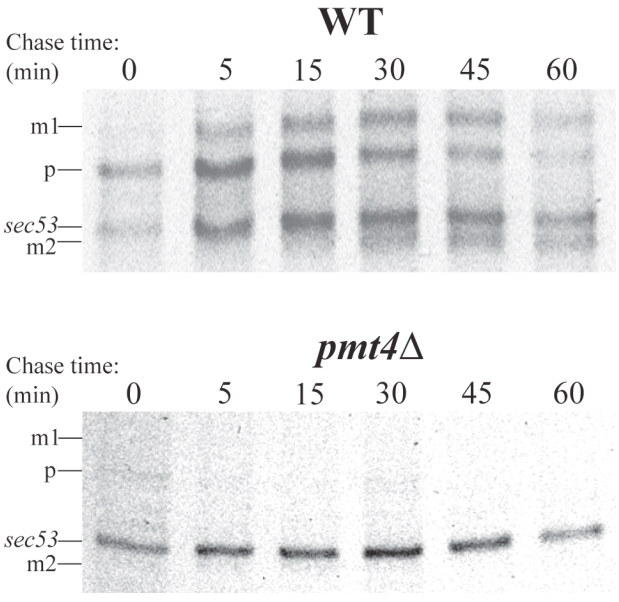


Figure 10. Processing of Fus1-GFP in wild-type and *pmt4Δ* cells. Cells were grown in medium containing raffinose as the carbon source and expression of Fus1-GFP was induced for 15 minutes by addition of galactose. The cells were then pulse-labeled with [^{35}S] methionine for 5 minutes and chased for various times as indicated. In wild-type cells, at the beginning of chase the sec53 and p forms of Fus1p were present. After 5 minutes the m1 form was visible and after 30 minutes the m2 form was generated. Throughout the chase period the p and sec53 forms were also detected. In *pmt4Δ* cells throughout the chase period Fus1 migrated with the same mobility as the unglycosylated sec53 form.

3.1.3. *pmt4Δ* cells do not show a general secretory defect.

In order to investigate whether the secretory block, which we observed in the *pmt4Δ* mutant, is specific for Fus1-GFP or also affects other proteins we examined the cell surface delivery of Mid2p. Mid2p, a cell wall integrity sensor, is a type I, O-glycosylated membrane protein (Philip and Levin, 2001; Rajavel et al., 1999). Several members of the PMT family are responsible for the glycosylation of Mid2p (Lommel et al., 2004). Mid2-GFP was efficiently delivered to the cell surface in wild-type and *pmt4Δ* cells (Fig. 11), indicating that in the *pmt4Δ* mutant there is no general block in exocytosis.

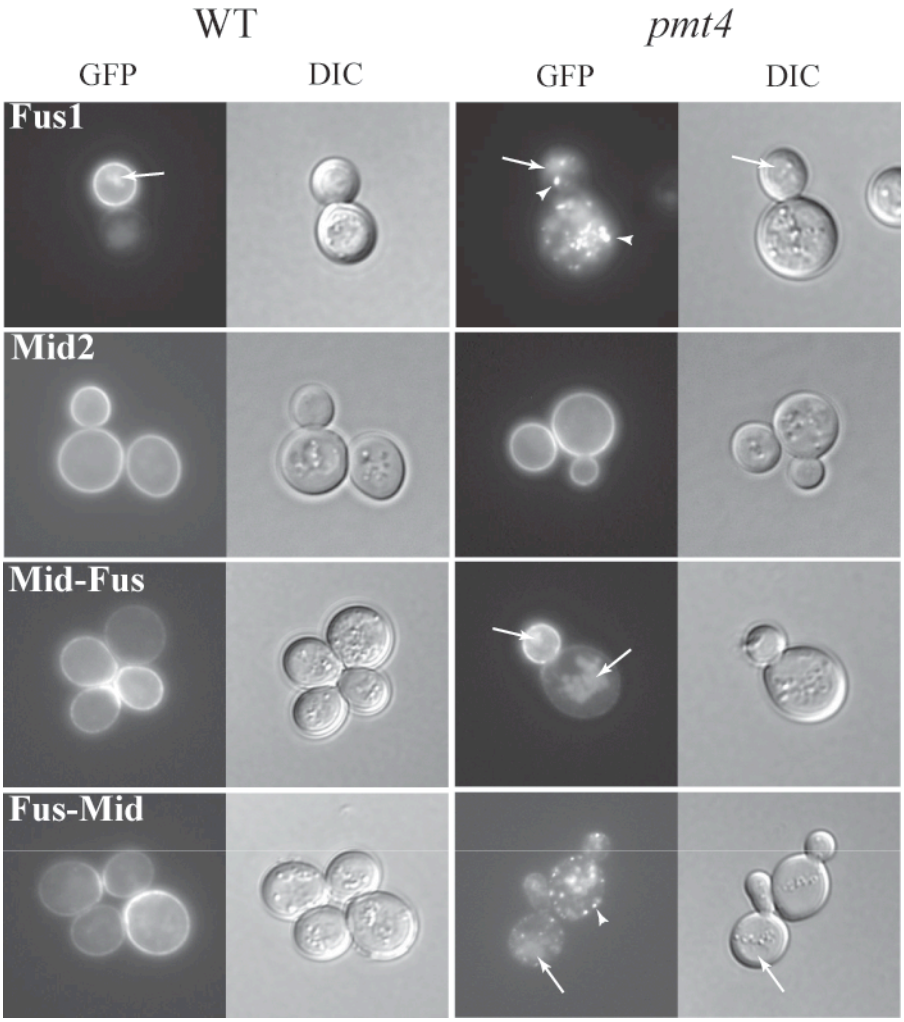


Figure 11. Cellular localization of Fus1-GFP, Mid2-GFP and chimeric proteins in wild-type and *pmt4* cells. In wild-type cells Fus1-GFP was delivered to the cell surface and to the vacuole (arrow). In *pmt4Δ* cells protein accumulated in dot-like structures (arrowhead) inside the cell and in the vacuole (arrow). Mid2-GFP was efficiently delivered to the cell surface in wild-type and *pmt4Δ* mutant.

Similarly Mid-Fus was delivered to the plasma membrane both in wild-type and *pmt4Δ* cells. In the *pmt4Δ* mutant the protein was also detected in the vacuole (arrow). Fus-Mid surface delivery was dependent on *PMT4*. In the *pmt4Δ* mutant the Fus-Mid protein accumulated in dot-like structures (arrowhead) inside the cell and in the vacuole (arrow). Quantitative fluorescence analysis confirmed increased intracellular accumulation of Fus-Mid in *pmt4Δ* cells as compared to wild-type cells (see Fig. 16).

3.1.4. O-glycans on the extracellular domain of Fus1p are important for surface localization.

Since the extracellular parts of both Fus1p and Mid2p are O-glycosylated and surface delivery of Fus1p but not Mid2p is affected in *pmt4Δ*, we decided to swap the extracellular domains of Fus1p and Mid2p to evaluate the role of the ectodomains in protein exocytosis (see cartoon in Fig. 12).

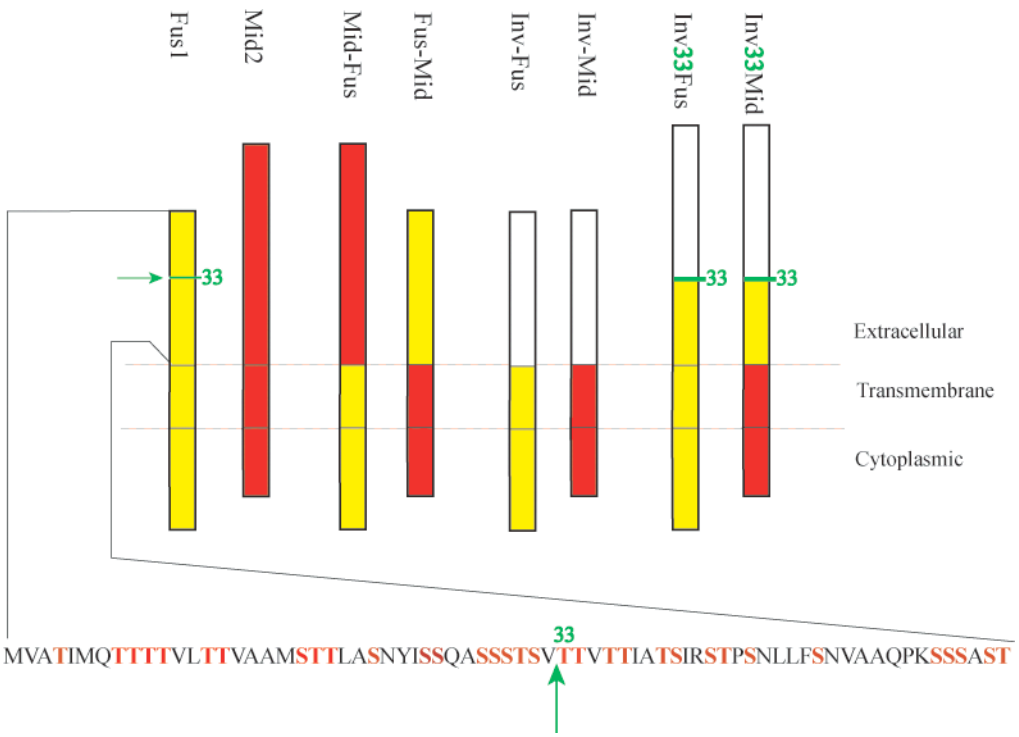


Figure 12. Schematic representation of constructs used in this study. The amino acid sequence of the extracellular domain of Fus1p is shown at the bottom. In red are shown potentially O-glycosylated amino acids. Green arrows and numbers indicate the length of the segment from the extracellular domain of Fus1p added to the invertase fusion constructs. Colours represent sequence from different proteins: yellow – Fus1p, red – Mid2p, white – invertase. All protein construct contain a C-terminal added GFP tag. Additionally, Fus1p was also tagged with the TAP-tag (see Fig 8A and 9).

The chimeric protein containing the extracellular domain from Mid2p fused to the transmembrane domain and cytoplasmic tail from Fus1p, followed by GFP was named Mid-Fus (Fig. 12). Similar to Mid2p, Mid-Fus was efficiently delivered to the cell surface but weak GFP fluorescence was also localized to the vacuole (Fig. 11). Conversely, the GFP-tagged chimeric protein containing the extracellular part from Fus1p fused to the transmembrane domain and cytoplasmic part of Mid2p was named Fus-Mid. Fus-Mid expressed in *pmt4Δ* mutant, accumulated intracellularly in dot-like structures and in the vacuole (Fig. 11). In SDS-PAGE Fus-Mid expressed in the *pmt4Δ* mutant cells migrated with the same mobility as the protein expressed in *sec53* cells at the restrictive temperature (data not shown), indicating that Fus-Mid was O-glycosylated in a *PMT4* dependent manner.

Next we asked in which compartments were the unglycosylated forms of Fus1p and Fus-Mid accumulated. To do so we introduced either Fus1-GFP or Fus-Mid-GFP in *pmt4Δ* cells expressing Sec7-DsRed, a late Golgi marker (Franzusoff et al., 1991; Rossanese et al., 2001; Rossanese et al., 1999). Most of the intracellular structures that accumulated GFP fusion proteins also showed intensive DsRed staining (Fig. 13), indicating that the unglycosylated Fus1p and Fus-Mid accumulated in the Golgi complex. Taken together our results indicate that the O-glycosylation of the extracellular part of Fus1p is required for surface delivery of Fus1p.

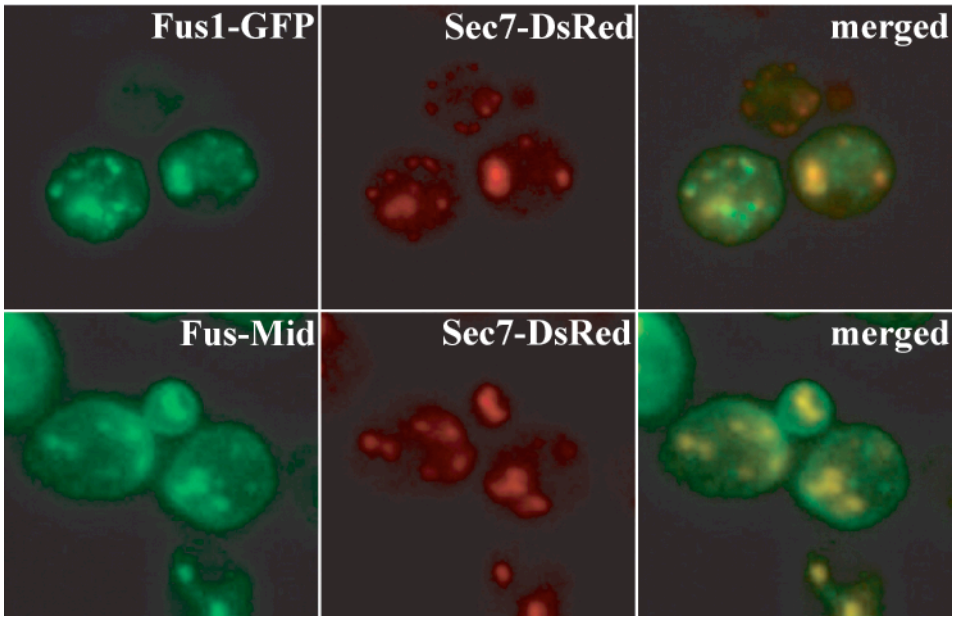


Figure 13. In *pmt4Δ* cells Fus1 and Fus-Mid accumulates in the late Golgi structures. In *pmt4Δ* mutant Fus1-GFP and Fus-Mid-GFP co-localized with Sec7-DsRed, a late Golgi marker.

3.1.5. *pmt4Δ* mutant cells have a strong unilateral mating defect.

Because in the *pmt4Δ* cells Fus1p, a protein involved in cell fusion during yeast mating, is not delivered efficiently to the cell surface we checked whether the *pmt4Δ* cells show a defect in mating. The *pmt4Δ Mat a* cells crossed to wild-type *Mat* α cells showed an eighteen fold reduced mating efficiency compare to control wild-type *Mat a* cells. *fus1Δ* cells show a bilateral mating phenotype that is pronounced only when *FUS1* is deleted in both parental strains (Berlin *et al*., 1991; Gammie *et al*., 1998). However, the mating defect of the *pmt4Δ* cells was unilateral and strongly pronounced when only one of the mating strains was missing the *PMT4* gene. Probably, in *pmt4Δ* cells not only Fus1p but also other proteins involved in mating were not glycosylated and therefore mislocalized and/or not functional.

3.1.6. Fus1 and Mid2 chimeric proteins lacking O-glycans are retained intracellularly

As Fus1p and Fus-Mid lacking O-glycans were blocked in their transport to the cell surface in *pmt4Δ* cells we analyzed whether the intracellular accumulation of both markers is due to the lack of O-glycosylation or to an indirect effect of the *PMT4* deletion. To do so we replaced the extracellular domain of both proteins with a sequence, similar in length, from the N-terminal part of invertase (Suc2p) (see Fig. 12). Invertase is a soluble secreted protein that is N-glycosylated, but not O-glycosylated. The protein has been used as a reporter fused to different membrane proteins to study membrane sorting (Darsow *et al*., 2000). Invertase contains a N-terminal signal sequence, which allows the correct translocation of the fusion constructs into the secretory pathway (Li et al., 2002a). We expressed the chimeric proteins named Inv-Fus and Inv-Mid containing a GFP tag in wild-type cells. Unlike the O-glycosylated Fus1p and Fus-Mid, both Inv-Fus and Inv-Mid were inhibited in their transport to the plasma membrane and were instead mis-sorted to the vacuole and accumulated in Golgi-like structures (Fig. 14A). Out of more than 300 cells we could not find any cell with surface staining.

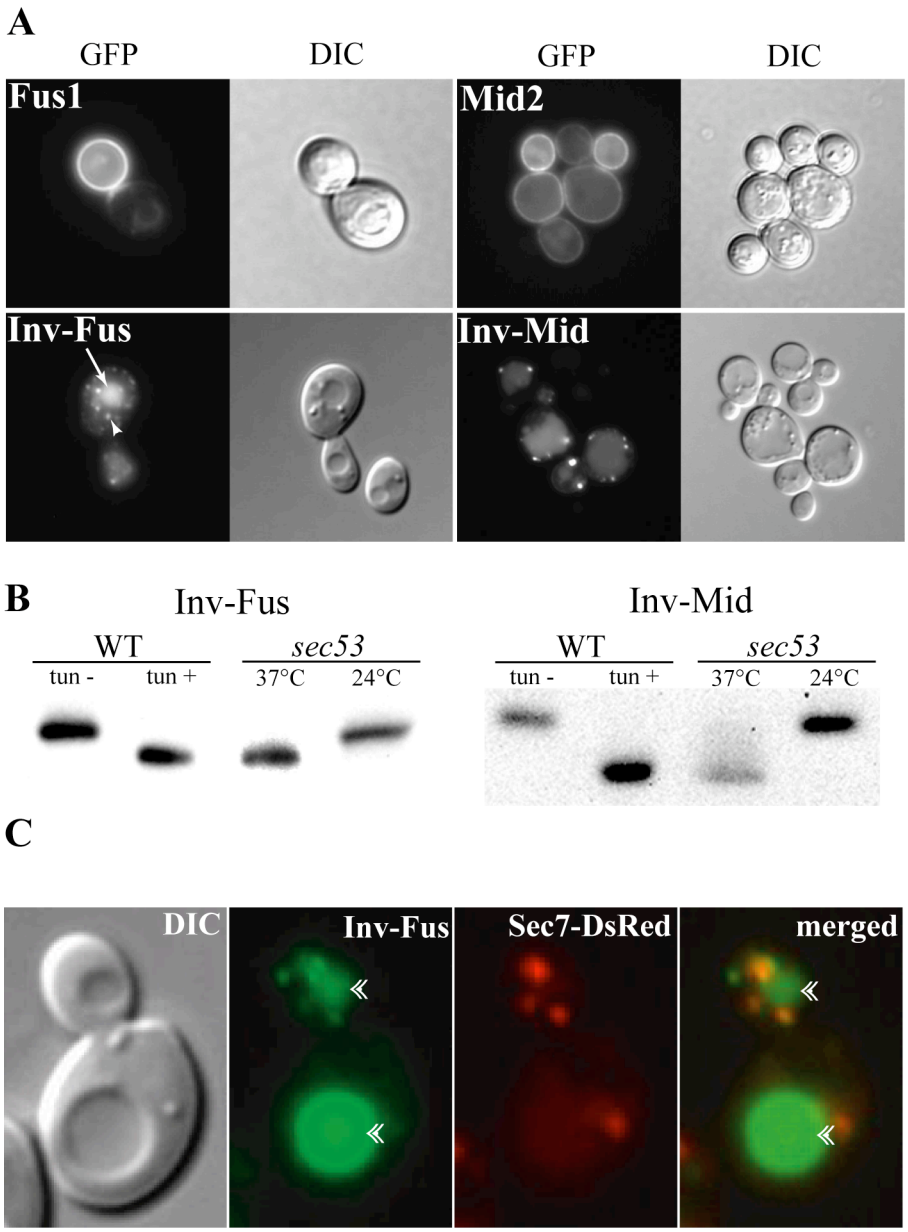


Figure 14. Inv-Fus and Inv-Mid are not delivered to the cell surface in wild-type cells and accumulate in late Golgi compartments. (A) Surface delivery of Fus1-GFP, Mid2-GFP and chimeric invertase constructs in wild-type cells. Both Fus1-GFP and Mid2-GFP were efficiently delivered to the cell surface. GFP fusion constructs of Fus1 and Mid2 carrying a portion of invertase sequence instead of their extracellular domain accumulated in dot-like structures (arrowhead) inside the cell and in vacuole (arrow). Quantitative analysis showed strongly reduced surface delivery of both invertase fusion constructs (Inv-Fus and Inv-Mid) compare to O-glycosylated constructs (Fus-GFP and Fus-Mid in wild type) (see Fig. 16). (B) Inv-Fus and Inv-Mid are N- but not O-glycosylated. Western blot analysis showed that Inv-Fus and Inv-Mid expressed in wild-type cells in presence of tunicamycin (tun) migrated faster than the proteins expressed in the absence of tunicamycin. After inhibition of N-glycosylation (WT +tunicamycin) both proteins migrated with the same mobility as the proteins produced in *sec53* cells at the restrictive temperature. (C) Inv-Fus was missorted to the vacuole (double

arrowhead) and also accumulated in late Golgi structures (marked by Sec7-DsRed). Note, that two different strains were used in Fig. A B and in Fig. C (see materials and methods).

Because invertase is an N-glycosylated protein, we checked, whether our fusion constructs containing 70 amino acids from invertase, including 3 potential N-glycosylated sites, were N-glycosylated. We expressed Inv-Fus and Inv-Mid in wild-type cells in the presence or absence of tunicamycin, an inhibitor that specifically blocks N-glycosylation (Lehle and Tanner, 1976). By Western blotting both proteins migrated faster when tunicamycin was added to the cell culture as compared to control cells not treated with the inhibitor (Fig. 14B). In tunicamycin-treated cells both proteins migrated with the same mobility as proteins produced in *sec53* cells cultured at the restrictive temperature, indicating that both Inv-Fus and Inv-Mid were N-glycosylated but not O-glycosylated.

Next we asked whether the dot-like structures that accumulated the invertase fusion construct were Golgi compartments. We expressed Inv-Fus in wild type cells producing Sec7-DsRed as before. As shown in figure 14C, there was partial co-localization of GFP and DsRed staining. Inv-Fus was mis-sorted to the vacuole and also localized in Golgi structures. Most likely, dots that were stained with GFP and did not co-localize with Sec7-DsRed marker were an intermediate structures on the way to the vacuole.

In summary these results show that Fus1 and Mid2 proteins fused to the N-terminal part of the invertase sequence containing N-glycans accumulated in Golgi structures and were also missorted to the vacuole.

3.1.7. Surface delivery of invertase chimeric proteins is rescued by addition of an O-glycosylated sequence from Fus1p.

The invertase fusion proteins were not delivered to the cell surface. This defect could be due to the lack of O-glycosylation. To test this hypothesis we tried to rescue surface delivery of Inv-Fus and Inv-Mid by addition of a portion (33 amino acids) of the O-glycosylated domain from Fus1p containing 15 potential O-glycosylation sites (see Fig. 12). We generated “rescue” constructs containing the same N-terminal part of invertase as used before fused to the 33 amino acid sequence of the juxtamembrane

part of the extracellular domain of Fus1p, followed by the transmembrane domain and the cytoplasmic tail from either Fus1p or Mid2p with the GFP tag at the C-terminus (see Fig. 12). The new constructs were named Inv33Fus and Inv33Mid. In wild type cells, unlike the parent invertase fusion proteins, Inv33Fus and Inv33Mid were efficiently delivered to the cell surface (Fig. 15A). Most of the cells expressing Inv33Mid showed GFP fluorescence on the plasma membrane but also a weak fluorescence signal was observed in the ER-like structures. The expression level of Inv33Fus was very low, but similar to Mid2p, most of the cells (64.1%; N°=256) expressing Inv33Fus showed a fluorescence signal on the plasma membrane.

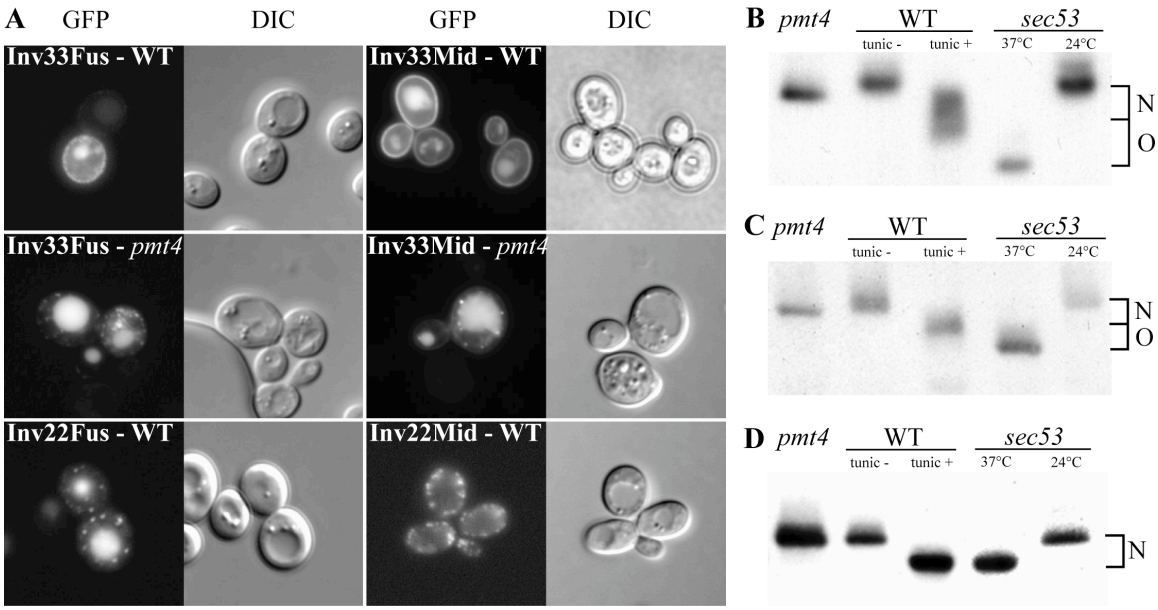


Figure 15. Surface delivery of invertase constructs can be rescued by addition of a portion of the Fus1p O-glycosylated extracellular domain and is dependent on *PMT4*. (A) Cellular localization of invertase rescue constructs containing portions from the extracellular domain of Fus1p. The constructs were expressed in wild-type and *pmt4Δ* cells as indicated. Quantification showed that the addition of 33 amino acids (but not 22 amino acids) from the extracellular part of Fus1p could restore the plasma membrane localization of Inv33Mid (see Fig. 16). Cells were grown in medium containing a raffinose as a carbon source and protein expression was induced overnight by addition of galactose. Western blot analysis of Inv33Mid (B), Inv33Fus (C) and Inv22Fus (D) expressed in *pmt4Δ* mutant, wild-type and *sec53* cells (temperature as indicated) in the presence or absence of tunicamycin (tun-/+). Differences in protein migration due to N- and O-glycosylation are indicated on the right side of the figure.

Interestingly, Inv33Fus and Inv33Mid expressed in *pmt4Δ* mostly accumulated inside the cells in dot-like structures and also in vacuoles (Fig. 15A), indicating that the surface delivery of "rescue" constructs was at least partially dependent on *PMT4*. Next we checked whether the "rescue" constructs were O-glycosylated. The Inv33Mid protein expressed in *pmt4Δ* cells migrated in SDS-PAGE with a slower mobility than the protein produced in wild type cells (Fig. 15B), suggesting that *PMT4* is involved in the O-glycosylation of Inv33Mid. The protein was also N-glycosylated, because we observed a shift in migration of the protein expressed in wild type cells in the presence of tunicamycin compared to the protein expressed in control cells without tunicamycin (Fig. 15B). There was an additional shift in migration of the protein between the form expressed in wild-type cells in presence of tunicamycin and the unglycosylated form produced in *sec53* at 37°C (Fig. 15B). These experiments show that Inv33Mid contains N-glycans and is also O-glycosylated in a *PMT4*-dependent manner. Similar results were obtained for Inv33Fus protein (Fig. 15C).

Therefore, addition of 33 amino acids from the glycosylated domain of Fus1p allowed surface delivery of the invertase fusion constructs. When we further shortened the 33 amino acid fragment by 10 amino acids (from the N-terminus) and inserted a 22 amino acid portion into Inv-Fus and Inv-Mid these chimeric proteins were not delivered to the plasma membrane and were not O-glycosylated (Fig. 15A and D).

Altogether these experiments show that the surface delivery of Inv-Fus and Inv-Mid could be rescued by addition of the 33 amino acids segment from the glycosylated domain of Fus1p. Inv33Fus and Inv33Mid were O-glycosylated and surface delivery of both proteins depended on *PMT4*. Thus, we conclude that O-glycosylation is required for surface delivery of Fus1p.

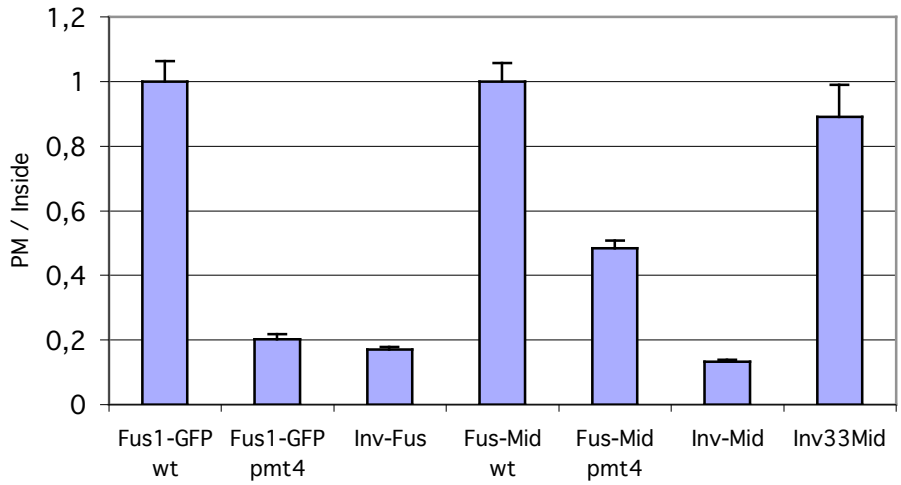


Figure 16. Quantification of the plasma membrane delivery of different protein constructs. Images of cells expressing different constructs were taken using the same conditions and the intensity of the fluorescence were measured for more than 40 cells in each experiment using the IpLab software (see Materials and Methods). The bars (with SEM) represent the ratio of the total fluorescence measured for the plasma membrane area and for the intracellular area of each cell.

It was reported many times in polarized epithelial cells that different types of glycosylation might function as a determinants for protein sorting to the apical plasma membrane. Here, the unglycosylated protein was not sorted properly to the cell surface suggesting that also in yeast O-glycosylation can function as a sorting determinant for plasma membrane delivery. The machinery that is responsible for glycosylation-dependent sorting at the Golgi remains to be identified.

3.2. A visual screen for sorting mutants in yeast biosynthetic pathways using the systematic deletion array

Amazingly, little is known about the genes that are responsible for sorting and packaging surface cargo into transport containers. Previous screens aimed at identifying this machinery relied e.g. on major growth defects and the internal accumulation of invertase, which has been later shown to be mainly transported by the minor one of the two populations of secretory vesicles (Gurunathan et al., 2002; Harsay and Schekman, 2002). These screens identified mutants that blocked ER-to-Golgi and Golgi-to-plasma membrane transport (Newman and Ferro-Novick, 1987; Novick et al., 1980; Novick and Schekman, 1979). However, the problem is that mutations in regulators of sorting and vesicle formation can be expected to exhibit only weak phenotypes in the transport of cargo to the cell surface due to partial rerouting from the affected to the undisturbed pathway (Gurunathan et al., 2002; Harsay and Schekman, 2002). We designed a novel screening procedure devised to circumvent this problem. We aimed at developing an assay sensitive enough to detect subtle kinetic delays or sorting defects within the secretory pathway and applicable to genome-wide screening.

We assumed that when one branch of the bifurcated exocytic pathway is not functional cargo should partially accumulate in the Golgi due to the kinetic delay in cargo transport through the remaining pathway. We expected that such a small defect in exit from the Golgi could be detected in the microscope when we express a marker protein fused to the GFP.

3.2.2. Characterization of the marker protein used for screening

The pre-requisite for a successful screen was to design a cargo protein that could be followed by microscopy after synthesis at the ER throughout the secretory pathway up to the cell surface. For the first screen we decided to use Fus-Mid-GFP (Fig. 17A) since we were interested in the identification of a machinery responsible for glycosylation dependent sorting. This protein was efficiently transported to the

plasma membrane and was more stable than Pma1-GFP or Fus1-GFP, making it a suitable probe for our screen. Fus-Mid-GFP was mostly recovered in a detergent resistant membrane (DRM) fraction in fractionation experiments (Fig. 17C). In a conditional mutant that fails in production of sphingolipids, *lcb1-100,* (Sutterlin et al., 1997), Fus-Mid was no longer transported to the cell surface at the restrictive temperature but was instead missorted to the vacuole (Fig. 17D). These results suggest that Fus-Mid requires raft-association for correct targeting.

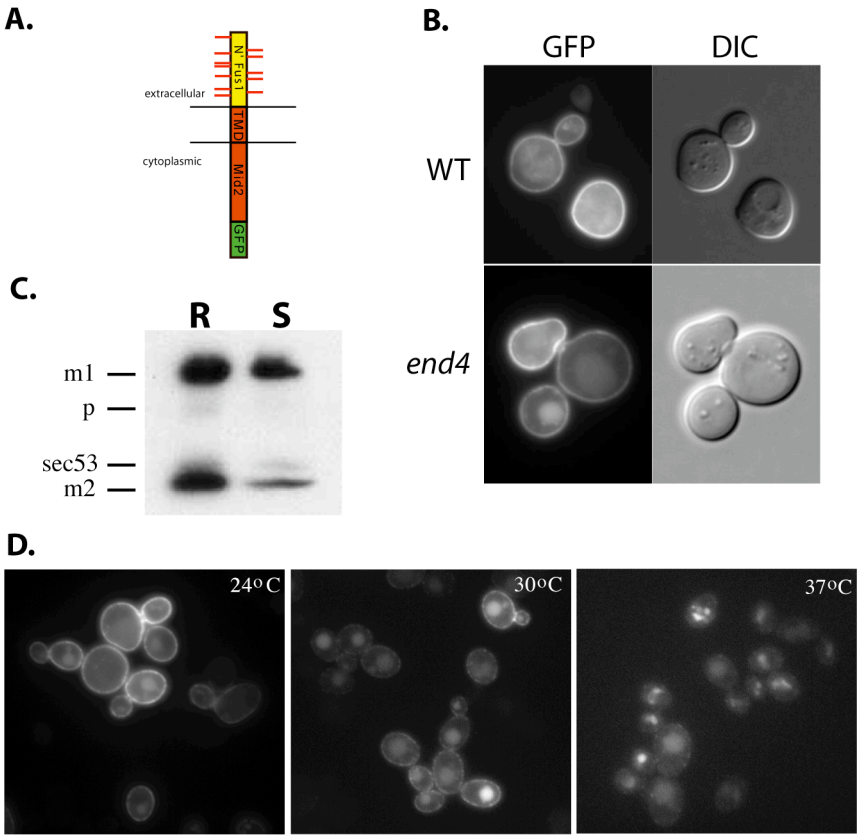


Figure 17. Characterization of the Fus-Mid-GFP marker. (A) Schematic representation of Fus-Mid-GFP. This construct consists of the extracellular portion of Fus1p (yellow) which is O-glycosylated (red lines) fused to the transmembrane domain (TMD) and cytoplasmic tail of Mid2p (red) followed by the GFP-tag (green). (B) Cellular localization of Fus-Mid-GFP in wild-type and *end4Δ* mutant cells. Note that in wild-type cells, subtle vacuolar labeling is observed. This is apparently a result of sorting of the probe directly to the vacuole as it is also observed in the endocytosis mutant. (C) Fus-Mid-GFP is raft-associated. Detergent-resistant, R, and soluble, S, membrane fractions were separated by Optiprep density centrifugation. The protein was recovered mostly in the detergent-resistant membrane (DRM) fraction. In Western blot analysis, Fus-Mid is detected as four different bands (see Fig. 9). Fus-Mid is generated as unglycosylated precursor (sec53 indicates the position of the unglycosylated form of the protein which is found in a *sec53* mutant blocking glycosylation), is then partially glycosylated

in the ER resulting in the precursor form (p). Mature, completely glycosylated protein migrates with lower mobility (m1) and is cleaved to yield higher mobility form (m2). (D) Cell surface delivery of Fus-Mid-GFP is blocked in a conditional-lethal sphingolipid mutant, *lcb1-101*. Cells were incubated at 24°C, 30°C and 37°C and processed for fluorescence microscopy. Fus-Mid-GFP expression was induced simultaneously with the temperature-shift. At restrictive temperature, the protein was no longer transported to the cell surface but instead accumulated in the vacuole.

A centromeric plasmid carrying Fus-Mid-GFP under the control of the inducible *GALs* promoter (used in previous project) was introduced into the entire deletion library encompassing about 4848 single knockouts of non-essential genes (European Saccharomyces cerevisiae Archives for Functional analysis or EUROSCARF; http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). For introduction of the plasmid, we developed a simple protocol for efficient, non-automated transformation in the 96-well format (see Materials and methods). We obtained 93% transformation efficiency using this procedure.

After selection of transformed cells, they were grown up overnight, and before microscopy expression of the GFP-tagged marker was induced for 3 to 4 hours. After this time of induction, robust and bright labeling of the plasma membrane was detected in wild-type cells (Fig. 17B and 18). We also found weak labeling of vacuoles. This labeling was at least in part due to direct biosynthetic transport of a fraction of the GFP-tagged marker to the vacuole because vacuolar staining was also observed in the endocytosis mutant *end4* (Fig. 17B). The vacuolar delivery turned out to provide additional value to the screen because this way we could also assess the role of genes regulating biosynthetic transport to the vacuole.

The entire deletion library was screened by individual microscopic inspection of each mutant. We were particularly interested in the identification of mutants defective in sorting and delivery of the probe to the cell surface. Because the exocytic pathways seem to be partially redundant we expected to observe only a partial redistribution of the marker, with internal fluorescence in addition to the expected plasma membrane labeling.

3.2.3. Phenotype classes and genes showing internal accumulation

Three distinct phenotypes of Fus-Mid mislocalization compared to wild-type cells were observed that we named type I, II and III (Fig. 18). Type I mutants show dot-like

intracellular labeling in addition to plasma membrane labeling. Type II mutants exhibit exaggerated vacuolar and reduced plasma membrane fluorescence probably due to increased missorting of Fus-Mid into a vacuolar rather than a surface-directed pathway. Type III mutants are characterized by a lack of vacuolar labeling indicating defective transport of the marker to the vacuole while plasma membrane delivery remains functional. Thus, by visual screening we could distinguish different sorting and transport defects.

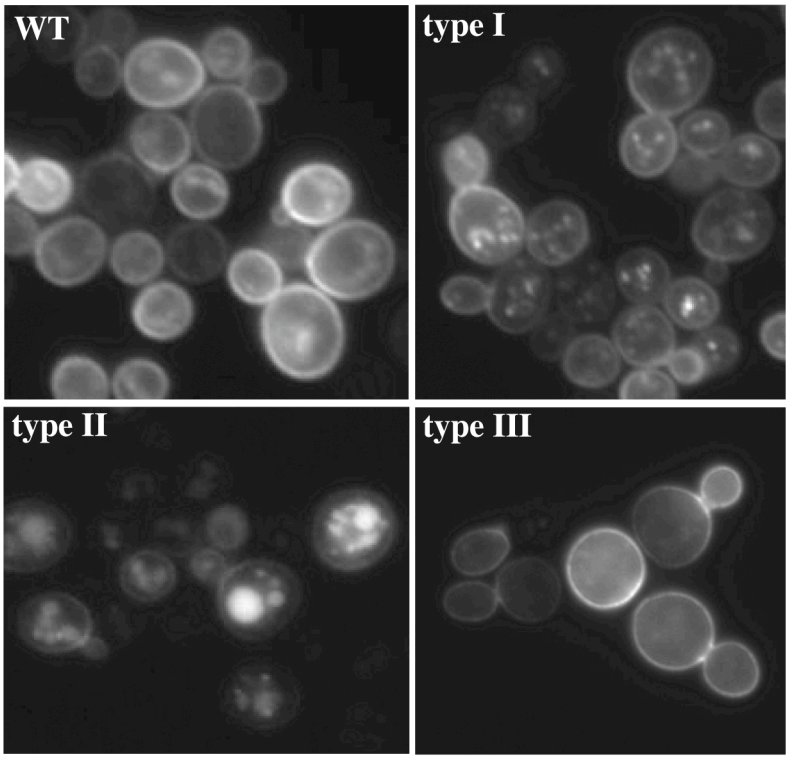


Figure 18. Different classes of phenotypes were observed in the screen. Cells transformed with Fus-Mid-GFP were grown overnight, transferred into inducing media, incubated for 4 h and observed by fluorescence microscopy. In wild-type cells, bright and robust plasma membrane labeling was observed. In addition, some vacuolar fluorescence was found. Type I mutants showed an internal dot-like staining in addition to plasma membrane fluorescence. In type II mutants, the probe was mostly accumulated in the vacuole coincident with reduced plasma membrane staining. Conversely, we also found mutants without vacuolar labeling and intense plasma membrane signal (type III).

We found 466 mutant strains that displayed phenotype I-III. Among these mutants there were genes that were involved in ribosomal function and translation, transcription and mitochondrial function. These were not looked at further because their role in post-Golgi traffic was bound to be mostly indirect. Also previous screens

have identified genes from these categories (Bonangelino et al., 2002; Davydenko et al., 2004; Muren et al., 2001). We also had a group of genes listed as 'Other functions'. Only 10 genes from this list showed a clearcut phenotype I or II. Also these mutant strains were not further pursued because they could not be placed in our functional context.

We had an additional category that displayed clearcut phenotype III lacking vacuolar staining (Fig. 18). These mutants represent for the most part genes previously linked to different aspects of vacuolar protein sorting and vacuole morphology or function: *vps* (vacuolar protein sorting) mutants, *vam* (vacuole morphology) mutants, *pep* mutants with altered vacuolar peptidase activity and *vac* or vacuole inheritance mutants (Bankaitis et al., 1986; Jones, 1977; Robinson et al., 1988; Rothman and Stevens, 1986; Wada et al., 1992; Weisman et al., 1990). A number of these mutants have also been isolated in a recent genomic screen for *VPS* genes (Bonangelino et al., 2002).

Since we were interested in sorting and membrane transport from the TGN to the cell surface, we focused our analysis on mutants of Type I and II that were classified as having membrane trafficking functions, affecting the cytoskeleton or lipid metabolism as well as those genes with unknown function. This list totaled 25 genes (Table 2). The images showing phenotypes for these mutants can be found at the website we created for the results of the visual screen (http://tds.mpi-cbg.de/yeast). In all these mutants, a majority of the cells showed the described phenotype. In order to understand in which compartment Fus-Mid accumulates, we next performed co-localization experiments using a second fluorescent marker. For this, we co-expressed in mutant cells either Sec7-DsRed to mark the TGN or DsRed-FYVE domain, a phosphatidylinositol 3-phosphate-binding domain found on endosomal membranes together with the Fus-Mid-GFP construct. TGN accumulation was observed in mutants of lipid metabolism (*ypc1, ayr1, erg4*) and cytoskeleton organization (*rvs161, pac10, vrp1*) (Table 2). In *sur4*/*elo3* and *sur2* mutants, Fus-Mid-GFP accumulated in vacuoles or vacuoles and at the TGN, respectively. Mutants in genes involved in endosome function as well as *rim8* and *rim21* accumulated Fus-Mid-GFP at endosomes (Table 2). Among mutants of uncharacterized ORFs either endosomal or

TGN accumulation of the GFP-tagged cargo was observed. The results of the colocalization studies are summarized in Table 2, and images can be found at the website (http://tds.mpi-cbg.de/yeast).

ORF	Gene name	Function	Cargo accumulation
YCL001W-A	**YCL001W-A**	Uncharacterized	Golgi
YGL015C	**YGL015C**	Uncharacterized	Golgi
YLR296W	**YLR296W**	Uncharacterized	Endosome
YLR338W	**YLR338W**	Uncharacterized	Golgi/Endosome
YOR318C	**YOR318C**	Uncharacterized	Endosome
YLR372W	**SUR4**	Lipid metabolism	Vacuole
YDR297W	**SUR2**	Lipid metabolism	Golgi/Vacuole
YBR183W	**YPC1**	Lipid metabolism	Golgi
YIL124W	**AYR1**	Lipid metabolism	Golgi
YML008C	**ERG6**	Lipid metabolism	ND
YGL012W	**ERG4**	Lipid metabolism	Golgi
YLR330W	**CHS5**	TGN exit	Golgi
YPL145C	**KES1**	TGN exit	Golgi
YCR009C	**RVS161**	Actin organization	Golgi
YLR337C	**VRP1**	Actin organization	Golgi
YNL153C	**GIM3**	Actin organization	ND
YGR078C	**PAC10**	Actin organization	Golgi/Endosome
YFL023W	**BUD27**	Actin organization	Golgi
YDR080W	**VPS41**	Vacuole transport	Endosome
YGL124C	**MON1**	Vacuole transport	Golgi/Endosome
YML001W	**YPT7**	Vacuole transport	Endosome
YFR019W	**FAB1**	Vacuole transport	Endosome
YOR306C	**MCH5**	Vacuole transport	Endosome
YGL045W	**RIM8**	Vacuole transport	Endosome
YNL294C	**RIM21**	Vacuole transport	Endosome

Table 2. List of mutants showing phenotypes I and II. Functional assignment for identified genes was based on description in YPD (www.incyte.com). The listed deletion strains display either strong or moderate phenotypes and were verified in four independent experiments. The preferred site of internal accumulation of the Fus-Mid-GFP probe was assessed in co-localization experiments where cells were in addition transformed with either Sec7-DsRed to label the TGN or DsRed-FYVE to label endosomes. All images can be found at the website (http://tds.mpi-cbg.de/yeast). In the *erg6* and *gim3* mutants co-localization experiments were unsuccessful due to poor growth of double transformants (ND - not determined).

3.2.4. Mutants in genes regulating synthesis of lipids

We identified 6 mutants in genes regulating synthesis of sphingolipids (*sur4*/*elo3*, *sur2*/*syr2*, *ypc1*, *ayr1*) and ergosterol (*erg6*, *erg4*) (Fig. 19). As shown in Figure 17D, mutation in *LCB1*, an essential gene required for sphingolipid synthesis, resulted in

missorting of Fus-Mid to the vacuole. The *sur4*/*elo3* mutant showed the most severe phenotype with an accumulation of the marker in the vacuole and an obvious reduction in plasma membrane fluorescence intensity (Fig. 19). According to our colocalization experiments, accumulation of Fus-Mid in *sur2*/*syr2Δ* and *ypc1Δ* was in vacuoles and TGN, or mostly TGN, respectively (Table 2). The ergosterol synthesis mutants *erg4* and *erg6* showed primarily an accumulation at the TGN (Table 2). Thus, defective sphingolipid and ergosterol synthesis results in inhibition of trafficking or sorting defects of Fus-Mid from the TGN. We also identified Ayr1p to give a phenotype I showing Fus-Mid-GFP accumulation in the late Golgi (see http://tds.mpi-cbg.de/yeast). This protein has a 1-acyldihydroxyacetone-phosphate-reductase activity and was also reported to interact genetically with YBR159W the major 3-ketoreductase important for fatty acid elongation (Han et al., 2002).

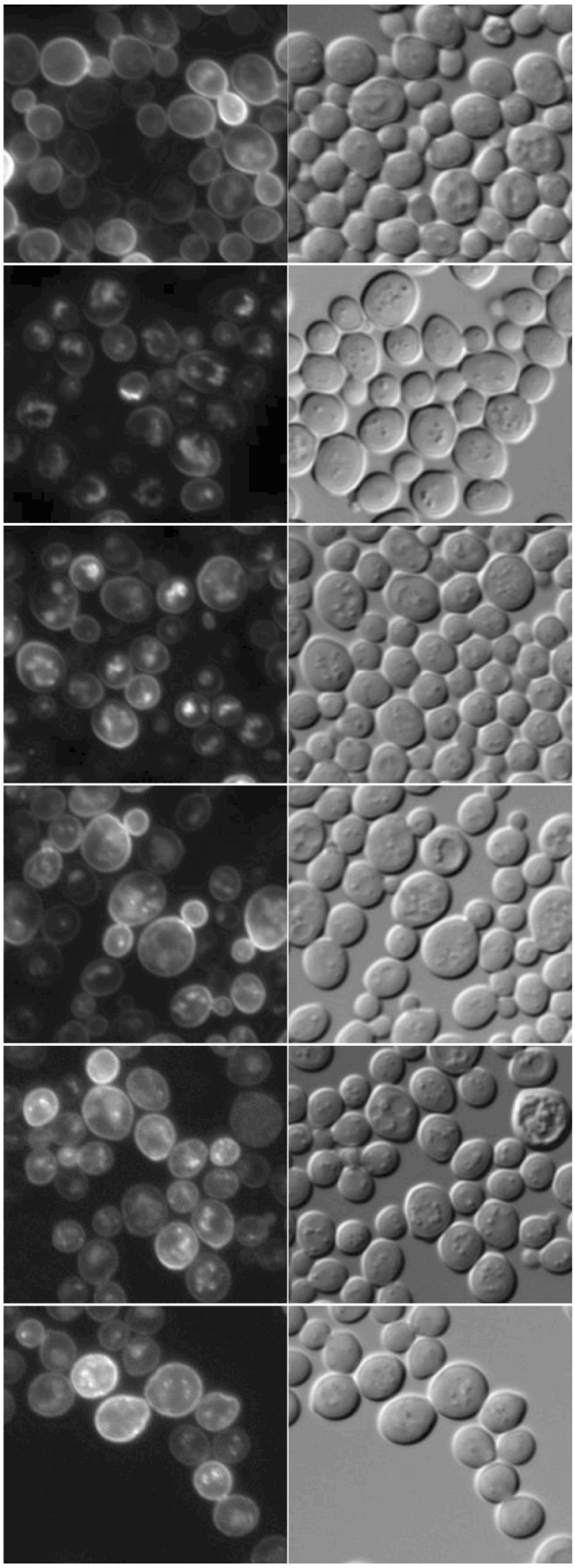


Figure 19. Deletion mutants of genes involved in lipid metabolism showing phenotype I and II. Cells transformed with Fus-Mid-GFP were grown overnight, transferred into inducing media, incubated for 4 h and observed by fluorescence microscopy. The deletion of *SUR4/ELO3* regulating sphingolipid metabolism resulted in a partial loss of plasma membrane labeling and accumulation of the marker in vacuoles. Deletion of other enzymes of this pathway (*SUR2* and *YPC1*, shown here and also *AYR1*, see http://tds.mpi-cbg.de/yeast) and of genes of ergosterol metabolism (*ERG6* and *ERG4*) showed phenotype I.

3.2.5. Mutants in genes of known membrane transport function

Phenotype I was also observed in deletion mutants of a couple of known proteins that participate in membrane transport from the TGN, namely *chs5* and *kes1* (Fig. 20). In agreement with a role of Chs5p and Kes1p at the late Golgi, we found the accumulation of Fus-Mid-GFP in the Sec7p-containing compartment (Table 2). Chs5p is a peripheral membrane protein of unknown molecular role important for Golgi to plasma membrane transport of Fus1p (Santos and Snyder, 2003) and chitin synthase III (Chs3p; (Santos and Snyder, 1997)), an enzyme required for synthesis of the polysaccharide chitin (Santos et al., 1997).

Phenotype I accumulation at the TGN was also observed in *kes1* mutants (Fig. 20). Kes1p/Osh4p is a member of the oxysterol binding protein family that localizes to the TGN via its PH and Oxysterol binding protein (OSBP) domain (Li et al., 2002b).

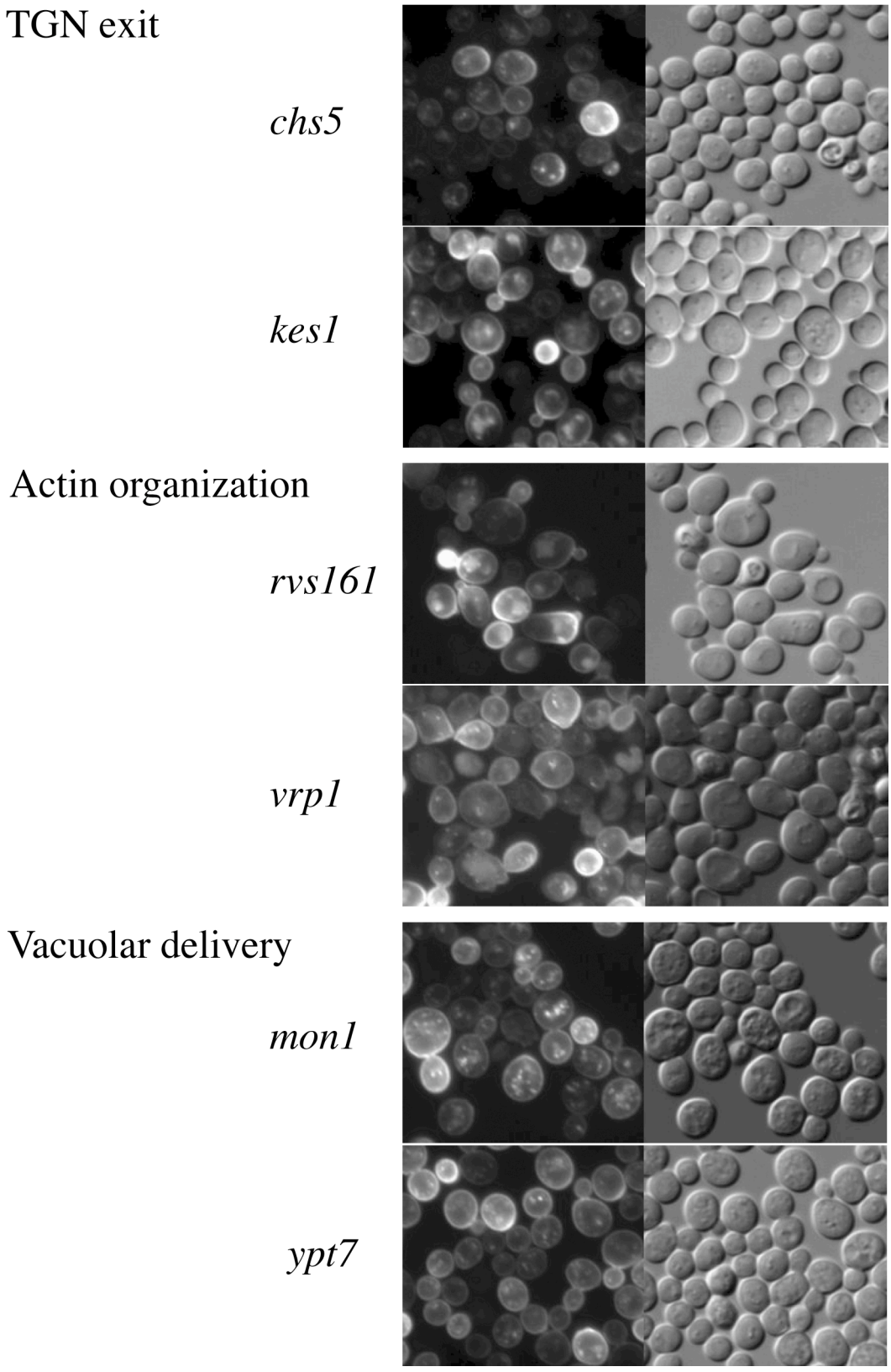


Figure 20. Deletion mutants of genes acting at the exit from the TGN, in actin organization and vacuolar delivery showing phenotype I. Cells transformed with Fus-Mid-GFP were grown overnight,

transferred into inducing media, incubated for 4 h and observed by fluorescence microscopy. Only examples for these functional groups are shown (see Table 2).

3.2.6. Cytoskeleton mutants and mutants of the prefoldin complex

We also found phenotype I in two mutants of genes involved in the organization of the cytoskeleton, *rvs161* and *vrp1* (Fig. 20). Vrp1p (verprolin, a homolog of mammalian Wiskott-Aldrich syndrome protein) is an actin-binding protein. Deletion of *VRP1* results in defects in growth (Donnelly et al., 1993), endocytosis and actin patch polarization (Munn et al., 1995; Naqvi et al., 1998; Vaduva et al., 1997).

Rvs161p is one of two yeast amphiphysin homologues (together with Rvs167p) that were first identified in a screen for mutations causing reduced viability upon nutrient starvation (Bauer et al., 1993; Crouzet et al., 1991). Rvs161p (and Rvs167p) play a role in cell polarity, actin polarization and endocytosis (Bauer et al., 1993; Durrens et al., 1995; Munn et al., 1995; Sivadon et al., 1995).

We further observed internal accumulation in mutants of the prefoldin complex: *gim3Δ* and *pac10Δ*, and of the prefoldin *bud27Δ* (Table 2, for pictures see http://tds.mpi-cbg.de/yeast) . The prefoldin complex acts as a chaperone for the assembly of actin and tubulin (Geissler et al., 1998; Siegers et al., 1999). We can only speculate that the trafficking defect observed in these mutants of the prefoldin complex is related to their role in organization of the cytoskeleton. Bud27/Uri (for Unconventional prefolding RBP5 Interactor) has been shown to be involved in the TOR pathway that coordinates nutrient availability with cell growth and proliferation (Gstaiger et al., 2003). The role of this protein in membrane transport will require further investigation.

In summary, several proteins involved in cytoskeleton organization affected TGN-to-plasma membrane transport of Fus-Mid. This could be due to a role of these proteins in vesicle formation at the TGN and/or transport of the secretory vesicles carrying the cargo in the case of Vrp1p and Rvs161p.

3.2.7. Mutants of genes involved in vacuolar sorting

Internal accumulation of Fus-Mid at endosomes was observed in mutants involved in biosynthetic traffic to the vacuole: *VPS41, MON1,MCH5, YPT7* and *FAB1* (Table 2,

Fig. 20, for pictures see http://tds.mpi-cbg.de/yeast).

The block of protein transport in these mutants apparently in a late step of TGN-to-vacuole delivery resulted in an accumulation of cargo in an endosomal compartment.

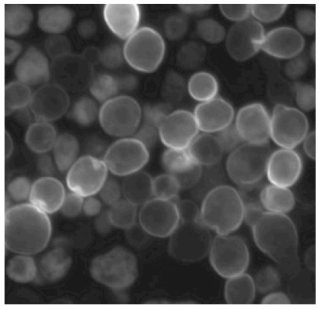
In addition, we also found endosomal accumulation of Fus-Mid-GFP in *Rim8* and *Rim21* mutants (Table 2, for pictures see http://tds.mpi-cbg.de/yeast). Rim 21 and Rim8 are members of the Rim pathway initially assigned to function in sporulation and invasive growth (Li and Mitchell, 1997). Recently, it has been reported that Rim pathway members could regulate cargo flux through the endosome by interacting with the ESCRT machinery (Xu et al., 2004).

3.2.8. N-glycosylation is involved in sorting to the vacuole

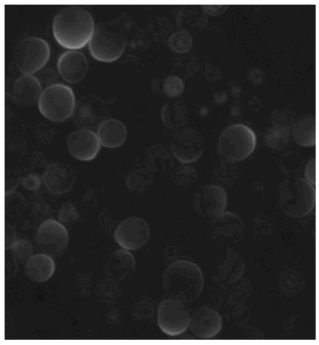
We also found blocked vacuolar transport (phenotype III) in mutants of genes involved in N-glycosylation or carbohydrate chain modification: *mnn10, mnn11* and *anp1* encoding for three components of a Golgi mannosyltransferase complex, and *och1*, an alpha-1,6-mannosyltransferase (Fig. 21). The phenotype observed for these mutants is similar to that found for some known *vps* mutants (Figure 22). Interestingly, Bonangelino et al. (Bonangelino et al., 2002) found that mutants affecting N-glycosylation missort the vacuolar marker carboxypeptidase Y (CPY), an N-glycosylated protein, to the cell surface. Our observations implicate that the addition of N-glycans is not only necessary for correct targeting of CPY but may be required for the proper functioning of the entire pathway because Fus-Mid-GFP itself is not N-glycosylated. How N-glycosylation plays a role in the CPY pathway is currently unknown.

Figure 21. Deletion mutants of genes regulating N-glycosylation showing phenotype III. Cells transformed with Fus-Mid-GFP were grown overnight, transferred into inducing media, incubated for 4 h and observed by fluorescence microscopy. Vacuolar labeling with the marker was absent in mutants of genes involved in N-glycosylation. Note that in deletion mutants of *OCH1* and *MNN10* some cells are showing autofluorescence. The *mnn9* mutant showed a weaker phenotype than the others.

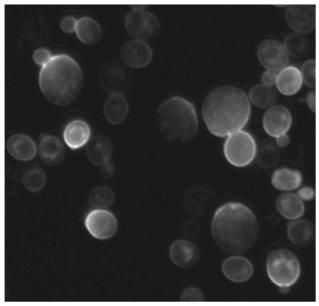
och1



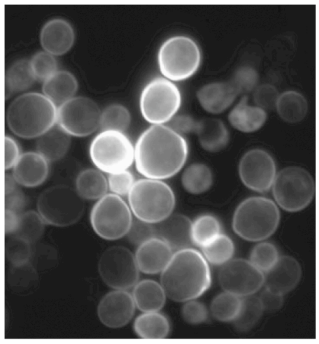
anp1



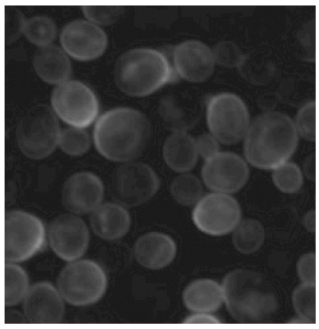
mnn11



mnn10



mnn9



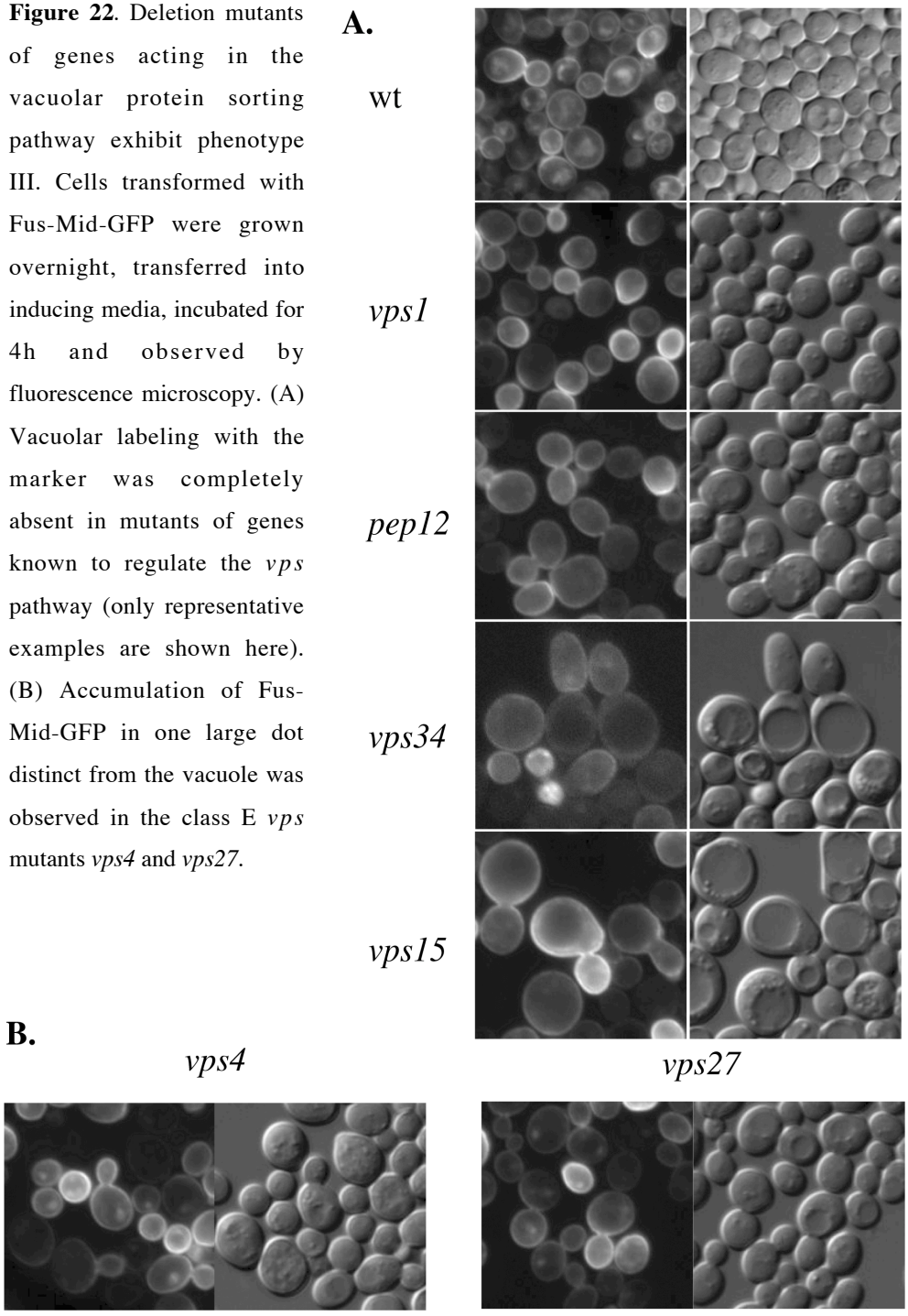


Figure 22. Deletion mutants of genes acting in the vacuolar protein sorting pathway exhibit phenotype III. Cells transformed with Fus-Mid-GFP were grown overnight, transferred into inducing media, incubated for 4h and observed by fluorescence microscopy. (A) Vacuolar labeling with the marker was completely absent in mutants of genes known to regulate the *vps* pathway (only representative examples are shown here). (B) Accumulation of Fus-Mid-GFP in one large dot distinct from the vacuole was observed in the class E *vps* mutants *vps4* and *vps27*.

3.2.9. Uncharacterized mutants

Five uncharacterized ORFs were among mutants exhibiting clearcut phenotype I (Fig. 23). Uncharacterized ORFs with phenotype I accumulated cargo at either Golgi (ycl001w-a, ygl015c, ylr338w) or endosomes (ylr296w, yor318c). Sequence analysis demonstated that the YLR338W locus overlaps with the Vrp1p coding region which was isolated in our screen as a mutant with phenotype I. YCL001W-A and YLR296W mutants show sensitivity to the anticholesterol drug Lovastatin (Giaever et al., 2004).

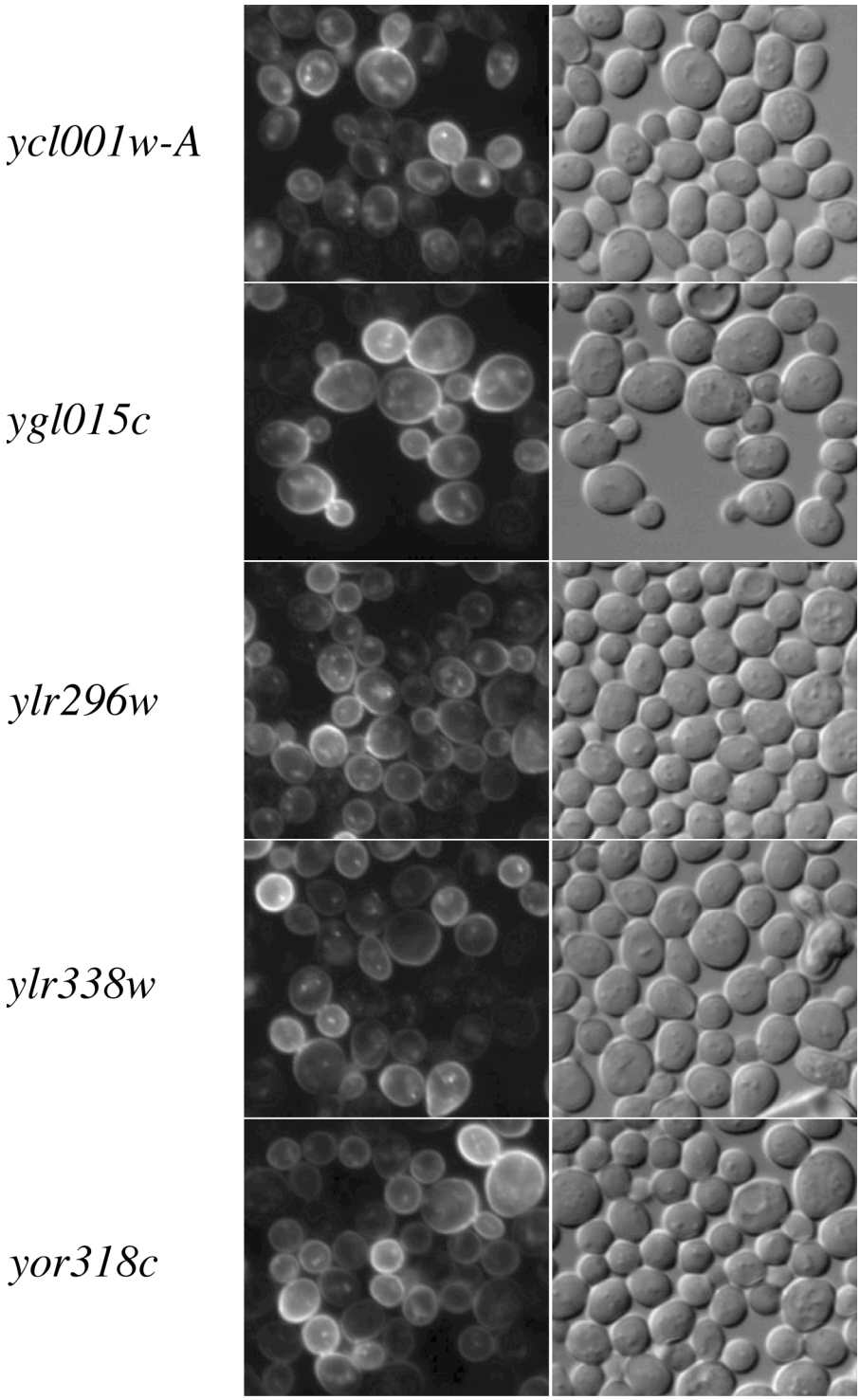


Figure 23. Deletion mutants of uncharacterized genes showing phenotype I. Cells transformed with Fus-Mid-GFP were grown overnight, transferred into inducing media, incubated for 4 h and observed by fluorescence microscopy. Note the dot-like internal accumulation of the probe.

3.2.10. Summary of results

We developed a new screening approach for non-essential genes involved in Golgi to plasma membrane sorting. The visual screening is sensitive enough to detect small defects in protein sorting expected to occur when only one branch of the bifurcated pathways is functional. This unbiased screen revealed a requirement of several enzymes regulating the synthesis of raft lipids (sphingolipids and ergosterol) in the correct and efficient delivery of Fus-Mid-GFP to the cell surface. Also, we found mutants of the cytoskeleton, several unknown genes and known membrane traffic regulators Kes1p, Rvs161p and Chs5p.

3.3. Mechanisms for cell surface polarization during yeast mating

Two different models for proteins localization to the tip of the mating projection were proposed. One model is based on lipid raft clustering of proteins involved in the cell-cell adhesion and fusion machinery responsible for mating (Bagnat and Simons, 2002a). Another model was recently put forward, employing an ongoing cycle of endocytosis and polarized delivery of membrane into tips of shmoos (Valdez-Taubas and Pelham, 2003).

3.3.1 Polarized delivery of membrane proteins in shmooing cells

To revisit the kinetic recycling model we have analyzed the role of polarized delivery and endocytosis in polarizing Fus1p, a type I transmembrane protein involved in cell fusion (Trueheart and Fink, 1989), to the tip of the mating projection. We first analyzed shmoo tip delivery of Fus1p and compared it to another marker protein that is distributed all over the plasma membrane of mating cells, Mid2p. Mid2p is a cell integrity sensor and similarly to Fus1p it is a type I transmembrane protein (Philip and Levin, 2001). One hour after induction of expression the marker proteins were delivered to the shmoo tip where both were localized at this point. However, 2 hours later Mid2p had diffused over the entire plasma membrane while Fus1p remained at the tip.

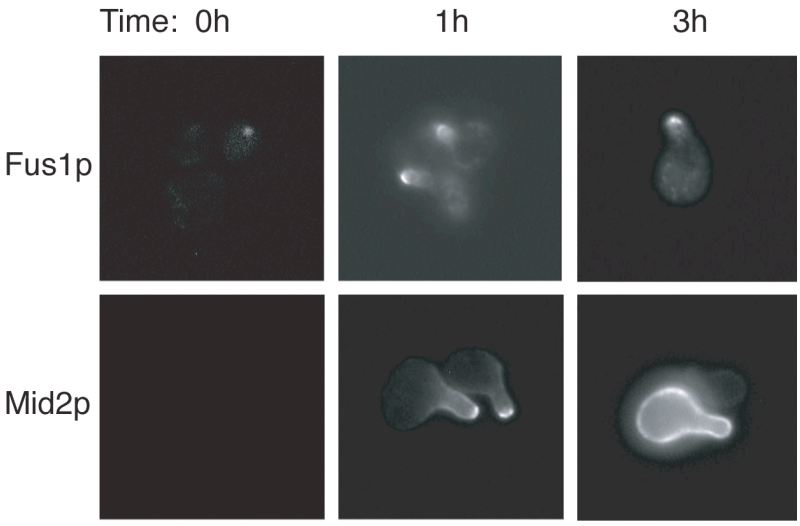


Figure 24. Polarized exocytosis to the tip of the mating projection. Localization of GFP-tagged Fus1p and Mid2p at different time points after expression induction in shmooing cells. Wild-type cells carrying plasmids MBQ30 or MBQ35 were treated with α-factor for 3h and after that, galactose was added to induce protein expression (For details see materials and methods).

3.3.2 Polarization of Fus1p to the tip of the mating projection is endocytosis independent

We then analyzed the effect of endocytosis on the process of Fus1p polarization. We also employed Snc1p, a yeast v-SNARE involved in post-Golgi plasma membrane transport, as a second marker protein that is tip localized. Valdez-Taubas had demonstrated that Snc1p polarization was abolished after inhibition of endocytosis (Valdez-Taubas and Pelham, 2003). In *end4*Δ cells in which endocytosis is blocked Snc1p was no longer polarized but was distributed over the plasma membrane of the shmooing cells. On the other hand, the polarization of Fus1p to the tip of the mating projection remained normal in *end4*Δ cells (Fig. 25). Thus, there must be a mechanism that maintains biosynthetically delivered Fus1p at the shmoo tip irrespective of ongoing cycles of endocytosis and exocytosis.

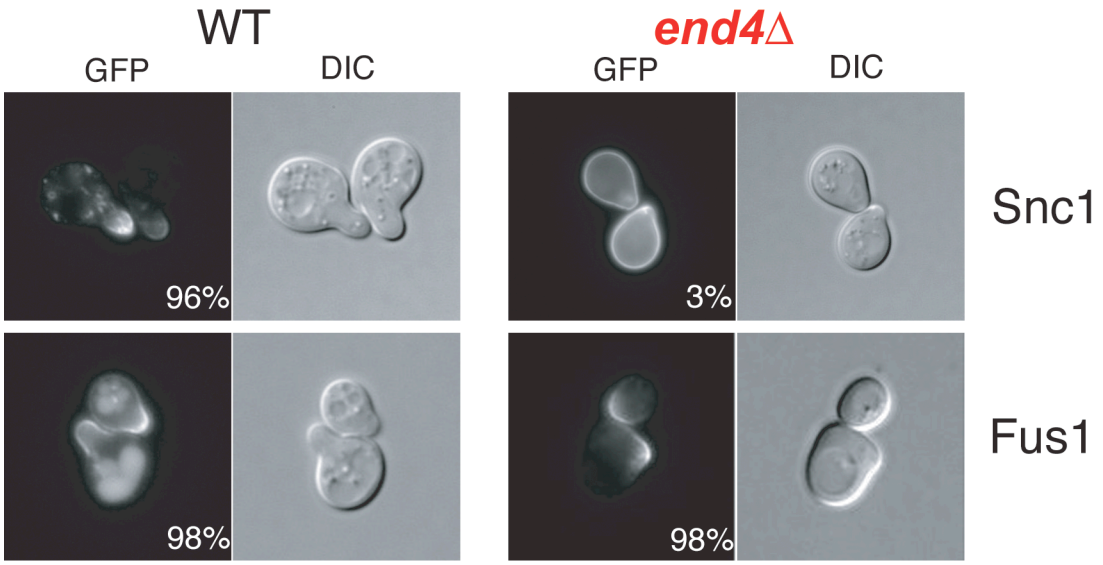


Figure 25. Polarized distribution of Snc1p but not Fus1p is dependent on endocytosis. Blocked endocytosis in *end4Δ* disrupted polarized distribution of Snc1p but did not affected tip localization of Fus1p compare to wild-type cells. The percentage of cells with fluorescence on the plasma membrane limited to the mating projection is given in the bottom right corner of the GFP images. For Fus1p in wild-type and end4Δ we counted 218 and 327 cells respectively, for Snc1p it was 220 and 100 cells respectively. *end4Δ* cells polarizes much slower and the mating projection is often less pronounced then in the wild-type cells but cells obtained their polarized shape what can be observed in the DIC images. In wild-type cells additional to plasma membrane Snc1p is found in endosomes and Golgi due to protein cycling. These structures were not visible in the endocytosis mutant because the protein was trapped on the plasma membrane. Fus1p in wild-type cells was found at the plasma membrane and in the vacuole where protein is sent for degradation.

3.3.3. Retention of Fus1p to the tip of shmoos is mediated by the cytoplasmic domain

One reason why Fus1p is retained at the mating tip could be due to interaction with the cell wall as was demonstrated for GPI-anchored proteins (De Sampaio et al., 1999). Thus, we constructed fusion proteins between Fus1p and Mid2p where we swapped the extracellular, the transmembrane and the cytosolic domains of the two proteins. Analysis of their surface distribution demonstrated that the information for mating tip retention was localized to the cytosolic tail (Fig. 26). We then analyzed how the chimeric protein containing the extracellular and transmembrane domains of Mid2p and the cytosolic tail of Fus1p behaved in mutants in which endocytosis was inhibited both in *end4*Δ cells and at the non-permissive temperature in *end4-1 ts* cells (Fig. 27). This chimeric protein behaved like Fus1p and maintained its polarization when endocytosis was inhibited (Fig. 27). Thus we concluded that the cytosolic tail of Fus1p mediates protein retention at the tip and that interactions with the cell wall cannot explain the polarization.

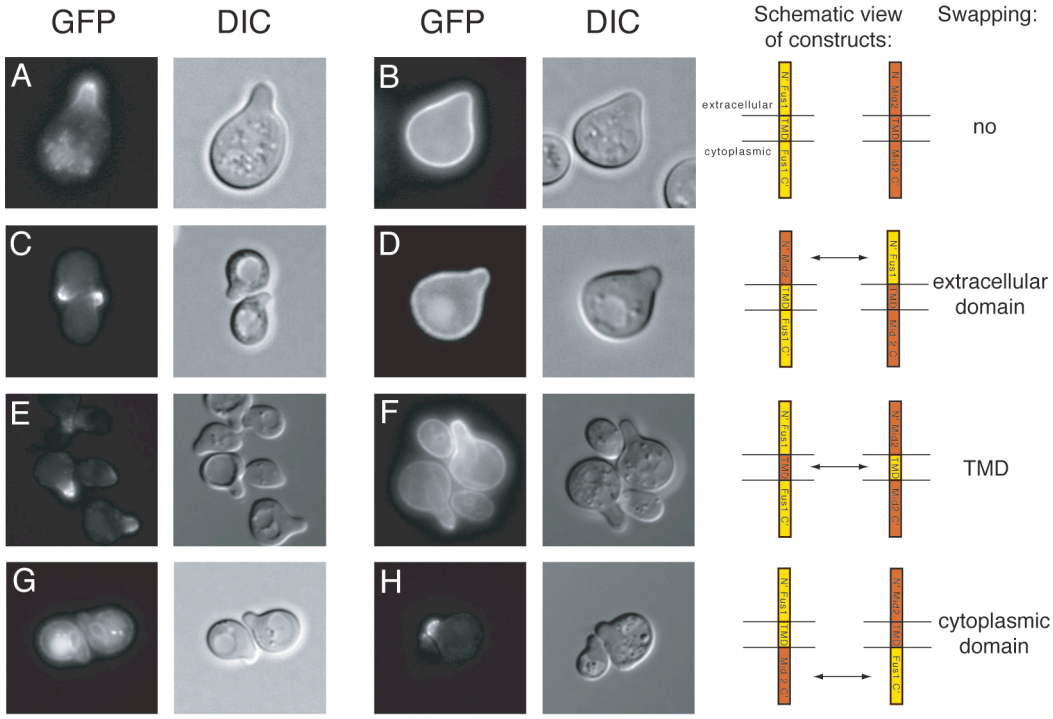


Figure 26. Cytoplasmic tail of Fus1p is responsible for proteins polarized localization. Fus1p, Mid2p and different chimeric proteins were expressed in wild-type cells treated with α-factor. The schematic representation of expressed fusion proteins is shown on the right side of the panel and swapped domains are indicated. The yellow and red colors specify *FUS1* and *MID2* sequence respectively. On

pictures F and G there is some internal membrane staining visible what might suggest that proteins sorting to the cell surface is compromised. However, it is important that fraction of protein that was delivered to the plasma membrane is equally distributed.

3.3.4. Fus1p is embedded in a network of protein-protein interactions responsible for Fus1p localization

The cytosolic tail of Fus1p is 416 amino acids long and contains close to the C-terminus a SH3 domain followed by a proline-rich domain both known to be responsible for protein-protein interactions (Tong et al., 2002). We deleted the SH3 domain from the chimeric protein Mid-Fus used in figure 27 or Fus1p (data not shown) and saw no effect on polarization. At this time, a report from Nelson et al. appeared, in which a detailed analysis of the cytoplasmic domain of Fus1p was described (Nelson et al., 2004). They showed that both domains were important for mating efficiency but even the double mutant protein was polarized normally in wild-type cells. Because mutations in these domains prevented protein interaction with the scaffolding machinery (Nelson et al., 2004) we considered the possibility that the double mutant of Fus1p could be polarized via endocytic recycling mechanism. To test this possibility we expressed the mutated Fus1p in the endocytosis deficient strain, nevertheless, protein polarization was still normal (Fig. 27). We concluded that additional sites on the cytoplasmic tail of Fus1p might contribute to Fus1p retention. By a detailed two-hybrid analysis it was demonstrated that the cytosolic tail of Fus1p interacts with several key players in mating polarity including the GTP-bound form of Cdc42p, with components of the polarisome Pea2p and Bni1p, Fus2p and the Ste5p, the scaffold protein for MAP kinase signaling (Nelson et al., 2004). From these data we propose that Fus1p is directly embedded in a dynamic network of protein-protein interactions that is responsible for scaffolding the protein as part of the mating machinery to the shmoo tip.

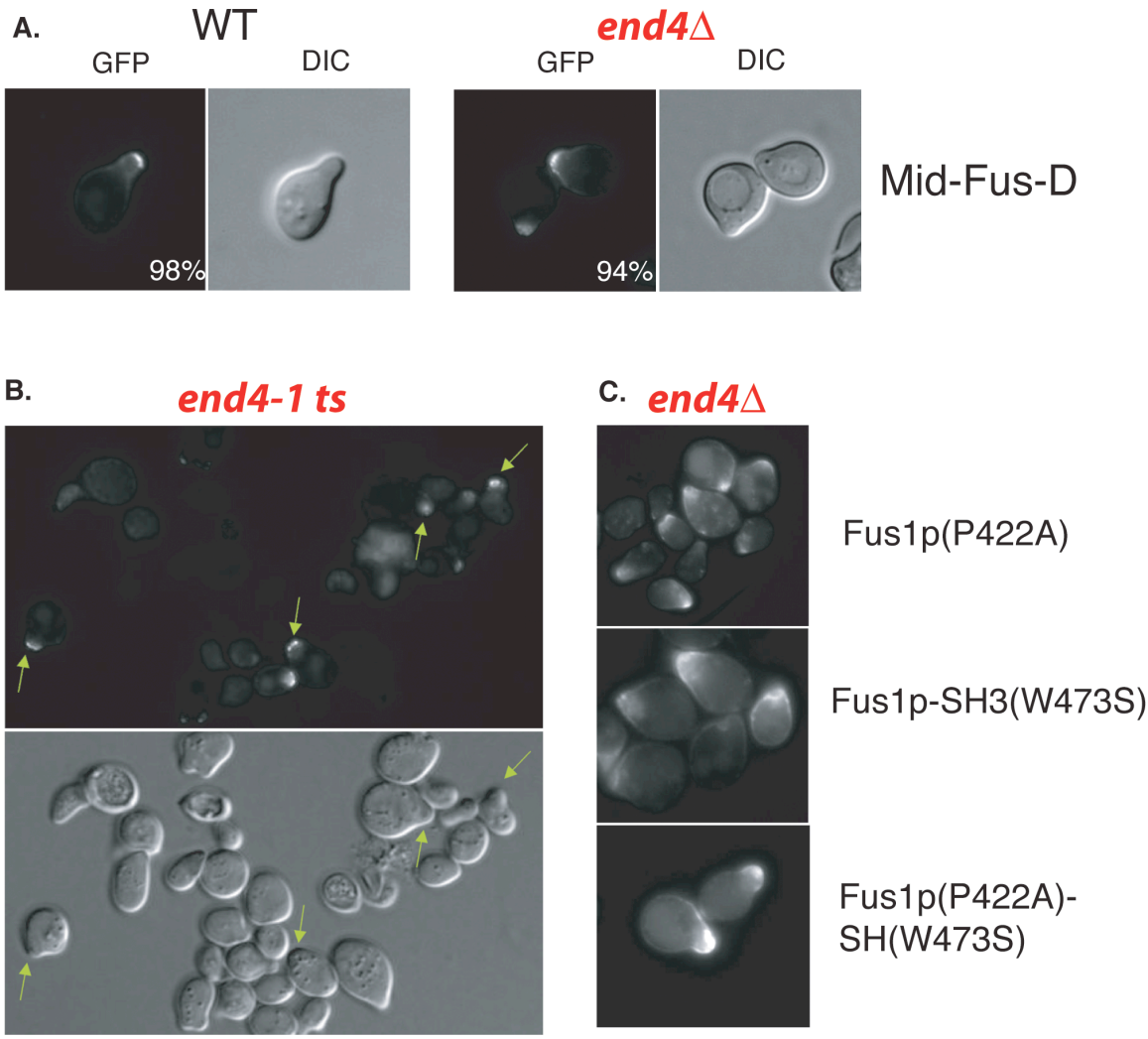


Figure 27. Fus1p does not require the SH3 nor the proline-rich domain to polarize. The chimeric Mid(cyt-FusΔSH3) protein which carries the cytoplasmic tail from Fus1p with deleted SH3 domain is equally well polarized in the wild-type and *end4Δ* cells. The percentage of cells with fluorescence on the plasma membrane limited to the mating projection is indicated (N=361 and 138 for the wild-type and *end4Δ* cells respectively). Protein polarization was also verified in *end4-1 ts* mutant cells. In this experiment galactose and the α-factor was added to the culture and cells were shifted from 24°C to 37°C for 3h (B). Similarly, Fus1p with point mutations that affect the function of the proline-rich region or the SH3 domain (Fus1p(P422A) and Fus1p-SH3(W473S) respectively), or the double mutant of Fus1p (Fus1p(P422A)-SH(W473S)) was polarized on the cell surface in the endocytosis independent manner.

3.3.5. Colnclusions:

Two different mechanisms operate in protein localization to the tip of the mating projection. The polarized distribution of Fus1p is independent of endocytic recycling. Instead Fus1p is linked to the scaffolding machinery via its cytoplasmic domain.

4. Discussion

4.1. O-glycans as a sorting determinant for cell surface delivery in yeast

4.1.1. Summary of results

In these studies we have analyzed the potential role of O-glycans as sorting determinants for surface delivery in yeast. We used Fus1p as our marker protein. Fus1p is an O-glycosylated integral membrane protein (Trueheart and Fink, 1989). Its O-glycosylation starts in the ER through the action of the O-mannosyl transferase Pmt4p (Fig. 8 and 9). Further mannose-groups are added to Fus1p in the Golgi complex and then the processed protein is transported to the plasma membrane (Trueheart and Fink, 1989) (Fig. 9). Even when *PMT4* is deleted a small amount of glycosylated Fus1p was detected (Fig. 8, 9 and 10). Probably in the absence of Pmt4p other members of the PMT family can glycosylate Fus1p but it occurs with low efficiency. We found that when O-glycosylation is blocked in *pmt4*Δ cells Fus1p accumulates intracellularly in punctate structures that co-localize with Sec7p, a Golgi marker (Fig. 13). The surface transport of another O-glycosylated plasma membrane protein, Mid2p was not affected in *pmt4Δ* cells (Fig. 11). Using chimeric constructs consisting of parts of Fus1p and Mid2p we demonstrated that the ectodomain of Fus1p was responsible for the blocked transport to the cell surface in *pmt4*Δ cells (Fig. 11). To find out whether O-glycans were indeed involved in Fus1 sorting and delivery to the cell surface we used a strategy previously employed by Rose and co-workers in mammalian cells (Guan *et al.*, 1985). They demonstrated that a non-glycosylated secretory protein, rat growth hormone was blocked in the Golgi complex when it was anchored to the membrane by fusing the hormone to the transmembrane and cytosolic domains of vesicular stomatitis virus G-protein. However, when one or two N-glycosylation sites were introduced into the growth hormone ectodomain, the N-glycosylated membrane protein was transported to the cell surface. Similarly, when we fused the N-terminal part of the invertase sequence to the transmembrane and cytosolic domains of Fus1p or of Mid2p, these chimeric proteins accumulated

intracellularly (Fig. 14). Next, we inserted segments from the Fus1p ectodomain between the invertase and the Fus1 transmembrane anchor (see cartoon in Fig. 12) in an attempt to find out, whether addition of O-glycans could influence transport to the plasma membrane. When a 33 amino acid segment containing several potential O-glycosylation sites was added to the fusion protein construct, the chimeric protein was transported to the plasma membrane (Fig. 15 and 16). In *pmt4Δ* cells the protein was blocked in the Golgi complex. Biochemical analysis demonstrated that O-glycans were present on the chimeric protein that was delivered to the cell surface in wildtype cells whereas O-glycans were lacking in *pmt4Δ* cells (Fig. 15). Our data suggest that O-glycosylation can serve as a signal for protein transport to the plasma membrane in *S.cerevisae*.

4.1.2. Unglycosylated Fus1p is not degraded

Another problem complicates the analysis of the role of glycosylation in secretion. Inhibition of glycosylation often leads to misfolding of the protein, accumulation and degradation in the ER (Ellgaard and Helenius, 2003; Hampton, 2002; Ng et al., 2000). In *S. cerevisae* also the Golgi complex seems to be a site for quality control (Hong *et al.*, 1996; Jorgensen *et al.*, 1999). Proteins that are not folded correctly or are incompletely oligomerized are singled out for the vacuolar delivery in the Golgi complex. Obviously therefore, one has to differentiate between effects due to quality control mechanisms or to sorting for surface delivery in the Golgi complex. It was reported that Axl2p, involved in bud site selection has altered glycosylation in *pmt4Δ* cells and is rapidly degraded before reaching cell surface, most probably in the Golgi, (Sanders et al., 1999). The kinetics of the degradation process of misfolded proteins is usually fast. The chimeric proteins composed of Fus1p ectodomain and of the Mid2p transmembrane and cytosolic domains in *pmt4Δ* cells were in fact more long-lived than the wild type Fus1p. Thus, we had no indication of misfolding or increased vacuolar delivery. Instead the chimeric protein mostly accumulated in the Golgi complex. We did not observe accumulation of our markers in ER structures (except for Inv33Mid which showed a faint staining of the ER but most of the fluorescence was localized to the plasma membrane) and we did not observe enhanced degradation as was the case for Axl2p (Sanders et al., 1999).

4.1.3. Glycans as a sorting determinants

The first suggestive evidence for carbohydrate side chains of proteins acting as sorting determinants for cell surface delivery came from the work of Rose and co-workers in fibroblasts (Guan *et al.*, 1985; Machamer *et al.*, 1985). Further support for a role of glycans as signals for transport from the Golgi complex to the plasma membrane emerged from studies of epithelial cells. Scheiffele et al. (1995) demonstrated that in epithelial MDCK cells N-glycosylated rat growth hormone was secreted apically while the non-glycosylated native form of the protein was secreted randomly, both apically and basolaterally . This was also the case when a GPI-anchor was added to the rat growth hormone (Benting et al., 1999). Gut and co-workers showed that a protein lacking its basolateral sorting determinants accumulated in the Golgi complex (Gut *et al.*, 1998). Addition of N-glycans to this mutant protein promoted its delivery to the apical surface. Several reports have also demonstrated that O-glycans can serve as apical sorting determinants (Alfalah et al., 1999; Spodsberg et al., 2001; Yeaman et al., 1997). In fact, also glycosylated basolateral proteins will be delivered apically if the basolateral sorting determinants in their cytosolic protein domains are mutated or deleted (Gut *et al.*, 1998). These data demonstrate that it is difficult to analyze sorting determinants for surface delivery in cells with two (or more) pathways from the Golgi complex to the plasma membrane. A protein can switch from one pathway to another.

4.1.4. Potential mechanisms for the glycosylation dependent sorting

If O-glycans of Fus1p function as sorting determinants for delivery to the cell surface the question arises how this signal functions mechanistically. Two models have been proposed for how carbohydrate sorting determinants function in apical transport in epithelial cells. Rodriguez-Boulan and Gonzalez (Rodriguez-Boulan and Gonzalez, 1999) have suggested that glycans change the biophysical properties of an apical protein such that the presentation of a proteinaceous sorting signal to a hypothetical sorting receptor is facilitated. Alternatively, the glycans contribute to a transport-permissive conformation of the apical protein that facilitate its incorporation into lipid rafts and thus into the apical targeting pathway. The latter possibility seems unlikely because it has been shown that raft association by itself is not sufficient for apical

delivery (Rietveld and Simons, 1998; Simons and Ikonen, 1997). Rafts are also routed basolaterally. The second model postulates the existence of apical lectins that bind to the glycans and sort the apical proteins into transport carriers in the trans Golgi network (Scheiffele et al., 1995). However, such a lectin has not yet been identified. According to this model O-glycosylated Fus1p would be bound by a lectin, which facilitates its surface delivery. Our findings that O-glycans promote plasma membrane transport of proteins in yeast provides all the tools that this model organism supplies for identification of the underlying sorting mechanisms.

4.1.5. Machinery for cargo sorting into exocytic routes is not known

As discussed in the introduction we know relatively little about the machinery responsible for sorting of proteins and lipids for cell surface delivery. Numerous genetic screens for secretion mutants identified genes required for ER to Golgi transport and fusion of secretory vesicles with plasma membrane, little is, however, known of how sorting of surface proteins occurs in the Golgi complex. Inactivation of one pathway may be rescued by routing cargo to the other pathway (Gurunathan et al., 2002; Harsay and Schekman, 2002) and does not block surface delivery. The major limitation in identification of genes involved in post-Golgi sorting is the lack of screening methods that would allow to identify mutants that cause missorting of exocytic cargo from one pathway to the other.

4.2. A visual screen for sorting mutants in yeast biosynthetic pathways using the systematic deletion array

4.2.1. A novel visual screening method for identification of secretion regulators

We aimed at developing an assay sensitive enough to detect subtle kinetic delays or sorting defects within the secretory pathway expected for mutants of specific regulators of the redundant exocytic routes from the Golgi to the cell surface. We introduce a novel visual screening strategy to search in a genome wide scale for genes regulating intracellular trafficking.

The probe that we used in our screen was DRM-associated (like Pma1p – marker for light density vesicles) and also carried O-glycans which have been shown to act as a sorting determinant for cell surface delivery (this work) (Proszynski et al., 2004). The chimeric protein Fus-Mid-GFP was missorted to the vacuole (Fig. 17D) like Pma1p in the sphingolipid mutant *1cb1-100* cells (Bagnat et al., 2001). Newly synthesized Fus-Mid-GFP was not only transported to the cell surface but a small fraction was also delivered to the vacuole. This "missorting" seemed to depend on an intracellular route from the Golgi because it was not inhibited by blocking endocytosis from the plasma membrane in the *end4*Δ mutant (Fig. 17B), excluding the plasma membrane as the only source for vacuolar targeting. The partial transport of Fus-Mid-GFP to the vacuole turned out to provide the interesting side product of our visual screen.

Phenotype III was characterized by a lack of vacuolar staining while plasma membrane delivery remained functional. We identified 98 genes exhibiting clear cut phenotype III. Many of these genes have been identified by previous screens for vacuolar protein sorting (Bonangelino et al., 2002; Rothman and Stevens, 1986). Interestingly, 37 mutants showing phenotype III overlapped with the genes obtained in the recent genome-wide screen of non-essential genes by Bonangelino et al. (Bonangelino et al., 2002) who screened for missorting of vacuolar carboxypeptidase Y to the cell surface. The gene products identified include proteins involved in Golgi-endosome transport, endosome maturation and multivesicular body formation and also endosome to Golgi transport. Some of these mutants seemingly block access of Fus-Mid-GFP from the Golgi to the endocytic system such as *vps1* (Fig. 22). Thus,

the only route remaining would be direct transport to the cell surface. Others block vacuolar entry from endosomes and in this case the endocytic system must be cleared of Fus-Mid-GFP by retrieval to the Golgi to give rise to phenotype III. Alternatively, these mutants block entry into the endosomal system like *vps1*. Mutants of genes involved in endosome-to-TGN retrograde transport might deplete the vacuolar delivery route of componenets required for forward transport. The fact that we could identify these mutants attests the power of the visual screen. This approach benefits from the fact that most genes that regulate vacuolar sorting and post-Golgi sorting in general are non-essential.

The major thrust of our screen were phenotypes I and II. Here, the idea was that if the pathway that Fus-Mid-GFP takes to the surface is blocked (or is even partially inhibited), then the remaining pathway gets overloaded and cannot clear the Golgi structures efficiently, thus leading to accumulation of the fluorescence probe in dot like structures inside the cells. An alternative possibility is that the cargo is missorted to the vacuole and cleared from the Golgi (phenotype II) like Fus-Mid-GFP does in *lcb1-100* cells (Fig. 17D).

We also found some mutant strains that led to fluorescence accumulation in the endosomes identified by colocalization with the endosomal marker DsRed-FYVE. The endosome-Golgi distinction was difficult because usually there was partial overlap with both the Golgi marker Sec7-DsRed and the endosomal marker DsRed-FYVE in most mutant strains with usually one location predominating. These compartments are tightly linked by anterograde and retrograde routes. Therefore, it is to be expected that a “traffic jam” in the Golgi leads to a backlog into endosomes.

Gene deletions that led to accumulation mainly in the endosomes included: *vps41*, *mon1*, *ypt7* and *fab1* (Table 2). They are all involved in late vacuole protein transport (Wickner, 2002). Vps41p, a class C Vps protein, is required for formation of AP-3 transport vesicles (Rehling et al., 1999) and is part of the HOPS (homotypic fusion and vacuole protein sorting) complex required for homotypic vacuole fusion (Price et al., 2000; Sato et al., 2000; Seals et al., 2000). Mon1p renders cells sensitive to brefeldin A and monensin when deleted (Muren et al., 2001). The class C Vps/HOPS complex regulates association of the Ccz1-Mon1 complex with the vacuole, which in turn is required for the Ypt7-dependent tethering/docking stage in vacuole homotypic fusion (Wang et al., 2003). Fab1p is a phopshatidylinositol 3-phoshate 5-kinase

involved in prevacuolar sorting and homeostasis (Odorizzi et al., 1998). It seems plausible that blocking of a late step in vacuolar delivery results in an endosomal accumulation of cargo en route to the vacuole, in this case Fus-Mid-GFP.

4.2.2. Lipid metabolism

The most striking result of our efforts to obtain a global view of the genes involved in the Fus-Mid-GFP pathway from the Golgi to the cell surface was the identification of mutant strains with defects in sphingolipid and ergosterol biosynthesis.

Sphingolipid synthesis up to ceramide involves 15 known enzymes (Funato et al., 2002) of which 5 are essential and therefore not subject of the screen. Of the remaining non-essential ones we identified 4 with a sorting phenotype (Fig. 28). It was clear from earlier work that mannosylation of sphingolipids was not important for exocytosis (our unpublished results and (Lisman et al., 2004)) Here, we could show now that alteration in the mixture of sphingolipid molecular species had impact on the sorting of Fus-Mid-GFP. First, *Elo3* mutants incapable of synthesizing C26:0 VLCFAs (Oh et al., 1997) missorted cargo to the vacuole and had weak PM staining. Thus, shortening of VLCFAs by only two carbon atoms results in defects in protein surface delivery. Second, deletion of Sur2p abolishes hydroxylation of the sphingosine backbone and accumulated cargo in the TGN and the vacuole.

Third, knockouts of Ypc1p an enzyme with reported ceramidase but also minor ceramide synthase activity with a substrate preference for phytoceramide (CER-B) showed defects in Golgi exit of our GFP-fusion construct. (Obeid et al., 2002). Intriguingly we also identified Ayr1p in the screen. This protein has been shown to have a 1-acyldihydroxyacetone-phosphate-reductase activity (Athenstaedt and Daum, 2000). However, in addition Ayr1p also seems to be involved in fatty acid elongation because of a 3-ketoreductase activity and could thus contribute to ceramide synthesis (Han et al., 2002). These data suggest that the fatty acid and the long chain base in the ceramide plays a crucial role for proper protein sorting. Such a model would also fit with the observation that the length of the transmembrane domain determines cell surface delivery (Munro, 1995; Rayner and Pelham, 1997). However, at this stage of our investigation we cannot exclude that the phenotypes of the mentioned mutants exhibit indirect effects. It is known i.e. for *elo3*/*sur4* mutants that they accumulate

free VLCFAs (C20:0 and C22:0), which are poor substrates for the ceramide synthase (H.Riezman, personal communication), thus lowering the amount of total synthesized ceramide. Furthermore, it is well known that a number of sphingolipids (i.e. ceramide itself and precursors like phytosphingosine) are potent effector molecules (Ogretmen and Hannun, 2004) and changes in their abundance might have an impact on the cell different than the biophysical effects on membrane domain formation that we discuss here.

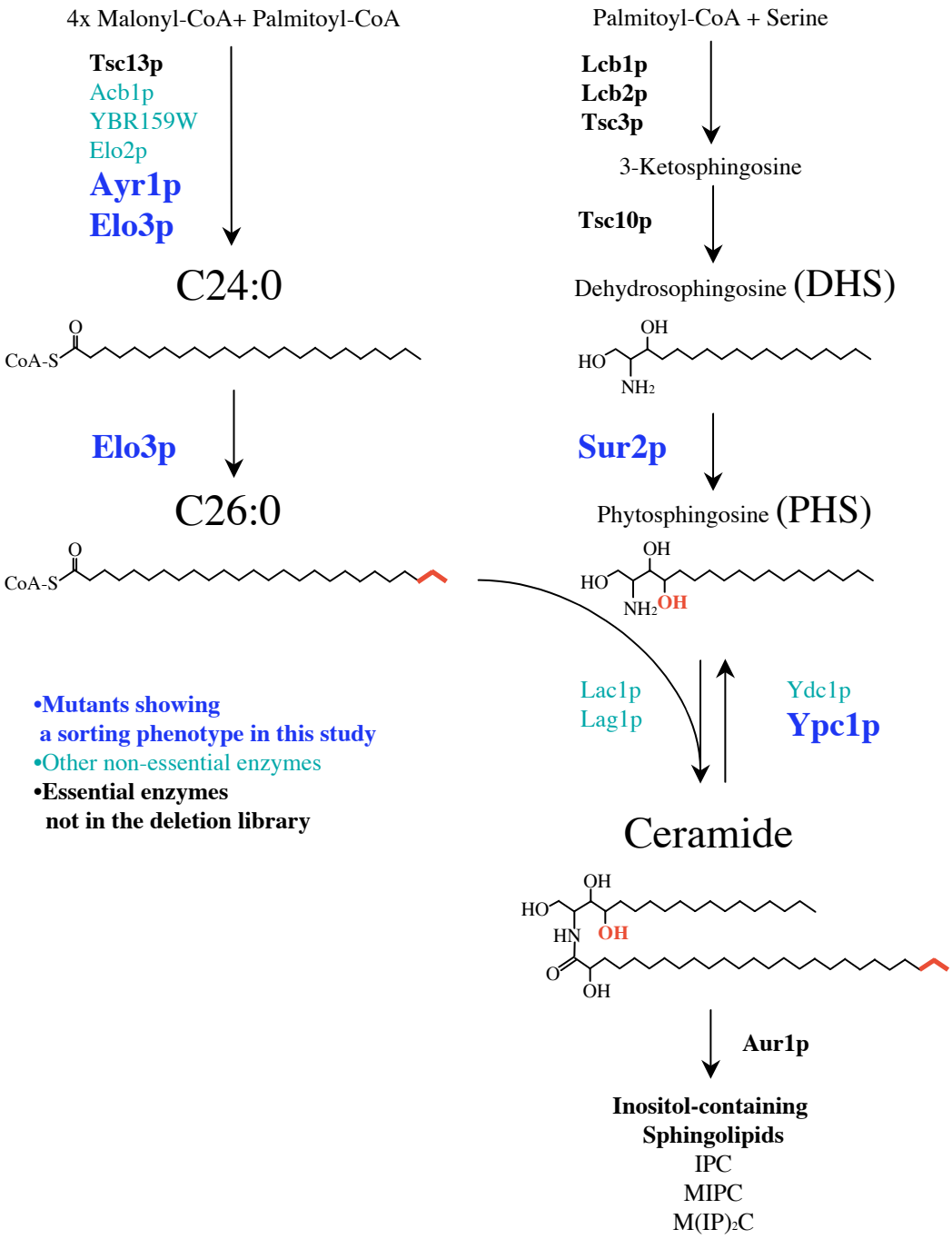


Figure 28. Biosynthetic pathway of sphingolipids in *S.cerevisiae*. Very long chain fatty acids (VLCFA: FA between C20:0 and C26:0) and long chain bases (LCB, which are dihydrosphingosine: DHS or phytosphingosine: PHS) get amide linked by the ceramide-synthase (containing Lag1p and Lac1p) which produces ceramides.

VLCFAs are synthesized starting from palmitoyl-CoA (C16:0) up to C26:0 by a concert of several enzymes. For the elongation process from C20:0 to C24:0 either Elo2p or Elo3p is sufficient. However, for the elongation of C24:0 to C26:0 Elo3p is necessary.
LCB synthesis starts with the condensation of L-serine and palmitoyl-CoA by the complex serinepalmitoyltransferase (SPT) comprising Lcb1p, Lcb2p and Tsc3p. The resulting 3-ketosphingosine gets reduced to DHS by Tsc10p and then hydroxylated on C-4 to PHS by Sur2p. Note that both, PHS and DHS can serve as substrate for Lac1p/Lag1p resulting in phytoceramide (PHC) and dihydroceramide (DHC), respectively. Dihydroceramide can then be converted to phytoceramide also by the C-4 hydroxylase Sur2p.
The hydrolysis of ceramides is carried out by two alkaline ceramidases: Ydc1p and Ypc1p with Ypc1p having higher specifity for PHC over DHC and Ydc1p preferring DHC over PHC.
From ceramide more complex sphingolipids are produced. Aur1p, the Inositolphosphorylceramide (IPC) synthase is essential. Importantly, none of the more complex sphingolipids (MIPC: mannosylPC; $M(IP)_2C$) is required for secretion (Lisman et al.JBC 2004).
Enzymes whose corresponding gene deletions gave phenotypes in our screen are highlighted in blue. All non-essential enzymes not found in the screen are typed in green and all essential enzymes (not present in the deletion library) are marked with bold black letters.

We further identified *erg4* and *erg6* as phenotype I strains (Fig. 19). These enzymes catalyze the late steps in ergosterol synthesis of which the last five involve non-essential genes. Both Erg6p and Erg4p regulate modification at position C-24 of the sterol back bone, being methyltransferases and reductases, respectively (Daum et al., 1998). Ergosterol, as previously shown, is also required for targeting of the tryptophan permease Tat2p to the cell surface under conditions of low external tryptophan concentration. This cargo switches to a TGN-endosome-vacuole pathway upon high tryptophan exposure in an ubiquitin-dependent sorting process that also takes place in an *erg6Δ* mutant indicating ergosterol dependence of the sorting of Tat2p to the cell surface (Umebayashi and Nakano, 2003).
Most interestingly, *ERG6* and *ELO3* show strong genetic interaction and they are believed to be required for formation of functional rafts (Eisenkolb et al., 2002). The maturation of GPI-anchored Gas1p is blocked in the ER in *elo3*Δ and more so in *elo3*Δ*erg6*Δ double mutant cells. Pma1p was rapidly routed for degradation in the vacuole in *elo3*Δ cells and this was not drastically enhanced in *elo3*Δ*erg6*Δ cells because the degradation is already rapid in the single mutant cells (Eisenkolb et al., 2002). Together, all these data point to an important role of sphingolipids and ergosterol in surface delivery of our DRM associated marker protein, Fus-Mid-GFP.

The emergence of most non-essential genes with specific non-reduntant function involved in both sphingolipid and ergosterol biosynthesis in a genome-wide non-biased screen suggests that assemblies of sphingolipids and ergosterol are involved as sorting platforms in Fus-Mid-GFP surface delivery.

4.2.3. Actin organisation

A gene *RVS161* with many proposed functions was identified in our screen. This gene has so far been implicated in endocytosis and in the generation of mating polarity in yeast (Brizzio et al., 1998; Munn et al., 1995). We suggest that Rvs161p plays a direct role in exocytosis and that the observed phenotype of *rvs161Δ* cells is not only an effect of impaired endocytosis. This view is based on the fact that in our screen no internalization mutant was showing TGN accumulation and phenotype I was not observed in *end4*Δ cells (Fig. 17B). Several other findings also speak for Rvs161p regulating exocytosis: First, *rvs* mutants accumulate late secretory vesicles at sites of membrane and cell wall construction (Breton et al., 2001). Second, Rvs161p occurs in a complex with Rvs167p, which interacts in a yeast two-hybrid assay with Exo70p and Sec8p (Bon et al., 2000), two components of the exocyst-complex involved in targeting and tethering of secretory vesicles to sites of polarized growth at the plasma membrane (Guo et al., 1999). Also, Rvs161p and Rvs167p interact with the Rab GAP Gyp5 and a GAP-related protein, Gyl1 (Talarek et al., 2004). These two Rab regulators are likely to be important for exocytosis, as they form a complex with GAP activity for Ypt1 and Sec4 (Chesneau et al., 2004), the major Rab proteins governing the secretory pathway.

Furthermore, Rvs161p is involved in actin regulation as it shows genetic and functional interaction with Myo2p, Myo1p (myosin motors) and with actin (Breton and Aigle, 1998). In yeast, the actin cytoskeleton is depolarized by NaCl stress. Rvs161p is required to repolarize the actin cytoskeleton (Balguerie et al., 2002). Interestingly, this requirement can be suppressed by mutations in sphingolipid biosynthesis including *SUR2* and *SUR4*/*ELO3*. Rvs161p was found to be DRM-associated and was delocalized from cortical actin patches in the sphingolipid mutants. These findings suggested a link between actin polarization, lipid rafts and Fus-Mid-GFP surface delivery (Balguerie et al., 2002).

It should be noted that Rvs161p is a BAR domain protein. BAR domains are sensors of membrane curvature and could potentially be involved in increasing membrane curvature in vesicle formation to drive fission (Peter et al., 2004).

The other gene involved in actin regulation that we identified was Vrp1p. This protein is part of the Arp2/3 machinery and localizes to cortical actin patches (Evangelista et al., 2000). In mammalian cells, a role of rafts and phosphatidylinositol 4,5-bisphosphate in formation of actin tails by the Arp2/3 complex has been suggested to drive the budding process of TGN-derived vesicles (Rozelle et al., 2000). In agreement with a role of Vrp1p in membrane transport events requiring ergosterol synthesis, it has been recently shown that the decrease in vacuole-associated actin turnover observed in a *vrp1Δ* mutant is recovered by overexpression of *ERG6*, connecting sterol metabolism and actin remodeling (Tedrick et al., 2004). Our finding that the raft-dependent cargo Fus-Mid is inefficiently transported from the TGN in *vrp1* mutants opens the question of whether Vrp1p-dependent actin assemblies could also play a role in vesicle formation at this compartment.

4.2.4. Golgi exit

Two other interesting genes remain to be discussed: *CHS5* and *KES1*. Both deletion strains lead to accumulation of Fus-Mid-GFP in the Sec7-containing compartment (Table 2). Chs5p is involved in transport of chitin synthase III (Chs3p), an enzyme required for synthesis of the polysaccharide chitin (Santos et al., 1997). Chs5p localizes to the TGN and is required for of the exit of Chs3p and also of Fus1p from the Golgi (Santos and Snyder, 1997). We now show that Fus-Mid-GFP requires functional Chs5p for undisturbed exit from the TGN en route to the cell surface. Interestingly, in a recent genome-wide screen *chs5Δ* was found to be synthetic lethal with *rvs161Δ*(Tong et al., 2004). Whether the raft- and cytoskeleton-dependent surface transport observed for Fus-Mid-GFP coincides with the surface delivery route for Chs3p remains to be established. In support of such a model, it has been shown that the GPI-anchored cell wall protein Crh2p also requires *CHS5* for plasma membrane delivery (Rodriguez-Pena et al., 2002). Some GPI anchored proteins are believed to depend on lipid rafts for surface delivery both in epithelial and yeast cells (Brown and Rose, 1992; Muniz and Riezman, 2000; Simons and Ikonen, 1997). One

aspect of Chs3p trafficking distinct from that of Fus-Mid is that Chs3p localizes to chitosomes, a specialized early endosomal compartment from where it is mobilized for surface delivery during bud formation or in response to activation of the cell wall integrity signaling pathway (Smits et al., 1999). This route could potentially involve clearance of Chs3p from chitosomes by retrograde transport to the Golgi (Valdivia et al., 2002), followed by delivery to the cell surface.

As an alternative to a common route for transport of Chs3p and Fus-Mid to the surface regulated by Chs5p, this protein could be a component required for multiple pathways from the TGN to the plasma membrane or have an indirect effect on Fus-Mid transport. Further work will be required to distinguish between those possibilities.

Another intriguing protein implicated in Golgi function that we identified in our screen was Kes1p (Fig. 20). It binds to the Golgi depending on Pik1p, a phosphatidylinositol 4-kinase required for normal Golgi structure and transport competence (Li et al., 2002b). Structurally Kes1/Osh4p belongs to a family of oxysterol binding proteins. Collectively, the seven yeast oxysterol protein homologue (Osh) genes are essential for yeast viability and affect sterol levels (Beh et al., 2001). There are indications that function of oxysterol-binding proteins is linked to control of cellular sterol homeostasis and endocytosis (Beh and Rine, 2004). However, Kes1p has been shown not to bind oxysterols but phosphoinositides including PI4P. Kes1p interacts genetically with Arf1p, Pik1p and the phospholipid transfer protein Sec14p that regulates Golgi lipid composition and formation of secretory vesicles from the TGN. These data suggest a role of Kes1p in exocytosis (Li et al., 2002b). Involvement in formation of transport carriers to the cell surface and dependence on phosphatidylinositol 4-phosphate and the small GTPase Arf1p has also been demonstrated for the mammalian proteins FAPP1 and FAPP2 (four-phosphate adaptor proteins; (Godi et al., 2004)). Recently, studies from our lab demonstrated that FAPP2 binds to PI4P in the Golgi of epithelial MDCK cells and is directly involved in the machinery responsible for sorting and delivery of raft-associated cargo to the apical surface (Vieira et al., submitted). Whether Kes1p plays a similar role in Mid-Fus-GFP transport to the cell surface in yeast remains to be shown.

4.2.5. O-glycosylation

Previously, glycosylation has been found to be an important determinant for sorting of biosynthetic cargo to the cell surface (Proszynski et al., 2004). Cells lacking Pmt4p enzyme necessary to initiate glycosylation of Fus-Mid-GFP accumulate unglycosylated Fus-Mid in late Golgi structures (Proszynski et al., 2004). Although O-glycosylation is required for correct sorting of Fus-Mid, our screen did not identify components regulating this modification. Unfortunately, the *pmt4Δ* mutant is missing from the deletion library used in the visual screen and enzymes involved in elongation of O-glycosylation including Ktr1p, Ktr3p and Kre2p have redundant function (Romero et al., 1999). In this screen, we did not identify any proteins that could be lectins responsible for glycosylation dependent sorting of Fus-Mid. It is possible that such lectins do not exist or there is more than one protein of this function and such redundant proteins would not be identified.

4.2.6. Genome-wide visual screen for nonessential exocytosis regulators

The screen presented here provides a sensitive assay that directly visualizes the distribution and trafficking of GFP-labeled cargo. Induced expression allows observation of the effects of single gene knockout on transport in a defined period of time and provides several read-outs. In previous screens for secretion mutants, the major criteria have been cell growth/viability and internal accumulation of vesicles (read out as increase in cell density) and cargo (e.g. internal accumulation of invertase). A genome-wide screen using a collection of essential genes under control of a deoxycycline-regulated promoter relied on growth defects and alterations in secretion of the heat shock protein Hsp150p (Davydenko et al., 2004). Our visual screen instead does not rely on growth defects or gross changes in overall secretion, as neither can be expected for sorting mutants regulating alternative pathways to the cell surface. Direct visual inspection of mutant phenotypes in cargo transport provides information about the site of transport delay (accumulation at the TGN or endosome identified by colocalization experiments) and about sorting (towards plasma membrane or vacuole).

The new approach presented here can be used for systematic screening of multiple cargoes in order to define the pathways and the machineries responsible for sorting

into different exocytic routes. The major outcome of our screen is that we identified gene products that implicate raft platforms in the sorting of proteins from the Golgi to the cell surface. As has been proposed for apical sorting in epithelial cells raft clustering could lead to domain-induced budding and formation of the transport carrier (Bagnat and Simons, 2002b; Schuck and Simons, 2004). What is missing are proteins – probably more than one- that regulate clustering. Future work will have to address this issue. Of particular interest is also to define molecular requirements of the route transporting invertase as it should be governed by different principles than sorting in the raft-dependent route studied here.

4.3. Mechanisms for polarized distribution of Fus1p to the shmoo tip

Additionally to our studies on protein sorting to the cell surface we were also interested in mechanisms responsible for polarized distribution of Fus1p on the plasma membrane of shmooing cells. Different models have been proposed for generating and maintaining cell surface asymmetry in mating cells. One model is based on lipid raft clustering of proteins involved in the cell-cell adhesion and fusion machinery responsible for mating (Bagnat and Simons, 2002a). Another model was recently put forward, employing an ongoing cycle of endocytosis and polarized delivery of membrane into tips of shmoos (Valdez-Taubas and Pelham, 2003).

Our results demonstrated that unlike Snc1p, Fus1p does not require endocytic cycling for polarized distribution. Instead, our marker protein was retained at the tip of the mating projection by the interaction with a multiprotein scaffolding machinery. Thus, there are different mechanisms for polarization of membrane components in shmooing cells. Important is also to note that most mutants that inhibit endocytosis, including *end3, end4, end6, end7* and *rvs167* mate with similar efficiency as wild-type cells (Brizzio et al., 1998). These findings also suggest that the kinetic polarization model employing endocytosis and polarized exocytosis that Valdez-Taubas and Pelham proposed is unlikely to explain how a shmoo cell effectively polarizes its mating machinery. Considering the functional role of Snc1p being a v-SNARE involved in vesicle fusion with the plasma membrane it is obvious that the protein has to cycle to fulfill its physiological role and is therefore not an optimal probe for studying mating cell polarity.

Based on previous findings that polarization of the mating machinery to the shmoo tip is inhibited in erg6 and in lcb1-100 cells, mutations that affect the synthesis of the major raft lipids in yeast reduced mating efficiency, it was postulated previously that raft lipid clustering plays a role in establishing and maintaining mating tip polarization (Bagnat and Simons, 2002a). More detailed analysis is clearly needed to

demonstrate that raft association is essential to localize the proteins involved in cell adhesion and fusion to the tip of the mating projection. The scaffolding of these proteins could be mainly through protein-protein interactions. However, also mammalian cells use actin-based raft clustering mechanisms to polarize their cell surfaces during cell migration or cell-cell contacting during immune recognition. In migrating neutrophils it was demonstrated that lipid raft clusters are localized to the rear of the cells in an actin-dependent manner (Seveau et al., 2001). In polarized T-lymphocytes Gomez-Mouton et al. showed that two types of raft clusters are assembled at opposite poles, at the leading edge and at the uropod (Gomez-Mouton et al., 2001). Each raft clustering process is specific in that a subset of raft components is included in the assembly. This could also be the case during yeast mating (Bagnat and Simons, 2002a). The coming together of raft-associated proteins at the mating tip could introduce specific lipid-protein interactions to activate the mating machine spatially and temporally for continuous control of this essential process.

These interactions could involve integral proteins binding to raft lipids in the bilayer and/or to peripheral proteins on the cytosolic side of the raft assembly. For instance the EGF receptor has been shown to be activated by interactions with the ganglioside Gd1a and the glutamate receptor by raft-cholesterol (Eroglu et al., 2003; Liu et al., 2004). By directing our attention to the role of both lipids and proteins in the membrane will be able to advance our understanding of the molecular interactions that drive cell surface polarization.

5. Materials and Methods

Table 3. Yeast strains used in these studies

Yeast strain	Genotype	Source
RH690-15D	Mata *his4 leu2 ura3 lys2 bar1*	H. Riezman lab
RH1965	Mata *his4, leu2, ura3, lys2, bar1, end4::LEU2*	H. Riezman lab
RH690-13D	Mata *lcb1-100 his4 ura3 leu2 lys2 bar1*	H. Riezman lab
RH268-1	Mata *his4, leu2, ura3, lys2, bar1, end4-1 (ts)*	H. Riezman lab
MBY249	RH690-15D *pmt5::LEU2*	This study
MBY254	RH690-15D *pmt4::LEU3*	This study
SEY6210	Mat alpha *ura3-52 leu2-3,112 his3D200 trp1D90 lys2-801 suc2D9*	W. Tanner lab
1403	SEY6210 *pmt1::HIS3*	W. Tanner lab
1405	SEY6210 *pmt2::LEU2*	W. Tanner lab
1407	SEY6210 *pmt3::HIS3*	W. Tanner lab
1409	SEY6210 *pmt4::TRP1*	W. Tanner lab
1421	SEY6210 *pmt6::LEU2*	W. Tanner lab
SFNY28-6C	Mata *sec53 ura3-52*	S. Ferro-Novick lab
H891	Mata *sec18-1 trp1-289 leu2-3,112 ura3-52 his-*	S.Keranen
NY430	Mata *sec14-3 ura3-52*	Ch. Walch-Solimena
W303	Mata ade2::ADE2 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psi+	W. Zachariae lab
MBY114	W303 TRP-1::SEC7-DsRed	This study
MBY119	MBY114 *pmt4::LEU2*	This study
AAY1017	Matα *his1*	CSH lab. course

Table 4. Plasmids used in these studies

Plasmid name	Expressing	Plasmid type	Source	Original name
TPQ67	Fus1-TAP	centromeric	This study	no
MBQ30	Fus1-GFP	centromeric	This study	no
MBQ35	Mid2-GFP	centromeric	This study	no
TPQ53	Mid-Fus	centromeric	This study	no
TPQ55	Fus-Mid	centromeric	This study	no
TPQ34	Inv-Fus	centromeric	This study	no
TPQ80	Inv-Mid	centromeric	This study	no
TPQ52	Inv33Fus	centromeric	This study	no
TPQ79	Inv33Mid	centromeric	This study	no
TPQ1	(vector)	centromeric	W. Zachariae lab	p416
TPQ61	(source of TAP-tag)	centromeric	B. Seraphin lab	pBS1539
TPQ76	Sec7-DsRed	integration	B. Glick lab	YIplac204/SEC7DsRed
TPQ127	DsRed-FYVE	centomeric	This study	no
TPQ128	Sec7-DsRed	centomeric	This study	no
TPQ63	Fus(TMD-Mid)	centromeric	This study	no
TPQ65	Mid(TMD-Fus)	centromeric	This study	no
TPQ72	Fus(cyt-Mid)	centromeric	This study	no

TPQ94	Mid(cyt-Fus)	centromeric	This study	no
TPQ97	Mid(cyt-FusΔSH3)	centromeric	This study	no
TPQ57	Fus_SH3	centromeric	This study	no
TPQ115	Fus1p(P422A)-SH3	centromeric	C. Boone lab	p4269
TPQ116	Fus1p-SH3(W473S)	centromeric	C. Boone lab	p4580
TPQ117	Fus1p(P422A)-SH3(W473S)	centromeric	C. Boone lab	p4667
TPQ109	Snc1-GFP	centromeric	H. Pelham lab	TPI-GFP-Snc1

5.1. Yeast strains

Yeast strains used in these studies are listed in Table 3 and construction of strains generated in these studies is described below. In the screening project we took advantage of the yeast deletion library encompassing 4500 single knockouts of non-essential genes (European Saccharomyces cerevisiae Archives for Functional analysis or EUROSCARF: http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Deletions are in BY strains derived from S288C (MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*).

5.2. Plasmid and Strain Construction

Plasmids used in this study are listed in Table 4. Most proteins used in this study were expressed from the centromeric plasmid p416 (Mumberg *et al.*, 1994). GFP-Snc1 and Sec7-DsRed (on integration plasmid) were expressed from the TPI promoter, DsRed-FYVE and SEC7-DsRed were expressed from the GPD and ADH promoters respectively. PCR products were integrated into the pGEM-T vector using the TA ligation kit (Promega, A3600), cut out using restriction sites introduced on primers and sub-cloned by the triple ligation method into *Xba*I/*Hin*dIII linearized p416. The *FUS1* sequence was amplified from genomic DNA using primers containing *Xba*I and *Bam*HI restriction sites and the TAP-tag sequence was PCR-amplified from pBS1539 (Puig *et al.*, 2001) with primers containing *Bam*HI and *Hin*dIII sites. Restriction digested *FUS1* and TAP-tag sequences were co-ligated into p416 to generate TPQ67. pMBQ30 has already been described (Bagnat and Simons, 2002a). MBQ35 containing Mid2-GFP construct was cloned by co-ligation of the PCR amplified *MID2* sequence from genomic DNA using primers containing *XbaI* and *Bam*HI sites and the GFP sequence was PCR amplified with primers containing *Bam*HI and *Hind*III sites. TPQ53, containing the Mid-Fus construct (Fig. 12) fused to GFP was

generated by co-ligation of a sequence encoding the extracellular domain of Mid2p amplified from MBQ35 using primers containing *Xba*I and *Bgl*II and the *FUS1-GFP* sequence amplified from MBQ30 with primers containing *Bam*HI and *Hin*dIII sites. TPQ55**,** containing the Fus1-Mid construct (Fig. 12) was generated by co-ligation of the DNA encoding the extracellular domain of Fus1p amplified from MBQ30 with primers containing *Xba*I and *Bgl*II sites and part of the *MID2-GFP* sequence amplified from MBQ35 with primers containing BglII and HindIII sites. The sequence encoding the N-terminal part of invertase (*SUC2*), present in four constructs (Fig. 12), was amplified from genomic DNA using primers containing *Xba*I and *Bam*HI sites. To generate TPQ34, containing the Inv-Fus (Fig. 12) portion of the invertase sequence was co-ligated with a sequence encoding portion of Fus1-GFP amplified from genomic DNA from the MBY229 strain (Bagnat and Simons, 2002a) with primers containing *Bam*HI and *Hin*dIII sites. To generate TPQ80, containing Inv-Mid, the invertase sequence was co-ligated with the part of *MID2-GFP* used to generate Fus-Mid construct described above. To generate TPQ52, containing Inv33Fus construct, part of invertase was co-ligated to the portion of *FUS1-GFP* amplified from MBQ30 with primers containing *Bgl*II and *Hin*dIII sites. To generate TPQ79, containing the Inv33Mid construct, the sequence encoding the extracellular part of Inv33Fus was amplified from TPQ52 with primers containing *Xba*I and *Bgl*II and co-ligated with the portion of *MID2-GFP* prepared as above to generate Fus-Mid. All protein constructs were membrane associated as determined by density gradient centrifugation (data not shown). To generate the TPQ128 we used YIplac204-T/C-SEC7-DsRed.T4 kindly provided by B. Glick (Chicago University) as a donor of the Sec7-DsRed gene. Sec7-DsRed is near 7 Kbp long and the attempt to clone the entire gene in single step cloning was unsuccessful. Instead, we removed the middle part of the gene by digesting the plasmid with SpeI restriction enzyme and re-ligating. This truncated version of the gene was PCR amplified with primers containing NheI and XhoI sites and introduced to the PCRII-TOPO vector (TOPO TA Cloning Kit, Invitrogen K4660-40). Then the insert was removed from the PCRII-TOPO vector with NheI/XhoI digestion and ligated to the pRS415 vector (Mumberg et al., 1995) linearized with XbaI/XhoI downstream of the constitutive ADH promoter. Next the plasmid was digested with SpeI and co-transformed with Yiplac204-T/C-SEC7-DsRed.T4 to yeast cells in order to recover the deleted fragment of Sec7-DsRed by

"gap-repair". The plasmid was isolated from yeast cells and amplified in bacteria. The correct sequence of the insert was verified by sequencing. To generate pTPQ127 we cut out the DsRed-FYVE encoding sequence from the pRS425 Met3-dsRed-FYVE plasmid kindly provided by S. Emr (Univ. of California – San Diego) and ligated downstream of the GPD promoter to the pRS415 using SpeI/NotI sites.

Plasmids p4269, p4580, p4667 containing mutants of *FUS1* under control of its own promoter were obtained from Ch. Boone lab (Nelson et al., 2004). The plasmid containing GFP-SNC1 under control of constitutive TPI promoter was obtained from H. Pelham lab (Lewis et al., 2000). Plasmids TPQ63, TPQ65, TPQ72 and TPQ57 were created using triple ligation method. To generate TPQ63 extracellular domain of Fus1p linked to TMD from Mid2p were PCR amplified from plasmid TPQ55 using primers containing XbaI and BamHI sites and fragment containing cytoplasmic tail of Fus1p fused to GFP was amplified from plasmid TPQ53 with primers containing BglII and HindIII sites. Both inserts were co-ligated to XbaI/HindII digested MBQ1 vector. To create TPQ65 the extracellular domain of Mid2p linked to TMD from Fus1p were amplified from plasmid TPQ53 using primers with XbaI and BamHI sites and fragment containing cytoplasmic tail of Mid2p fused to GFP was amplified from plasmid MBQ35 with primers containing BglII and HindIII sites. Both inserts were co-ligated to XbaI/HindII digested MBQ1 vector. To make TPQ72 the extracellular domain and TMD of Fus1p were PCR amplified from plasmid MBQ30 using primers containing XbaI and BamHI sites and fragment containing cytoplasmic tail of Mid2p fused to GFP was prepared as for construction of TPQ65 and inserts were co-ligated to XbaI/HindII digested vector.

Plasmid TPQ57 was made by co-ligation of GFP sequence flancked by BamHI/HindIII sites with sequence coding truncated (SH3Δ) version of Fus1p amplified by PCR from MBQ30 using praimers containing XbaI/BglII sites. Vector was prepared as above.

The TPQ94 and TPQ97 were constructed through the homologus recombination in yeast RH690-15D cells. To generate TPQ94 the DNA fragment containing TMD of *MID2* and cytoplasmic tail of *FUS1* followed by the GFP was cut with BglII/HindIII from TPQ63 and co-transforemed with BamHI linearized MBQ35. In result of recombination within TMD and GFP sequence the cytoplasmic tail of *FUS1* was introduced to the *MID2* sequence. To create TPQ97, fragment coding truncated

cytoplasmic tail of Fus1p followed by the GFP sequence was amplified from TPQ57 and co-transformed with NheI linearized TPQ97. The successful recombination was verified by observation of fluorescence in microscope. The plasmids were purified and amplified in bacteria. Plasmids were purified and the restricion digestion analysis was done to confirm expected recombination. Inserts were sequenced.

5.3. Gene disruption in the genome

Disruption of *PMT4* and *PMT5* was done by integrating PCR-amplified *C. glabrata LEU2* into the *PMT4* or *PMT5* locus. To express SEC7-DsRed, a YIplac204/-SEC7DsRed plasmid, kindly provided by Benjamin Glick, was integrated into the genome of wild-type or *pmt4Δ* cells in the W303 background.

5.4. Growth Conditions

Yeast cells were grown in yeast extract/peptone/dextrose (YPD) medium or yeast extract/peptone (YP) medium containing 2% raffinose (YPRaf) as a carbon source at 24°C or at the indicated temperature. For induction of expression from the GALS promoter, cells from YPD media were washed twice with YP media containing 2% galactose (YPGal) and incubated for 3h (or as indicated) in YPGal; cultures in YPRaf was supplemented with 2% galactose (final concentration).

5.5. Western Blot

Western blot analysis was performed according to standard procedures. Cells were disrupted by beating with glass beads for 5 min at 4°C and boiled with SDS-PAGE sample buffer containing 5% mercaptoethanol. To detect Protein A present in the TAP-tag we used a Peroxidase-Anti-Peroxidase antibody (Sigma, P-2026). For Western blot analysis of GFP fusion proteins we used a mouse monoclonal anti-GFP antibody (B-2) (Santa Cruz Biotechnology, sc-9996).

5.6. Metabolic labeling and Immunoprecipitation

Cells were grown to mid log phase in complete synthetic medium without methionine containing 2% raffinose as carbon source and expression of Fus1-GFP was induced for 15 minutes by addition of 2% galactose. Then the cells were pulse-labeled with 1 mCi of [^{35}S] methionine for 5 minutes and chased for various times. At the indicated times samples were taken and cells were killed in 0.2% sodium azide on ice. Then the cells were lysed in lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and CLAP protease inhibitors mix: 0.1% chymostatin, 0.1% leupeptin, 0.1% antipain and 0.1% pepstatin) by shaking with glass beads for 5 min at 4˚C. After removing cell debris lysates were adjusted to 1% NP40 and 0.1% SDS and warmed at 37˚C for 5 min. Insoluble material was removed by centrifugation (1 min, 6000g) and samples were diluted 2 fold in IP buffer (10 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA,1% NP40, 0.1% SDS). Then samples were incubated with Protein A and a rabbit anti-GFP antibody (Santa Cruz Biotechnology, sc-9996) for 3 h at room temperature. Immunoprecipitates were washed 4 times with IP buffer, once with Tris 20 mM pH 7.4, and subjected to SDS-PAGE. Protein bands were analyzed by autoradiography.

5.7. Tunicamycin treatment and mating assay

Cells were grown overnight in raffinose-containing medium and expression was induced by addition of galactose (2%). Tunicamycin was added (10 μg/ml) and cells were incubated for 3h at 24°C. Mating assay was performed as described (Bagnat and Simons, 2002a). 0.5 ml of the over night culture was diluted 20 times in YPD and incubated for 3h. The density of cells in the culture was assessed by cell counting in the counting chamber and 1x10^7 of each mating type cells were mixed together and collected on a nitrocellulose filter. Filters were incubated for 3h at 24˚C on YPD plates to allow for cell fusion. Finally cells were resuspended in water, diluted 100x and equal amount was plated on SD (selection for diploids) and YPD (non selective) plates. Mating efficiency was calculated as a ratio of number of colonies from SD plates to number of colonies from YPD plates. MBY1102 (AAY1017) was used as a wild-type Mat alpha strain for mating with either RH690-15D (wild-type) or MBY249 (*pmt4Δ*).

5.8. Microscopy

Microscopy was performed on live cells that were washed twice in water and resuspended in water for imaging using an Olympus BX61 microscope, RT Slider SPOT camera (Diagnostic Instruments inc.) and MetaMorph software.

5.9. Quantification of the fluorescence microscopy

Cells were grown until middle log phase in YPD media, washed twice and resuspended in YPGal media following by incubation for 3h at 24˚C to induce protein expression. Images of cells expressing different constructs were taken using the same conditions. Stacks of the pictures were converted to the TIFF format using ImageJ program (Wayne Rasband NIH, USA) and the fluorescence intensity was analysed using the IpLab (Scanalytics, Inc.) software. For each image the background fluorescence was measured and subtracted. The area representing plasma membrane of each cell was marked as a ring-like segment. The DIC images were used to localize the periphery of the cells when fluorescence from the plasma membrane was too weak to localize it. The total fluorescence of the plasma membrane was measured from the encircled area. The fluorescence corresponding to the intracellular space was measured from the area inside the ring-like segment. The bars represent the ratio of the total fluorescence measured for the plasma membrane and for the intracellular area. The data were standardized to O-glycosylated constructs (Fus1-GFP and Fus-Mid) expressed in wild type cells.

5.10. Induction of mating response

To induce mating response α-factor (5 μM) (T-6901; Sigma-Aldrich, St. Louis, MO) was added and cells were incubated for 3h at 24°C (or as indicated).

5.11. Protocols used in the screening project

5.11.1. Tools used to transfer yeast cells

The yeast deletion library is organized in 96-well plates. To transfer cells we used a 96-floating pin replicator containing 23 mm long pins with 1.58 mm in diameter (V&P Scientific, Inc. San Diego, CA 92121, VP 408FH). We also constructed 96-fixed pin replicators with pins 16 mm long and 4 mm of diameter that is able to transfer more material. Both types of pin replicators were sterilized by incubation two times for one minute in sterile water, one minute in bleach (10% sodium hypochloride) and next two times in water. After this the pin replicators were transferred to ethanol and flame sterilized three times.

For growing cells from the deletion library liquid media standard 96-well plates were used. To grow on solid media we used Single Well OmniTrays (Nunc International, 242811).

5.11.2. Transformation protocol, Media and Growth condition

Strains of the deletion library were thawed and transferred from 96-well plates to solid Yeast extract/Peptone with 2% Dextrose (YPD) media using the floating pin replicator. Plates were incubated for three days at 24°C. The sterile 96-well plates were filled with (100µl/well) transformation solution containing (per well): 30µg Sonicated Salomon Sperm DNA (Stratagene, 201190-81) denatured by incubation for 5 minutes at 95°C, 3µg of the TPQ55 plasmid in 40% PEG, 1mM EDTA, 10mM Tris-Cl (pH=7.5), 100mM Lithium acetate. The transformation solution was mixed well before use. The yeast cells were collected with fixed pin replicator and transferred to the 96-well plate containing the transformation solution. The pin replicator was gently agitated when pins were in the wells to remove yeast cells from the pins to the liquid. Next, plates were incubated at room temperature over night, the edges of the plates were wrapped up with parafilm (American National Can, Chicago Il. 60631) and incubated in a water bath for 15 minutes at 45°C. The plates were dried and incubated for one hour at room temperature. The liquid was gently removed using an 8-channel pipet and transformed cells were collected with the fixed pin replicator and transferred to selective plates containing solid Synthetic Dextrose Minimal

Medium without Uracil (SD-Ura). The plates were incubated for four days at 24°C and cells were transferred with the fixed pin replicator for a second round of selection on SD-Ura (3-4 days of incubation).

Next, cells were transferred with floating pin replicator to 96-well plates containing Yeast extract/Peptone with 2% Raffinose (YPRaf) and incubated over night at 24°C. For the induction of Fus-Mid-GFP expression YPRaf was gently replaced with YPRaf containing 2% galactose. Plates were incubated at 24°C for four hours. For taking final images, cells were grown over night in SD-Ura or SD-Ura-Leu media containing 2% raffinose as a source of carbon. To induce the protein expression this media was exchanged with fresh SD-Ura or SD-Ura-Leu media containing 2% raffinose and 2% galactose.

5.11.3. Preparing samples for microscopy

2 μl of live cells were collected by pipeting from the bottom of the well, placed on the glass microscopy slide and gently covered with cover-slip. It is important to avoid pressing the cover-slip down or immobilizing cells in agar since these treatments generate artifacts. Also, too little material taken for slide (especially when big cover-slips are used) might damage cells because of surface tension generated by liquid on the slide.

5.11.4. Microscopy

Microscopy was performed using an Olympus BX61 microscope, Olympus PlanApo 60x/1.10 oil LSM objective, RT Slider SPOT camera (Diagnostic Instruments inc.) and MetaMorph software.

5.11.5. Co-localization experiments

For co-localization experiments cells containing pTPQ55 were transformed with either pTPQ127 or pTPQ128 as described above. Expression of both co-localization markers (Sec7-DsRed and DsRed-FYVE) slightly affected protein transport. To avoid

potential artifacts all pictures of GFP fluorescence showing phenotype were taken when cells were containing plasmid pTPQ55 alone.

5.11.6. Detergent Resistant Membrane (DRM) association.

DRM association of Fus-Mid-GFP was done essentially as described previously (Bagnat et al., 2001). Briefly, cells (20 optical density units at 600 nm) were lysed, treated with Chaps and subjected to Optiprep density gradient centrifugation. After centrifugation, a floating fraction and a soluble fraction were obtained. The floating fraction corresponds to detergent-resistant membrane (DRM). Presence of Fus-Mid-GFP in different fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with anti GFP antibody (Roche Applied Science, Indianapolis IN, 11814460001).

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I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 15.09.2005 to 20.05.2005 under the supervision of Prof.Dr.Kai Simons at Max-Planck Institute of Molecular Cell Biology and Genetics.

I declare that I have not undertaken any previous unsuccessful doctorate proceedings.

I declare that I recognize the doctorate regulations of the Faculty of Sciences of the Dresden University of Technology.

Dresden, 26.05.2005, Tomasz J. Prószyński