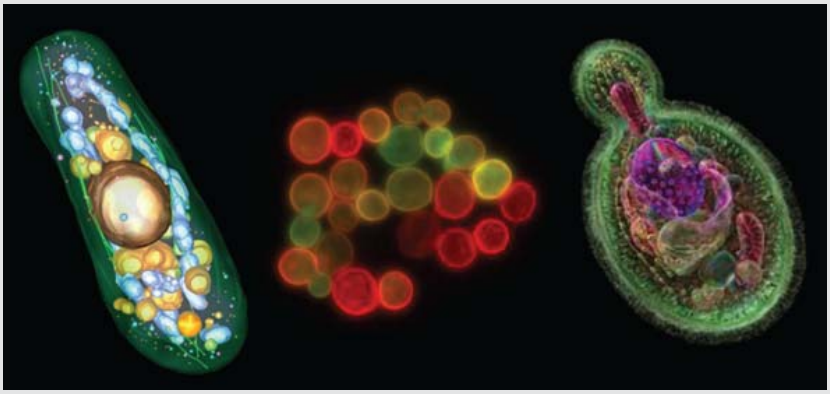

Functional Dissection of Alternative Secretory Pathways in the Yeast *S. Cerevisiae*

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Science may set limits to knowledge, but
should not set limits to imagination.

Bertrand Russell (1872 - 1970)

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List of original publications

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I) Karhinen L, **Bastos RN**, Jokitalo E, Makarow M. (2005).
Endoplasmic reticulum exit of a secretory glycoprotein in the absence of sec24p family proteins in yeast.Traffic. 2005 Jul;6(7):562-74.

II) **Bastos RN**, Suntio T, Jokitalo E and Makarow M. (2008).
A selective protein transport route for secretion of Hsp150 occurs in the post-Golgi secretory mutant *sec15-1*, Manuscript resubmitted to Traffic, 2008.

III) Makarow M, Hanninen AL, Suntio T, **Bastos RN**. (2006)
Production of heterologous proteins in yeast with the aid of the Hsp150Δ carrier. Methods Mol Biol. 2006;313:333-43.

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	ATP phosphatase
ARF	ADP ribosylation factor
BiP	Immunoglobulin heavy chain binding protein
COP	Coat protein
CPY	Carboxypeptidase Y
Dol-P	Dolichol pyrophosphate
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GalNAc	N-acetyl galactosamine
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	Guanidine diphosphate
GEF	guanine nucleotide exchange factor
GFP	Green fluorescent protein
GMP	Guanidine monophosphate
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GPI	Glycosylphosphatidylinositol
GTP	Guanidine triphosphate
GTPase	Guanidine triphosphatase
HDSV	Heavy density secretory vesicle
HRP	Horse radish peroxidase
Hsp	Heat shock protein
kDa	Kilodalton
LDSV	Light density secretory vesicle
Man	Mannose
M-Pol	Mannosyl polymerase
NSF	N-ethylmaleidimide-sensitive factor
OST	Oligosaccharyl transferase
PDI	Protein disulfide isomerase
PLD	Phospholipase D
PMT	Protein-mannosyl transferase
RNA	Ribonucleic acid
RNC	RNA-nascent chain complex
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electroforesis
Sfb	Sed5 binding protein
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SPC	Signal peptidase complex
SR	SRP receptor
SRP	Signal recognition particle
TEM	Transmission electron microscopy
TGN	*trans*-Golgi network
TRAPP	Transport protein particle
UDP	Uracil diphosphate
UGGT UDP-Glc::	glycoprotein transferase
UPR	Unfolded protein response

Single letter code	Three letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Summary

Eukaryotic cells are characterized by having a subset of internal membrane compartments, each one with a specific identity, structure and function. Proteins destined to be targeted to the exterior of the cell need to enter and progress through the secretory pathway.

Transport of secretory proteins from the endoplasmic reticulum (ER) to the Golgi takes place by the selective packaging of proteins into COPII-coated vesicles at the ER membrane. Taking advantage of the extensive genetic tools available for *S. cerevisiae* we found that Hsp150, a yeast secretory glycoprotein, selectively exited the ER in the absence of any of the three Sec24p family members. Sec24p has been thought to be an essential component of the COPII coat and thus indispensable for exocytic membrane traffic.

Next we analyzed the ability of Hsp150 to be secreted in mutants, where *post*-Golgi transport is temperature sensitive. We found that Hsp150 could be selectively secreted under conditions where the exocyst component Sec15p is defective. Analysis of the secretory vesicles revealed that Hsp150 was packaged into a subset of known secretory vesicles as well as in a novel pool of secretory vesicles at the level of the Golgi. Secretion of Hsp150 in the absence of Sec15p function was dependent of Mso1p, a protein capable of interacting with vesicles intended to fuse with the plasma membrane, with the SNARE machinery and with Sec1p.

This work demonstrated that Hsp150 is capable of using alternative secretory pathways in ER-to-Golgi and Golgi-to-plasma membrane traffic. The sorting signals, used at both stages of the secretory pathway, for secretion of Hsp150 were different, revealing the highly dynamic nature and spatial organization of the secretory pathway.

Foreign proteins usually misfold in the yeast ER. We used Hsp150 as a carrier to assist folding and transport of heterologous proteins though the secretory pathway to the culture medium in both *S. cerevisiae* and *P. pastoris*. Using this technique we expressed Hsp150Δ-HRP and developed a staining procedure, which allowed the visualization of the organelles of the secretory pathway of *S. cerevisiae*.

INTRODUCTION

1. Principles of intracellular membrane traffic

1.1 The exocytic pathway: an introduction

Eukaryotic cells contain membrane-enclosed compartments, called organelles, which have specialized functions and contain a unique combination of proteins, lipids and cofactors. In order to conserve their identity, structure and functional dynamics, there has to be an organized intracellular traffic to and from these organelles. Only a few proteins can enter these organelles directly from the cytosol. Thus, most proteins have to be sorted and further distributed to their final destination (1, 2). They need to contain a signal peptide in order to accomplish the first step of the secretory pathway, this is, translocation into the endoplasmic reticulum (ER). From this point on they are transported in transport vesicles along the secretory pathway, while being modified, and finally delivered to their destinations. In addition to maintaining the cell dynamics, the secretory pathway is used for the delivery of proteins, membrane and cell wall components to the growing bud area as the cell divides, and for secreting proteins to the exterior of the cell.

The intracellular route of secretory proteins was originally defined in mammalian cells (3), and was shown to be basically the same in *S. cerevisiae* by using genetic analysis (4). The yeast secretory pathway was initially defined using temperature-sensitive secretory mutants (*sec*) that reversibly accumulate exocytic proteins at the restrictive temperature of 37°C, whereas at the permissive temperature (25°C) intracellular transport is normal (5, 6). These conditional mutants have provided the tools for investigating

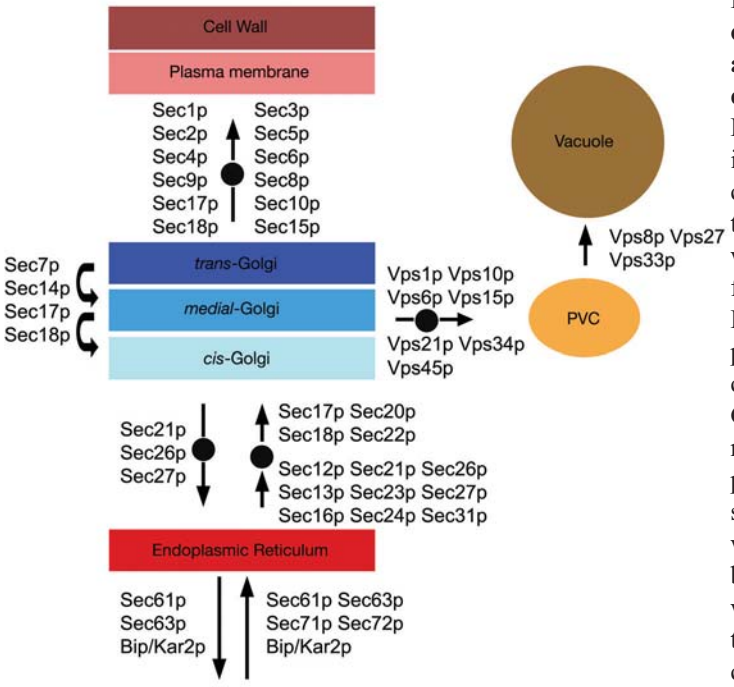


Figure 1. A schematic illustration of the yeast secretory pathway and key proteins involved in each transport step.
Protein translation from mRNA, is initiated on free ribosomes in the cytoplasm. Polypeptides are then translocated into the ER lumen, where they are modified and folded into an active conformation. For ER exit to occur proteins and packaged into vesicular transport carriers. Vesicles migrate to the Golgi and release their cargo after membrane fusion. In the Golgi proteins are further modified and sorted to their final destinations, which can be either to the growing bud (Plasma membrane, cell wall or culture medium) or to the vacuole. (PVC) prevacuolar complex.

the molecular mechanisms that regulate and allow traffic through the secretory pathway. Since then, the genes have been identified and divided into functional groups according to the transport step in which they are involved (see **Figure 1**).

1.2 Membrane-enclosed compartments with individual properties

Eukaryotic cells have membrane-enclosed compartments. The existence of a membrane separating the lumen of the organelles from the cytosol permits the creation of unique and individual environments, which allow special reactions to occur that would not be capable of occurring in the cytosol. In **Figure 2**, transmission electron microscope pictures of a yeast cell can be found depicting the different organelles typically found within the cell.

Nucleus. The nucleus is the largest organelle in the eukaryotic cell and is easily visible with the light microscope. It is separated from the rest of the cell by an envelope consisting of an outer and inner nuclear membrane. The region between the two is the perinuclear space and is continuous with the lumen of the ER. Almost the entire DNA of the cell is located within the nucleus, which is the compartment for storage, replication and expression of genetic information. Nuclei often contain a nucleolus, or some times several, which are the sites of ribosome formation.

Mitochondria. Mitochondria are the energy power station of the cell. They are delimited by two membranes, a smooth outer membrane and a folded inner membrane that encloses the matrix. The folds are referred as cristae and the space between membranes is called the intermembrane space. Components of the respiratory chain and ATP synthesis reside within the inner mitochondrial membrane. The main function of the mitochondria is the oxidative degradation to CO_2 and H_20 of energy-yielding substrates coupled with the synthesis of ATP, which is the major energy storage form used by the cell to drive reactions. Mitochondria probably evolved from aerobic prokaryotic bacteria living in symbiosis with anaerobic host cells (endosymbiotic theory) and hence have their own DNA (mtDNA), which is used to synthesize some of their own proteins.

Endoplasmic Reticulum (ER). The ER is a large intracellular compartment

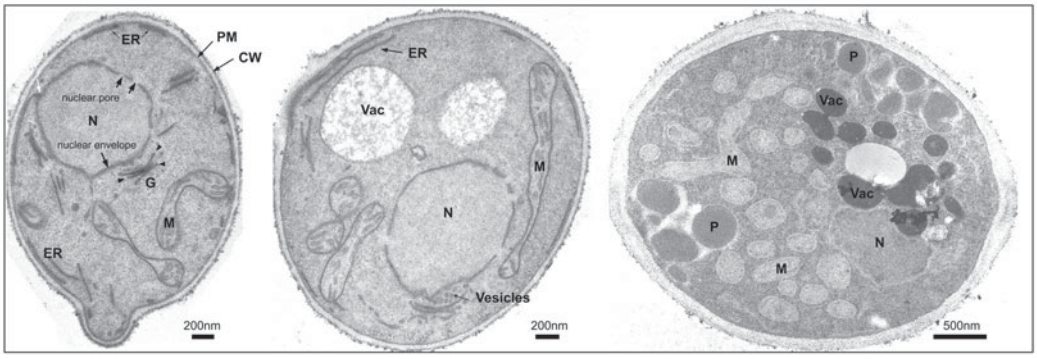


Figure 2. Electron micrographs depicting the different organelles within a yeast cell. Nucleus (N), endoplasmic reticulum (ER), Golgi complex (G), mitochondria (M), peroxisomes (P), vacuole (Vac), plasma membrane (PM) and cell wall (CW).

that extends throughout the cytoplasm. It can be subdivided into to two types, the rough ER (RER) and smooth ER (SER). The RER have many attached ribosomes, and is thus the site of protein biosynthesis. Most of the proteins synthesized at the SER undergo post-translational modifications and are transported to their final destination by transport vesicles, or then remain within the ER.

Golgi Apparatus. Like the ER, the Golgi is a complex network of membrane-enclosed sub-compartments. There are *cis*, *medial* and *trans*-Golgi regions. The main function of the Golgi is protein maturation (phosphorylation, modification of glycans, and processing by proteolytic cleavages for example) and sorting of proteins to the various targets within the cell.

Vacuole. The main function of the vacuole is the enzymatic degradation of various cellular components, as well as nutrients taken up from the surrounding environment via endocytosis. For this purpose vacuoles are filled with different types of degradative enzymes, hydrolases with acidic pH optima. Vacuoles also act as storage compartments of amino acids and detoxification components.

1.3 A model for vesicle-mediated transport

It often takes only seconds for a secretory vesicle to move between intracellular organelles. But this very rapid traffic is also very selective. Only a subset of the proteins and lipids in the donor membrane are allowed into the transport vesicle, thus permitting membraneous organelles to maintain their identity. During the formation of the vesicle, a specific set of proteins, including the coat proteins COPI, COPII and clathrin, carries out a sequential set of actions that lead to the budding of vesicles. Coat components are required to generate highly curved membrane and to select cargo into them. After vesicle fission, uncoating takes place and the naked vesicle is allowed to fuse with the target membrane. The budding reaction is generally regulated by the small G proteins of the ARF family, which in the GTP-activated state initiates coat assembly at the bud site. Coat disassembly then results when GTP is hydrolyzed to GDP (reviewed in (7, 8)) (**Figure 3**).

COPI and COPII coated vesicles are used in the anterograde and recycling pathways between the Golgi and the ER - COPI from the Golgi to the ER and intra-Golgi cisternae, and COPII from ER to the Golgi. The clathrin-coated vesicles are used in two major routes, from the plasma membrane to the early endosome and from the Golgi to the endosome. It should be noticed that tubular transport containers also exist in traffic between organelles (9, 10). Their formation should follow the same principles as explained above.

1.4 Rab GTPases as molecular switches

The Rab proteins constitute a family of GTPase proteins, that together with other proteins take part in the transport of vesicles, controlling the docking and fusion of vesicles to the target membranes. Many of the transport vesicles only form if specific Rab and SNARE proteins are coupled on their membrane, thus allowing the vesicle to correctly fuse. Rab proteins play an important role in the specificity of vesicular transport. As with the soluble NSF attachment protein receptor (SNAREs), each Rab protein has a unique distribution at the cellular membranes and each organelle has at least one type of Rab on its cytosolic surface. Rab proteins are known for facilitating and regulating the tethering and fusion of the vesicles, cargo sorting and cytoskeleton-dependent transport.

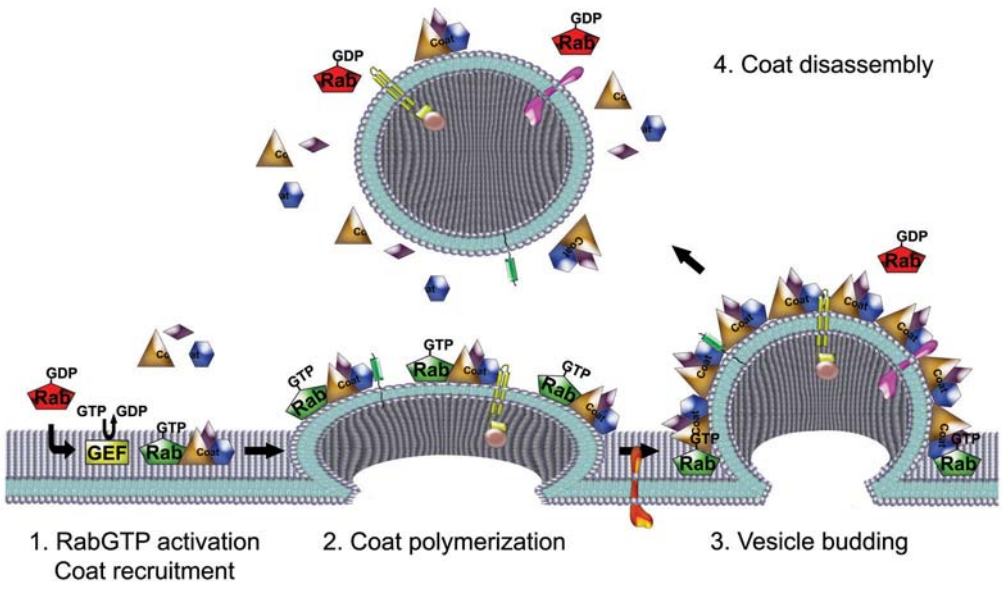


Figure 3. Model for the sequential assembly and formation of coated vesicles.
1. RabGTP activation and coat recruitment. The guanyl exchange factor (GEF) recruits and activates the Rab by exchange of the bound nucleotide. Activated Rab in the GTP form then recruits set of protens that function as a coat. **2. Coat polymerization**. Additional coat component are recruited and cargo intended for exit are packaged into the forming vesicle at this stage. Transmembrane cargo directly interact with the coat proteins, while soluble cargo interact via adapter proteins. **3. Vesicle budding.** Polymerization of the coat leads to membrane deformation and formation of a vesicle. Vesicles scission between the neck of the vesicle and the donor compartment is severed either by direct action of the coat or by accessory proteins. **4. Coat disassembly**. After the vesicle has been released from the membrane, various events including inactivation of the small GTPase, phosphoinositide hydrolysis, and the action of uncoating enzymes lead to uncoating of the vesicle thus exposing proteins on the membrane, which are involved in subsequent membrane fusion reaction.

1.4.1 The Rab GTPase cycle

As with all the other GTPases, Rab proteins circulate between membrane and the cytosol. When in the GDP form, they are inactive and present in the cytosol. In the GTP form, they are active and coupled to membranes. An enzyme, guanine nucleotide exchange factor (GEF), catalyzes the change of GDP for GTP, which leads to a conformational change in the Rab (exposure of two isoprenoid lipids) that allows it to anchor itself to the membrane (**Figure 4**). Once in the GTP form it recruits a specific subset of proteins, called effectors, to particular sites on the membrane and facilitate their assembly into larger complexes. These effectors carry out the downstream functions associated with the Rab protein. The GTP hydrolysis is facilitated by GTPase activating proteins (GAPs) and returns the Rab into the GDP form. A protein called Rab GDP dissociation inhibitor (GDI) removes the Rab GDP from the membrane and solubilizes it, restoring the cytosolic reservoir. Reattachment of the Rab to the membrane for a new cycle is facilitated by GDI displacement factors (GDF)

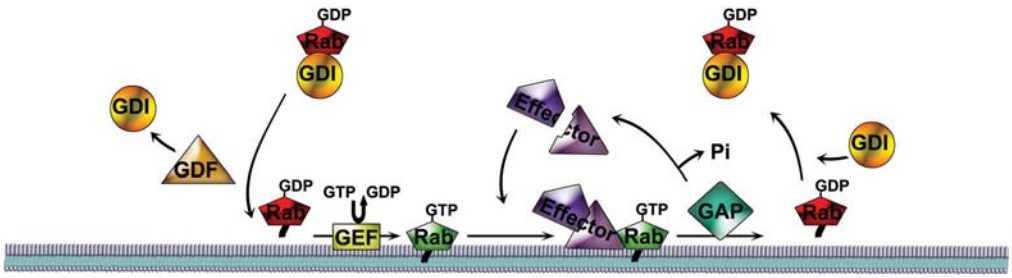


Figure 4. Model of the RabGTP cycle
RabGTPases cycle between the cytosol and the membrane where they recruit a subset of effector proteins, which carry downstream events. See text for details. Guanine nucleotide exchange factor (GEF), GTPase activating proteins (GAPs), Rab GDP dissociation inhibitor (GDI), GDI displacement factors (GDF).

(reviewed in (11)). For this controlled mechanism that leads to a downstream action triggered by the hydrolysis of GTP, Rab proteins are often seen as molecular switches, which regulate and synchronize the sequential steps required in all aspects of intracellular membrane traffic.

1.5 Tethering factors

In addition to SNAREs, other proteins participate in the pairing of two opposing membranes. Tethering factors recognize two target membranes and help bring them into close proximity, thus allowing pairing of the t- and v-SNAREs. Their ability to interact with Rabs and SNAREs, together with their restricted subcellular localization, suggest that tethers help determine the specificity of membrane fusion (reviewed in (12, 13). As examples we can find the transport protein particle TRAPP I in ER to Golgi, TRAPP II in intra-Golgi, GARP in early endosome/PVC to Golgi and the exocyst in Golgi to plasma membrane fusion events.

Tethering factors may be divided into two families: long coiled-coil proteins such as p115, or large multisubunit complexes such as the exocyst complex. Unlike the Rab and SNARE families where different members share a common mechanism of action, the different tethers may facilitate membrane traffic through distinct mechanisms. Tethering factors have been implicated in different aspects of intracellular traffic such as bridging of membranes, SNARE complex assembly, cargo selection, coat dynamics, cytoskeleton-linking and signaling events.

1.5.1 Bridging of membranes

One feature associated to tethers is the physical association of transport intermediates to the target membranes, in a step that precedes SNARE paring and membrane fusion. As the vesicle approaches, tethering factors (exemplified in **Figure 5** by TRAPPI complex) can bridge two opposing membranes at distances more than 200 nm, where t- and v-SNAREs cannot physically interact yet. The tethering complex may then bring the vesicle into close proximity to the target membrane, where the SNARE pairs form a complex. The assembly of the SNARE complexes then leads to the fusion of the transport vesicle with the target membrane.

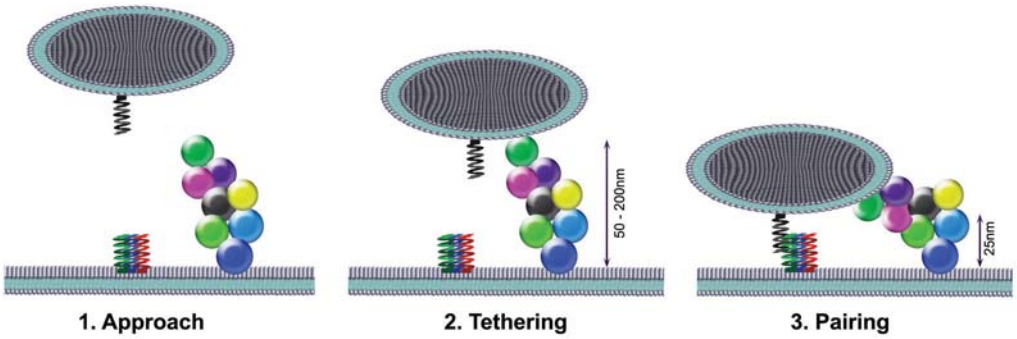


Figure 5. Schematical representation of the role of tethering complexes.
1. Approach. The vesicle moves close to the target membrane compartment, possibly guided by the action of the cytoskeleton. **2. Tethering.** The vesicle then becomes tethered to the acceptor compartment by the combination of a GTP bound Rab and a tethering factor. This step occurs at a distance where v- and t-SNAREs do not interact. **3. Pairing.** The tether then brings donor and target membrane into close proximity where the SNAREs can assemble into a four-helix bundle. This "trans-SNARE complex" then promotes fusion of the vesicle and acceptor lipid bilayers.

1.6 SNARE hypothesis and specificity of vesicular transport

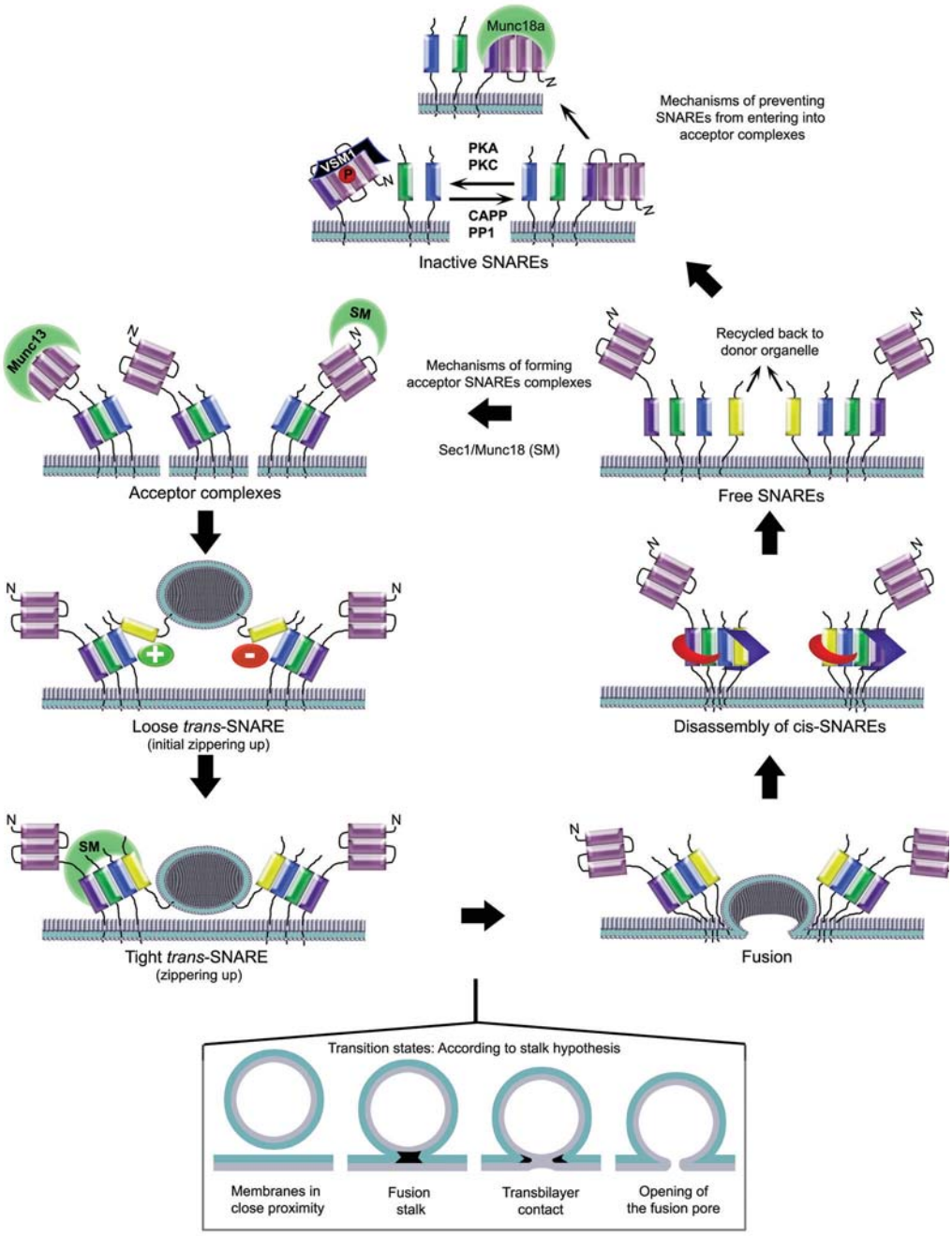
SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins are considered to be the key components that drive membrane fusion in all trafficking steps of the secretory pathway (reviewed in (14)). SNARE proteins form a superfamily of small proteins with a characteristic SNARE motif (stretch of 60-70 amino acids arranged in heptad repeats). At the C-terminus, most SNAREs have a single transmembrane domain, or then are subject to post-translational modifications that lead to their insertion into the membrane. Variations in the N-terminal region lead to the classification of the SNAREs into different subgroups.

SNAREs mediate membrane fusion by forming a helical complex of elongated coiled-coils of four parallel α-helices, each

Figure 6. Membrane fusion is mediated by the formation of SNARE complexes between donor and acceptor compartments.
A general model for the regulation of SNARE assembly in membrane fusion events is shown. Prior to membrane docking, donor and acceptor bilayers undergo a tethering process, mediated by Rab GTPases and tethering complexes (see Figure 5), thus bringing *trans*-SNARE complexes on apposed membranes within close proximity. At this stage members of the Sec1/Munc18 family are recruited, which might promote the formation of a trans-SNARE complex between the two membranes or maintain the primed complexes in a metastable intermediate (represented as a plus or minus signals respectively). After input by some fusogenic signal, the SNARE regulators are released, which allows for the full zippering of the trans complexes from their membrane-distal to membrane proximal ends, this bringing the two membranes into close proximity. Bilayer fusion between the two membranes then occurs (depicted here according to the stalk hypothesis). Subsequently the four-helix *cis*-SNARE bundle is disassembled by NSF (Sec18p in yeast) and its partner α-SNAP (Sec17p in yeast) and the v-SNARE is recycled back to the donor compartment. See text for further details concerning the regulatory mechanisms. SNAREs (v-SNARE = yellow; SNAP-25-like t-SNARE = green and blue; syntaxin-like t-SNARE = purple).

one contributed by a different SNARE, between two opposing membranes. At the central layer of this complex, the "0" layer, there are 4 conserved residues, 3 glutamines (Q) and one arginine (R). The contributing SNARE motifs are thus classified as Qa-, Qb, Qc- and R-SNAREs.

SNAREs are broadly distributed throughout the secretory pathway and at the membrane they target for. NSF (*N*-ethylmaleimide-sensitive factor, or Sec18p in yeast) guarantees that a free pool of SNAREs is always present. Before a SNARE complex between donor and acceptor can form, the SNAREs on



the target membrane form a partially helical Qabc intermediate (see **Figure 6**). The formation of this intermediate is rate limiting, but highly reactive after formed. Factors such as Sec1p/Munc18 (SM) family members may bind to these intermediates and stabilize them until a R-SNARE is incorporated into them. SNAREs are thought to drive membrane fusion through the formation of the 4 helical bundle, starting at the N-terminus and proceeding to the C-terminus, and hence the term Zippering. Central to this zipper-like assembly model, is the formation of the SNARE in *trans*-configuration; i.e. where SNAREs participating in the SNARE complex reside in two opposing membranes. As the SNARE complex assembles in the N to C terminus direction, "zippering up", they exert mechanical forces on the membranes bringing them into close proximity while deforming there normal curvature, thus facilitating the formation of the stalks required for fusion. After fusion, the SNARE complexes are now in the *cis*-orientation, i.e. they reside on the same membrane. These complexes remain biologically inactive until they dissociate. The disassembly requires energy (ATP), NSF and SNAPs (soluble NSF attachment proteins or Sec17p in yeast) that together dissociate the complex. After disassembly, some of the SNAREs are returned to their original compartment by intracellular membrane traffic. As a consequence, SNAREs exist not only on the organelle for which they mediate membrane fusion, but also on the organelles involved in their recycling. Thus, there needs to be a temporal and spatial regulation of the events leading to membrane fusion, to avoid incorrect membrane fusion events.

1.6.1 SNARE regulation

SNAREs are considered to be the elements that drive membrane fusion, and hence the names fusogens or engines for membrane fusion. Thus, while being a central element to the fusion of two membranes, they alone are insufficient. Additional proteins are required to mediate membrane fusion *in vivo*. Many of these factors, which can be called SNARE regulators, play a role in the assembly and ability of SNAREs to form such complexes (reviewed in (15)). Regulation of SNARE activity is crucial for the maintenance of the organelle identity. In this sense, regulators may bind to SNAREs early in the secretory pathway to avoid cognate SNAREs from forming until they reach their appropriate destination. SNAREs tend to be broadly distributed, but on the other hand fusion events tend to occur in localized regions, for example in polarized delivery to the bud tip of the yeast. In this sense only SNAREs in the fusion region need to be activated. Thus some factors may promote the formation of these *trans*-complexes in these regions while others may restrict the activation of SNAREs in regions distant to the site of fusion. When SNARE complexes form, they exist in a metastable state (loose trans-complex). SNARE regulators may bind at this stage and either promote the zippering up or then stabilize this state until certain stimulus occurs (e.g. regulated exocytosis). Thus, SNAREs may be seen as switches that favour (match-makers) or inhibit (match-breakers) the assembly of SNARE complexes (15).

Sec1/Munc18 (SM) family. SM family members bind to the syntaxin (Qa-) class of t-SNAREs in both yeast and mammalian cells. A feature of the syntaxin class is that they have a N-terminal regulatory domain (Habc) folded as a three helice bundle that may fold back

onto the SNARE motif, located in the C-terminus, and form an intra-molecular four-helix bundle, called the "closed" conformation (16). In yeast, SM family members (Sec1p for example) may bind to preassembled SNARE complexes activating them, and prevent the folding back of the Habc domain. In cases where the syntaxin N-terminus does not form a closed conformation (Sed5p or Tlg2p), SM family members (Sly1p or Vps45p) may bind to the N-terminal peptide of the regulatory domain (17, 18). Yeast SM family members favour the transition of inactive to active t-SNARE and thus promote the assembly of the SNARE complex. In contrast, in mammalian cells, SM family members (Munc18a) bind to the closed conformation of syntaxin and were therefore initially thought to prevent SNARE assembly. Although SM family members appear to play opposing roles in yeast and mammalian cells, recent evidence suggest a positive role for them, by preventing for example oligomerization between syntaxins, or by inhibiting their association with other SNAREs while trafficking to their final destination (19).

Synaptotagmins and Munc13/Unc-13. These regulators are found exclusively in higher eukaryotes and mediate stimulus-coupled exocytosis.

Synaptotagmins have a C2 domain that works as a calcium sensor and hence mediates stimulus-coupled secretion. Binding of synaptotagmins to the metastable SNARE complexes inhibits complete zippering up until the influx of calcium. Upon calcium influx synaptotagmins facilitate assembly of the SNARE complex leading to fusion of the docked vesicles (15).

Munc13/Unc-13 contains a C1 domain that is sensitive to diacylglycerol and binds to the N-terminal regulatory domain of syntaxins. Munc13/Unc-13 may favour SNARE complex assembly by promoting the change from closed to the open state of syntaxin (15).

LMA1. Lma1p binds to the t-SNARE (Vam3p) involved in vacuolar fusion in yeast that is normally found in *cis*-complexes. After disassembly of the *cis*-SNARE complexes by Sec18p, Lma1p is transferred from Sec18p to the t-SNARE Vam3p, and prevents Vam3p from reentering into a *cis*-complex. Vam3p- Lma1p thus enters a fusion cycle, when the SNAREs form a tight *trans*-complex. The concerted action of calcium, calmodulin and phosphatase activity lead to the release of Lma1p from Vam3p, and the bilayers fuse (20).

Phosphorylation as a mechanism of regulation. Internal and external signals in the cell lead to signaling cascades that culminate in the activation or repression of kinases and phosphatases. Regulation of intracellular transport by protein phosphorylation allows the cell to couple vesicular traffic with the prevailing conditions. Recently many of the elements involved in the fusion event, such as Rabs, tethering factors, SNAREs and their regulators are subject to phosphorylation. In general phosphorylation seems to favour the binding of the SNARE regulator at the expense of their binding SNARE, thus disrupting the formation of acceptor complexes, and hence inhibiting the fusion of two membranes. For example phosphorylation of the NH2-domain of syntaxin (Sso1/2) inhibits the binding of its partner t-SNARE SNAP-25 (Sec9) and thus prevents the formation of acceptor complexes (21).

Vsm1. Vsm1p is capable of binding to the yeast t-SNARE syntaxin Sso1/2p, after the latter has been phosphorylated (22). The binding of Vsm1p to Sso1/2p

prevents binding of the t-SNARE partner Sec9p, thus preventing the formation of acceptor complexes for posterior fusions events to occur (23).

2. Endoplasmic Reticulum

2.1 Entry into the exocytic pathway

Proteins destined to enter the ER can be divided into two groups: a) soluble proteins that are secreted to the exterior of the cell or remain in the lumen of an organelle such as the ER, Golgi or vacuole and b) membrane proteins, such as those present in the plasma membrane and those on the membranes of the secretory organelles.

The synthesis of most exocytic proteins begins on free ribosomes in the cytosol. The presence of a 15 to 50 residue signal peptide at the N-terminus of the nascent polypeptide chain directs the ribosome to the ER membrane and initiates transfer of the growing polypeptide across the ER membrane (24). The newly synthesized polypeptide thus enters the exocytic pathway. Translocation of the polypeptide into the ER lumen can occur either cotranslationally or posttranslationally. In mammalian cells, translocation of proteins into the ER occurs primarily cotranslationally, i.e. simultaneously with protein synthesis, whereas yeast is capable of using both pathways (25). The reason for this may be that in fast growing cells translocation may not always keep pace with translation (26).

2.1.1 Signal peptides

The decision on the translocation mode is determined by the hydrophobicity of the signal peptide. The signal peptide (from 15 up to more than 50 amino acids) is composed by an essential hydrophobic central region, called the h-region of 6 to 15 amino acids. The h-region is flanked by two polar regions, the n-region located N-terminally and the c-region located to the C-terminus. Within the c-region, a cleavage site for the ER-located signal peptidase is present, resulting in removal of the signal peptide (27).

The more hydrophobic the signal peptide, more likely the polypeptide will be translocated cotranslationally. This is mainly due to the signal recognition particle (SRP), which mediates cotranslational translocation, associates with the h-region within the signal peptide. Less hydrophobic cores do not bind SRP, and thus these polypeptides use the post-translational route (28). Both translocation pathways require specific targeting of the polypeptide to the site of translocation

2.1.2 Co-translational translocation

In cotranslational translocation, synthesis of the polypeptide is coupled to translocation or insertion into the ER membrane. Essential to this type of translocation is the presence of the signal recognition particle (SRP) and its ER membrane-bound receptor (SR).

When a protein is being synthesized, the SRP recognizes the signal peptide that emerges from the ribosome and directs the polypeptide with the ribosome to the ER membrane (for review see (29)). Two domains, the S domain and the Alu domain constitute the SRP (**Figure 7**). The S domain mediates signal peptide binding and docking to the SR, while the Alu domain is responsible for a transient delay in translation, i.e. elongation arrest (30, 31). The SRP of higher eukaryotes is composed of six proteins SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72 assembled on the SRP RNA 7SL (25). The yeast SRP homolog is also composed of 6 proteins (Srp14p, Srp21p, Srp54p, Srp65p, Srp68p and Srp72p) assembled on scR1

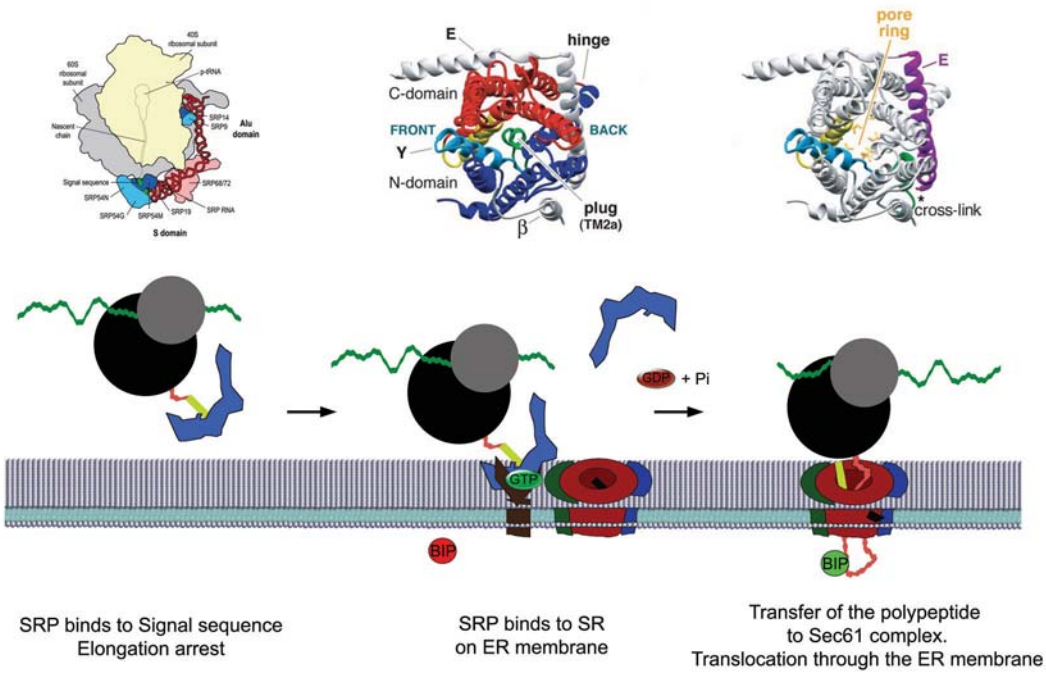


Figure 7. SRP-mediated co-translational translocation.
In cotranslational translocation, the ribosome is bound to the translocon (Sec61 complex; Sec61p, Ss1p and Sbh1p), and translocation and translation occur simultanously. The N-terminal signal peptide (yellow box) is recognized by the signal recognition particle (SRP) as soon as it emerges from the ribosomal polypeptide exit tunnel. In eukaryotes peptide elongation is temporarily arrested upon the formation of the SRP-RNC complex (RNC, ribosome nascent chain complex). This complex is then targeted to the ER membrane by binding to its receptor SR. The nascent polypeptide is then transferred to the Sec61 complex and translocated into the ER lumen as translation of the remaining mRNA (green) proceeds. The ER chaperone Kar2p/BiP, in the ADP from, binds to the emerging polypeptide. Thereafter the polypeptide looses its signal peptide.
Structures: Schematic overview of the mammalian SRP bound to the signal sequence carrying 80S ribosome (RNC) based on a cryo-EM structure (Wild *et al.*, 2004). The S and Alu domains of SRP are illustrated. The 40S and 60S ribosomal subunits are yellow and grey respectively. Reprinted, with permission, from Nature Structural & Molecular Biology, Volume 11, Number 11 (c) 2004.
The *Methanococcus jannaschii* SecY complex viewed from the cytoplasm. (Osborne *et al.*, 2005). The N-terminal domain of SecY (TMD1–5) is shown in dark blue with TMD2b in bright blue. The C-terminal domain (TMD6–10) is shown in red, with TMD7 shown in yellow. The plug that blocks the pore of the closed channel (TMD2a) is shown in green. The SecE and Secβ subunits are shown in white.
Cytosolic view of *E. coli* SecY channel, with the plug modeled in its open position (Osborne et al., 2005). The asterisk indicates the region where introduced cysteines resulted in cross-links between the plug and the TM segment of SecE.
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RNA, except that there is no SRP9 and there are two copies of Srp14p in yeast (31). SRP54 constitutes the universally conserved region of the S domain and is responsible for the signal sequence binding (32, 33) and the GTP-dependent SR interaction (34, 35). The SRP9, SRP14 and the SRP RNA 7SL form the Alu domain (36, 37).

The SRP binds to the signal peptide as it emerges from the ribosome and then targets the ribosome-nascent chain (RNC)-SRP complex to the ER by interacting with ER-membrane bound SR. At this stage the elongation arrest induced by the Alu domain provides a longer time frame for the interaction to take place. The elongation arrest is not essential for translocation to occur but increases translocation efficiency (38).

The ER membrane-bound receptor SR is a heterodimeric complex composed of SRα and SRβ in mammals (39, 40) and its homologues Src101p and Src102p in yeasts (41, 42). SRα/Src101p is a GTPase and SRβ/Src102p is responsible for anchorage to the ER.

Binding of GTP by SRP54 and SRα, helps stabilize the complex formed between SRP and SR and initiates the transfer of the signal peptide from the SRP54 subunit to the Sec61α component of the Sec61 complex at the translocation site. Hydrolysis of GTP by both SRP54 and SRα is required to dissociate the SRP-SR complex and to resume polypeptide synthesis (43).

The Sec61 complex is composed of three subunits, Sec61α, Sec61β and Sec61γ in mammals. The yeast homolog counterparts are called Sec61p, Sbh1p and Sss1p in *S. cerevisiae* (44). Ss1p in yeast is also present in a second trimeric complex, called the Ssh1p complex together with Ssh1p and Ssh2p, homologues of Sec61p and Sbh1p, respectively. Ssh1p has been proposed to function in the cotranslational pathway since it interacts with membrane-bound ribosomes but not with components involved with the posttranslational pathway (45).

The largest subunit of the Sec61 complex is the Sec61α subunit and spans the membrane ten times (44). The protein-conducting channel formed by the Sec61 complex is a passive pore, i.e. the polypeptide can slide in either direction, and therefore the channel requires accessory proteins to provide the driving force to ensure that the polypeptide slides into the ER. In the case of cotranslational translocation the force is provided by GTP hydrolysis that occurs during translation (46). Although the channel is passive, a tight seal ensures that no ions or proteins traverse freely the pore. There is a short helix in the Sec61α-subunit (TM2α) that functions as a plug. It slides back upon binding of a ribosome and the signal peptide to the α-subunit, opening the pore for translocation (47). Once the signal peptide is connected to the Sec61 channel walls, the peptide region distant to the signal sequence is pushed through the pore and prevents the plug from returning to the closed state. Although translocation occurs through one pore of the Sec61 complex, the actual translocation complex is composed of four Sec61 complexes. Two of them are associated side by side, with each dimer being packed in a back-to-back fashion (48). The oligomerization of the translocation channel may recruit additional factors such as TRAM (membrane chaperone) and TRAP (unknown function), that may play important roles for example in translocation of transmembrane proteins, or increase the surface area available to

bind the ribosome, increasing the stability of the connection (48, 49).

The polypeptide chain then elongates into the ER lumen where the signal peptide is cleaved off by the signal peptidase and is rapidly degraded. The polypeptide continues to elongate through the translocon into the ER lumen.

After the translation is completed, the ribosomes are released, the C-terminus of the protein is drawn into the ER lumen, the short α-helix that constitutes the plug slides black closing the pore, and the translocated protein assumes its conformation.

Transmembrane proteins may be orientated differently depending on the flanking amino acids of the first transmembrane region (for a more detailed explanation see (50)). The transmembrane region is composed of hydrophobic amino acids usually arranged as α-helical regions of 20 to 25 residues. This sequence, called stop-transfer sequence, blocks further translocation of the polypeptide into the ER, the ribosome is released from the translocation apparatus and finishes its job in the cytosol. In the case of a multispanning protein, the first transmembrane region often determines the orientation of the subsequent ones, which alternate correspondingly. During the synthesis of a membrane protein, the transmembrane segments move from the aqueous interior of the Sec61channel through a lateral gate into the lipid phase of the ER membrane (47). Proteins can also be anchored in the ER membrane by internal signal peptides that are not cleaved by signal peptidase. Proteins that span the membrane multiple times may result from alternating series of internal signal peptides and stop-transfer sequences.

Cleavage of the signal peptide, *N*-linked glycosylation and folding of the polypeptide are essential cotranslational events. Since folding of many protein precursors occurs simultaneously with cotranslational translocation, the activity of luminal proteins such as protein disulfide isomerase (PDI) and the chaperone BiP/Kar2p may be added to the list of functions required for successful translocation (51).

2.1.3 Post-translational translocation

Eukaryotes have the capacity of translocating proteins post-translationally, i.e. after they have been fully synthesized in the cytosol and released from the ribosome. These proteins have a less hydrophobic signal peptide, and may therefore not interact with the SRP during their synthesis (28). Posttranslational translocation was determined in *S. cerevisiae* (52, 53) and requires a seven-component Sec complex constituted by the Sec61 complex (the same used in cotranslational-translocation), and the Sec62/63 complexes together with the luminal BiP/Kar2p, a member of the Hsp70 family of ATPases (54, 55). The Sec62/63 complex is composed of Sec62p and Sec63p, and the nonessential components Sec71p and Sec72p. The yeast Sec61 complex components are Sec61p, Sbh1p and Sss1p, and those of the Sec62/63 subcomplex are Sec62p, Sec63p, Sec71p and Sec72p. The mammalian homologue of Sec62/63 subcomplex lacks Sec71p and Sec72p homologs (56, 57). Presumably, the completed polypeptide chain is presented to the ER membrane in a complex with cytosolic chaperones that cycle on and off, and like in cotranslational translocation, the Sec61 complex serves as the channel. The signal peptide binds initially to the Sec61p component of the Sec61 complex and directs the polypeptide to the translocation channel (58). While bound to Sec61p, the signal peptide simultaneously

contacts Sec62p of the Sec62/63 complex (59) (**Figure 8**). When the signal peptide is bound to the posttranslational translocation complex, the cytosolic chaperones are released, thus helping the passive forward movement of the polypeptide (60). Since the polypeptide in the channel can slide in either direction, the driving force to ensure the correct direction is provided by BiP/Kar2p. Binding of BiP/Kar2p to the polypeptide prevents its sliding back, and hence posttranslational translocation is thought to occur through a ratcheting mechanism (53). BiP/Kar2p has a peptide-binding pocket that is open in the ATP form and closed in the ADP form. BiP-ATP binds to a lumenal region of Sec63p called the J domain that is close to the incoming peptide. The interaction of BiP with the J domain activates ATP hydrolysis and the peptide pocket closes, capturing a region of the incoming polypeptide. The peptide-binding pocket shows no sequence specificity when activated by the J domain and the location of the J domain guarantees that BiP/Kar2p activation only occurs in close proximity to the incoming polypeptide (61, 62), ensuring that no backsliding occurs. The polypeptide moves forward by Brownian motion, other molecules of BiP/Kar2p bind to it and this process continues until the whole polypeptide has been translocated. After the polypeptide has moved away from

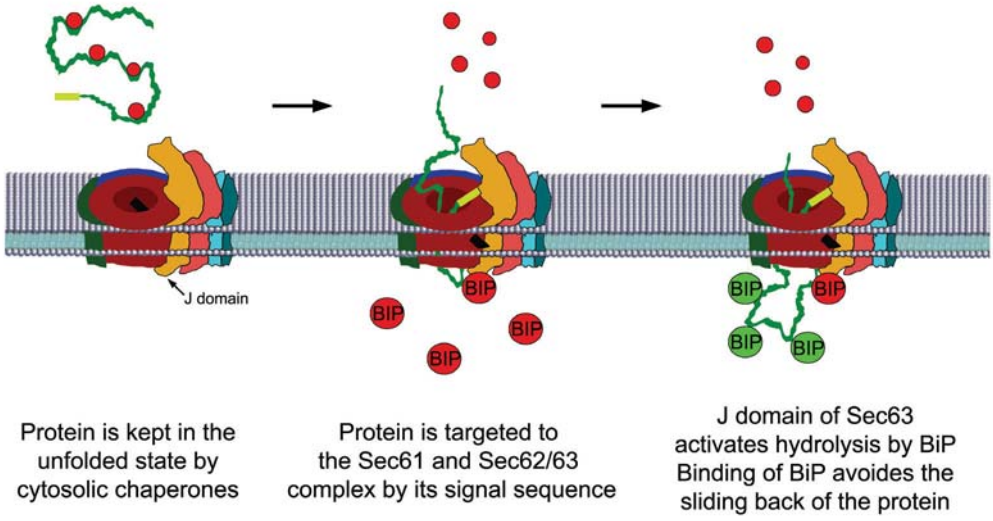


Figure 8. Post-translational translocation.
After it is synthesized in the cytosol, the polypeptide is released from the ribosome. The polypeptide is kept in solution in a loosely folded state by cytosolic chaperones of the Hsp70 family. The signal peptide targets it to the translocation channel, which is formed by the Sec61 complex and the Sec62/63 complex. Polypeptide binding to Sec62/63 induces conformational changes that lead to the transfer of the signal peptide to the Sec61 complex and the polypeptide is initially translocated through the channel. The J-domain of Sec63 stimulates ATP hydrolysis by the ER chaperone Kar2p, which binds to the translocating polypeptide in the ADP bound form and prevents it from slipping back into the cytosol. When the polypeptide has moved a sufficient distance into the ER lumen, another Kar2p molecule can bind to it. This process is repeated until the polypeptide chain has passed through the channel. Kar2p is released from the polypeptide upon exchange of ADP for ATP, which opens the peptide-binding pocket.

the channel, exchange of ADP for ATP on BiP/Kar2p releases it for a new cycle and rebinding to the polypeptide does not occur (61).

2.2 Protein maturation in the ER

After translocation of the polypeptide into the ER, the protein needs to adopt the correct final conformation to be fully functional. For this purpose the polypeptide undergoes a series of post-translocation modifications that are initiated in the ER, such as signal peptide cleavage, glycosylation and formation of disulfide bonds. All of these processes are required for the protein to fold into its correct and active three-dimensional conformation.

2.2.1 Signal peptide cleavage

Maturation in the ER begins by the removal of the signal peptides while the protein is being translocated or then shortly after. In the case where the signal peptide serves as the membrane anchoring transmembrane domain it remains uncleaved. The signal peptidase is a complex called the signal peptidase complex (SPC). The mammalian SPC is composed of five components SPC18, SPC21, SPC22/23, SPC25 and SPC12 (63). The yeast SPC consists of four components, Sec11p, Spc1p, Spc2p and Spc3p (64, 65), where the core catalytical domain is constituted by the essential Spc3p and Sec11p components (66, 67). Spc1p and Spc2p are non-catalytical and are not essential for the overall function. They are tightly associated in the SPC and their role may be in assisting the interaction with the translocation channel and in enhancing the overall activity of the SPC (68, 69).

When a protein is being translocated, the SPC is recruited to the translocation channel and interacts with members of the Sec61 complex. In mammalian cells, SPC21 interacts with the Sec61β-subunit (70), whereas in yeast the interaction with Sbh1p and Sbh2p (homologues of Sec61β-subunit) is mediated by the Spc2p component (69).

2.2.2 Core-glycosylation

The most common modification of proteins that enter the ER is glycosylation. The addition of glycans has been shown to be important for a variety of functions. The glycans are important in the folding of the protein and serve as signals for quality control. They play a role in conferring stability to proteins, by protecting them from pH-inflicted denaturation and from proteases. They can also be involved in targeting and signaling events. Addition of different glycan chains may also help creat different isoforms of the same protein, which would allow an increase in their specificity. From yeast to mammalian cells, glycans are generally added to amino (N-glycosylation) or hydroxyl groups (O-glycosylation) of specific amino acid residues (reviewed by (71-73).

2.2.2.1 N-linked glycosylation

In general, *N*-glycosylated proteins are secretory proteins that are either exported to the plasma membrane, or to the extracellular matrix, or then to the cell wall (72). *N*-linked core glycans are added from a lipid carrier (dolichol-pyrophosphate) to the protein *en bloc* in the lumen of the ER. In **Figure 9** the biosynthesis of the core oligosaccharide Dol-PP-$GlcNAc_2Man_9Glc_3$ is shown. The core glycan, which has a defined structure in virtually all eukaryotes, results from the sequential addition of monosaccharides in a reaction catalyzed by monosaccharyltransferases in the ER membrane (74-76). Synthesis starts

in the cytosol by the transfer of N-acetylglucosamine phosphate from UDP-GlcNAc to the carrier Dol-P, creating GlcNAc-PP-Dol, in a step that is inhibited by tunicamycin. After addition of 6 more monosaccharides (one GLcNAc and five mannose residues) donated by UDP-GLcNAc or GDP-Man, the glycan chain moiety is flipped across the ER membrane to its luminal side, by an ATP-independent bi-directional flipase. In yeast Rft1p is responsible for this event (73). In the lumen, four additional mannoses and three glucoses are added sequentially and specifically creating the final branched glycan core. In the ER lumen, the lipid-activated sugars Dol-P-Man and Dol-P-Glc serve as donors. The addition of the terminal α-1,2 linked glucose residue is of special importance, since it is required for efficient recognition by the ER-resident oligosaccharyl transferase (OST) (71, 77). OST is responsible for the transfer of the oligosaccharide core to the amino group of the asparagine residue of the consensus sequence Asp-X-Ser/Thr, where X may be any amino acid except proline. OST is composed of 8 proteins, Wbp1p, Swp1p, Ost2p, Ost1p, Ost5p, Stt3p, Ost3p/Ost6p and Ost4p arranged in 3 sub-complexes. Wbp1p, Swp1p, Stt3p, Ost1p and Ost2p are essential, but cells lacking the other three genes exhibit defects in N-glycosylation and growth (reviewed by (78)). All yeast OST components, except Ost4p and Ost5p, have mammalian homologues (79). The yeast Stt3p has two homologues in mammalian cells, STT3-A and STT3-B, and the Ost3p/Ost6p are homologous to the mammalian N33 and IAP. The formation of the OST complex with these different proteins creates different isoforms that differ in activity, composition and tissue specificity (80). Similarly in yeast the presence of either Ost3 or Ost6 subunits in the OST complex modifies the transfer specificity towards proteins to be glycosylated and they specify the interaction with different translocation complexes (81). The Stt3p subunit appears to compose the active site of the OST (82) and Wbp1p interacts directly with the Sss1p component of the Sec61 translocation complex (83), which results in close positioning of the OST to the translocon complex where N-glycosylation occurs as the nascent polypeptide emerges. The potential N-glycosylation site is approximately 65 amino acids away from the ribosomal peptidyl–tRNA binding site and hence interacts with the polypeptide chain shortly after it has entered the ER lumen (84). After the oligosaccharide core is transferred to the polypeptide, in yeast and in mammalian cells, some initial trimming occurs where the terminal ⟨1,2-glucose and the remaining two ⟨1,3-glucoses residues are removed by ⟨-glucosidase I (CWH41 in yeast) and ⟨-glucosidase II (ROT2 in yeast), respectively (reviewed by (85)). Further trimming may occur later in the Golgi or in the ER (86). In **Figure 9** examples of possible trimming events is depicted on the core of the oligosaccharide as well as the enzymes involved.

2.2.2.2 O-linked glycosylation

Unlike its higher eukaryotic partners, yeast only possess one type of O-glycosylation, which is typically known as O-mannosylation, since it refers to the addition of mannoses (Man) to the hydroxyl groups of certain serine or threonine residues of the polypeptide. For approximately 30 years since their discovery, protein mannosylation was thought to be a fungal specific modification. However In the late 90s α-dystroglycan was isolated from muscle

and nerve cells and showed to have mannose residues (87).The biosynthesis of *O*-glycans starts in the yeast ER lumen during translocation. The first mannose residue is linked to a serine or threonine in a reaction catalyzed by protein *O*-mannosyl transferases (PMTs). The activated sugar is supplied by Dol-P-Man, resulting in α-D-mannosyl linkage (reviewed by (88)). The PMT family can be subdivided into three subfamilies PMT1, PMT2 and PMT4 (88, 89), each one with different substrate specificity (90). Depletion of any one of the PMT genes is viable, although cells lacking multiple PMT genes exhibit severe defects (91). In yeast the active enzyme consists actually of a dimer of different members of the PMT family, which is responsible for different substrate specificities even within the same protein (92, 93). The seven potential protein O-mannosyl transferases (Pmt1 to Pmt7) have 50-80% homology (88). Addition of mannose residues continues in the Golgi apparatus. Higher eukaryotes on the other hand, have the ability of O-glycosylating other residues such as hydroxylysine (Hyl), hydroxyproline (Hyp) and tyrosine (Tyr) with a variety of sugars such as N-acetylglucosamine (GlcNAc), N-

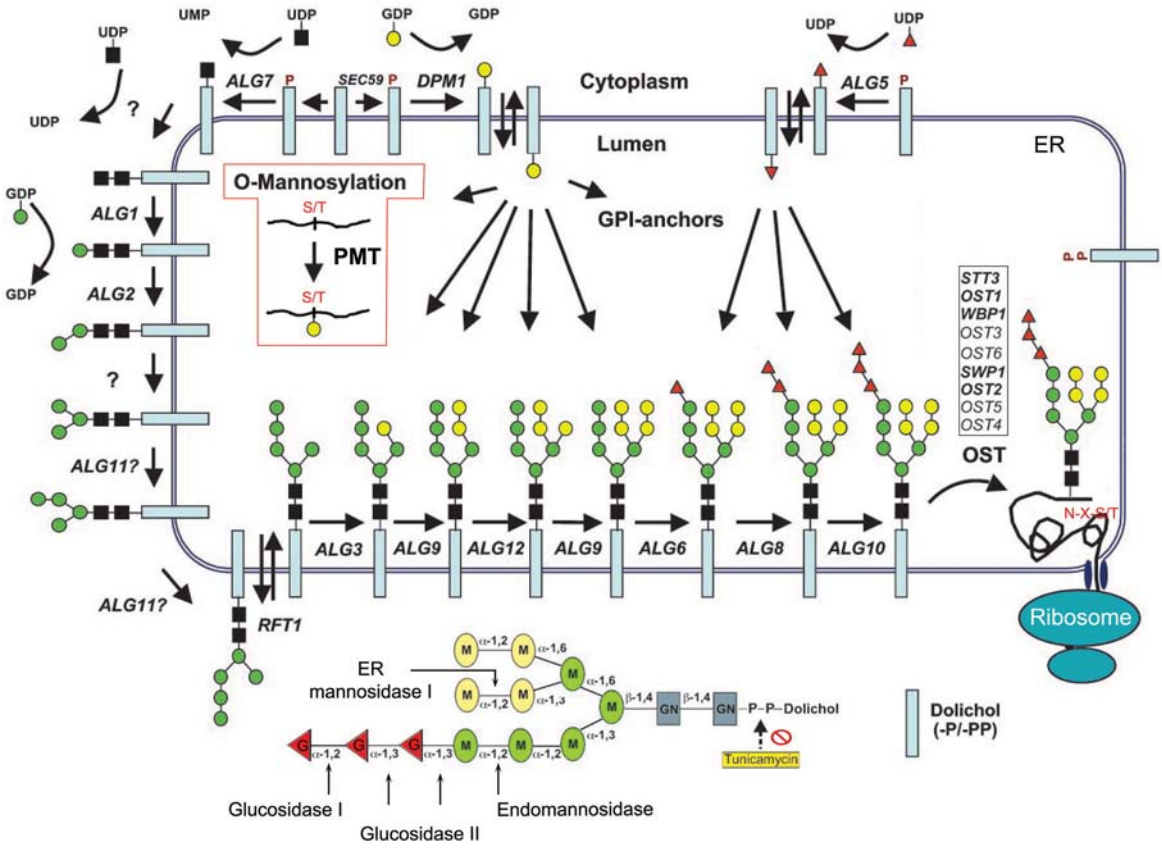


Figure 9. Glycan biosynthesis in the ER.
The yeast proteins known to be involved in each step are indicated. Adapted from Helenius and Aebi, 2004. In the lower panel the cleavage sites on the N-glycan core by the ER-resident trimming glycosidases are indicated.

acetylgalactosamine (GalNAc), glucose (Glu), galactose (Gal), rhamnose (Rha), xylose (Xyl), arabinose (Ara) and fucose (Fuc) (71).

It has been proposed that O- and N- glycosylation compete for the same substrate in the ER and that O-glycosylation precedes N-glycosylation (94). In this study they show that cell wall protein 5 (Ccw5) could only be N-glycosylated when O-glycosylation of certain sites was abolished. For proper N-glycosylation to occur, the region recognized by the OST, the sequon (N-X-S/T, where X can be any amino acid except proline) has to have a hydroxy group donated by serine or threonine, to mechanistically allow N-linkage between the oligosaccharide and the peptide (95). In this sense by modifying the hydroxy group in the sequon it is possible to regulate N-glycosylation.

2.2.3 Protein folding and quality control in the ER

The ER provides an optimized environment for folding, oxidation and assembly of oligomeric proteins. The composition of the ER lumen, as well as its redox conditions favors the fold of proteins to native conformations. A set of proteins such as chaperones and folding enzymes stabilize the partially folded protein during the process of folding and assembly. If the protein fails to achieve its correct folded state, it is detected by the ER quality control machinery and is either refolded or then targeted to ER-associated degradation (ERAD).

2.2.3.1 Chaperones and folding enzymes

The formation of disulphide bonds between the correct cystein residues plays an important role in the stability of the final protein structure. In average a protein has a disulphide bond for every 500 amino acids, and the formation of such a bond is spontaneous. Acquisition of correct disulphide bonds is crucial for the protein. In eukaryotic cells oxidoreductin 1 (Ero1p) and protein disulphide isomerase (PDI) enzymes ensure correct formation of disulphide bonds (for review see (96)). Ero1p is an ER membrane-associated protein that has seven conserved cysteine residues that are thought to be involved in catalyzing the electron transfer. In agreement with its function in assisting the folding of the protein in the ER, expression of both yeast Ero1p and human hERO1-L are induced by the unfolded protein response (UPR) (97). PDI is an abundant soluble ER protein and contains two thioredoxin-like Cys-Gly-His-Cys (CGHC) active sites. Depending on the reduction state of PDI, it may aid in the formation, removal or isomerization of the disulphide bond (98). Formation of the disulphide bond is thought to occur through a relay mechanism with the transfer of oxidizing equivalents. In this mechanism flavin adenine dinucleotide (FAD)-bound Ero1p oxidizes PDI directly though disulfide exchange, PDI then catalyzes the formation of disulfides in folding proteins. The ability of FAD-bound Ero1p to rapidly pass electrons directly to the terminal acceptor O_2 provides the driving force for disulfide formation (99, 100). Formation of disulphide bonds requires that the oxidizing conditions be maintained in the ER. Glutathione is the major redox buffer in eukaryotic cells, and exists in two states reduced (GSH) and oxidized (GSSG) (101). The ratio of GSH/GSSG in the ER is much more oxidizing than the ratio in the cytosol (3:1 vs. 100:1) and hence for a long time it was thought that glutathione was the source of oxidizing equivalents for disulphide

bond formation, but the Ero1p-PDI mechanism is independent of glutathione (99). The function of glutathione appears to provide the net reducing equivalents to the ER that buffer the ER against transient hyperoxidizing conditions (102). PDI has four homologues in yeast (EUG1, MPD1, MPD2 and EPS1) and Ero1p specifically oxidizes only PDI (96). PDI and its homologues are required to rearrange/ isomerase the disulphide bonds since many of them occur simultaneously in the protein. But to fulfill this function PDI and its homologues have to exist in the reduced state. Thus the redox-exchange between cytosol and ER ensures that some PDI can exist in the reduced state for proper isomerization of the polypeptides (96, 103).

The ER has additional proteins in the lumen that assist the folding of the protein, like BiP (Kar2p in *S. cerevisiae*), an Hsp70 family molecular chaperone. BiP like other HSP70 proteins is composed of two domains, an N-terminal ATPase domain and a C-terminal substrate domain, whose affinity is regulated by nucleotide binding to the ATPase domain. (ATP low affinity, rapid and ADP high affinity and slow) (104). BAP (Sil1p in yeast) is the nucleotide exchange factor of BiP mediating the exchange of ADP for ATP (105, 106). The difference of exchange rate is the basis of the posttranslational mechanism, where ADP-BiP remains attached to the incoming polypeptide preventing its sliding back (discussed above). BiP does not bind to proteins in their native state but instead binds temporarily to newly synthesized proteins and more permanently to misfolded, under-glycosylated or unassembled proteins that are captured by the ER quality control machinery and hence cannot exit the ER (107). BiP performs its action by recognizing unfolded polypeptides and inhibiting molecular aggregation, thus maintaining them in a form capable for correct folding and oligomerization (104). BiP is an essential protein with multiple functions that is conserved in eukaryotes. It has a role in translocation of polypeptides, in their folding and assembly (104), in ER quality control (108), in ER-associated protein degradation (109), in sensing ER stress (110) and is also required for fusion of nuclear membranes (karyogamy) during cell mating in yeast (111).

The ER has another Hsp70 family molecular chaperone called GRP170 (Lhs1p in yeast) that shares some overlapping functions with BiP. Lhs1p does not interact significantly with the J-domain of Sec63 but is required for correct folding of polypeptides translocated into the ER as well as in the refolding and processing of misfolded proteins (105, 112, 113). Lhs1p and BiP are thought to interact with each other coordinating their ATP cycles (114) where Lhs1p stimulates the nucleotide exchange phase of BiP, while BiP favors the ATP hydrolysis of Lhs1p. This mutual mechanism may allow coordinated binding and release of Kar2p and Lhs1p to and from different regions of the unfolded polypeptide. This might enhance native folding of the released region while minimizing the possibility of aggregation with other non-native sequences.

2.2.3.2 Quality control and ER-associated degradation

Before a protein can exit the ER, it has to be correctly assembled in its native conformation. If the protein fails to achieve its correct conformation the ER quality control machinery (ERQC) detects the protein and targets it either for degradation or then attempts to refold it

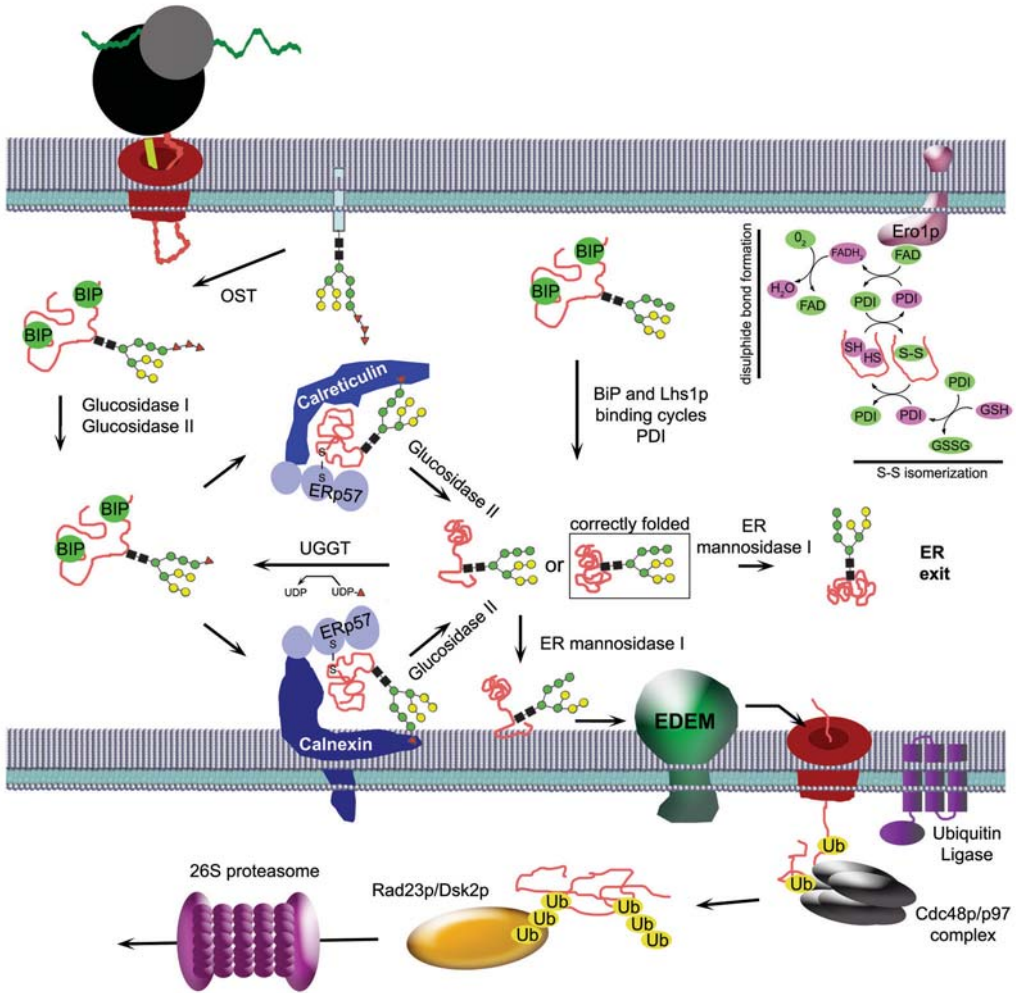


Figure 10. ER quality control.
Calnexin and calreticulin assist in the folding of glycoproteins in the ER. After the N-glycan core is transferred to the nascent chain of the protein two glucose residues (red) are removed by glucosidase I and II. The protein in the monoglucosylated form can then interact with calnexin and callreticulin. Correct disulphide-bond formation is promoted by the thiol-disulphide oxidoreductase ERp57. In addition, the enzymes oxidoreductin 1 (Ero1p) and protein disulphide isomerase (PDI) ensure that the correct disulphide bonds are formed. Cleavage of the remaining glucose by glucosidase II terminates the interaction of the polypeptide with either calnexin or calreticulin. If the protein is correctly folded it is allowed to exit the ER. GRP170 (Lhs1p) and BiP coordinately bind to and are released from different regions the folding polypeptide, which is thought to enhance native folding while preventing aggregation. However in the scenario the protein is not in the native state it is a substrate for the UDP-glucose:glycoprotein glucosyltransferase, which adds a single glucose and the polypeptide may enter a new folding cycle attempt. If the polypeptide remains too long in the unfolded state, the ER α1,2-mannosidase I removes one mannose residue. This leads to recognition by the ER degradation-enhancing 1,2-mannosidase-like protein (EDEM), which targets these proteins for the ER-associated degradation (ERAD) pathway. The unfolded protein is retro-translocated back into the cytosol, where it is ubiquitinated and degraded by the proteasome.

(**Figure 10**). In general the other organelles to which exocytic proteins are destined do not support proper folding. The ERQC guarantees that unfolded proteins are not targeted to their terminal destinations where they might potentially damage the cell (108). The cell uses two mechanisms to distinguish between the native and incorrect conformation, one is the use of a variety of sensor molecules, such as chaperones, and the second is by tagging them with ubiquitin or glucose. The tags are added to the protein when hydrophobic residues, unpaired cysteine residues and aggregates are detected. In the majority of the cases, there are no specific signals or motifs that surrender the protein to the ERQC, so all proteins that pass through the ER are subjected to it (108).

In mammalian cells one of the best-characterized ERQC systems is the calnexin/calreticulin cycle. Calnexin and calreticulin are members of the legume lectin family and are homologous to each other. They bind to the N-glycan core. Calnexin is a type I transmembrane protein, while calreticulin is soluble (115). Although a functional calnexin/calreticulin cycle has not been characterized fully in yeast, some homologues can be found in the yeast ER, for example CWH41/glucosidase I and GLS2/glucosidase II (116, 117), KRE5/UGGT (118) and Cne1p/Calnexin (119). Imperfect polypeptides must be eliminated to avoid injury to the cell. For this purpose the cell has developed a subset of mechanisms that correctly recognize a protein, which should be destroyed. Such a system has to be capable of distinguishing between a protein to be destroyed from a protein that is in the process of acquiring the correct form. The mechanism adopted appears to be the residence time in the ER, thus giving the glycopeptide sufficient time to fold and assemble before it is targeted for degradation (73). The cycle starts when the N-glycan core is added to the nascent polypeptide by the OST (reviewed in (73, 108)). Two glucose residues are removed by the action of glucosidase I and glucosidase II, respectively (**Figure 10**). The presence of a monoglucosylated core serves as a ligand and is recognized by calnexin or calreticulin. The binding of calnexin/calreticulin prevents aggregation, degradation and export of the non-native polypeptide. The binding of calnexin and calreticulin recruits ERp57, a thiol-disulphide oxido-reductase with homology to PDI. Release of the glycopeptide from the complex is provided by the action of glucosidase II. The glycopeptide is now free in the lumen and does not bind to lectins. If the protein is correctly folded, it is allowed to be exported out of the ER. If the glycoprotein has hydrophobic residues exposed, it is recognized by UDP-Glc::glycoprotein glucosyltransferase (UGGT), and the glycoprotein reglucosylates. UGGT only reglucosylates improperly folded proteins and hence serves as a folding sensor in the cycle. The glycoprotein can reenter a new cycle in an attempt to be properly folded. If the glycoprotein remains too long in the ER, which is a signal that the protein is improperly folded, the ER manosidase I removes a mannose residue. The ER manosidase I has a very low activity, thus giving a chance to the polypeptide to adopt its native conformation. After the ER mannosidase I has removed a mannose residue, the membrane bound ER protein called EDEM (ER degradation-enhancing-mannosidase-like protein, Htm1p/Mnl1p in yeast) binds to it. Once the mannose residue has been removed, the affinity of both UGGT and glucosidase II is reduced, thus preventing the entry of the unfolded

protein into a new folding cycle. EDEM and calnexin can associate with each other forming a complex. Presumably EDEM then targets misfolded $Man_8GlcNAc_2$-glycoproteins for degradation. The non-native glycoprotein is removed from the ER by retrotranslocation into the cytosol and is subsequently targeted to ubiquitin-dependent degradation by the proteasome, in a process called ER-associated degradation (ERAD). The ERAD system involves three steps: recognition of misfolded proteins, retrograde transport or dislocation back to the cytoplasm, and ubiquitin-dependent degradation involving the proteasome (reviewed in (73, 120).

The sequential trimming of N-linked glycans is not the only mechanism that targets misfolded proteins for degradation. The ER chaperones associate with hydrophobic surfaces of unfolded proteins, whereas oxido-reductases bind to them and control the formation/isomerization of disulphide bonds. Eps1p, a homolog of PDI, for example targets misfolded membrane protein for degradation (121). In the second step these unfolded proteins are dislocated back to the cytosol through a putative channel like the heterotrimeric Sec61 complex, or then possibly through a channel formed by Der1p (Derlin-1 in mammalian cells) (122). The driving force for the retrotranslocation seems to be provided by the sequential ubiquitination of the cytoplasmic exposed lysine residues by ubiquitin ligases and the binding of ubiquitin binding factors. An example of such ubiquitin ligases is Ubc6p, Ubc7p and Cue1p. Ubc6p is and ER membrane protein, while Ubc7p relies on Cue1p for ER membrane recruitment (120). Another ubiquitin ligase complex present on the ER is composed of Hrd1p/Der3p ligase that is localized to the ER through Hrd3p, Ubc7p and the ubiquitin conjugating enzyme Ubc1p and is responsible for the regulated degradation of HMG reductase and CPY (123, 124). A third ubiquitin ligase present at the ER membrane is Doa10p that works together with Ubc6p and Cue1p/Ubc7p to remove integral membrane proteins and is capable of targeting soluble proteins also (125). Therefore it seems that different substrates use different degradation targeting mechanisms and may use different ERAD (126). In the third step, targeting to the ubiquitin-dependent degradation involving the proteasome starts with binding of Cdc48p–Npl4p–Ufd1p to monoubiquitinated proteins. Then Rad23p and Dsk2p bind to polyubiquitinated proteins through a UBA domain, and deliver the protein to the proteasome system due to the presence of a UBL (ubiquitin-like) region that mediates its interaction with the 26S proteasome.

2.3 Exiting the ER: COPII vesicles

Transport vesicle-mediated traffic provides a versatile and dynamic connection between two organelles, and allows specific cargo to be selected from the donor compartment and delivered to the target compartment. The formation of these transport vesicles depends on molecular coats that cover the cytosolic face of the vesicles. In this section we will discuss the features of the coat proteins involved in exit of cargo proteins from the ER.

2.3.1 Molecular features of COPII proteins

Three protein components are required to form COPII-coated vesicles. These include Sar1p in its GTP-bound state, and the heterodimers Sec23p/24p and Sec13p/31p (127). These proteins are sufficient to reconstitute the physical characteristics of the COPII coated vesicles *in vitro*.

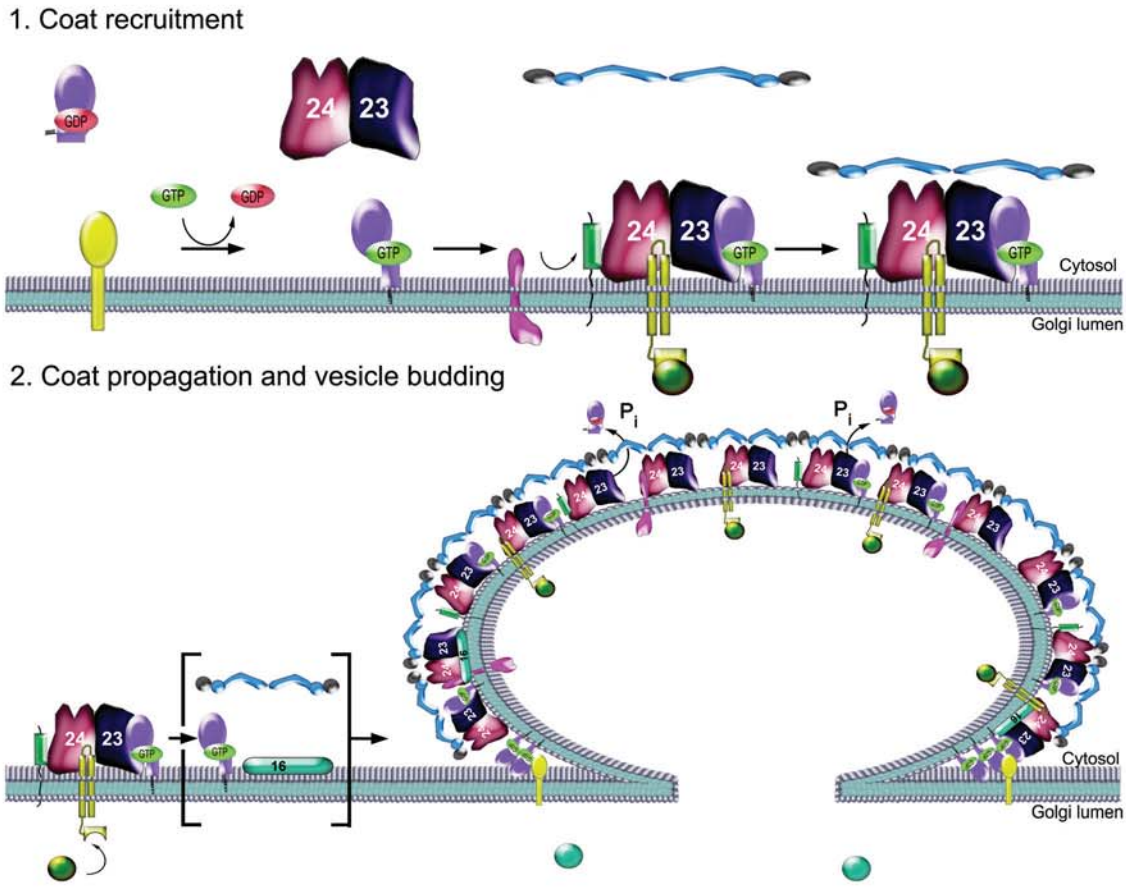
Given that more than one third of the proteome is targeted to the ER, probably additional components assist the core COPII components in functions related to specialized cargo sorting (128). Recent studies carried out on each of these components have revealed the molecular features that allow these proteins to fulfill their functions.

2.3.1.1 Sar1p, Sec12p and Sec16p

Formation of the coat starts with the recruitment of cargo proteins to ER exit site. Recruitment of coat proteins starts by change of the secretion associated and Ras-related protein-1 (Sar1p) from the inactive GDP-bound state to the activated GTP-bound state. The GEF of Sar1p, Sec12p, is a 70 kDa type II transmembrane protein localized to the ER membrane, with a large cytosolic domain that catalyzes the exchange of nucleotide on Sar1p (129). Upon activation, Sar1p becomes localized to the ER membrane due to a conformation change, where a 20-23 amino acid amphipathic helix is exposed. A hydrophobic patch at the N-terminus of the helix contains a STAR (Sar1 N-terminal activation recruitment) motif that mediates its partitioning into the ER membrane (130, 131). Sar1p is a GTPase belonging to the Ras superfamily (132). The Sar1p GTP hydrolysis is activated by binding of Sec23p, which contributes a key arginine to the active site of Sar1p, accelerating the slow intrinsic GTPase activity of Sar1p (133). Further binding of the Sec13p/31p complex accelerates the GTPase activity of Sec24p-Sec23p-Sar1p (134). GTP hydrolysis is required for cargo sorting (135) and for COPII vesicle disassembly. Generation of COPII vesicles occurs at subdomains of the ER membrane called ER exit sites or transitional ER sites, which are ribosome-free subdomains. These specialized domains have been shown to be stable, but very dynamic structures in mammalian cells (136) and in the yeast *P. pastoris* (137). The components responsible for the formation of these subdomains are not known, but may involve scaffold proteins on the cytoplasmic side of the ER that would recruit the components required for formation of COPII vesicles. One such possible scaffold protein and COPII interacting protein is Sec16p. *SEC16* is an essential gene in *S. cerevisiae* that encodes a 240 kDa hydrophilic protein that associates peripherally, yet tightly with the ER membrane. Sec16p is capable of interacting with Sec23p (138) and with Sec31p (139). The function of Sec16p appears to involve the nucleation and stabilization of the COPII vesicle coat at the ER membrane. The ability of Sec16p to localize to the ER and to interact with both of the COPII heterodimers, together with the ability of Sec23p/24p and Sec13p/31p to polymerize, could create such a specialized domain at the ER membrane (140). Although Sec16p has been proposed to play a role in scaffolding, the presence of such ER domains remains to be verified.

2.3.1.2 Sec23p/24p complex

Sec23p (84 kDa) and Sec24p (104 KDa) come together to form a heterodimer of 195 kDa. Sec23p is structurally homologous to Sec24p, each one is composed of an α-helical region, a β-barrel region, a zinc-finger domain, a gelsolin-like domain and a trunk domain (141). The 3D structure of Sec23p/Sec24p in complex with Sar1p has been solved (133, 142)(**Figure 11**). The ER membrane interface region of the assembled Sec23p/24p/Sar1p complex forms, a concave and positively charged surface that fits to the shape and charge


1. Coat recruitment
GDP
24
23
GTP
GDP
GTP
24
23
GTP
24
23
GTP
Cytosol
Golgi lumen
2. Coat propagation and vesicle budding
Pi
Pi
24
23
GTP
GTP
16
Cytosol
Golgi lumen
3. COPII coat structures
Sar1p
Sec12p
Sec23p
Sar1p
A
B
C
Sec24p
Sec13p
Sec13p
Sec31p
Cuboctahedral structure
Sec12p
Sar1p-GDP
Sar1-GTP
SNARE
Transmembrane cargo
Soluble cargo receptor
Soluble cargo
Golgi resident protein
Heterodimeric Sec23p/Sec24 complex
Heterotetrameric Sec13p/Sec31p complex
16
Sec16p

of a standard COPII vesicle. Although structurally related, Sec23p and Sec24p have specific roles. Sec23p is the specific GAP of Sar1p (143), contributing a specific arginine residue to the active site of Sar1p, while Sec24p is the cargo adaptor subunit. The GTPase activity of Sec23p is independent of Sec24p, and the cargo selection capacity of Sec24p is independent of Sec23p (140, 144).

Detailed analysis of the Sec23p/Sec24p complex interaction with cargo molecules, have revealed the presence of at least three binding pockets in Sec24p (A, B and C sites) that allow the accommodation of both cargo and SNARE (145, 146). Binding of proteins to the COPII coat takes place though sorting motifs. These sorting motifs are present in the cytosolic SNAREs and in the cytoplasmic tails of transmembrane cargo proteins. In the case of soluble cargo proteins, the sorting motifs are apparently located in the cytosolic tails of their putative transmembrane receptors (146, 147) These motifs can be subdivided into 4 general categories: Di-acidic motifs such as [DE]X[DE], di-basic motifs such as RR, di-hydrophobic motifs such as FF,FY,LL or VV and then other motifs (146-148).

An interesting feature of the COPII coat can been observed in reference to SNARE selectivity. The binding pocket A can recognize an YNNSNPF sequence present in the N-terminal regulatory domain of the SNARE protein syntaxin Sed5p, the B site can recognize Lxx-L/M-E on the N-terminus of Bet1p and Sed5p, and the C site recognizes Sec22p (146). Since a fusion event between two membranes can only occur if the SNAREs are pre-disposed, this is if they are in the acceptor complex form, the COPII coat recognizes the assembly state of

Figure 11. COPII coat vesicle formation.
1. Coat recruitment. At the endoplasmic reticulum Sec12p recruits and activates Sar1p, which results in the insertion of the N-terminal helix of Sar1p–GTP into the membrane bilayers. This stabilizes Sar1p on the ER membrane which in turn recruits the Sec23p/24p complex through Sar1p–GTP binding to the Sec23 subunit. Cargo is packaged at this stage. Transmembrane cargo directly interacts with the COPII coat, meanwhile soluble cargo interact via adapter proteins. Finally, the Sec13p/31p complex binds to the forming coat resulting in completion of the coat at the ER membrane. **2. Coat propagation and vesicle budding.** The Sar1p–Sec23p/24p cargo complex becomes concentrated in local scaffolds such as in increased regions of Sar1p, Sec16p and also by binding to the self-assembled Sec13p/31p cage. These properties result in the construction of a cuboctahedral cage. Sar1p displacement is accelerated by both cargo- and Sec31p-mediated stimulation of the GAP activity of Sec23p. Insertion of Sar1p into the membrane together with its interaction with the concave face of Sec23p/24p help curve the membrane. Recruitment of an active ring of newly recruited Sar1p-GTP to the vesicle neck may help membrane curvature and formation of the fission pore. **3. COPII coat structures.** The molecular structures of the core components involved in COPII-coated-vesicle budding from the ER are shown. Left panel: structures of Sar1p represented by ribbon diagrams, showing the transition from the GDP- to GTP-bound states. Structural prediction of Sec12p modeled as a β-propeller WD40 domain. Middle panel: Structure of the Sar1p–Sec23p/24p complex showing the multivalent cargo adaptor platform sites. Right panel: model of the Sec13p/31p complex. It is composed of β-propeller WD40 domains and α-solenoid motifs. In green the self-assembled Sec13p/31p heterotetramers are shown without Sec23–Sec24, revealing that Sec13p/31p is necessary and sufficient to generate the COPII cage. Structures reprinted, with permission, from the Nature Reviews in Molecular Cell Biology, Volume 7 (c) 2006 by Nature publishing group.

the SNAREs and binds preferentially to fusogenic SNAREs. Thus the B-site preferentially binds to the free v-SNARE Bet1p and the A-site to Sed5 within the t-SNARE acceptor complex composed of Sec22p, Sed5p and Bos1p.

In mammalian cells there are two isoforms of Sec23p, called hSec23Ap and hSec23Bp, which are 85% identical to each other and 48% to yeast Sec23p. The expression profile varies between cell types, with hSec23Ap being the functional counterpart of yeast Sec23p (149). Sec24p has four isoforms in mammalian cells (Sec24A to Sec24D), each one with tissue-specific expression (150). In addition to Sec24p, yeast has two homologous proteins Sfb2p/Iss1p and Sfb3p/Ist1p that are 56% and 23% identical to Sec24p, respectively. Both homologues can form a complex with Sec23p and appear to be involved in cargo packaging (151-155). The Sec23p/Sfb3p complex is capable of driving COPII coat formation and generate vesicles, but is unable to package the v-SNARE Bet1p and hence these vesicles are not capable of fusing to the target membrane (155). Thus it seems that the function of Sfb3, is to confer cargo-sorting diversity, since the COPII coats formed exclusively of Sec23p/Sec24p are not capable of sorting the plasma membrane ATPase Pma1p into vesicles (154). In a similar way, COPII coats formed of Sec23p/Sfb2p are capable of driving vesicle formation and promote the recruitment of pro-α-factor (153, 156). Unlike the complex formed with Sfb3p, Sec23p/Sfb2p is capable of packaging the correct pair of SNAREs, and further more overexpression of Sfb2p is able to suppress the lethality of Δ*sec24* deletion *(151)*. Thus, COPII-coated vesicles may be formed by all of these combinations, this is, Sec23p with Sec24p, Sfb2p or Sfb3p, thus maximizing the sorting capacity of the budding vesicle (140).

2.3.1.3 Sec13p/31p complex

The Sec13p/31p complex is composed of two copies of Sec13p and two copies of Sec31p that come together to form a stable asymmetric heterotetramer. Similar to *S. cerevisiae*, there are two mammalian Sec13 isoforms called SEC13-like-1 protein or SEC13R and SEC13-like, or SEH1 (157). Sec13p is composed of a WD40 domain, a structural arrangement of several blades arranged radially around a central axis. Each blade consists of four-stranded anti-parallel β-sheet formed by WD40 repeats. The WD40 repeats are 40 amino acid motifs that often terminate with the conserved Trp-Asp dipeptide (158).

Like for Sec13p, there are also two mammalian SEC31 isoforms, called A and B (159). Structure prediction of Sec31p suggests a WD40 domain in its N-terminus, two regions of α-solenoid structure separated by a region with low complexity (158). Previous studies have suggested that the heterotetramer arranges itself in an elongated globular domain with two-fold symmetry with the following organization Sec31p-Sec13p-Sec13p-Sec31p (160), and that formation of the COPII cage requires both Sec23p/24p and Sec13p/31p (161). But recent studies suggest that Sec13p/31p can self-assemble into a cage with a novel cuboctahedral structure with the subunits arranged in the Sec13p-Sec31p-Sec31p-Sec13p order (162). This self-assembling cytosolic cage would act as a scaffold agent recruiting the Sec23p/24p-cargo to the emerging vesicle. Sec13p and Sec31p interact through the common WD40 domains and the Sec31p-Sec31p interaction results from the dimerization of the C-terminal α-solenoid domains, with each vertices of the cage being formed by four Sec13p molecules. The same type of interactions, i.e. the β-propeller-α-solenoid scaffold, are found in the clathrin cage, reflecting

an evolutionarily conserved feature of coat protein complexes (144). The arrangement of the cage in a cuboctahedral design, instead of the icosahedral symmetry in clathrin-coated cages, results in a less rigid structure of the COPII cage. This would allow the cage to be more flexible for the incorporation of a wide variety of cargo, some of which do not fit in the typical COPII cage, like procollagen and chylomicrons (144).

2.3.1.4 Accessory proteins

Even though the purified coat components and guanine nucleotides are sufficient to drive vesicle formation *in vitro*, additional proteins may be involved in the budding event to ensure appropriate spatial-temporal regulation. There is a subset of proteins such as Shr3p, Chs7p and Vma22p that are capable of interacting with secretory cargo proteins and facilitate their incorporation into COPII vesicles (128). They may either assist the formation of a secretion-competent form or by sorting the cargo proteins into the vesicles. ER exit of GPI-anchored proteins is dependent of Uso1p, the tethering factor required to fuse COPII vesicles with the Golgi membrane. Sec34p and Sec35p are members of the conserved oligomeric Golgi (COG) complex, required for tethering functions in retrograde traffic within the Golgi. Incorporation of GPI-anchored proteins into COPII vesicles is also dependent on the Rab GTPase Ypt1p as well as the SNAREs Bos1p, Bet1p and Sec22p (163-165). Although these proteins play a crucial role in the sorting of GPI-anchored proteins, they are not required for the uptake of other types of cargo molecules.

Another protein required to for the biogenesis of COPII vesicle is the Ypt1p-interacting protein, Yip1p, which is an integral membrane protein that cycles between the ER and the Golgi (166). Yip1p together with Yif1p, another Ypt1p-interacting protein, form a heteromeric complex (167). Ypt1p and Yip1p may work as a diffusion barrier and increase the possibility of forming a vesicle at a certain region of the ER membrane. Mechanistically, Uso1p and Ypt1p may bind to sorting motifs of specific cargo molecules, whereafter Yip1p and Yif1p may cluster the secretory cargo together, until the coat proteins initiate their polymerization at these sites (168).

2.3.2 Formation of COPII transport vesicles

In previous models of COPII cage vesicle assembly, the first step is the activation of Sar1p by its GEF, Sec12p present on the ER membrane, allowing Sar1p to become ER membrane-attached due to exposure and membrane insertion of an N-terminal α-helix. In a second step, Sar1p recruits the heterodimeric complex Sec23p/24p, which captures cargo destined from the ER to the Golgi. In a third step, the Sec13p/31p heterotetramer together with Sec23p/24p drive cage assembly, leading to vesicle budding and formation of a COPII-coated transport vesicle (169). But recent studies suggest a novel model for formation of COPII-coated vesicles. This model is in agreement with models of clathrin-coated vesicle formation, and suggest a common mechanism for formation of coated transport vesicles (144). In the subsequent section we will address these new features and their implications.

In addition to the function of Sar1p-GTP in recruiting the Sec23p/24p complex to the ER membrane, Sar1p may also facilitate generation of membrane curvature and vesicle fission (131, 170). Sar1p-GTP, in the absence of Sec23p/24p

and Sec13p/31p appears to be capable of inducing membrane curvature, resulting in the formation of tubules at ER exit sites. Additionally, Sar1p-GTP seems to be recruited to the fission pore to form a ring of activated molecules. Fission of the vesicle requires GTP hydrolysis.

The GEF activity of Sec12p is 10-fold faster than the GAP activity of Sec23p, and thus continuous charging of the growing bud with Sar1p-GTP would help propagate coat assembly, stabilizing the coat at sites where GTP hydrolysis has occurred (171) by the cross-linking functions of Sec13p/31p (162). Formation of a ring of activated Sar1p is essential to control the balance between positive and negative curvature of the membrane prior to vesicle fission (131, 170, 172). Novel and important information came from the fact that Sec13p/31p can self-assemble into a cuboctahedral cage, thus providing a potential local scaffold where COPII components can assemble (162). In addition to the pre-assembled Sec13p/31p cage, additional scaffold agents may be involved such as Sec16p (144). The intrinsic GAP activity of the Sec23p/24p/Sar1p was elicited approximately 30 seconds after the addition of Sar1p-GTP to Sec23p/Sec24p. This complex may diffuse along the ER membrane capturing cargo. When Sec24/24/Sar1p come into contact with the preassembled Sec13p/31p cage, they become stably associated to it, forming the final COPII cage.

Structural analysis has revealed that the cargo sorting Sec24p subunit associated to the middle domain of Sec31p, closer to the vertices formed by Sec13p whereas Sec23p associates to the C-terminal domain of Sec31p, which is relatively accessible and in the center of the cage (162). This would allow independent control of the release of Sar1p by the GAP activity of Sec23p, which is further stimulated by the binding of Sec31p from the cargo related packaging activity of Sec24p (144). The formation of multivalent interaction between the different coat components as well with the transmembrane cargo or receptors would help stabilize the coat until vesicle budding has occurred (144). The COPII Sec13p/31p cage is formed by the four vertices at each intersection, this arrangement would allow the cage to be relatively flexible and assume different sizes depending on the angles formed between the four vertices (144, 162). Interestingly the Sec24p subunit of the Sec23p/24p complex, the one responsible for cargo sorting is localized in the region proximal to the vertices formed by Sec13p and hence may influence the geometry and the size of the cage accordingly to the cargo incorporated. In **Figure 11** the interactions and a schematic overview of the molecules that drive COPII-coated-transport carriers can be seen.

2.3.3 Fusion of vesicles with the Golgi membrane

After budding of the COPII transport vesicle from the ER membrane, GTP hydrolysis leads to the uncoating of the vesicle, preparing it for fusion with the Golgi membrane. Although in yeast little evidence supports a role for the cytoskeleton in delivery of transport vesicles to the Golgi membrane, in mammalian cells the ER to Golgi transport is dependent on the microtubule cytoskeleton and the motor protein dynein/dynactin (173-175). In mammalian cells, an ER to Golgi intermediate compartment (ERGIC) exists between the ER and the Golgi. This compartment is defined by the presence of the lectin ERGIC-53

(176). Analysis of the ERGIC dynamics in mammalian cells revealed that uncoated vesicles migrate forward to the Golgi complex and COPI-coated vesicles derived from the Golgi are directed to the ER (See chapter in molecular features of COPI coats). ERGIC may represent the first compartment that discriminates between anterograde and retrograde transport (177). The existence of ERGIC in mammalian cells could be due to the fact that vesicles have to be transported across long distances from dispersed ER exit sites to the central peri-nuclear Golgi (169). Since yeast cells are small compared to mammalian cells, traffic between the ER and Golgi may occur in the absence of an ERGIC-like compartment by simple diffusion (178).

After the vesicle is relatively close to the membrane of the Golgi, two separate processes ensure that only the correct vesicles fuse. Vesicle tethering and SNARE assembly, ensure the specificity of vesicle traffic. The first interaction between the transport vesicle and the Golgi membrane is provided by tethering factors, these include Uso1p and the TRAPP I complex (179-181). TRAPP I (transport protein particle I) is a large oligomeric complex composed of seven subunits (Bet5p, Bet3p, Trs20p, Trs23p, Trs31p, Trs33p, Trs85p) that helps bridge the distance between the Golgi membrane and the vesicle (182). Besides functioning in tethering TRAPPI also functions as a GEF for the RabGTPase Ypt1p (183), a yeast Rab1 homolog that is present on ER-to-Golgi transport vesicles. Once these vesicles arrive at the Golgi membrane Ypt1p recruits Uso1p to the membrane (180). Uso1p is the yeast homolog of mammalian p115 and is a large coiled-coil protein that helps bridge the target membrane and the vesicle, and possibly also facilitates SNARE complex assembly (184).

When the vesicle is sufficiently close to the Golgi membrane to allow SNARE assembly, fusion of the two membranes is initiated. The SNAREs involved in this fusion reaction are Bet1p (Qc) present on the vesicle and Sed5p (Qa), Bos1p (Qb) and Sec22 (R) present on the target Golgi membrane (185). The yeast Sly1p belongs to the Sec1p/Munc18p family that is involved in modulating SNARE activity. The yeast Sly1p interacts with the N-terminal regulatory domain of the syntaxin Sed5p and helps promote the assembly state of the SNARE-complex and consequently fusion (17, 186). After fusion of the two membranes, soluble and transmembrane cargo proteins are delivered to the Golgi, and the *cis*-SNAREs are disassembled by the action of Sec18p and Sec17p.

3. Golgi: The major sorting station of the cell

The next compartment of the secretory pathway is the Golgi complex. In this compartment exocytic proteins are subjected to modifications of their glycans and to proteolytic processing of precursor proteins. In addition to these functions, the Golgi is involved in sorting of proteins to proceed through the secretory pathway or to be retrieved to the ER or to remain in the Golgi. Thus the Golgi is a highly specialized sorting organelle that is capable of maintaining its structural organization while sorting proteins to the cell surface or the endosome/vacuole. In this section we will address briefly the features and characteristics that allow the Golgi to perform these functions.

3.1 Structure and function

3.1.1 Topology of the Golgi

The general image of a Golgi is a stack of flat cisternae that can be subdivided into different sub-compartments like *cis-, medial-* and *trans*-Golgi. The number of cisternae within an individual stack varies, but in animal cells it is approximately seven (187). In a typical mammalian cell the Golgi occurs normally in the perinuclear region. In contrast, in the yeast *S. cerevisiae* the Golgi is scattered throughout the cytoplasm, and usually occurs as single cisternae (178). On the other hand, the yeast *P. pastoris* appears to have Golgi structures organized as stacks (see **Figure 2**) (137).

3.1.2 Modification of protein-bound glycans

In the ER a universal glycan core was added to the polypeptide and further trimmed. It functioned as a sensor for folding state of the polypeptide. This core-N-glycan is further modified in the Golgi by glycosidases and glycosyltransferases that generate the diversity of protein-bound N-glycans. Although the core structure is universally conserved from yeast to human, the modifications that occur in the Golgi are very different. Mammalian cells have a wide variety of structures where a core consisting of N-acetylglucosamine and mannose is decorated with more N-acetylglucosamine, galactose, sialic acid and fucose. In contrast, the yeast N-glycans are extended with only mannose residues (188). Extension may give rise to a smaller ($hex_{<15}$) core or to a larger ($Hex_{>15}$) outer chain (188). Many of the glycoproteins incorporated into the cell wall and periplasmic space contain a large mannose backbone composed of approximately 50 mannose residues. Meanwhile proteins that remain intracellularly, like for example CPY, have smaller glycan chains where only a small amount of mannose residues are added (189).

Elongation of the N-glycan core starts with the *cis*-Golgi resident α1,6-mannosyltransferase, Och1p, that extends $Man_8GlcNAc_2$ by one single mannose residue (190) (See **Figure 12**). Further elongation occurs by sequential addition of mannose residues by two enzyme complexes called mannan polymerase (M-Pol) I and II, creating the backbone of α1,6-linked mannose residues. M-Pol I is responsible for addition of the first 10 mannose residues (191), where after, M-Pol II elongates the chain by approximately 40 more mannose residues. This backbone is then branched by the sequential addition of more mannose residues by Mnn2p, Mnn5 and Mnn1p. Some of the branches may acquire a phosphomannose in a reaction catalyzed by Mnn4p and Mnn6p (192, 193). The N-glycan core of intracellular proteins is extended by only three mannose residues. The first is added by Och1p, then an unidentified ⟨1,2-mannosyltransferase adds the second mannose and the third is added by Mnn1p (188).

All of the mannosyl transferases are putative type II membrane proteins with a short cytoplasmic N-terminus, a short membrane-spanning region and a conserved catalytic lumenal domain. This type II orientation is relatively uncommon in membrane proteins of other compartments than the Golgi, and their transmembrane domain is significantly shorter than that of other membrane proteins (188). These features seem to be involved in retention of the glycan-modifying enzymes in the Golgi, as well as in targeting them to specific cisternae.

In the Golgi the single O-linked mannose residue attached in the ER is

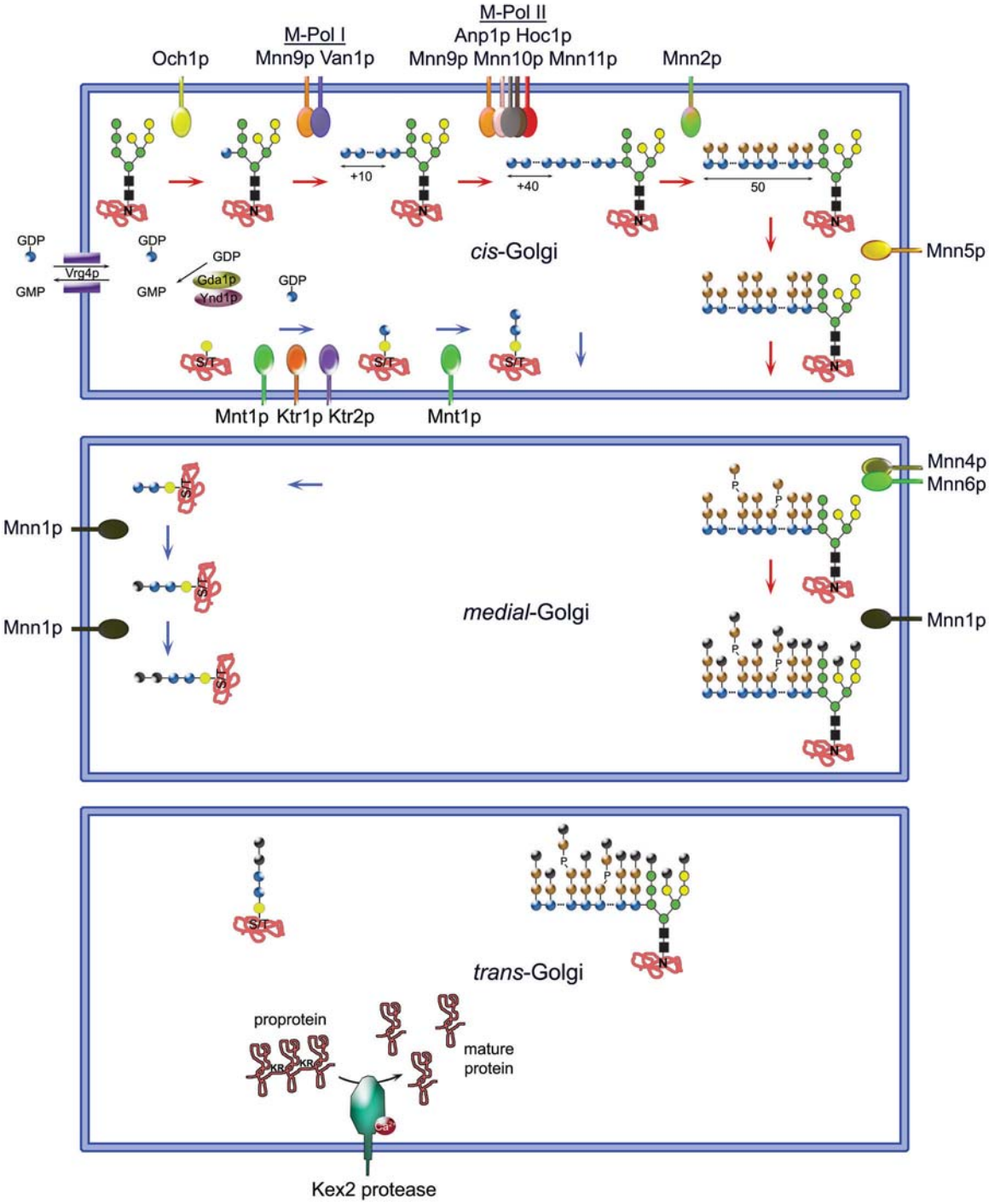


Figure 12. Golgi *post*-translational modifications.
In the *cis*- and *medial*-Golgi O- and N-glycosylation proceeds. These are carried out by Golgi cisternae resident enzymes, which catalyze different steps of the glycan elongation process. In the *trans*-Golgi for example proproteins may be processed into the final mature form by action of cleaving enzymes such as Kex2p.

extended by up to five mannose residues. Like in N-glycosylation, the donor is GDP-mannose, and the mannosyl transferases are type II transmembrane proteins. The transfer of the second α1,2-linked mannosyl residue is catalyzed by either Ktr1p, Ktr3p or Mnt1p/Kre2p (194). Mnt1p/Kre2p adds the third mannose residue. The fourth and fifth mannose residues are added by Mnn1p resulting in α1,3-linked mannose (88). Interestingly only proteins, transported to the exterior of the cell are O-mannosylated. When mannosylation of externalized proteins is disturbed, detection of these proteins in the extracellular space is greatly reduced. Since no intracellular accumulation of these proteins is detected in O-mannosylation mutants, it is not certain if O-mannosylation is a prerequisite for secretion or if these proteins are just more susceptible to protease degradation, (88). Almost all of the cell wall proteins show this type of modification, hence mutations disturbing O-glycosylation disturb cell wall stability by either reducing their secretion or then the glycan side chain residues are important for conferring structural stability (88). One possibility that could explain their reduced secretion and the lack of intracellular accumulation is if reduced O-mannosylation functions as a signal for vacuole sorting (the main proteolytic compartment) and consequently their degradation. Yeast cells with mutations in the O-glycosylation pathway show decreased cell wall stability as well as cell growth and multiplication vulnerability (91).

3.1.3 Precursor processing

Some proteins are synthesized as larger precursors that are proteolytically cleaved to a mature protein by specific endoprotease in late compartments of the secretory proteins, such as the *trans*-Golgi. Examples of such proteins include the yeast pheromone α-factor, killer toxin K1, Hsp150/Pir2p, proinsulin and digestive enzymes. The yeast *trans*-Golgi harbors three enzymes responsible for proteolytic precursor cleavage of these proproteins, carboxypeptidase Kex1p, the serine protease Kex2p and the dipeptidyl aminopeptidase Ste13p (195). The first and best-characterized proprotein processing protease is the Kex2 protease, so we will address this one as an example. Kex2p itself is synthesized as a zymogen (proenzyme), which itself is autocatalytically processed after its role in folding (196). Kex2p is composed of an N-terminal signal sequence, followed by the pro-domain, a catalytic domain belonging to the subtilase superfamily of Ca^{2+}-dependent proteases, a unique P domain, a transmembrane domain and a C-terminal cytosolic tail (197). The C-terminal extensions are required for proper cycling among late compartments of the secretory pathway (TGN and endosomes) (198). The unique P domain is responsible for stabilization and activity. Swapping this domain within the different members of the family of serine proteases results in differences in substrate recognition, stability and pH optimum (199). Kex2p uses the serine protease mechanism, where the catalytic triad composed of serine, histidine and aspartate are involved (198). The same catalytic triad is present in the enzymes of the trypsin superfamily (200), so it is essential to the processing protease to be capable of discriminating the correct substrates. It is also important that they are very efficient since they are only exposed to their substrates briefly during their passage through the secretory pathway. Enzymes such as Kex2p have a high catalysis rate with high specificity

and do not show rate-limiting acylation (197). In contrast, the related subtilisins, which are digestive enzymes, have low catalysis rates with broad specificity, since they are exposed to the substrate for long periods of time and their role is to degrade the whole protein. One well known substrate of Kex2p is the pheromone precursor pro-α-factor, which contains 2 to 4 copies of the α-factor pheromone separated by Kex2 cleavage sites (201). This structural organization is similar to that of the mammalian neuropeptide precursor proopiomelanocortin (POMC), which is processed by the yeast Kex2p, when expressed in yeast cells (202). An other substrate of yeast Kex2p is pro-killer-toxin. Similar to proinsulin it is a folded precursor with multiple chains connected by disulphide bonds (203). Studies on different potential substrates showed that Kex2p prefers dibasic sites like (K/R)-R with Arg (R) at the P1 site (198). Shortly after the discovery of the yeast Kex2 protease, several mammalian homologues were found, including human furin, the related prohormone convertases (PC) PC1/3, PC2, PC4, PC6A, PC6B and PC7, and the paired base amino acid-cleaving enzyme (PACE) 4 (197). All of these proteins have the same overall structure and features of the yeast Kex2p, with the exception of enzymes like PC2 that are soluble proteases.

3.2 Golgi maintenance and ER retrieval

The Golgi is organized in three regions, the *cis*-, *medial*- and *trans*-Golgi. Currently there are two models that explain how protein trafficking occurs through the Golgi apparatus in the *cis* to *trans* direction (204, 205). The vesicular transport model predicts that the Golgi cisternae are stable and distinct compartments that are connected by means of vesicular traffic (206). The cisternal progression and maturation model suggests that the cisternae are transient structures that form *de novo* and progress in the *cis* to *trans* direction while maturing, and then dissipate at the *trans*-Golgi (207). Both models have their limitations and are not mutually exclusive, since the cisternal progression and maturation model cannot explain the presence of anterograde cargo detected in the COPI vesicles, or the different transport rates of anterograde cargo. On the other hand, the vesicular transport model cannot explain the transport of large molecules that would not fit in the typical COPI vesicle (208). Recent imaging data support the maturation model for yeast cells, where individual *cis*-Golgi cisternae maturate in a very dynamic way to form *trans*-Golgi cisternae (209, 210). While the cisternal maturation model can explain rapid protein secretion in yeast, it cannot explain the transport rate of proteins in higher eukaryotes. In higher eukaryotes the Golgi cisternae are tightly stacked to each other, hence maturation of individual cisternae occurs at a relatively slow rate. The discussion of the biogenesis and maintenance of the Golgi compartment is beyond the scope of this study but a comprehensive discussion of the models can be found in two excellent reviews (205, 211), where a revision of the models is proposed in order to include the novel findings found in the structural organization and traffic of cargo within this organelle. These recent findings include the presence of inter-cisternal connections that connect adjacent cisternae (212, 213), which would be responsible for the transport within the different Golgi cisternae, and hence lead to the formation of the continuity-based models (211). In addition, they refer to the possible presence

of *peri*-Golgi vesicles that emerge at the tips of the different cisternae and traffic cargo in a bidirectional fashion. In order to maintain the dynamics and functionality of the cisternae, resident cisternae proteins that mature to later cisternae have to be returned to the new forming *cis*-cisternae. This is postulated to occur by retrograde transport by means of vesicles that capture components from later cisternae and then transport them back to earlier ones (214, 215). Similar to the vesicles that form at the ER membrane, these Golgi-derived vesicles are also formed by cytosolic coat proteins that capture cargo and deform the membrane. In this case, specific coat proteins form the so called COPI coat. Due to membrane traffic from the ER to the Golgi performed by COPII vesicles, the ER membrane is gradually consumed and some ER-resident proteins may escape to the Golgi. This retrograde traffic also requires COPI-coated vesicles, which are responsible for returning from the Golgi to the ER SNAREs, cargo adaptor proteins and membrane components.

3.2.1 Molecular features of COPI proteins

The minimal components required to form COPI-coated vesicles *in vitro* are the COPI coatomer proteins, the small Rab family GTPase ADP-ribosylation factor 1 (ARF1) and nucleotides (216). The COPI coatomer is a complex composed of seven different subunits α, β, β', γ, δ, ε and ζ subunits in mammalian cells, and Ret1p, Sec26p, Sec27p, Sec21p, Ret2p, Sec28p and Ret3p, respectively, in yeast (169). Even though under certain conditions the coatomer may exist as two separate complexes, the F-COPI subcomplex (β', γ,δ,ζ) and the B-COPI subcomplex (α, β, ε), *in vivo* it appears to exist solely as a completely formed complex (217). Similar to COPII coats, the COPI coatomer has N-terminal β-propeller WD40 domains and α-solenoid motifs (158). Some of the COPI subunits are structurally related to those of the clathrin coat and appear to fulfill the same functions (218-220). The B-COPI subunit may function as the outer surface layer of the coat, similar to the role played by the polymerized clathrin triskelions. Meanwhile, the F-COPI subunit may form the surface of the coat proximal to the membrane, similar to the adaptins of the clathrin coat.

The function of the GTPase ARF1, or yARF1/2/3 in yeast, is similar to that of Sar1p in COPII cage assembly. ARF1 in its GDP-bound form is inactive and soluble, and upon binding of GTP, a conformational change in the N-terminus leads to exposure of a myristoylated group, which allows its membrane association, and activates ARF (221). Two additional factors, like in the case of COPII assembly, modulate the activity of ARF1 and assist in the assembly/disassembly of the COPI coat. These are the GTP exchange factor ARFGEF (in yeast Gea1p and Gea2p) and the GTPase-activating protein ARFGAP (in yeast Glo3p, Gcs1p) (222, 223). The ARFGEFs share a conserved 200 amino acid catalytic Sec7 domain (221). Unlike Sec12p (GEF in COPII assembly), ARFGEFs are soluble proteins that localize transiently to the Golgi membrane by an unidentified receptor. ARFGAPs contain a conserved zinc finger motif catalytic domain with a conserved arginine residue that is involved in GTPase activation (223). Initially, ARFGAP was thought not to structurally make part of the coat, and the primarily function of ARFGAP was thought to be to induce vesicle uncoating by stimulating GTP hydrolysis (224, 225). Recent results suggest an involvement of ARFGAP as a component of the coat due

to its requirement in cargo sorting and vesicle formation (226-228).

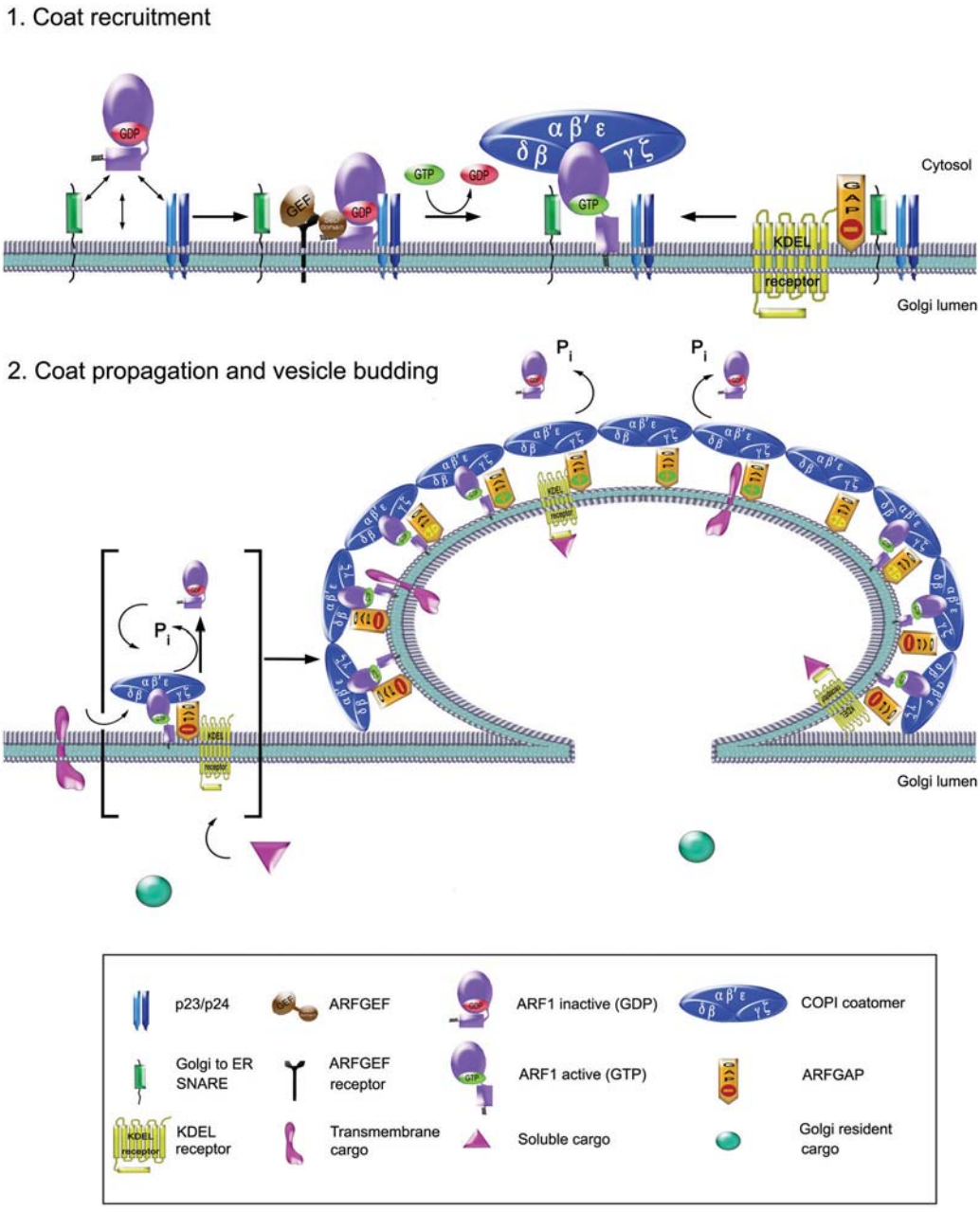
3.2.2 Formation of COPI transport vesicles

The formation of the COPI transport carrier is initiated by recruitment of ARF1 to the Golgi membrane (see **Figure 13**). This involves both the recruitment of ARFGEF (soluble) to an unknown receptor and ARF-GDP to the Golgi membranes. Targeting of ARF1-GDP to the Golgi membrane may be assisted by its interaction with the cytoplasmic region of p23, a member of the abundant p24 type I transmembrane Golgi-cargo receptors (229, 230). In addition, ARF1 may also be targeted to the Golgi membrane though its interactions with SNAREs that are normally present in COPI vesicles (231, 232). Following nucleotide exchange on ARF1 by its GEF, ARF-GTP is released from p23/p24 and becomes associated to the Golgi membrane though membrane insertion of the myristoylated group. At this stage recruitment of the COPI coatomer takes place and coat assembly and cargo packaging is initiated (233). Simultaneously, ARFGAP1 is initially recruited to the Golgi membrane though its interaction with KDEL transmembrane receptors (Erd2p in yeast) (234, 235), with transmembrane proteins with dilysine motifs (228), through the cytoplasmic tail of p23/p24 (222) and its interactions with v-SNAREs involved in Golgi to ER traffic (236). The complex of ARF1, ARFGAP and coatomer together with cargo, start assembling on the Golgi membrane. At this stage GAP activity on ARF1 is still low and the coat assembly and cargo sorting still goes on. As the coat assembles and the COPI lattice is formed, the GAP activity increases at regions distal to the forming bud by a membrane curvature-dependent mechanism (224, 237). In these studies, GAP activity of ArfGap1 was shown to be sensitive to membrane curvature. ArfGAP1 has a lipid-packaging sensor that recognizes when the membrane is positively curved, as is the case in the outermost region of the budding membrane vesicle (238). An increase in GAP activity was detected when the artificial used liposomes where similar in size to that of an authentic COPI vesicle (60 nm), suggesting that coat formation should be stable at the bud neck, where the membrane curvature is negative, allowing formation of the COPI coated vesicle. In support of these observations, a ring of ARF-GTP forms at the vesicle neck. As the GAP activity on ARF1 increases at regions distal to the vesicle neck, GTP hydrolysis takes place and ARF1-GDP is released from the coat lattice. These dynamics between ARF1 and ARFGAP1 activation allows specific and accurate spatiotemporal regulation of the initiation of GTP hydrolysis (239).The polymerized coatomer may be maintained by multiple lateral interactions between its subunits that occur within the lattice even in the absence of Arf1-GTP (238).

3.2.3 Sorting signals in COPI-mediated traffic

In order to proper sort proteins within the COPI vesicles that are destined to be transported back to the ER, or from later Golgi cisternae to previous ones, the cell uses a mechanism that relies on the presence of sorting signals on the cargo proteins. These sorting signals specify the intracellular localization of the protein and bind directly to the coat subunits, or to adaptor proteins. One such sorting signal that mediates ER retrieval of transmembrane proteins is the cytosolic canonical KKXX motif signature (where K

is lysine and X is any amino acid), which binds to the α- and β'-COP subunits of the coat using two distinct but overlapping binding sites (240-242). As mentioned previously, the p24 protein family can bind to the γ-COP coatomer (243). For this they use mainly the di-phenylalanine motif but may also use two basic residues KK(X)n or KR(X)n where n≥2 (217). Luminal ER resident proteins that escape the ER must be distinguished and efficiently retrieved from Golgi proteins and newly synthesized proteins on their way beyond the Golgi. For this purpose the cell uses a KDEL sequence (HDEL in yeast) that is typically found in the C-terminal end of luminal



ER-resident proteins. The presence of this sequence allows recycling from early Golgi back to the ER (244). This sorting sequence binds in the Golgi to the KDEL receptor (Erd2p in yeast) (245-247), a transmembrane protein that spans the membrane 7 times (248) and is transported from the Golgi to the ER by means of the COPI pathway (249, 250). The binding of the ER proteins to the KDEL receptor is pH-dependent, which ensures the capture of the ligand in the Golgi and release in the ER (251). Additionally, ligand binding by the KDEL receptor induces oligomerization of the receptor, which may have an important role in its recruitment to COPI vesicles (235). The presence of a double lysine signal sequence together with a phosphorylated serine residue (in mammalian cells) is responsible for the recruitment of the KDEL receptor to the COPI-pathway (252).

Another ER retrieval signal present in the cytoplasmic tail of transmembrane proteins, such as Sec71p, is the δL motif (WXXW/Y/F). This sorting motif allows its association to δ-COP (253). One interesting ER retrieval-sorting signal is the RXR sequence, which can be found in the cytoplasmic loops or C-terminal tails of the subunits of the ATP-sensitive K^+ channel (254). These subunits assemble in the ER to a fully functional receptor. The binding of COPI coatomer to the individual subunits ensure that they remain in the ER until fully assembled. Upon assembly, multiple RXR motifs are exposed, and as a consequence it can bind to the 14-3-3 dimer proteins, which successfully mask the retention sequence and successfully compete for binding instead of COPI coatomer proteins, thus allowing transport of the fully assembled receptor to the plasma membrane (255).

3.3 Station for sorting of proteins

The transport of newly synthesized proteins through the ER and Golgi cisternae occurs in a vectorial manner with virtually no diversion to alternative routes. However once proteins reach the *trans*-Golgi they may be directed to various destinations. They may be targeted to the extracellular space, to the plasma membrane and to the vacuole. Therefore the Golgi must be capable of sorting secretory proteins and cell surface proteins from proteins destined for endosomes or vacuoles (256, 257). The *trans*-Golgi also receives extracellular content and recycled proteins//lipids from the endosomal/ vacuolar compartments. The late Golgi compartment is for this reason regarded as the major protein sorting station and

Figure 13. COPI coat vesicle formation.
1. Coat recruitment. Arf1 is recruited to the Golgi membranes by its interaction with members of the p23/p24 family and with Golgi SNAREs involved in intra-Golgi and Golgi to ER transport. At the same time its GEF, ARFGEF is recruited to the ARFGEF receptor. Nucleotide exchange on ARF is mediated by the Sec7 domain of the GEF, which results in change of the bound nucleotide and insertion of an α-helix into the membrane bilayers. When ARF is in the activated GTP form, it recruits the COPI coatomer. **2. Coat propagation and vesicle budding.** At this stage, cargo is packaged into the forming vesicle. Transmembrane cargo are capable of interacting directly with the COPI coat, meanwhile soluble cargo interact via adapter proteins, such as the HDEL receptor. In the initial phase of the budding, the GAP activity is relatively low, insuring that the coat remains sufficient time to stabilize membrane deformation and cargo recruitment. As the bud grows, GAP activity increases at the distal sites of the forming vesicle by a membrane curvature dependent mechanism.

is responsible for the correct targeting of newly synthesized proteins to their final destinations. To correctly fulfill this role, the Golgi must be capable of successfully discriminating between different sorts of proteins and be capable of segregating them into specific sets of vesicles. Two different populations of secretory vesicles may deliver secretory and cell wall cargo proteins to the cell surface (258). One population originates directly from the *trans*-Golgi membrane and delivers its cargo to the plasma membrane, while the other, appears to transit through an endosomal intermediate compartment "*en route"* to the plasma membrane (259, 260). In the following sections we will address how these pathways function and are organized.

4. The endosomal/vacuolar system: a second sorting station

The vacuole is responsible for enzymatic degradation of cellular components, as well as breakdown of nutrients taken up from the surrounding environment. The endosomal/vacuolar system is subdivided into biochemically distinct compartments such as the early endosome, late endosome and vacuole (261, 262). Yeast genetic studies have helped elucidate the molecular details underlying the traffic to these compartments. Instrumental for this understanding were large-scale screens that identified several mutants defective in different steps of this pathway. This lead to the classification of the mutants according to the observed phenotype. For example, *vps* (vacuolar protein sorting) mutants typically secrete the vacuolar carboxypeptidase Y (CPY), *pep* mutants are defective in vacuolar protease activity, *vam* mutants have unusual vacuole morphology, *end* mutants are defective in endocytosis, and *vac* mutants are defective in vacuolar segregation (263).

Cargo intended for delivery to the vacuole may follow two distinct pathways. They may be targeted directly to the vacuole (ALP pathway) or they may transit through endosomes from where they are then further sorted and finally delivered to the vacuole (CPY pathway) (263-265).

4.1 The CPY Pathway

Research on the delivery of carboxypeptidase Y (CPY) to the vacuole elucidated the first pathway that cells employ to deliver proteins to the vacuole and hence the name of this pathway (266-269). CPY is synthesized as a prepro form and translocated across the ER membrane. After signal peptide cleavage by signal peptidase in the ER, the precursor form (p1, 67kDa) is glycosylated and delivered to the Golgi. Here it acquires sugar modifications and becomes the Golgi (p2, 69kDa) form. Exit of CPY from the Golgi occurs by its receptor, Vps10p/PEP1(270). The crucial residues required for proper sorting of proCPY to the vacuole are the residues QRPL (271). The receptor and its bound cargo, the Vps10-CPY complex, are transported from the *trans*-Golgi to the late endosome (also known as pre-vacuolar/endosomal compartment, PVC) by vesicular traffic mediated by clathrin-coated vesicles and involves the dynamin related GTPase Vps1p (272). In the endosome, CPY dissociates from its receptor and is transported to the vacuole where it is proteolytically cleaved to the active mature form (mCPY, 61kDa). Meanwhile the receptor Vps10p is recycled back to the Golgi through the retrograde transport pathway and is available for a new sorting cycle (273). Vps10p is a type I transmembrane protein that is localized

predominantly to late Golgi membranes. The signal that mediates and controls the cellular localization of Vps10p is found in the cytosolic domain of the protein, where two aromatic-based signals, YSSL and FYVF, enable the protein to cycle between the *trans*-Golgi and the endosome (274, 275). The luminal portion of the receptor recognizes cargo, where binding occurs between the cystein-rich domain and the transmembrane domain of the receptor (276). When Vps10p is absent, CPY is secreted to a large extent to the culture medium. In addition, other vacuolar hydrolases of the proteinase A family (PrA, Pep4p) and aminopeptidase Y (APY) are also secreted, although to a lower extent, and are thus believed to be ligands for the receptor Vps10p (263, 275, 277).

Two other proteins that cycle between the late Golgi and the endosomal compartments are Kex2p, and the dipeptidyl aminopeptidase A (DPAP A or more commonly known as Ste13p)(263). Recycling is achieved by aromatic-based amino acid motifs that associate with the retromer complex (278, 279). However, in contrast to Vps10p, these proteins have *trans*-Golgi retention signals that increase their local concentration in the late compartments of the Golgi (280).

4.1.1 Adaptor proteins: AP-1 complex and GGAs

Transport of cargo between the endosome and the late-Golgi requires clathrin and clathrin-adaptor proteins. The function of clathrin is to provide a scaffold for vesicle budding, similar to the function of the COPI and COPII coats. Meanwhile, the adaptor proteins link vesicle formation with protein sorting due to the ability to bind both to cargo and clathrin (281). Transport along the CPY pathway involves three such adaptor proteins, the heterotetrameric adaptor-protein (AP)-1 complex and the Golgi-localized, γ-ear containing, Arf-binding family of proteins, commonly referred as GGAs, (Gga1p and Gga2p).

In addition to the AP-1 complex (TGN to late endosome sorting) (282), three additional AP complex exist that participate in the sorting events along different pathways, namely AP-2 (endocytosis) (283, 284), AP-3 (endosome/ TGN sorting to the vacuole) (285) and AP-4 which is present only in mammals and plants (TGN sorting to vacuole and basolateral sorting to plasma membrane) (286, 287). Besides these ubiquitous AP complexes, two additional complexes exist in higher eukaryotes that are cell-type specific. These are AP-1B (polarized epithelial cells, used in basolateral sorting) (288) and AP-3B (neurons, used for synaptic vesicle biogenesis) (281, 283).

The AP-1complex similar to the other AP complexes consists of two large subunits, Apl2p (a homologue of the mammalian β1 adaptin) and Apl4p (a γ adaptin homologue), one medium subunit Apm1p (a μ1 chain homologue) and one small subunit Aps1p (a σ1 chain homologue). The AP-1 complex is organized into three domains (289). The core domain is responsible for the recruitment of the complex to membranes by binding to Arf-GTP, to phosphoinositides and to sorting signals present on the cytoplasmic side of transmembrane cargo proteins. The hinge segments contain clathrin-box sequences that mediate the binding of clathrin. The third domain of the AP-1 complex is organized into two ear or appendage domains that are responsible for the recruitment of accessory proteins. AP complexes selectively recognize and

bind to sorting signal(s) usually present in the cytoplasmic region of transmembrane proteins (290). Such signals include the tyrosine signal such as the NPXY sequence present on proteins destined for endocytosis, which is recognized by the AP-2 complex and the YXX∅ motif (where Y is tyrosine and ∅ is a bulky hydrophobic residue) (290, 291). Another type of sorting signal to which AP complexes attach is the dileucine signal D/EXXXLL (where D/E is aspartate or glutamate and L is leucine) (290).

The other group of adaptor protein that are recruited to the late-Golgi membranes by Arf-GTP are the GGA proteins (292). The GGA proteins are organized into three folded domains and are arranged in a fold that is similar to the overall structure of the AP-1 complex (289, 293). The VHS (Vps27, Hrs, Stam) domain functions as a recognition module for sorting signals that are exposed on the cytoplasmic tails of transmembrane cargo (294-297). Several of the transmembrane protein that traffic between the late Golgi and endosomes have a DXXLL signal that interact with the VHS domain of GGAs. The key D (aspartate) and LL (leucine) of the signal bind to an electropositive pocket and two shallow hydrophobic pockets, respectively on the surface of the VHS domain of GGAs (298, 299). In contrast, yeast GGAs do not have the specific residues in the VHS domain that recognize the canonical DXXLL signal of the transmembrane cargo. Thus, the sorting determinant of yeast GGAs appears to be different. The GAT (GGA and TOM) domain is responsible for the ability of GGAs to bind specifically to the GTP-bound form of Arf1 (300, 301). The GAT domain is subdivided into two subdomains, an amino-terminal hook that interacts with specific regions of Arf and is capable of discriminating between the GTP and GDP forms (302), and a carboxy-terminal triple helix bundle that possibly may interact with SNAREs and with Rabaptin-5 that together with Rabex-5 participate in endosomal tethering and fusion events (303-305). The GAE (γ-adaptin ear) domain shares structural similarities with the ear domains of the γ1- and γ2 adaptin-subunit isoforms (Apl4p in yeast) of AP-1. These domains interact with a specific set of accessory proteins all of which share a canonical peptide motif DFGX∅ (where ∅ is a bulky hydrophobic residue) (306). Differences in the peptide motifs and in their overall shape may account for the distinct binding preferences of a subset of specific accessory proteins for either GGAs or γ-adaptins (289). Another significant different between yeast and mammalian GGAs is that yeast GGAs are less dependent on Arf and clathrin to fulfill their function, as compared to their mammalian homologues. Deletion of the genes encoding Gga1p or Gga2p individually result in minor phenotypic changes but deletion of both genes, similar to the sorting defects observed in Δ*vps10* mutants cells, result in inappropriate processing of the inactive precursors of vacuolar hydrolases such as CPY, PrA and the transmembrane carboxypeptidase S (CPS) (307). Pro-CPY and pro-PrA are sorted to the endosome by binding to the transmembrane receptor Vps10p. Meanwhile, sorting of pro-CPS (transmembrane protein) is Vps10p-independent and correct sorting to the vacuole is dependent upon ubiquitination of the pro-CPS tail that occurs in the late-endosome/PVC (308). A Pro-CPS sorting defect is more severe than the sorting defects of pro-CPY and pro-PrA in *gga1*Δ*gga2*Δ cells (307). Taken together, pro-CPS appears to be more dependent on

GGAs for proper sorting, and for example the late endosomal SNARE Pep12p, the transmembrane endopeptidase Kex2p and the transmembrane receptor Vps10p are missorted in *gga1*Δ*gga2*Δ cells. Thus, it appears that GGA proteins are involved in proper sorting of transmembrane proteins to the late endosome (307, 309, 310). The differences in sorting defects observed between the distinct cargo proteins may be due to the recruitment of a diverse subset of accessory proteins, by either AP-1 complex or GGAs, that recognize a specific set of cargo proteins/ transmembrane receptors.

4.1.2 Formation of functional transport vesicles

So how does a transport vesicle carrying cargo destined to the vacuole through the CPY pathway form at the *trans*-Golgi membrane? As in the case of COPI and COPII coats, the first step in vesicle formation is membrane recruitment of the individual coat components. This involves localized activation of Arf by Arf-GEF, which converts Arf-GDP to Arf-GTP. This change of nucleotide induces a structural rearrangement with exposure of a myristoylated amino-terminal α-helix and a change of the spatial rearrangement of the "switch 1" and "switch 2" regions of Arf. Exposure of the myristoylated helix allows tethering of the Arf-GTP to the membranes of the late-Golgi, while the spatial rearrangement of the switch regions allows the recruitment of Arf effectors (301, 311, 312). The insertion of Arf-GTP to the membrane thus allows the recruitments of either GGAs or/and AP-1 complex to the membrane. The precise mechanism of action of GGA and AP-1 binding to Arf-GTP and cargo molecules is not fully elucidated and it is not known if binding of GGA is a prerequisite for binding of AP-1 complex or if these adaptors mediate transport in different directions, act in parallel pathways, or cooperate in the same transport steps (282, 293, 313). Given that Arf-GTP is also found associated with the *cis*-Golgi membranes, the specificity of binding of the adaptor proteins to the late-Golgi membranes may be accomplished by either Arf-GEFs, Arf-GAPs or in particular the local enrichment of phosphoinositide in the late-Golgi membranes. For instance, in yeast Laa1p is required for correct localization of AP-1 to the late Golgi membranes, while no significant effect is seen in GGA protein distribution (314). The subsequent binding of GGAs through their GAT domain to Arf-GTP may stabilize this complex and hinder the activation action of the Arf-GAP on Arf-GTP, thus allowing the subsequent assembly steps (315). The binding of the adaptor proteins to Arf-GTP present on the late-Golgi membrane places them in close proximity to the membrane, where they can interact with specific signals exposed on the cytosolic side of transmembrane cargo/receptor proteins. The membrane-bound adaptors then initiate recruitment of clathrin to the TGN through interactions between the clathrin heavy chain and clathrin-box-like sequences present in the hinge segments of GGA and AP-1 complex (300, 316). At this stage the ear domain of the γ-adaptin subunit of AP-1 complex and the GAE domain of GGAs initiate the recruitment of a subset of accessory proteins that mediate or regulate, and are required for the formation of a functional clathrin-coated vesicle. Such accessory proteins are for example the phosphoinositide-binding epsin-like proteins Ent3p and Ent5p that might help curve the membrane to allow vesicle budding and sort cargo (317, 318). These proteins, in addition to assisting

the function of GGA and AP-1 complex, may also be classified as adaptor proteins themselves, due to the ability to interact with clathrin and cargo proteins. Besides the above functions, accessory proteins are involved in vesicle budding, disassembly of the coat, vesicle targeting/fusion by establishing functional links to tethers and SNAREs on the target membrane, and may also provide interactions with the cell cytoskeleton (289). One such example of the involvement of accessory proteins in vacuolar protein sorting is the Vps15p/Vps34p complex. Vps15p is a myristoylated serine/threonine protein kinase that interacts with the GDP-bound form of Gpa1p and recruits the phosphatidylinositol 3-kinase, Vps34p, to Golgi membranes (319, 320). This membrane-associated complex, which is activated by the GTP-bound form of Gpa1p, regulates vacuolar protein sorting.

4.1.3 SNAREs involved in late-*Golgi to PVC vesicle fusion*

After the vesicle pinches off from the late-Golgi it has to fuse with the appropriate target membrane. This specificity is achieved due to the presence of a specific subset of SNAREs on the target and donor membranes that help dock and fuse the vesicle with the endosomal target membrane. Mutant cells that lack these proteins accumulate a large number of 40-60 nm vesicles in the cytoplasm and display a single large round and poorly acidified vacuole that is incapable of forming the segregation structures required for vacuolar inheritance (264). These mutant genes are commonly referred as members of the class D *VPS* genes. Characterization of these genes revealed that many of their products are components of the SNARE complex machinery (267). These include the SNAREs Pep12p/Vps6p (t-SNARE, Q_A) and Vti1p (v-SNARE) that functions in both retrograde transport within the Golgi and transport to the PVC) (321, 322). SNARE complex formation is regulated by Vps45p (Sec1p like function) and the Rab GTPase Vps21p/Ypt51p (a Rab5 homologue that functions in endosome fusion in mammalian cells) and by Sec17p (yeast α-SNAP) and Sec18p (NSF) (323, 324). An additional Sec1p/Munc18 family protein, Vps33p, is required for fusion of late Golgi-derived vesicles with the late endosome (325). Vps33p is part of the large class C Vps protein complex (addressed in the following sections) and is required for fusion of transport intermediates from early endosomes to late endosomes, as well as of late endosome-derived membranes with the vacuole through interactions with the vacuolar t-SNARE Vam3p (326, 327). Both Vps33p and Vps45 interact with the t-SNARE present on early endosome membranes Pep12p (325, 328). Similar to Vps33p, Vps45 is capable of interacting with another Q_A-SNARE, Tlg2p, where this (Q_A) t-SNARE appears to be responsible for membrane association of Vps45p during the cycle of membrane fusion. Vps45p is thought to act as a molecular switch for the formation of the SNARE complex between Tlg2p/Tlg1p/Snc2p and Vti1p, since prior to membrane fusion Vps45p dissociates from Tlg2p and only reassociates with the *cis*-SNARE complex after membrane fusion (329-331). Additional accessory/regulatory proteins include Vps9p, Vps8p and Vps19p/Pep7p/Vac1p (263, 264, 332, 333). Vps9p functions as a GEF for Vps21p (Rab5 homologue), and is homologous to the mammalian Rab5 regulator, rabex-5 (332). Vps8p belongs to the class D *VPS* genes and is conserved across species. It is essential for sorting proteins to the endosome but dispensable

for delivery of proteins through the AP-3 pathway to the vacuole (333-335). Vps8p is possibly a functional homologue to the mammalian rabaptin-5, and hence in analogy to the functional Rabex-5/ rabaptin-5 pair may work together with Vps9p in intra-endosomal membrane traffic. It is also a component of the CORVET complex. Vps19p/Pep7p/Vac1p is the effector molecule of the RabGTPase Vps21p and hence facilitates/regulates vesicle-mediated vacuolar protein sorting (336).Tethering of transport intermediates to the endosomal membranes is provided by the class C Vps complex proteins, which bind to Vps8p instead of the vacuolar membrane partners Vps41p and Vam6 (13, 325, 337).

4.2 The ALP Pathway

Studies on the secretion of another membrane vacuolar protein, alkaline phosphatase (ALP) revealed that this protein is correctly localized to the vacuole when transport into or out of the PVC is defective, such as in class D and class E *vps* mutants (338, 339). In these studies the vacuolar maturation of ALP was followed and compared with the maturation of the vacuolar ATPase subunit (Vbh1p) and with the maturation of the vacuolar membrane protein carboxypeptidase S (CPS) under conditions where transport through the PVC is blocked, such as in *vps27*, *vps45* and *pep12* mutants. Under these conditions transport of ALP is unaffected, while transport of other cargo along the CPY pathway was blocked. Correct delivery of ALP to the vacuole was not due to bypass to the cell surface and subsequent endocytosis, since additional late secretory mutants did not effect the correct sorting of ALP (338, 339). Due to the ability of ALP to follow an alternative transport pathway to the vacuole that completely bypasses the PVC, this pathway is commonly referred as the ALP pathway.

4.2.1 The AP-3 adaptor complex provides sorting into the ALP pathway

Since ALP is not found in Golgi-derived 40-60 nm vesicles that accumulate in *vps45* mutant cells, it appears that ALP and other proteins that follow this pathway such as Vam3p and the vacuolar t-SNARE are sorted into a different class of transport vesicles that emerge from the late-Golgi (339). The vacuolar-sorting signal was identified in the cytoplasmic domain of ALP (13 to 16 amino acid sequence, rich in lysine and arginine residues). Domain swapping experiments showed that this region is sufficient to provide VPS27-independent sorting of the hybrid Dap2p and CPS to the vacuole (264, 338, 339). The correct sorting of ALP to the vacuole is mediated by the binding of the AP-3 adaptor complex to the cytosolic sorting sequence (285, 340, 341). Similar to the other family of AP complexes, the AP-3 complex consists of two large subunits, Apl6p (a homologue of the mammalian β3 adaptin) and Apl5p (a δ adaptin homologue), one medium subunit Apm3p (a μ3 chain homologue) and one small subunit Aps3p (a σ3 chain homologue) (281, 282, 342, 343). However, unlike the related AP-1 and AP-2 clathrin adaptor complexes that recognize sorting sequences exposed on the cytosolic tail of transmembrane proteins and recruit them into clathrin-coated pits, the AP-3 complex is not associated with clathrin. Deletion of any one subunit of the AP-3 complex delays transport of ALP to the vacuole through the ALP pathway. Under these conditions ALP is transported by default through the CPY pathway, which is unaffected by mutations in the AP-3 complex (340, 341). The precise mechanism and place of action

of the AP-3 complex is still not clear. One model suggests that the AP-3 complex recognizes the sorting sequence present in the cytoplasmic tail of transmembrane cargo protein in membranes of the *trans*-Golgi, and selectively recruits them into a distinct class of non-clathrin-coated vesicles (340). This model is based on the observations that GFP-ALP accumulates in the Golgi and cofractionates with Golgi markers. Due to the increased residence time in the Golgi and hence exposure to Golgi-localized glycosyltransferases, proALP is hyperglycosylated in AP-3 mutants. A second model suggests that instead of the AP-3 complex working at the *trans*-Golgi level, it may be working in a *post*-Golgi compartment, where it would recycle certain ALP pathway proteins back to the Golgi (341). This model is based on the observations that ALP is found in a distinct population of small vesicle-like structures that appear to be incapable of fusing with their target membrane. In AP-3 mutant cells, due to the inability of recycling the corresponding SNAREs back to the Golgi, the transport vesicles lack the SNARE machinery required for fusion, preventing their subsequent fusion. This block on the pathway could indirectly lead to missorting at the *trans*-Golgi, and subsequent accumulation in the Golgi and leakage to the CPY pathway.

Unlike the AP-1 complex, the AP-3 complex is not associated to clathrin, since in yeast AP-3-dependent transport of ALP to the vacuole, is not impaired in clathrin mutant cells (344). However the β3A subunit (Apl6p in yeast) is capable of interacting with clathrin through the clathrin-binding domain, and AP-3 colocalizes with endosomal membranes in HeLa cells (345). Although it is possible that AP-3 is capable of functionally associating with clathrin to form clathrin-coated vesicles *in vivo,* the only protein reported to be preferentially involved in the ALP pathway is Vam2/Vps41p (338, 346), which contains a highly conserved sequence related to the linker region of the clathrin heavy chain. Interestingly, Vps41p can be cross-linked to Vam6/Vps39p, and these two proteins appear to be involved in the same role, and part of a subcomplex that functions *in vivo*. Although these proteins would be good candidates for alternative proteins that could fulfill the role of clathrin, they are localized to one or two distinct patches on the vacuole membrane, and hence in principle should not mediate sorting at the Golgi (264, 347).

4.3 Sorting into the CPY vs. ALP pathway

As was discussed in the previous sections, two pathways target proteins to the vacuole. So how does the cell know which proteins are to be sorted into these pathways? The list of proteins to be transported to the vacuole through the CPY pathway includes a large number of proteins, while the ALP pathway includes mainly two reported proteins. Studies carried out on the sorting signals of these two pathways lead to the suggestion that entry to the ALP pathway is a signal-mediated sorting event and hence saturable, since overexpression of ALP or in the absence of AP-3, ALP is delivered to the vacuole through the CPY pathway (338). Vacuolar delivery of Dap2p (dipeptidyl aminopeptidase B, DPAP B) through the CPY pathway is unaffected by removal or replacement of either the cytoplasmic, transmembrane or lumenal domains (348). Moreover, when the localization signals of resident ER or Golgi membrane proteins are mutated, these are directly transported to the vacuole (349). These data suggest

that transport of membrane proteins through the CPY pathway in yeast occurs by default. However recent data suggest that entry into the CPY pathway may be mediated by certain signals that selectively target these proteins to the different pathways. One such example includes the general amino acid permease Gap1p. The final destination of Gap1p, changes according to physiological clues, namely the available nitrogen source (350). In cells grown under rich nitrogen conditions, such as glutamate medium, Gap1p is transported directly from the Golgi to the vacuole. Change to suboptimal nitrogen concentrations, such as urea, causes Gap1p to be transported to the plasma membrane (351). Proper sorting of Gap1p to the plasma membrane is dependent on the COPII complex component Sec13p, the ER-resident membrane protein Shr3p, the GSE complex, as well as of Npr1p, which is involved in stability and retention of Gap1p at the plasma membrane (351-354). Transport of Gap1p to the vacuole is dependent on the ubiquitination of Gap1p by the Rsp5p E3-ubiquitin ligase complex together with Bul1p/Bul2p (355), and occurs through the CPY pathway since it is dependent on Pep12p and facilitated by Gga1p and Gga2p (351, 356). The second example includes the selective transport of a mutant form of plasma membrane H^+-ATPase (Pma1p). In wild type cells Pma1p is transported from the *trans*-Golgi to the plasma membrane through selective packaging into a specific subset of secretory vesicles (258). When a mutant form of this protein is used, *pma1-7*, the normal delivery to the cell surface is inhibited and the protein is rerouted to the endosomal/vacuolar compartment for degradation (357-359). Transport to the vacuole occurs through the CPY pathway, since mutations that affect the transport between Golgi and the PVC, such as *vps1*, *vps8*, *pep12*, *gga1* and *gga2* mutant cells, block the transport of *pma1-7*p for degradation and instead *pma1-7p* is rerouted to the cell surface (357, 358, 360). Similar to Gap1p, transport of mutant *pma1-7* for degradation is dependent of Rsp5p-Bul1p-Bul2p ubiquitin ligase protein complex (360). Thus it appears that ubiquitination is one regulatory mechanism that serves to target proteins to different pathways, and understanding how these proteins specifically recognize and control the fate of exocytic proteins is object for further investigation (361).

4.3.1 Separate pathways with common principles: The dynamin-like protein Vps1p

If indeed proteins that follow these pathways are separated into distinct vesicles, how are these regulated and how does the cell distinguish these vesicles from the secretory vesicles targeted to the cell surface? Functional studies on the formation of vesicles at the Golgi implicated one *VPS* gene encoding the dynamin-like GTPase Vps1p, in the budding of vesicles transporting vacuolar cargo from the Golgi apparatus (362, 363). In addition to *VPS1*, clathrin has also been implicated in such vesicle formation events, since a sudden loss of clathrin function results in mislocalization of vacuolar proteins to the cell surface (364). Even though clathrin plays a fundamental role in sorting into the CPY pathway, the ALP pathway is relatively unaffected by clathrin mutations, so the role of clathrin will not be addressed in this section (364). Thus, we will focus on Vps1p, due to its importance in both pathways. Mutations that lead to the inactivation of Vps1p result in missorting of proteins destined to the vacuole into secretory vesicles destined

for the cell surface. Vps1p is an 80-kDa protein with homology to the mammalian GTPase dynamin, which is required for endocytosis and appears to be involved in the pinching off of vesicles from the plasma membrane (362, 365-367). Mammalian dynamin is thought to act as a regulatory GTPase and as a mechanochemical fission factor, by accumulating at the emerging bud necks, where it initiates tubulation and vesiculation of membranes (368, 369). Dynamin and its homologues share three common domains, an amino-terminal GTPase domain, a middle domain and a GTPase effector domain (370). Dynamin itself contains two additional domains, the pleckstrin homology domain, which is responsible for the binding to phosphoinositides, and a proline-rich domain. Yeast cells lacking Vps1p do not display any defect in endocytosis but instead secrete the Golgi form of CPY (p2 form). In vps1 mutants, Golgi and vacuolar membrane proteins are delivered to the vacuole via the plasma membrane (363, 371). Vps1p is required in yeast for the transport of proteins through both the CPY and ALP pathway. This observation and the homology to dynamin suggest a similar role for Vps1p in the pinching off of vesicles (vesicle scission) that divert proteins intended for the vacuole from the secretory pathway in wild type cells. Vps1p is also required for maintenance and stability of Golgi-localized proteins, since in *vps1*Δ cells Kex2p and Vps10p are also delivered to the vacuole *via* the plasma membrane (272, 363, 371). Moreover, Vps1p appears to be involved in vesicle fission at vacuolar membranes. The yeast vacuole is a highly dynamic organelle that undergoes constant cycles of fission and fusion during both vacuole inheritance, and due to changes in osmolarity of the environment. When a yeast cell is exposed to hyperosmotic stress the vacuoles fragment, and when Vps1p is mutated the vacuoles lose the ability to fragment and enlarge (372, 373). Surprisingly, Vps1p also appears to be involved in regulating vacuolar membrane fusion since antibodies against Vps1p and temperature-sensitive mutations in Vps1p block vacuole fusion (373). Studies carried out on the localization of Vps1p revealed that the protein localizes to vacuoles, and to be exact, to vacuole membrane constrictions, and to a number of spots on vacuole membranes (373). The presence of Vps1p to membrane constrictions is in agreement with the role of Vps1p in vesicle fission. The presence of Vps1p in discrete spots appears to be linked to formation of complexes of Vps1p and the vacuolar SNARE Vam3p (373). The release of Vps1p from the Vps1p-Vam3p complex required the yeast NSF Sec18p, but did not its co-factor Sec17p (the yeast α-SNAP) (373). So how does Vps1p coordinate vacuole fusion? Vps1p may trap Vam3p on the vacuole membrane making it a rate-limiting factor for fusion, thus favoring fission events. Alternatively, it may be involved as a sorting factor by separating v- and t-SNAREs or it may support the enrichment of Vam3p at docking sites (374, 375). The release of Vps1p at the onset of vacuole membrane fusion by the SNARE-activating ATPase Sec18p releases the t-SNAREs that may now initiate fusion events, bringing to an end the intrinsic fission activity of Vps1p. Alternatively, it is known that actin plays an active role in vacuolar fusion (376, 377) and dynamins as well as dynamin-like proteins, such as Vps1p, control actin dynamics. Consequently, it is possible that release of Vps1p from the Vps1p–Vam3p complex allows Vps1p to interact with actin or actin-regulating proteins, such as

Sla1p (378), and thus regulate a later step in vacuole membrane fusion.

In contrast to the mammalian homologue dynamin, the yeast Vps1p does not appear to be required for endocytosis, as in *vps1*Δ cells proteins are delivered to the vacuole *via* the plasma membrane (371). When a temperature-sensitive mutant allele of *END4,* which specifically blocks the internalization step of endocytosis (379), is combined with *vps1*Δ cells, vacuolar proteins are no longer delivered to the vacuole but accumulate at the plasma membrane (371). In support of the idea that Vps1p does not play an essential role in endocytosis at the plasma membrane, the internalization kinetics of uracil permease Fur4p in *vps1*Δ cells (at 30°C) is similar to that of wild type cells (378). Fur4p is localized at the plasma membrane and mediates the specific uptake of uracil. Similar to Gap1p, its expression and localization are tightly regulated by the uracil levels and environmental clues. Under normal conditions Fur4p is delivered directly to the cell surface *via* the secretory pathway. However in the presence of excess uracil, newly synthesized Fur4p can be directed to the degradative vacuolar pathway without ever passing through the plasma membrane (380). Normal degradation of plasma membrane Fur4p occurs through phosphorylation, which in turn facilitates ubiquitination of Fur4p, a process that is dependent on the Npi1p/Rsp5p ubiquitin-protein ligase (381, 382). Fur4p is then internalized, and following endocytosis it is targeted to the vacuole for proteolysis (383). Three possible explanations for the observation that Vps1p is not required for endocytosis include that a dynamin-like GTPase is not required for the endocytosis step, or then other dynamin related proteins might be involved in the scission of endocytic vesicles (263). These may include for example Mgm1p or Dnm1p, which coordinate mitochondrial fission and fusion (384). In addition to the above functions, Dnm1p is involved in the endocytic system although at a *post*-internalization step, namely before fusion to the late endosome (385). Alternatively, Vps1p may be required under normal conditions, whereas under conditions where Vps1p is nonfunctional, other pathways, perhaps clathrin-independent, may compensate.

4.3.2 The CPY and ALP pathways converge at the vacuole

The CPY and ALP transport pathways, which start diverging at the level of the late Golgi and follow two separate and distinct pathways to the vacuole, intersect only at a late stage, namely at the fusion with the vacuole. Class B and C *vps* mutants generally have a fragmented vacuole with more than 20 small vacuole-like compartments, or lack any identifiable vacuoles, respectively. Many of these genes encode components of the SNARE machinery, or components of a very large vacuole-associated complex that appears to be required for vacuolar membrane fusion events. These mutants typically show defects in ALP maturation and CPY processing. Delivery of CPY and ALP occurs by two parallel pathways, which meet at the vacuole. One such class B *VPS* gene encodes Vam3p, which is a vacuole-localized t-SNARE (Q_A) that is delivered to the vacuole through the ALP pathway and is required for the fusion of multiple transport intermediates with the vacuole (339, 386). In *vam3* mutants correct processing of both CPY and ALP is blocked. Although mature CPY is formed over time, this is due to aberrant intermediate processing of p2-

CPY that occurs outside of the vacuole, since proALP processing does not occur in these cells, suggesting that ALP is trapped in a intracellular compartment distinct form the one which contains CPY (326, 387). In addition to Vam3p, the other proteins involved in the formation of the SNARE complex are Vti1p (Q_B) and Vam7p/Vps43p (Q_C) exposed on the vacuole membrane, and Ykt6p (R) present on the transport intermediate membrane (386, 388-391). Membrane fusion to the vacuole is regulated by the Rab-GTPase Ypt7p and by Sec17p, Sec18p (392, 393). Other mutants such as the members of the class C *VPS* genes encode components of a large detergent-insoluble complex that is partially associated with vacuolar membranes known as the class C Vps complex. The class C Vps complex functions at two distinct protein trafficking steps. It has been reported to be required for fusion of late Golgi vesicles with the late endosome, and to be involved in fusion events at the vacuole (325, 337). In the latter case the class C Vps complex is also known as the homotypic fusion and vacuole protein sorting (HOPS) complex (327, 337). The HOPS/class C Vps complex is composed of six different subunits, the class B proteins Vps41p (Vam2p) and Vam6p (Vps39p) and the class C subunits Vps11p (Pep5p), Vps16p, Vps18p (Pep3p) and Vps33p (327, 347, 394, 395). This complex is thought to mediate the transition from tethering to *trans*-SNARE pairing at the membrane due to its ability to interact with the GTP form of Ypt7 (the yeast Rab7) and to the SNAREs Vam3p/Vam7p (396-398). Each of the subunits of the HOPS/Class C Vps Complex appear to have specialized domains that contribute to the correct tethering function of this complex. Vps33p is homologous to the Sec1/Munc18 (SM) family proteins (325, 399). Vps11p and Vps18p have essential RING finger zinc binding domains at their C-terminus (327). Vam6p (Vps39p) acts as a GEF on the Rab GTPase Ypt7p thus stimulating the nucleotide exchange on Ypt7p (395). Finally Vps41p appears to be specialized in two functions one is in tethering as a component of the HOPS complex and the second appears to be in the biogenesis of the AP-3 vesicles that transport cargo such as ALP from the Golgi to the vacuole (341, 394, 400, 401). Due to the specific involvement of Vps41p in the biogenesis of the AP-3 vesicles and the localization of Vps41p/Vps39p to vacuolar membranes these two proteins are thought to be preferentially involved in the ALP pathway possibly by stabilizing the interaction between the vacuolar t-SNARE Vam3p and an ALP pathway-specific v-SNARE (263, 347). Within all of the known class B and C mutants no synaptobrevin-like molecules are found. One potential protein, Nyv1p, is required for fusion of transport intermediates containing ALP in *vam3* mutant cells and is transported to the vacuole through the ALP pathway, where it works as a specific v-SNARE (392, 402). A null mutant of *vps41* typically has phenotypes similar to class B/C mutants with fragmented vacuoles, defects in CPY maturation and ALP processing (347, 403). Many of these defects are thought to be an indirect effect of blocking the ALP pathway, since incubation of *vps41-ts* mutant cells at the restrictive temperature lead to a rapid block in ALP processing, while maturation of both CPY and CPS remained unaffected, as well as vacuole morphology. The long-term effect of the *vps41* null mutation could be explained taking into count that the vacuolar t-SNARE Vam3p, which is required for the fusion of transport intermediates of the

CPY pathway to the vacuole, is delivered to the vacuole through the ALP pathway (347, 403). When the ALP pathway is blocked, newly synthesized Vam3p is no longer delivered to the vacuole and as soon as the free/active Vam3p present on the vacuole membrane is consumed the fusion of transport intermediates from the CPY pathway starts to be defective, and culminates with the appearance of fragmented vacuoles.

4.3.3 The CORVET complex mediates intra-endosomal tethering

The class C proteins that constitute the previously mentioned HOPS complex that functions at the vacuolar membrane interface are also present on endosomal membranes (337, 387). Here they form a novel-tethering complex referred as the class C core vacuole/endosome tethering (CORVET) complex (335). The CORVET complex is composed of the class C subunits Vps11p (Pep5p), Vps16p, Vps18p (Pep3p) and Vps33p together with the class D subunits Vps8p and Vps3p (325, 335, 404). CORVET and HOPS complex thus share a great degree of similarities. Both of these complexes have the class C core proteins as a common platform and both are composed of six subunits with approximately the same size. The differences appear concerning the remaining two components. In the HOPS complex Vps41p/Vam6p functions as an effector of the Rab Ypt7p (393, 395, 399), while in the CORVET complex Vps8p/ Vps3p functions as an effector of the Rab GTPase Vps21p (yeast Rab5 homologue) (333, 335). Vps3p is very similar to hVam6p with exception of the n-terminus. Additionally, Vps3p can bind to Vps21p-GDP and promote nucleotide exchange in a GEF-like function similar to the role of Vam6p in promoting nucleotide exchange on Ypt7-GDP (335, 395). Thus it appears that the HOPS and CORVET complex are structurally organized into three parts: the class C core, a GEF (Vam6p or Vps3p) and an effector protein (Vps41p or Vps8p). Since the HOPS complex is present at the vacuole interface and the CORVET complex at the endosomal interface, endosomal-vacuolar biogenesis might be regulated by which tethering agents is recruited. From elegant studies carried out on the dynamics and presence of these complexes at each step of the endosomal vacuolar pathway (335), it was possible to perceive that these complexes interchange according to their respective site of action and that a intermediate complexes exist. The first step in the conversion of these complexes appears to be the exchange of the putative GEF subunit, which binds to the class C subunit Vps33p (335, 395). Both Vps3p and Vam6p compete for binding to the effector subunit Vps41p at this stage. The next step is the exchange of the potential Rab effector. When the intermediate complex Vps3p-Vps41p-Class C core is formed Vps8p can replace Vps41p and the CORVET complex is formed. One important observation in the inter-conversion of these complexes is that Vps41p can replace Vps8p; but Vps8p cannot displace Vps41p without first Vps3p being brought to the system (335). The order of conversion is especially interesting since the GEF would recruit the next Rab, which would bind to the next effector, and in this way ensure appropriate membrane fusion events. In summary there appears to be a direct role of the tethering complexes in the way the endosomal/vacuolar organization is regulated possibly by controlling the RabGTPase switching and stability (335).

4.4 Proteins are sorted from each other along the endosomal/ vacuolar pathway

Multiple pathways emerge from the late-Golgi, which transport proteins and lipids to the vacuole. In addition to this pathway, membranes and proteins are continuously internalized through endocytosis. These two pathways intersect at the level of the early/late endosome. The late endosome is morphologically characterized by the presence of membrane-bound inner membranes or vesicles, and for this reason is also known as the multivesicular body (MVB). At the late endosome/ MVB, proteins are sorted to their correct destination through the presence of sorting signals similar to what occurred at the *trans*-Golgi. At this stage certain proteins such as transmembrane receptors and proteins that occasionally enter these transport intermediates and endocytic vesicles need to be correctly returned to their right place of function in order to maintain the appropriate trafficking dynamics in the cell. In the following sections we will address briefly how the cell recycles cargo back to the plasma membrane and how proteins are recycled back for further cycles of protein sorting.

4.4.1 Protein sorting at the late endosome / MVB.

Once proteins arrive at the MVB, they are once again sorted from each other (405). It appears that one of sorting signals for transport to the vacuole is the addition of ubiquitin (406). For example addition of monoubiquitin to the G-protein-coupled pheromone receptors, Ste2p and Ste3p present at the cell surface, acts as a signal for internalization, and also functions as a sorting determinant at the late endosome/MVB (308, 407, 408). Other proteins travel from the late Golgi to the vacuole and are ubiquitinated either at the late Golgi or at the late endosome/ MVB. The addition of monoubiquitin to certain proteins allows them to interact with the ubiquitin-binding domain of the GGA proteins that mediate sorting at the late-Golgi to the MVB (356). For the addition of ubiquitin to the respective substrates the cell relies on Rsp5p, an ubiquitin ligase of the Nedd4 family that is localized to diverse sites such as the plasma membrane, Golgi and endosomes (409, 410). Rsp5p is responsible for the ubiquitination of proteins at the surface prior to internalization, and is involved in the ubiquitination of biosynthetic cargo destined to the vacuole, a step required for cargo to enter the CPY pathway. (352, 409, 411-413). The broad range of action of Rsp5p in the cell appears to be accomplished by specific domains within Rsp5p, since different mutations in Rsp5p affect different cargos (414). For example the C2 domain is responsible for the binding of Rsp5p to phosphoinositides and may be the key feature required for recruitment of Rsp5p to Golgi/endosomal membranes (415). When the C2 domain of Rsp5p is deleted, ubiquitination of biosynthetic cargo such as carboxypeptidase S is affected, while the ubiquitination and internalization of Ste2p at the plasma membrane is unaffected (409, 412, 415).

4.4.1.1 The ESCRT complexes mediate sorting of ubiquitinated cargo at the late endosome / MVB

The class E *vps* mutants are characterized by the presence of a large and aberrant late endosome/MVB, where proteins that follow the CPY pathway and endocytic pathway accumulate (416). The class E *VPS* family is composed of 18 genes, 17 of which encode soluble proteins form membrane-associated complexes with each other on the MVB membrane.

Biochemical and genetic analyze have revealed the formation of four endosomal sorting complexes required for transport, commonly referred as ESCRT complexes: ESCRT-0 (Vps27p and Hse1p); ESCRT-I (Vps23p, Vps28p and Vps37p); ESCRT-II (Vps22p, Vps25p and Vps36p) and finally ESCRT-III (Vps2p, Vps20p, Vps24p and Vps32p) (407, 417-421). Vps44p/Nhx1p is the only member of the class E *VPS* genes that encodes a transmembrane protein, namely a sodium/proton exchanger localized to the MVB membrane (422). Although its precise function still remains unclear, it appears that its ion exchange activity is essential for late endosome/MVB sorting, possibly by regulating the lumenal MVB ionic/pH environment. This in turn could affect the recruitment of ESCRT or ESCRT-associated proteins to the membrane and consequently affect protein sorting (263, 423).

The ESCRT-0 complex provides the sorting receptor for ubiquitinated cargo into lumenal vesicles at the late endosome/MVB. This feature is provided by the presence of ubiquitin interacting motifs (UIMs) in both Vps27p and Hse1p (419, 424). The ESCRT-0 complex is recruited to the membranes through the interaction of the FYVE domain of Vps27p with phosphatidyl inositol 3-phosphate (PI3P) (425). This recruitment is further enhanced by the interaction of Vps27p with the ENTH domain proteins Ent3p and Ent5p (426). These proteins also bind phosphatidyl inositol-3,5-bisphosphate, and are required for correct sorting of proteins to the lumenal membranes (426). When the ESCRT-0 complex is correctly positioned, it may recruit the ESCRT-I complex through the interactions between the PTAP-like motifs present on the C-terminal region of Vps27p and Vps23p (425, 427). In addition to recruiting ESCRT-I to the MVB membranes, it is possible that it also recruits the ubiquitin ligase Rsp5p, thus allowing the ubiquitination of cargo that would then be closely positioned to ESCRT-0 complex for MVB sorting (263, 420). ESCRT-I is also capable of binding to ubiquitinated cargo, but this interaction is provided by the ubiquitin E2 variant (UEV) domain (407). After ESCRT-I is correctly positioned, ESCRT-II and ESCRT-III are recruited to the late-endosome/MVB membrane (417, 418, 420). ESCRT-II associates with both ESCRT-I and with ESCRT-III and thus helps increase the membrane-binding/recruitment of ESCRT-IIII (428). Additionally, ESCRT-II appears to have the ability to bind to ubiquitinated cargo, since point mutations in the NZF-ubiquitin-binding domain of Vps36p abolish the correct sorting of GFP-CPS at the late endosome/MVB (429). ESCRT-III complex is made itself from two subcomplexes composed of Vps2p/Vps24p and Vps20p/Vps32p (417). ESCRT-III is recruited to the late endosome membranes through interactions between Vps20p subunit and ESCRT-I and –II (420). At this stage ESCRT-III recruits accessory proteins such as Vps4p, Vps31p/Bro1p, Vps46p, Vps60p and Vta1p (263, 420, 430). Bro1p in turn recruits the deubiquitinating enzyme Doa4p to the ESCRT-III complex through interactions of Vps31p/Bro1p with Vps32p (420, 431, 432). Doa4p removes ubiquitin from the cargo molecules prior to their incorporation into the budding lumenal vesicles and is essential for proper maintenance of the normal intracellular ubiquitin levels (433). Vps4p is an AAA-ATPase that is required for membrane dissociation of the ESCRT complexes thus allowing recycling of the ESCRT complexes for further cycles of late endosome/MVB sorting (407, 434, 435).

In summary, the ESCRT complexes mediate sorting into the lumenal vesicles through the following steps: ESCRT-0 is recruited to the MVB membrane *via* PI3P-binding that is generated by the Vps34p/Vps15p complex. Rsp5p is recruited to the membrane by ESCRT-0 and may ubiquitinate cargo. ESCRT-0 recruits ESCRT-I and ESCRT-II, and ubiquitinated cargo binds to these complexes in this order (429). In turn, ESCRT-III is recruited through interactions with ESCRT-I and –II, and ESCRT-III-associated proteins are recruited leading to deubiquitination of cargo to be incorporated into the lumenal vesicle through the action of Doa4p. ATP hydrolysis by Vps4p leads to disassociation of the ESCRT complexes from the membrane and cargo is sorted into the inward budding vesicle. (234)

4.4.1.2 The Retromer mediates recycling of late-Golgi proteins

The transport of vacuolar proteins from the *late*-Golgi to the PVC requires the efficient recycling of many *late*-Golgi membrane proteins that cycle between these two organelles and have to return to the Golgi. Such proteins include the acid hydrolase receptor Vps10p, the enzymes Kex2p and Ste13p and the SNAREs Snc1p and Tlg1p. This is accomplished by the presence of critical aromatic amino acids in the cytosolic tail of these transmembrane proteins that function as sorting signals for their retrieval from a *post*-Golgi compartment (274, 275, 278, 279). The mechanism of signal recognition and retrieval is still not well understood but involves a number of genes, including *VPS35*, *VPS29*, *VPS26*, *VPS5* and *VPS17*, which are thought to encode components of the recycling machinery namely the Retromer complex (273, 436-439).

Two subcomplexes constitute the retromer complex, the Vps35p–Vps29p–Vps26p subcomplex that has a role in cargo selection, and the Vps5p–Vps17p subcomplex that has a structural role namely in membrane recruitment and vesicle formation. With exception of the Vps17p subunit, which seems to be specific to fungi, retromer subunits are conserved in all eukaryotes. Vps35p appears to be the platform onto which the remaining components of the retromer associate. This observation came from studies that revealed that Vps35p could interact directly with cargo proteins such as Vps10p (273, 440, 441). Membrane binding of Vps35p to the late endosome membrane/MVB is further promoted by Vps26p, which also mediates the interaction with the Vps5p-Vps17p dimer, thus linking cargo selection and self-assembly (442). Association of the retromer to endosomal membranes appears to result from a direct interaction of the retromer with lipids of the endosomal membrane. Vps5p and Vps17p are both members of the sorting nexin (SNX) family, which are characterized by the presence of a conserved Phox homology (PX) domain, a BAR (Bin, amphiphysin, Rvs) domain and a function in the sorting of membrane proteins namely by avoiding that the protein reaches the vacuole (443, 444). The PX domain is responsible for the ability to bind to the endosomal lipid phosphatidylinositol 3-phosphate (PtdIns3P) (445). Since in yeast there is only one phosphatidylinositol 3-kinase, the product of the VPS34 gene, this protein regulates the action of the retromer by regulating the level of PtdIns3P (446, 447). Thus, Vps34p together with an adaptor complex composed of Vps30p/Vps38p, which is required for stimulation of kinase activity, regulates the production

of PtdIns3P, which in turn is required for proper localization of Vps5p-Vps17p and the remaining components of the retromer complex (446, 447). The function of the retromer is to associate to endosomal membranes and selectively sort cargo proteins into the transport vesicle. But does the retromer function as a vesicle coat? Two characteristics define a vesicle coat, the ability to select cargo and the ability to self-assemble thus driving vesicle formation that selectively encloses cargo within. The retromer appears to fulfill these characteristics, since Vps35p provides the cargo selection function, while the self-assembly function is provided by the BAR domains of Vps5p/Vps17p, which dimerize and bind to highly curved membranes (448, 449). If the retromer can be classified as a typical vesicle coat, like clathrin or adaptor coats is still not fully clear. However it appears that the transport intermediate that emerges from endosomes, and closely positioned to the observed retromer site of action, may indeed be tubules rather than vesicles (449-451). The retromer has not been shown to form electron dense coats as the classical vesicles coats do, and the mechanism of sorting cargo appears to be distinct from that of classical membrane coats (147, 452). A recycling pathway between the endosome and the Golgi also exits in yeast. This pathway is involved in the specific retrieval of the endocytosed exocytic SNARE Snc1p, and is mediated by another set of sorting nexins, namely *SNX4*, *SNX41* and *SNX42* (453). These sorting nexins might constitute another sorting complex that is involved in retrograde transport.

The nature and properties of the transport carriers that mediate the retrograde transport of cargo to the late-Golgi is still unknown. Nevertheless for fusion to occur with the late-Golgi, tethering factors have to be recruited from the cytosol. These tethering factors facilitate the assembly of SNARE complexes between the v-SNAREs on the retrograde carriers and the t-SNAREs on the late-Golgi. One such tethering factor implicated in endosome-to-late Golgi transport is the hetero-oligomeric complex GARP/VFT (Golgi-associated retrograde transport / Vps fifty three). The GARP/VFT complex is constituted by four subunits Vps51p, Vps52p, Vps53p and Vps54p and is recruited to the late-Golgi membranes through interaction with the Rab-GTPase Ypt6p. Vps51p interacts with the t-SNARE Tlg1p, thus it may coordinate tethering events with membrane fusion (454-456). The GARP/VFT complex is involved in tethering of transport carriers that originate from both the early and late endosome (454-457). Interestingly two other hetero-oligomeric complexes, where recently reported to be involved in tethering of retrograde transport carriers (458). These are the COG complex and the TRAPP-II complex. The COG complex is composed of eight subunits Cog1p to Cog8p, organized in two domains and is recruited to the late-Golgi by the Rab-GTPase Ypt1p, and interacts with the t-SNARE Sed5p (459-461). The main function of the COG complex appears to be in *intra*-Golgi membrane fusion events. However mutations in individual COG subunits, revealed impaired retrograde transport from both early and late endosomes to the Golgi (459, 460). The other hetero-oligomeric complex is the transport protein particle (TRAPP) complex (181). This complex is present in the cell in two forms, TRAPP-I and TRAPP-II (462). While TRAPP-I acts at the ER-Golgi interface and mediates tethering of

COPII to the *cis*-Golgi membrane face, TRAPP-II acts at the *intra*-Golgi level and mediates the tethering of COPI vesicles (462-465). These two TRAPP forms share seven subunits, whereas three subunits are specific to TRAPP-II (Trs130p, Trs120p and Trs65p) (462). Mutations in Trs130p disrupt Golgi traffic, while mutations in Trs120p interrupt recycling of proteins from the early endosomes to the late-Golgi (463). Taking into count, that Trs120p colocalizes with the late-Golgi marker Sec7p and Trs120p mutants reveal a defect in the recruitment of COPI subunits to the early endosome, it appears that Trs120p and the TRAPP-II complex are required for tethering of vesicles that traffic from the early endosome to the late-Golgi (463).

5. Transport to the plasma membrane, the last step of the exocytic pathway

In the previous sections we addressed how proteins destined to the secretory pathway enter the ER and are transported through the Golgi apparatus. During these transport events little differences are observed between exocytic cargo and proteins that have an intracellular function such as the carboxypeptidase Y receptor Vps10p. At the late-Golgi proteins intended for delivery to the plasma membrane, to be incorporated into the cell wall, or secreted to the medium, are sorted from proteins destined to remain intracellular. Meanwhile, proteins intended for delivery to the endosomal membrane system are sorted into a separate pathway. In some but not all cases, the N- or O-glycans added to the newly synthesized proteins provide sorting information. Proteins to be exocytosed are delivered to the cell surface in a specific set of vesicular carriers, which enclose a unique set of proteins in their membrane, although the coat proteins that participate in the formation of these carriers have not yet been fully identified (2, 258, 260, 466). In the following sections we will address how exocytic cargo is targeted to the plasma membrane and the fusion machinery involved in the regulation, fusion and delivery of these transport intermediates to the plasma membrane.

5.1 Different vesicles transport distinct proteins

In the majority of the eukaryotic cells, exocytic cargo can reach the cell surface by multiple pathways (256, 257). For example in polarized epithelial cells distinct vesicles with specific subsets of cargo are targeted preferentially to the apical or basolateral membranes (467-470). This suggests a unique sorting mechanism for the different classes of cargos. This idea is further supported by the fact that when apical and basolateral proteins are expressed in fibroblasts (i.e., nonpolarized cells), the sorting machinery of the cell segregates these cargos into different populations of vesicles using the same sorting signals as in polarized cells (471, 472). Similar to their higher eukaryotic partners, the yeast *S. cerevisiae* also has the ability of packaging exocytic cargo into two classes of secretory vesicles, which differ in their cargo (258-260, 473). These two populations of vesicles were first identified using an isolation procedure that allowed the specific enrichment of secretory vesicles, followed by further separation according to their densities (258, 474). Thus, the vesicles classes were designated as LDSV (light density secretory vesicle) and the HDSV (heavy density secretory vesicle). The LDSV class carries the cell wall component Bgl2p (endo-β-1,3-glucanase),

Pma1p (the major plasma membrane ATPase) and Gas1p (GPI anchored β-1,3-glucanosyltransferase) (258). On the other hand, invertase (a periplasmic secreted sucrose hydrolyzing enzyme) and acid phosphatase (PHO11/PHO12/PHO5) are found in the HDSV class of vesicles (258). These two classes of vesicles also differ in their transit times through the secretory pathway. While the LDSV cargo is secreted in 30 minutes, the HDSV cargo is transported in only 5 minutes (4, 258, 475, 476). The asynchrony between the different cargos develops before the final exocytic step. One possible explanation suggested for this difference in secretion kinetics is transported through different compartments (4, 258). Indeed, cargo found in the HDSV class of vesicles is dependent on a functional endosomal pathway, and hence thought to transit through an intermediate endosomal compartment (259, 260). In mutants deficient in the endocytic pathway such as *vps1*Δ, *vps4*Δ and clathrin mutants, the biogenesis of HDSV is abolished and HDSV cargo is now found in LDSV (259, 260). Meanwhile, the biogenesis and sorting of cargo into LDSV appears unaffected in these mutants. Thus, it appears that LDSV are generated directly at the late-Golgi membrane, while HDSV are generated at endosomal membranes (258-260, 477, 478).

Although the best-known mechanism of transport from the early endosomes to the plasma membrane is the recycling of the transferrin receptor and synaptic receptors, newly synthesized proteins are also capable of entering this pathway (479-481). This is illustrated by the trafficking of newly synthesized transferrin receptor, asialoglycoprotein receptor H1 and the major histocompatibility complex class II molecules (482-485). It appears that in yeast, an endosomal to plasma membrane pathway is also functional. Mutant plasma membrane ATPase, *pma1-7p*, is normally delivered to the vacuole for degradation, however when delivery to the vacuole is blocked *pma1-7p* is rerouted to the plasma membrane (357, 358). *Vps* mutant cells are characterized by the missorting of the vacuolar protein carboxypeptidase Y to the cell surface (371). This feature can be observed by the packaging and accumulation of CPY into LDSV in *vps1*Δ *sec6-4* cells, since this mutant appears to lack dense secretory vesicles due to the block of all trafficking events to the vacuole (260). However, in *vps10*Δ *sec6-4* mutant cells, where traffic to the vacuole is normal, but the receptor for CPY is lacking, CPY is found in the HDSV class of vesicles (259, 260). The reason for two divergent pathways for delivery of proteins to the plasma membrane is not fully understood. Perhaps certain proteins may need special processing requirements or alternatively need to be regulated differentially.

Another difference between the LDSV and the HDSV is the nature of their cargo. While the LDSV transports cargo involved in cell surface expansion, the HDSV transports newly synthesized soluble exocytic proteins that appear to be required under certain physiological conditions (260). The cargo transported in the HDSV is under transcriptional regulation, e.g. invertase and acid phosphatase. Packaging these proteins separately from constitutively expressed ones that are required for cell surface expansion, may add one layer of regulation at a *post*-translational level. One such example is the approach yeast uses to regulate the levels of active general amino acid permease, Gap1p, whose localization to the plasma membrane is regulated by

the available nitrogen source (350, 486, 487). Gap1p is capable of transporting all the naturally occurring amino acids into the yeast cell. Under poor nitrogen conditions, Gap1p is transported to the plasma membrane to increase amino acid uptake. On the other hand, under nitrogen-rich conditions, the presence of Gap1p on the membrane is downregulated through the action of Rsp5p ubiquitin ligase, which targets Gap1p for degradation in the vacuole (355).

The rapid relocation of Gap1p to the plasma membrane appears to involve a novel complex called the GSE complex (GTPase-containing complex required for Gap1p sorting in the endosome) that recognizes Gap1p in the endosomes (354). The GSE complex is composed of two small GTPases (Gtr1p and Gtr2p) and three additional components (Gse1p, Gse2p and Ltv1p). The Gtr2p subunit provides the Gap1p recognition by binding to a di-aromatic motif (Trp-Tyr) exposed on the cytosolic domain of Gap1p (354). Whether the GSE complex is indeed a coat complex, which mediates the sorting of proteins into vesicles for transport to the plasma membrane, still remains to be fully addressed. The GSE complex is capable of binding cargo but the second feature of a coat complex, the ability to oligomerize and remodel the membrane thus driving vesicle budding, still remains to be elucidated. The GSE complex appears to form a complex with an M_r of approximately 600K, though its components have a total M_r of approximately 170K. Thus, the GSE complex could possibly mediate its self-assembly, or then associate with an accessory protein that would mediate the oligomerization and remodeling of the membrane. One such candidate is Mvp1p, which is capable of interacting with Gse1p (488). Mvp1p is a member of the sorting nexin family of proteins, which are characterized by the presence of a p40 Phox homology (PX) domain that allows the binding to phosphatidyl-inositol-3-phosphate present in the endosomal membranes (445, 489). The ability of sorting nexins to bind to membrane lipids, together with the feature that many sorting nexins contain BAR (Bin/Amphiphysin/Rvs) domains that may mediate tubulation, raises the possibility that the GSE complex may fulfill the role of coat complexes (444, 448, 489).

From the previous example we can see that the existence of a parallel pathway to the cell surface with an additional level of regulation, would be especially useful in adjusting the levels of external proteins that are required only under certain physiological conditions, or for soluble proteins, which cannot be efficiently retrieved (258, 260). Due to the complexity of the late secretory pathway, it has been difficult to characterize the transport routes involved and the machinery responsible for sorting and packaging cargo into these vesicles. Nevertheless, these two classes of vesicles do reveal some distinct features that may allow, better understanding of how these parallel pathways work and are regulated.

5.2 Polarized delivery of secretory cargo to the plasma membrane

The major mechanism for delivery of new membrane components to the cell surface involves the delivery, docking and fusion of secretory vesicles with the plasma membrane. This process is essential since it allows the cell to transport proteins to the cell surface that will permit it to interact with the surrounding environment. Polarized growth is a phenomenon that occurs in almost all of the cells, from prokaryotic to specialized eukaryotic

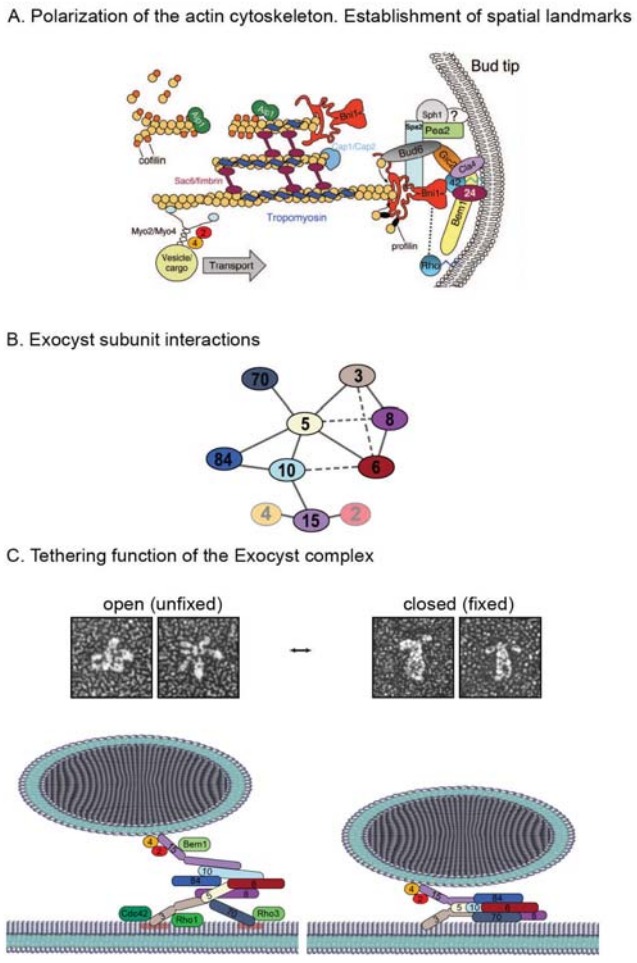
tissues and plays an important role in diverse cellular events such as organism development and cell movement. In the majority of eukaryotic cells this process occurs at discrete sites of the cell surface and is tightly coordinated with the overall polarity of the cell. For example, in epithelial cells, polarized delivery of basolateral and apical proteins to the correct membranes has to be spatially regulated to allow correct morphology and physiology (468, 469). Yeast cells display a high degree of polarized traffic during their life cycle, where the sites of active vesicle fusion to the plasma membrane change during the progression through the life cycle. This is accomplished by a shift in the protein machinery involved in cell polarity and exocytosis to discrete sites of the plasma membrane (490, 491). During vegetative growth yeast cells undergo polarized growth by budding. When a haploid cell is at the end of the G1 phase of the cell cycle, secretory vesicles are directed to the emerging bud tip. As the bud grows in the G2 phase, vesicle fusion becomes transiently isotropic (unpolarized) within the bud. During cytokinesis the machinery is relocated to the mother-bud neck until cell separation occurs. In yeast cells the budding pattern depends on their mating type. In haploid cells, the next round of budding occurs in an axial fashion, where the bud forms at the pole adjacent to the previous bud site. Diploid cells show a bipolar budding pattern, where daughter cells bud opposite to the previous budding event (490). In yeast, the polarized delivery of secretory vesicles to the site of active membrane growth involves three steps: 1) polarized delivery of vesicles along actin cables towards sites of polarized growth; 2) Docking of secretory vesicles with the plasma membrane, and 3) Fusion of secretory vesicles at sites of polarized growth. In the following sections we will address these issues separately.

5.2.1 Motor molecules, actin cytoskeleton and spatial landmarks

During the different cell cycle stages, the yeast *S. cerevisiae* spatially and temporally regulates exocytosis by directing surface growth and secretion to distinct sites on the plasma membrane. The accurate delivery of secretory vesicles to the sites of active membrane expansion involves both an actin-dependent vesicle transport, and actin-independent establishment of the machinery involved in receiving the vesicle at the cell surface (for reviews see (492-494)). In animal cells, transport of secretory vesicles to the cell surface relies primarily on microtubule-based transport, which is accomplished by kinesin-dependent transport along microtubules, followed by actomyosin-dependent transport (495, 496).

Actin filaments are dynamic polymers whose ATP-driven assembly in the cell cytoplasm drives shape changes, cell locomotion and chemotactic migration. Actin is the most abundant protein in the eukaryotic cell, accounting for about 15% in some cell types. The protein is highly conserved, and forms a huge variety of structures in cells in concert with a huge number of actin-binding proteins. The yeast actin cytoskeleton is organized into at least four biochemically and morphologically distinct structures: cortical patches, actin cables, the cytokinetic ring and the cap (494). Actin cables are highly dynamic structures containing actin (Act1p), fimbrin (Sac6p), and tropomyosin (Tpm1p, Tpm2p). The actin present in the actin cables is capable of rapid turnover (497). The dynamics of the actin cables appear to result from equilibrium between

protection/stabilization through the action of tropomyosin and disassembly by the cofilin (Cof1p)-Aip1p complex (498, 499). The polarization of the actin cable arrays is intimately linked to a group of proteins called the "polarity cap" (490, 493, 494). This term refers to a group of interacting cellular factors, shaped into a cap-like structure that localize during the bud emergence and apical growth and have a role in the regulation of the overall cytoskeleton polarity thus directing cell growth (**Figure 14**). The polarity cap is thought to function as a nucleation/anchor site for actin cables and is composed of proteins such as Cdc42p, Cdc24p, Bem1p, Ste20p, Cla4p, formins and the polarisome (490, 493, 500). One of the most crucial elements required for the polarization of the actin cytoskeleton is the small GTPase Cdc42p of the Rho/Rac subfamily of Ras-GTPases, and its GEF Cdc24p (501-504). A key event in the polarization function of Cdc42p is its recruitment to the plasma membrane, where it can locally activate its downstream effectors that signal to the actin cytoskeleton (505, 506). The association to the membrane is mediated by a geranylgeranyl anchor, which is transferred to Cdc42p through the action of Cdc43p, a type I geranylgeranyl transferase (507). The local cycles of hydrolysis and relocalization are facilitated by the activating effects of the GAPs (Bem3p, Rga1p and Rga2p) as well as by the action of the GDI Rdi1p (508-511). On



the plasma membrane Cdc24p and Cdc42p form complexes with a subset of different effectors, forming putative scaffolds that function as clusters transmitting signals to the cell and orientating the actin cytoskeleton (493). Two such effector molecules essential in the Cdc42p-actin signaling and are involved in all stages of growth (bud emergence, bud growth and cytokinesis), are the two p21-activated kinases (PAKs) Ste20p and Cla4p (512, 513). Cdc42p in the GTP-loaded form binds to the CRIB domain of the PAKs, that functions as an N-terminal PAK inhibitory domain, preventing the PAK from entering into the auto-inhibited conformation and thus activates these kinases that then mediate further signaling that control actin organization and polarized growth (514). The initial polarization of Cdc42p/Cdc24p to the sites of active membrane expansion is strongly affected by Bem1p, which plays a crucial role in maintaining a polarized cell, since it links the Cdc42p/Cdc24p complex to the polarity determinants involved in shmoo- and bud-site selection (515-517). For example during early bud emergence pre-existing cortical cues mark the proper site to initiate bud emergence. These cues are left by the previous budding events, which resulted from a set of actions controlled by the *BUD* gene products. These cues lead to the local activation of the Ras-related protein Bud1p/Rsr1p GTPase by its GAP Bud2p and GEF Bud5p (518, 519). At this stage Bud1p is capable of binding to Cdc24p and to Bem1p, which in turn recruits Cdc42p to a discrete region of the plasma membrane where the nascent bud

Figure 14. Spatial landmarks for polarized delivery of secretory vesicles.
A. Polarization of the actin cytoskeleton. Establishment of spatial landmarks. Actin cable assembly is regulated by the action of the polarity cap components, which are localized to the bud tip. The polarity cap may be further linked to the plasma membrane via components that directly associate with phospholipids. Nucleation of the actin filaments is provided by the formin Bni1, which uses profilin- and Bud6-bound actin subunits as substrates. Bni1 is typically associated with the fast-growing (barbed) end of the actin filament it nucleates, facilitating insertional growth while protecting ends from the capping protein (Cap1/2). Cables are stabilized along their sides by tropomyosin, which competes with cofilin for binding F-actin. Whereas individual actin cables are connected to each other by cross-linking proteins such as Sac6 and Abp140. Actin cables are disassembled, by the action of cofilin and Aip1. Transport of secretory vesicles and other cargo along actin cables is provided by the action of the barbed-end-directed type V myosins. Reprinted, with permission, from the Microbiology and Molecular Biology Reviews, Volume 70 (c) 2006 by American Society for Microbiology.
B. Genetic interactions between exocyst components. Solid lines depict physical interactions between each of the exocyst subunits that have been shown to occur either by co-immunoprecipitation or by two hybrid screens Dashed lines reveal possible interactions hypothesized by their loss when the respective interacting partner is mutated. **C. The tethering function of the exocyst.** After the vesicle has been properly marked by the active form of the RabGTPase Sec4 and transported to the sites of active membrane fusion, Sec15p interacts with Sec4-GTP. The remaining components of the exocyst arrive to these sites either due to the polarization of the secretory pathway or are individually localized due to their interaction with the polarization machinery. The exocyst subunits assemble into the multisubunit complex at the interface between the secretory vesicle and the plasma membrane, helping to bridge a distance where the SNAREs cannot yet interact. At this stage the exocyst appears to be in a starfish shape. As the exocyst subunits start to assemble and pack there coiled coil regions against each other, they bring the vesicle closer to the plasma membrane where SNAREs on opposing membranes may interact. The exocyst in the closed form shows a T-shape.

site will be marked and bud emergence will begin (520). One additional set of proteins required for maintaining the polarized state of the cell, is the polarisome, which is a complex formed by Spa2p, Pea2p, Bud6p and the additional proteins Sph1p and Bni1p (521). In polarisome mutants shmoo growth is affected and the mother-daughter necks are enlarged, suggesting that initial bud emergence is not focused (522). Taking into count that Cdc42p/Cdc24p remains clustered during apical growth and the polarisome proteins are required for apical actin organization, the polarisome proteins may function as apical scaffolds that help maintain a tight cluster of Cdc42p/Cdc24p during the elongation of bud (493). The polarisome appears to play a central role in the integration of the different signals inside the cells that help regulate polarization. Such signals are mediated by a family of Rho GTPases such as Cdc42p, and additional family members such as Rho1p, Rho3p and Rho4p, which in the activated state bind to the central element of the polarisome, Bni1p, and link Rho GTPase signaling to actin filament assembly (523-526). Actin assembly is further promoted by the ability of Bni1p to bind to profilin (Pfy1p), which stimulates actin polymerization, and to the actin bundling proteins Tef1p/Tef2p (527, 528). Proper localization of the polarisome to the growth sites is provided by Spa2p, Sph1p and Pea2p that provide the docking site for Bni1p and Bud6p (521, 523, 529-533).

Regulation of the overall polarity state in *S. cerevisiae* is under the coordinated control of Rho GTPases and cyclin-dependent protein kinases. The Rho family GTPases, including Rho and Rac GTPases, are small monomeric GTPases primarily involved in polarization, control of cell division, and reorganization of cytoskeletal elements (534). Yeast has six Rho family members RHO1-5 and CDC42. Rho1p and Rho2p are partially redundant GTPases that are involved in a variety of roles within the cell (535, 536). In particular, they are key proteins in maintaining cell integrity by stimulating directly two β-1,3-glucan synthases (Fks1p and Fks2p) which participate in the construction of the cell wall at the sites of polarized growth (537). Since some *rho1* alleles cause depolarization of actin cytoskeleton it is thought that Rho1p activity promotes polarity (538). This role is accomplished by signaling through the Pkc1p and Mpk1p MAPK cascade, but the precise mechanism of signaling remains unclear (539). The Rho GTPases Rho3p and Rho4p play an important role in growth polarization by acting on the cytoskeletal polarity (493). Genetic evidence suggest that Rho3p/Rho4p and Cdc42p share a common polarizing function, although Rho3p/Rho4p appear to be involved after initiation of the bud formation and are required to maintain cell polarity during the maturation of the daughter cells (540).

5.2.1.1 Targeting secretory vesicles for transport along actin cables

Besides the role in actin polarization, Rho3p appears to have a function in targeting of secretory vesicles to the sites of active plasma membrane fusion by positively regulating actin cable-based vesicular transport (541, 542). This function can be subdivided into two steps: the transport of exocytic vesicles from the mother cell to the bud, and in a second step docking and fusion of vesicles with the plasma membrane. In the first stage, Rho3p directly interacts with the unconventional Myo2p motor. Myo2p is an myosin V heavy chain and constitutes

a type V myosin motor, which is involved in actin-based transport of cargos (543). Class V myosins are characterized by a particular domain architecture and distinct modes of regulation. Myo2p has a N-terminal actin-binding motor domain, a neck region that contains six IQ motifs that can bind calmodulin, and a globular C-terminal tail domain separated by a coiled-coil dimerization domain (544, 545). The globular C-terminal domain mediates the association to the vesicle to be transported and the C-terminal tail appears to be required for bud localization (546, 547). Rho3p regulation of Myo2p is mediated by its direct association with the coiled-coil region of Myo2p (542). Myo2p is additionally regulated by, and physically associated to the myosin light chain 1, Mlc1p (548). Mlc1p belongs to a branch of the calmodulin superfamily and is essential for vesicle delivery at the mother-bud neck during cytokinesis due to is ability to bind to the IQ motifs of the class V myosin Myo2p (549). Although Mlc1p belongs to the calmodulin superfamily, it is unable to bind calcium and the precise mechanism of its interaction with target motifs is not clear. Myosin motors polarize transport of secretory vesicles by translocating them along actin cables in direction of the cap. Unidirectional transport is ensured by the fact that class V myosins are processive, barbed end-directed motors, and by the fact that the filaments in the cables appear to be of uniform polarity with their barbed ends directed towards the cap (550, 551). Myo2p-driven transport of secretory vesicles along actin cables is very rapid, which results in the accumulation of these vesicles in the cap (552). Under conditions where actin cables are lost, such as in tropomyosin mutants, or in cases where the motor activity of Myo2p is lost, vesicles are no longer delivered to the cap and cell growth becomes depolarized, the cells enlarge isotropically (552-554).

The formation of a functional complex between Myo2p and the vesicle appears to require three proteins, Smy1p, Sec2p and Sec4p. Defects in these proteins are synthetically lethal, which suggested that their functions are interrelated (547, 552, 555). This is further supported by the fact that Rho3p, which is directly associated with Myo2p, also interacts genetically with *SEC4* and *TPM1*(542, 556). Smy1p is a non-essential kinesin-related heavy chain homologue that is capable of interacting directly with the C-terminal tail of Myo2p and is polarized to the cap by the motor activity of myosin (557). Although Smy1p is non-essential, overproduction of this protein enhances polarization of Myo2p to sites of polarized growth and can partially compensate for defects in the *myo2-66* mutant (555). Although Smy1p is a kinesin-related protein, it appears that its function does not rely on microtubules or on the kinesin motor activity but rather is associated to an activity in promoting the assembly of the transport complex (493, 558).

Sec4p is a Rab GTPase that controls the final stage of the exocytic pathway in the yeast *S. cerevisiae (559-561)*. Like above, activation of Sec4p involves exchange of GDP for GTP in a reaction mediated by its guanine nucleotide exchange factor (GEF) Sec2p (562, 563). Sec4p and Sec2p are found on the surface of secretory vesicles and escort the vesicle to the sites of active membrane fusion (562). The proper recruitment of Sec4p to the vesicle membrane appears to involve a Rab cascade where Sec2p is both a GEF for one Rab and an effector for the Rab that acts upstream (564). The first step of this cascade is the recruitment of Sec2p to the vesicle in a reaction catalyzed by the

Rab GTPases Ypt31p/Ypt32p, which is homologous to the mammalian Rab11 that regulates export from the Golgi apparatus (565, 566). When Sec2p is on the vesicle membrane it recruits the downstream Rab GTPase Sec4p (567). The ability of Sec2p to interact with these two Rab GTPases in a step-wise process is provided by the structural organization of Sec2p. The C-terminal region (amino acids 450-508), designated the localization domain, is required for Sec2p to associate to the vesicle; the exchange activity for Sec4p is localized in the N-terminus region (amino acids 1-160), and the Rab GTPase Ypt32p binds to a region localized within these two (567, 568).

Sec15p is a component of the exocyst complex that provides the tethering force required to bring two membranes into close proximity prior to fusion (see next section for further details) (569, 570). The exocyst is an effector of Sec4p and the direct effector of this interaction is mediated by the subunit Sec15p (571-573). Interestingly, Sec2p also binds to Sec15p, which means that Sec2p binds to the Rab GTPase and to the effector of the GTPase they activate (568). This interaction occurs on secretory vesicles and couples nucleotide exchange on Sec4p to the recruitment of the downstream Sec4p effector. These interactions are particularly useful to increase signaling specificity or to establish a positive-feedback loop that maintains a localized domain of activated GTPase that would in turn help maintain a polarized cell (564). It appears that the Sec15p and Ypt32p binding sites on Sec2p overlap to some extent (amino acids 161-258) and these two proteins compete with each other for the binding to Sec2p (567, 568). Thus it appears that the following sequence of events takes place to correctly deliver the vesicle to the plasma membrane: Ypt32p-GTP recruits Sec2p to the vesicle; the exchange activity domain of Sec2p activates Sec4p by exchanging the bound nucleotide. Taking into count that Sec4p is polarized to the cap in a Myo2p-dependent manner and that Sec2p is required for the delivery of vesicles along actin cables, it appears that one of these proteins promotes binding of Myo2p-Smy1p to the vesicle, which in turn, carries the vesicle along the actin cables to the sites of polarized growth (547, 562) (**Figure 14**). Overexpression of Ypt32p in a constitutively activated form stimulates the interaction between Mlc1p and Myo2p (549). As the vesicle approaches the site of active membrane fusion, Sec15p, which has a higher affinity for Sec2p than Ypt32p, displaces Ypt32p (568). The interaction between the Rab GTPase and its effector Sec15p helps establish and maintain a domain of highly activated Sec4p on the vesicle, which then triggers downstream events. After the vesicle is tethered at the plasma membrane, the localization domain of Sec2p falls back and helps displace bound Sec15p, which releases Sec2p for further rounds of vesicle transport (574). Sec4p in turn relies on two accessory proteins to ensure its proper recycling (575). These are Gdi1p (GDP dissociation inhibitor) and Dss4p (dominant suppressor of the *sec4-8* temperature-sensitive mutation). Gdi1p slows down the dissociation rate of GDP from Sec4p after hydrolysis has occurred on the membrane, and releases the GDP-bound form from the yeast membranes, thus ensuring a soluble pool of Sec4p (576). Dss4p in turn is a nucleotide release factor that assists the dissociation of GDP from Sec4p thus stimulating the activation of Sec4p by Sec2p (563, 577).

5.3 The Exocyst provides the tethering force at the plasma membrane

The exocyst complex is an evolutionarily conserved multiprotein complex composed of eight components, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (5, 570, 578, 579). Although the yeast and mammalian exocyst components share a limited sequence homology (17-24%), both complexes contain eight subunits and are of similar molecular weights (569). Morphological characterization of exocyst mutants indicate that this complex functions at a step after vesicles have been delivered to the site of active membrane fusion, but prior to the formation of SNARE complexes (570, 571, 580, 581). Exocyst mutants typically accumulate a pool of secretory vesicles at exocytic sites, which supports the notion that the vesicles are correctly targeted to the plasma membrane, but fail to successfully fuse with it. The yeast exocyst is concentrated in sub-regions of the plasma membrane that correspond to sites of active vesicle fusion (581, 582).

5.3.1 Spatial regulation of the exocyst complex

During progress through the cell cycle, the exocyst complex changes its localization and is found specifically in the bud tip or in the mother/daughter connection, which represent regions of active membrane growth. Thus, the exocyst complex must be capable of integrating itself into the cell cycle and re-localizing to the appropriate sites on the plasma membrane. One such component proposed to function as a spatial landmark allowing the proper positioning of the exocyst is Sec3p. Sec3p appears to be the component of the exocyst that is most proximal to the plasma membrane and was suggested to localize to the plasma membrane independently of an ongoing secretory pathway and of mutations in the remaining exocyst complex (582, 583). However, Sec3p many not be the only landmark for exocytosis. For example, deletion of *SEC3* yields viable cells at 24^{o}C, so additional factors must ensure that the exocyst is correctly localized (584, 585).

One component that has been suggested to be responsible for the Sec3p-independent exocyst localization is Exo70p (583, 586). Both Sec3p and Exo70p are capable of responding to signals coming from the Rho family of small GTP-binding proteins, which are master regulators of a wide range of cellular processes (569, 587). Sec3p appears to use a vesicle-independent mechanism, which appears to be dependent on the ability of Sec3p to interact with the polarity-establishing protein Cdc42p and Rho1p in the activated GTP form (582, 586, 588, 589). Both Rho1p-GTP and Cdc42p-GTP interact with the N-terminus of Sec3p and truncation of this binding domain (*sec3*Δ*N*), leads to its depolarized localization within the cell (586, 588). The N-terminus of the exocyst component Sec3 also has the ability to directly interact with phosphatidylinositol 4,5-bisphosphate (589). The interaction of Sec3p with Cdc42p, which is essential for establishment of yeast polarity, and with Rho1p, which is important for maintenance of polarized growth, may help restrict the exocyst and hence exocytosis to the sites of polarized cell growth, where new plasma membrane components are intended to be delivered. The additional component that appears to help localize the exocyst is Exo70p. Exo70p relies on both a vesicle-dependent and on a vesicle-independent mechanism to be correctly placed and is capable of interacting with Rho3p (542, 583, 590). The binding region

of Rho3p has been mapped to the domain C of Exo70p (590), however recent studies suggest that the polarized localization of the exocyst is not controlled by Rho3p (591), and this is further supported by the fact that a mutant form of Exo70p that does not have the ability to bind to Rho3p, *exo70-1521*, did not display any growth defects and neither did the double mutant *exo70-1521/sec3ΔN* (592). The domain D, localized in the C-terminus of Exo70p, was crucial for its ability to associate to the plasma membrane (592). This association seems to be mediated between a positively charged surface patch of domain D and the phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] in the plasma membrane. Despite the low sequence homology between mammalian and yeast exocyst subunits, this C terminal region of Exo70p is the most conserved and fulfills the same function in mammalian cells (593). It appears that Exo70p and Sec3p work together to correctly place the exocyst at the vesicle/plasma membrane interface. Defects in either of these subunits are insufficient to affect membrane targeting of the exocyst, and do not give rise to any severe growth or secretion defects. But when these two are combined, the exocyst can no longer be anchored to the plasma membrane, resulting in severe growth and secretion defects and inviability (592). In order to describe the mechanism of vesicle tethering we need to understand how the exocyst components themselves are targeted to the plasma membrane.

Using techniques such as FRAP (fluorescence recovery after photobleaching), GFP-tagged proteins and immunoelectron microscopy, it was shown that the yeast exocyst subunits Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo84p travel to exocytic sites by association with exocytic vesicles, transport which is dependent on actin cables (571, 582, 583). Meanwhile a portion of Exo70p and Sec3p become localized to the exocytic regions of the plasma membrane by associating themselves to patches of activated Cdc42p-GTP and Rho3p-GTP (591). The association of Rho3p to Exo70p may induce structural changes that expose the surface patch involved in phospholipids binding and thus promote anchoring to the plasma membrane (592, 593). At this stage the activated patch of Rhop/Cdc42p proteins would work as allosteric regulators on the initially unpolarized late secretory machinery targeting an initial fraction of these vesicles to this region of the plasma membrane. Since many components of the docking and fusion machinery, as well as Cdc42p and Rho1p, are associated with the secretory vesicles, this would result in the reinforcement of allosteric regulation by depositing an increased amount of exocyst components. The increased docking and fusion rates at these sites would be expected to lead to the polarization of the secretory pathway by an positive feedback mechanism (591). This proposed model is called the localized activation / allosteric model. It suggests that the polarization of the secretory pathway is the consequence, rather than the cause of ongoing polarized delivery of secretory vesicles to the plasma membrane. This model suggests that the exocyst is locally activated rather than sequestered, and functioning as a spatial landmark as proposed by the landmark / recruitment model (591, 594).

5.3.2 Function of the exocyst complex

The exocyst complex is essential for the ability of secretory vesicles to interact with the plasma membrane, in a process that is called tethering. This process occurs through a physical interaction, at some distance, between the vesicle and

the target membrane. Tethering events occur at a stage after vesicles have been delivered by cytoskeletal motors, but prior to the engagement of SNARES on opposing membranes. This is supported by the fact that upon loss of exocyst function, secretory vesicles are still delivered to exocytic site but SNARE complexes do not form and hence fusion is blocked (466, 562). The working model for assembly of the exocyst is that this complex is only fully assembled once the vesicles arrive at the sites marked by Sec3p and Exo70p (583). The component of the exocyst that links the complex to the vesicle is Sec15p (571). Not only is Sec15p an effector of the Rab GTPase Sec4p but it also interacts with Bem1p, a downstream effector of the Cdc42p-mediated polarity establishment pathway, which plays an essential role in the initial localization of Sec15p to the early bud (571, 595, 596). Although the yeast exocyst and the mammalian exocyst interact with a somewhat different set of proteins, the primary mechanism and function appears to be the same (579, 597). An insight into the mechanism of how the exocyst may function is provided by availability of the structures of some of the exocyst subunit domains, namely the C-terminal domains of Sec15p, Sec6p and Exo84p, as well as nearly full length Exo70p (590, 598-600). Despite the very low sequence similarity between the different subunits, all of them share a similar motif – a tandem repeat of helical-bundle units, arranged in a mixed antiparallel-parallel right-handed bundle, which appear to be packed together in an end-on manner, forming elongated rod-like structures (599, 601). Taking into count that the interaction of Exo70p with Sec8p and Sec10p are distributed along the length of the structure of Exo70p, some of the exocyst subunits may pack together in an elongated side-to-side fashion (590, 601). Images of the mammalian exocyst complex have been obtained by quickfreeze/Deep-etch EM, under conditions where the sample was either unfixed or prefixed in glutaraldehyde (602). In the unfixed state, the complex appears in different conformations usually as a set of 4-6 arms that radiate outwards from a central point in an arrangement similar to an open flower (**Figure 14**). The arms are about 4-6 nm in width and 10-30 nm in length. For comparison, the elongated Exo70p structure is 3 nm by 16 nm (598). After fixation, the exocyst adopts a less variable structure that assembles the letter T or Y, which is composed by an elongated body (13 nm wide and 30 nm long) and two arms (15 nm and 6 nm) that spread outwards and appear to be connected to the body by an flexible hinge region (602). The change of exocyst structure from "open flower" to the "T-Y" shape may reflect the normal function of the exocyst. In the open conformation the exocyst may initially tether the vesicle at long distances, but as the subunits pack together in an elongated side-to-side fashion, the vesicle is drawn closer to the plasma membrane where it may promote SNARE complex formation and membrane fusion. The precise functioning of the exocyst still remains to be fully described (601, 602).

In addition to the association of the exocyst with the plasma membrane, the exocyst is capable of interacting with earlier compartments of the secretory pathway. Such interactions include for example the association of the exocyst with Seb1p, a component of the ER translocon (584, 603-605). In yeast, overexpression of *SEB1* suppressed mutant alleles of the exocyst components (604). Overexpression of certain exocyst subunits increased overall protein synthesis (603). The interaction

between exocyst and ER is further supported by the fact that a prominent patch of Sec61p-GFP is observed at the bud tip when Sec3p is overexpressed, whereas *sec3*Δ cells show a defect in inheritance of the cortical ER into the bud (584, 606). This association might ensure the optimal function of the secretory pathway by balancing protein synthesis and secretory capacity. In this scenario the translocation machinery would modulate vesicular transport, and the downstream tethering proteins would themselves in turn regulate upstream protein synthesis by a feedback loop mechanism (605).

5.3.2.1 The exocyst in higher eukaryotes

Although the yeast exocyst and the mammalian exocyst interact with a somewhat different set of proteins, the primary mechanism and function appears to be the same, although some notable differences should be mentioned (579, 597). For example in polarized epithelial cells the exocyst is required for delivery of secretory cargo to the basolateral membrane, but apparently not to the apical membrane (607). In neurons, the exocyst has been shown to be required for neurite branching and syntaptogenesis. However, it is not required for synaptic vesicle release at mature synapses (608-610). Additionally the exocyst has also been associated to the endocytic-recycling pathway that operates between endosomes and the plasma membrane and is used to return internalized receptors to the cell surface (600, 611, 612). Therefore it appears that in higher eukaryotes, depending on which cell type and in which developmental phase they are, the exocyst responds to a number of different signaling pathways and determines where and when vesicles may fuse to the plasma membrane. In support of this idea, in animal cells the exocyst interacts with a subset of different GTPases. For example, Sec15p interacts with Rab11p, a Rab GTPase involved in the regulation of the recycling pathway (600, 613), and Sec10p interacts with Arf6p, a small GTP-binding protein of the ADP-ribosylation factor family that regulates membrane recycling to the plasma membrane through the endocytic pathway (611); Sec5p and Exo84p both interact with the RalA GTPase, which is required for regulated exocytosis and for neurite branching (610, 614, 615). Exo70p is recruited by the G protein TC10 to the plasma membrane after insulin activation, and this interaction is required for targeting of the glucose transporter GLUT4 from the adipocytes to the cell surface (616-618).

5.4 SNAREs and the fusion regulation machinery

The yeast exocytic SNARE complex that forms at the plasma membrane is composed of one molecule each of the Sso1/2p t-SNAREs (Qa type), Sec9p t-SNARE (Qbc type) and Snc1/2p v-SNAREs (R type), which form a fusion complex that is conserved throughout evolution. Besides the interaction of the exocyst with polarity establishment proteins and phospholipids of the plasma membrane, certain exocyst subunits are also capable of interacting either with SNAREs or with SNARE regulators. This suggests that the exocyst might promote SNARE complex assembly, or possibly assist in the process of SNARE-mediated membrane fusion (564, 619, 620). The first observations of the exocyst possibly linking tethering and fusion events at the plasma membrane came from data showing that Sec6p in the dimer form is capable of interacting with the t-SNARE Sec9p, which in turn inhibits the association of the two t-SNAREs Sso1p and Sec9p (621). Although the interaction

between Sec6p and Sec9p proposes a negative regulator function, this is not in agreement with the role of the exocyst, which has a positive role in SNARE complex assembly and membrane fusion. The interaction studies between Sec6p and Sec9p were performed in the absence of Sec6p partners and therefore the scenario with the entire exocyst present may be different.

The N-terminal autoinhibitory domain of Sso1p, which is capable of folding back and forming a stable complex, inhibits the binding to its partner t-SNARE Sec9p (622, 623). It is thought that this inhibition is one of the mechanisms the cell adopts to spatially and temporally regulate the places where membrane fusion events may occur. SNARE complex assembly can only occur after relieve of the regulation domain, which is thought to be released by a regulator/opener that localizes to sites of secretion. The exocyst complex is one the best candidates as regulator of SNARE complex formation. It is localized to the sites of active membrane fusion, interacts genetically with SNAREs and has a function prior to SNARE complex assembly (624-626). One possible mechanism explaining how the exocyst may function, is that upon vesicle arrival at the plasma membrane, the fully assembled exocyst would interact with the SNAREs. At this stage, Sec6p would interact with Sec9p and together with additional regulatory proteins would promote Sec9p-Sso1p assembly, which then would lead to the subsequent ternary SNARE complex formation (together with the v-SNARE Snc1p) and vesicle fusion at the plasma membrane (621). Possible regulator candidates of the transition from tethering to SNARE complex assembly are Sec1p, Sro7p and Mso1p (627-629).

5.4.1 Sec1/Munc18-like family: Sec1p

Sec1p is a member of the Sec1/Munc18-like (SM) family of proteins, which are involved in all SNARE-mediated fusion events. In yeast, Sec1p is localized to the sites of active vesicle fusion and is capable of binding to the pre-assembled t-SNARE complexes and to the fully assembled SNARE complex, with higher affinity to the final one (628, 630, 631). The association of Sec1p with the SNARE complexes directly stimulates SNARE-mediated membrane fusion. For this reason it is thought to be a key regulator in fusion events at the plasma membrane. The exocyst complex is composed of eight subunits, three of which (Sec3p. Sec5p and Exo70p) appear to be specifically involved in regulation of exocyst function, by integrating signals originating from different signaling pathways within the cell. Interestingly, *sec3*Δ, *sec5*Δ and *exo70*Δ cells are viable and these proteins appear not to be essential for growth or secretion under conditions where the upstream Rab GTPase Sec4p or the downstream SNARE-binding fusion enhancer protein Sec1p are overexpressed (619). Overexpression of Sec1p in these cells resulted in an increased level of assembled SNARE complexes over the wild type levels, suggesting a possible mechanism by which the defect in exocyst assembly can be compensated (619). Since a fraction of Sec1p could be coprecipitated with the exocyst (619), and the normally polarized distribution of Sec1p is lost when the exocyst function is lost (466, 628), the interaction between exocyst and Sec1p may serve to localize and possibly activate Sec1p at exocytic sites where it can promote the transition from tethering to SNARE-mediated vesicle docking and fusion.

5.4.2 Lethal giant larvae family: Sro7p/ Sro77p

A screen for suppressors of the growth defect observed in cells with loss of Rho3 GTPase function identified two proteins, Sro7p and Sro77p (632). They are yeast homologs of the lethal giant larvae (Lgl) family of proteins identified as tumor suppressors in *Drosophila melanogaster* and implicated in cell polarity in animal epithelial cells (633). The first functional evidence that revealed a direct role of Lgl in exocytosis was provided by the ability of Sro7p and Sro77p to interact with the plasma membrane t-SNARE Sec9p (634). This finding was further supported by the fact that Sro7p is found in the cell periphery associated to Sec9p, and is also associated to the ternary SNARE complex formed by Sec9/Sso/Snc (634). The fact that *sro7*Δ*/sro77*Δ cells have a severe exocytic defect at restrictive temperature and act downstream of Rho3 GTPase, suggests that members of the lethal giant larvae/tomosyn/Sro7 family play an important role in polarized exocytosis by regulating SNARE function at the plasma membrane (634). Structural analysis of Sro7/Lgl proteins revealed the presence of two seven-bladed WD40 β-propellers followed by a 60-residue-long 'tail', which is bound to the surface of the amino-terminal propeller, and turned out to be a conserved SNARE regulatory domain (635-637). The binding site for the 'tail' on Sro7 is largely hydrophilic, which suggests that the tail serves a regulatory rather than a structural role. Studies on the interaction between Sro7p and Sec9 suggest that Sro7p may exist in two conformational states, one where Sro7p binds preferentially to the Qbc-SNARE domain of Sec9p, and another where this domain of Sec9p is displaced by the Sro7p tail (636). The decision between which state is preferred may be affected by factors involved in Sro7p regulation.

Taking into count the structural arrangement of Sro7p, the regulation appears to occur by an allosteric mechanism, where interactions with bound ligands lead to rearrangements in the β-propeller domain interface, which are then propagated to the tail (636). One such potential regulator is Sec4p, which can directly interact with Sro7p. This interaction is dependent of the nucleotide state of the Rab GTPase (629). Furthermore, a ternary complex is formed between Sec4p-GTP, Sro7p and the t-SNARE Sec9p, which suggests that Sro7p is an effector of the Rab GTPase Sec4p and may act in parallel to the exocyst to promote SNARE complex formation or SNARE-mediated fusion in response to Sec4-GTP (629). In yeast, Sro7p and Sro77p are also capable of interacting with the exocyst subunit Exo84p, and this interaction may promote SNARE-mediated membrane fusion at specific regions of the plasma membrane (638). Genetic data collected on Sro7p suggest that this member of the Lgl family has many of the properties required of a key downstream effector in transmitting Rab GTPase function onto the SNARE assembly process (629, 634, 638). Thus it appears that Sec4p, Sro7p and Sec1p work in concert with the exocyst to ensure that the vesicle fuses correctly with the plasma membrane. This regulatory mechanism is further supported by the finding that overexpression of Sec4p, Sro7p and Sec1p, which enhance SNARE-mediated function, can overcome the partially defective exocyst complex in *sec3*Δ and *exo70*Δ cells (619, 629, 638). Suppression by Sec4p, however, requires that its downstream effector Sro7p/Sro77p is fully functional. Meanwhile Sec1p, which

works downstream or independently of Sro7p, does not require Sro7p (629).

5.4.3 The multicopy suppressor of Sec1p Mso1p

Mso1p was found in a screen for suppressors of the temperature-sensitive mutation *sec1-1*, and was found to directly interact with Sec1p (639). Deletion of *MSO1* yielded viable haploid cells with no obvious growth phenotypes except a mild accumulation of secretory vesicles in the small bud tip (639, 640). Deletion of *MSO1* in diploid cells resulted in sporulation defects, namely in formation of the prospore membrane, which is the precursor of the spore membrane, forming on the cytoplasmic side of the spindle pole bodies (641-643). In addition to the association of Mso1p to Sec1p, Mso1p is capable of interacting with the SNARE complex, Sec15p and it appears to functionally interact with Sec4p GTPase and its exchange factor Sec2p (642).

Although the precise function of Mso1p is not fully understood, the set of interacting partners of Mso1p places it in the interface between the exocyst complex and the exocytic SNARE machinery (**Figure 14**), where it may bridge the connection between Rab GTPase and Sec1p function facilitating the binding of Sec1p to the SNARE complex prior to membrane fusion (627, 642). On the other hand, overexpression of *MSO1* inhibited growth of *sec4-8*, *sec8-9* and *sec15-2* mutants even at permissive and semirestrictive temperature, which suggest that Mso1p may have a second role in attenuating Sec4p function (627). One model for the function of Mso1p in secretory vesicle membrane fusion would be that it coordinates the recruitment and subsequent removal of Sec1p in response to the GTPase cycle of Sec4p (627). According to this model, the pair Mso1p-Sec1p is recruited by activated Sec4-GTP to the assembling v-SNARE and t-SNARE complexes, formed between membranes tethered by the exocyst complex. GTP hydrolysis on Sec4p would trigger dissociation of Mso1p and Sec1p from the preassembled *trans*-SNARE complex, leading to full SNARE assembly and membrane fusion. In support of the regulatory function of Mso1p, local sequence alignments revealed that the C-terminus of Mso1p is highly homologous to the Munc13/Mint family members of SNARE regulators, and that this region is required for proper localization of Mso1p to the plasma membrane (627, 642). Both Mso1p and Munc-13 members are connected with exocytosis, interact with Sec1p/Munc18p and with the SNARE complex (15, 644). Furthermore, in *Caenorhabditis elegans* and in *Drosophila* melanogaster UNC13/Munc13 displaces Unc18/Munc18 from the SNARE complex prior to exocytosis (644-646). Therefore, the role of Munc13 and Munc18 proteins in SNARE-mediated fusion corresponds well with the binding of Mso1p and Sec1p to yeast SNAREs and the proposed regulatory mechanism.

AIMS OF THE STUDY

The aims of the present study was to elucidate whether Hsp150 is capable of entering parallel or alternative post-ER routes in the yeast secretory pathway.

First the role of the COPII coat member Sec24p and its two homologues Sfb2p and Sfb3p in ER exit of Hsp150 was elucidated. Second, the dependence of Hsp150 secretion in *post*-Golgi secretory mutants was studied as well as the structural signatures that guided Hsp150 to these alternative pathway. In addition we also wanted to further clarify the role of the exocyst components and accessory regulatory proteins.

While searching for the signature that guided Hsp150 exit in the yeast secretory pathway, we found that Hsp150Δ was capable of working as a carrier, allowing the fused protein to proper fold and be secreted. Therefore we explored the potential of using Hsp150Δ as a carrier for the expression of recombinant heterologous proteins in both *S. cerevisiae* and *P. pastoris*. The same technology was to be used in the development of a staining procedure to be used to visualize secretory organelles at the electron microscopy level.

MATERIALS AND METHODS

A summary of the experimental methods used in this study, together with the references to the respective publications in which they are described can be found in Table 1. The yeast strains used in this study are listed in Table 2, together with the publications in which they are described. Table 3 highlights the relevant features of the yeast mutants used. A schematic representation of the recombinant proteins used throughout this study can be found in **Figure 15**.

Table 1. Experimental methods used in this study

Method	Publication
β-lactamase activity assay	II, III
Calcofluor staining of bud scars	I
Fermentationof yeast cells	III
Immunoprecipitation	I, II
Invertase activity assay	II
Invertase activity staining in non-denaturing gels	I, II
Metabolic labeling of proteins	I, II
Nucleotide sequencing	I, II
Plasmid constructions	I, II, III
HRP staining method developed for yeast electron microscopy	I, II
Scanning electron microscopy	I
SDS-PAGE	I, II, III
Secretory vesicle analysis by density gradients	II
Subcellular fractionation	II
TCA precipitation of secretory proteins	II
Transmission electron microscopy	I, II
Western blot analysis	I, II, III
Yeast mating and tetrad dissection	I, II
Yeast strain construction	I, II, III
Yeast transformation	I, II, III

Table 2. Yeast strains used in this study

Strain	Relevant mutant genotype	Used in Publication	Source/reference
	Control cells		
H1	None	I	R.Schekman
H245	None	I, II	K. Kuchler & J. Thorner
H247	None	I, II	K. Kuchler & J. Thorner
H335	*URA3::Hsp150Δ–β-lactamse*	II	Simonen *et al.*, 1994
H1718	*LEU2::Hsp150Δ–HRP*	II	This study
H306	*Δhsp150*	II	This study
H2260	*sec15-1 SEC15*	II	This study
	ER to Golgi transport		
H1101	*sec24-1*	I	C. Kaiser
H1735	*sec24-1 LEU2::Hsp150Δ-HRP*	II	This study
H1866	*sec24::kanMX4/SEC24*	I	Euroscarf
H1914	*sec24-1/SEC24 Δsfb3/SFB3 Δsfb2/SFB2*	I	Karhinen *et al., 2005*
H1895	*sec24-1 Δsfb3*	I	Karhinen *et al., 2005*
H1930	*sec24-1 Δsfb3 Δsfb2*	I	Karhinen *et al., 2005*
H1927	*Δsec24 URA::SEC24-HIS$_6$ LEU2::PCM244 CEN*	I	Karhinen *et al., 2005*
H1996	*Δsec24 URA::SEC24-HIS$_6$ LEU2::PCM244 CEN Δsfb3*	I	Karhinen *et al., 2005*
H2025	*Δsec24 URA::SEC24-HIS$_6$ LEU2::PCM244 CEN Δsfb2*	I	Karhinen *et al., 2005*
H2023	*Δsec24 URA::SEC24-HIS$_6$ LEU2::PCM244 CEN Δsfb3 Δsfb2*	I	Karhinen *et al., 2005*
H1141	*Δsec24b (Δsfb2)*	I	J.P. Paccaud
H1142	*Δsec24c (Δsfb3)*	I	J.P. Paccaud
H2006	*Δsfb3 Δsfb2*	I	Karhinen *et al., 2005*
H4	*sec18-1*	II	R.Schekman
H1735	*sec18-1 LEU2::Hsp150Δ-HRP*	II	This study
	***Intra*-Golgi transport and *trans*-Golgi sorting**		
H3	*sec7-1*	II	R.Schekman
H1732	*sec7-1 LEU2::Hsp150Δ-HRP*	II	This study
H10	*sec7-1*	II	R.Schekman
H1734	*sec7-1 LEU2::Hsp150Δ-HRP*	II	This study
H206	*sec14-3*	II	This study
H1720	*sec14-3 LEU2::Hsp150Δ-HRP*	II	This study
H815	*vps10Δ::HIS3*	II	Scott Emr
H2335	*vps10Δ::LEU2*	II	This study
	Endosomal / Vacuolar pathway		
H2079	*vps1Δ::LEU2*	II	A. Chang
H2077	*vps8Δ::LEU2*	II	A. Chang

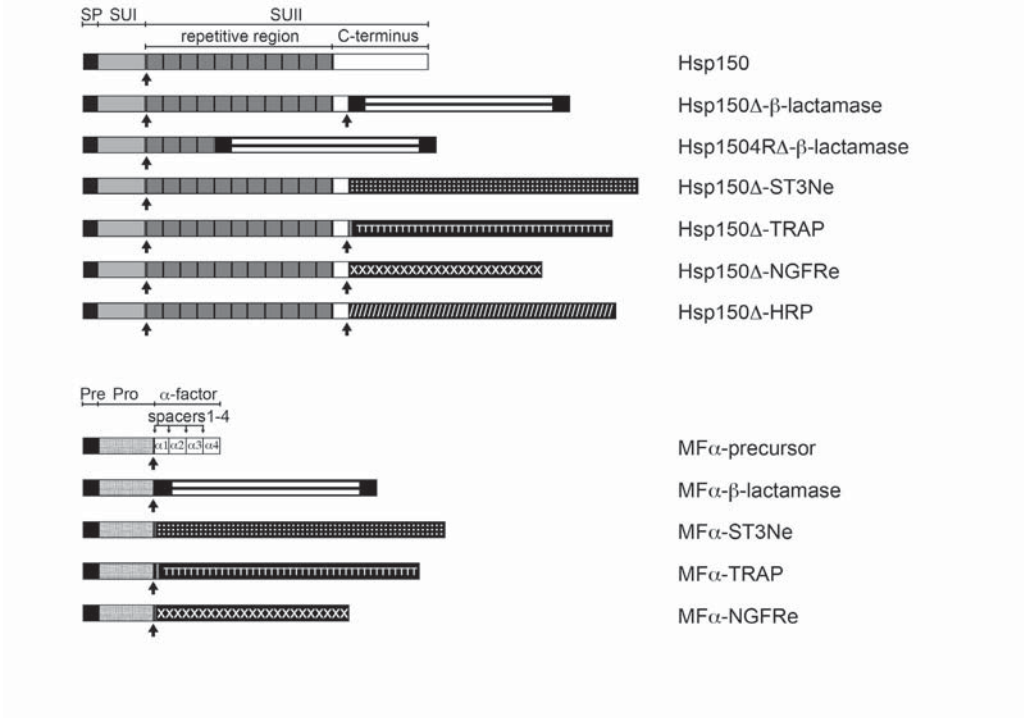
Table 2 continuing

Tethering of Secretory vesicles / Exocyst regulation			
H211	*sec2-56*	II	R.Schekman
H213	*sec4-2*	II	R.Schekman
H212	*sec3-1*	II	R.Schekman
H214	*sec5-24*	II	R.Schekman
H216	*sec8-6*	II	R.Schekman
H2129	*sec10-2*	II	P. Novick
H2074	*sec6-4*	II	P. Novick
H2253	*sec6-4 LEU2::Hsp150Δ–β-lactamase*	II	This study
H2255	*sec6-4 LEU2::Hsp150Δ–HRP*	II	This study
H2409	*sec6-4 vps1Δ::LEU2*	II	This study
H2075	*sec15-1*	II	P. Novick
H2087	*sec15-1 LEU2::Hsp150Δ–β-lactamase*	II	This study
H2257	*sec15-1 LEU2::Hsp150Δ–HRP*	II	This study
H2182	*sec15-1 Δmso1::URA3*	II	This study
H2319	*sec15-1 Δmso1::URA3 LEU2::Hsp150Δ–HRP*	II	This study
H2188	*sec15-1 Δmso1::LEU2*	II	This study
H2336	*sec15-1 vps10Δ::LEU2*	II	This study
H2328	*sec15-1 vps1Δ::LEU2*	II	This study
H2356	*sec15-1 vps8Δ::LEU2*	II	This study
Fusion of secretory vesicles with the plasma membrane			
H2	*sec1-1*	II	R.Schekman
H217	*sec9-4*	II	R.Schekman
H2181	*Δmso1::URA3*	II	This study
H2316	*Δmso1::URA3 LEU2::Hsp150Δ–HRP*	II	This study
H2187	*Δmso1::LEU2*	II	This study
Actin cytoskeleton			
H2311	*tpm1Δ::LEU2*	II	A. Bretsher
H2312	*srv2Δ::HIS3*	II	D.G. Drublin
***Pichia pastoris* strains**			
P714	Control	III	Invitrogen
P1405	*Hsp150Δ-Kex2p-β-Lactamase*	III	This study
P1407	*MFαΔ-Kex2p-β-Lactamase*	III	This study
P1402	*Hsp150Δ-ST3Ne*	III	This study
P1403	*MFαΔ-ST3Ne*	III	This study
P1476	*Hsp150Δ-Kex2p-β-NGFR*	III	This study
P1477	*MFαΔ-Kex2p-β-NGFR*	III	This study
P1478	*Hsp150Δ-Kex2p-β-TRAP*	III	This study
P1479	*MFαΔ-Kex2p-β-TRAP*	III	This study

Table 3. Relevant defects of the mutants used in this study

Mutation	Description of gene product	Phenotype	Reference
sec24-1	Component of the COPII coat. Role in cargo selection. Required for recruitment of the Sec13/31 complex to ER membranes	Defect in formation of COPII coat leading to ER accumulation	Hicke *et al.,1992*
Δ*sec24b/* Δ*sfb2/lss1*	SEC24 family member. Mutant phenotype found only in combination with other mutants.	Altered ER to Golgi transport when in combination with other mutants	Peng *et al.,* 2000
Δ*sec24c/* Δ*sfb3/lst1*	SEC24 family member. Secretion defect observed at elevated temperatures.	Growth and Pma1p secretion defects observed at elevated temperature	Roberg *et al.,* 1999
sec18-1	ATPase required for the release of Sec17p during the priming step of vesicle fusion.	Vesicles cannot fuse to their target membane. ER accumulation	Kaiser & Schekman, 1990
sec7-1	GEF for ADP ribosylation factores. Required for ER to Golgi and Intra-Golgi transport	Protein accumulation in ER and Golgi. Formation of Berkley bodies	Franzusoff & Schekman, 1989
sec14-3	PI/PC transfer protein involved in phospholipid metabolism. Regulates Golgi to PM transport	Protein transport from the *trans*-golgi is inhibited	Hama *et al.,*1999
*vps10*Δ	Transmembrane sorting receptor. Required for proper targeting of CPY to the vacuole	CPY is not targetted to the vacuole. Secretion of CPY to the medium	Marcusson *et al.,* 1994
*vps1*Δ	Dynamin-like GTPase. Required for protein targeting from the Golgi to the vacuole	Golgi to endsome traffic is blocked. Proteins normally targetted to the vacuole via endosome are secreted	Vater *et al.,* 1992
*vps8*Δ	Membrane protein that interacts with the small GTPase Vps21p. Required for protein transport from the late endosome to vacuole	Endosome to vacuole traffic is blocked	Chen *et al.,* 1996
sec2-56	Sec4 GEF. Essential for *post*-Golgi secretory vesicle transport	Unpolarized secretory vesicle accumulation	Walch-Solimena *et al.,* 1997
sec4-2	Secretory vesicle associated RabGTPase. Required for proper delivery of the vesicle to the sites of membrane fusion	Unpolarized secretory vesicle accumulation	Goud *et al.*, 1988
sec3-1 *sec5-24* *sec6-4* *sec8-6* *sec10-2* *sec15-1*	Exocyst subunits. Required for tethering of secretory vesicles to the sites of active membrane fusion on the plasma membrane	Accumulation of secretory vesicles at the sites of active membrane fusion	Novick *et al.,* 1980
sec1-1	Binds to assembled SNARE complexes. Required for docking and fusion of exocytic vesicles.	Secretory vesicle accumulation	Novick & Schekman, 1979
sec9-4	PM t-SNARE. Important for fusion of secretory vesicles	Secretory vesicle accumulation	Brenwald *et al.,* 1994
Δ*mso1*	Possible component required for vesicle docking. Interacts with Sec1p and SNAREs. Required for prospore membrane formation	Secretory vesicle accumulation in the small bud. Required for sporulation	Aalto *et al.,* 1997
*tpm1*Δ	Binds and stabilizes actin cables and filaments	Loss of actin cables	Liu *et al.,* 1992

Figure 15. Hsp150 and Mating factor α constructs used in this study.



RESULTS AND DISCUSSION

1. Role of the family members of the COPII coat component Sec24p in ER exit of Hsp150 (I)

The *HSP150* gene and its products, were first identified and characterized in our laboratory in 1992 (647). Hsp150 is a secretory glycoprotein expressed in several yeast strains, like *S. cerevisiae*, *P. pastoris* (647-653). It consists of a cleavable signal peptide of 18 amino acids, subunit I and subunit II (**Figure 15**). The two subunits are separated by a Kex2p recognition site, which is presumably cleaved in the late-Golgi, yielding two subunits, (SUI of 54 amino acids and SUII of 341 amino acids) that remain noncovalently attached to each other. Subunit II contains a repetitive region of 11 tandem repeats of mostly 19 amino acid peptides, and a unique C-terminal region containing four cysteine residues, which form at least one disulfide bond (647-649, 651). Hsp150 lacks any N-glycosylation sites but is extensively O-glycosylated (647). The *HSP150* gene is constitutively expressed, however the promoter of the *HSP150* gene includes heat-inducible elements (heat shock elements, HSE) and accordingly, the expression of *HSP150* is upregulated by shift of the cells from physiological temperature 25^{o}C to heat shock temperature 37^{o}C (647, 648). Hsp150 was found to be secreted to the culture medium with a half-time of about 2 minutes in wild type *S. cerevisiae* (647, 649). We have previously found that normal function of Sec13p was not required for ER exit of the Hsp150 (654). Sec13p is a functional component of the COPII coat and is thought to be required for vesicle formation at the ER membrane and therefore essential for protein transport from the ER to the Golgi. Sec13p function is linked to Sec31p where together they can self-assemble into a cytosolic cage and possibly function as an scaffold agent recruiting the Sec23p/24p-cargo to the emerging vesicle. We also found that the C-terminal domain of Hsp150 harbors an active mediator of Sec13p-independent secretion, and when fused to invertase was capable of recruiting invertase out of the ER (654). Since Sec24p functions as the cargo selection subunit of the COPII coat, we were interested in exploring the role of Sec24p and its two homologues Sfb2p and Sfb3p, in the exit of proteins from the ER.

1.1 Hsp150 is secreted in the absence of functional Sec24p family proteins

Sec24p is an integral component of the COPII coat, which has thought to be essential in the formation of functional COPII-coated vesicles that emerge at the ER membrane, and transport cargo to the next compartment of the secretory pathway, the Golgi. Incubation of the temperature sensitive strain *S. cerevisiae sec24-1* at 37^{o}C turns this component of the COPII dysfunctional, and ER exit of CPY and invertase is blocked under these conditions (655). To verify the block in ER exit, CPY pulse chase experiments with [^{35}S]-methionine/cystein were carried out under the permissive temperature (24^{o}C) and restrictive conditions (37^{o}C), whereafter cell lysates were analyzed by immunoprecipitation with CPY antiserum (655). At 24^{o}C the vast majority of CPY was detected in the mature form as soon as 10 minutes after chase. At 37^{o}C, CPY remained in the ER-specific p1 form even after chase periods up to 120 min. In order to follow invertase, the *sec24-1* cells were shifted to low glucose medium

to derepress the synthesis of invertase, followed by incubation either at 24^{0}C or 37^{0}C (655). Under permissive conditions, invertase was secreted to the periplasmic space, because it was detected by non-denaturating gel electrophoresis in the periplasmic space in the mature form, i.e. with similar mobility as control cells. In contrast, at the restrictive temperature, invertase remained intracellular and in the ER-specific form co-migrating with invertase blocked in the ER at 37^{0}C in *sec18-1* cells. When the experiment was repeated at 37^{0}C in *sec24-1* cells Hsp150 exited the ER and was efficiently secreted (655). After immunoprecipitation of cell lysates and culture medium samples using Hsp150 antiserum, the majority of the signal was found to reside in the culture medium and due to the mature form of Hsp150 (655). Therefore, while CPY and invertase accumulated in the ER under conditions where the Sec24p function was impaired, Hsp150 was selectively exported to the medium.

1.1.1 Hsp150 is secreted in sec24-1 Δsfb2 and sec24-1 Δsfb3 mutants

In this previous study, the roles of Sfb2p and Sfb3p, the two homologues of Sec24p in ER exit of Hsp150, was not addressed. Cells lacking either of these genes or both of them, are viable and have no observable phenotype. However there is a possibility that in the absence of Sec24p, Sfb2p or/and Sfb3p may contribute to the formation of the COPII coat, and therefore be functionally redundant. Indeed *in vitro*, both of these members are capable of forming COPII vesicles when Sec24p was missing, but the size of the vesicles and the nature of the cargo selectively incorporated into them appeared to differ from Sec24p COPII-coated vesicle (151, 152). To verify if Sfb3p was responsible for the exit of Hsp150 in the absence of functional Sec24p, the double mutants *sec24-1 Δsfb2p* and *sec24-1 Δsfb3p* were constructed, and secretion of Hsp150 was followed by pulse-chase experiments. Immunoprecipitation experiments with Hsp150 antiserum revealed that Hsp150 was efficiently secreted in the double mutants, and with kinetics similar to the *sec24-1* strain (**I**, Figure 2A and Fig 7 of (655)). During the chase period, the apparent molecular weight of the ER form of Hsp150 increased from 86 to 105 kDa. This was due to O-glycan extension, carried out in the ER by Golgi mannosyl-transferases that normally recycle between the Golgi and the ER, but became trapped in the ER in COPII mutants (656). Thus, it appears that Sfb2p and Sfb3p are dispensable for ER exit of Hsp150 in the absence of functional Sec24p.

To verify that ER exit of Hsp150 was the result of active and specific recruitment rather than to bulk flow, the fate of two other reporter proteins known to be dependent of Sec24p was followed. Pulse-chase analysis of cell lysates using CPY antiserum showed that after approximately two hours, CPY still persisted in the ER p1 form (67 kDa) in the double mutants *sec24-1 Δsfb3* and *sec24-1 Δsfb3,* and in the parental *sec24-1* cells, and failed to evolve to the p2 Golgi form (69 kDa) or mature form (62 kDa) (**I**, Figure 3B). The overall intensity of the CPY ER-specific form decreased with chase time, and this coincided with the appearance of an unreported 59 kDa form, which was not detected in *sec18-1* cells which accumulate CPY in COPII vesicles due to the incapacity of fusing to the Golgi membrane. We suspect that the faster migrating molecules represent a partially degraded form of CPY.

Next we followed the maturation of Gas1p under the same conditions described previously. Gas1p is a glycolipid-anchored plasma membrane protein whose exit from the ER is slowed down in cells lacking Sfb3p (153, 657, 658). Immunoprecipitation experiments using Gas1p antiserum revealed that Gas1p was translocated into the ER (86 kDa) and acquired glycans in both the parental *sec24-1* strain as in the double mutant *sec24-1* Δ*sfb3* but failed to achieve the mature form (92 kDa) detected under permissive temperature (**I**, Figure 3A). To further study whether Hsp150 was the only protein secreted to the culture medium in *sec24-1, sec24-1* Δ*sfb2* and *sec24-1* Δ*sfb3* under restrictive conditions, TCA precipitates of culture medium samples of ^{35}S-labelled cells were analyzed by SDS-PAGE. One protein, of 150 kDa corresponding to the mature form of Hsp150, was detected in the culture medium (unpublished data). This suggests that no other proteins, detected under our experimental conditions, were secreted in these cells. We therefore concluded that neither Sfb2p nor Sfb3p compensated for the nonfunctional Sec24p in ER exit of Hsp150. Under these conditions, functional transport vesicles were still generated at the ER membrane and delivered, at least Hsp150 cargo, to the next compartment of the secretory pathway.

1.1.2 Hsp150 is secreted under conditions where all Sec24p family proteins are absent

Since deletion of *SFB2* or *SFB3* had no severe effect on the secretion of Hsp150 in the *sec24-1* background, and to ensure that the possible third member of the family was not compensating in the double mutants, we set out to construct the triple mutant *sec24-1* Δ*sfb2* Δ*sfb3*. The strain was viable but had a very long generation time (6,5 h) when compared to the parental *sec24-1* (3 h) and to the double mutants *sec24-1* Δ*sfb2 and sec24-1* Δ*sfb3* (4 h). Immunoprecipitation of Hsp150 from lysates and culture medium samples of the triple mutant revealed that Hsp150 was capable of exiting the ER even in the absence of all three family members, albeit slowly. Under these conditions proteins corresponding to the mature form of Hsp150 could be observed in the culture medium as early as after 15 min of chase. After 2 hours, approximately 45% of Hsp150 had been exported from the ER (**I**, Figure 5). Therefore it appears that Sfb3p and Sfb2p are not required for ER exit of Hsp150 in the *sec24-1* background. In the above experiments we used *sec24-1*, where the last 35 C-terminal amino acids of Sec24p(ATLRLWASST LVEDKILNNESYREFLQIMKARISK) are replaced by an unrelated 8 amino acid peptide (VNAKTMGF). Therefore, it could be possible that the mutated Sec24 protein still allowed the formation of semi-functional COPII-coated vesicles that could eventually allow the ER exit of Hsp150. To address this issue we decided to construct a strain that lacked the *SEC24* gene. To overcome the fact that deletion of *SEC24* is lethal, a mutant strain lacking the original *SEC24* gene but carrying *SEC24* under a controllable tetracycline-regulated dual system promoter was used. In the absence of tetracycline, the *tetO* promoter expresses *SEC24*, however when cells are grown in the presence of tetracycline (or its derivative doxycycline), tetracycline-activable repressor elements bind to the promoter and expression of *SEC24* is turned off (659). To follow the expression of *SEC24* and find out the optimal time period where no Sec24p is

present, but protein synthesis goes on, an epitope-tagged version of Sec24p was used. Results collected from the assays of depletion of Sec24p-HA *versus* protein synthesis revealed that after 24 hours of addition of doxycycline, Sec24p-HA had been completely depleted as tested by Western blot assay using anti-HA antibody (**I**, Figure 6). Under these conditions, Hsp150 was still efficiently synthesized as could be verified by immunoprecipitation ^{35}S- Hsp150 (**I**, Figure 7).

To analyze the influence of complete absence of Sec24p on ER exit of Hsp150, the Δsec24 cells were incubated for 24 hours in the presence or absence of doxycycline to turn off expression of *SEC24*-HA. At this stage cells were shifted to 37^{o}C and labeled with [^{35}S]-methionine-cystein. The pulse chase experiment revealed that secretion of Hsp150 to the medium in the absence of the antibiotic was very efficient (**I**, Figure 7A). In the presence of the antibiotic, Hsp150 could be secreted to the culture medium (**I**, Figure 7C). After 15 min of chase a small amount of Hsp150 was detected in the culture medium, and after 1 hour, about 40% was detected. The secretion kinetics of Hsp150 in the absence of Sec24p were slightly slower than in *sec24-1* cells. But the experimental conditions were also different, so no direct relationship between these two independent experiments could be done. To verify that secretion of Hsp150 was specific, CPY and invertase were studied. Under the same conditions as above, in the presence of doxycycline, CPY remained in the ER after 2 hours chase (**I**, Figure 8A). Similarly, after incubation of cells in the presence of doxycycline for 24 hours and shift to low glucose conditions, invertase remained quantitatively in the ER (**I**, Figure 8B). Thus it appears that Hsp150 was secreted to the culture medium in cells lacking Sec24p, while two other exocytic proteins, invertase and CPY, remained trapped in the ER.

After establishing the conditions in which Sec24p was absent, we set out to investigate the role of its two homologues Sfb2p and Sfb3p. For this purpose we constructed strains lacking either or both of the homologues in the Δ*sec24* background. Secretion analysis was carried out as previously and the same controls were used. Secretion of Hsp150 in Δ*sec24* Δ*sfb2p* and Δ*sec24* Δ*sfb3p* cells was similar to that in the parental Δ*sec24* strain (data not shown). In Δ*sec24* Δ*sfb2p* Δ*sfb3p* cells, a small fraction of Hsp150 was secreted to the culture medium, as early as 15 min, and this fraction increased up to 30% after 2 hours of chase (**I**, Figure 7E). Taking into count that under the same experimental conditions CPY and invertase remained trapped in the ER (**I**, Figure 8) it appears that all Sec24 family proteins are dispensable for ER exit of Hsp150. The decrease in Hsp150 secretion kinetics in the Δ*sec24* and Δ*sec24p* Δ*sfb2p* Δ*sfb3p* mutants may be the result of a diminished Golgi complex as a result of the continuous Golgi-to-ER traffic mediated by COPI retrograde traffic, which gradually depletes the Golgi membranes and hence the secretion capacity of Hsp150.

1.2 Deletion of all SEC24 family genes results in morphological changes within the cell

Due to the proposed role of the Sec24p family members in ER exit of secretory cargo we set out to study the subcellular morphology of the mutants. Deletion of *SFB2* or *SFB3* individually in the *sec24-1* background yielded viable cells with a slightly prolonged generation time.

Deletion of both *SFB2* and *SFB3* from *sec24-1* cells also gave viable cells, but with a very long generation time. First we analyzed the overall shape of the mutants by scanning electron microscopy (SEM) at the permissive temperature 24°C. Structural abnormalities were observed in *sec24-1* Δ*sfb3* cells, as compared to parental strain *sec24-1* (**I**, Figure 1A). The double mutant was irregularly shaped with small depressions in the cell surface. Bud scars appeared to be localized on opposite sides, whereas in the parental strain they were adjacent to each other. Calcofluor staining and confocal image analysis revealed that in the double mutant, bud scars were randomly distributed around the cell surface (**I**, Figure 1B). Furthermore, the triple mutant *sec24-1* Δ*sfb2p* Δ*sfb3p* revealed an even more severe phenotype (**I**, Figure 4A and B). These cells displayed an irregular shape; the cell wall appeared collapsed and had even more severe depressions. A significant number of cells had undetached daughter cells. These cells also displayed a random budding pattern (**I**, Figure 4B). Interestingly, deletion of *SFB3*, *SFB2,* or even both, in a cell carrying a normal copy of *SEC24* yielded no defects in the budding pattern. Therefore, it appears that the combination of *sec24-1* with either Δ*sfb3p* or Δ*sfb2p* Δ*sfb3p,* but neither deletion alone, resulted in a random budding pattern observed.

Next, the cells were incubated for 1 hour at the permissive (24°C) or restrictive (37°C) temperature prior to fixing and processing for transmission electron microscopy (TEM) (**II**). The parental *sec24-1* strain at both 24°C and at 37°C displayed no severe ultrastructural abnormalities (**I**, Figure 1C-1 and C-2). At both temperatures, structures corresponding to what appears to be a cluster of vesicles surrounded by a coat were detected. In *sec24-1* Δ*sfb3* mutants incubated at the permissive temperature displayed some proliferated ER (**I**, Figure 1C-3). Incubation of these cells at 37°C further increased this phenotype, and ring-like structures that appear to be composed of 1 to 3 layers of membranes juxtaposed to each other were also detected (**I**, Figure 1C-4). None of these structures were observed in the parental *sec24-1* cells. Analysis of the triple mutant *sec24-1* Δ*sfb2* Δ*sfb3* revealed severe morphological defects that supported the observations collected from the SEM analysis (**I**, Figure 4C). At the permissive temperature segments of cell wall projecting into the cytosol, indicative of abortive daughter cell budding were detected. This was further supported by the finding of multiple or fragmented nuclei within the same cell. Alternatively, the abnormal cell shape may cause unusual shapes of the nuclei, which in thin sections appear as a fragmented nucleus. In addition, the cell wall depressions detected previously by SEM were detected as donut shape cells (data not shown).

The triple mutant *sec24-1* Δ*sfb2* Δ*sfb3*, had already at 24°C ER proliferations, similar to the double mutant *sec24-1* Δ*sfb3* and this phenotype was further exacerbated after shift of the cells to the restrictive temperature (**I**, Figure 4C). Extensive ER accumulation was observed throughout the cell and multiple ER membranes (4 to 8) were detected closely to each other, positioned in a stack-like manner (**I**, Figure 4C-3). They appeared to extend throughout the cell and make connections with cortical ER. This suggests that the Sec24p family members are involved in ER-to-Golgi traffic, and have overlapping functions in export of secretory cargo from the ER.

One additional observation that deserves to be mentioned is that the depressions observed in the SEM samples of *sec24-1 Δsfb3 and sec24-1 Δsfb2 Δsfb3* cells, in cells in early logarithmic growth phase, were also observed in control cells grown to late stationary phase and therefore were in senescence. Interestingly, aged cells also appear to have fragmented nucleolus, display a symmetrical cell division and increased generation time and cell size (660, 661). All of these aging symptoms were also observed in *sec24-1 Δsfb3 and sec24-1 Δsfb2 Δsfb3* cells at the permissive temperature in early logarithmic phase. How exactly the Sec24 proteins influence the aging of yeast cells is unknown. It is known that deletion of *SFB2* and *SFB3* reduces the cargo repertoire selected into the COPII-coated vesicles. Hence proteins such as the plasma membrane ATPase (Pma1p), the GPI-anchored protein Gas1p, invertase, CPY and alkaline phosphatase (ALP) are blocked or display show delay in ER exit (151, 153-155). Therefore one possibility for the early aging phenotype may be that the Sec24 proteins and perhaps other proteins not yet identified, may play an important role in the normal yeast life cycle by affecting either the cell wall stability or intracellular regulatory mechanisms.

1.3 Hsp150 secretion and formation of the carrier in the absence of Sec24p family members

In summary we have shown that the soluble secretory glycoprotein Hsp150, is secreted under conditions in which other secretory cargo proteins remain trapped in the ER. This suggests that sorting of Hsp150 is selective rather due to bulk flow. Since Hsp150 is a soluble protein, and the coat components are cytosolic proteins, it has to interact with a putative transmembrane receptor, for ER exit to occur. The sorting signal responsible for the selective ER exit of Hsp150 in the absence of Sec24p family members was mapped to the C-terminal domain of Hsp150 (**Figure 15**, amino acids 299-413). The same region of Hsp150 has also been shown to mediate Sec13p-independent ER exit (654). This transmembrane adaptor protein may possibly have on its cytosolic side, ER exit motifs, which ensure its active packaging into COPII-coated vesicles, similar to what is found for Emp24p. Emp24 together with Erv25p forms a heteromeric transmembrane protein complex, and mediates the active incorporation of Gas1p into COPII vesicles (662, 663). Emp24p/Erv25p heteromeric complex cycles between the ER and the Golgi, where it is capable of binding to both coat proteins and cargo molecules, thus mediating their active incorporation into the COP vesicles. Emp24p and Erv25p have a di-aromatic motif on their cytoplasmic sequences that bind to COPII coat subunits and promote their export from the ER. The Erv25p tail sequence, which binds to COPI coat subunits, is responsible for returning this complex to the ER (662). Since Hsp150 contains one signature for ER exit in COPII mutants and another for ER exit in COPI mutants, perhaps more than one cargo receptor exist for Hsp150. In ongoing work in our laboratory, candidates for an Hsp150 receptor are being searched for.

In vitro, the minimal components required to drive the formation of the COPII coated vesicle are Sar1p, Sec23p/24p complex and Sec13p/Sec31p complex (127, 161). Sec24p is thought to act as the cargo sorting subunit of the COPII coat (145). However, Sec24p is not absolutely indispensable, since vesicles can still be

generated in the mutant *sec24*Δ strain. Under these conditions the homologues Sfb2p and Sfb3p compensate. But these vesicles only contain a subset of the cargo proteins packaged into Sec24p containing vesicles (151-155). Furthermore, Sfb3p generated vesicles are not capable of packaging SNARE proteins and can therefore not fuse with the Golgi membrane. In normal cells, these homologues may co-exist with Sec24p in the formation COPII-coated vesicles, and thus extend the repertoire of the cargo sorting machinery. So how can an ER-derived carrier vesicle be formed in the absence of all Sec24p family members? Perhaps *in vivo*, unusual transport vesicles are formed in such conditions, which are still capable of recruiting Hsp150, but do not recruit other secretory cargo. We would however like to suggest some possible mechanisms for how ER export may possibly be mediated in the absence of Sec24p family proteins. In the first model, an incomplete COPII coat might be formed, where the Sec23p binding partner, Sec24p would be missing. This coat could possibly be temporarily stabilized by Sec16p, which is capable of interacting with Sec23p and with Sec31p, for sufficient time to allow budding of the vesicle (138, 139, 142). Alternatively, since Sec23p and Sec24p are structurally related to each other (133, 141, 144, 162), it may be possible that the COPII coat formed in the absence of Sec24p, includes two copies of Sec23p. This could possibly provide some structural stabilization to the deforming membrane. However, these two models have two major problems. How exactly would the incomplete COPII coat recruit secretory cargo and SNAREs in the absence of the cargo sorting Sec24p family members. A novel uncharacterized component X may possibly interact with Sec23p and form a complex, thus supporting COPII formation, by providing coat stabilization and recruiting cargo, such as Hsp150 and SNAREs. Alternatively, if the putative transmembrane receptor is long enough, it may recruit Hsp150 and allow local deformation of the ER membrane, by enrichment of this receptor in a specific sub-region of the ER membrane. The remaining COPII coats may then provide some additional stabilization. Alternatively Hsp150 may use an ER exit route that is completely independent of COPII, where a novel set of proteins would recruit Hsp150 and the necessary SNAREs into the budding membrane. However, successful ER exit of Hsp150 required functional Sec12p, Sec23p and Sec31p, thus making the last model unlikely.

Future work will be required to address all this unanswered issues. For instance, the identification of the putative transmembrane receptor for Hsp150, may allow the subsequent identification of interacting proteins, which may give some insight into the ER exit mechanism. Additionally through a detailed TEM analysis, it may be possible to elucidate if indeed COPII vesicles are formed in cells lacking all Sec24 family members, and if Hsp150 incorporated into them.

2. The yeast secretory glycoprotein Hsp150 is selectively secreted in a subset of post-Golgi secretory mutants (II)

Our previous results suggested that Hsp150 is selectively secreted to the culture medium in cells with a deficient COPII subunit Sec13p (654), and in the absence of all Sec24p family members (655, 664), or in cells with a deficient COPI component Sec21p (665, 666).

Therefore we wanted to explore if Hsp150 was capable of bypassing additional *post*-Golgi secretory mutants. To investigate this possibility a subset of different post-Golgi temperature sensitive mutants (see **Table 2** for details) were shifted to the restrictive temperature and metabolically labeled. After 60 minutes of incubation, TCA precipitation of culture medium samples was performed (**II**, Figure 2A and B). Under these conditions no proteins were detected in the cases of mutants such as *sec4-2* (RabGTPase) or *sec2-41* (Sec4p GEF) that work prior to vesicle docking, and neither in mutants that affect SNARE-mediated membrane fusion such as *sec1-1* (SM family member) or *sec9-4* (t-SNARE). Interestingly, we found that one specific and significant band with similar electrophoretic mobility as mature Hsp150 was detected in the culture medium of *sec15-1* exocyst subunit mutant cells (lane 8). Meanwhile, in the other exocyst mutants *sec5-24*, *sec6-4*, *sec8-6* and *sec10-2* secretion of all proteins was severely or completely blocked. It thus appeared that Hsp150 bypassed the *sec15-1* mutation. To further validate this, a pulse-chase experiment was performed. ^{35}S-labelled cells were chased in the presence of cycloheximide for 60 minutes at the restrictive temperature, and cell lysates and culture medium samples were analyzed by immunoprecipitation with Hsp150 antiserum. In wild type cells approximately 80% of Hsp150 was found in the culture medium, meanwhile, in *sec18-1* cells Hsp150 was detected solely in the intracellular fraction and in the ER form. Analysis of the remaining *post*-Golgi temperature-sensitive secretory mutants revealed that Hsp150 appeared to be significantly secreted (>50%) only in *sec15-1* mutant cells. In all the mutants screened, secretion of proteins appeared to be blocked with exception of the *sec3-1* mutant, where a small amount of Hsp150 (approximately 20%) was secreted. Taking into count that deletion of *SEC3* yielded viable cells (584) it appears that under certain conditions, additional spatial landmarks such as Exo70p may compensate absence of Sec3p in the exocyst complex (583, 619). In view of the fact that Hsp150 appears to be secreted in *sec15-1* cells while other proteins fail to be secreted, we decided to further investigate the role of the exocyst in secretion of Hsp150.

2.1 Hsp150 is secreted in the absence of functional Sec15p

Sec15p is the exocyst subunit that interacts with the approaching secretory vesicle, marked by the RabGTPase Sec4p, and bridges the vesicle to the plasma membrane through the action of the tethering exocyst complex (569, 571, 573). Besides the interaction of Sec15p with the secretory vesicle and with the remaining exocyst complex subunit, Sec10p, Sec15p is also capable of interacting physically with Bem1p (595). The polarity establishment protein Bem1p is considered to be required for proper localization of several proteins involved in polarity including Cdc42p and its exchange factor Cdc24p (596, 667). The interaction of Sec15p with Bem1p may provide an additional link that helps coordinate the polarity machinery and the secretory pathway components (595). The interaction of Sec15p with Bem1p is thought to occur though the C-terminus of Sec15p, and this interaction is required for proper localization of Sec15p, even under conditions where actin is disrupted (595). Interestingly, in the case of the temperature-sensitive mutant form of Sec15p in the *sec15-1* mutant, the last 76

C-terminal amino acids are missing due to premature termination. This shorter version of Sec15p fails to interact with Bem1p (595). This truncation did not affect the binding of Sec15p with Sec10p. This interaction has been shown to be due to the first 82 amino acids localized in the N-terminus of Sec15p (595).

To address the functional importance of the C-terminal region of Sec15p, wild type Sec15p and the truncated form were tagged with GFP and compared with an other exocyst component Sec8p-GFP (595). Here they showed that wild type cells show a bright and proper localization of both Sec15-GFP and Sec8-GFP to sites of active membrane expansion at both permissive and restrictive temperature. In contrast, truncated *sec15*-GFP and Sec8-GFP localization was faint and appeared to be cytoplasmic even at permissive temperature. Shift to the restrictive temperature lead to the appearance of disorganized punctate-staining over both mother and daughter cells. From these studies it was concluded that the C-terminal region of Sec15p, which is lacking in *sec15-1* cells, is important for proper localization of both Sec15p and the remaining exocyst components. In support to these observations, the exocyst complex was found to be disrupted in lysates of *sec15-1* cells (580, 581, 595, 668). Furthermore, the level of exocyst assembly is reduced significantly in the *sec15-1* strain even at permissive temperature. When *sec15-1* cells were shifted for 30 minutes to the restrictive temperature, the isolated complex was dramatically reduced to almost non-detectable levels (581). In addition to the instability of the exocyst complex in the *sec15-1* strain, Sec4p appeared to be unable to associate to Sec15 and to the remaining exocyst complex (668). Taking together, the data collected on the *sec15-1* mutant, it appears that at the restrictive temperature the *sec15-1* mutation severely affects the overall stability and integrity of the exocyst complex, and consequently fusion of secretory vesicle to the plasma membrane. Therefore, the *sec15-1* mutation seems to be a viable platform to carry out secretory studies to understand the possible mechanism by which Hsp150 appears to be selectively secreted.

2.1.1 Hsp150 is secreted in sec15-1 cells

To verify if indeed *sec15-1* is a tight temperature-sensitive secretory mutant, growth at permissive and restrictive temperature was studied and compared with that of known secretory mutants (**II**, Figure 2 E and Supplementary Figure S1). Cells were grown overnight to early logarithmic phase in either rich medium (YEPD) or synthetic complete medium (SC). Samples from each strain were taken and standardized to Od_{600}=0,2 and incubated either at permissive or at restrictive temperature. Samples were collected hourly and cell density measured. Growth of the *sec15-1* strain at the permissive temperature (24^{o}C) was indistinguishable from control cells or from the remaining temperature-sensitive mutant strains addressed (**II**, Supplementary Figure S1). However at the restrictive temperature (37^{o}C) these cells failed to grow. Similar results were observed for *sec18-1* and *sec6-4* mutant cells. In contrast, control cells incubated at the restrictive temperature divided steadily Next aliquots of 10^4 cells and 4 tenfold dilutions were carried out for each strain and spotted onto YEPD or SC plates. The samples were then incubated at 24^{o}C or 37^{o}C for 2 to 3 days. All temperature-sensitive *post*-Golgi secretory mutants analyzed failed to grow at the restrictive

temperature reflecting the importance of the respective proteins in the late stages of the secretory pathway. In conclusion *sec15-1* is a non-leaking temperature-sensitive mutant and the resulting Sec15p is nonfunctional at 37°C.

To analyze the secretion kinetics of Hsp150 in the *sec15-1* mutant, cells were preincubated at the restrictive temperature and pulse-chase experiments were performed. ^{35}S-labelled cells were chased in the presence of cycloheximide for up to 60 minutes. We have previously reported that a small fraction of secreted Hsp150 remains cell wall-associated in normal cells in a non-covalent fashion, and can thus be released by SDS treatment (654, 669). Covalently bound cell wall proteins can be released by mild-alkaline treatment. Using this technology we investigated if the cell-associated pool of Hsp150 was extracellular or intracellular. These cell wall samples together with the intracellular and culture medium samples were immunoprecipitated with Hsp150 antiserum and subjected to SDS-PAGE analysis. After a preincubation period of 15 minutes at restrictive temperature, the secretion kinetics of Hsp150 was very rapid and some Hsp150 could be detected in the culture medium already after a 5 minute chase (**II**, Figure 3Ba). After 60 minutes chase, 60% of Hsp150 was found in the medium, 16% was associated to the cell wall and only 22% remained intracellular. For comparison, in control cells, after 60 minutes of chase 79% of Hsp150 was in the medium, 10% remained associated to the cell wall and a very low amount was detected intracellular (**II**, Figure 3Aa). In contrast, when using another exocyst subunit mutant, *sec6-4*, Hsp150 was found solely as an intracellular form and no Hsp150 was secreted to the cell exterior (**II**, Figure 3C). Similar results were obtained for the exocyst mutants *sec5-24*, *sec8-6* and *sec10-2* (data not shown). It has been reported that incubation of *sec15-1* cells, for 30 minutes at the restrictive temperature damages the integrity of the exocyst complex and its ability to interact with the RabGTPase Sec4p (581, 668). To ensure that the preincubation period was sufficient to render *sec15-1* defective, we extended it to 30 and 60 minutes. When cells were preincubated for 30 minutes, 48% of the labeled Hsp150 was found in the culture medium, 22% was cell associated and 30% remained intracellular (**II**, Figure 3Bb). After 60 minutes of preincubation at 37°C lead to the following distribution of Hsp150, 28% in the culture medium, 37% in the cell wall and 35% intracellular (**II**, Figure 3Bc).

Increase of the preincubation period appeared to increase the relative amount of covalently cell wall-attached Hsp150, relative to secreted and noncovalently attached. The faster migrating Hsp150 bands detected in the lanes subjected to alkaline treatment (**II**, Figure 3 lanes 3, 7, 11, 15 and 19) are the result of release of O-glycans by β-elimination (669). Also in control cells increase of the preincubation period lead to a greater fraction of Hsp150 (approximately 29%) being covalently attached to the cell wall (**II**, Figure 3AB). In summary, in control cells 90% and 85% of Hsp150 was secreted to the cell exterior when cells where either preincubated at 37°C for 15 or 60 minutes, respectively. Meanwhile *sec15-1* cells externalized 78%, 70% and 65%, when the preincubation periods lasted 15, 30 and 60 minutes, respectively. In other exocyst mutants such as *sec6-4*, *sec5-24*, *sec8-6* and *sec10-2*, Hsp150 remained quantitatively intracellular.

TCA precipitation of culture medium samples of *sec15-1* cells revealed that Hsp150 was the only labeled protein detected. To verify if indeed the ability to be secreted in *sec15-1* was unique to Hsp150, secretion of other reporter proteins was assed in the same set of secretory mutants. Invertase, a periplasmic secreted glycoprotein, is often used as a reporter to measure secretory defects in *S. cerevisiae*. To derepress the synthesis of invertase, cells were shifted to pre-warmed low glucose growth medium and incubated at the restrictive temperature for up to 120 minutes, followed by quantification of intra- and extracellular activity (**II**, Fig 5). In control cells over 80% of invertase activity was external, meanwhile in *sec18-1* and *sec6-4* cells the activity remained intracellular. In *sec15-1* cells an initial secretion of invertase (<20%) to the extracellular space was detected in the first 30 minutes, followed by no increase during the following 90 minutes. Incubation of these strains at permissive temperature yielded a similar distribution as the control cells. To further analyze invertase traffic in these strains, samples were collected at 90 minutes and intracellular and periplasmic samples were subjected to native gel electrophoresis, followed by invertase activity staining (**II**, Fig 5). Under these conditions we found invertase in the fully mature form in control, *sec6-4* and *sec15-1* cells, meanwhile in *sec18-1* cells, invertase remained trapped in the ER and failed to extend its N-glycans. In control cells the vast majority of invertase was in the periplasmic space. In contrast, in *sec6-4* and *sec18-1* cells the entire detected amount of invertase was trapped intracellular. In *sec15-1* cells a small amount of Hsp150 was also found in the periplasmic space, thus verifying the results obtained for invertase above.

Next we addressed the secretion of Bgl2p, Gas1p and Pma1p in the same secretory mutants. Secretion analysis revealed that these proteins completely failed to be secreted in *sec18-1*, *sec6-4* and *sec8-6* cells, as well as in the *sec15-1* mutant (data not shown).

Thus it appears that secretion in *sec15-1* cells at the restrictive temperature is a unique feature of Hsp150. However, a small fraction of invertase was also secreted in these cells. One possibility for the differences of secretion ratios observed between these proteins may reside in the different kinetics in translation/processing/transport through the secretory pathway. To address this issue we carried out a comparative secretion kinetics assay on invertase and Hsp150 in control cells, in a subset of exocyst mutants and in the *post*-Golgi secretory mutant *sec14-1* (**II**, supplementary figure S2). At permissive temperature, invertase secretion to the periplasmic space was similar in all the mutants observed where extracellularly invertase activity could already be detected, as early as 5 minutes after shift to low glucose conditions. At the restrictive temperature secretion of invertase was completely blocked in *sec14-1*, *sec5-24*, *sec6-4* and *sec8-6* cells. However in *sec3-1*, *sec10-1* and *sec15-1* cells a small amount of activity (typically below 20%) was detected extracellular. This partial secretion occured in the first 30 minutes of incubation and failed to increase after that. For comparison, wild type cells incubated at the restrictive temperature efficiently secreted invertase and in average more than 80% was found in the exterior of the cell. In parallel with the above experiments a pulse-chase experiment was performed to address the secretion kinetics of Hsp150. Similar to invertase, secretion of Hsp150 to the culture medium was completely blocked

in *sec5-24*, *sec6-4* and *sec8-6* cells. In addition, in *sec3-1* and *sec10-1* mutants the same low secretion ratio and kinetics for Hsp150 and invertase was observed, where approximately 20% of the labeled Hsp150 was secreted to the culture medium in the first 30 minutes. However in the case of *sec14-1* and *sec15-1* mutants, an increased ratio of Hsp150 secretion was observed in comparison to the amount of invertase secreted. In the case of Hsp150, a total of approximately 50-60% of Hsp150 was secreted. TCA precipitation of culture medium samples revealed only one protein that migrated in SDS-PAGE like mature Hsp150. Thus it appears that Hsp150 is specifically and more efficiently secreted in *sec15-1* and *sec14-1* mutants than invertase. The secretion kinetics of invertase was also addressed by Harsay *et al.*, (1995). They concluded that invertase was rapidly secreted after 5 minutes of derepression (258). We have also shown that Hsp150 can be found in the culture medium as soon as 5 minutes after pulse (649). Therefore it appears that both these proteins are secreted with the same kinetics in control and in a subset of exocytic mutants other than *sec15-1*. The differences between invertase and Hsp150 in *sec15-1* cells possibly reflect a selective secretion pathway for Hsp150, instead of a difference in the kinetics of progression of Hsp150 and invertase through the secretory pathway.

2.1.2 The sorting signal for bypass of the sec15-1 mutation resides in the N-terminal/RR region of Hsp150

As previously referenced, Hsp150 is selectively secreted to the culture medium in cells that have a deficient COPII subunit Sec13p (654), and in the absence of all Sec24 family members (655, 664), and even in cells with a deficient COPI component Sec21p (665, 666). We have previously found that ER exit of Hsp150 in COPII-deficient mutant cells is dependent on a molecular signature that resides in the unique C-terminal domain of Hsp150 (**Figure 15**) (654, 655), and in the case of COPI-independent ER exit, to the repetitive region of SUII (666). In search of the molecular signature guiding secretion of Hsp150 in *sec15-1* cells, variants of Hsp150 lacking either the C-terminal domain (Hsp150Δ) or having a shorter version of the repeat domain of SUII (Hsp1504RΔ) were to β-lactamase and expressed in *sec15-1* cells (**Figure 15**). The β-lactamase portion originating from *E. coli* folds in the yeast ER to a bioactive conformation and in normal conditions the fusion protein is efficiently secreted to the medium (650, 651). A pulse chase experiment of the variant lacking the unique C-terminal fragment was first carried out to see if this region was required for bypass of Sec15p. Cells were preincubated at the restrictive temperature, labeled, and chased for 60 minutes in the presence of cycloheximide. After cell fractionation, Hsp150Δ–β-lactamase was immunoprecipitated with β-lactamase antiserum. In *sec15-1* cells, after a 5 minutes chase, we could already detect Hsp150Δ-β-lactamase in the culture medium and after 1 hour of chase, 50% was found in the culture medium, 16% was non-covalently attached to the cell wall and 7% was covalently cell wall-bound, while only 30% remained intracellular (**II**, Figure 4C). In control cells similar secretion results were obtained for Hsp150Δ–β-lactamase (**II**, Figure 4A). In *sec6-4* cells Hsp150Δ–β-lactamase failed to be secreted and accumulated intracellularly. At the permissive temperature the fusion protein is efficiently secreted to the culture medium.

To further elucidate the sorting determinant we analyzed the secretion of Hsp1504RΔ–β-lactamase (**Figure 15**). Pulse-chase experiments revealed that analogous to Hsp150Δ–β-lactamase (**II**, Figure 4), this shorter version of the fusion protein was efficiently secreted in wild type cells and in *sec15-1* cells, but failed to be secreted in *sec6-4* cells incubated at restrictive temperature (data not shown).

From these results it is apparent that the molecular signature that mediates Hsp150 secretion in *sec15-1* mutant cells does not reside in the unique C-terminal fragment and neither in the full repetitive region of Hsp150, but rather appears to reside in either subunit I or in the first 4 repeats of SUII (**Figure 15**). Alternatively, since Hsp150 is heavily O-glycosylated in both SUI and SUII (651, 666), the molecular signature may reside in the oligosaccharides (670). However, deletion of the protein O-mannosyltransferase gene *PMT4*, which is responsible for the transfer of mannose residues from the dolichylphosphate-D-mannose to serine or threonine residues on the target protein (90, 91, 93) yielded no secretory defects in these cells (unpublished data).

2.2 Hsp150 is selectively packaged into a subset of secretory vesicles

Secretory cargo destined to the exterior of the cells is packaged into secretory vesicles, which selectively concentrate cargo and transport them to the plasma membrane. Two pools of *post*-Golgi vesicles, which differ in respect to their cargo, have been identified by isodensity gradient centrifugation (258, 260, 474). The vesicles detected in the lighter region of the gradient were classified as LDSV (low density secretory vesicle) and contain proteins such as the endo-β-1,3-glucanase Bgl2p, the plasma membrane ATPase Pma1p and the GPI-anchored β-1,3-glucanosyltransferase Gas1p. Meanwhile, the vesicles detected in the heavier region of the gradient HDSV (heavy density secretory vesicles) contained proteins such as invertase, exo-β-1,3-glucanase Exg1p and alkaline phosphatase ALP. Both vesicle populations transport cell wall-modifying enzymes such as glucanases, which are thought to be involved in the softening of the yeast cell wall in order to allow its expansion and insertion of new wall materials. Since Hsp150 is secreted to the culture medium in wild type and *sec15-1* cells, we set out to investigate in which population of secretory vesicles was Hsp150 transported.

2.2.1 Hsp150 is packaged into a novel class of secretory vesicles

To address into which population of secretory vesicles Hsp150 was guided, cells were grown to early logarithmic phase and shifted to restrictive temperature for 2 hours to allow accumulation of secretory vesicles. The cells were then lyzed, and after differential centrifugation steps, the secretory vesicles were isolated as an individual pellet (for a detailed description see **II**, Methods and materials). This pellet was then loaded on the bottom of an Optiprep density gradient and centrifuged for 19 hours at 100.000 x g, which allowed the vesicles to migrate to their corresponding densities. Subsequently aliquots were sequentially removed and analyzed for the presence of different proteins by either activity measurements or by SDS-PAGE analysis. Unlike previous reports (258-260), which used Nycodenz to construct the density gradients, we used Optiprep for the following reasons. Similar to Nycodenz/Iohexol, Optiprep is a nonionic derivate of metrizoic acid which is constituted by

Iodixanol, essentially a dimer of iohexol. The main differences between Nycodenz and Optiprep is that Nycodenz gradients are hyperosmotic at densities above 1,16g/mL even when inverse gradients are used to balance the osmolarity. In contrast Optiprep is capable of forming isoosmotic solutions at all densities (671, 672). Also a better performance/resolution by Optiprep *versus* Nycodenz has been observed in the separation/purification of organelles that fractionate close to this density limit (671, 672). Thus, in an attempt to preserve the structural properties and identity of the vesicles we used Optiprep in this study. We isolated vesicles from *sec15-1*, *sec6-4* and control cells at both permissive and restrictive temperatures and loaded them on the bottom of a 12-30% Optiprep gradient constructed in 0.8 M Sorbitol / TEA. Control cells and temperature-sensitive mutant cells, incubated at the permissive temperature accumulated very little secretory vesicles, as confirmed by transmission electron microscopy. Therefore, detection of proteins by SDS-PAGE analysis was difficult under these conditions. For this reason vesicle analysis was carried out in the temperature-sensitive *sec6-4* and *sec15-1* mutants which accumulate a significant number of secretory vesicles (258). The aliquots collected from the gradient were subjected to SDS-PAGE analysis and probed by western blotting for the presence of a subset of different marker proteins. To verify the purity of the isolated secretory vesicles and to address the integrity of both experimental sets we first followed the ER marker Kar2p/BIP. In both *sec6-4* and *sec15-1* cells Kar2p/BIP was found on the top of the gradient together with the syntaxin Pep12p, which is commonly used as a late endosomal marker (**II**, Figure 6 A and B). When probing for proteins such as Bgl2p, Gas1p and Pma1p, which are found in the LDSV population, we found that these proteins were detected in the same fractions (1-7) in both *sec6-4* and *sec15-1* cells (**II**, Figure 6, A and B). The accumulation of Gas1p, Pma1p and Bgl2p in the LDSV population was similar in *sec6-4* and *sec15-1* cells (**II**, Figure 6E). When the samples were assayed for invertase activity, we found that the distribution profile was different. Invertase could be detected in fractions 5-15 (HDSV, see next section) and at a very high density (fractions 24-28) (**II**, Figure 6 C and D). The total amount of invertase activity in the gradient was in the case of sec15-1 cells about 20% lower than in *sec6-4* cells (**II**, Figure 6 E).

In the case of *sec6-4* cells, invertase and Hsp150 were detected in the same fractions (**II**, Figure 6C), this is in the HDSV (C3-17) as well as in the very heavy density fractions (C23-27). In contrast, in the case of *sec15-1* cells (**II**, Figure 6D), Hsp150 was found in the HDSV population only (D3-15). Less than 50% of Hsp150 was found throughout the gradient in the *sec15-1* strain as compared to *sec6-4* cells (**II**, Figure 6 E)

Previous studies carried out on the identity of the secretory vesicles using Nycodenz, placed the LDSV at a density equal to 1.14 g/mL and the HDSV at a density of 1.16 g/mL (258, 260). In our experimental conditions using Optiprep we found LDSV at a density approximately of 1.11 g/mL and the HDSV at 1.13 g/mL. This difference in density between Optiprep and Nycodenz has previously been reported, and appears to be due to the different ability of these two media in forming isoosmotic solutions (672). In this study they found that for example that the endoplasmic reticulum, mitochondria and lysosomes sediment at significantly lower

densities in Optiprep when compared to Nycodenz. This may be the reason why vesicles detected in the very heavy density fractions (with a density of 1.198 g/mL) were not detected in the previous studies, which had a maximum density range of 1.195 g/mL. This also explains the differences observed between the densities found for LDSV and HDSV in our *versus* previous studies.

In summary, we have detected Hsp150 together with invertase in the HDSV population of secretory vesicles, and in a novel population of vesicles, which sedimented in the very heavy density fractions of the gradient. In the *sec15-1* strain, where much of Hsp150 is secreted, its amount within the secretory vesicles, accumulated during restrictive temperature, was much lower than in *sec6-4* cells, where Hsp150 was found only in the HDSV population. Invertase, however, was found in *sec6-4* and *sec15-1* mutants, in both the HDSV population and in the novel very heavy density fractions. Proteins carried in LDSV accumulate to similar levels internally in both *sec6-4* and *sec15-1* cells. Conclusion: a novel type of vesicles was identified, in which part of the Hsp150 is transported.

2.2.2 Mutations affecting the Endosomal/vacuolar pathway do not affect Hsp150 secretion

Previous reports have documented that mutants that disturb the endosomal/vacuolar pathway also disturb the formation of the HDSV population of vesicles (259, 260). Under these conditions, cargo typically found in the HDSV is rerouted to the LDSV population (260). In addition, when the pathway that targets CPY from the Golgi to the vacuole is blocked, like in *vps1* and *vps10* mutants, CPY is targeted to the plasma membrane through the LDSV population of vesicles. Thus, to further elucidate the pathway through which Hsp150 is secreted in *sec15-1* cells, we set out to investigate the role of the endosomal/vacuolar pathway in exocytosis of Hsp150.

To address this issue we crossed *vps10*Δ, *vps1*Δ and *vps8*Δ mutants with either *sec15-1* or *sec6-4* cells. Spores were dissected and scored for the appropriate genotype (see Table 2 for details). Crossing of the different *vps* mutants with either *sec15-1* or *sec6-4* yielded viable cells with no severe effects on growth (**II**, Supplementary Figure S4.1-S4.3 C). Next secretion of Hsp150 and invertase was followed in the single and double mutants by pulse chase experiments and invertase activity assays (**II**, Supplementary Figure S4.1 to S4.3). Under these conditions no secretion defects were observed for either protein in *vps10*Δ, *vps1*Δ or *vps8*Δ strains. Furthermore, in the double mutants no effects on secretion could be observed when compared to the parental strains *sec15-1* and *sec6-4*. To verify that the endosomal/vacuolar delivery pathway was indeed affected in these mutants, maturation of CPY was followed. In the case of the *vps* deletants, CPY was found intracellularly in the P2-Golgi form and failed to acquire the mature form, which occurs by protease cleavage in the vacuole. Therefore it appears that the combination of mutations that block traffic through the endosomal/vacuolar pathway with *sec15-1* had no effect on the secretion of Hsp150.

Vps mutants have been shown to abolish the formation of the HDSV population of secretory vesicles while the formation of the LDSV remains intact (260). For this reason we decided to isolate the different population of secretory vesicles that accumulate in *sec6-4 vps1*Δ, *sec15-1 vps1*Δ, *sec15-1 vps10*Δ and

*sec15-1 vps8*Δ mutants, and investigate the effect these double mutations have on the biogenesis of the different population of vesicles. Cell growth, isolation and isodensity centrifugation of the secretory vesicles was conducted as previously with the exception that the gradient covered a broader region (10-35%) in an attempt to increase the resolution. Similar to the previous fractionations carried out, the distribution of the ER marker Kar2p/BiP throughout the gradient was similar in all of the mutants, as well as the total amount of protein detected (**II**, Figure 9 and Supplementary Figure S5). In addition, fractionation of the congenic *sec6-4* and *sec15-1* cells yielded comparable results to those obtained previously, thus reflecting the reproducibility of the fractionation and density gradient analysis.

When *post*-Golgi secretory mutants are combined with endosomal mutants that block the delivery of CPY to the vacuole, CPY is no longer delivered to the vacuole and since fusion to the plasma membrane is blocked, CPY accumulates intracellularly (260) (**II**, Supplementary Figure S5 I). Combination of *sec15-1* or *sec6-4* with the absence of the dynamin-like GTPase Vps1p lead to relocalization of cargo normally transported by the HDSV into the LDSV population of secretory vesicles, as previously mentioned by Harsay *et al.*, 2002 and blocks traffic through the endosomal pathway (260, 358, 362)(see **Figure 16**). This relocalization can be detected by a shift in the peak of invertase activity, which runs in the HDSV region in the case of the *sec15-1* and *sec6-4* cells, to the LDSV region in the case of the *sec15-1 vps1*Δ and *sec6-4 vps1*Δ mutants (**II**, Figure 9B and D). In addition the majority of the unprocessed P2-Golgi form of CPY cofractionated with the LDSV population of secretory vesicles. On the other hand, invertase activity detected in the very heavy density fractions remained unaffected by the absence of the dynamin-like GTPase Vps1p. Interestingly, packaging of Hsp150 into the LDSV population did not abolish its secretion capacity in *sec15-1 vps1*Δ cells, since approximately 50% of the total amount of Hsp150 was secreted in this strain. Meanwhile, other proteins such as Pma1p, Gas1p and to a significant degree invertase, remained intracellular (**II**, Supplementary Figure S5 I). In contrast, in the single and double *sec6-4* mutant strains Hsp150 failed to be secreted and accumulated intracellularly. In conclusion, disturbing the endosomal pathway did not block the secretion of Hsp150 in *sec15-1* cells, neither did it block the formation of the novel pool of vesicles detected in the very heavy density fractions of the gradient.

Next, we analyzed the secretory vesicles that could be found in the *sec15-1 vps10*Δ mutant strain. Since Vps10p is the sorting receptor for carboxypeptidase Y, we expect that its absence would not affect the biogenesis of the HDSV population, while CPY would still be missorted into the secretory vesicles (**Figure 16**). Indeed we found that formation of HDSV prevailed and CPY accumulated within the HDSV population (**II**, Figure 9E). The unprocessed p2-Golgi form of CPY was found together with invertase and Hsp150 in HDSV. In addition a significant amount of p2 CPY was found in the very heavy density region of the gradient together with invertase. Hsp150 was efficiently secreted in the double mutant *sec15-1 vps10*Δ, and was not detected in the very heavy density fractions of the gradient. Externalization of Gas1p or Pma1p was not observed during the time course of the experiment. In conclusion absence of

VPS10, lead to specific packaging of CPY into the HDSV. Results collected from the vps1Δ mutant suggest that HDSV are formed from endosomal membranes. This raises the questions where indeed does the CPY receptor function. Is it at the *trans*-Golgi, or does it sort CPY for transport from early to late endosome (**Figure 16**). We expected that *vps10* deletion would lead to the packaging of CPY into LDSV, since vps10p is proposed to function at the *trans*-Golgi. However this was not the case, since CPY was detected together with HDSV cargo. Thus either Vps10p functions in early to late endosome traffic or alternatively HDSV may originate directly from late-Golgi membranes.

Next we studied the secretory vesicles that could be detected when the CORVET component Vps8p was missing. The CORVET complex is responsible for *intra*-endosomal tethering and when *VPS8* is deleted, delivery of proteins form the early endosome to the late endosome is blocked (334, 335, 358). Biogenesis of the HDSV and LDSV population prevailed in *sec15-1 vps8*Δ mutants, since Gas1p and Pma1p profiles were distinct from that of Hsp150 (II, Figure 9I). In addition, CPY coincided in the *sec15-1 vps8*Δ with the HDSV population, with the majority of CPY being found in the mature M form (**II**, Figure 9I). The mature CPY is thought to be due to processing of CPY in a pre-vacuolar compartment, due to accumulation of CPY and proteases in the same compartment. In the case of the double mutant *sec15-1 vps8*Δ, invertase was found in the gradient fractions containing the very heavy density vesicles, with only a small amount occurring in the HDSV region. In contrast, Hsp150 was not detected in the heavy density vesicles. Thus Hsp150 was selectively secreted in *sec15-1 vps8*Δ, while other proteins analyzed were trapped intracellularly. Invertase was detected in the novel very heavy density vesicles when traffic from early- to late-endosome was blocked. This suggests that the HDSV population may preferentially be formed on the membranes of late-endosomes, while the novel very heavy density vesicles are formed on the early-endosomal membranes (**Figure 16**).

In summary, we found that blocking the endosomal/vacuolar pathway had no inhibitory effect on secretion of Hsp150 in the *sec15-1* background. Our results support previous studies (260), which suggest that the biogenesis of HDSV population of vesicles takes place on the membranes of a *post*-Golgi compartment, most probably of endosomal nature (**Figure 16**). When the endosomal/vacuolar pathway was blocked (for example *vps1*Δ cells), formation of the HDSV population was inhibited, and when traffic from an early endosomal compartment to the vacuole was disturbed (such as in *vps8*Δ cells), proteins intended for delivery to the vacuole were packaged into HDSV. In *vps10*Δ *sec15-1* cells CPY was selectively packaged into the HDSV population and into the novel very heavy density vesicles, but remained absent from the LDSV population, which is puzzling. The CPY receptor Vps10p selectively exports CPY from the late-Golgi (270, 275). According to this traditional perception CPY should be found in the LDSV population of vesicles in the *vps10*Δ *sec15-1* cells. However, the detection of CPY in the HDSV class of vesicles in the absence of Vps10p suggests that CPY may have a Vps10p-independent step. Thus, an alternative view is that CPY may undergo two sorting steps, one at the Golgi (Vps10p-independent) and a second at the early endosomes (Vps10p-dependent) (260).

Both invertase and CPY appear to be transported to the early endosomes, but once there, proteins intended to the vacuole have to be separated from proteins intended for delivery to the exterior of the cell. The active involvement of Vps10p at the early endosomal membranes would help ensure that only the appropriate cargos are selected, and thus explain why in its absence, CPY is packaged into HDSV. Thus, the function of Vps10p may be similar to that of the mannose 6-phosphate receptor, which is involved mainly in *trans*-Golgi to early endosome sorting, but has also been suggested to be required for early to late endosome sorting prior to recycling back to the late-Golgi (673-675).

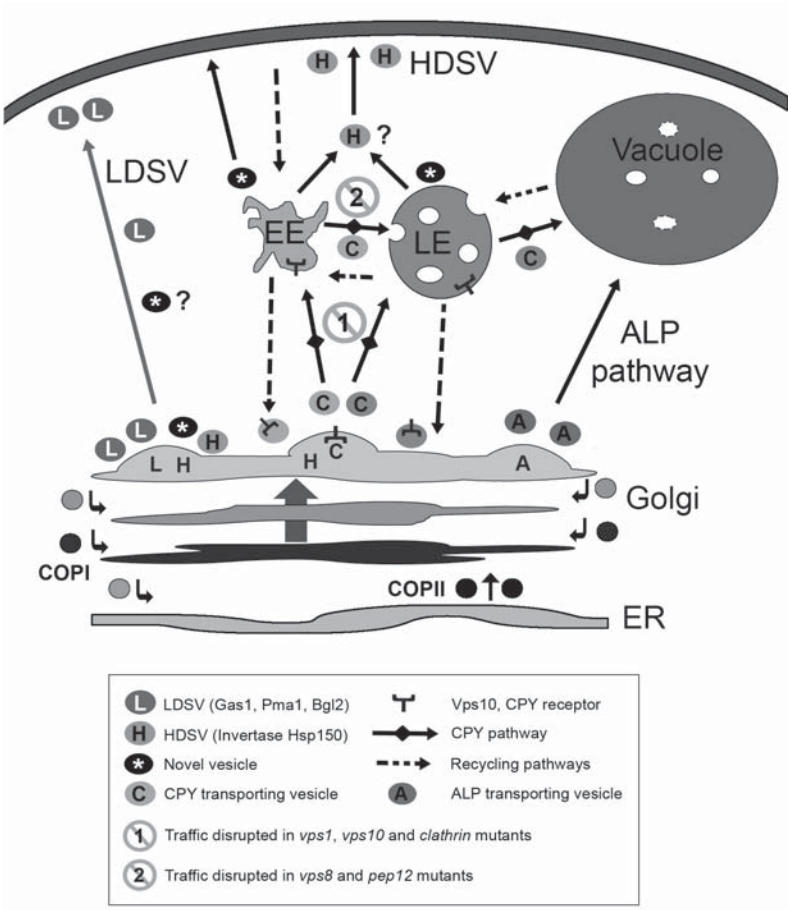


Figure 16. Post-Golgi sorting pathways. From the Golgi a multitude of different pathways emerge, each transporting proteins and membranes to diverse targets. There are two pathways for targeting proteins and membranes to the vacuole. One of these, the CPY pathway transits through the endosomes prior to reaching the vacuole, meanwhile the ALP pathway appears to be mediated in one unique direct step. Similarly there appears to be at least two pathways for targeting proteins to the cell surface. One is targeted directly from the Golgi to the PM (demonstrated by the LDSV), and a second transits through an endosomal compartment, prior to be targeted to the cell surface (demonstrated by the HDSV). It is possible to block specific transport events in these pathways and analyze how cargo is incorporated into the different populations of secretory vesicles. See text for further details.

2.2.3 Morphological characterization of the different secretory vesicles: development of a novel HRP staining procedure for TEM

The results collected so far suggest that Hsp150 is selectively secreted in *sec15-1* cells at restrictive temperature. Next, a morphological approach was developed. We took advantage of the fact that the molecular signature that guides Hsp150 to bypass the *sec15-1* block does not reside in the unique C-terminal fragment of Hsp150, and that the repetitive region of Hsp150 (Hsp150Δ, see **Figure 15**) promotes correct folding of heterologous proteins fused to its C-terminus (see further sections). We decided to test whether the Hsp150Δ carrier would assist proper folding in the yeast ER of Horseradish peroxidase (HRP). HRP is commonly used in morphological studies, due to its ability to react with the substrate diaminobenzidine (DAB) when in the presence of hydrogen peroxide. This reaction yields an insoluble precipitate in the lumen of the intracellular compartment where HRP is located. This precipitate becomes electron dense upon subsequent reaction with reduced osmium tetroxide, and is visible in TEM as a dark staining pattern (**II**, Material and methods). This method that we have adapted now for yeast, was previously developed for mammalian cells (676).

The first step in the development of this method was the construction of the fusion protein Hsp150Δ-HRP, where the unique C-terminus of Hsp150 was replaced by HRP (**Figure 15**). This construct was then expressed in wild type *S. cerevisiae* cells to verify that indeed Hsp150Δ was able to confer secretion competence to the fused HRP protein. Immunoprecipitation experiments with either Hsp150 or HRP antiserum verified that the fusion protein was efficiently secreted to the culture medium with kinetics similar to those of wild type Hsp150. Next, we took advantage of the fact that the C-terminal fragment of Hsp150 is required for the Hsp150 secretion in *sec24-1* and *sec7-1* cells, and therefore in these mutants Hsp150Δ-HRP should remain trapped in the ER and Golgi, respectively (655). In addition, we used control cells and *sec18-1* for comparison. This allowed the development and optimization of the conditions for application of this staining procedure for yeast. Incubation of these cells at the restrictive temperature resulted in different morphologies that were characteristic to each block of the secretory pathway in the different mutants (**Figure 17**). In control cells no significant staining was observed due to the fact that the protein is efficiently secreted. Since Hsp150Δ-HRP is not capable of exiting the ER in *sec24-1* and in *sec18-1* cells, in these cells a membrane-staining characteristic of typical ER structures was observed (**Figure 17**). In the *sec7-1* cells a different staining pattern was observed suggestive of Golgi-stacks. Thus when all the morphological data collected from the different *sec* mutants was assembled; a framework of the staining pattern expected along the secretory pathway was constructed.

Using this staining procedure we set out to analyze whether any morphological differences could be observed within the secretory vesicles that accumulate in *sec6-4* and *sec15-1* cells. To be able to distinguish secretory vesicles that transported Hsp150 we constructed *sec6-4* and *sec15-1* cells expressing a fusion protein where the C-terminal fragment of Hsp150 was replaced by horseradish peroxidase (HRP). These mutants, accumulate secretory vesicles,

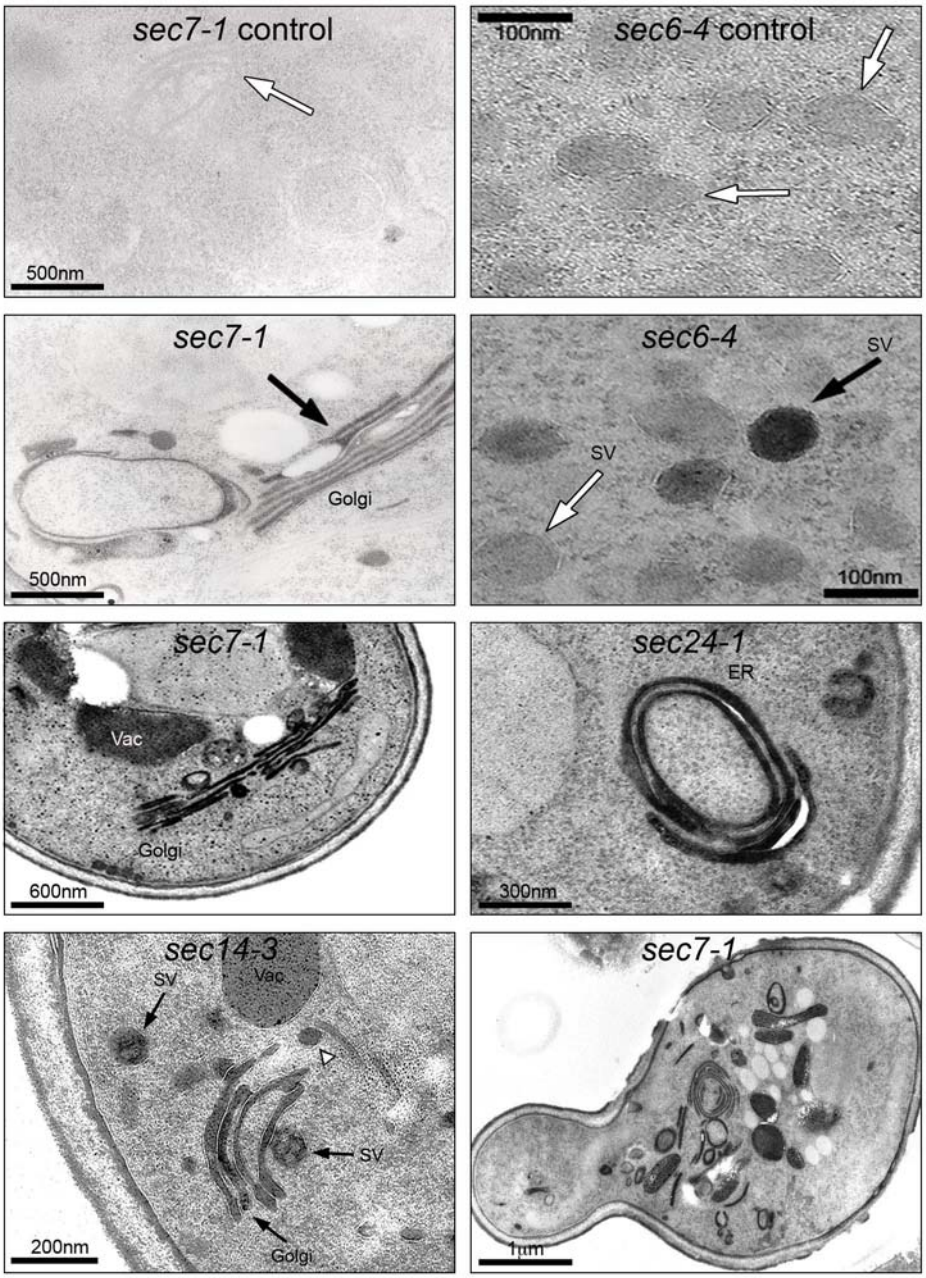


Figure 17. Development of a novel HRP staining method for TEM. Taking advantage of the dark precipitate formed by HRP when H_2O_2 is added, we developed a novel HRP staining procedure. To assist the proper folding and transport of HRP through the secretory pathway we exploited the carrier ability of the Hsp150 fragment. When the H_2O_2 treatment is omitted, no precipitate is observed as can be observed in the control panels. However when H_2O_2 is added, a precipitate visible under the TEM can be observed in the secretory compartments where the fusion protein Hsp150Δ–HRP is localized.

therefore we reasoned that Hsp150Δ-HRP containing vesicles should have electron dense material within them upon DAB and H_2O_2 treatment (**II**, Figure 7B). Since in *sec6-4 cells* Hsp150 accumulates intracellularly, a difference at the level of quantifiable HRP-positive vesicles should be obvious as compared to *sec15-1,* which efficiently secretes Hsp150. Thus, Hsp150Δ-HRP was expressed in *sec15-1* and *sec6-4* cells, and secretion assays at permissive and restrictive temperature were carried out. Immunoprecipitation of Hsp150Δ-HRP using HRP antiserum showed that Hsp150Δ-HRP is secreted in *sec15-1* cells with similar kinetics to those of the endogenous Hsp150. In contrast, in *sec6-4* cells, it remained quantitatively intracellular. Since Hsp150D-HRP behaved like Hsp150, the next step was to visualize these proteins using TEM. Cells were incubated for 90 minutes at the restrictive temperature (to accumulate secretory vesicles) and chased for 10 minutes in the presence of cycloheximide (to enrich secretory proteins at the final stages of the secretory pathway). Vesicle were counted and scored for the presence or absence of HRP. Both *sec15-1* and *sec6-4* mutants accumulated a significant number of 100 nm secretory vesicles, however quantification showed that almost 4-fold more HRP-stained vesicles were detected in *sec6-4* than in *sec15-1* (**II**, Table 2). Sections collected from *sec6-4* mutants, revealed that they accumulated a significant number of both stained and unstained vesicles, whereas sections collected from *sec15-1* mutants, showed that the secretory vesicles accumulated in these cells were in their majority unstained (**II**, Figure 7).

Parallel samples of these cells were collected and subjected to vesicle isolation to verify if the HRP stained vesicles corresponded to the region where Hsp150 was detected. After isodensity centrifugation and fixation, the samples were subjected to the HRP staining (**II**, material and methods). Analysis by TEM revealed a homogeneous population of 100 nm vesicles that corresponded to the size of typical post-Golgi secretory vesicles (**II**, Figure 8). A positive HRP-staining pattern was observed within the vesicles that were collected from fractions of the gradient corresponding to vesicles that were previously shown to transport Hsp150, i.e., within the HDSV region, as well as from the very heavy density regions of the gradient. In contrast, no significant staining pattern was observed within the LDSV region. Once again, much less stained vesicles were observed in the samples collected from the *sec15-1* cells in comparison to the number detected in the *sec6-4* cells. In addition, no HRP-stained very heavy density vesicles were detected in *sec15-1* cells.

2.3 Mso1p is required for fusion of secretory vesicles in *sec15-1* cells

Thus it appears that in *sec15-1* cells, Hsp150 is selectively and efficiently transported to the cell exterior, while other secretory proteins are retained intracellularly. Sec15p is the exocyst subunit that responds to the activated state of the RabGTPase Sec4p, and is responsible for the connection between exocyst and the incoming secretory vesicle (571, 580, 581, 613, 668). The later is a crucial step for vesicle tethering at the plasma membrane. So how does the vesicle carrying Hsp150 then fuse with the plasma membrane in *sec15-1* cells? In theory two alternatives could possibly take place: A) fusion of these vesicles with the plasma membrane is completely independent of the exocyst function, or

alternatively, B) The RabGTPase Sec4p may recruit an alternative effector protein in the absence of functional of Sec15p, which may or may not use an alternative set of accessory proteins.

Hsp150 was not secreted in the following exocyst subunit mutants *sec3-1*, *sec6-4*, *sec5-24*, *sec8-6* and *sec10-1* (**II**, Figure 1). Consequently it appears that these components are essential for the fusion of secretory vesicles carrying Hsp150. Nevertheless, it is possible that in the absence of *sec15-1*, an alternative route may come into play, which would direct the fusion of these vesicles to the plasma membrane without the interplay of the remaining exocyst subunits. However due to the lethality of double temperature-sensitive mutant strains, and a strain that has a combination of *sec15-1* with a deletion of an additional exocyst subunit, we could not address this question to rule out the first hypothesis.

RabGTPases are key regulators of intracellular events. Once in the activated state, they generally interact with a multitude of different effector proteins, which may either contribute to a specific cellular event or may have different roles within the cell (11, 677-679). However, only one effector has been found for Sec4p, namely the exocyst complex, and to be more precise, through the interaction with the Sec15p subunit. Recently however, Sro7/77p has been suggested to function as a new effector of the RabGTPase Sec4p (629, 636). Sro7/77p appears to provide one additional regulatory link between the secretory vesicle and the fusion machinery by responding to activated Sec4p and interacting with the t-SNARE Sec9p (636). In a screen performed to test which proteins confer fusion competence to the secretory vesicles carrying Hsp150, one specific protein, Mso1p, emerged as a key component. Mso1p was initially identified in a multi-copy suppressor screen for *sec1-1* temperature-sensitive mutants (639) and has recently been proposed to play a key role in the dynamic interface that takes place between the RabGTPase Sec4p, Sec1p and the exocytic SNARE machinery (627, 642).

2.3.1 Deletion of MSO1 blocked fusion of Hsp150 transporting vesicles in sec15-1 cells

Deletion of the *MSO1* gene is not lethal in vegetatively growing cells, although they do show a slight reduction in growth rate (639). In the *mso1* deletant, a slight accumulation of small secretory vesicles is detected during the early stages of bud expansion, which suggests a positive role for Mso1p in the last stages of the exocytic pathway, namely in the fusion of secretory vesicles with the plasma membrane. Although Mso1p appears not to be essential in haploid cells, it plays a crucial role in sporulation (641). At the end of the second meiotic division that occurs during sporulation, *de novo* plasma membrane is generated by the fusion of the prospore membranes to the meiotic plaque, which is localized on the cytoplasmic side of the spindle pole bodies (SPB) (680). This meiotic plaque functions as a recruiting platform for the fusion of further prospore membranes, culminating in the protrusion of this membranous structure through the cytoplasm. The tips of the prospore membrane are marked by the leading edge coat proteins, which advance and encapsulates the material required to form a viable haploid cell. When *MSO1* was deleted in diploid cells, the prospore membranes failed to fuse to the meiotic plaque. Instead numerous 60 to 70-nm vesicles accumulated at the SPB. Consequently, no prospore formation was observed in these cells (641, 642).

Taking into count the accumulation of secretory vesicles in Δ*mso1* cells (639), and the fact that Mso1p interacts with exocytic SNARES, Sec1p and also with Sec15p (641, 642), we investigated if Mso1p played a role in the secretion of Hsp150 in *sec15-1* cells. To address this issue, *sec15-1* cells were crossed with *mso1*Δ cells, sporulated and the resulting haploid cells were scored for their respective genotype. Deletion of the *MSO1* gene in the *sec15-1* background yielded viable cells that grew slightly slower than the parental strains (II, Supplementary Figure S6). We set out to analyze if any secretion differences could be observed between the double mutant and the background *sec15-1* mutant. Pulse chase experiments were carried out at permissive (24°C) and restrictive (37°C) conditions, followed by immunoprecipitation of Hsp150. In *mso1*Δ cells Hsp150 was efficiently secreted at both the permissive and restrictive temperature, with kinetics similar to those of wild type cells (**II**, Figure 10A). TCA precipitation of medium samples from *mso1*Δ cells, revealed the presence of the same set of proteins as in wild type cells. As shown before, in *sec15-1* cells, approximately 60% of Hsp150 was secreted after 60 minutes of chase at the restrictive temperature. However when the *MSO1* gene was deleted in the *sec15-1* background, no secretion of Hsp150 to the medium or to the cell wall was detected at the restrictive temperature. TCA precipitation of medium samples and SDS-PAGE analysis revealed the total absence of any labeled proteins in the double mutants. When *sec15-1 mso1*Δ cells were incubated at the permissive temperature, a wild type secretion profile was observed. Next we compared the secretion of invertase in *mso1*Δ, *sec15-1* and *sec15-1 mso1*Δ mutants (**II**, Figure 10B). In *mso1*Δ cells at permissive and restrictive temperature, invertase was efficiently externalized to the periplasmic space, with secretion kinetics similar to those of the wild type cells, which suggests that Mso1p is not a key factor under these conditions in the secretion of either invertase or Hsp150. Similar to previous results, in *sec15-1* cells, secretion of invertase was significantly impaired, with only a very small portion (<20%) of the active protein being externalized. In contrast in the double mutant *sec15-1 mso1*Δ secretion of invertase was completely blocked and accumulated intracellularly at the restrictive temperature, analogous to the secretion block observed for Hsp150. At the permissive temperature, the double mutant *mso1*Δ *sec15-1*, efficiently secreted invertase to the periplasmic space. In addition, secretion of other secretory cargo such as Pma1p, Bgl2p and Gas1p were also analyzed, and under the same experimental conditions no secretion of these proteins was detected (data not shown).

Since protein secretion was completely blocked in the *mso1*Δ *sec15-1* strain, we next investigated which secretory vesicles accumulated in this double mutant and compared it to the *sec15-1*, *mso1*Δ and *sec6-4 mutants*. Cells were grown to early logarithmic phase and shifted to restrictive temperature for 2 hours to allow accumulation of secretory vesicles. The cells were then lyzed, and after differential centrifugation steps, the secretory vesicles were isolated as an individual pellet and loaded on to the gradient as previously..

Secretion in the *mso1*Δ strain is not blocked at the restrictive temperature, therefore they accumulated a very small number of secretory vesicles. As a consequence, detection of secretory cargo

carried by these vesicles was hard to follow due to their low signal.

Analysis of LDSV secretory cargo such as Gas1p, Pma1p and Bgl2p, revealed that they, accumulated intracellularly in the same relative amounts and hence failed to be secreted in either *sec15-1*, *sec6-4* or *sec15-1 mso1*Δ cells (data not shown). As before, in both *sec6-4* and *sec15-1* mutants, invertase activity was found in two peaks, within the region corresponding to HDSV and in the very heavy density vesicles. In the case of the *sec15-1 mso1*Δ mutant invertase activity was also detected in the same regions. However, while the total intracellular invertase activity from *sec15-1* mutants was slightly lower than in *sec6-4* mutants, in the double mutant *sec15-1 mso1*Δ the invertase activity levels were approximately equal to those detected in *sec6-4* mutants. This suggests, that invertase secretion is completely blocked in the double mutant and further supports the invertase secretion assays carried out previously (**II**, Figure 10B).

As previously, we found that Hsp150 was detected in significantly lower levels in the *sec15-1* mutant than in *sec6-4* mutants, and that in *sec15-1* mutants it was detected only in the region corresponding to the HDSV (**II**, Figure 10C). However when *MSO1* was deleted in the *sec15-1* background, a significant higher amount of Hsp150 was detected in the gradient, which supports the fact that Hsp150 is not secreted in *sec15-1 mso1*Δ mutant. Furthermore, in the double mutant, Hsp150 was found in two regions, the one corresponding to the HDSV and now also in the region corresponding to the very heavy density vesicles. Both the distribution profile of the different reporter proteins, as well as the total amount detected in the double mutant *sec15-1 mso1*Δ were similar to *sec6-4* mutants, where secretion of all cargo is blocked. This suggests that Mso1p plays a key role in the ability of vesicles to fuse in the *sec15-1* background.

In summary, deletion of *MSO1* alone apparently had no effect on the secretion of Hsp150, invertase or other secretory cargo analyzed. But, in a *sec15-1* background, secretion of all proteins to the culture medium was completely blocked at the restrictive temperature including that of Hsp150 which was selectively secreted in *sec15-1* cells. To further verify the secretory block imposed on Hsp150 and other proteins in the double mutant *sec15-1 mso1*Δ, we decided to study the accumulation of secretory vesicles using the novel staining procedure described above. For this end, Hsp150Δ-HRP was expressed in *mso1*Δ, *sec15-1* and in *sec15-1 mso1*Δ cells. Cells were incubated at the restrictive temperature for 90 minutes and chased in the presence of cycloheximide as previously. As reported previously, *mso1*Δ cells accumulated a significant number of secretory vesicles in the early bud tip, but as this bud expanded and grew in size, the number of vesicles decreases (**II**, Figure 11B). Similar to previous results, the *sec15-1* sister cell accumulated a significant number of secretory vesicles, nevertheless the majority of them lacked HRP staining, because both Hsp150 and Hsp150Δ-HRP were secreted. In contrast, about half of the secretory vesicles that accumulated within the *sec15-1 mso1*Δ cells at the restrictive temperature, were HRP stained. The staining pattern and distribution ratio observed in the double mutant *sec15-1 mso1*Δ cells were very similar to those in *sec6-4* cells, where secretion of all proteins was also completely blocked.

In conclusion we found a role for Mso1p in the fusion of Hsp150-

containing secretory vesicles with the plasma membrane, under conditions where the exocyst is impaired due to the *sec15-1* mutation. Deletion of *MSO1* in the *sec15-1* background blocked secretion of all proteins, including that of Hsp150. Furthermore under these conditions, where Hsp150 detected in the HDSV and in the very heavy density vesicles, similar to what was found in *sec6-4* cells.

2.3.2 The role of Mso1p in the vesicle plasma membrane interface

Mso1p is capable of interacting directly with proteins , which have a key role in exocytosis events at the plasma membrane. Immunoprecipitation experiments revealed that Mso1p coprecipitated with the Sec1/Munc18 family member Sec1p, with the exocytic SNARE complex Sso1/2p-Sec9p-Snc1/2p and specifically with the exocyst subunit Sec15p (642). Interaction with the remaining exocyst components like Sec8p was not detected in the complexes containing Mso1p-Sec1p. Thus, it appears that the role of Mso1p at the plasma membrane interface may be to temporally regulate tethering of the secretory vesicle with the pairing of the exocytic SNAREs, which subsequently leads to fusion of the vesicle with the plasma membrane. The precise mechanism that allows this regulatory interaction to take place is not fully understood. However, there are regions within the C-terminus of Mso1p that are highly homologous to a conserved region in the Munc13 family members (627) and to the common phosphotyrosine binding (PTB) domain in Mint proteins (642). These proteins are proposed to work as SNARE regulators in the stimulus-coupled secretion pathway that takes place in higher organisms, namely at the vesicle "priming" stage (15, 644). Since both Mso1p and the Munc13 family members play a role in exocytosis, interact with Sec1/Munc18 and are components of the exocytic SNARE complex, it is possible that Mso1p may indeed represent a yeast ortholog of Munc13-related proteins.

When *sec15-1* cells are incubated at the restrictive temperature, association of the mutant form of Sec15p with the incoming secretory vesicle, as well as assembly of the remaining exocyst complex are impaired (570, 573, 580, 581, 595). Under these conditions secretory vesicles fail to fuse to the plasma membrane due to the inefficient tethering function of the exocyst complex (**Figure 18**). Nevertheless, secretory vesicles transporting Hsp150 are still capable of fusing to the plasma membrane and deliver their cargo to the culture medium, while other secretory cargo accumulates intracellularly. Thus, additional complexes may exist in yeast that play an important role in the fusion of subclasses of vesicles to the plasma membrane in order to ensure that specific proteins are delivered to their final destination. Since Hsp150 is a soluble protein, it must interact with a transmembrane receptor protein for packaging into the forming secretory vesicle. This putative receptor may recruit either directly or indirectly a set of alternative accessory proteins, or interact directly with Mso1p. This interaction with Mso1p at the plasma membrane may help stabilize the secretory vesicle at the plasma membrane, even if temporarily, until a functional SNARE fusion complex is formed. According to this hypothesis, when the *MSO1* gene is deleted in the *sec15-1* background, these secretory vesicles are no longer capable of being stabilized at the plasma membrane interface and fail to fuse. Consequently secretion is blocked. When the *MSO1* gene is deleted in a wild type background,

no severe deficiencies are observed, since, under these conditions, the fully functional exocyst complex provides sufficient stabilization of the vesicle at the plasma membrane. During the early stages of bud tip expansion that occur in *mso1*Δ cells, a significant number of secretory vesicles accumulate at the tip region. During this early phase, a large amount of secretory vesicles are targeted to a relatively small region within the bud tip. This phenotype may arise due to the fact that in the absence of Mso1p function, stabilization and temporal regulation may be slightly impaired. As a consequence of this somewhat lower stabilization or increase in the transition time from tethering to fusion events, secretory vesicles start to accumulate due to the higher residence time of the vesicle at this interface. As the bud tip grows and vesicle fusion events become broader, the secretory vesicles are distributed to different regions of the plasma membrane and the exocyst complex becomes capable of handling this load efficiently, hence the lower accumulation of secretory vesicles and almost wild type phenotype observed.

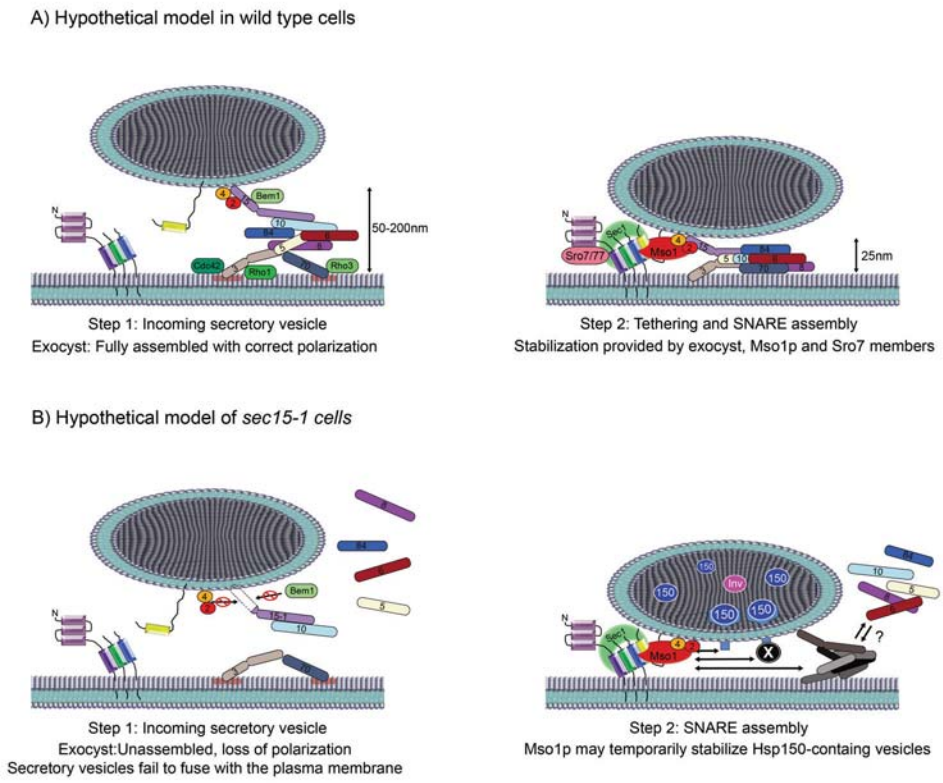


Figure 18. Tethering and SNARE assembly at the plasma membrane. A) Hypothetical description of how the transition from tethering to SNARE assembly occurs in wild type cells with some of the key protein depicted. **B)** Illustration of how the shift to 37ºC of *sec15-1* cells disrupts the exocyst integrity and how possibly the cell may adapt to such changes. See discussion for details.

Alternatively, in the absence of Sec15p function, Sec4p may recruit an alternative effector protein, such as a possible Sec15p homologue, that would partially compensate for the lack of functional Sec15p. Although an additional isoform of Sec15p was found in the extracts of rat brain (681), in yeast cells the presence of isoforms has not been reported.

In conclusion, isolation of mutants that abolish the secretion of Hsp150 in the *sec15-1* background may help uncover the identity of additional proteins that possibly play a role at the plasma membrane/ secretory vesicle fusion interface.

2.4 Multiple pathways lead to the cell surface: Why the need for divergent pathways?

Two reported pathways for delivery of exocytic cargo to the plasma membrane exist, which use the LDSV and HDSV populations of secretory vesicles (258, 260). These vesicle populations can be distinguished on one hand by the presence of specific secretory cargo, and on the other hand by their secretion kinetics, where HDSV cargo is secreted quickly. Expression of cargo proteins transported in the HDSV are typically regulated at the transcriptional level. Perhaps these proteins require specific processing. In agreement with this, Hsp150 has a Kex2p cleavage site and is transported by the HDSV population of secretory vesicles. The HDSV exocytic branch transits through the early-endosome, where the Kex2p protease is believed to function. Another difference between these two exocytic branches was observed during the early stages of the cell cycle of *exo70* mutant cells (682). The *exo70* mutant strain accumulated a significant amount of Bgl2p (LDSV-specific cargo), while secretion of invertase (HDSV-specific cargo) appeared unaffected. Furthermore, the combination of the *vps1*Δ mutation, which causes a defect in invertase vesicle trafficking with the *exo70* mutation was detrimental to the cell. One additional difference observed between the behavior of these different vesicle populations appeared from studies carried out on actin cytoskeleton mutants (551, 552, 554, 683). These cells accumulate a considerable number of *post*-Golgi secretory vesicles, but do not accumulate significantly invertase. Interestingly, when Hsp150 secretion assays were carried out in *tpm1*Δ or *srv2*Δ mutants, which disturb the actin cytoskeleton, we observed that these mutations had no effect at all on the secretion kinetics of Hsp150. Under these conditions more than 90% of the total labeled pool of Hsp150 was found in the culture medium after 30 minutes chase (**II**, Supplementary Figure S8).

Yeast have myriad ways to respond to the changing surroundings, to ensure that they are operating at their peak efficiency. At least two parallel routes for targeting proteins to the cell surface have evolved to ensure delivery of proteins to their final destination. One of these pathways, the HDSV pathway may function as a rescue route, since proteins transported in this class of secretory vesicles are generally expressed when the cell is exposed to non-physiological conditions. This pathway appears to be less susceptible to changes in growth conditions and unfavorable mutations, such as disruption of the actin cytoskeleton. When yeast cells are shifted to adverse growth conditions, growth is temporarily slowed down, which in turn leads to a delay or block in polarized delivery of secretory vesicles to the emerging bud tip of the dividing cell. Since the HDSV population appears to be less dependent on an active cytoskeleton

polarization for their delivery to the sites of active membrane fusion. This suggests that HDSV vesicles may fuse all around the cell surface. This would allow essential enzymes, such as Invertase and alkaline phosphatase, to be secreted, which in turn would provide the cells with the tools to quickly adapt to the changing environment and gain competence to continue the normal cell cycle.

Studies carried out on the transport of the general amino acid permease Gap1p further supports the notion that proteins required under certain growth conditions, transit through the endosomal/Vacuolar pathway. This permease is regulated both transcriptionally, and posttranslationally by differential sorting in the late secretory pathway (351, 413, 487, 684). When cells are grown on a nitrogen-limiting media, such as urea, Gap1p is bound by a membrane coat complex, the GSE complex, present on the endosomal membranes and transports Gap1p to the plasma membrane (354). However, when cells are grown under nitrogen-rich conditions, for example on glutamate, the Rsp5p ubiquitin ligase ubiquitinates Gap1p, and the modified protein is targeted to the vacuole for destruction thus downregulating the levels of Gap1p on the cell surface (355). Thus, it appears that proteins that are only required under certain growth conditions are first transported to the early endosomes. The endosome as a compartment, which receives both exocytic and endocytic material, may respond to clues from the external environment, and alter its sorting properties in order to direct various cargoes either to the plasma membrane or to the vacuoles for degradation (260).

Several different types of early endosomes occur in mammalian cells, which can be recognized by their distinct morphologies and functions (479, 685). Although yeast endosomes are clearly less characterized, it is emerging that they share a significant amount of similarities (686). Biogenesis of the HDSV population of secretory vesicles is proposed to occur on the membranes of the early endosome, since both *vps1*Δ and *vps27*Δ mutations abolished their formation (260). However, a strong effect on invertase sorting into the HDSV population of vesicles has been reported in *pep12* mutants, thought to block vesicle fusion with the late endosome (260). Therefore it is possible that invertase (and secretory cargo typically found in the HDSV population) is first transported from early to late endosomes, from which it is then selectively incorporated into the HDSV vesicles that reach the cell surface (**Figure 16**). Due to the fact that certain v*ps* mutations abolish the formation of the HDSV population of secretory vesicles, while the LDSV population appears rather unaffected by such disturbances in the endosomal/vacuolar pathway, lead to the proposal that invertase is first sorted into endosomes and packaged into HDSV vesicles for exocytosis (258-260). However, studies carried out on *drs2/dnf* and clathrin mutants have suggested that the HDSV population of secretory vesicles may indeed arise directly from the *trans*-Golgi membranes (477, 478). One possible interpretation forwarded for the mechanism by which the endosomal mutants affect the traffic of HDSV cargo was based on the fact that late-Golgi resident proteins are in continuous movement between the late-Golgi and the endosomes (264, 365). Thus when a strong endosomal block is on, the continuous vesicle formation without appropriate retrieval to the Golgi through the endosomes, would eventually

deplete the late-Golgi membranes of the appropriate resident proteins required for sorting invertase into clathrin coated vesicles (259, 260, 478), and hence in their absence formation of the HDSV population of vesicles is abolished. If indeed biogenesis of HDSV takes place on the late-Golgi membranes, this hypothesis provides some explanation for the fact that in *sec15-1 vps10*Δ mutant cells, CPY is detected in the HDSV population of secretory vesicles (**II**, Figure 9E).

An alternative explanation forwarded for the observation of CPY in the HDSV region in *sec6-4 vps10*Δ mutants, is if CPY is sorted both at the late-Golgi and at the endosome. But only the endosomal CPY sorting step would be Vps10p-dependent (260). Several different proteins have been shown to cycle from endosomes back to the plasma membrane (354, 358, 405, 482, 687). Therefore proteins intended for delivery to the vacuole have to be efficiently sorted from those intended to be recycled to the plasma membrane. Therefore, although Vps10p has been proposed to mainly function at the Golgi (270, 275), it may also play an important role in sorting cargo at early endosomes (**Figure 16**).

A significant amount of data shows that secretory cargo transported in the HDSV population is rerouted to the LDSV vesicles upon disruption of the endosomal/vacuolar pathway, and that a functional delivery pathway for newly synthesized proteins operates from the endosomes to the plasma membrane. Further work will need to be done to clearly define from which membranes the HDSV vesicles originate.

To add one more layer of complexity to the parallel pathways found in the yeast secretory pathway, we found a third distinct population of secretory vesicles in *sec6-4* and *sec15-1* mutant cells. This novel population of secretory vesicles was found in the very heavy density fractions of the gradient (d=1,198±0,003 g/mL) and typically had diameters between 80 and 120 nm (**II**, Figure 6 and 8). No Golgi or vacuolar/endosomal markers were found in these vesicles and thus we suggest that they indeed represent a new population of secretory vesicles that transport cargo typically found in the HDSV population of secretory vesicles. These novel vesicles appeared to display selectivity in their cargo selection as could be observed when they were HRP stained (II, Figure 8). While the HDSV population (that transport both invertase and Hsp150) revealed a homogeneous staining pattern, part of the vesicles in the very heavy density fractions were stained and part was not.

This selective packaging may be the key feature behind the secretion differences observed between Hsp150 and invertase. In all the mutants analyzed, which secreted Hsp150, such as *sec15-1* cells, Hsp150 was not detected in the very heavy density fractions, while invertase which failed to be secreted was found in significant levels in these vesicles. In contrast when *MSO1* was deleted in the *sec15-1* mutant background, Hsp150 failed to be secreted (as in the case of *sec6-4* cells) and accumulated in two populations: in the HDSV and in the very heavy density vesicles. Since disturbing the endosomal/vacuolar pathway did not disturb the biogenesis of the very heavy density vesicles we proposed that these vesicles might arise from the *trans*-Golgi membranes. However when the biogenesis of the different secretory vesicles was addressed in the *vps8*Δ

mutant, which blocks traffic from early to late endosome and therefore is similar in nature to the *pep12* mutant described previously, we found that the majority of the invertase activity was in the very heavy density vesicles, with very little being detected in the HDSV (**II**, Figure 9F). Thus one possibility is that these very heavy density vesicles may form on the membranes of the early endosomes, whereas the HDSV form on the membranes of the late-endosome (**Figure 16**). This possibility would explain the preferential packaging of invertase into the very heavy density vesicles in *vps8*Δ *sec15-1* cells. Alternatively, in the double mutant *vps8*Δ *sec15-1*, invertase may just show a preferential packaging at the *trans*-Golgi into the very heavy density vesicles, if indeed they form at the *trans*-Golgi. At this point we cannot rule out the possibility that both HDSV vesicles and very heavy density vesicles originate from the same compartment. Hsp150 as a soluble secretory cargo relies on the interaction with a transmembrane receptor (who's nature is unknown so far) to be selectively packaged into the different secretory vesicles. This receptor may have the ability to recruit additional accessory proteins or even coat proteins, (as in the case of GSE complex-mediated transport of Gap1p to the plasma membrane) which could possibly ensure efficient vesicle formation and transport of Hsp150 to the cell surface. We are currently aiming to address the nature of the putative receptor and identify proteins it may interact with. This would allow us to get a deeper understanding of the partners involved, and possibly the key features characteristic of the different exocytic pathways that operate in yeast.

3. Hsp150 as a carrier for secretion of heterologous protein in yeast (III)

3.1 Aspects of recombinant protein expression in yeast

Many factors have to be considered when designing protein production in yeast cells. The main objective of heterologous expression is the production of secretory proteins of mammalian origin. Therefore the host must be capable of providing eukaryotic-specific post-translational modifications, which are required for proper folding and full activity of the recombinant protein. In contrast to prokaryotes, yeast as a eukaryotic cell is capable of executing many, but not all of the required post-translational modifications that take place during transit through their native secretory pathway. A second reason for using yeast as an expression system is that the amount of endogenous proteins in the yeast medium is relatively low (<0,5%). Thus, secretion of the protein product to the cell exterior facilitates down stream processing and purification (688). Other advantages of using yeast as a recombinant expression system are the well-established techniques for genetic engineering, large-scale protein production by fermentation, the capability of the organism to grow in inexpensive culture media, and the lack of ethical concerns.

However, in order to achieve extracellular production, it's necessary to fuse a functional signal peptide to the protein of interest to direct the protein for translocation across the ER membrane, and thus to allow the protein to access the yeast secretory pathway. Although signal peptides are recognized with low specificity in yeast, it is preferable to choose a sequence of yeast origin to ensure efficient translocation (689). The most commonly used yeast species

for heterologous protein expression are *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia Pastoris*.

3.2 Hsp150 promotes the folding of heterologous fused proteins to active and secretion competent forms

Usually secretion of heterologous proteins in yeast requires a 'carrier protein", whose role is to guide the foreign protein to the ER translocation channel and to smuggle the protein through the ER quality control machinery thus facilitating ER exit and secretion of the protein (688). Taking into count the high secretion efficiency of Hsp150 and its bypass of secretory blocks in several temperature-sensitive mutants under restrictive temperatures, we wanted to explore the possibility of using Hsp150 as a carrier in the production of different heterologous proteins and to compare *S. cerevisiae* and *P. pastoris* as hosts (647, 654, 655, 664, 666). The Hsp150Δ carrier fragment which was previously shown to confer secretion competence to *E. coli* β-lactamase in S. cerevisiae contains the 321 N-terminal amino acids of the endogenous Hsp150 protein and consists of the signal peptide for ER targeting, subunit I and the repetitive region of the subunit II (651) (**Figure 15**). The carrier portion has 95 potential O-glycosylation sites, but it lacks N-glycosylation sites. All of the O-glycosylation sites of the first 53 amino acids of the Hsp150 are used (666). In order to release the fused protein from the carrier, a Kex2p cleavage site can be introduced between the carrier and the foreign protein. Several recombinant proteins such as *E.coli* β-lactamase, rat nerve growth factor receptor and rat alpha-2,3-sialyltransferase have been successfully expressed using the Hsp150Δ – carrier system (651, 652, 690-692). For this reason we were interested in further analyzing the repertoire of heterologous proteins that could be expressed using the Hsp150Δ carrier method, and to compare the performance of the Hsp150Δ carrier to that of the mating factor (MF) α carrier (688, 693-695).

The MFα carrier consists of the ⟨-factor preproprotein of the *S. cerevisiae* mating factor α and it is composed of the 19 amino acid signal peptide followed by a 66 amino acid fragment (pro) with three consensus N-glycosylation sites, and a dibasic Kex2 endopeptidase-processing site (696) (**Figure 15**). Upon post-translational translocation into the ER, the signal peptide is removed by the signal peptidase and in the late-Golgi, the Kex2p endoprotease removes the pro-fragment at the C-terminal side of the Kex2p site (697). For the purpose of heterologous expression, the foreign protein is fused to the C-terminus of the MFα carrier, either with or without the Kex2p cleavage site. In the first case the protein of interest is released into the medium free of its carrier. Whereas in the second case, the fusion protein is secreted in the unprocessed form (698).

Despite the successes of *S. cerevisiae* as a host organism the product yield is usually low. In general, higher protein yields can be obtained by the methylotrophic yeast *Pichia pastoris*. The simplicity and similarity of techniques to those used in *S. cerevisiae* combined with the higher ability to produce foreign proteins have made *P. pastoris* the preferred option for production of the recombinant protein (693, 694, 699). The high level of expression in *P. pastoris* results from the use of a commercially available expression system that is based on the alcohol oxidase (AOX) promoter. Alcohol oxidase catalyses the first step in methanol utilization pathway by

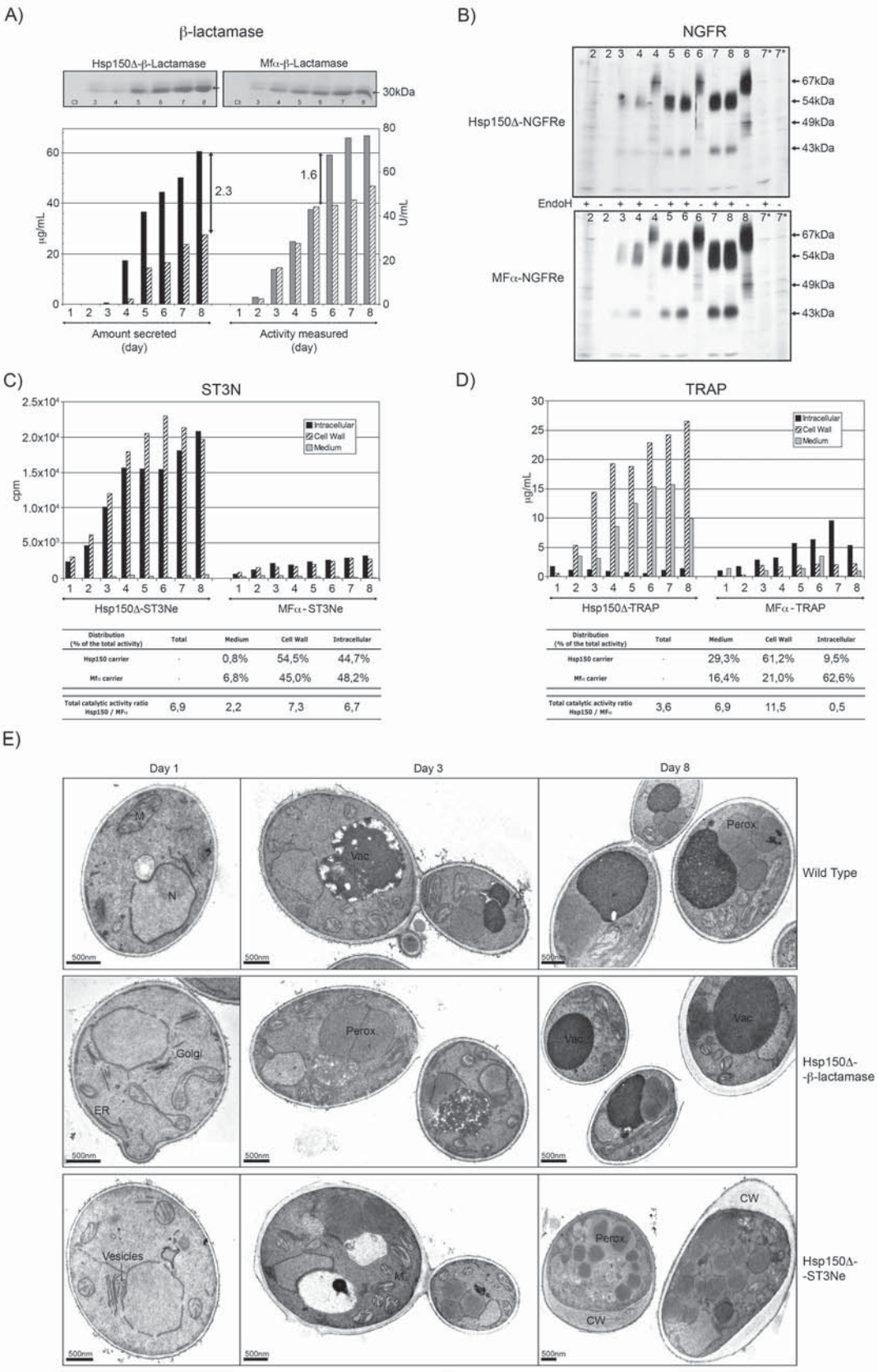
oxidation of methanol to formaldehyde and hydrogen peroxide (699). To avoid hydrogen peroxide toxicity, this first step in methanol metabolism takes place within a specialized organelle, the peroxisome, where the resulting hydrogen peroxide is degraded to oxygen and water by catalase. Alcohol oxidase has a poor affinity for oxygen and the methylotrophic yeasts appear to compensate for this deficiency by synthesizing large amounts of the enzyme in the presence of methanol. The regulation of the promoter of *AOX1* is similar to that of the *GAL1* gene of *S. cerevisiae,* in the sense that the control appears to involve two mechanisms: a repression / derepression mechanism plus an induction mechanism (700). However, unlike *GAL1* regulation, the absence of a repressing carbon source, such as glucose or glycerol in the medium, does not result in substantial transcription of the *AOX1* gene. The presence of methanol appears to be essential to induce high levels of transcription. One additional advantage of *P. pastoris* over *S. cerevisiae* is that it is a poor fermenter. Since it prefers aerobic growth, it can reach extremely high cell densities when the conditions are optimized (701). In contrast, *S. cerevisiae,* when grown to high cell densities, produces ethanol as fermentation product, which in turn at toxic levels inhibits cell growth and production of recombinant proteins. This quality of *P. pastoris*, is particularly useful, when the secreted protein is proportional to cell density.

Therefore the method we adopted for recombinant protein production was to insert our different fusion protein constructs (**Figure 15**) under control of the *AOX1* promoter, induce their expression by methanol and collect samples at diverse time points for comparison.

3.2.1 Secretion efficiency of the Hsp150Δ carrier is higher than the commonly used MFα

As a starting point for comparison of the efficiency of the Hsp150Δ and MFα carriers, we used the following recombinant proteins, which included *E.coli* β-lactamase, the ectodomain of rat nerve growth factor receptor (NGFRe), the ectodomain of rat alpha-2,3-sialyltransferase (ST3Ne), and rat bone tartrate-resistant acid phosphatase type 5 (TRAP). The constructs were inserted into the pPICZα vector under the control of the *AOX1* promoter, and the resulting plasmids were transformed by electroporation into *P. pastoris* (for strain list see table 2). Strains were grown initially in BMGY overnight at 30ºC. On the next day the cell density was measured and a new shake flask grow period was now started using BMMY as growth medium. On the following day recombinant protein production was initiated by the addition of methanol (0,5% V/V). Samples were collected daily and analyzed for the expression of the proteins.

In the case of both carriers, *E. coli* β-lactamase, folded correctly to an enzymatically active form and the fusion protein was efficiently secreted to the culture medium (**III**, Figure 4 and **Figure 19A**). When using the Hsp150Δ carrier 2,3 fold more protein was secreted in comparison to the MFα carrier. In the case of the MFα carrier a substantial amount of the protein remained intracellular. On the other hand, when we analyzed the activity of the secreted protein we only observed a 1,6 fold increase when using the Hsp150Δ carrier. The difference observed between the amounts of protein secreted *versus* active protein secreted may reflect that the secretory pathway is overloaded. For example if the ER protein disulphide



Distribution (% of the total activity)	Total	Medium	Cell Wall	Intracellular
Hsp150 carrier	-	0,8%	54,5%	44,7%
Mfα carrier	-	6,8%	45,0%	48,2%
Total catalytic activity ratio Hsp150 / Mfα	6,9	2,2	7,3	6,7

Distribution (% of the total activity)	Total	Medium	Cell Wall	Intracellular
Hsp150 carrier	-	29,3%	61,2%	9,5%
Mfα carrier	-	16,4%	21,0%	62,6%
Total catalytic activity ratio Hsp150 / Mfα	3,6	6,9	11,5	0,5

isomerase (PDI) is saturated, it can no longer form all of the disulphide bridges. Since correct disulphide bridges are essential for the activity of β-lactamase, if PDI is saturated, we would expect a reduction in the total amount of active β-lactamase secreted. Alternatively the protein may misfold while in the medium, and in turn become inactive. TEM analysis of *P. pastoris* strains expressing Hsp150Δ-β-lactamase, showed heavily stained vacuoles (**Figure 19E**). This suggests that a significant amount of proteins are targeted for degradation, which is probably due to the overload placed on the secretory pathway.

So to further optimize the production of β-lactamase we decided to use a fermentor, where the growth conditions can be regulated and optimized to best suit the expression of the protein(701). Under these conditions the cells reached a high density (OD_{600}=400/ wet weight= 330g/L), and 450 mg/L of β-lactamase was secreted to the culture medium with a peak activity of 200 U/mL (**III**, Figure 5).

Although β-lactamase was secreted efficiently, the main interest is to produce proteins of mammalian origin, which can be used for therapeutic purposes. One benefit of *P. pastoris* over *S. cerevisiae,* is that *P. pastoris* does not hyperglycosylated proteins or add terminal α-1,3-linked mannose residues, which are highly immunogenic in humans (702, 703). α2,3-Sialyltransferase (ST3N) is a type II transmembrane protein of the Golgi complex of mammalian cells. It transfers sialic acid form CMP-NeuNAc to terminal galactose residues of Galβ1-3-GlcNAc or Galβ1-4-GlcNAc, creating a α-2,3 linkage. Sialylation of glycoproteins is particularly important since proteins circulating in the blood plasma that lack this modification are removed by the hepatic asialoglycoprotein receptor (687). Production of the soluble ectodomain of ST3N would allow large-scale sialylation of deficiently sialylated recombinant proteins, thus increasing their half time in circulation. Additionally, the α2,3-linked sialic acid is a component of the tetrasaccharide sialyl Lewis X exposed on lymphocytes (704). This epitope is recognized by selectins present on endothelial cells that line the blood vessel walls and is required for tissue invasion and inflammation.

In order to facilitate folding and ER exit the cytosolic and transmembrane domain of ST3N were omitted and the soluble catalytic ectodomain ST3Ne was fused to the Hsp150Δ and MFα carriers. Both carriers were equally effective in promoting the folding of the ST3Ne portion to a catalytically active and secretion-competent conformation in the ER of *P. pastoris*. Approximately 50% of

19. Production of recombinant proteins in *P.pastoris.* A) Medium **s**amples corresponding to OD_{600}=2 were collected from strains expressing β-Lactamase, which was fused either to the Hsp150Δ or to the MFα carrier. Quantification of the secretion of β-lactamase to the medium as well as activity measurements was carried out. **B)** Medium samples from strains expressing NGFRe were collected and analyzed by SDS-PAGE followed by silver staining. Endoglycosidase H treated cells are referenced. C) Activity measurement of ST3N fused to either carrier using a radioactive substrate. The donor was sialic acid (CMP[^{14}C]NeuNAc) and the acceptor was lacto-N-tetraose (LNT). The final product was [^{14}C]NeuNAc α 2,3-Galβ1-4GlcNac. D) TRAP activity test was carried out using either the Hsp150 or the MFα carrier and the distribution of the activity was measured. E) Electron microscopy pictures of *P.pastoris* expressing Hsp150Δ-β-lactamase or Hsp150Δ-ST3N and control were collected at diverse time points and prepared for TEM.

the total active protein remained attached to the cell wall, the other half remained intracellular in both cases (**Figure 19C**). The Hsp150Δ carrier produced 6,7 fold more protein than the MFα carrier. TEM analysis of *P. pastoris* strains expressing Hsp150Δ-ST3Ne, showed enlargement of the cell wall during the induction period (**Figure 19E**). This cell wall thickening may be caused by the increased deposit of Hsp150Δ-ST3Ne.

This data suggest that the yeast strain carrying Hsp150Δ–ST3Ne could serve as a self-perpetuating and inexpensive source of α2,3-sialyltransferase activity. By immobilizing ST3Ne on the cell wall, substrates may diffuse through the cell wall and gain access to the cell wall-bound enzyme. After sialylation the product is released back into the medium and therefore easily purified afterwards (652, 705). This straightforward method could potentially allow the large-scale sialylation of glycoproteins and oligosaccharides produced either in yeast or by other hosts. By adding these mammalian specific modifications, it may be possible to increase the circulation half-time or even use it for enzyme assisted synthesis of glycodrugs for use in anti-inflammatory therapy (706). Furthermore it is possible to co-express both α-2,3-sialyltransferase and α-1,3-fucosyltransferase VII ectodomains in *S. cerevisiae* (707). Both of these enzymes are actively targeted to the cell wall and were capable of producing the tetrasaccharide sialyl Lewis X epitope. Thus suggesting, that yeast cells can indeed be used as a self-perpetuating source of glycosyltransferase activity.

Next, we attempted to express rat bone tartrate-resistant acid phosphatase type 5 (TRAP), which is highly expressed by osteoclasts, activated macrophages and neurons (708). TRAP is associated with osteoblast migration to bone resorption sites, and once there it initiates osteoblast differentiation, activation and proliferation by secreting TRAP into the bone area, where resorption occurs by dephosphorylation of the bone matrix phosphoproteins (709). TRAP is commonly used as a histochemical and biochemical marker for osteoclasts and bone resorption. It is also used to diagnosis hairy cell leukemia and signals of bone breakdown/destruction by cancer. TRAP is translated as a monomeric polypeptide, but the purified enzyme is composed by two disulfide-linked subunits (20 and 16 kDa) (710, 711). The deduced amino acid sequence of human, rat and mouse TRAPs, show a high degree of similarity to mammalian purple acid phosphatases. TRAP is composed of 327 amino acids with a putative cleavable signal peptide of 21 amino acids. The mature form of TRAP was joined to the Hsp150Δ and MFα carrier, after the Kex2p cleavage site (**Figure 15**).

In the case of the Hsp150Δ carrier, the vast majority of active TRAP produced was secreted to the cell exterior (61% in the cell wall and 29% in the culture medium) and only a very small portion remained intracellular (<10%) (**Figure 19D**). In contrast, when using the MFα carrier, a lower amount was secreted (21% in the cell wall and 16% in the culture medium), with the majority of the protein accumulating intracellular (62%). Western blot analysis confirmed that the Hsp150Δ carrier was more skilled than it's rival MFα carrier, in conferring secretion competence to fused TRAP. The Hsp150Δ carrier produced 3,6 fold more protein than the MFα carrier. Using our experimental conditions we managed to secrete a total of 40,6 mg/L of active TRAP (15,6 mg/L

in the culture medium and 25mg/L in the cell wall), which can be classified as an efficient secretion rate for shake flask growth. For comparison, a previous study using the baculovirus expression system, obtained a maximal level of 4.3 mg/L of medium (712).

Producing TRAP using this relatively straightforward and inexpensive procedure, could potentially allow its use, for example, in tissue regeneration therapy. Where application of a thin layer of TRAP on the surface of the new biomaterials being discovered, may promote their use as artificial bone substitutes. The intrinsic bone matrix remodeling activity of TRAP, may promote the integration of the new material with the pre-existing material, mimicking the natural process of bone deposition on an osteoclast resorbed bone surface (713, 714).

To further test the ability of the Hsp150Δ carrier to promote correct folding and secretion of the heterologous protein fused to it, we decided to choose a protein that would challenge the yeast chaperone machinery. For this purpose we selected the rat nerve growth factor receptor ectodomain (NGFRe). NGFRe has 41 potential O-glycosylation sites, one N-glycosylation site and 24 cysteine residues, and it is structurally arranged into four domains, each containing three disulfide bonds. (690, 692, 715). Furthermore, heterologous proteins with complex conformations or multiple disulfide bonds are often misfolded and targeted for degradation in yeast (688). When expressing this protein in *P. pastoris* NGFRe was secreted to the culture medium revealing that the protein adopted in the ER the correct conformation (**Figure 19B**). However in this case, the MFα carrier was more efficient in promoting the secretion of NGFRe (approximately 2 fold more) than the Hsp150Δ carrier was. Our previous studies on the expression of NGFRe in *S. cerevisiae* revealed that NGFRe was N-glycosylated, but not O-glycosylated (690, 692) whereas in *P. pastoris* the NGFRe portion is also O-glycosylated.

There is no consensus primary amino acid sequence for O-glycosylation. Hence it should not be assumed that *P. pastoris* will not glycosylate a heterologous protein just because that protein is not glycosylated by its native host, neither should it be assumed that the specific Ser and Thr residue(s) selected for O-glycosylation by *P. pastoris* will be the same as their native host (702).

In conclusion it appears that the Hsp150Δ carrier was efficient in promoting proper folding of heterologous proteins. The ability of Hsp150Δ to function as a good carrier appears to reside in the fact that it does not interfere with the folding of the fused protein. Structural analysis showed that the repetitive region within SUII occurs as random coil. A prediction that is supported by the fact that this regions is heavy O-Glycosylated (647, 666) and extensive O-glycosylation cause peptides to adopt extended rod-like configurations due to steric interference (716).

Thus it appears that the lack of structure in the repetitive region of Hsp150 allows the fused protein to adopt its native conformation and therefore bypass the quality control machinery in the ER, resulting in efficient ER exit and secretion.

4. Final remarks

This present study provides evidence that *in vivo*, COPII vesicles are covered by different compositions of the COPII components. By alternating Sec24p and its homologues Sfb2p and Sfb3p, the cargo repertoire that the COPII coat recognizes is altered. In the absence of Sec24p, Sfb2p or Sfb3p may replace Sec24p in the COPII coat. However in the absence of all Sec24 members, we found that Hsp150 was specifically recruited for ER exit, while other exocytic proteins remained in the ER. Under these conditions an aberrant coat was evidently formed. The signature guiding Hsp150 for ER exit under these conditions is located in the C-terminal domain of the protein, which suggests an active and specific recruitment rather than bulk flow.

In this study we also showed that Hsp150 was secreted under conditions where the exocyst component Sec15p was defective, meanwhile other secretory cargo remained intracellularly. We found that Hsp150 is transported in HDSV vesicles and in a novel class of very heavy density vesicles. The selective incorporation of Hsp150 into this novel class of vesicles may be responsible for its secretion in *sec15-1* mutant cells. Moreover, we found that mutants that abolish the formation of the HDSV population of secretory vesicles by disturbing the endosomal/vacuolar pathway, did not affect the secretion of Hsp150. However, secretion of Hsp150 was dependent on Mso1p, which may play a role in stabilization of secretory vesicles at the plasma membrane interface, and hence allow secretion of a subset of secretory vesicles. The signature guiding Hsp150 exit in *sec15-1* cells did not reside in the C-terminal domain of Hsp150, like in the case of COPII independent pathway, but rather in subunit I or in the first 4 repeat (**Figure 15**). Thus it appears that Hsp150 uses different putative receptors along the secretory pathway. This feature may allow its efficient sorting and rapid movement through the yeast exocytic pathway

Although Hsp150 is not an essential protein, its ability to escape multiple secretory blocks, while other proteins are trapped, suggests that it may have an important biological function. As secretion of Hsp150 is rapid and the *HSP150* gene is strongly expressed at 37°C, it may play a role under heat shock conditions. However, deletion of *HSP150* yielded no obvious phenotype (647). In yeast there are three proteins homologous to Hsp150, *PIR1/CCW6*, *PIR3/CCW8* and *PIR4/CCW5*, but none of them are upregulated at 37°C. Deletion of all family members is non-lethal, but since the quadruple mutant exhibits cell wall defects, it appears that one function of the PIR family proteins is in cell wall stabilization (717). Taking into count the ability of Hsp150 to escape multiple secretory blocks, Hsp150 may have a more sophisticated role. The Hsp150Δ carrier confers secretion competence to fused heterologous proteins, which indicates a possible chaperoning role for Hsp150. Thus, Hsp150 might escort proteins through the secretory pathway under stress conditions.

Understanding how parallel secretory pathways are coordinated is a key challenge for future research and will allow us to understand the dynamic nature of the yeast secretory pathway, and possibly how more complex secretion pathways evolved in higher eukaryotes.

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