Functional and structural characterization of a yeast membrane protein involved in the secretory pathway

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1 Introduction

1.1 Newly synthesized proteins enter the secretory pathway through translocation into the ER

A typical yeast cell contains about 5000 different kinds of proteins. For a cell to function properly, each of its numerous proteins must be localized to the correct internal sites. The process of directing each newly made polypeptide to a particular destination – referred to as protein targeting – is critical to the organization and functioning of eukaryotic cells. A key choice for protein targeting is made soon after synthesis of a protein begins at a cytosolic ribosome. Protein synthesis continues in the cytosol unless the nascent polypeptide chain contains a specific signal or targeting sequence that directs the ribosome to the endoplasmic reticulum (ER). Most nuclear, mitochondrial and peroxisomal proteins are synthesized by free ribosomes in the cytosol and are posttranslationally imported into the organelles, whereas the polypeptide chains that are synthesized at ribosomes bound to the ER membrane are cotranslationally threaded through the membrane. This translocation process is stopped only for generating integral membrane proteins that contain a membrane anchor sequence. After translocation the ER signal sequence is cleaved off and heat shock proteins in the ER lumen serve as chaperones that bind the translocated polypeptide chains and assist in their folding. Glycoproteins acquire in the ER from a dolichol pyrophosphate donor the core of their *N*-linked sugars and the C-terminus of proteins destined for the plasma membrane is covalently attached to a sugar residue of a glycolipid, the glycosylphosphatidylinositol (GPI) anchor.

1.2 The biosynthetic-secretory pathway leads outward from the ER via the Golgi to the cell surface or to the lysosomes

All proteins that cross the ER membrane from the cytosol enter the secretory pathway. Quality control pathways in the ER ensure that only properly folded proteins are allowed to exit the ER (Molinari and Helenius, 2000). The outward traffic is highly organized and leads from the ER towards the Golgi apparatus and cell surface, with a side route leading to lysosomes. It is mediated by transport vesicles, which transfer proteins from membrane to membrane by cycles of vesicle budding from a donor compartment and vesicle fusion to an acceptor compartment. Correctly folded

proteins are transferred first from the ER to the *cis*-Golgi by COPII coated vesicles, whereby ER resident proteins, such as chaperones, are recognized by their carboxy-terminal KDEL or di-lysine motif and are retrieved to the ER by COPI vesicles (Pelham, 1995). The Golgi apparatus consists of a stack of membranous cisternae that are separated into *cis*-, *medial*- and *trans*-compartments. Distinct coated vesicles deliver cargo proteins from the *cis*- to the *medial*- and finally to the *trans*-Golgi compartments. The different cisternae of the Golgi contain diverse sets of enzymes that introduce various modifications to cargo proteins. For example, carbohydrate units of glycoproteins are modified in each of the compartments of the Golgi dependent on the sequence of the protein undergoing glycosylation and on the glycosyl transferase composition of the respective Golgi cisterna. The oligosaccharides promote stability of the glycoproteins and serve as a targeting determinant. In mammalian cells glycoproteins destined for lysosomes are, for example, phosphorylated in the *cis*-Golgi, yielding multiple mannose 6-phosphate (M6P) residues. M6P receptors bind the phosphorylated proteins in the *trans*-Golgi and direct their transfer to the late endosomes, where receptors and proteins dissociate (Kirchhausen et *al.*, 1997). The late endosomes then fuse to the lysosomes. Other vesicles budding from the *trans*-Golgi deliver secretory proteins to the plasma membrane. In these transport vesicles most secretory proteins undergo proteolytic cleavage that yields the active proteins.

The molecular mechanisms of budding and fusion are the key events of the vesicular protein transport. Nowadays, its critical players - coat proteins, small GTP-binding proteins (Arf and Ypt proteins) and SNARE proteins - are well characterized. The event of vesicle budding is mainly controlled by GTPases. The idea for vesicle fusion is that GTPases drive the association of tethering proteins situated on each membrane (Whyte and Munro, 2001). These tethering proteins interact with SNAREs, which have a helical structure. It is the formation of four-stranded helical bundles between membrane-anchored SNAREs on vesicles (*v*-SNAREs) and their cognates on target membranes (*t*-SNAREs) that is thought to drive membrane fusion (Pelham, 2001). Up to now it is not clear whether SNARE compatibility or previous assembling of tethering factors gives specificity in membrane fusion.

1.3 ER-Golgi transport is mediated by COPII vesicles

In yeast cells proteins are transported from the endoplasmic reticulum (ER) to the Golgi apparatus through membrane-bounded vesicles. During the budding process a set of soluble cytosolic proteins forms a coat, termed COPII, around these vesicles. The small GTPase Sar1p binds, after switch of its GDP-bound to its GTP-bound form under the stimulation of the guanine nucleotide exchange factor Sec12p, directly to the lipid surface of the ER membrane (Barlowe et *al.*, 1994). Integral membrane cargo is then selected for incorporation into COPII vesicles by the specific, Sar1p•GTP-dependent binding to the cytosolic heterodimer Sec23p-Sec24p (Kuehn et *al.*, 1998; Springer and Schekman, 1998). It has been proposed that the nonessential Sec16p facilitates, like a scaffold, the assembling of Sar1p and Sec23p-Sec24p at the ER membrane by direct interaction with the three components as well as with the lipid bilayer (Shaywitz et *al.*, 1997). Sec16p also interacts with Sec31p of the Sec13p-Sec31p heterotetramer (composed of two copies of Sec13p and Sec31p each), which after its recruitment to the ER membrane (Shaywitz et *al*., 1997; Lederkremer et *al.*, 2001) initiates formation of the budding vesicle. After budding from the ER, the next steps in vesicle movement are the inactivation of Sar1p through the GAP-activity (GTPase activating protein) of Sec23p and the subsequent release of the COPII coat proteins from the vesicle membrane (Rothman and Wieland, 1996). So, nucleotide exchange and GTP hydrolysis are spatially and temporally organized to coordinate coat assembly with cargo selection and coat disassembly with vesicle fission (Antonny and Schekman, 2001). Binding of Sar1p to the membrane depends upon neutral phospholipids (Gorelick and Shugrue, 2001), whereas the efficient recruitment of the Sec23/Sec24p complex requires the presence of acidic phospholipids such as phosphatidylinositol 4,5-diphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PI4P) (Matsuoka et *al.*, 1998). Budding seems to occur at distinct regions on the ER, which have been termed “privileged sites” (Hobman et *al*., 1998). Although the mechanisms responsible for establishing these sites are unknown, it is possible that selectively concentrating phospholipids may play a role in establishing these sites. Morphological studies have provided convincing evidence that also cargo proteins are concentrated during vesicle budding (Hong, 1998). Integral membrane cargo is enriched up to 10-fold as it exits the ER (Balch et *al.*, 1994). The binding to the COPII coat components Sec23p-Sec24p causes membrane proteins, such as VSV-G in mammalian cells (Aridor et *al.*, 1998)

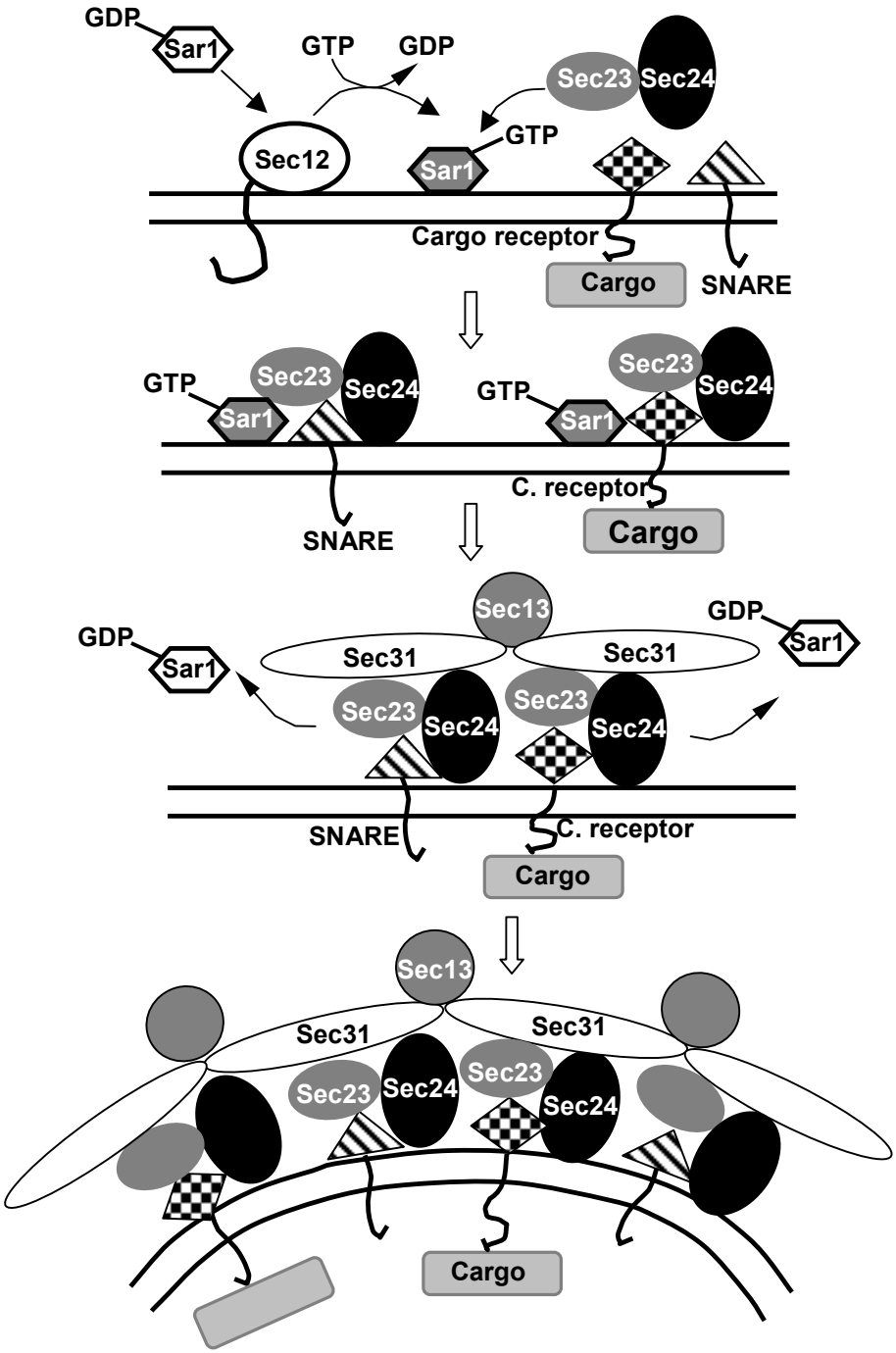

Figure 1. Scheme of COPII vesicle formation [modified from (Schekman and Orci, 1996); kindly provided by A. De Antoni].

and the *t*-SNARE Sed5p in yeast cells (Peng, 1999), to segregate into a complex that excludes ER resident proteins such as the ER chaperone BiP, the mammalian homologue of the yeast Kar2 protein (Aridor et *al.*, 2001). The specificity of the cargo-vesicle coat interaction implies the presence of sorting signals in the cytosolic sequence of membrane cargo. Two signals involved in the selection of cargo have already been described for mammalian cells. First, a di-acidic sorting signal (DXE,

where X is any amino acid) in the cytosolic tail sequence of VSV-G has been reported to take part in concentrating the protein to the bud site and in accelerating its ER export (Nishimura et *al.*, 1997 and 1999; Aridor et *al.*, 2001). The importance of this sequence has been confirmed in two studies on the transport of the inwardly rectifying potassium channel proteins Kir1.1, Kir2.1 and Kir4.1, which contain the related ER export sequences EXE and EXD (Ma et *al.*, 2001; Stockklausner et *al.*, 2001). The membrane associated protein PRA1 also is transported to the Golgi complex through its DXE motif at the carboxyl terminus (Abdul-Ghani et *al.*, 2001). Secondly, a C-terminal di-phenylalanine (FF) motif mediating binding to the COPII coat is important for efficient ER-to-Golgi transport of ERGIC-53, a receptor for glycoproteins and of cargo receptors of the p24 family (Fiedler et *al.*, 1996; Kappeler et *al.*, 1997; Dominguez et *al.*, 1998). A recent study suggests that the FF motif interacts with the Sec13p-Sec31p heterotetramer (Belden and Barlowe, 2001) and not, as the DXE motif does, with Sec23p-Sec24p (Votsmeier and Gallwitz, 2001). Examples for soluble cargo selection are the binding of the membrane receptors ERGIC-53 and Erv29p to coagulation factors V and VIII, and pro-α-factor gpαf, respectively, that are sorted out of the ER through this binding (Belden and Barlowe, 2001; Nichols and Ginsburg, 1999). The concentration of soluble proteins like albumin to ER exit sites has also been described (Mizuno and Singer, 1993).

Further refinement of the selection process has been observed with isoforms of Sec24p. In addition to Sec24p, the yeast *Saccharomyces cerevisiae* expresses two non-essential Sec24p-related proteins, termed Sfb2p and Sfb3p/Lst1p (Peng et *al.*, 2000). The deletion of Sfb3p/Lst1p results in a selective deficiency in delivery of the plasma membrane ATPase Pma1p, but not other plasma membrane proteins (Roberg et *al.*, 1999). The vesicles formed with a mixed coat of Sec23p-Sec24p and Sec23p-Sfb3p are larger and therefore show structural differences compared to standard COPII vesicles (Shimoni et *al.*, 2000). Nevertheless, these homologues show overlapping functions, since Sfb2p and, with lower efficiently, Sfb3p/Lst1p are, like Sec24p, able to bind the integral membrane cargo protein Sed5p (Peng et *al.*, 2000).

1.4 The Ypt family of small GTPases

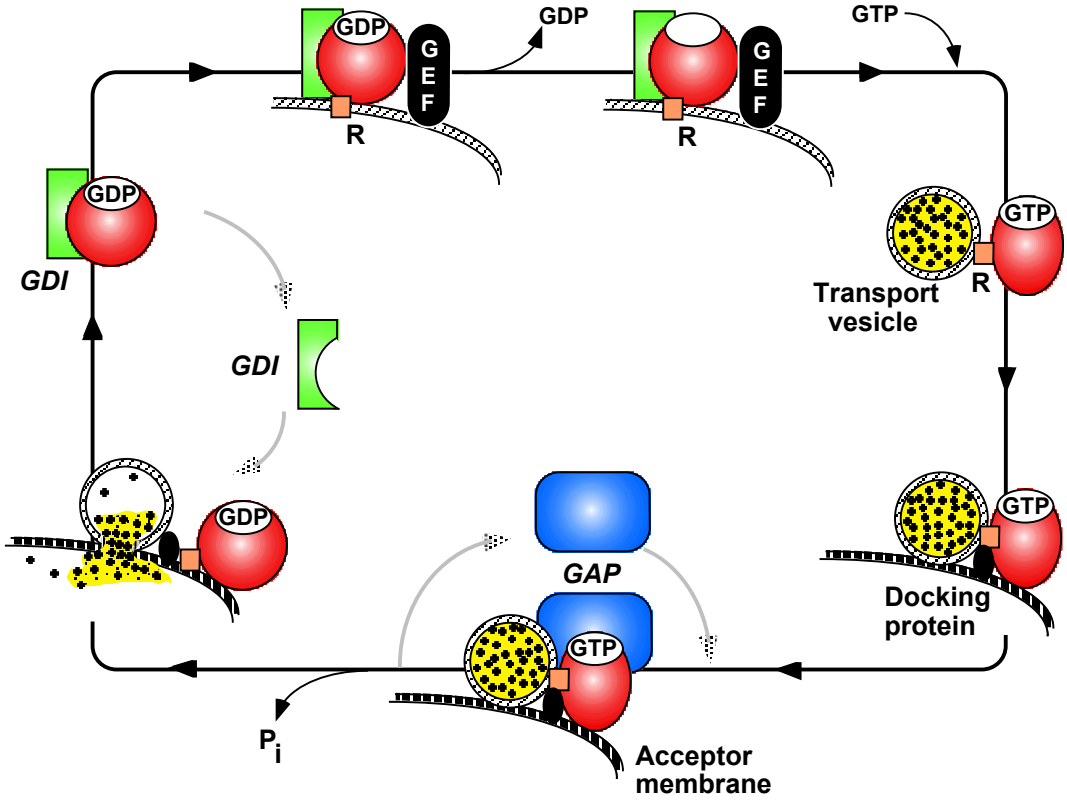

Figure 2. Model of the Ypt GTPase cycle. The inactive GDP-bound form of the Ypt protein is kept in a soluble state by the GDP-dissociation inhibitor (GDI). After docking to a putative membrane receptor (R), GDI dissociates from transport GTPases. Upon membrane binding, a guanine nucleotide exchange factor (GEF) catalyzes GDP/GTP exchange. The activated Ypt GTPase most likely acts in the assembly of a "docking protein complex", which facilitates membrane docking. A GTPase activating protein (GAP) accelerates the GTP hydrolysis and the GDP-bound form of the Ypt can be solubilized by GDI and used in a new cycle. (Kindly provided by D. Gallwitz)

A family of GTP-binding proteins participates in the control of vesicular traffic in eukaryotic cells. All of these Rab (in animal cells) and Ypt (in yeast cells) proteins contain around 200 amino acids and have an overall structure similar to the Ras protein. Ypt/Rab GTPases and their involvement in membrane traffic were first discovered by the characterization of Ypt1p and Sec4p in yeast (Gallwitz et *al.*, 1983; Salminen and Novick, 1987). Like Ras, purified Ypt1p binds and hydrolyzes GTP, and the cycle of GTP binding and hydrolysis regulates the rate of COPII vesicle fusion to the Golgi membrane (Figure 2). Its GTP-bound form is considered as its active state and the GDP-bound form as its inactive state. The inactive GDP-bound form of the Ypt protein is kept in a soluble state by the GDP-dissociation inhibitor (Sec19p=GDI). Docking of the GTPase to a membrane receptor induces a

conformational change, which results in the dissociation of Sec19p (Ullrich et *al.*, 1994). Recently, the Yip1p-Yif1p complex has been described as a candidate membrane receptor for Ypt1p on Golgi membranes (Yang et *al.*, 1998; Matern et *al.*, 2000). Once a GDP-bound GTPase associates with the membrane, GDP has to be exchanged for GTP, in order to activate the protein (Soldati et *al.*, 1994). The exchange reaction is catalyzed by a guanine nucleotide exchange factor (GEF) (Cherfils and Chardin, 1999). For Ypt1p the TRAPP complex has been proposed to fulfill this function (Jones et *al.*, 2000; Wang et *al.*, 2000). Since Ypt1p acts in the docking event of ER-to-Golgi vesicular transport, it was suggested that it recruits the vesicle docking factor Uso1p or the Sec34p-Sec35p-complex to the membrane and promotes, directly or indirectly, SNARE-complex formation (Cao et *al.*, 1998; Allan et *al.*, 2000; VanRheenen et *al.*, 1999; Whyte and Munro, 2001). Finally, a GTPase activating protein (GAP) accelerates the GTP hydrolysis, and the GDP-bound form of the Ypt can be solubilized by GDI and used in a new cycle. GAPs for Ypt1p are Gyp1p (Albert et al., 1999; Du and Novick, 2001) and two newly discovered members of the GYP family, Gyp5p and Gyp8p, of which Gyp5p has a remarkable specificity for Ypt1p (De Antoni et *al.*, submitted for publication).

1.4.1 Ypt6p is involved in retrograde transport to the *trans*-Golgi

In *Saccharomyces cerevisiae* there are 11 Ypt GTPases with distinct sites of action and they all regulate transport between the various organelles by cycling

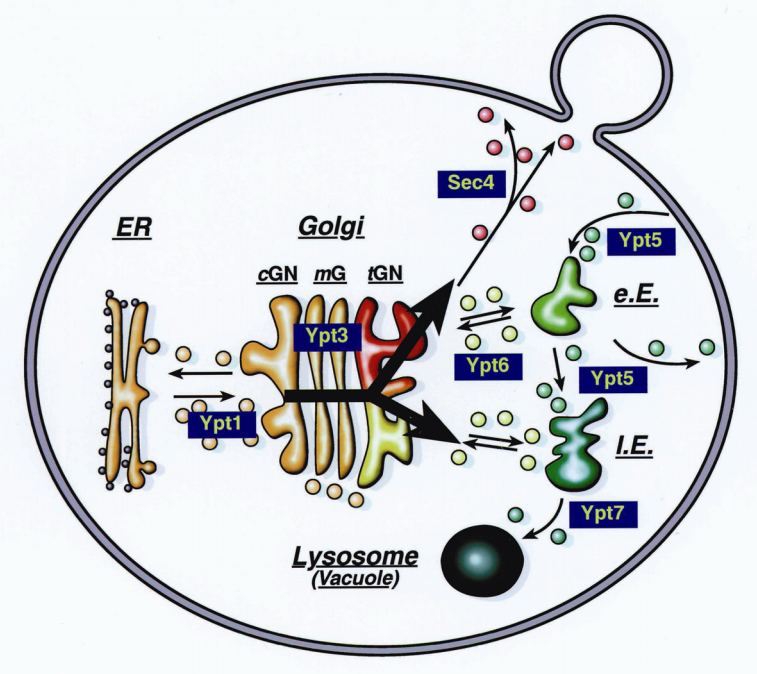

Figure 3. Scheme of intracellular transport in the yeast cell. GTPases of the Ypt family acting at different transport steps in yeast are indicated. (Kindly provided by D. Gallwitz)

between the GDP- and the GTP-bound state (Figure 3) (Lazar et *al.*, 1997; Segev, 2001). Transport to and from the Golgi requires the function of several GTPases. As mentioned above, Ypt1p is required for docking of ER-derived vesicles with early Golgi compartments containing the SNARE Sed5p (Cao and Barlowe, 2000). The related pair Ypt31/32p has been suggested to act on exit from the Golgi (Benli et *al.*, 1996). Another Golgi-associated GTPase is Ypt6p, the yeast homologue of mammalian Rab6. This protein is thought to be required for the delivery of vesicles from endosomes to the late Golgi (Tsukada and Gallwitz, 1996). In yeast, all known membrane proteins of the late Golgi cycle through an endocytic compartment and have to be retrieved constantly. In *ypt6* mutants, this process fails, and Golgi proteins such as Kex2p and the sorting receptor Vps10p are mislocalized to the vacuole. The vacuolar protease carboxypeptidase Y, which is sorted by Vps10p, is partially secreted as a result (Tsukada et *al.*, 1999). Recently some accessory proteins of Ypt6p were elucidated (Figure 4). First, the guanine nucleotide exchange factor (GEF) Ric1p-Rgp1p has been shown to activate Ypt6p to its GTP bound form. The heterodimer Ric1p-Rgp1p is a peripheral membrane protein restricted to the Golgi (Siniossoglou et *al.*, 2000). Secondly, the conserved trimeric protein complex VFT (Vps52/53/54p) binds directly to Ypt6p•GTP and to the *v*-SNARE Tlg1p and has been proposed to direct Tlg1p-containing vesicles to their target, which is defined by the local activation of Ypt6p (Siniossoglou and Pelham, 2001; Conibear and Stevens, 2000). Also Sgm1p binds Ypt6p and may provide an additional tethering function (Will and Gallwitz, unpublished result; Siniossoglou and Pelham, 2001). Third, Gyp2p, Gyp6p and Gyp8p have been shown to be the primary GAPs for the acceleration of the slow intrinsic GTPase activity of Ypt6p (Strom et al., 1993; Albert and Gallwitz, 1999; Will and Gallwitz, 2001).

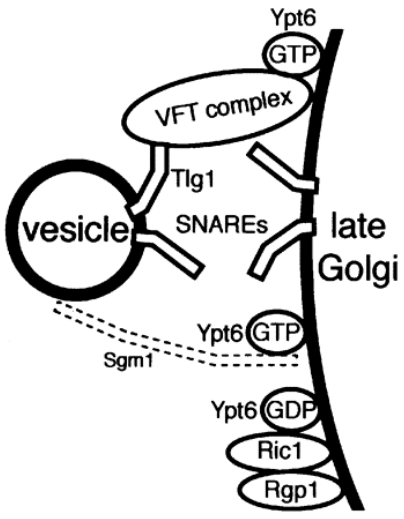

Figure 4. Model of the fusion event of endosome-derived vesicles with the late Golgi. The Ric1p–Rgp1p complex activates Ypt6p by nucleotide exchange at the late Golgi membrane. Ypt6p•GTP recruits the VFT complex, which also interacts with the N-terminal domain of Tlg1p on vesicles (and/or on the Golgi membrane). SNARE engagement and fusion follow. Sgm1p also binds Ypt6p and may provide an additional tethering function, though this remains to be shown (Siniossoglou and Pelham, 2001).

1.4.2 High expression of the Sys proteins suppresses the perturbations of endosome-to-Golgi transport in *ypt6* mutant cells

Sys1p, Sys2p, Sys3/Imh1p and Sys5p were identified by their ability to suppress at high intracellular concentration the perturbations of endosome-to-Golgi transport in *ypt6* mutant cells (Tsukada and Gallwitz, 1996), whereby Sys3/Imh1p was already identified before as a low-copy suppressor of a truncation allele of *YPT6* (Li and Warner, 1996). All four Sys proteins can complement the temperature-sensitive growth phenotype of the *ypt6* null mutant and, when overexpressed, even partially suppress the missorting of vacuolar enzymes. Gene disruption of *SYS1*, *SYS2/SRO9* and *SYS3/IMH1* did not result in a significant growth defect, whereas the deletion of each *SYS* gene in the *ypt6* null mutant enhances defects in cell growth and also in vacuolar protein sorting. The *ypt6*/*sys1* double and the *ypt6*/*sys1*/*sys2* triple mutants show a severe fragmentation of the vacuole, and the *ypt6*/*sys3* double mutant accumulates 40- to 50-nm vesicles (Tsukada and Gallwitz, 1996; Tsukada et *al.*, 1999). The Ric1p component of the heterodimeric Ypt6p-GEF shows genetic interactions with *SYS3/IMH1* (Siniossoglou et *al.*, 2000).

1.4.3 Ypt6p could also be involved in retrograde transport to the *cis*-Golgi

According to the obtained data, Ypt6p has been suggested to act in retrieval of proteins from endosomes to the *trans*-Golgi. However, defects caused by the absence of either Ric1p or Ypt6p can be suppressed by multicopy plasmids expressing the *v*-SNAREs Ykt6p and Gos1p, as well as *SLY1-20*, which is an activator of the *cis*-Golgi *t*-SNARE Sed5p (Bensen et *al.*, 2000). These results suggest that the Ypt6p pathway leads to the *cis*-Golgi rather than the *trans*-Golgi, as the *v*-SNAREs Ykt6p and Gos1p form complexes with the *cis*-Golgi *t*-SNARE Sed5p (McNew et *al.*, 1997). Consistent with this function, the overexpression of Ypt1p can suppress *ypt6* mutant defects *in vivo*, suggesting that Ypt1p can substitute for Ypt6p (Li and Warner, 1998).

SYS1 and *SYS2* show also a genetic interaction with *SEC7*, a protein involved in intra and post Golgi transport (Murén et *al.*, 2001; Tsukada and Gallwitz, 1996). Wiederkehr and co-workers suggest an involvement of Sys1p in endocytosis (Wiederkehr et *al.*, 2001).

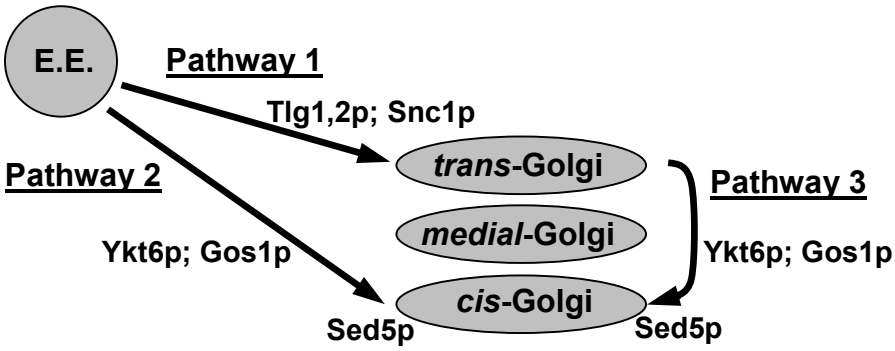

Figure 5. Models for Ypt6p-mediated retrieval of proteins from the early endosome. The arrows indicate possible Ypt6p-dependent pathways. Ypt6p has been suggested to act in retrieval of proteins from endosomes to the *trans*-Golgi (Pathway 1) (Tsukada and Gallwitz, 1996; Siniossoglou and Pelham, 2001). *cis*-Golgi-targeted vesicles containing Gos1p and possibly Ykt6p may originate from the early endosomes (Pathway 2) or from the *trans*-Golgi (Pathway 3; Bensen et *al.*, 2001).

1.5 Objective of the thesis

Previously, Sys1p was identified as a high copy suppressor of Ypt6 GTPase-deficient yeast mutants that are defective in endosome-to-Golgi transport. The amino acid sequence of Sys1p suggests that it is an integral membrane protein. Although no other protein with significant homology was found in the databases, Sys1p with its four putative membrane-spanning domains appears to be structurally related to the synaptic vesicle protein synaptogyrin. The subsequent idea was that Sys1p is, like synaptogyrin, a resident of transport vesicles carrying cargo proteins to or from the Golgi that might have a function within the Ypt6p-mediated pathway (Tsukada and Gallwitz, 1996). First, the membrane topology and the intracellular localization had to be examined. It was thought that the termini of Sys1p face the cytosol and N- and C-terminal truncation mutants could reveal which domain is important for proper suppressor function. Such a presumed functional domain could be screened for putative interaction partners by two-hybrid- and affinity chromatographical studies. The resulting Sys1p-interacting proteins could add to an understanding of the Ypt6p-mediated pathway and the functioning of the growing family of tetraspanning membrane proteins that reside on different organelles involved in several transport steps. Their cytoplasmic tails might play an important role in the regulation of anterograde and/or retrograde vesicular traffic within the cell.

2 Materials

2.1 Strains

2.1.1 Bacterial strains

Table 1

Strain	Genotype	Source
DH5α	F⁻ϕ80 d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 supE44 λ⁻*gyr*A96 *rel*A1	Gibco-BRL (Karlsruhe, Germany)
DH5α F´IQ	F´*pro* AB⁺*lac*I^q ZΔM15 zzf::Tn5[Kan^r]/ ϕ80 d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *sup*E44 λ⁻*gyr*A96 *rel*A1	Gibco-BRL
BL21	*F*⁻ ompT hsd*S* $_{(rB\text{-}mB\text{-})}$ gal dcm	Novagen
BL21(DE3)	*F*⁻ ompT hsd*S* $_{(rB\text{-}mB\text{-})}$ gal dcm *(DE3)*	Novagen

2.1.2 Yeast strains

Table 2 – Strains not generated in this work

Strain	Genotype	Source
cl3-ABYS-86	MATα *ura3-Δ5 leu2-3 112 his3 pra1-1 prb1-1 prc1-1 cps1-3 can^R*	(Heinemeyer et *al.*, 1991)
SEY6210	MATα *leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	(Robinson et *al.*, 1988)
MT1	MATα *his3 leu2 ura3 ypt6::HIS3*	(Tsukada and Gallwitz, 1996)
ADY6	MATα *ura3-Δ5 leu2-3 112 his3 pra1-1 prb1-1 prc1-1cps1-3 can^R SFB2-6His-3HA-loxP-KanMX-loxP SFB3-6His-2MYC-loxP*	(Peng et *al.*, 2000)
SSY78	MATa *trp1 his4 ura3 leu2 EMP47-MYC::LEU2 pHA-PMR1::URA3 sec23-1*	(Ossipov et *al.*, 1999)
RH1491	MATa *ura3 leu2 his3 sec12-4*	(Schröder et *al.*, 1995)
Y190	MATa *gal4 gal80 trp1-901 ade2-101 ura3-52 leu2-3,112 ura3::GAL→lacZ lys2::GAL→HIS3 cyh^r*	(Elledge et *al.*, 1991)
L40	MATa *his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_8$-lacZ gal4 gal80*	(Hollenberg et *al.*, 1995)
MSUC-3D	MATα *ura3 trp1 leu2 his3 lys2*	This Department
MSUC-IA	MATa *ura3 trp1 leu2 his3 ade8*	This Department
Y20242	MATa/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 WBP1::kanMX4/WBP1*	Euroscarf, Frankfurt
RH270-2B	MATa Gal⁺ *ura3 leu2 his4 lys2 bar1-1*	(Schröder et *al.*, 1995)
GFUI-5B	MATα *his3 leu2 ura3*	(Dascher et *al.*, 1991)

2.2 Plasmids

2.2.1 *E. coli* cloning and expression vectors

Table 3

Vector	Description	Source
pGEX-TT	Expression vector for production of GST fusion proteins. Contains the polylinker from pEG-KT, tac promoter, *lacI*^{q} repressor, ampicillin resistance	(Albert, et *al.*, 1999)
pQE30	Expression vector for production of N-terminus 6xHis tagged proteins. T5 promoter, *lac* operator, ampicillin resistance	QIAGEN
pMAL-c2	Expression vector for production of MBP fusion proteins,. Ptac promoter, *lacI*^{q} repressor, ampicillin resistance	New England Biol. (Frankfurt, Germany)

2.2.2 Yeast vectors

Table 4

Vector	Description	Source
pUG6	Plasmid containing the loxP-*KanMX*-loxP gene disruption cassette.	(Güldener et *al.*, 1996)
pUG24	Plasmid containing the *loxP-GFP-KanMX-loxP cassette* for PCR mediated tagging	(Güldener et *al.*, 1996)
pUG36		(Güldener et *al.*, 1996)
pU6H2MYC	Plasmid containing the *6His-2MYC-loxP-KanMX-loxP* cassette for PCR mediated epitope tagging.	(De Antoni and Gallwitz, 2000)
pU6H3HA	Plasmid containing the *6His-3HA-loxP-KanMX-loxP* cassette for PCR mediated epitope tagging	(De Antoni and Gallwitz, 2000)
pYX213	2µ, *URA3*, GAL1-promoter	R&D-Systems
pAS2	2µ, *TRP1*, ADH1-promoter; Gal4-DNA-BD	(Elledge et *al.*, 1991)
PACTII	2µ, *LEU2*, ADH1-promoter; Gal4-AD	(Elledge et *al.*, 1991)
pBTM116	2µ, *TRP1*, ADH1-promoter; LexA-DNA-BD	(Hollenberg et *al.*, 1995)
pRS315	Cen, *LEU2*	(Sikorski and Hieter, 1989)
pRS325-*SYS1*	Contains the complete reading frame of Sys1p	(Tsukada and Gallwitz, 1996)

2.3 Oligonucleotides

Table 5 – Oligonucleotides for PCR mediated epitope tagging

Name	Sequence	Purpose / Vector
(The underlined sequences anneal to the corresponding pUtag vector)		
SYS1-tag1_f	5'-GAG CAA TCA CCA ATA CAA CTA AAA GAC TTA GAA AGC CAA ATA TCC CAC CAC CAT CAT CAT CAC-3'	*SYS1*-HA-tagging pUGH2MYC/pUGH3HA
SYS1-tag1_r	5'-TTA AAT ATA GAT TGT GTA TAA ATA AGA AAT AGG ATC TGT TTT ACT ATA GGG AGA CCG GCA GAT C-3'	*SYS1*-HA-tagging pUGH2MYC/pUGH3HA

SYS1(1-186)-tag_f	5'-GAA GTT GGA TTG GTT ACG CCT AGT CAG CAG CAT AGT AAT CAT TCC CAC CAC CAT CAT CAT CAC-3'	*SYS1(1-186)*-tagging pUGH2MYC/pUGH3HA
SYS1(1-186)-tag_r	5'-AAA TTG GCC AAA AAG GCA GAT TTA TTA AGC CTA TAG AGA TAA ACT ATA GGG AGA CCG GCA GAT C-3'	*SYS1(1-186)*-tagging pUGH2MYC/pUGH3HA
SYS1(D198A; E200A)-tag_f	5'-AGT AAT CAT TCA GAA TTG GAG CAA TCA CCA ATA CAA CTA AAA **GCC TTA GCA** AGC CAA ATA TCC CAC CAC CAT CAT CAT CAC-3'	*SYS1(D198A;E200A)*-tagging ; pUGH3HA In bold letters: Sequence coding for **(ALA)** instead of **(DLE)**
SYS1(D198A; E200A)-tag_r	5'-TTA AAT ATA GAT TGT GTA TAA ATA AGA AAT AGG ATC TGT TTT ACT ATA GGG AGA CCG GCA GAT C-3'	*SYS1(D198A;E200A)*-tagging pUGH3HA
SYS1-tag2_f	5'-GAG CAA TCA CCA ATA CAA CTA AAA GAC TTA GAA AGC CAA ATA ATG TCT AAA GGT GAA GAA TTA TTC AC-3'	*SYS1*-GFP-tagging pUG24
SYS1-tag2_r	5'-TTA AAT ATA GAT TGT GTA TAA ATA AGA AAT AGG ATC TGT TTT GCA TAG GCC ACT AGT GGA TCT G-3'	*SYS1*-GFP-tagging pUG24
YDL173-tag_f	5'-CAA CAA GTT CAA CAA ATG GTA GAA ATG CAA GGA AAA ATG AGA TCC CAC CAC CAT CAT CAT CAC-3'	*YDL173w*-tagging pUGH2MYC/pUGH3HA
YDL173-tag_r	5'-GAC AAA CAG TAT GTT GCG ATG GGG TTG CAT GTA TAT AGA TAC ACT ATA GGG AGA CCG GCA GAT C-3'	*YDL173w*-tagging pUGH2MYC/pUGH3HA
YDL173-del_f	5'-CGA GTA GCG TAA GAC CAA GTT AGT ATG GCT ACG TTC AAC CCG CAT AGG CCA CTA GTG GAT CTG-3'	*YDL173w*-knockout pUG6
YDL173-del_r	5'-GGC TCC TTG TAG CGC TGA TAC GAG ACC TGG TGT TGG TCA CCG GGC AGC TGA AGC TTC GTA CGC-3'	*YDL173w*-knockout pUG6

Table 6 – Oligonucleotides for generation of pYX213-*SYS1* constructs

Name	Sequence	Annealing sequence
Bold letters: Restriction site; Underlined: Homologues sequences to SYS1		
*SYS1*_f1	5'-CCG **GAA TTC** ATG GTT TCG ATA AGA AGG TAT-3'	***Eco*RI** Underlined: aa (1-7)
*SYS1*_f2	5'-CCG **GAA TTC** ATG AAT GAG TTG AAA CCT TCC CAG-3'	***Eco*RI** Underlined: aa (12-18)
*SYS1*_f3	5'-CCG **GAA TTC** ATG AAA CAA GAT TCT CTC TCT-3'	***Eco*RI** Underlined: aa (21-27)
*SYS1*_r1	5'-CCC GGG **AAG CTT** TCA TAT TTG GCT TTC TAA GTC-3'	***Hind*III** Underlined: aa (203-197)
*SYS1*_r2	5'-CCC GGG **AAG CTT** TCA ATG ATT ACT ATG CTG CTG ACT-3'	***Hind*III** Underlined: aa (186-180)
*SYS1*_r3	5'-CCC GGG **AAG CTT** TCA TTC ATT AGG ATC AAC CAA GCC-3'	***Hind*III** Underlined: aa (171-165)
*SYS1*_r4	5'-CCC GGG **AAG CTT** TCA TGT TGT CCA TGT CCC CAA AAA-3'	***Hind*III** Underlined: aa (153-147)

Table 7 – Oligonucleotides for generation of pUG36-*SYS1* and pUG36-*SNC1*:

Name	Sequence	Annealing sequence
Bold letters: Restriction site; Underlined: Homologues sequences to *SYS1* or *SNC1*		
*SYS1*_f4	5'-CCG **GAA TTC** ATG GTT TCG ATA AGA AGG TAT-3'	***Eco*RI** Underlined: aa (1-7)
*SYS1*_r5	5'-CCC GGG **AAG CTT** TCA TAT TTG GCT TTC TAA GTC-3'	***Hind*III** Underlined: aa (203-197)
*SNC1*_f1	5'-CCG **GAA TTC** ATG TCG TCA TCT ACT CCC TTT-3'	***Eco*RI** Underlined: aa (1-7)
*SNC1*_r1	5'-CCC GGG **AAG CTT** CTA TCG ACT AAA GTG AAC AGC-3'	***Hind*III** Underlined: aa (117-112)

Table 8 – Oligonucleotides for generation of pGEX-TT-*SYS1* constructs

Name	Sequence	Annotations
Bold letters: Restriction site or mutation; Underlined: Homologues sequences to SYS1		
*SYS1*_f5	5'-TTT TTG **GGA TCC** TGG ACA ACA AGA TGG AGA GAG-3'	***Bam*HI** Underlined: aa (151-157)
*SYS1*_r6	5'-CCC GGG **AAG CTT** TCA TAT TTG GCT TTC TAA GTC-3'	***Hind*III** Underlined: aa (203-198)
*SYS1*_f6	5'-GA GAG CTT AGA GAC ACC **GC**T **GC**T GAG GGC TTG GTT GAT C-3'	FF(162,163)AA mutation
*SYS1*_r7	5'-G ATC AAC CAA GCC CTC A**GC** A**GC** GGT GTC TCT AAG CTC TC-3'	FF(162,163)AA mutation
*SYS1*_f7	5'-GA TTG GTT ACG CCT AGT **GC**G **GC**G CAT AGT AAT CAT TCA G-3'	QQ(181,182)AA mutation
*SYS1*_r8	5'-C TGA ATG ATT ACT ATG C**GC** C**GC** ACT AGC GGT AAC CAA TC-3'	QQ(181,182)AA mutation
*SYS1*_f8	5'-**GA TCC** TGG ACA ACA AGA TGG AGA GAG CTT AGA GAC ACC TTT TTT GAG GGC TTG GTT GAT CCT AAT GAA **A**-3'	***Bam*HI (5'); *Hind*III (3')** aa (151-171)
*SYS1*_r9	5'-**AG CTT** TTC ATT AGG ATC AAC CAA GCC CTC AAA AAA GGT GTC TCT AAG CTC TCT CCA TCT TGT TGT CCA **C**-3'	***Bam*HI (3'); *Hind*III (5')** aa (171-151)
*SYS1*_f9	5'-CCG **GAA TTC** CGG AGG GCT TGG TTG ATC CTA ATG AAG GAG	***Eco*RI** Underlined: aa (164-172)
*SYS1*_r10	5'-CCC GGG **AAG CTT** TCA TAT TTG GCT TTC TAA GTC-3'	***Hind*III** Underlined: aa (203-198)
*SYS1*_f10	5'-**GA TCC** GGA GAA GTT GGA TTG GTT ACG CTA AGT CAG CAG CAT AGT AAT CAT TCA GAA TTG GAG CAA TCA CCA ATA CAA CTA AAA GAC TTA GAA AGC CAA ATA TGA **A**-3'	***Bam*HI (5'); *Hind*III (3')** aa (172-203)
*SYS1*_r11	5'-**AG CTT** TCA TAT TTG GCT TTC TAA GTC TTT TAG TTG TAT TGG TGA TTG CTC CAA TTC TGA ATG ATT ACT ATG CTG CTG ACT AGG CGT AAC CAA TCC AAC TTC TCC **G**-3'	***Bam*HI (3'); *Hind*III (5')** aa (172-203)
*SYS1*_f11	5'-TCA CCA ATA CAA CTA AAA G**C**C TTA G**C**A AGC CAA ATA TGA AAG CTT-3'	DLE(198-200)ALA mutation
*SYS1*_r12	5'-AAG CTT TCA TAT TTG GCT T**G**C TAA G**G**C TTT TAG TTG TAT TGG TGA-3'	DLE(198-200)ALA mutation
*SYS1*_f12	5'-CAG CAT AGT AAT CAT TCA G**C**A TTG G**C**G CAA TCA CCA ATA CAA CTA-3'	ELE(188-190)ALA mutation

*SYS1*_r13	5'-TAG TTG TAT TGG TGA TTG C**G**C CAA T**G**C TGA ATG ATT ACT ATG CTG-3'	ELE(188-190)ALA mutation
*SYS1*_f13	5'-TCA CCA ATA CAA CTA AAA G**C**C TTA GCA AGC CAA ATA TGA AAG CTT-3'	D(198)A mutation
*SYS1*_r14	5'-AAG CTT TCA TAT TTG GCT TGC TAA G**G**C TTT TAG TTG TAT TGG TGA-3'	D(198)A mutation
*SYS1*_f14	5'-TCA CCA ATA CAA CTA AAA GCC TTA G**C**A AGC CAA ATA TGA AAG CTT-3'	E(200)A mutation
*SYS1*_r15	5'-AAG CTT TCA TAT TTG GCT T**G**C TAA GGC TTT TAG TTG TAT TGG TGA-3'	E(200)A mutation

Table 9 - Oligos used to generate pGEX-TT-*BAP2, -PTR2, -TAT1 and -TAT2* constructs:

Name	Sequence	Annotations
Bold letters: Restriction site or mutation; Underlined: Homologues sequences to SYS1		
*BAP2*_f1	5'-TTT TTG **GGA TCC** TAC AAC CGA GAT TTT ACG CTA-3'	***Bam*HI** Underlined: aa (557-609)
*BAP2*_r1	5'-GA GAT **AAG CTT** TTA ACA CCA GAA ATG ATA AGC-3'	***Hind*III** Underlined: aa (557-609)
*PTR2*_f1	5'-TTT TTG **GGA TCC** AGG AAG TAT AAT GAT ACA GAG-3'	***Bam*HI** Underlined: aa (553-601)
*PTR2*_r1	5'-GA GAT **AAG CTT** CTA ATA TTT GGT GGT GGA TCT-3'	***Hind*III** Underlined: aa (553-601)
*TAT1*_f1	5'-TTT TTG **GGA TCC** ATA TAC TTC AAA TCA TGG AGC-3'	***Bam*HI** Underlined: aa (550-619)
*TAT1*_r1	5'-GA GAT **AAG CTT** TTA GCA CCA GAA ATT GGT CAT-3'	***Hind*III** Underlined: aa (550-619)
*TAT2*_f1	5'-TTT TTG **GGA TCC** ATT TAT TAC AAG TGT CAA ACA-3'	***Bam*HI** Underlined: aa (534-592)
*TAT2*_r1	5'-GA GAT **AAG CTT** TTA ACA CCA GAA ATG GAA CTG-3'	***Hind*III** Underlined: aa (534-592)

Table 10 – Oligonucleotides for generation of the two-hybrid constructs

Name	Sequence	Annotations
Bold letters: Restriction site or mutation; Underlined: Homologues sequences to SYS1		
*SYS1*_f15	5'-CTG TA**C CAT GG**C TTG GAC AAC AAG ATG GAG AGA G-3'	***Nco*I** Underlined: aa (151-157)
*SYS1*_r16	5'-CTG **GGA TCC** TCA TAT TTG GCT TTC TAA GTC-3'	***Bam*HI** Underlined: aa (203-198)
*SYS1*_f16	5'-CCG **GAA TCC** TGG ACA ACA AGA TGG AGA GAG-3'	***Eco*RI** Underlined: aa (151-157)
*SYS1*_r17	5'-GA GAT **AAG CTT** CTA ATA TTT GGT GGT GGA TCT-3'	***Bam*HI** Underlined: aa (203-198)
*SYS1*_f17	5'-TTT TTG **CCA TGG** TTT CGA TAA GAA GGT AT-3'	***Nco*I** Underlined: aa (1-7)
*SYS1*_r18	5'-TTT TTG **CTC GAG** AAG TCC AAT CTT ACT TGG AGA-3'	***Xho*I** Underlined: aa (32-26)
*GOS1*_f1	5'-TTT TTT **CAT ATG** AGC TCA CAA CCG TCT TTC-3'	***Nde*I** Underlined: aa (1-7)
*GOS1*_r1	5'-TTT TTG **GGA TCC** TTA CCA TGT GAA AAA CAA AAA-3'	***Bam*HI** Underlined: aa (223-217)

*YKT6*_f1	5'-TTT TTG **CCA TGG** GAA TGA GAA TCT ACT ACA TCG GT-3'	***Nco*I** Underlined: aa (1-7)
*YKT6*_r1	5'-TTT TTG **GTC GAC** CTA CAT GAT GAT GCA ACA CGA-3'	***Sal*I** Underlined: aa (201-195)

Table 11 – Oligonucleotides for generation of pQE30-*GOS1* and pQE30-*YKT6*

Name	Sequence	Annotations
Bold letters: Restriction site or mutation; Underlined: Homologues sequences to SYS1		
*YKT6*_f2	5'-TTT TTG **GGA TCC** ATG AGA ATC TAC TAC ATC GGT-3'	***Bam*HI** Underlined: aa (1-/)
*YKT6*_r2	5'-GA GAT **AAG CTT** CTA CAT GAT GAT GCA ACA CGA-3'	***Hind*III** Underlined: aa (201-195)
*GOS1*_f2	5'-TTT TTG **GGA TCC** ATG AGC TCA CAA CCG TCT TTC-3'	***Bam*HI** Underlined: aa (1-7)
*GOS1*_r2	5'-TTT TTG **GGT ACC** TTA CCA TGT GAA AAA CAA AAA-3'	***Kpn*I** Underlined: aa (223-217)

Table 12 – Oligonucleotides for generation of *WBP1-SYS1* fusion constructs

Name	Sequence	Annotations
Bold letters: Restriction site or mutation; Underlined: Homologues sequences to SYS1		
*SYS1*_f18	5'-GA GAT **AAG CTT** AGA TGG AGA GAG CTT AGA GAC-3'	***Hind*III** Underlined: aa (154-201)
*SYS1*_r19	5'-TTT TTG **GGA TCC** GAG ATA AGG TTC TTG CCA TTT-3'	***Bam*HI** Behind the reading frame
*WBP1*_f1	5'-CCC **CTC GAG** CAC CAC CAA CCC TAT TCG AAA-3'	***Xho*I** Anneals 1500 bp before ATG
*WBP1*_r1	5'-GT TTC **AAG CTT** CTT GCC AAC AGA GGA AGT CGT AA-3'	***Hind*III** Anneals 1 bp before STOP codon

Table 13 – Oligonucleotides for sequencing

Name	Sequence	Annotations
Bold letters: Homologues sequences to the sequenced open reading frame		
*SYS1*_seq1	5'-**GAT** GGA GAG AGC TTA GAG-3'	Bold: bp 150-148 behind STOP codon (Reverse)
*SYS1*_seq2	5'-**AAG** AAC GAT CGC TGT GGT-3'	Bold: bp 148-150 before ATG codon
*SNC1*_seq1	5'-**AAA** TAA ACA CGA AGA ACG T-3'	Bold: bp 211-209 (Reverse)
*SNC1*_seq2	5'-**GAA** TAA TGA GAG ATA ACA T-3'	Bold: bp 235-237
*SNC1*_seq3	5'-**TGT** ACA GTC TAA GTC AAG G-3'	Bold: bp 66-69
*WBP1*_seq1	5'-**AGA** GGT GTA TAG TCA ACT-3'	Bold: bp 94-96 before ATG codon
*WBP1*_seq2	5'-**AGC** GAG ACA AAT CCC AGT-3'	Bold: bp 270-272
*WBP1*_seq3	5'-**ACC** AGC GGA TCT CAA GGC-3'	Bold: bp 628-630
*WBP1*_seq4	5'-**GCC** AAG CGG AAA CGA TTC-3'	Bold: bp 984-986
*WBP1*_seq5	5'-**AGC** GCC ATT TGT GGC GTC-3'	Bold: bp 1189-1191

pGEX-TT_seq1	5'-GAC CAT CCT CCA AAA TCG G-3'	
pGEX-TT_seq2	5'-GAG GCA GAT CGT CAG TCA G-3'	(reverse)
pMAL-c2_seq1	5'-GGT CGT CAG ACT GTC GAT GAA GCC-3'	
pMAL-c2_seq2	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	(reverse)
pQE30_seq1	5'-CTC GAG AAA TCA TAA AAA ATT-3'	

2.4 Antibodies

2.4.1 Primary antibodies

Table 14

Antibody	**source**
Rabbit anti-Sys1p, polyclonal	This department, unpublished
Rabbit anti-Sys2p, polyclonal	This department, unpublished
Rabbit anti-Sys3p, polyclonal	(Tsukada et *al.*, 1999)
Rabbit anti-Sec23p, polyclonal	(Peng et *al.*, 1999)
Rabbit anti-Sec24p, polyclonal	(Peng et *al.*, 1999)
Rabbit anti-Emp47, polyclonal	(Schröder et *al.*, 1995)
Rabbit anti-Sed5p, polyclonal	(Grabowski and Gallwitz, 1997)
Rabbit anti-Kar2p, polyclonal	(Benli et *al.*, 1996)
Rabbit anti-Bos1p, polyclonal	(Ossipov et *al.*, 1999)
Rabbit anti-Wbp1p, polyclonal	(te Heesen et *al.*, 1993)
Rabbit anti-Sar1p, polyclonal	(Saito-Nakano and Nakano, 2000)
Rabbit anti-Sec22p, polyclonal	(Ballensiefen et *al.*, 1998)
Rabbit anti-CPY, polyclonal	This Department, (Benli et *al.*, 1996)
Rabbit anti-GST, polyclonal	Pharmacia (Freiburg, Germany)
Mouse anti-6xhis, monoclonal	Gibco (Karlsruhe, Germany)
Mouse anti-HA (12CA5), monoclonal	Roche (Mannheim, Germany)
Mouse anti-c-myc (9E10), monoclonal	Santa Cruz Biotech. (USA)

2.4.2 Secondary antibodies

Table 15

Antibody	**source**
Sheep anti-mouse-IgG, HRP conjugated.	Amersham-Buchler (Braunschweig, Germany)
Donkey anti-rabbit-IgG, HRP conjugated	Amersham-Buchler

2.5 Growth media

2.5.1 Media components

Bacto-agar, bacto-peptone 140, bacto-yeast-extract, and bacto-yeast nitrogen base w/o amino acids from Difco (Detroit, USA). D-glucose, D-raffinose, D-galactose, ammonium sulfate, potassium acetate and amino acids from SERVA (Heidelberg, Germany). Geneticin G418 Sulfate from Calbiochem (La Jolla, CA, USA), Ampicillin Na-salt and kanamycin sulfate from SERVA. 3-Amino-1,2,4-Triazole (3-AT) and 5-Fluoroorotic acid (5-FOA) from Sigma-Aldrich (Deisenhofen, Germany).

2.5.2 Bacterial media

All media were autoclaved for 20 min at 120°C and stored at 4°C. Solid media were obtained adding bacto-agar at the final concentration of 1.5 % (w/v).

LB (Luria Bertani)	5 g/l	Yeast extract
	10 g/l	Bacto-peptone
	5 g/l	NaCl
	5 ml/l	NaOH 1N
Additives		
IPTG	0.1 1	
Antibiotics:	100 μg/ml	Ampicillin
	50 μg/ml	Kanamycin

2.5.3 Yeast media

All media were autoclaved for 20 min at 120°C and stored at 4°C. Solid media were obtained adding bacto-agar at the final concentration of 2 % (w/v).

YEPG (YEPGal)	10 g/l	Yeast extract
	20 g/l	Peptone 140
	20 g/l	D-glucose (D-galactose)
	20 mg/l	Uracil
	20 mg/l	Adenine sulphate
PM-glucose	1.7 g/l	Yeast nitrogen base w/o amino acids
(PM-galactose)	5 g/l	Peptone 140
(PM-raffinose)	5 g/l	Ammonium sulfate
	20 g/l	D-glucose (D-galactose) (D-raffinose)
SD	1.7 g/l	Yeast nitrogen base w/o amino acids
	5 g/l	Ammonium sulfate
	20 g/l	D-glucose
Additives		
Amino acids:	20 mg/l	Uracil
	20 mg/l	Adenine sulphate
	20 mg/l	L-tryptophan
	20 mg/l	L-histidine
	30 mg/l	L-leucine
	30 mg/l	L-Lysine/HCl
Antibiotics:	200 mg/l	geneticin G418

2.6 Chemicals

All chemicals used were of analytical grade and were purchased from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany), Sigma Deisenhofen, Germany) or Serva (Heidelberg, Germany) unless otherwise stated. Tran^{35}S-label from ICN (Meckenheim, Germany) and Amplify fluorografic reagent from Amersham (Braunschweig, Germany). Glutathione sepharose 4B, protein A sepharose fast flow and protein G sepharose fast flow were from Pharmacia (Freiburg, Germany). Ni-NTA agarose from QIAGEN (Hilden, Germany). Bradford protein assay reagent and Chelex 100 resin from Bio-Rad (Munich, Germany). Protease inhibitors cocktail tablets and PefablocSC (4-2-Aminoethyl-benzenesulfonyl fluoride) from Roche (Mannheim, Germany). Prestained protein ladder BenchMark from Gibco Brl (Karlsruhe, Germany), Rainbow protein marker from Amersham (Braunschweig, Germany).

2.7 Enzymes

Restriction endonucleases were from Boehringer (Mannheim, Germany), New England Biolabs (Frankfurt, Germany), and Promega (Mannheim, Germany). T4-polymerase, T4-DNA ligase and RNase were from Boehringer (Mannheim, Germany). Taq DNA polymerase was from Perkin Elmer (New Jersey, USA), Deep Vent DNA polymerase from *Thermococcus litoralis* was from New England Biolabs (Frankfurt, Germany), Pfu DNA polymerase from Stratagene (Heidelberg, Germany). Zymolyase 100T from *Arthrobacter luteus* was from Seikagaku Corp. (Tokyo, Japan), Lyticase partially purified from *Arthrobacter luteus* was from Sigma (Deisenhofen, Germany), ß-glucuronidase/arylsulfatase from *Helix pomatia* was from Boehringer (Mannheim, Germany). Thrombin was from Sigma (Deisenhofen, Germany).

2.8 Reaction systems, Kits

Plasmid DNA extraction from *E. coli*, DNA extraction from agarose gels and purification of PCR products were performed with a QIAGEN Spin Miniprep or Midiprep kit, a QIAquick Gel Extraction kit, a QIAquick PCR purification kit respectively, from QIAGEN (Hilden, Germany).

ECL western blotting detection reagents, ECL direct nucleic acid labeling, 3'-oligo-labeling and detection system, and Lumi-LightPLUS western blotting substrate from Amersham-Buchler (Braunschweig, Germany).

QIAexpressionist (His-tag) from QIAGEN, GST-fusion system from Pharmacia (Freiburg, Germany) and MBP-fusion system from New England Biolabs (Frankfurt, Germany).

2.9 Diverse materials

Autoradiography films X-Omat from Kodak-Eastman (Rochester, New York, USA). Nitrocellulose membrane filters BA 85 from Schleicher and Schuell (Dassel, Germany). Whatman 541 Filter and Whatman 3MM paper from Whatman (Maidstone, UK). Filtropur BT50 0.2µm 500ml from Sarstedt (Nümbrect, Germany), non pyrogenetic 0.22 µm filter Millex-GS from Millipore (Molsheim, France). Centricon concentrators from Amicon (Beverly, USA), and membra-spin PES columns (membraPure, Bodenheim, Germany). Reaction vials 0.5 ml, 1.5 ml and 2 ml from Eppendorf (Hamburg, Germany). Polypropylene Falcon vials 15 ml and 50 ml from Becton –Dickinson (Heidelberg, Germany). Petri dishes from Nunc (Wiesbaden, Germany). Electroporation cuvettes from Invitrogen (Leek, The Netherlands). All other materials including glassware were purchased from Schütt (Göttingen, Germany).

2.10 Laboratory hardware equipment

Centrifuges: Eppendorf bench centrifuge 5415 (Eppendorf, Hamburg, Germany), Hereaus Laborfuge GL, Sorvall RC-5B with rotors HS-4, HB-4, GSA, SS-34 and SA600 (DuPont Instruments, Bad Homburg, Germany), Ultracentrifuges TL-100, L7, L8-M with rotors TLA100.3 45Ti 70Ti SW40Ti and SW60 Ti (Beckman, Munich, Germany). Electroporation chamber BioRad Gene-Pulser with pulse-controller from BioRad Laboratories (Munich, Germany). Gel dryer BioRad Slab Dryer 443 and 448 from BioRad Laboratories (Munich, Germany). Homogenizer Gaulin Micron Lab 40 “French Press” from APV Gaulin (Lübeck, Germany). HPLC (High performance liquid chromatography) “System GOLD” from Beckman (Munich, Germany). Incubators: Gyrotory Shaker and controlled environment incubators from New Brunswick (Edison, NJ, USA). Lumi-imager from Boehringer (Mannheim, Germany). Micromanipulator from Singer Instruments (Watchet, GB). Spectrophotometer Uvikon 860 from Kontron instruments (Eching, Germany). Microscopes: Zeiss Photomicroscope Axiophot (Zeiss, Oberkochen, Germany), Leitz Laborlux (Leitz, Bad Bensheim, Germany), Confocal Leica TCS NT laser scanning microscope. PCR thermocycler devices PTC-100 from MJ Research Inc. (MA, USA), and RoboCycler gradient 40 from Stratagene (Heidelberg, Germany). Radiography developing machine Gevamatik 60 from AGFA Gevaert (Hannover, Germany). DNA Sequencer 373A from Applied Biosystems (Weiterstadt, Germany). Transilluminators 302 and 366nm from Bachofer (Reutlingen, Germany). Sonicator: sonifier-B15 and 250 from Branson ultrasonics (Schwäbisch Gmünd, Germany)

3 Methods

3.1 DNA preparation

3.1.1 Bacterial plasmid DNA preparation

Small-, medium- and large-scale plasmid extractions were performed using Plasmid mini-, midi- and maxi-prep from QIAGEN according to the manufacturer's recommendations.

3.1.2 Analytical plasmid isolation from yeast cells

Yeast cells were grown in appropriate medium overnight to stationary phase at suitable temperature in a shaking incubator. 1,5 ml of the overnight culture was transferred to a microcentrifuge tube and spun 15 seconds at high speed. The cell pellet was then disrupted by vortexing for 4 min with glass beads in 300 µl of breaking buffer (phenol/chloroform/isoamyl alcohol, 25/24/1 (v/v/v)). The mixture was then centrifuged at the highest speed in an Eppendorf centrifuge for 5 min at room temperature. 2-3 µl of the aqueous layer was then used for direct transformation of competent *E. coli* to amplify the amount of plasmid extracted from the yeast cells.

3.1.3 Rapid isolation of yeast chromosomal DNA

The procedure for rapid plasmid isolation could easily be scaled up to prepare chromosomal DNA for use in PCR amplification or restriction enzyme digest. For that purpose the aqueous solution with chromosomal DNA recovered after phenol-extraction generally required further purification by ethanol precipitation. A volume of the TE buffer equal to the volume of the aqueous DNA solution was added, vortexed briefly, spun in a microcentrifuge and the clear aqueous layer was then transferred to a new microcentrifuge tube. The genomic DNA was subsequently precipitated with ethanol, the pellet was dried and dissolved in TE buffer. The procedure typically gave a yield of chromosomal DNA in a range of several hundred nanogramms from 1 OD_{600nm} of yeast cells.

TE-buffer	10 mM	Tris/HCl, pH 7,5
	1 mM	EDTA

3.2 Enzymatic manipulation of DNA

3.2.1 Restriction enzyme digestion

Restriction endonuclease cleavage is accomplished simply by incubating the enzymes with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction varies depending upon the specific application. The conditions were chosen as proposed in the manufacturer’s manual. Restricted DNA fragments were purified either by gel electrophoresis and extraction using a QIAGEN gel extraction kit, or by using a QIAGEN PCR and nucleotides purification kit.

3.2.2 Ligation

The T4 DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and joins duplex DNA restriction fragments having either blunt or cohesive ends.

Sticky-end ligations were carried out for 20 minutes at 37°C using a 1:1 - 1:5 vector:insert molar ratio. A typical 20 μl reaction mixture contain: 50-100 ng insert DNA, 10-50 ng vector DNA, 1x ligase buffer, 0.5-1 mM ATP, 1U T4 DNA ligase, water.

3.2.3 QuickChange Site-directed mutagenesis

The in vitro site-directed mutagenesis was used for studying the protein structure-function relationship of Sys1p by identifying single amino acids whose substitution results in the loss of binding to COPII. The basic procedure starts with a supercoiled, dsDNA vector, with an insert of interest and two long oligonucleotide primers (30-45 bases) containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by Pfu-Turbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid. After temperature cycling, the product is treated with DpnI. The DpnI is used to digest the methylated non-mutated parental DNA template since DpnI is specific for methylated and hemimethylated DNA. The nicked vector DNA incorporating the desired mutations is then transformed into E. coli.

3.3 PCR amplification of DNA

The Polymerase Chain Reaction is an iterative process, consisting of three elements: denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence, and extension of the annealed primers by a thermostable DNA polymerase.

A typical 50 μl reaction mixture consist of:

1-10 ng plasmid DNA or 50-100 ng genomic DNA
20 pmol forward primer
20 pmol reverse primer
1x nucleotide mix (200 μM of each dNTP)
1x PCR buffer with $MgCl_2$ (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$)
1 U DNA polymerase (Taq or a mixture Taq/Deep-Vent 5/1)
dH_2O

The reaction is incubated in a thermocycler device. Usually there is a preheating step of 3 min at 93°C during which the template DNA is denatured. This is followed by 30-32 cycles of:

denaturing	30-60 sec at 92°C
annealing	30-60 sec at 45-60°C
elongation	30-120 sec at 72°C

(the last cycle is followed by an extra elongation step of 5-10 min at 72°C)

PCR products were purified by using a QIAGEN PCR purification kit according to the manufacturer's instructions.

3.4 Electrophoretic DNA separation

Agarose gel electrophoresis is used for separation, purification and identification of plasmid DNA and DNA fragments. Because of the negative charge of their phosphate groups, the DNA molecules move towards plus-pole in the electric field. Under constant voltage the migration speed of linear, double-stranded DNA in agarose gels is proportional to the logarithm of its molecular weight. The size of a

DNA fragment can be determined by comparison with standard DNA fragments of identified size (DNA-marker). Rough estimation of the DNA-concentration can be done by comparison of band intensity of the sample and marker DNA upon staining with ethidium bromide.
Agarose horizontal gels were used for analytical and preparative separation of DNA fragments. Depending on the size of the DNA molecules the agarose concentration was chosen between 0.7% and 2.0% (w/v). The agarose was molten by heating and, after addition of DNA-intercolating stain ethidium bromide, poured into electrophoresis chamber. Then a comb was inserted to create the wells for DNA loading. DNA-samples were mixed with one-third volume of loading buffer and applied into the wells.

In parallel a marker (e.g. 1 μg *EcoRI*/*HindIII*-cut double-stranded DNA of phage lambda) was loaded. TAE buffer was used for agarose solution and as electrophoretic buffer. The electrophoretic separation was done at 3 to 5 volts/cm. Separated fragments were visualized by UV-light (λ = 302 nm). Preparative gels were exposed to UV-light (λ = 366 nm) as shortly as possible to avoid photocrosslinking of DNA molecules.

TAE-buffer	40 mM	Tris-acetate, pH 7,2
	20 mM	Sodium acetate
	1 mM	EDTA
Loading-buffer	0,25% (w/v)	bromphenolblue
	0,25% (w/v)	xylencyanol
	25% (w/v)	ficoll (Type 400) in H_2O
Ethidium bromide	1 μg/ml	ethidium bromide in H_2O

3.5 *E. coli* transformation

Electroporation with high voltage is currently the most efficient method for transforming *E. coli* with plasmid DNA. With freshly grown cells, it routinely gives more than 10^9 bacterial transformands per microgram of input plasmid DNA.

For preparing cells competent for electroporation the bacteria culture was grown to OD_{600nm} of 0,4-0,6 in 500 ml LB medium in a 2-liter flask at constant shaking at 37°C. Cells were then chilled for 10-15 min in an ice-water bath and centrifuged 15 min at 4500 rpm in Beckman J-6M at 2°C. The cell pellet was subsequently washed 3 times with ice-cold sterile double distilled water. It was important to keep cells at 2°C at all steps. If frozen cells were to be used for electroporation, 40 ml ice-cold 10% glycerol was commonly added to the bacterial pellet after the washing step and mixed thoroughly. Cells were then centrifuged at 4000 rpm for 5 min at 2°C and dissolved in the volume of ice-cold 10% glycerol roughly equal to the one of cell pellet. Aliquots of 50-100 μl of cells were then transfered into prechilled Eppendorf tubes and frozen in liquid nitrogen. The cells could be stored at –80°C for many months without losing their high competence for transformation. Immediately before electroporation 50 μl of cells was thawed on ice and mixed with a volume of plasmid DNA not exceeding 2,5-3 μl, which usually contained from several nanogramms to 1 μg of DNA. The mixture of cells and DNA were placed subsequently into prechilled cuvette for electroporation and subjected to a pulse of a strong electric field in the electroporation apparatus (Gene Pulser, Bio-Rad, USA) set to 2,5 kV, 25 μF and

200-400 ohms. Right after the electrical shock, cells were placed on agar plates with LB media containing appropriate antibiotics for the selection of transformants.

3.6 Recombinant plasmids created in this work

Table 16

***SYS1* constructs for the suppressor screen**		
Construct	**Insert preparation**	**Vector preparation**
pYX213-*SYS1*	PCR template: pRS325-*SYS1* Primer: *SYS1*_f1 / *SYS1*_r1 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(12-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f2 / *SYS1*_r1 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(21-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f3 / *SYS1*_r1 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(1-186)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f1 / *SYS1*_r2 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(1-171)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f1 / *SYS1*_r3 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(1-153)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f1 / *SYS1*_r4 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(12-186)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f2 / *SYS1*_r2 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
pYX213-*SYS1*$_{(21-171)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f3 / *SYS1*_r3 Restriction: *Eco*RI/*Hind*III	pYX213 *Eco*RI/*Hind*III digested
***SYS1 and SNC1* constructs for GFP-fusions**		
Construct	**Insert preparation**	**Vector preparation**
pUG36-*SYS1*	PCR template: pRS325-*SYS1* Primer: *SYS1*_f4 / *SYS1*_r5 Restriction: *Eco*RI/*Hind*III	pUG36 *Eco*RI/*Hind*III digested
pUG36-*SNC1*	PCR template: pRS325-*SYS1* Primer: *SNC1*_f1 / *SNC1*_r1 Restriction: *Eco*RI/*Hind*III	pUG36 *Eco*RI/*Hind*III digested

GST-*SYS1* fusion constructs for binding assays		
Construct	**Insert preparation**	**Vector preparation**
pGEX-TT-*SYS1*$_{(151\text{-}203)}$ = **pGEX-TT-WT**	PCR template: pRS325-*SYS1* Primer: *SYS1*_f5 / *SYS1*_r6 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-WT$_{(FF162\text{-}3AA)}$ = **pGEX-TT-C1**	PCR template: pGEX-TT-WT Primer: *SYS1*_f6 / *SYS1*_r7 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-WT$_{(QQ181\text{-}2AA)}$ = **pGEX-TT-C2**	PCR template: pGEX-TT-WT Primer: *SYS1*_f7 / *SYS1*_r8 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-C1$_{(QQ181\text{-}2AA)}$ = **pGEX-TT-C3**	PCR template: pGEX-TT-C1 Primer: *SYS1*_f7 / *SYS1*_r8 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-*SYS1*$_{(151\text{-}171)}$ = **pGEX-TT-C4**	Hybridization of primers: *SYS1*_f8 / *SYS1*_r9 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-*SYS1*$_{(171\text{-}203)}$ = **pGEX-TT-C5**	PCR template: pRS325-*SYS1* Primer: *SYS1*_f9 / *SYS1*_r10 Restriction: *Eco*RI/*Hind*III	pGEX-TT *Eco*RI/*Hind*III digested
pGEX-TT-C5$_{(QQ181\text{-}2AA)}$ = **pGEX-TT-C6**	PCR template: pGEX-TT-C1 Primer: *SYS1*_f7 / *SYS1*_r8 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-*SYS1*$_{(151\text{-}170)}$ = **pGEX-TT-C7**	Hybridization of primers: *SYS1*_f10 / *SYS1*_r11 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-C7$_{(D198A;E200A)}$ = **pGEX-TT-C8**	PCR template: pGEX-TT-C7 Primer: *SYS1*_f11 / *SYS1*_r12 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-C7$_{(E188A;E190A)}$ = **pGEX-TT-C9**	PCR template: pGEX-TT-C7 Primer: *SYS1*_f12 / *SYS1*_r13 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-C7$_{(D198A)}$ = **pGEX-TT-C10**	PCR template: pGEX-TT-C7 Primer: *SYS1*_f13 / *SYS1*_r14 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
pGEX-TT-C7$_{(E200A)}$ = **pGEX-TT-C11**	PCR template: pGEX-TT-C7 Primer: *SYS1*_f14 / *SYS1*_r15 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR
GST-*BAP2, -PTR2, -TAT1 and TAT2* fusion constructs for binding assays		
Construct	**Insert preparation**	**Vector preparation**
pGEX-TT-*BAP2*$_{(557\text{-}609)}$	PCR template: Genomic DNA Primer: *BAP2*_f1 / *BAP2*_r1 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-*PTR2*$_{(553\text{-}601)}$	PCR template: Genomic DNA Primer: *PTR2*_f1 / *PTR2*_r1 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-*TAT1*$_{(550\text{-}619)}$	PCR template: Genomic DNA Primer: *TAT1*_f1 / *TAT1*_r1 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested
pGEX-TT-*TAT2*$_{(534\text{-}592)}$	PCR template: Genomic DNA Primer: *TAT2*_f1 / *TAT2*_r1 Restriction: *Bam*HI/*Hind*III	pGEX-TT *Bam*HI/*Hind*III digested

Two hybrid constructs		
Construct	**Insert preparation**	**Vector preparation**
pAS2-*SYS1*$_{(151-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f15 / *SYS1*_r16 Restriction: *Nco*I/*Bam*HI	pAS2 *Nco*I/*Bam*HI digested
pBTM116-*SYS1*$_{(151-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f16 / *SYS1*_r17 Restriction: EcoRI/*Bam*HI	pAS2 EcoRI/*Bam*HI digested
pAS2-*GOS1*	PCR template: Genomic DNA Primer: *GOS1*_f1 / *GOS1*_r1 Restriction: *Nde*I/*Bam*HI	pAS2 *Nde*I/*Bam*HI digested
pAS2-*YKT6*	PCR template: Genomic DNA Primer: *YKT6*_f1 / *YKT6*_r1 Restriction: *Bam*HI/*Hind*III	pAS2 *Nco*I/*Sal*I digested
pACTII-*SYS1*	PCR template: pRS325-*SYS1* Primer: *SYS1*_f16 / *SYS1*_r17 Restriction: EcoRI/*Bam*HI	pACTII EcoRI/*Bam*HI digested
pACTII-*SYS1*$_{(1-32)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f17 / *SYS1*_r18 Restriction: *Nco*I/*Xho*I	pACTII *Nco*I/*Xho*I digested
HIS-*GOS1*, HIS-*YKT6*, HIS-*SYS1* and MBP-*SYS1* fusion constructs		
Construct	**Insert preparation**	**Vector preparation**
pQE30-*GOS1*	PCR template: Genomic DNA Primer:GOS*1*_f2 /GOS*1*_r2 Restriction: *Bam*HI/*Kpn*I	pQE30 *Bam*HI/*Kpn*I digested
pQE30-*YKT6*	PCR template: Genomic DNA Primer: *YKT6*_f2 / *YKT6*_r2 Restriction: *Bam*HI/*Hind*III	pQE30 *Bam*HI/*Hind*III digested
pQE30-*SYS1*$_{(151-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f5/*SYS1*_r6 Restriction: *Bam*HI/*Hind*III	pQE30 *Bam*HI/*Hind*III
pMAL-c2-*SYS1*$_{(151-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f5/*SYS1*_r6 Restriction: *Bam*HI/*Hind*III	pMAL-c2 *Bam*HI/*Hind*III digested
***WBP1*-*SYS1* fusion constructs**		
Construct	**Insert preparation**	**Vector preparation**
pRS315-*SYS1*$_{(151-203)}$	PCR template: pRS325-*SYS1* Primer: *SYS1*_f18/ *SYS1*_r19 Restriction: *Bam*HI/*Hind*III	pRS315 *Bam*HI/*Hind*III digested
pRS315-*WBP1-SYS1*$_{(151-203)}$ **=pRS315-*WBP1*-(DXE)**	PCR template: Genomic Primer: *WBP1*_f1/ *WBP1*_r1 Restriction: *Xho*I/*Hind*III	pRS315-*SYS1*$_{(151-203)}$ *Xho*I/*Hind*III digested
pRS315-*WBP1*-(AXA)	PCR template: pGEX-TT-C7 Primer: *SYS1*_f11 / *SYS1*_r12 Site-directed mutagenesis	Digestion of the methylated DNA template by *Dpn*I after PCR

3.7 Yeast genetics

3.7.1 *S. cerevisiae* transformation

Transformation of yeast was carried out using a modified lithium acetate method (Schiestl and Gietz, 1989).

3.7.2 PCR-targeted gene disruption using the *kanMX* module

The *kanMX* module was used in a transformation with a PCR-generated DNA fragment designed to replace most of *YDL173w*, resulting in strain CV7. The method is based on direct homologous recombination of a PCR-product into the yeast genome (Güldener et *al.*, 1996). The selectable module loxP-*kanMX*-loxP was amplified by PCR from the pUG6 vector, using two primers with tail sequences homologous to the yeast genomic sequences flanking the ORF to be deleted. After transformation the PCR product is introduced into the yeast genome by homologous recombination (Figure 6). The transformants were selected on YEPG plates containing 200 μg/ml G418.

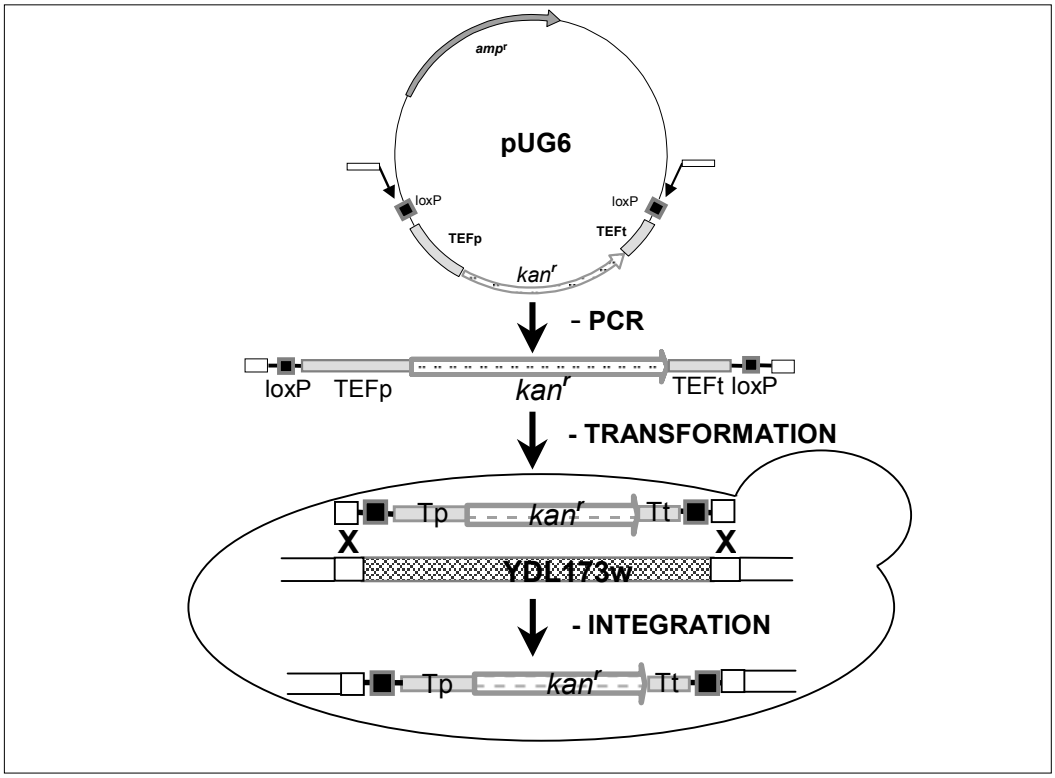

Figure 6. PCR-targeted gene disruption using the *kanMX* module. The *kanMX* module is transformed in yeast strain GFUI-5B with a PCR-generated DNA fragment designed to replace *YDL173w*. The primers for the amplification of the loxP-*kanMX*-loxP cassette from pUG6 are designed with tail sequences homologous to yeast genomic sequences flanking the ORF to be deleted. (Modified from Anna De Antoni, Ph.D. thesis).

3.7.3 Yeast PCR-mediated epitope tagging

It was made use of a method for PCR-mediated C-terminal epitope tagging, that allows the tagging of chromosomal genes with sequences expressing the MYC, HA or GFP epitopes (Güldener et *al.*, 1996; De Antoni and Gallwitz, 2000). The plasmids pU6H2MYC, pU6H3HA and pUG24 (pU-tag vectors) contain the modules 6His-2MYC-loxP-*kan*MX-loxP, 6His-3HA-loxP-*kan*MX-loxP or GFP-loxP-*kan*MX-loxP that allow tagging of different genes by using the *kan*^{r} marker (Figure 7). The method relays on PCR amplification of the *tag-loxP-kanMX-loxP* cassettes with chimeric primers. The forward primer is composed of 42-45 nucleotides derived from the 3'-end of the gene of interest (excluding the stop codon, and in frame with the epitope-encoding sequence), plus 21 nucleotides, that anneal to the 5'-end of the cassette. The backward primer is composed of 42-45 nucleotides derived from the sequence downstream of the gene of interest (starting at either the stop codon or 50-100 nucleotides after the stop codon) plus 22 nucleotides annealing to a sequence downstream of the cassette (Figure 7). The primers used in this study are listed in

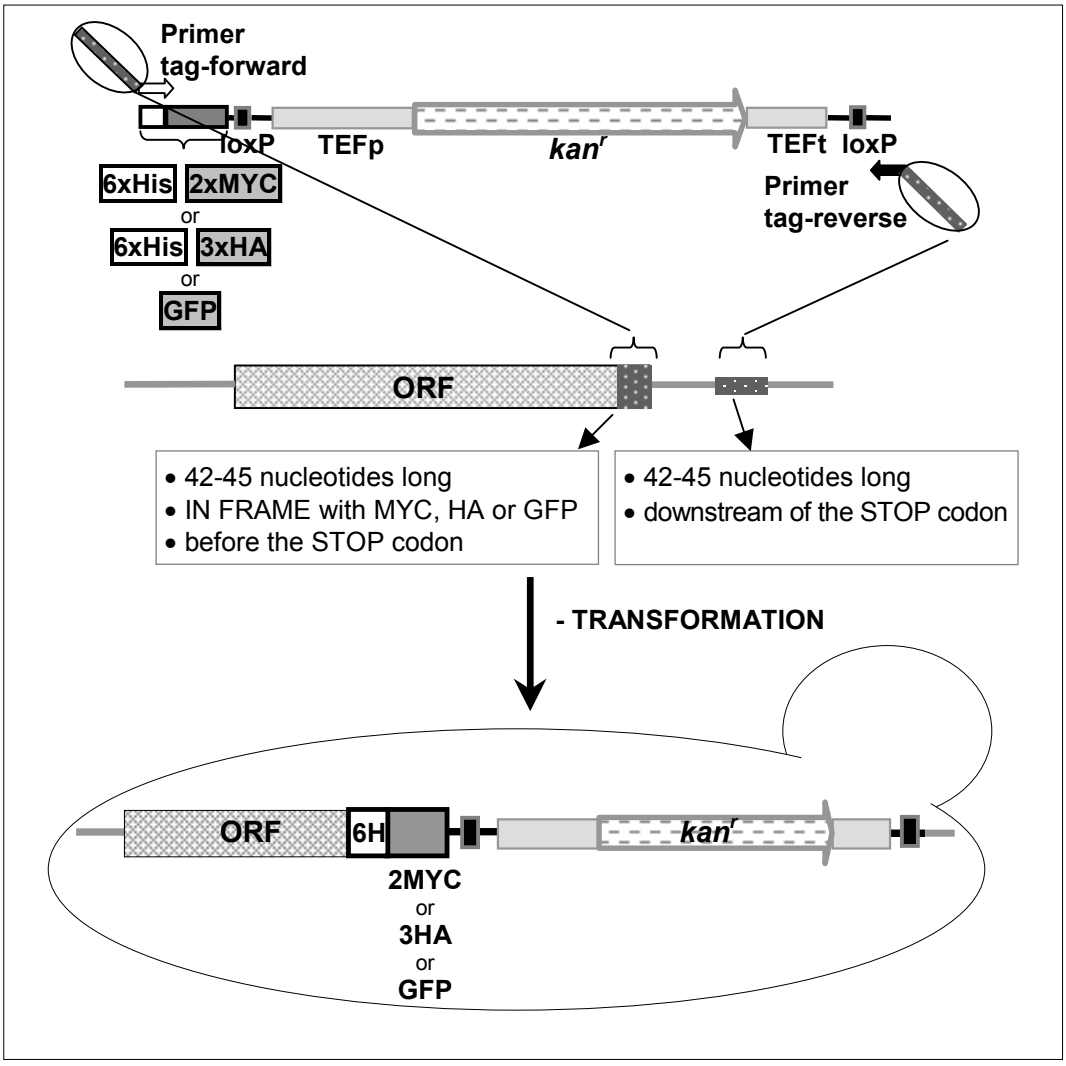

Figure 7. PCR-targeted epitope tagging using the *kanMX* module. The tag-loxP-*kanMX*-loxP module is transformed in yeast with a PCR-generated DNA fragment designed to tag the selected reading frame with HA, MYC or GFP. The primers for the amplification of the loxP-*kanMX*-loxP cassette from pU6H2MYC, pU6H3HA and pUG24 are designed with tail sequences homologous to yeast genomic sequences proximal of the selected reading frame. (Anna De Antoni, Ph.D. thesis).

Table 5. A 100 μl preparative PCR contains: 5-10 ng template (pU6H2MYC or pU6H3HA), 30 pmol of each primer (tag-forward and tag-reverse), 200 μM of each dNTP, 1.5 U AmpliTaq (Perkin Elmer), 0.4U Deep Vent DNA polymerase (NEB) and 10 μl 10X PCR buffer (Perkin Elmer, containing 15 mM $MgCl_2$). PCR conditions were: denaturation at 93° C for 3 min, followed by 32 cycles (93° C for 1 min, 55° C for 1 min, 72 °C for 1.5 min) and a final elongation step at 72 °C for 10 min. The PCR products were purified with QIAquick PCR columns and 1-5 μg were used to transform yeast cells. Transformants were plated onto YEPG plates containing 200 μg/ml G418. Plates were incubated at 30° C until colonies appeared. Well grown colonies were re-streaked onto YEPG/G418 plates.

With this method the HA epitope was added to the C-terminus of Sys1p in strains CV1, CV2 and CV3, in strains CV5 and CV6 Sys1p was fused to GFP, and in strain CV4 the Ydl173w-MYC fusion protein was created.

Table 17 – Strains generated in this work

Strain	Genotype	Generation
CV1	MATα *leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 SYS1-6HIS-3HA-loxP-KanMX-loxP*	Template: pU6H3HA Primer: *SYS1*-tag_f *SYS1*-tag_r; Strain: SEY6210
CV2	MATα *leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 SYS1-Δ(188-203)-6HIS-3HA-loxP-KanMX-loxP*	Template: pU6H3HA Primer: *SYS1*(1-186)-tag_f *SYS1*(1-186)-tag_r; Strain: SEY6210
CV3	MATα *leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 SYS1-(D198A;E200A)-6HIS-3HA-loxP-KanMX-loxP*	Template: pU6H3HA Primer: *SYS1*(1-186)-tag_f *SYS1*(1-186)-tag_r Strain: SEY6210
CV4	MATa Gal$^+$ *ura3 leu2 his4 lys2 bar1-1 YDL173W-6HIS-2MYC-loxP-KanMX-loxP*	Template: pU6H2MYC Primer: *YDL173*-tag_f *YDL173*-tag_r Strain: RH270-2B
CV5	MATα *ura3 trp1 leu2 his3 lys2 SYS1-GFP-loxP-KanMX-loxP*	Template: pUG24 Primer: *SYS1*-tag2_f *SYS1*-tag2_r Strain: MSUC-3D
CV6	MATa *ura3 trp1 leu2 his3 ade8 SYS1-GFP-loxP-KanMX-loxP*	Template: pUG24 Primer: *SYS1*-tag2_f *SYS1*-tag2_r Strain: MSUC-IA
CV7	MATα *ura3 leu2 his3 YDL173W::KanMX*	Template: pU6 Primer: *YDL173*-del_f *YDL173*-del_r Strain: GFUI-5B

3.7.4 Crossing of yeast cells

Yeast laboratory strains can be cultivated in the haploid as well as the diploid state. Haploid cells possess the mating type a or α. Haploids of opposite mating types can mate and make a diploid cell. Diploid cells can be induced by starvation to enter a meiotic cell cycle, first of all by a lack of nitrogen. During this process, four haploid spores, called a tetrad, are formed and surrounded by a thick-walled sac. This structure is called ascus.

5 ml of liquid culture of a diploid strain in complete medium was grown until the logarithmic phase. Cells were pelleted by centrifugation for 5 minutes at 2.000*g* and the supernatant decanted. Cells were resuspended in the rest of liquid medium and dropped on sporulation potassium acetate plates. Plates were incubated at 30°C for at least three days.

During tetrad analysis, the spores from one ascus are separated using the glass tip of the micromanipulator. To 0.5 ml of the sterile water, 10 µl of β-glucuronidase (Boehringer) was added. Cells scraped from the sporulation plate were resuspended in this solution. During subsequent incubation for 10 minutes at room temperature, the asci are partially digested by the enzyme.

The cell suspension was streaked out onto YEPG plates. Under the light microscope at 100 times magnification, four spores of one tetrad were dissected separately at four different locations of the plate (mostly on one line). After dissection of several (~20) tetrads, the plates were incubated at 25°C to germinate the spores. After 3 days, the spores formed colonies that could be tested for auxotrophy and further characterization.

3.8 Protein analysis

3.8.1 Alkaline lysis for total protein extraction from yeast

One OD_{600} unit of cells (~10^7 cells) is centrifuged (2 min at 10, 000 rpm), and the cell pellet is washed with cold water, resuspended in 180 µl lysis buffer (2 M NaOH / 0.5% ß-mercaptoethanol) and incubated on ice for 5 min. Proteins are precipitated by adding 20 µl 100% TCA. After incubation on ice for 5 min, proteins are pelleted by centrifugation (10 min at 14, 000 rpm). The pellet was then washed with acetone, left to air dry thoroughly and resuspended in 100 µl Laemmli loading buffer. After boiling for 5 minutes the proteins are separated by SDS-PAGE.

3.8.1.1 Denaturing (SDS) discontinuous gel electrophoresis (SDS-PAGE)

One dimensional gel electrophoresis under denaturing conditions (in presence of 0.1% SDS) separates proteins on the base of their molecular size. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel line, and the individual proteins are separated electrophoretically. The mobility of the proteins is inversely proportional to the logarithm of their molecular mass. SDS is employed to effect denaturation of the proteins, to dissociate protein complexes and to impart upon the polypeptide chains net negative charge densities proportional to the length of the molecule. A reducing agent as ß-ME is used to reduce any existing disulphide bond.

For an 8x10x0.1 cm gel the following volumes were used:

5% Stacking gel (5 ml)	3.4 ml	H_2O
	0.83 ml	30% Acrylamide stock solution (see 2.2)
	0.63 ml	1M Tris-HCl, pH 6.8
	50 µl	10% SDS
	50 µl	10% APS
	5 µl	TEMED

Separating gel (10 ml)	10%	12%	15%	
	4 ml	3.3 ml	2.3 ml	H_2O
	3.3 ml	4 ml	5 ml	30% Acrylamide stock sol
	2.5 ml	2.5 ml	2.5 ml	1.5 M Tris-HCl, pH 8.8
	100 µl	100 µl	100 µl	10% SDS
	100 µl	100 µl	100 µl	10% APS
	4 µl	4 µl	4 µl	TEMED

3.8.2 Protein detection in gels using fixation

3.8.2.1 Coomassie blue R-250 staining

Coomassie brilliant blue R-250 binds nonspecifically to almost all proteins, which allows detection of protein bands in polyacrylamide gels. Gels are soaked in fixating solution with gentle shaking for 10 min and stained in staining solution for 1 hour or for longer time at room temperature. The background was subsequently reduced by soaking the gel in 10% acetic acid solution in which the gels can be kept for several days. After that gels can be dried.

Fixating solution	25% (v/v)	Isopropanol
	10% (v/v)	Glacial acetic acid
Staining solution	10% (v/v)	Glacial acetic acid
	60 mg/l	Coomassie brilliant blue R250

3.8.2.2 Silver staining

Silver staining is about 10 to 100 times more sensitive than Coomassie blue staining. Gels are first fixated in fixating solution for 1 hour at room temperature, then soaked for 30 min in incubation solution and washed 3 times 10 min in distilled water. After that the gels are placed in binding solution for 20 min. Finally the gels are washed with water and put into developing solution till brown bands appear. Washing with 50 mM EDTA stops the staining reaction and subsequently the gels are soaked in 10% acetic acid. All the solutions should be freshly prepared.

Fixing solution	50%	Ethanol
	10%	Acetic acid
Incubation solution	30%	Ethanol
	0.83 M	NaOAc
	13 mM	$Na_2S_2O_3$ (sodium thiosulfate)
	0.25%	Glutaraldehyde (to be added just before use)

Binding solution	6 mM	$AgNO_3$ (silver nitrate)
	0.02%	Formaldehyde
Developing solution	0.25 M	Na_2CO_3
	0.01%	Formaldehyde

3.8.3 Electroblotting from polyacrylamide gels on nitrocellulose filters

Electroblotting of proteins from polyacrylamide gels onto retentive membranes is usually performed for immunoblotting (or Western blotting). Proteins are separated by SDS-PAGE and electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane at 100 mA constant current for 1 hour or at 30 mA overnight (Burnette, 1981).

Transfer buffer	20 mM	Tris-base
	150 mM	Glycine
	20% (v/v)	Methanol

After the transfer onto nitrocellulose the proteins are stained with Ponceau S solution. Major advantages of Ponceau S staining are that it is simple, rapid, and reversible as all of the stain can be removed by extended washing with buffer-A. This is particularly advantageous as the blot can be used for a second detection by immunoblotting. After washing the blot is treated with blocking solution and incubated with the primary antibody in blocking solution for 1 h at RT each. After 2x 5 min washing in buffer-A, 2x 5 min in buffer-B and once again for 5 min in buffer-A, the membrane is incubated for 1 h at RT with the horseradish peroxidase-coupled secondary antibody (1:10.000 dilution). Finally, the membranes were washed as described above. Detection by chemiluminescence was performed using the ECL detection system as recommended by the manufacturer.

Ponceau S solution	2.5 g/l	Ponceau S
	15% (v/v)	Glacial acetic acid
	40% (v/v)	Methanol
Blocking solution	5% (w/v)	low fat milk powder in washing buffer A
Washing buffer-A	10 mM	Tris-HCl, pH 7.4
	0.9% (w/v)	NaCl
	0.05% (v/v)	Tween 20
Washing buffer-B	0.2% (w/v)	SDS
	0.9% (w/v)	NaCl
	0.5% (v/v)	Triton X-100
	0.5% (w/v)	BSA

3.8.4 Measurement of protein concentration

3.8.4.1 Coomassie Dye-Binding assay to measure total protein

The color change produced when the Coomassie dye binds to proteins provides a measure of total protein. The absorbance for the protein-specific dye, Coomassie brilliant blue G-250, shifts from 465 nm to 595 when binding to protein occurs. Therefore, the A_{595} yields a good linear concentration dependence for most soluble proteins. 800 µl of a protein solution of unknown concentration are mixed with 200 µl of the dye solution (BioRad) and the measured A_{595} is blotted against a reference curve obtained with known concentrations of BSA.

3.8.5 Spectrophotometric determination of protein concentration

Determination of protein concentration by measuring absorbance at 280 nm (OD_{280nm}) is based on the absorbance of UV light by aromatic amino acids in protein solutions. This is primarily due to tryptophan and tyrosine residues and to lower extent due to phenylalanine residues. The measured absorbance was used to calculate the concentration by comparison with a calibration curve prepared from measurements with standard protein solutions. This assay was applied to solutions with protein concentrations of 20 to 3000 μg/ml.

3.9 Studies on intracellular localization and membrane topology

3.9.1 Subcellular Fractionation

The pellet of a 300 ml culture (1 OD_{600} unit/ml) of SEY6210 was transferred to a mortar containing liquid nitrogen and was smashed until a fine powder was obtained. This powder was dissolved in 4 ml of buffer A (20 mM Hepes-KOH pH 7.2/ 150 mM KOAc/ 1 mM MgOAc/ 2mM Pefabloc/ proteinase inhibitor tablets cocktail (Boehringer)) and centrifuged for 15 min at 500*g*. The supernatant was divided into 4 tubes (600 µl each), and to each tube were added 600µl of buffer A, B, C ,D, respectively. Buffers B,C and D had the same composition as buffer A except for 2% Triton X-100 (B), 2M NaCl (C), or 8M urea (D). After incubation for 15 min on ice, the samples were centrifuged at 4° C for 3 min at 10, 000*g* and the pellet (p10) was resuspended in 1.2 ml buffer A. The supernatant (1 ml) was subjected to further centrifugation for 1h at 100, 000*g* at 4° C and the pellet was resuspended in 1 ml of buffer A. All samples were taken up in 2X SDS/PAGE buffer and were subjected to SDS/PAGE, followed by immunoblotting.

3.9.2 Gradient fractionation

For gradient fractionation of cell organelles, the pellet of a 100 ml culture (1 OD_{600} unit/ml) of SEY6210 was taken up in 5 ml 100 mM 2-mercaptoethanesulfonic acid/ 1,2 M sorbitol/ 10 mM Hepes pH 7.2/ 1 mM $MgCl_2$ for 15 min. The cells were washed in 1,2 M sorbitol/ 10 mM Hepes pH 7.2/ 1 mM $MgCl_2$ and spheroplasted in 800µl of the same buffer with an addition of 150 µl lyticase(10 mg/ml) and 100µl EDTA-free protease-inhibitor cocktail for 30 min at 30° C. The spheroplasted cells were osmotically lysed in water and the soluble proteins were seperated of the cell debris by centrifugation (500*g*). The cleared lysate was loaded onto a manually generated 12-step sucrose gradient (1ml 60%, 54%, 50%, 46%, 42%, 38%, 34%, 30%, 26%, 22% and 18% sucrose in 10mM Hepes pH7.2, 1 mM $MgCl_2$) and centrifuged at 100, 000*g* for 3h at 4° C in a Beckman SW40 rotor. Twelve fractions were collected manually from the top to the bottom and were processed for Western blot analysis. Band intensities were calculated with a Lumi-Imager (Boehringer).

3.9.3 Fluorescence microscopy

To view live cells expressing the various GFP fusions, cells were grown in the appropriate selective medium at 30°C to early log phase, and examined with a Zeiss Photomicroscope.

3.9.4 Proteinase protection assay

Logarithmically grown cells (100 OD_{600}) were spheroplasted for 30 min at 30° C in 800 µl of 1.2 M sorbitol, 10 mM Hepes-KOH (pH 7.2), 1 mM $MgCl_2$ after adding 150 µl lyticase (10 mg/ml) and 100 µl EDTA-free protease-inhibitor cocktail. Spheroplasts were osmotically lysed in 1 ml of 50 mM Tris-Cl (pH 7.5), and the cell debris was removed by centrifugation at 500g. Fifty µl of the cell lysate were incubated with proteinase K (50 µg/ml) for 30 min on ice in a total volume of 100 µl. The reaction was stopped by the addition of 1 mM PMSF. The proteins were precipitated on ice with 10 % TCA, resuspended in 80 µl loading buffer and subjected to SDS-PAGE and immunoblot analysis.

3.10 Screening for protein interactions

3.10.1 Chromatography with affinity matrices

3.10.1.1 Protein purification from yeast extract

Cells of the protease-deficient strain c13-ABYS-86 were grown over night in 4l of standard yeast extract/peptone/dextrose (YEPD) medium. The harvested cells were washed in 20 mM HEPES (pH 7.2). 50g washed cells were resuspended in 50 ml lysis buffer (20 mM HEPES, pH 7.2/ 3 mM Pefabloc/ protease inhibitors cocktail) and disrupted by high-pressure homogenization (French-Press). After lysis the proteins were isolated from cytosolic extracts by the addition of detergent buffer (20 mM HEPES, pH 7.2/ 0,2 M NaCl/ 2% CHAPS/ 3 mM Pefabloc/ protease inhibitors cocktail) up to a concentration of 1% CHAPS and a subsequent high-speed centrifugation (100, 000*g*). The supernatant was precleared by the addition of 300 µl glutathione-Sepharose (Pharmacia) to separate the proteins with an affinity for the matrix itself. Finally $MgCl_2$ and $CaCl_2$ were added to a concentration of 2.5 mM.

3.10.1.2 Expression of GST-fusion proteins

The different GST-Sys1p constructs were expressed from the pGEX-TT vector (Amersham Pharmacia). After transformation of the vector in the protease deficient strain BL21, 500 ml of an overnight culture were diluted to a volume of 5l and incubated at 37° C to an OD of 0.6. For the protein expression IPTG was added to a final concentration of 1mM and the culture was incubated for another 6 hours. 6 g cells were washed and resuspended in lysis buffer (50 mM Tris, pH 7,5/ 100 mM NaCl/ 2.5 mM $MgCl_2$/ 2.5 mM $CaCl_2$). After sonification this cell suspension was adjusted to a Triton X-100 concentration of 1% for the complete solubilization of the proteins. The cell debris was separated from the solubilized proteins by high speed centrifugation (100, 000*g*) and the supernatant was incubated with 1 ml of glutathione-Sepharose beads by end-over-end rotation for 4 hours at 4° C. The beads were twice washed with detergent buffer (50 mM Tris, pH 7,5/ 100 mM NaCl/ 2.5 mM $MgCl_2$/ 2.5 mM $CaCl_2$/ 1% Triton X-100) and with high salt buffer (50 mM Tris, pH 7,5/ 1 M NaCl/ 2.5 mM $MgCl_2$/ 2.5 mM $CaCl_2$). The washed beads were loaded on a column.

3.10.1.3 Incubation with yeast extract and elution

The glutathione-Sepharose beads loaded with the different GST fusion proteins were incubated with the yeast extract. The unspecific bound proteins were washed of the column with a 150 mM salt buffer (50 mM Tris, pH 7,5/ 150 mM NaCl/ 2.5 mM $MgCl_2$/ 2.5 mM $CaCl_2$/ 0.1% CHAPS). The remaining proteins were eluted with 1M NaCl, 5 mM EDTA, 0,5% CHAPS or directly with SDS/PAGE buffer and were fractionated by SDS/PAGE. The protein detection was performed by silver staining or by immunoblotting using the enhanced chemiluminescence system (Amersham Pharmacia).

3.10.2 Two-hybrid analysis

3.10.2.1 Principal of the two-hybrid system

The basis of the two-hybrid system relies on the structure of particular transcription factors that have two physically separable domains: a DNA-binding domain and a transcription activation domain. The fact that a functional transcription factor can be reconstituted through noncovalent interaction of two independent hybrid proteins containing either a DNA-binding domain or an activation domain constitutes the basis of the two-hybrid approach (Fields and Song, 1989). A number of different DNA-binding domain and transcription activation domain vectors have been successfully employed in this system.

3.10.2.2 The system based on yeast Gal4p

The most extensively used vectors are Gal4p-based. Examples are the Gal4p DNA-binding domain vector pAS2 and the Gal4p activation domain vector pACTII. The reporter strain must contain a reporter gene that is under the control of the upstream activation sequence (UAS) corresponding to the DNA-binding domain vector. A number of reporter strains are available in which the bacterial gene *lacZ* is the reporter, for example strain Y190. It also carries the yeast biosynthetic gene *HIS3* under the control of the *GAL* UAS so that interacting clones can be screened by both nutritional selection for histidine and an assay for ß-galactosidase activity. The most commonly used two-hybrid cDNA libraries are made as Gal4p transcription activation domain fusion libraries.

3.10.2.3 The system based on the *Escherichia coli* LexA protein

In another implementation of the two-hybrid system, fusion proteins to the DNA-binding domain of the *Escherichia coli* LexA protein and to the activation domain of the herpes simplex virus VP16 protein are expressed on 2µ high copy number plasmids in the *Saccharomyces cerevisiae* L40 reporter strain (Hollenberg et *al.*, 1995). The bait plasmid, pBTM116, contains the entire coding region of the *Escherichia coli* LexA protein, expressed from the yeast alcohol dehydrogenase I (*ADHI*) promotor. The library plasmid, pVP16, contains the VP16 acidic activation domain, expressed from the yeast *ADHI* promotor. The L40 strain contains two integrated reporters: the yeast *HIS3* gene and the bacterial *lacZ* gene. The use of the heterologous binding domain in the yeast system leads to a reduction of the artificial activation of the reporter gene.

3.10.2.4 Protocol of a two-hybrid screen

First, the gene to examine must be subcloned into pAS2 and introduced into a reporter strain such as Y190 by transformation and nutritional selection using synthetic complete media lacking *TRP*. Some proteins that are not transcription factors themselves activate reporter gene transcription when fused to the Gal4p-DNA-binding domain, which will severely interfere with the two-hybrid system. Prior to beginning a screen, the obtained transformants carrying the bait protein have to be checked for *lacZ* activation and growth properties on SC-HIS plates containing different concentrations of 3-AT. 3-AT, an inhibitor of *HIS3*-encoded IPG-dehydratase, is used because the basal level of *HIS3* expression from the reporter construct, in which the *HIS3* UAS sequences have been replaced by the *GAL1-10* UAS, is sufficient to allow growth on SC-His media. 3-AT concentrations of 25 to 50 mM are typically sufficient to select against the growth of strains bearing pAS2 subclones that fail to activate transcription on their own.

If the Y190 reporter strain expressing the protein of interest (pAS2-X) does not activate *HIS3* or *lacZ*, this strain can be used for library transformation. Positive transformants are screened on (Leu+,Trp+;His-,3-AT+)-plates. Since only one out of six cDNAs is in the correct reading frame with the activation domain, a large number of independent yeast transformants need to be screened. The following protocol gives greater than 10^6 transformants with 50 µg library plasmid. The recipient strain Y190 (pAS2-X) is grown to mid-log ($1x10^7$ cells/ml) in SC(-Trp). With the above culture 1 liter of YEPD is inoculated such that the cell density becomes $1x10^7$ cells/ml in two generations. The cells are pelleted and resuspended in (100 mM LiOAC, 10 mM Tris pH8, 1 mM EDTA). Again the cells are pelleted and resuspended in 5 ml of (100 mM LiOAC, 10 mM Tris pH8, 1 mM EDTA, 1M Sorbitol) for every 200 ml of starting culture. Then the cells are incubated for 30 minutes at 30°C with shaking. As above the cells are pelleted and resuspended in 500 µl of (100 mM LiOAC, 10 mM Tris pH8, 1 mM EDTA, 1M Sorbitol) per 200 ml of culture. 2 µg pf pACTII library DNA and 200 µg of ssDNA for every 100 µl of cells are added. After mixing, the cells are incubated for 10 minutes at 30°C without shaking. After adding 900 µl of (100 mM LiOAC, 10 mM Tris pH8, 1 mM EDTA, 40% polyethylene glycol) for each 100 µl of cells the cells are mixed again. The tubes are heat shocked for 12 minutes in a 42°C water bath. At this point, cells can be plated out to check the transformation frequency. Five µl on a SC(-Trp,-Leu)-plate should give 1000 or more transformants. The rest is stricked on (+Leu,+Trp,-His,+3-AT)-plates. Colonies that grow after four to five days are then tested for ß-galactosidase activity using the X-Gal colony filter assay as follows: Schleicher and Schuell BA85 45 µm circular nitrocellulose filters (cat. 20440) are laid onto the plates of the yeast colonies resulting from the library transformation. The filters have to be completely wet. Then they are carefully lifted off the plate to avoid smearing of the colonies. To permeabilize the cells, the filter is placed for five to ten seconds in liquid nitrogen. The filter is carefully removed from the liquid nitrogen, thawed and placed cell side up in a petri dish that contains 3MM chromatography paper circles soaked with 0.3 ml/square inch of (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2.7 ml/l ß-mercaptoethanol) containing 1 mg/ml X-Gal-STL. The filters are incubated overnight at 30°C for the development of the color. Blue colonies are taken for further study and they can often be recovered directly from the filters.

3.11 Protein maturation assays

3.11.1 Invertase assay

The different stages of glycosylation and secretion of active invertase in *Saccharomyces cerevisiae* were analyzed as follows:

Cells of different strains were grown in YEPG medium at 30°C to mid–log phase and 10 OD units of cells were collected by centrifugation at 4.000*g* for 5 min at room temperature. Cells were washed with YEPG 0.1% (YEPG medium containing 0.1% glucose) resuspended in 10 ml of YEPG 0.1% and incubated at the desired temperature for 1-6 hours (1h at 37° C or 30° C, 2h at 25 ° C, 4h at 20°C, 6h at 15°C) (Esmon et *al.*, 1981). After induction, cells were collected, washed in 10 mM NaN_3 and resuspended in 100 µl lyticase buffer. Spheroplasts were formed by incubating the cells at 30°C for 1 hour with 200 units Lyticase (Sigma). Subsequently, to isolate periplasmic and intracellular invertase, spheroplasts were gently centrifuged at 1.000*g* for 5 min at RT and the supernatant containing the periplasmic invertase was transferred to a new tube. The pellet, containing the intracellular invertase, was gently washed with 1M sorbitol, resuspended in 100 µl lysis buffer and vortexed for 5 min at 4° C, finally the samples were centrifuged to eliminate cell debris, and the supernatant (containing the intracellular invertase) was transferred to a new tube. 20 µl of each sample were loaded onto a non-denaturing 7% polyacrylamide gel. The gel was soaked in 200 ml sucrose buffer for one hour at 37°C, washed with water and soaked in 150 ml staining buffer over a boiling bath till a red staining appeared. The reaction was blocked washing the gel with cold water and 10% acetic acid.

Lyticase buffer	1.4 M	Sorbitol
	50 mM	K_2HPO_4/KH_2PO_4, pH 7.5
	10 mM	NaN_3
	80 mM	β-mercaptoethanol
Lysis buffer	50 mM	K_2HPO_4/KH_2PO_4, pH 7.5
	10 mM	NaN_3
	0.1%	Triton X-100
Sucrose buffer	0.1 M	Sucrose
	0.1 M	NaOAc
	(pH 4.5-5.0)	
Staining buffer (150 ml)	300 mg	Triphenyltetrazoliumchloride
	4 g	NaOH
	(dissolve separately then mix)	
7% Polyacrylamide gel	5 ml	0.6M Tris-HCl pH 8.4
(non denaturing)	8.7 ml	30% Acrylamide stock solution
	26 ml	H_2O
	0.3 ml	10% APS
	17 µl	TEMED
Running buffer (1l)	5.19 g	Tris-HCl
	2.5 g	Glycine
	(pH 9.5)	

3.11.2 Carboxypeptidase Y assay

Overnight cultures of the appropriate strains were used to inoculate fresh SMM medium and grown at the required temperature to reach an OD_{600} of 0.6-1.2. Six OD units of cells were harvested, resuspended in 500 µl SMM medium containing 1 mg/ml ovalbumin and incubated at the appropriate temperature for 30-60 min. After that cells were pulsed for 10 min with 250 µCi Trans[^{35}S]-label mix (a mix of [^{35}S]-cysteine and [^{35}S]-methionine) followed by a chase of 30 minutes with methionine and cysteine (1.5 mg/ml each). The incubation time for both pulse and chase was longer if the temperature was lower than 30 °C. To stop the reaction the samples were placed on ice and NaN_3, PMSF and pefabloc were added (final concentration was: 10 mM NaN_3, 1 mM PMSF, 2 mM pefabloc). The labeled samples were fractionated into intracellular and extracellular fractions by centrifugation (1, 000*g* for 5 min). 250 µl medium (the extracellular fraction) was collected in new tubes and 10 µl 10% SDS were added, the pellet (intracellular fraction) was washed once with PBS containing 10 mM NaN_3 and resuspended in 100 µl 1% SDS, both fractions were frozen in liquid nitrogen (they can be stored several days at -80°C). The pellet samples were subsequently lysed by vortexing for 5 min in the presence of 0.1 g glass beads, boiled for 5 min and centrifuged at 14.000 rpm for 5 min. The supernatant (~100 µl) containing the intracellular fraction was transferred to new tubes and 500 µl 2x IP buffer plus 400 µl water were added. The 250 µl extracellular fractions were treated in a similar way. They were boiled for 5 min, centrifuged at maximum speed for 5 min and the supernatants transferred to new tubes containing 500 µl 2x IP buffer plus 250 µl water. The samples so treated were ready for the immunoprecipitation with anti-carboxypeptidase Y (CPY). 5-10 µl antiserum and 5 mg protein-A sepharose CL-4B were added to each sample. The samples were incubated overnight at 4°C (end-over-end rotation). After that the sepharose "beads" were sequentially washed with 1 ml ice-cold washing buffer-1, washing buffer-2, and washing buffer-3. The "beads" were resuspended in 50 µl Laemmli buffer and boiled for 5 min at 95°C. The immunoprecipitated proteins were separated by SDS-PAGE on 10% Acrylamide gels. After electrophoresis the gels were fixated for 20 min in a [10% acetic acid / 25% methanol] solution, treated with Amplify (Amersham) according to the manufacturer's instruction, dried and exposed to Kodak X-Omat AR films at -80°C for 2-10 days.

CPY is a soluble vacuolar hydrolase. It leaves the ER as a core-glycosylated precursor protein of 67 kDa, is further glycosylated in the Golgi apparatus (69 kDa), and finally reaches the vacuole, where after a short proteolytic truncation, it becomes active. This mature form has a molecular mass of 61 kDa.

SMM		
	0.1%	KH_2PO_4
	0.1%	NH_4Cl
	0.02%	$CaCl_2$
	0.06%	$MgCl_2$
	0.05%	NaCl
	0.3%	Yeast extract
	2%	Glucose
	Amino acids (according to the auxotrophy requirement)	

2x IP buffer	100 mM	Tris-HCl, pH 7.5
	2%	Triton X-100
	0.2%	SDS
	300 mM	NaCl
	2 mg/ml	Ovalbumin
	10 mM	EDTA
	4 mM	Pefabloc
	1 tablet/5ml	protease inhibitors
Washing buffer 1	1x	IP buffer
	2M	Urea
Washing buffer 2	1x	IP buffer
	500 mM	NaCl
Washing buffer 3	150 mM	NaCl
	5 mM	EDTA
	50 mM	Tris -HCl, pH 7.5

4 RESULTS

4.1 Studies on localization and topology of Sys proteins

Sys1p is a protein of 23,7 kDa containing hydrophobic regions over its entire length except for the N- and C-terminal regions. According to the MEMSAT (Jones et *al.*, 1994) model four putative transmembrane domains are predicted, the two domains residing in the C-terminal half of Sys1p with a composition that is characteristic for Golgi membrane proteins (Tsukada and Gallwitz, 1996).

```
1   MVSIRRYLRV PNELKPSQIF KQDSLSPSKI GLQIVLLQIF YYTTAIVLFY
51  CWAKLAGYDL NIKEWLFSWE NIDFTNAYGL SISLLWLLDS LICVFFLTVI
101 VGRSKLAWDF AITIHAINFI VVFLYTRKFP SFSWFFLQIL SSLILIFLGT
151 WTTRWRELRD TFFEGLVDPN EGEVGLVTPS QQHSNHSELE QSPIQLKDLE
201 SQI*
```

Figure 8. Amino acid sequence of Sys1p. The putative transmembrane regions are underlined.

SYS3 encodes a 105-kDa protein with a predicted high level of α-helices. It is related to a variety of mammalian Golgi-associated proteins and according to chemical cross-linking analyses, it forms dimers and larger protein complexes (Tsukada et *al.*, 1999). The DNA sequence of *SYS2* predicts a hydrophilic protein of 51.8 kDa. As there is a genetic interaction between *SYS2* and *SEC7*, which is involved in transport within and from the Golgi complex, Sys2p might also participate in a Golgi-associated transport reaction. It seems reasonable to assume then that the role of all the three proteins is somehow linked to the Golgi compartment

4.1.1 Sys1p is a late Golgi/endosomal transmembrane protein and Sys3p is associated to *cis*-Golgi membranes

To gain further insight into the intracellular localization of the Sys proteins, their distribution was first examined by differential centrifugation of cell lysates. To limit proteolysis, lysates were prepared by disrupting logarithmically grown cells in a mortar after they were frozen in liquid nitrogen. The frozen material was dissolved in proteinase inhibitor-containing buffer and successively subjected to a 10, 000*g* and 100, 000*g* centrifugation to enrich for ER and Golgi membranes, respectively. As shown in Figure 9, Kar2p (Rose et *al.*, 1989), a hydrophilic protein located in the ER

lumen, is almost completely pelletable with the 10, 000*g* centrifugation step. Emp47p (Schröder et *al.*, 1995), a protein that cycles through the ER, is evident in the ER as well as in the Golgi fraction. Sys1p also divides between these two fractions, but in contrast to Emp47p, a larger part co-sediments with the Golgi membranes. Treatment of the cell fractions with high salt or urea, conditions expected to dissociate protein complexes, did not result in the solubilization of Sys1p. Instead, detergents were able to solubilize it. Therefore, Sys1p appears to be a membrane protein and not to be part of a multiprotein complex. Sys2p could exclusively be detected in the Golgi fraction, whereas Sys3p was present in the soluble fraction as well. Treatment of the cell lysate with either Triton X-100, high salt or urea established that Sys2p and Sys3p behaved like proteins present in larger pelletable protein complexes, and were solubilized with salt and urea only.

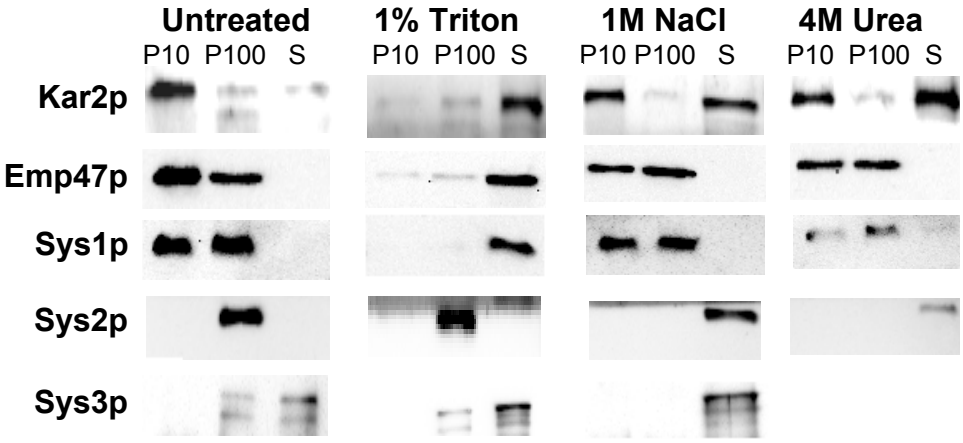

Figure 9. Sys1p is an integral membrane protein. A cleared yeast cell lysate (strain SEY6210) was divided into four aliquots that were treated for 15 min on ice with either lysis buffer (untreated), detergent, high salt or urea as indicated. After consecutive centrifugation a 10, 000*g* and 100, 000*g*, the pellets (P10, P100) and the supernatants (S100) were subjected to immunoblot analysis with anti-Kar2p (ER marker), anti-Emp47p (Golgi marker), anti-Sys1p, anti-Sys2p and anti-Sys3p antibodies.

To separate Golgi membranes from ER and vacuolar compartments more effectively, the cell lysate was subjected to velocity centrifugation through sucrose gradients. 1-ml fractions were collected from the top (1) to the bottom (12), and aliquots of these fractions were subjected to SDS-PAGE, Western blotting and immunodetection with the indicated antibodies (Figure 10). The band intensities were quantified with a Lumi-Imager and calculated as percentage of specific protein detected in all 12 fractions. As shown in Figure 10, Sys2p (B) and Sys3p (C) overlapped with the *cis*-Golgi membrane protein Sed5p. Sed5p and the late Golgi

marker Kex2p were at least partially separated and peaked at different densities. Sys1p cofractionated reproducibly with the late Golgi marker Kex2p (A). But most importantly, the Sys proteins did not co-migrate with the ER resident Kar2p.

As an independent approach to confirm the localization of Sys1p, a green fluorescent protein (GFP) tag was added to the C-terminus of Sys1p and the resulting

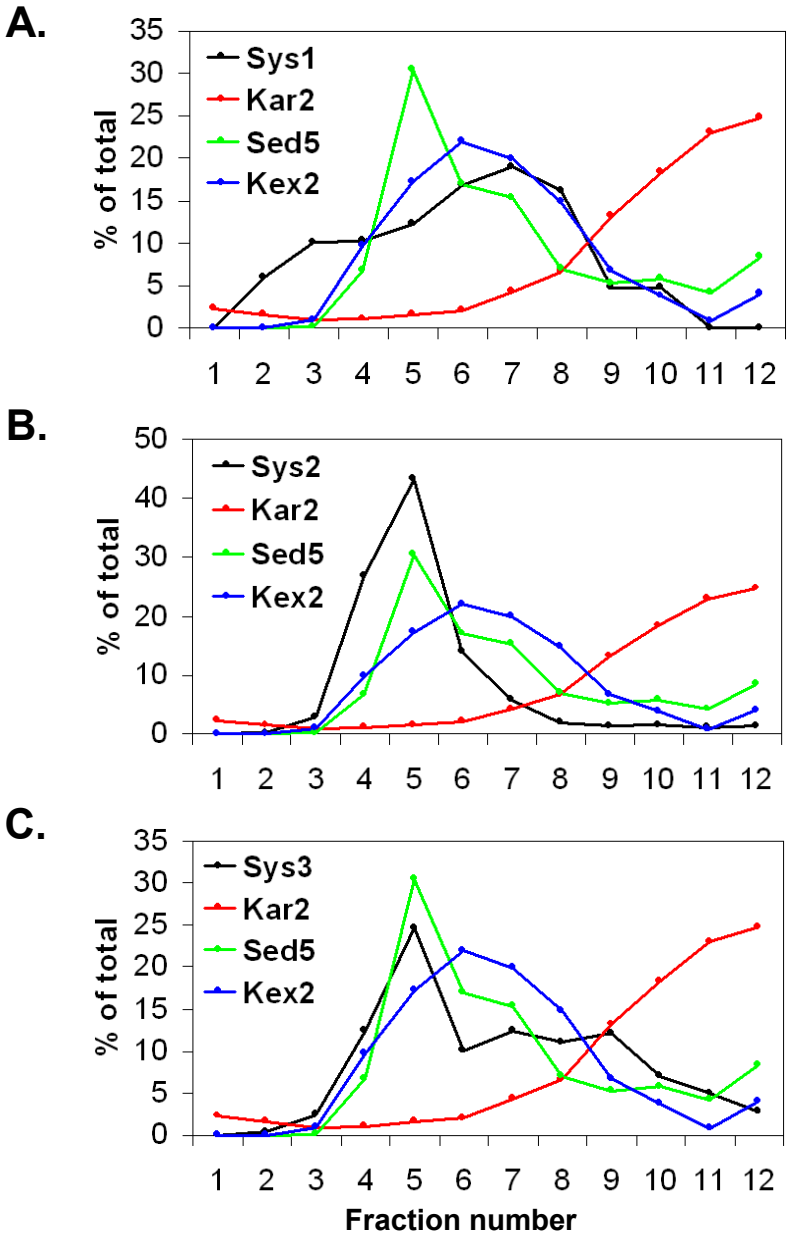

Figure 10. Cleared lysate was fractionated by velocity sedimentation on a 18-60% sucrose gradient. The fractions (1-12, from top to bottom) were collected and subjected to immunoblot analysis with antibodies to the proteins shown to the left. The band intensities were quantified using a LumiImager and presented as percentage of the loaded protein. Sys1p (A) colocalizes with the trans-Golgi protein Kex2p, Sys2p (B) and Sys3p (C) with the *cis*-Golgi protein Sed5p.

GFP-tagged protein (Sys1p-GFP) was expressed under control of the *SYS1* promoter. To generate this chimeric protein, it was made use of the plasmid pUG24 containing the cassette (GFP-*kanMX*) for integrative PCR-mediated GFP tagging. This integration cassette was amplified by PCR with the primers *SYS1*-tag2_f and *SYS1*-tag2_r containing homologous sequences to the C-terminus of genomic *SYS1*. After transformation of the PCR product into yeast strain Sey6210, the transformants were grown on geneticin-containing plates. Only cells with homologous recombination of the integration cassette into the genome were able to grow due to the obtained *kanMX* gene locus. These cells were grown to mid-logarithmic phase but the observed fluorescent signal was too weak to determine the intracellular localization of GFP-Sys1p.

The GFP-tag was also added to the N-terminus of Sys1p and expressed under control of the strong, regulated *MET25* promoter. *SYS1* was amplified by PCR with primers *SYS1*_f4 and *SYS1*_r5 and integrated into the vector pUG36. After transformation of pUG36-*SYS1* into strain SEY6210, cells were grown in SD medium without methionine. Lysates of these cells were separated through sucrose gradients.

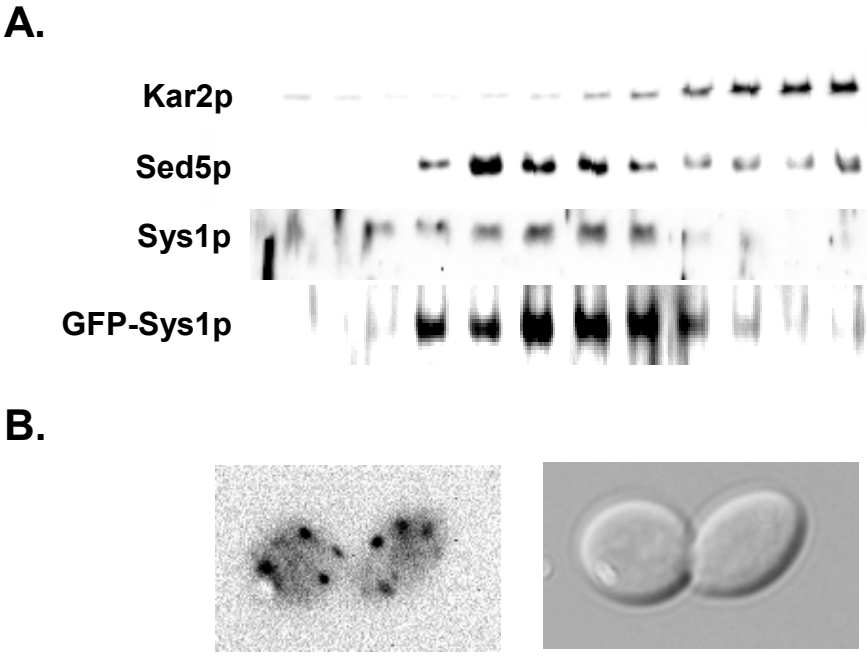

Figure 11. (A) Subcellular fractionation of cells expressing GFP-tagged Sys1p. Cell lysate of SEY6210 harboring pUG36-*SYS1* was separated on a sucrose-density gradient, and fractions were probed by immunoblotting (anti-Sed5p; anti-Kar2p; anti-Sys1p). All data are from a single gradient. Note that the GFP-Sys1p profile overlaps completely with wildtype Sys1p and both are compared to the *cis*-Golgi protein Sed5p displaced to slightly higher sucrose concentrations. (B) Visualization of GFP-Sys1p by fluorescence microscopy.

The distribution of the different markers was similar to those described above (Figure 11A). GFP-Sys1p only partially colocalized with the early Golgi marker Sed5p, but it was found exactly in those fractions, which contained wildtype Sys1p. Thus, overexpression of GFP-Sys1p does not seem to lead to an artificial distribution of the chimeric protein within cellular membranes. To test whether GFP-Sys1p also suppresses the temperature sensitivity of the *ypt6* deletion mutant, *ypt6* knockout strain MT1 (Tsukada and Gallwitz, 1996) was transformed with pUG36-*SYS1*. The overexpression of the chimeric protein enables strain MT1 to grow at 37°C. As expected from the fractionation studies, visualization of GFP-Sys1p by fluorescence microscopy revealed a punctate pattern characteristic of yeast endosomes or Golgi (Figure 11B).

According to subcellular fractionation studies and visualization of GFP-Sys1p, Sys1p resides mostly on late Golgi cisternae and/or on endosomes. The ability of Sys1p to suppress defects in Ypt6 GTPase-regulated endosome-to-Golgi transport, therefore, supports the conclusions drawn.

Sys3p, which is functionally linked to Ypt6p, appears to predominantly reside on *cis*-Golgi-membranes.

4.1.2 Sys1p does not cycle through the ER

To ensure that Sys1p does not recycle through the ER as many *cis*-Golgi proteins do, it was made use of *sec12* and *sec23* mutants. In *sec12* and *sec23* mutant cells secretion is blocked before the budding step of transport vesicles from the ER at non-permissive temperature (Schröder et *al*., 1995; Barlowe and Schekman, 1993). If Sys1p cycles through the Golgi and the ER, it should be possible to redistribute Sys1p to the ER and to deplete the Golgi pool after elevating the temperature. Using these mutants, Schröder et *al.* (1995) were able to trap Emp47p in the ER although at steady-state Emp47p localizes to Golgi membranes nearly exclusively (Figure 12).

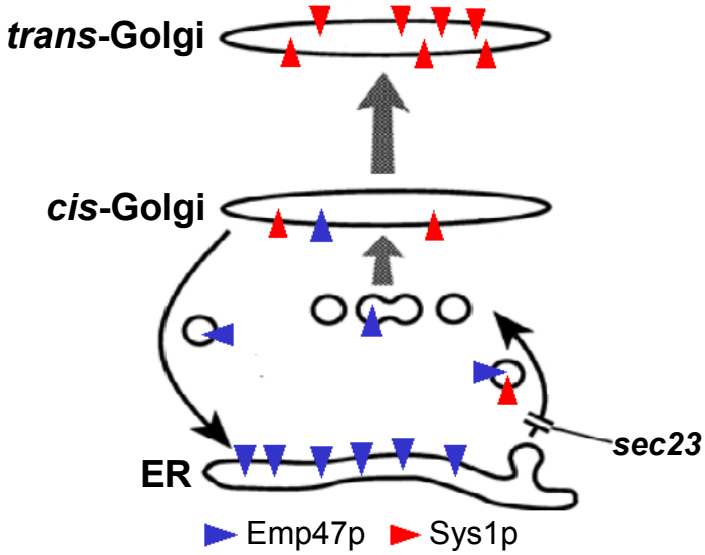

Figure 12. Cycling of Emp47p through the ER could be demonstrated by trapping it in the ER after a block of export in a temperature–sensitive *sec23* mutant (Schröder et *al.*, 1995). If Sys1p does not cycle through the ER, it maintains its wildtype localization after shifting the cells to non-permissive temperature.

sec23 cells were pre-cultivated at permissive temperature (24° C). To prevent an accumulation of newly synthesized proteins in the ER, cycloheximide was added to limit protein biosynthesis. Then the culture was divided and incubated at permissive (24° C) and non-permissive temperature (36° C) for 1h. The cells were spheroplasted, lysed and the cell lysates were fractionated on a sucrose gradient. Immunoblot analysis of the gradient fractions confirmed that the *cis*-Golgi-localized Emp47p and Sys1p partially overlapped, but the peak of Sys1p was at higher density than that of Emp47p. At non-permissive temperature, Emp47p accumulated in the Kar2p-containing ER fractions, whereas Sys1p still remained in Golgi fractions (Figure 13B). This result clearly shows that Sys1p does not cycle through the ER. In another experiment, *sec12* cells were treated in the same way as described above, but the synthesis of new proteins was not blocked after the pre-cultivation at permissive temperature. The spheroplasted cells were submitted to differential centrifugation as described before. The time of the 10, 000*g* centrifugation was reduced to 1 min to obtain a better differentiation between ER and Golgi membranes. At non-permissive temperature, Emp47p accumulated in the ER fraction but Sys1p mainly stayed in the Golgi fraction (Figure 13A), supporting the conclusion that Sys1p does not recycle to the ER.

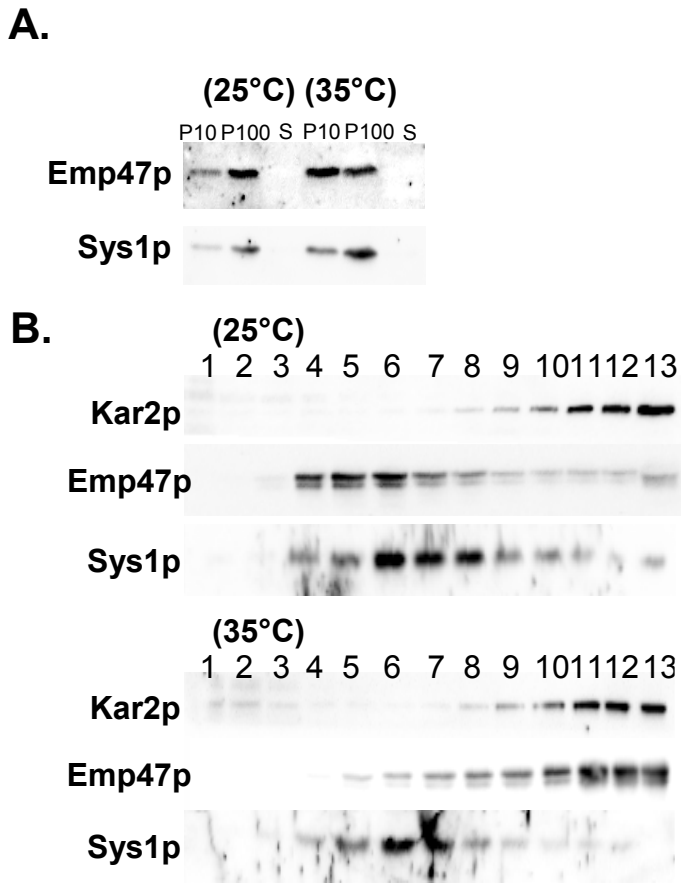

Figure 13. Sys1p does not cycle through the ER. Cleared lysates of *sec12* (A) or *sec23* (B) mutant cells, grown at either permissive (24°C) or non-permissive temperature (36°C), were subjected to differential centrifugation (A) or sucrose gradient centrifugation (B) and subsequently to immunoblot analysis as described in the legend to Figure 9.

4.1.3 The C-terminus of Sys1p faces the cytosol

According to differentiell centrifugation analysis Sys1p is an integral Golgi/endosomal membrane protein with four predicted transmembrane regions. For a rough topological study, cell membranes and organelles were treated with proteinase K. Proteins surrounded by membranous structures are protected against degradation in this experiment. To analyze either degradation or protection, proteins were subjected to Western blot analysis. Sys1p was probed with an antibody directed against its hydrophilic C-terminus. Emp47p, a type I integral Golgi membrane protein (Schröder et *al*., 1995), served as a control and was identified with an antibody to its lumenal part. Also the hydrophilic ER lumen protein Kar2p (Rose et *al*., 1989) should be protected against the protease. As a type II transmembrane protein, Sec22p's complete N-terminus is exposed to proteinase K and therefore is an ideal control (Ballensiefen et *al*., 1998). Whereas the C-terminal hydrophilic domain of Sys1p and

the N-terminal domain of Sec22p were at least partially digested by the protease, the lumenal part of Emp47p and Kar2p were protected (Figure 14). This shows that the C-terminus of Sys1p faces the cytosol.

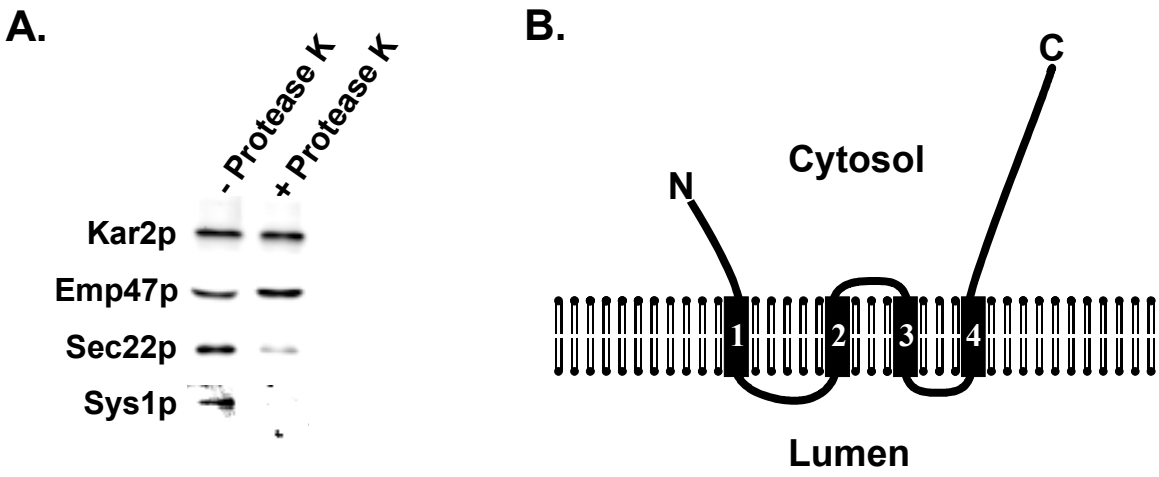

Figure 14. (A) Cell membranes and organelles of gently lysed cells were treated with proteinase K. Emp47p and Sec22p were then identified by Western blot analysis using antibodies against its lumenal or its cytosolic part, respectively. Sys1p was probed with an antibody to its C-terminus. Kar2p was chosen as reference protein because it is a hydrophilic protein of the ER lumen and so completely protected from digestion. (B) Model of the resulting membrane topology of Sys1p. The four transmembrane regions are indicated by numbers (1-4), the terminal regions by N and C.

4.2 Lack of expression of both Sys1p and Ypt6p disturbs the recycling of GFP-Snc1p from the plasma membrane to the Golgi

The protein sorting events in the prevacuolar compartment in yeast are relatively well characterized, because defects at this point have an easily scorable “vacuolar protein sorting” (*vps*) phenotype (Rothman et *al*., 1989). In contrast, sorting defects in early endosomes have much more subtle phenotypes, and this process has been harder to detect and study. As Ypt6p and Sys1p are implicated in the retrograde transport from early endosomes to the Golgi, direct evidence for a precise functional involvement in this transport step is difficult to obtain. Recently, a new assay was described, that uses the change in localization of GFP-Snc1p as readout for a functional recycling pathway (Lewis et *al*., 2000). In wildtype cells, the protein travels from the Golgi to the plasma membrane, is endocytosed and returns to the Golgi for reuse. At steady state, GFP-Snc1p accumulates at the plasma membrane,

with some intracellular punctate staining which corresponds to endosomal and Golgi compartments (Lewis et *al*., 2000). When GFP-Snc1p was expressed in cells lacking the Golgi/endosomal SNAREs Tlg1p and Tlg2p, no cell surface but punctate fluorescence was detectable which is typical for endosomal structures (Lewis et *al*., 2000). As Sys1p and Ypt6p are assumed to be involved in the same transport step like Tlg1p and Tlg2p, the intracellular localization of GFP-Snc1p in *sys1* and *ypt6*

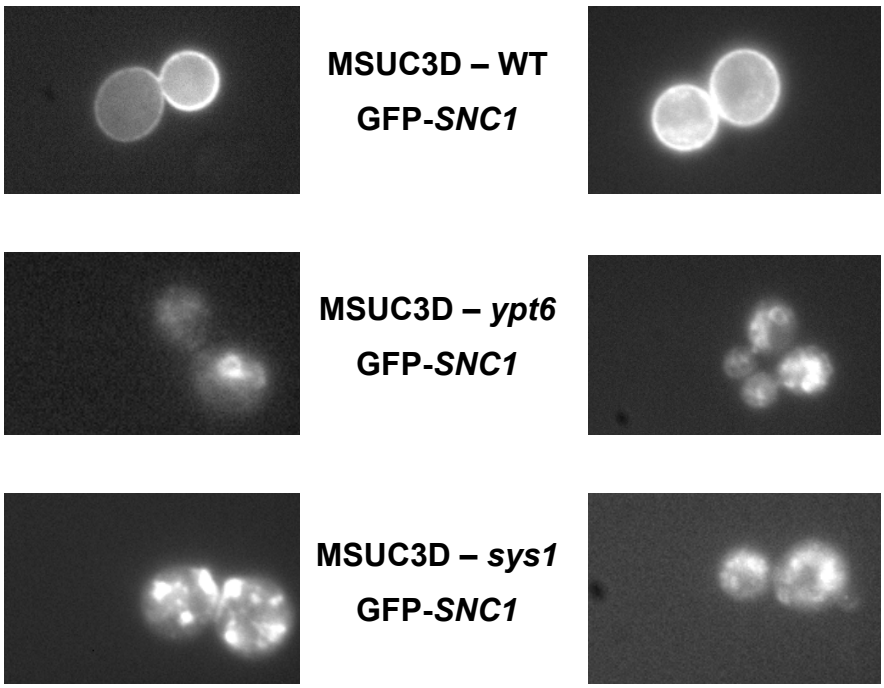

Figure 15. Defects in GFP-Snc1p recycling in *ypt6* and *sys1* deletion mutants. The *ypt6* and *sys1* deletion mutants and the wildtype strain were transformed with pUG36-*SNC1*. The transformants were grown to exponential phase at 30°C and inspected by fluorescence microscopy.

single deletion mutants was analyzed. Therefore, Snc1p was N-terminally fused to GFP and expressed from the recombinant vector pUG36-*SNC1* under control of the strong methionine promotor (detailed information in *Table 16*). *sys1* and *ypt6* mutant cells, transformed with pUG36-*SYS1*, were grown without the selection marker uracil and methionine to induce the negatively regulated methionine promotor. Lack of Sys1p or Ypt6p caused GFP-Snc1p to accumulate preferentially in punctate internal structures, most likely early endosomes. Additionally, in *ypt6* mutants circular structures were detectable. Since the Ypt6p deletion phenotype characteristically shows a severe fragmentation of the vacuole, the circular fluorescence may correspond to vacuolar structures (Tsukada and Gallwitz, 1996). The block of

retrograde transport from endosomes to the Golgi in *sys1* and *ypt6* mutants can be taken as evidence for an involvement of both proteins in this transport step.

4.3 Screening for functional domains in the hydrophilic termini of Sys1p

To define specific domains in the cytoplasmic tails of Sys1p that may contribute to the suppressor function, mutants with truncations in the hydrophilic N- or C-terminus of the protein were generated.

4.3.1 Expression of Sys1p mutants with successive truncations in the hydrophilic termini of Sys1p

Therefore, *SYS1* and the different 5´- and 3´-terminal truncations of *SYS1* were amplified by PCR. The oligonucleotides used for the amplification of the complete reading frame and for the truncated versions contained an *Eco*RI and a *Hind*III

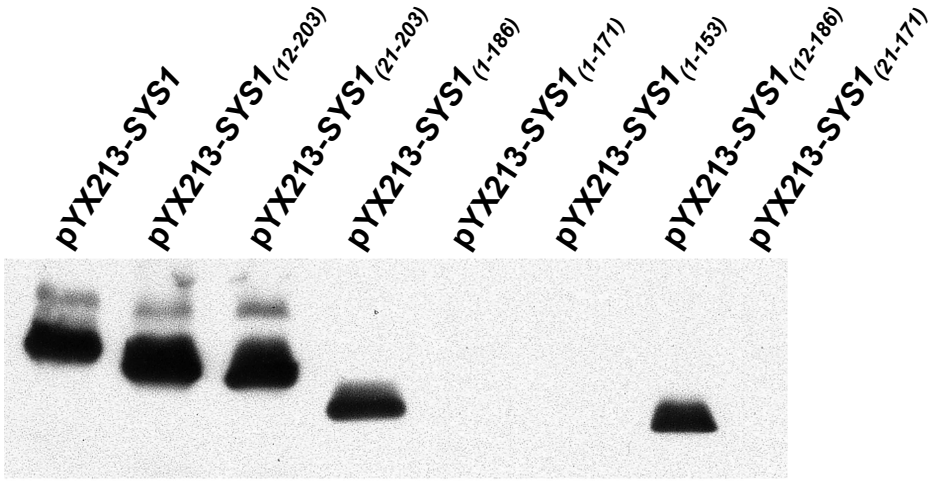

Figure 16. Western blot showing Sys1p expression in a *sys1* knockout strain containing pYX213-*SYS1* or the indicated constructs that encode the Sys1p truncation mutants.

restriction site. The resulting PCR products encoding the truncated versions of *SYS1* were digested by *Eco*RI and *Hind*III and integrated into the predigested high-expression vector pYX213 to place them under transcriptional control of the galactose-inducible *GAL10* promotor (details in *Table 16*). After transforming a *sys1* deletion strain with different pYX213-*SYS1* constructs, the transformants were pre-cultivated in PM-raffinose medium and then shifted to YEPGal medium to induce the Gal-promoter. Aliquots of total cell extracts were subjected to SDS-PAGE and

Western-blotting. The wildtype protein, the N-terminal truncation mutants and the C-terminal mutant with a deletion of the last 15 amino acids could be detected with an antibody directed against the C-terminal tail of Sys1p. The other C-terminal truncations could not be detected due to deletion of the recognition sequence of the antibody (Figure 16).

4.3.2 A fragment of the hydrophilic C-terminus of Sys1p is required for *ypt6* suppressor function.

The eight constructs were then tested for their ability to suppress the *ypt6* deletion phenotype. Yeast Ypt6p is a non-essential protein, but *ypt6* deletion mutants are temperature-sensitive (Figure 17D). Therefore, a *ypt6* deletion strain was transformed with the different pYX213-*SYS1* constructs and the transformants obtained were pre-cultivated as described above. The ability of cells to grow was scored on galactose-containing YEPGal plates at permissive (25° C) and non-permissive temperature (37° C). As shown in Figure 17A, the N-terminus and the last 30 amino acids of the C-terminal tail of Sys1p were not of relevance for its suppressor ability, whereas a deletion of the complete C-terminal domain resulted in a total loss of this property. This result suggests that the 20 amino acids downstream of the last transmembrane domain are of special importance for the suppressor function of Sys1p or contain sequence elements that ensure the correct intracellular localization of the protein.

Overexpression of constructs with both N- and C-terminal truncations does not compensate the loss of Ypt6p. This may be due to failures in folding or incorrect membrane insertion.

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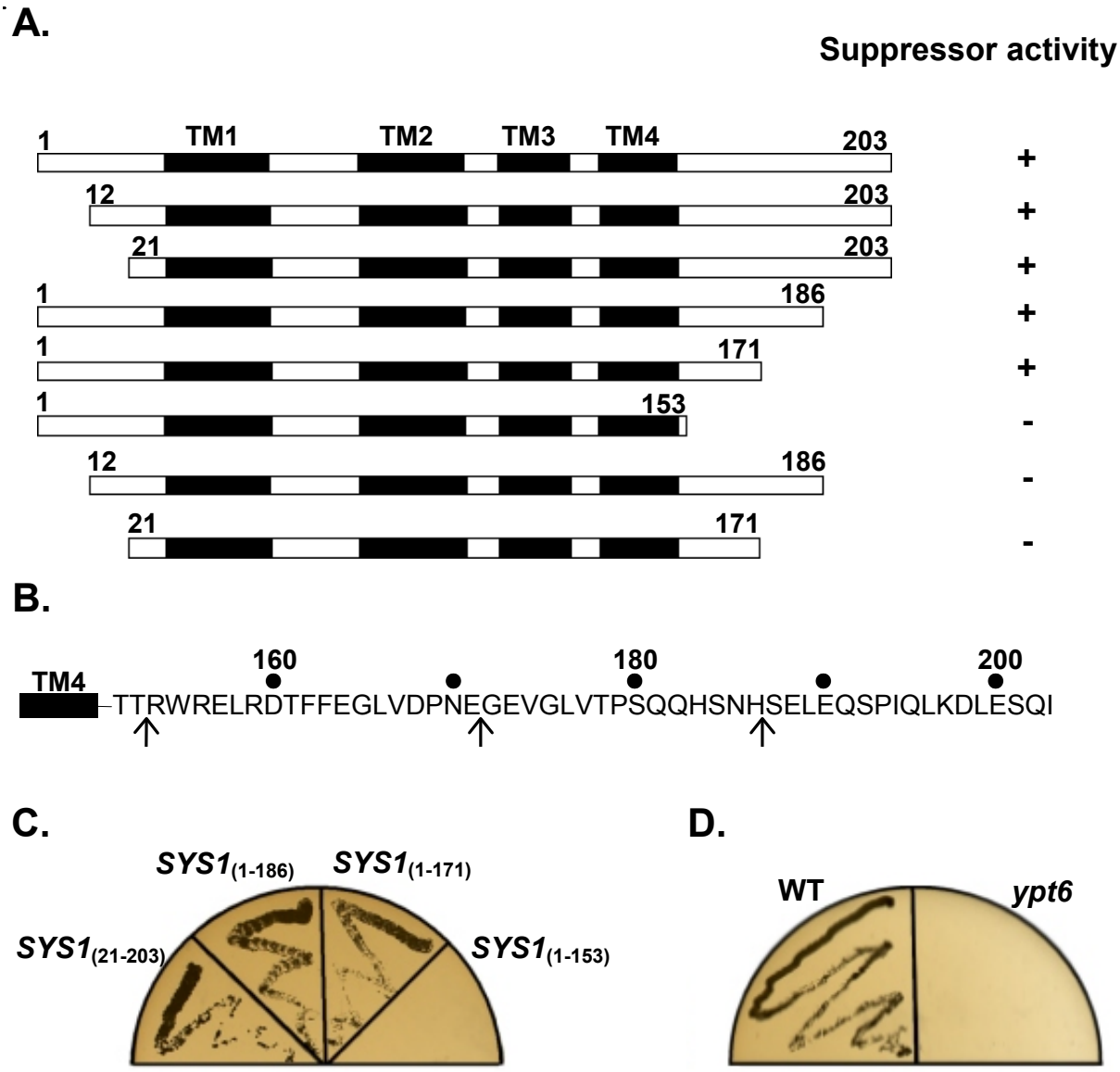

Figure 17. Delineation of *ypt6* mutant suppressor activity of Sys1p. (A) Schematic representation of N- and C-terminally truncated Sys1 mutant proteins tested for suppressor activity. The four putative transmembrane domains (TM1-4) and the first and last amino acids of the various Sys1 proteins are indicated. (B) The sequence of the C-terminal tail of Sys1p is shown with arrows pointing to the end points of truncations. (C) Deletion of the complete C-terminal hydrophilic domain (*SYS1*$_{(1\text{-}153)}$) unables the protein to rescue the growth defect of the *ypt6* mutant at 37°C. (D) Wildtype strain and the *ypt6* mutant were incubated at 37°C.

4.4 Screening for proteins that interact with the C-terminus of Sys1p

According to the results of the *ypt6* suppressor test, the C-terminal region of Sys1p downstream the fourth transmembrane domain appeared to be the prime candidate for serving a functional role in the context of the Ypt6-GTPase. Therefore, the major goal was now to screen for potentially interacting proteins both by two-hybrid analysis and affinity chromatography.

4.4.1 The C-terminal domain of Sys1p itself activates the transcription of *GAL4* in the yeast two-hybrid system

The two-hybrid system is an efficient method to analyze known or to find new protein-protein interactions *in vivo*. It takes advantage of the properties of the Gal4 protein of *Saccharomyces cerevisiae*. This protein is a transcriptional activator required for the expression of genes encoding enzymes of galactose utilization. It consists of two separable and functionally essential domains: An N-terminal domain which binds to specific DNA sequences [DNA-BD(omain)] and a C-terminal domain containing acidic regions, that are necessary to activate transcription [AD(omain)]. To screen for potential interacting partners the bait gene is expressed as a fusion to the DNA-BD of *GAL4* in vector pAS2, while a cDNA library is expressed as a fusion to the AD of *GAL4* in vector pACTII. Interaction of the fusion proteins brings the DNA-BD and AD into close proximity, thus reconstituting *GAL4* and activating transcription of the reporter genes *LacZ* and *HIS3*. The expression of the reporter genes results in prototrophy for histidine and expression of ß-galactosidase, the gene product of

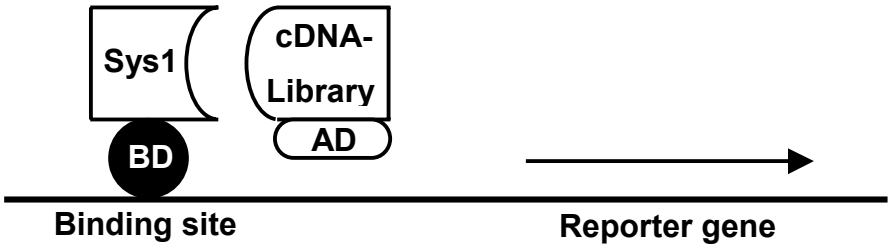

Figure 18. The two-hybrid system. Interaction between the “bait” protein Sys1p and a potential binding partner (“prey”) out of a cDNA library brings the activation domain (AD) into close proximity to the DNA-binding site and results in transcription of the reporter gene. The acidic C-terminus of Sys1p itself activates transcription and hinders library screening. Binding domain (BD); Activation domain (AD).

LacZ. ß-Galactosidase activity is measurable by a colorimetric filter assay using X-Gal (Breeden and Nasmyth, 1985) or ONPG (Miller, 1972) as substrate.

The result of the *ypt6* suppressor test (Figure 17D) indicated that the C-terminus of Sys1p downstream the putative transmembrane domain TM4 is an important functional domain. Therefore, the complete C-terminal tail of Sys1p (amino acids 151 to 203) was fused to the DNA-binding domain of *GAL4* (amino acids 1 to 147) in vector pAS2 and the resulting fusion protein was expressed in strain Y190

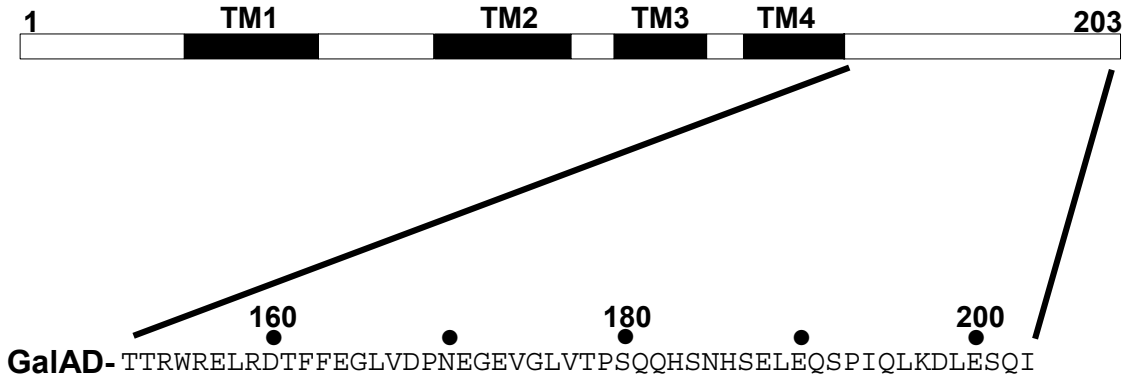

Figure 19. The complete C-terminal tail of Sys1p was fused to the DNA-binding domain of *GAL4* (amino acids 1 to 147) in vector pAS2. The vector was expressed in strain Y190 with the goal to identify interacting proteins in a two-hybrid screen. The expression of pAS2-*SYS1* resulted in a strong transcription of the reporter gene, whereas the empty plasmid pAS2 in the same assay did not show activation at all.

with the goal to identify interacting proteins in a two-hybrid screen (Figure 19). For the two-hybrid screen it is essential that this hybrid is not active on its own. Even a low level of transcription due to activation through the bait protein can severely complicate library screening. The expression of pAS2-*SYS1* resulted in a strong transcription of the reporter gene, whereas the empty plasmid pAS2 in the same assay did not show activation at all. In a modified two-hybrid system (Hollenberg et *al.*, 1995), one hybrid is a fusion between the *E.coli* LexA DNA-binding domain (amino acids 1 to 211) and the bait protein. The use of a heterologous binding domain in the yeast system can result in a reduction of artificial activation of the reporter gene. *SYS1* was also integrated into the plasmid pBTM116 and transformed into yeast strain L40 to express the LexA-Sys1 fusion protein (detailed information about the plasmid construction in *Table 16*). But also expression of the Sys1p C-terminal tail fused to the prokaryotic binding domain LexA resulted in strong activation of the reporter genes.

Many proteins, including those not known to be involved in transcription, activate transcription when fused to a DNA-binding domain (Ma and Ptashne, 1988), and this activation makes a library screen impossible. Therefore, the two-hybrid system could not be used for interaction analysis of the Sys1 C-terminal tail.

4.4.2 On an affinity column, Sys1p binds to the COPII heterodimer Sec23p-Sec24p

Based on the results of the suppressor test, the complete C-terminal tail of Sys1p (amino acid residues 151-203) was fused to GST and used as an affinity matrix to screen for interaction partners in total yeast cell extract. *SYS1* was amplified with primers containing restriction sites for *Bam*HI and *Hind*III. The purified PCR product and the vector pGEX-TT (Amersham Pharmacia Biotech) were cut with *Bam*HI and *Hind*III and were ligated (compare *Table 16*). For protein expression, the vector was transformed in the protease-deficient *E.coli* strain BL21. After lysis and detergent solubilization of the proteins, recombinant GST-Sys1p was immobilized on glutathione-Sepharose and used as an affinity matrix for solubilized *Saccharomyces cerevisiae* proteins. Unspecifically bound proteins were washed off the column with 150 mM salt buffer. The remaining proteins were eluted with 1M NaCl (Figure 20A) or directly with SDS/PAGE buffer (Figure 20B), fractionated by SDS/PAGE and detected by silver staining.

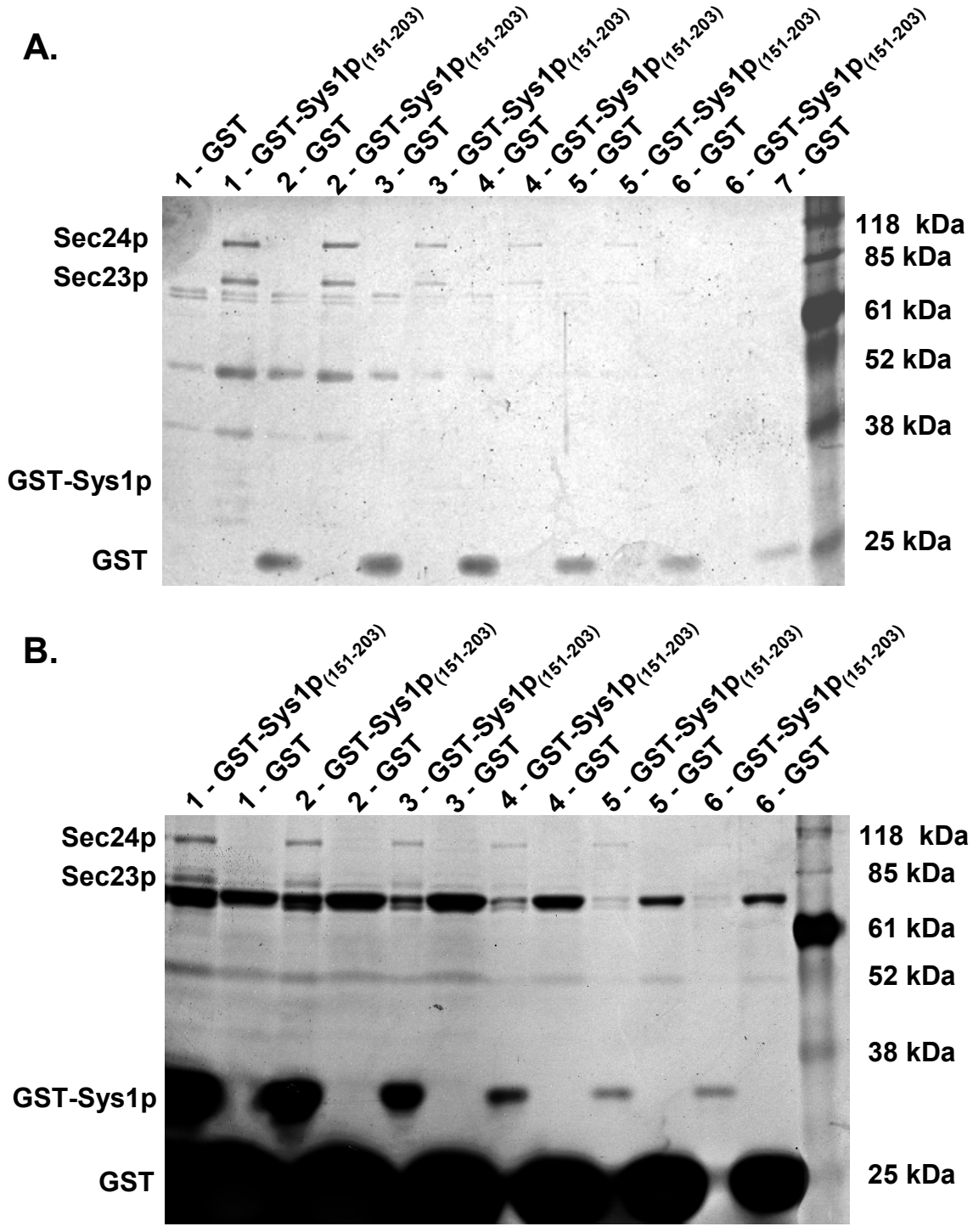

Figure 20. Sys1p binds specifically to the COPII heterodimer Sec23p-Sec24p. GST or GST-Sys1p was bound on a glutathione-Sepharose column and incubated with CHAPS-solubilized proteins from protease-deficient yeast cells in HEPES-buffer. After washing with 150 mM NaCl/ 0,1% CHAPS, the bound proteins were eluted in 1 ml fractions of 1M NaCl/0,5% CHAPS (*A*) or SDS/PAGE buffer (*B*). The proteins were separated by SDS/PAGE and detected by silver staining.

Two proteins larger than the 30 kDa fusion protein and exhibiting a molecular mass of about 85 and 100 kDa, respectively, were observed reproducibly to bind to GST-Sys1p, but not to GST alone (Figure 20). Immunoblotting with the polyclonal anti-Sec24p and anti-Sec23p antibodies identified these proteins to be the COPII

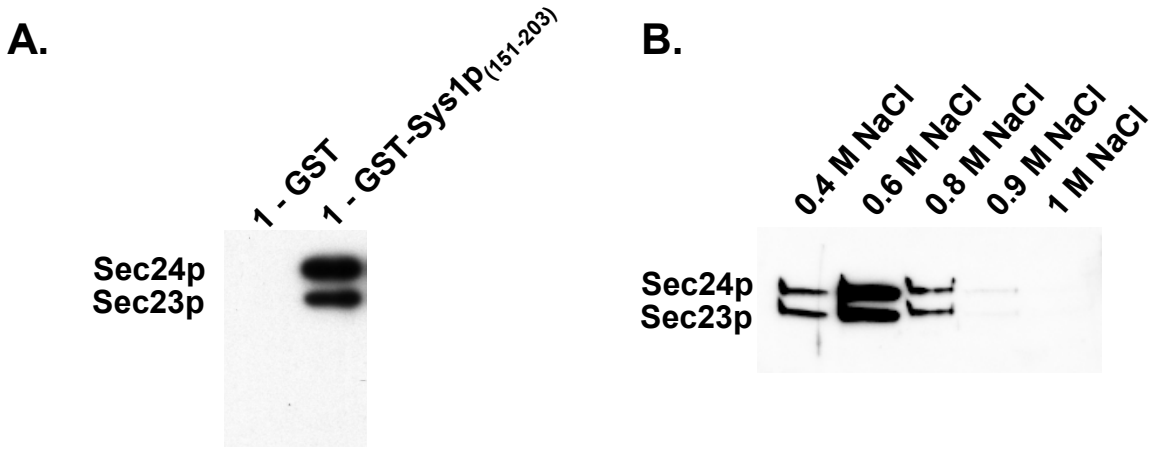

Figure 21. GST and a GST-Sys1 tail fusion protein, coupled to agarose beads, served as affinity matrix for detergent-solubilized total yeast protein. Beads were washed with buffer, and bound proteins eluted in 1 ml fractions of 1M salt buffer. (A) Immunoblot analysis with antibodies against Sec23p and Sec24p. (B) Successive elution with the indicated salt concentrations and immunoblot analysis as in (A).

heterodimer Sec23p-Sec24p (Figure 21A). In another experiment, the bound proteins were successively eluted from the GST-Sys1p matrix with accelerating salt concentrations. The majority of the two proteins eluted at a NaCl-concentration of 0.6 M (Figure 21B), a concentration where the Sec23p-Sec24p complex itself is still stable.

These results suggest that Sys1p is a protein with four transmembrane spanning domains, whose hydrophilic C-terminus directs the protein into COPII vesicles for transport to the Golgi apparatus.

To determine which component of the COPII heterodimer binds Sys1p, GST-Sec23p and GST-Sec24p were bound to beads which then were incubated with bacterial extract of cells expressing the C-terminus of Sys1p fused either to the maltose binding protein (MBP) or to six histidines (His_6-Tag). For GST-Sec23p and GST-Sec24p expression, the protease-deficient yeast strain cl3-ABYS-86 was transformed with pEG-KT-*SEC23* and pEG-KT-*SEC24*, respectively (Peng et *al.*, 1999). Transformants were first grown in glucose medium to midlog phase, and then

shifted to PM-galactose medium for induction of protein expression. Cells were broken with glass-beads and the GST fusion proteins were purified with glutathione-Sepharose. The beads were washed with 1M KCl and the purified GST fusion proteins were eluted with reduced glutathione. The expression of both fusion proteins has already been described (Peng et *al.*, 1999). MBP-Sys1(151-203)p was expressed from pMal-c2 containing the whole coding region of the C-terminus of *SYS1* (Figure 22A). Therefore, the plasmid was transformed into the protease-deficient bacterial strain BL21, and protein expression was performed as described before. Alternatively, the C-terminus of Sys1p was expressed as His_6 fusion protein from the vector pQE30 (Figure 22B).

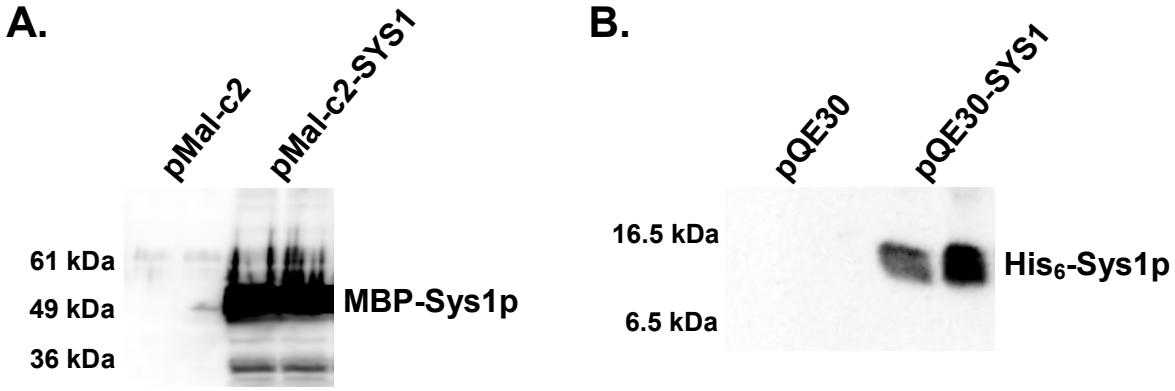

Figure 22. Western blots showing MBP-Sys1p (A) and His_6–Sys1p (B) expression in the bacterial strain BL21 containing the expression vectors pMal-c2-*SYS1* and pQE30-*SYS1*, respectively. For the immunoblot, anti-Sys1p antibodies were used.

To test for binding to Sys1p, GST-Sec23p or GST-Sec24p were bound to glutathione-Sepharose beads and incubated with detergent-solubilized MBP-Sys1p or His_6-Sys1p. After extensive washing, the bound proteins were eluted with 1M NaCl, separated by SDS-PAGE and detected by immunoblot analysis with anti-His_6 or anti-MBP antibodies, respectively. In both experiments, no binding of Sys1p to Sec23p or to Sec24p could be detected (data not shown). This might mean that the Sys1 C-terminal tail only binds to the Sec23p-Sec24p COPII subcomplex.

4.5 *In vitro* characterization of the COPII binding motif in Sys1p

The main purpose of studying Sys1p was its ability to suppress the deletion phenotype of *ypt6* mutants. Because the C-terminal tail of Sys1p is required for this activity, interacting proteins were screened for. The unexpected observation that the Sys1p C-terminal tail tightly interacts with Sec23p-Sec24p, the COPII subcomplex required to form transport vesicles at the ER membrane, suggested that Sys1p is sorted into ER export vesicles. There is a deep current interest in signals for ER exit, as this area has been neglected for a long time due to the suggestion in earlier models that proteins simply leave the ER by "bulk flow" (Pfeffer and Rothman, 1987). Nowadays, there is growing evidence that exit from the ER is selective rather than by default (Barlowe et *al.*, 1994). The specificity of the interaction of Sys1p to the COPII coat proteins Sec23p-Sec24p implies the presence of sorting signals in its cytosolic sequence. A double phenylalanine (FF) and an Asp-Leu-Glu (DXE) motif have already been proposed to be involved in the selection of the mammalian proteins ERGIC-53 and VSV-G, respectively, to the COPII coat and are also present in the C-terminus of Sys1p (Kappeler et *al*., 1997; Nishimura and Balch, 1997; Figure23). The

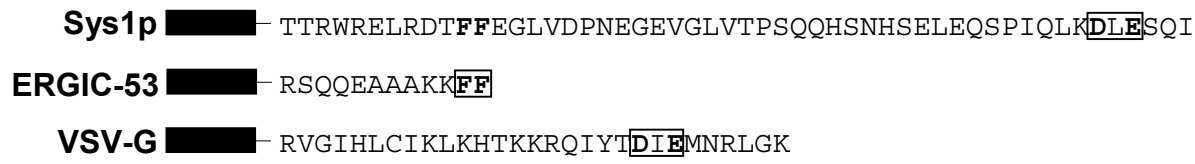

Figure 23. Comparison of C-terminal sequences of yeast Sys1p, mammalian ERGIC-53 and viral VSV-G proteins. The (FF) and the di-acidic motifs effective in efficient ER export in mammalian cells are highlighted.

experimental result described above suggested that the truncation of the hydrophilic C-terminal domain of Sys1p could have disrupted forward trafficking and therefore eliminated suppressor function. To examine whether the loss of suppressor function correlates with the absence of trafficking signals, alanine substitution mutants of the putative export motifs were generated and tested for their ability to bind to the heterodimer Sec23p-Sec24p. GST fusions of the Sys1p C-terminal tail, with and without various amino acid substitutions, were analyzed for their interaction with the heterodimer.

4.5.1 The di-phenylalanine motif is not relevant for ER export of Sys1p

It was previously shown for the mammalian protein ERGIC-53 that, together with the two phenylalanine residues of the (FF)-motif, a second targeting determinant composed of two glutamines is essential for binding to COPII. Therefore, site-directed mutagenesis was used to substitute with alanines the two conserved phenylalanines Phe-162 and Phe-163 and the two glutamine residues Gln-181 and Gln-182, alone or in combination, in the GST-Sys1 fusion protein (WT; amino acid residues 152-203; C1-C3 – Figure 24). In another construct, only the last 39 amino acids of the C-terminal tail of Sys1p were fused to GST to eliminate the whole domain around the (FF)-motif (C5 – Figure 24). In this shortened fusion construct again, the two glutamine residues were substituted by alanines (C6 - Figure 24). In construct C4, amino acids 152 to 171 were fused to GST, to test whether the domain around the (FF)-motif alone is capable of binding Sec23p-Sec24p.

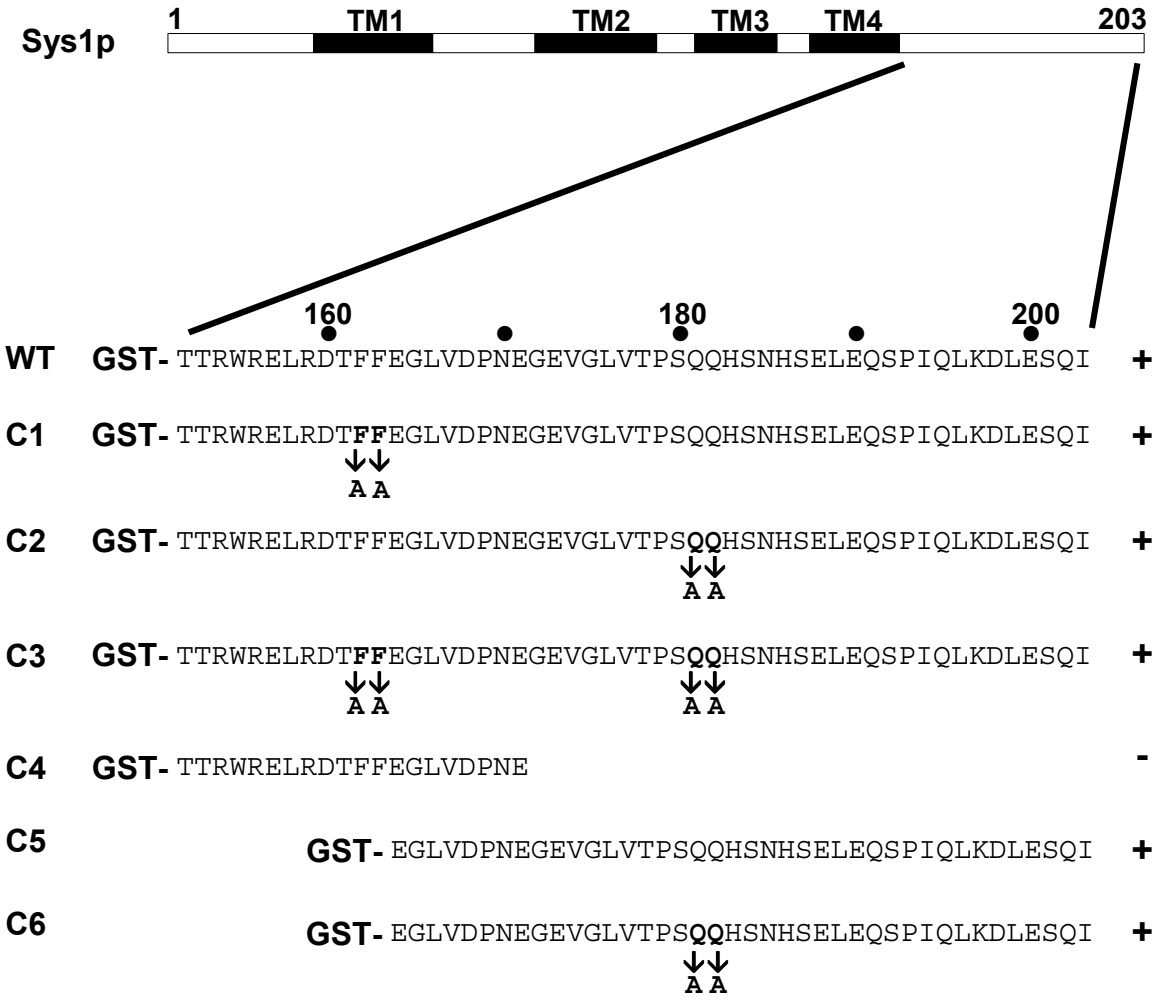

Figure 24. The hydrophilic tail of Sys1p (WT) and of six mutants (C1 to C6) thereof were fused to GST in the vector pGEX-TT and expressed in the protease-deficient *E.coli* strain BL21. “+” indicates binding, “-“ no binding to Sec23p-Sec24p. Experiment as described in Figure 20.

All constructs were bound to glutathione-Sepharose beads and were analyzed for their interaction with Sec23p-Sec24p as described above in Figure 20. Substitution with a double alanine or deletion of the (FF)-motif did not affect the binding affinity for the Sec23p-Sec24p heterodimer in comparison to the binding observed with the wildtype construct (GST-WT), whereas the domain around the (FF)-motif alone was not able to mediate binding (C4). Also, the additional replacement of the two glutamines did not result in a reduced binding affinity of the chimeric Sys1 protein to the COPII proteins (C6; Figure 25).

In conclusion, the (FF)-motif is negligible for interaction of Sys1p to Sec23p-Sec24p and consequently is not its targeting determinant for ER export.

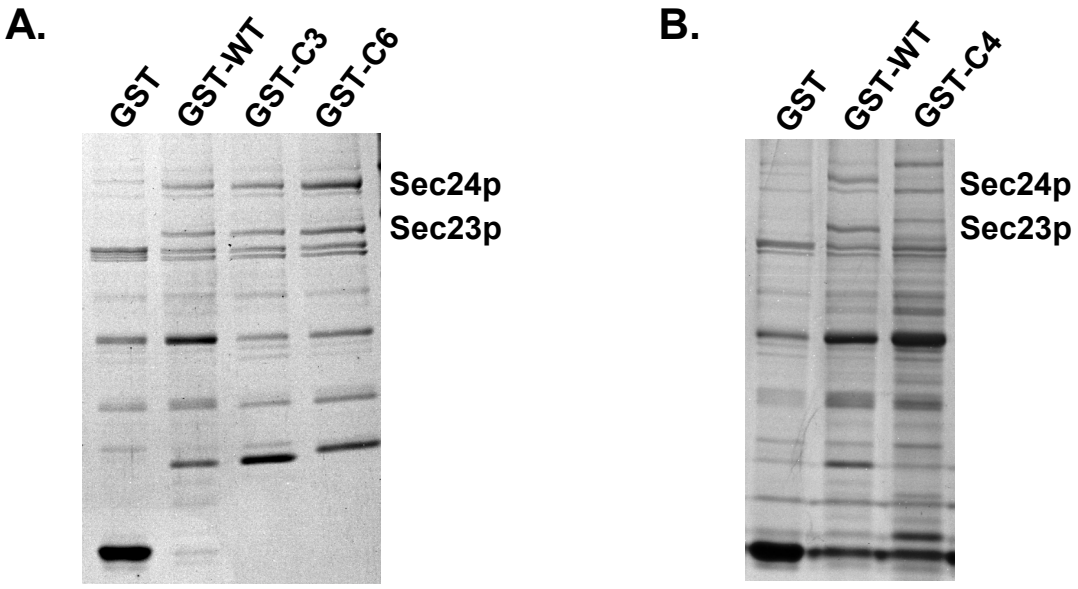

Figure 25. The hydrophilic tail of Sys1p (WT) and three mutants (C3, C4 and C6) were probed for Sec23p-Sec24p interaction. COPII subcomplex binding was investigated by an affinity approach as described in the legend to Figure 20. Constructs C3 and C6 bind the COPII heterodimer as efficiently as the wildtype construct, whereas construct C4 does not bind at all.

4.5.2 Binding to Sec23p-Sec24p depends on a (DXE)-motif in the C-terminus of Sys1p

A sequence comparison of the C-terminal hydrophilic domain of Sys1p with the cytoplasmic tail of other transmembrane proteins displayed an additional candidate binding motif for the binding of Sec23p-Sec24p. The di-acidic signal (DXE) that contains the amino acids aspartic and glutamic acid separated by a variable residue is present once in the C-terminus of Sys1p (Figure 23). This motif was first described for the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) to be required for efficient recruitment to COPII-vesicles in baby hamster kidney cells (Nishimura and Balch, 1997). To test for the importance of the di-acidic motif for the binding of Sys1p to Sec23p-Sec24p, the last 32 amino acids of the C-terminal tail of

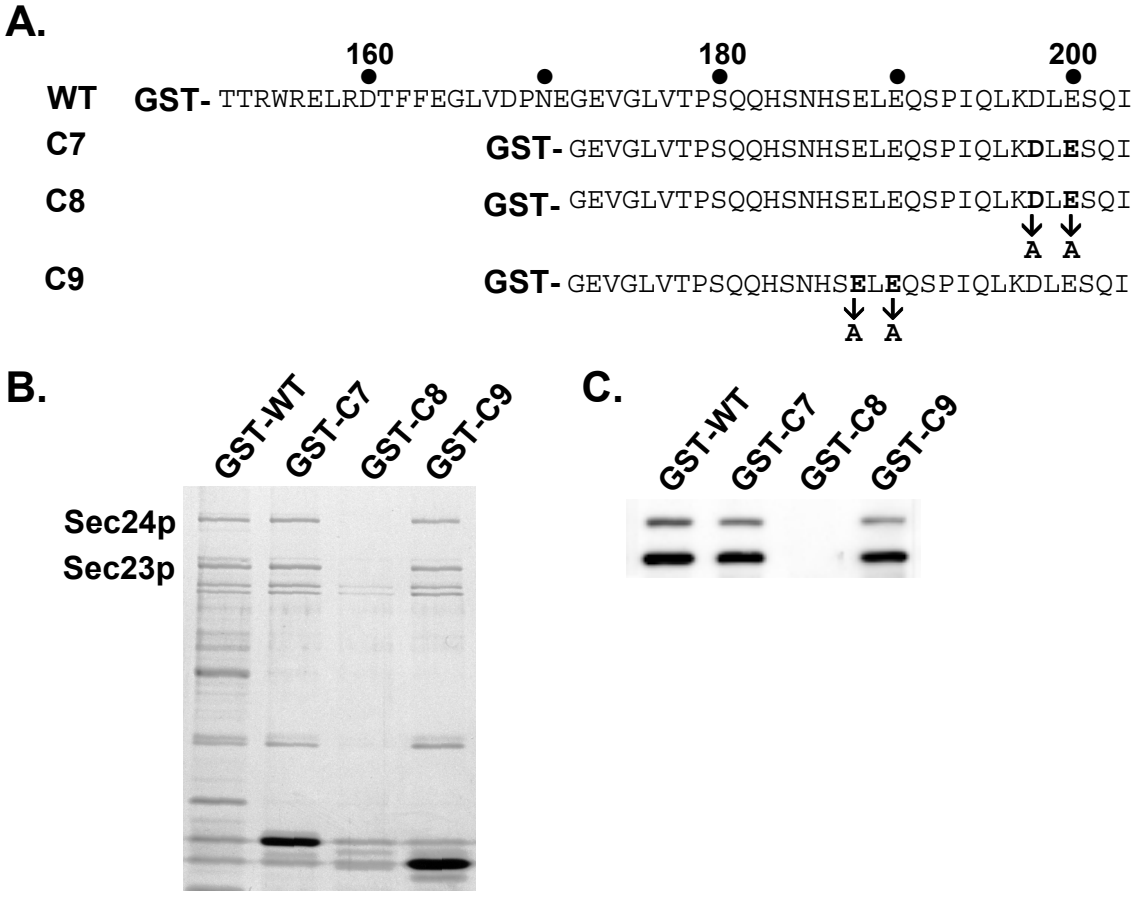

Figure 26. A di-acidic motif of the Sys1p tail is responsible for Sec23p-Sec24p binding. (A) The hydrophilic tail of Sys1p (WT) and several deletion and substitution mutants (C7-C9) were fused to GST and probed for Sec23p-Sec24p interaction. COPII subcomplex binding was investigated by an affinity approach as described in the legend to Figure 20 and is shown for some tail fusions in (B). Some amount of aliquots of the eluted samples were subjected to SDS-PAGE and proteins were identified by silver staining. Sec23p and Sec24p were verified with affinity-purified antibodies (C).

Sys1p were fused to GST and expressed from the pGEX-TT vector (GST-C7 – Figure 26A). The GST-C7 fusion protein contains the (DXE)-motif and the related sequence (EXE). To narrow down the COPII-binding motif, D-198 in combination with E-200 (GST-C8 – Figure 26A) and E-188 and E-190 (GST-C9 – Figure 26A) were replaced with alanines by site-directed mutagenesis at the DNA-level. To test whether these mutations affected the binding to the Sec23p-Sec24p complex, the mutant proteins fused to GST were produced in *E.coli* BL21 cells and used for affinity binding studies with total yeast cell extracts. Silver staining or immunoblotting with specific antibodies determined the relative amounts of Sec23p or Sec24p recovered on various affinity beads. Under these conditions, GST-tail fusion proteins containing either the complete C-terminus (GST-WT) or only the last 32 amino acids (GST-C7) interacted with similar efficiency with Sec23p-Sec24p (Figure 26B and 26C). The chimeric GST-C9 fusion protein with the $A_{188}XA_{190}$ substitution was still capable to recruit the COPII proteins Sec23p and Sec24p from solubilized total yeast cell extract (Figure 26B and 26C). This result implied that Sys1p containing the $A_{188}XA_{190}$ mutation was as efficiently collected into budding vesicles as the wildtype protein and that the (EXE) sequence had no influence on COPII subcomplex binding. Recombinant GST-C8 with the $A_{198}XA_{200}$ substitution failed to recruit Sec23p-Sec24p (Figure 26B). Neither silver staining of the eluted proteins separated by SDS-PAGE nor Western blotting with subsequent immunodetection showed any specific signal for Sec23p or Sec24p (Figure 26B and 26C). This result suggests that the (DXE)-motif of Sys1p is indispensable for the binding to the COPII-heterodimer and that it might be required for the efficient incorporation of Sys1p into COPII vesicles.

In further experiments, the sequence requirements of the (DXE)-motif for binding to COPII were characterized in more detail. Individual substitutions, either D198A or E200A (mutants C10 and C11 – Figure 27) abolished Sec23p-Sec24p binding (Figure 27).

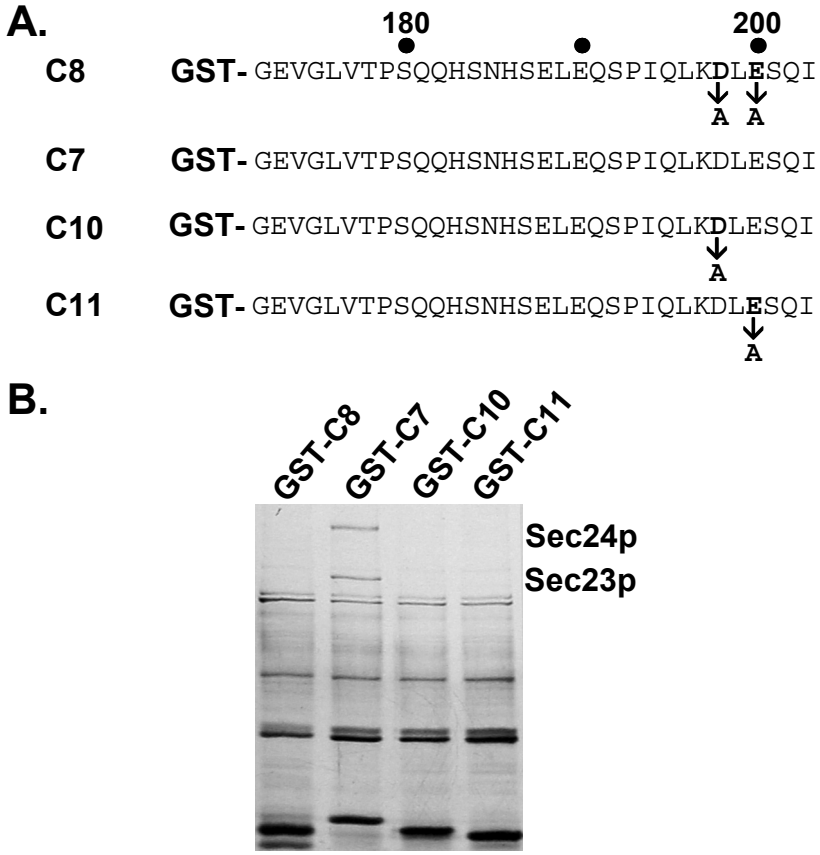

Figure 27. Detailed characterization of the sequence requirements of the DxE-motif for COPII binding. GST-Sys1p fusions (A) in which the two residues, D198 and E200, were mutated separately to alanine were tested for binding to Sec23p-Sec24p as described in the legend to Figure 20 (B).

4.5.3 Sys1p also interacts with Sfb2p and Sfb3p (Lst1p) in a (DxE)-dependent fashion.

Like Sec24p, two non-essential Sec24p-related proteins, termed Sfb2p and Sfb3p/Lst1p, form a complex with Sec23p and might facilitate the packaging of cargo proteins into COPII vesicles. Proteins from protease-deficient yeast cells expressing Sfb2p-HA and Sfb3p-Myc instead of the respective wildtype proteins were incubated with GST alone, GST-Sys1p (amino acid residues 151-203) and the GST-Sys1p fusion construct with alanine substitutions in the di-acidic motif. The elution profile revealed that both, Sfb2p and Sfb3p/Lst1p, are able to bind the C-terminus of Sys1p but not the construct which carries a mutation in the (DxE)-motif (Figure 28). Thus, the interaction of Sys1p and Sfb2p/Sfb3p is also mediated by the (DXE)-motif in Sys1p.

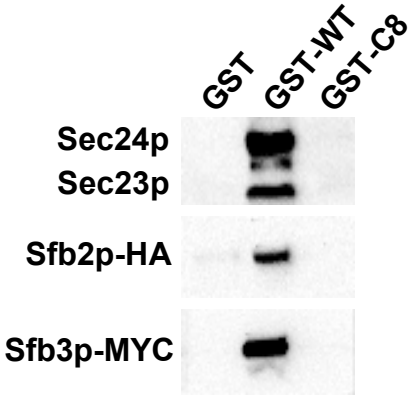

Figure 28. Sec24p-related proteins Sfb2p and Sfb3p bind to the Sys1p C-terminal tail. GST, and GST-Sys1 tail fusions (complete tail, GST-WT; C-terminal 32 amino acids with alanine-substituted (DXE)-motif, GST-C8) were used in an affinity binding experiment as outlined in legend to Figure 20. Detergent-solubilized total cell protein from strain ADY6 was used to identify tagged Sfb2-HA and Sfb3-MYC proteins by pull down experiments.

4.5.4 Sar1p does not interact with the C-terminus of Sys1p

COPII coat assembly at the ER membrane is initiated by the binding of the small GTPase Sar1p to the lipid surface of the membrane and the Sec12p-mediated switch to its GTP-bound conformation. Once Sar1p is associated with the membrane, Sec23p and Sec24p are recruited from the cytosol. Recently, it was proposed that an interaction of Sar1p with VSV-G might impose a conformational change in Sec23p-Sec24p that allows it to interact with the (DXE)-signal of VSV-G (Aridor et *al*., 2001). Consequently, the interaction of cargo with the COPII heterodimer is thought to be Sar1p-dependent, since Sec23p-Sec24p alone failed to bind the wildtype VSV-G tail including the (DXE)-motif.

Figure 29. Sar1p does not interact with the Sys1p cytoplasmic tail. Yeast cell lysate was incubated with GST-Sys1p (C7) and GST-Sys1(D198A;E200A)p (C8) fusions bound to agarose beads. After stringent washing steps, bound proteins were eluted with 1 M NaCl buffer. Proteins of eluted samples and those of yeast lysate were loaded on SDS-PAGE and transferred to nitrocellulose filters. Sar1p was identified with a polyclonal antibody.

I therefore analyzed whether the binding of Sys1p to Sec23p-Sec24p might be Sar1p-dependent. Recombinant GST chimeras containing the last 32 amino acid residues of the wildtype Sys1p tail (GST-C7) or of the Sys1p tail with alanine substitutions in the (DXE)-motif (GST-C8) were bound to glutathione-Sepharose beads and incubated with solubilized total yeast protein. The amount of Sar1p recovered on the beads was determined by immunoblotting with specific antibody. As seen in Figure 29, no evidence for a direct interaction of Sys1p and Sar1p was obtained.

4.5.5 Computer search for proteins with a di-acidic motif in the proteom of yeast

To screen for other yeast transmembrane proteins containing a C-terminal di-acidic motif, a simple computer analysis was performed. The “Yeast Genome Pattern Matching” program available on the SGD homepage (http://genome-www.stanford.edu/Saccharomyces) enables the user to define an algorithm. In order to search for proteins with a (DXE)-motif in close proximity of a C-terminal transmembrane domain, the following algorithm was defined:

B{10,}X{6,}D[IPL]E

B:	Any hydrophobic residue (IFVLWMAGCY)
X:	Any amino acid residue
{10,}	At least 10 times
{6,}	At least 6 times
[IPL]	Isoleucine, proline or leucine

To derive only sequences that fulfill the search criteria, the data obtained were analyzed for proteins whose membrane topology has already been solved. The following proteins have short C-terminal hydrophilic tails including a (DXE) motif in close proximity to a C-terminal transmembrane region:

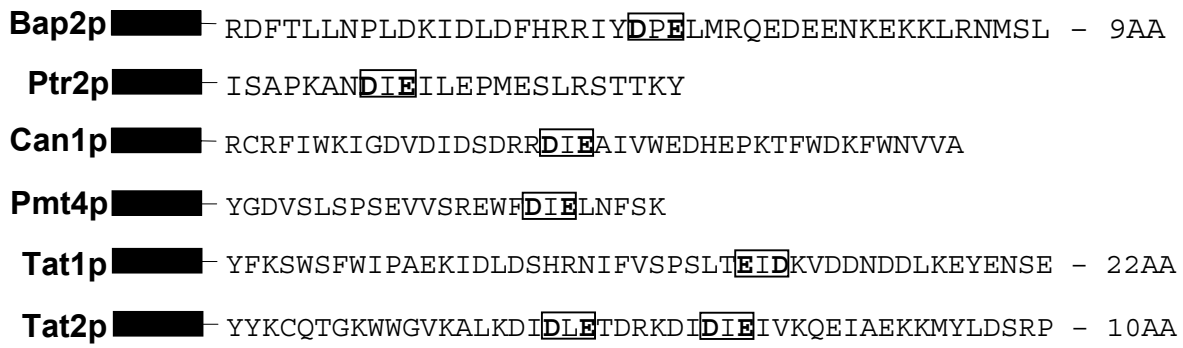

Figure 30. Like Sys1p, the amino acid permeases Bap2p, Can1p, Tat1p and Tat2p, the peptide transporter Ptr2p and the mannosyltransferase subunit Pmt4p contain at least one di-acidic motif in their C-terminal hydrophilic tail. Each motif is highlighted. AA – number of amino acid residues preceding the C-terminal end.

As can be seen in Figure 30, the C-terminal tails of all six proteins are rich in acidic residues. The amino acid permeases Bap2p (Grauslund et *al.*, 1995), Can1p (Opekarova et *al.*, 1998) and Tat2p (Schmidt et *al.*, 1994), the peptide transporter Ptr2p (Perry et *al.*, 1994) and the mannosyltransferase subunit Pmt4p (Immervoll et

al., 1995) contain a typical (DXE)-motif within their C-terminal tail. Tat1p, a structural homologue of Tat2p, contains an inverted (DXE)-motif.

The C-terminal tails of Bap2p, Tat1p, Tat2p and Ptr2p, each beginning at the end of the last membrane-spanning domain, were fused to GST and analyzed for their ability to retain the COPII components Sec23p-Sec24p on glutathione-Sepharose beads. GST-Sys1p served as positive control and GST alone as negative control. GST and the five chimeras were bound to beads and incubated with detergent-solubilized total yeast protein. Sec23p and Sec24p recovered on the beads was detected by immunoblotting with specific anti-Sec23p or anti-Sec24p antibody, respectively. As already found in other experiments, Sys1p was capable of efficiently recruiting Sec23p and Sec24p (Figure 31). In contrast to Sys1p, Bap2p and Ptr2p gave significantly weaker signals, and the permeases Tat1p and Tat2p showed no

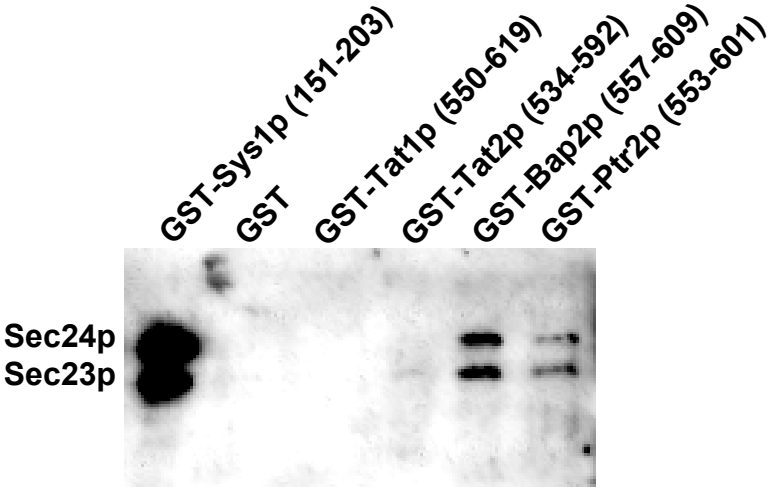

Figure 31. Sec23p-Sec24p interacts with the cytoplasmic tails of Sys1p, Bap2p and Ptr2p. GST or the GST fusions to the cytoplasmic domains of Sys1p, Tat1p, Tat2p, Bap2p and Ptr2p were incubated with total yeast extract. The experiment was performed exactly as described in Figure legend 19.

binding at all (Figure 31). This result is in line with previous studies showing that binding of VSV-G to Sec23p-Sec24p can be detected by Western blotting but not by direct protein staining.

In conclusion, this experiment suggested that a number of yeast proteins uses the (DXE)-motif for COPII interaction and, possibly, as a signal for ER export.

4.6 *In vivo* characterization of the COPII binding motif in Sys1p

The results described in the previous section provide the first and compelling evidence for a direct interaction of an integral membrane protein´s (DXE)-sequence motif and the COPII subcomplex Sec23p/Sec24p. Is this interaction of COPII coat and a potential membrane cargo of relevance for ER-export? Since a deletion of more than 50% of the C-terminal tail of Sys1p including the (DXE)-motif did not affect *ypt6* suppressor activity (see Figure 17), this signal sequence obviously cannot severely limit the extent of transport. In line with this conclusion are the results of an examination of transport of VSV-G over a longer time course. They revealed that the transport defect resulting from mutation of the (DXE)-motif predominantly affected the rate, but less strongly the extent of export outward from the ER (Nishimura and Balch, 1997).

To demonstrate rigorously that the (DXE)-motif controls the efficiency of ER export of Sys1p, following the transport kinetics of different Sys1 mutant proteins would have been the perfect choice for an experimental protocol. This, however, is hampered by the fact that Sys1p is not N-glycosylated, a prerequisite for pulse-chase experiments.

It was therefore decided to investigate the steady-state localization of Sys1p. To do this, Sys1 mutant proteins without the (DXE)-motif were examined for a possible ER retention.

4.6.1 Deletion of the (DXE)-motif results in partial mislocalization of Sys1p to the ER

To study the localization of different Sys1 mutant proteins, wildtype Sys1p, Sys1(D198A;E200A)p and Sys1(1-186)p were C-terminally tagged with six consecutive histidines followed by three copies of the HA epitope (YPYDVPDYA) by integrative PCR-mediated tagging. For this purpose, the expression cassette 6His-3xHA-*KanMX* was amplified with the vector pUG6H3HA (EMBL accession no. AJ132966) as template and with the primer pairs *SYS1*-tag1_f/*SYS1*-tag1_r (Sys1p), *SYS1*(D198A;E200A)-tag_f/*SYS1*(D198A;E200A)-tag_r [Sys1(D198A;E200A)p] and *SYS1*(1-186)-tag_f/*SYS1*(1-186)-tag_r [Sys1(1-186)p] containing homologous sequences to the C-terminus of genomic *SYS1*. After transformation of the PCR product into yeast strain Sey6210, the transformants were grown on geneticin-

containing plates. Only cells with homologous recombination of the integration cassette into the genome were able to grow due to the obtained *kanMX* gene locus.

To test whether substitution of the (DXE)-motif [Sys1(D198A;E200A)p] results in a partial mislocalization of Sys1p to the ER, sucrose gradient centrifugations were performed (Figure 32). A culture of the strain expressing Sys1(D198A;E200A)p was spheroplasted and the cleared lysate was fractionated on sucrose-gradients as described in Materials and Methods. The substitution mutant showed in addition to the Golgi co-localization, a peak co-migrating with the ER-marker Kar2p. The deletion of the (DXE)-motif resulted in a partial block of anterograde transport from the ER to the Golgi, since nearly one third of the Sys1 protein was retained in the ER in comparison to wildtype protein.

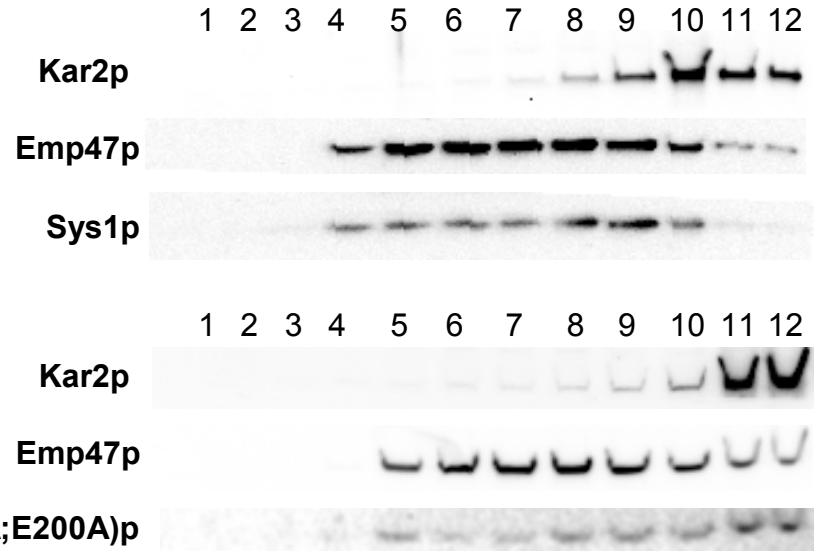

Figure 32. Substitution of the di-acidic motif results in a partial mislocalization of Sys1p to the ER. The wildtype protein and a mutant variant of Sys1p with substitutions D198A and E200A were C-terminally tagged with three copies of the HA epitope. Spheroplasted and lysed cells of these two strains were fractionated by sucrose gradient centrifugation. Aliquots of the obtained fractions were subjected to SDS/PAGE and Western blot analysis with antibodies against the proteins shown to the left. The bulk of Sys1p migrated to a Golgi co-localized position. The Sys1 mutant protein, in addition to the Golgi co-localization, shows a peak co-migrating with the ER-marker Kar2p.

In the second mutant, Sys1(1-186)p, the (DXE)-motif along with surrounding sequences was deleted since a recent study had shown that the signal responsible for efficient ER export of VSV-G protein in mammalian cells includes four additional amino acids proximal to the di-acidic motif (Sevier et *al.*, 2000). Like Sys1(D198A;E200A)p, the shortened variant of Sys1p lacking the (DXE)-motif also showed a peak co-migrating with the ER-marker Kar2p (Figure 33A). Quantification of band intensities with the Lumi-Imager revealed that this peak comprised about 30% of the total Sys1p. Nearly one third of total Sys1(1-186)p was retained in the ER (Figure 33B), suggesting that the (DXE)-sequence is in fact a determinant for efficient ER export of Sys1p and possibly other membrane proteins containing this signal sequence.

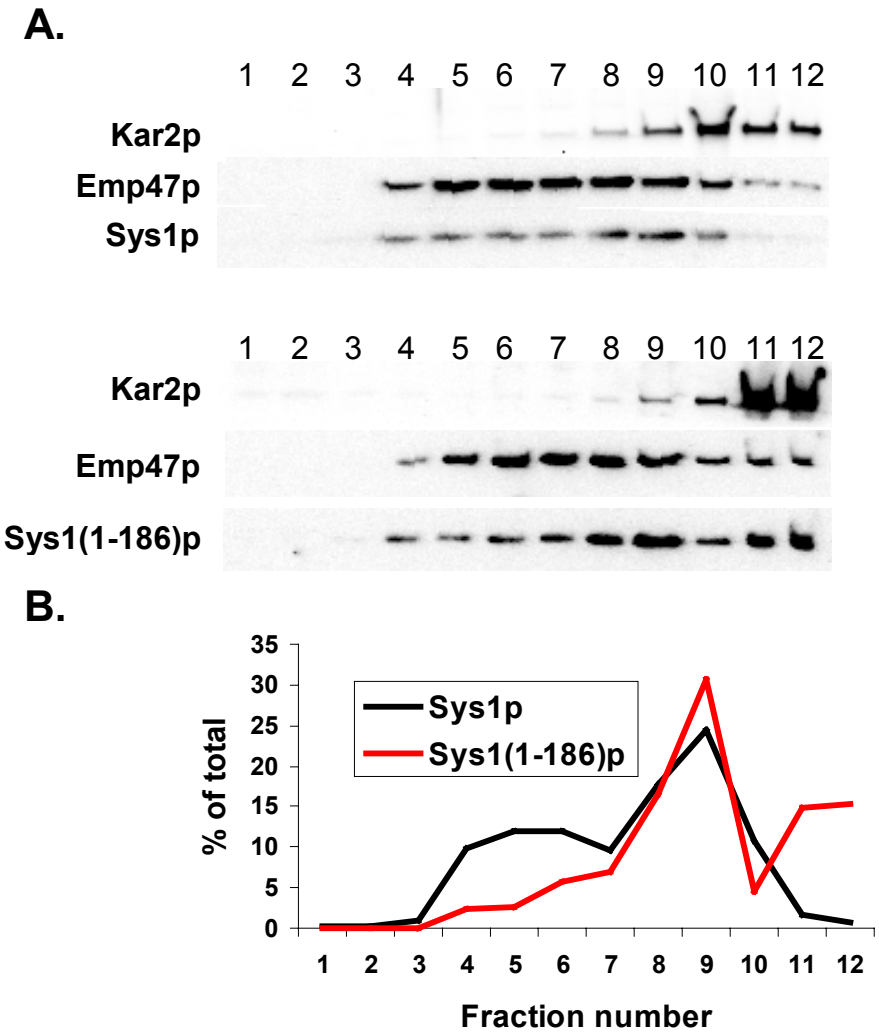

Figure 33. The deletion of the (DXE)-motif along with the surrounding sequences retains around 30% of the mutant Sys1(1-186)p in the ER. (A) The experiment was performed exactly as described in Figure legend 32. (B) The quantification of the band intensities with the NIH Image 1.59 software reveals that nearly 30% of the total Sys1p are mislocalized to the ER.

4.6.2 Engineering of the (DXE)-signal into an artificial cargo protein enhances ER exit

If, as suggested by the investigation described above, the (DXE)-motif of the Sys1p tail has a role in the efficient export from the ER of this Golgi/endosomal membrane protein, it should be possible to make an ER resident membrane protein available to the export machinery by fusing the (DXE)-motif-containing tail to its C-terminus. Such an experimental strategy has already been used for mammalian cells: VSV-G chimeras were generated in which the transmembrane and cytoplasmic domains of a type I transmembrane protein (TCRα) were replaced by those of VSV-G. The chimeric protein was efficiently exported from the ER, whereas a chimera with a mutated di-acidic motif stayed completely in the ER (Nishimura and Balch, 1997). Furthermore, the ion channel proteins Kir4.1 (Stockklausner et *al.*, 2001) and Kir6.2 (Ma et *al*., 2001) are largely ER-localized due to the lack of forward-trafficking signals, but their steady-state localization changes to the cell surface after fusion with an exogenous di-acidic signal. These results seem to exclude less efficient folding or assembling as the prime reason for ER retention of mutants in the di-acidic motif.

I chose to fuse the C-terminal tail of Sys1p to an ER type I membrane protein, the essential oligosaccharyl transferase subunit Wbp1p (te Heesen et *al*., 1993). For engineering the sequence around the (DXE)-signal of Sys1p into the C-terminus of Wbp1p, $SYS1_{(154\text{-}203)}$ and $WBP1_{(1\text{-}423)}$ were amplified with two sets of primers and the digested PCR-products were integrated into the centromeric vector pRS315 (Figure 34). Since it has been shown for Kir6.2 that the di-acidic motif cannot override an RXR(R)-mediated ER retention and retrieval (Ma et *al*., 2001), the 10 C-terminal, cytoplasmically oriented residues of Wbp1p, including the ER retrieval sequence (KKXX), were deleted. Wbp1p does not require the dilysine motif (KKXX) for proper function and localization nor does it aquire α1,6 mannose modification indicative of transit to an early Golgi compartment (Gaynor et *al*., 1994). The chimeric fusion between Wbp1p, lacking its short C-terminal tail, with the last 52 amino acids of Sys1 protein was termed Wbp1-$Sys1p_{(DXE)}$ (Figure 34B). Through site-directed mutagenesis, the two acidic residues of the di-acidic motif were substituted by alanine resulting in construct Wbp1-$Sys1p_{(AXA)}$ (Figure 34C). The recombinant vectors containing the two fusion genes were transformed into the protease-deficient yeast strain cl3ABYS, and organelles of gently lysed cells were separated through sucrose gradients. In untransformed cells, Wbp1p perfectly colocalized with the ER

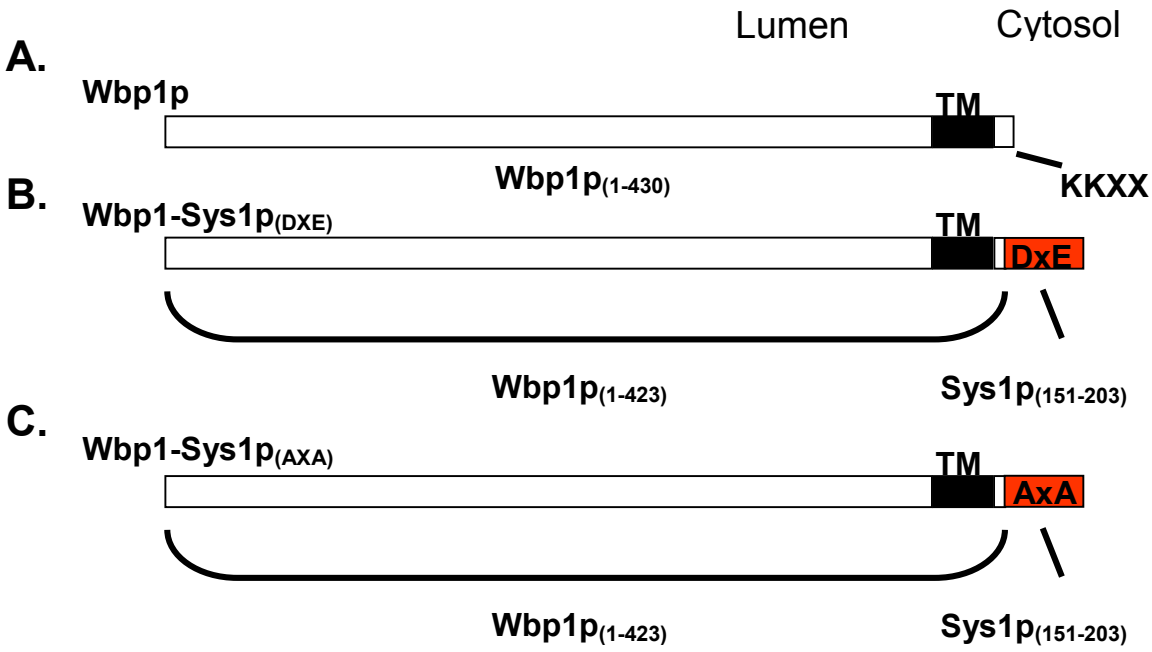

Figure 34. For engineering the sequence around the DxE-signal into the C-terminus of the ER-resident protein Wbp1p (A), the 7 C-terminal, cytoplasmically oriented residues of Wbp1p were deleted and replaced by the 53 amino acid-long Sys1p tail including the (DXE)-motif. The resulting construct was termed Wbp1-Sys1$_{(DXE)}$ (B). Through site-directed mutagenesis the two acidic amino acid residues of the (DXE)-motif were substituted by alanines resulting in the construct Wbp1-Sys1p$_{(AXA)}$ (C).

marker Kar2p (Figure 35A). In transformed cells, Wbp1-Sys1p$_{(DXE)}$ was shifted to late Golgi fractions (Figure 35B). The additional substitution of the two acidic residues of the di-acidic motif to alanine resulted in a redistribution of the chimeric Wbp1-Sys1p$_{(AXA)}$ to ER fractions (Figure 35C). Although wildtype and chimeric Wbp1p theoretically should show differences in their electrophoretical mobility due to a slightly divergent molecular mass, both proteins were indistinguishable on Western blots. In those cells that were transformed with the chimeric Wbp1-Sys1p$_{(DXE)}$, the ER-localized wildtype Wbp1p gave a quite weak signal on blots (Figure 35B). The expression of Wbp1-Sys1p$_{(DXE)}$ might therefore result in a down-regulation of the expression of wildtype Wbp1p, which is known to be under complex transcriptional control (Knauer and Lehle, 1994; Silberstein et *al*., 1995). Thus the fusion of the 52 amino acids of the C-terminal tail of Sys1p to Wbp1p was indeed sufficient for its translocation apparently into the Golgi complex. This was not due to deleting a putative ER retention signal in the Wbp1p C-terminus as referred to before.

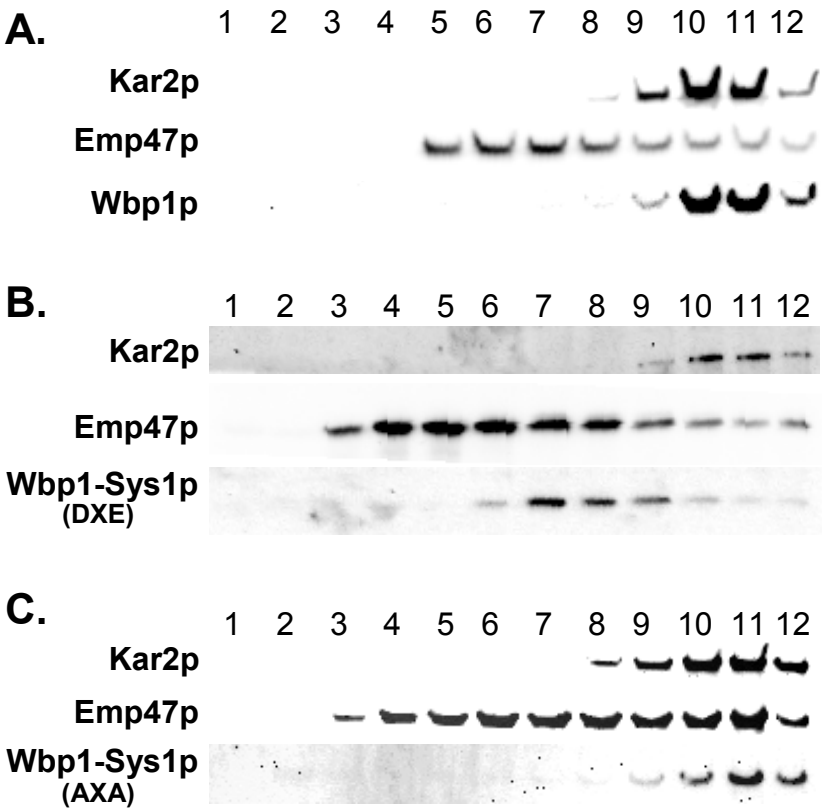

Figure 35. Engineering of the (DXE)-motif into the C-terminus of ER-resident protein Wbp1p directs it out of the ER. The additional substitution of the two acidic amino acid residues from the motif redistributes the chimeric protein back to the ER. (A), cleared lysates from wildtype yeast (strain CV1), (B) from yeast cells expressing chimeric Wbp1p such that its 10 C-terminal, cytoplasmically oriented residues were deleted and replaced by the 53 amino acid-long Sys1p tail and (C) from yeast mutant cells expressing Wbp1-Sys1p with D198A and E200A substitutions were subjected to sucrose gradient centrifugation, followed by immunoblot analysis of the proteins in gradient fractions 1 to 12 using the Wbp1 antibody.

To test whether the lethal phenotype of the *WBP1* deletion can be rescued by a plasmid that expresses the chimeric Wbp1-Sys1$_{(DXE)}$ fusion protein, it was made use of a diploid strain heterozygous for the *WBP1* deletion. The EUROSCARF strain Y20242 (*WBP1*/*WBP1*::*KanMX*) was transformed with the centromeric plasmid pRS315(*WBP1-SYS1*). Tetrad dissection of the transformants showed that haploid segregants containing the wildtype *WBP1* locus were completely viable, whereas the deletion-containing spores were able to germinate but to form micro-colonies only (Figure 36A). The colonies were replica-plated on YEPG(G418) medium to determine their phenotype (Figure 36B). All growing colonies on these plates corresponded to

the micro-colonies on non-selective medium, indicating that these were the haploid spores carrying G418 at the chromosomal locus of *WBP1*.

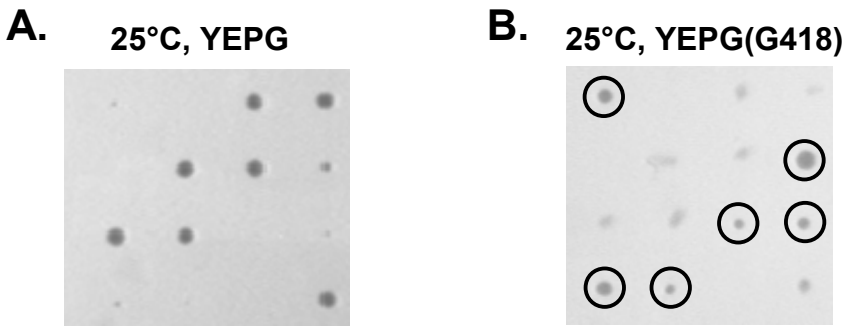

Figure 36. Tetrad analysis of a diploid strain, heterozygous for the *WBP1* deletion and transformed with a plasmid expressing the Wbp1-Sys1 fusion protein described in the legend to Figure 35. Tetrads obtained from sporulation of the strain Y20242 (*WBP1*/*WBP1*::Kan), carrying pRS315(*WBP1-SYS1*), were dissected on YEPD medium, and the four spores of one tetrad were grown for four days at 25°C (A). The colonies were replica-plated on geneticine-containing medium to determine their phenotype (B). All growing colonies (featured through circles) on geneticine resulted form spores with *WBP1* deletion.

The lethal phenotype of the *WBP1* deletion is weakly rescued by the Wbp1-Sys1 fusion protein. Wbp1p is an essential component of the oligosaccharyl transferase complex, which transfers a core oligosaccharide to newly synthesised proteins in the ER. If the Wbp1-Sys1 chimeric protein is transported out of the ER due to the (DXE)-motif present in the cytoplasmic tail of Sys1p, only a very small part of the fusion protein is retained in the ER and can fulfil its enzymatic function.

These findings strongly suggest that the Sys1 protein cytoplasmic tail fused to the C-terminus of an ER resident membrane protein can force the chimeric protein to leave the ER in a (DXE)-dependent fashion.

4.7 Sys1p interacts with the uncharacterized protein p32 of reading frame *YDL173w*

In two independently performed two-hybrid screens (Will and Gallwitz, personal communication; Uetz, personnal communication), p32 (corresponding to the gene product of *YDL173w* in the EMBL/GenBank/DDBJ databases) was identified as an interaction partner for some Ypt-GTPases, among them Ypt6p. In a subsequent two-hybrid assay with *YDL173w* as "bait" and *SYS1* as "prey", an interaction between these two proteins could also be shown (Will and Gallwitz, personnal communication). Although the two-hybrid interaction indicated a stable complex between Sys1 and Ydl173w proteins in the yeast nucleus, the complex may contain additional components contributed by the yeast system. In particular, this protein might be of significance for the *ypt6* suppressor function of Sys1p.

Therefore, the 53 amino acids of the C-terminal tail of Sys1p were fused to GST, bound to glutathion-sepharose beads and incubated with total yeast extract from cells that expressed a MYC-tagged version of Ydl173w instead of the wildtype protein. After extensive washing, bound proteins were eluted from the affinity

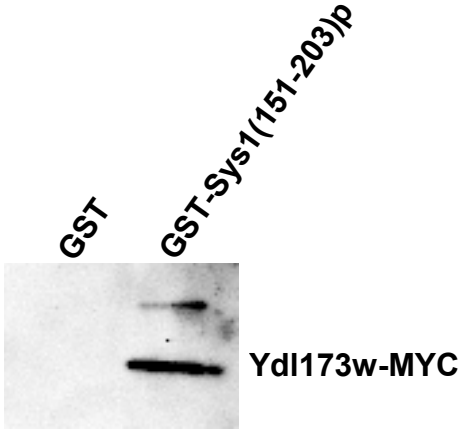

Figure 37. The C-terminal tail of Sys1p (amino acids 151-203) interacts with Ydl173w. GST and a GST-Sys1 tail fusion protein, coupled to agarose beads, served as affinity matrix with detergent-solubilized total yeast protein from cells expressing p32-MYC protein instead of the Ydl173w wildtype protein. Beads were washed with buffer, and bound proteins eluted in 1 ml fractions of 1M salt buffer and 20µl of each fraction were loaded on SDS-gels. Immunoblot analysis with an antibody against MYC is shown.

columns with 1 M NaCl buffer. Immunoblotting with polyclonal anti-MYC antibody verified the interaction of Sys1p with Ydl173w-MYC protein (Figure 37). Nevertheless, the observed interaction is rather weak and it is questionable whether it is of physiological relevance.

4.7.1 Ydl173w is an ER membrane-associated protein

To investigate the intracellular localization of Ydl173w, subcellular fractionations were preformed. As suggested by the primary structure (Figure 38A), the Ydl173w protein is hydrophilic and appears not to be an integral membrane protein. To prove this, Ydl173w was searched for in membrane fractions sedimenting either at 10, 000*g* (P10) or 100, 000*g* (P100). After both centrifugation steps, a significant fraction of Ydl173w was detected in P10 and P100, indicating that this protein is membrane-associated. Bos1p, a membrane protein involved in ER-to-Golgi vesicular transport and known to cycle between these two organelles, was also found in P10 and P100 (Figure 38B). High salt and 6M urea treatment, conditions known to dissociate protein-protein interactions, solubilized Ydl173w, but not the membrane protein Bos1p. Sucrose gradient centrifugation of cell lysates revealed that most of Ydl173w co-sedimented with Kar2p, a protein of the ER-lumen (Figure 38C). This could indicate that Ydl173w is associated with ER membranes. Preliminary indirect immunofluorescence analyses with an antibody directed against the MYC-epitope revealed no specific signal.

A.

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1   MATFNPQNEM ENQARVQEYK VSTGRGGAGN IHKSMSKPSP VLLPLKSNSK
51  TVANNNNNGS NQEKVPRFAI GRGGAGNIFH DPHLTRSAQQ LDSNDNINYN
101 DVINDIDDYI SPITSDMVDE GGSNPVTNTR SRISATRSHQ SLHATTSSPN
151 NNAPIVVGRG GAGNIFFNKK KVASNGGNEE DEIRGGNIED EDTINANEDN
201 LFVVTSNGNA LAAIKSTSKK PKNKLKGKSA PEKFAIGRGG AGNIISPKSS
251 RNTINHNLND DDEDKFNLKD DNGKEKKKKK KKKSGFFSSL KTMFN
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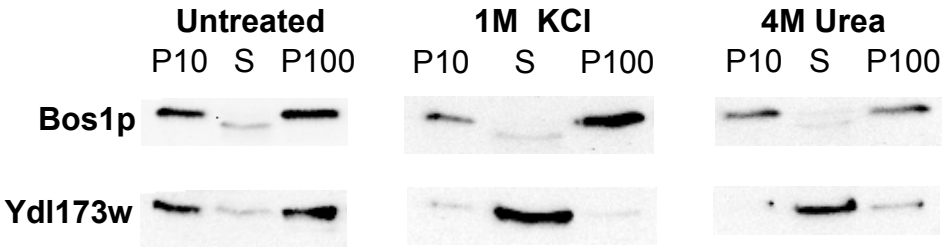
B.



C.

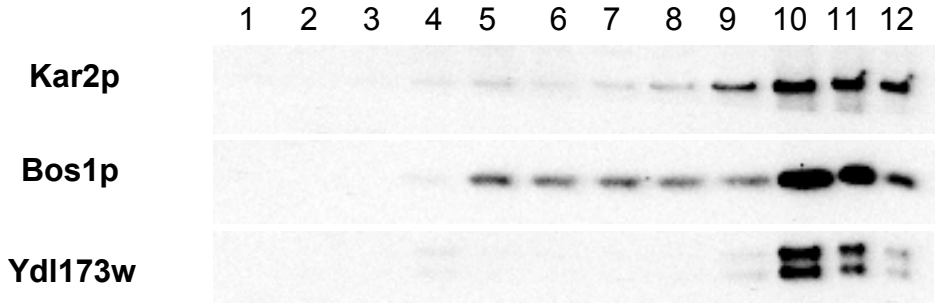


Figure 38. Primary sequence of Ydl173w protein. (A) A sequence of eight amino acid residues repeated four times is underlined. Ydl173w is an ER membrane-associated protein. Cells expressing a MYC-tagged version of Ydl173w instead of wildtype protein were frozen in liquid nitrogen, disrupted in a mortar and subjected to centrifugation at 500*g* to remove unbroken cells and cell debris. The supernatant was fractionated by differential centrifugation at 10, 000 and 100, 000*g* (B) or by sucrose gradient centrifugation (C). Aliquots of fractions were subjected to SDS-PAGE and Western blot analysis with antibodies against the marker proteins shown to the left. The gradient fractions in (C) are numbered from the lowest (fraction 1) to highest (fraction 12) sucrose density.

4.7.2 Characterization of a *ydl173w* deletion mutant

A gene disruption experiment was performed in order to investigate the importance of the Ydl173w protein for correct intracellular transport. Therefore, *YDL173w* was deleted in the haploid strain GFUI-5B using the loxP-*kanMX*-loxP cassette and the PCR-based method described in section 3.7.2 on page 27. The correct gene deletion was checked by PCR. Since the knock-out of *YDL173w* did not result in slowed growth or temperature-sensitivity, it was searched for protein targeting failures of the two marker proteins invertase and carboxypeptidase Y.

Invertase, induced in low glucose medium, is a commonly used marker protein in studies of the yeast secretory pathway. The secreted form of invertase is core-glycosylated in the ER, further glycosylated during its passage through the Golgi compartments and is then transported to the periplasmic space. Secreted invertase migrates in non-denaturing polyacrylamide gels as heterogeneous species with an apparent molecular mass of 100-140 kDa, whereas the glycosylated ER-form has an apparent molecular mass of 80-88 kDa.

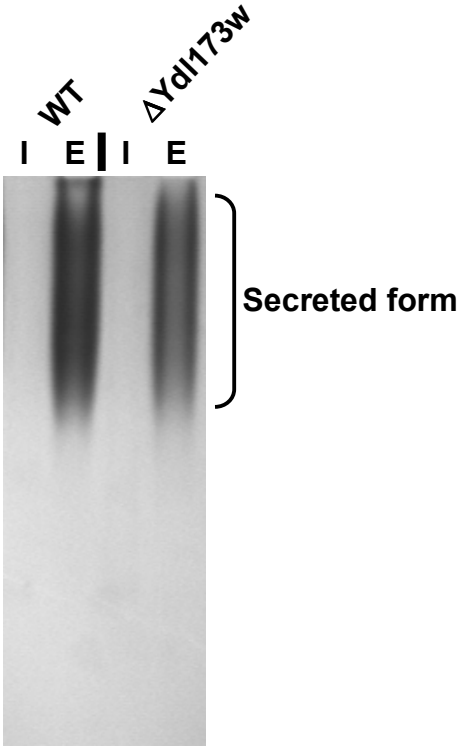


Figure 39. Staining of active invertase in non-denaturing gels. MSUC-3D based strains *Δydl173w* and the wildtype were grown at 25°C and induced for secreted invertase synthesis at 37°C for 1h. “I”=intracellular fraction; “E”=periplasmic form.

To assess whether the deletion of *YDL173w* inhibits proper function of the secretory pathway, glycosylation and secretion of invertase were followed in wildtype or *Δydl173w* mutant cells. As can be seen in Figure 39, highly glycosylated invertase

is secreted and there is no indication of an intracellular accumulation of the core-glycosylated ER forms or the Golgi form of the enzyme.

To follow the intracellular traffic of another marker protein, processing and maturation of the soluble vacuolar proteinase CPY was analyzed. The transport of newly synthesized CPY through the secretory pathway can be monitored by the appearance of three forms, a core-glycosylated 67 kDa ER form (p1CPY), a 69 kDa Golgi-modified form (p2CPY) and the 61 kDa mature form (mCPY) found in the vacuole. As demonstrated in Figure 40, an ER and a Golgi precursor form of CPY can be distinguished after a short pulse with radioactive amino acids, and these can be chased to the mature form of the enzyme within 30 minutes at 30°C. For *ypt6* and *sys1* null mutants it has been reported that small but clearly detectable portions of p2CPY were missorted to the medium (Tsukada and Gallwitz, 1999). As shown in Figure 40, no missorting to the extracellular space could be detected in the *ydl173w* null mutant.

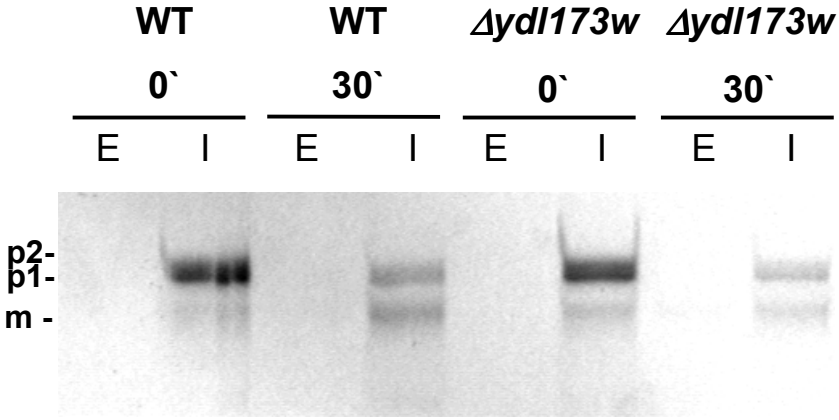


Figure 40. CPY transport in MSUC-3D wildtype and *ydl173w* null mutant strain. Cells of both strains were grown to an OD_{600} of 0.5, incubated for 1 h at 20°C, pulsed with ^{35}S-methionine for 10 min and chased for 30 min at 30°C. p1, ER core-glycosylated CPY; p2, Golgi-modified CPY; m, mature CPY.

Since Ydl173w and Ypt6p localize to different intracellular organelles, they do not appear to play a role in the same functional context. Therefore, further studies as double gene disruptions of *YDL173w* in combination with *SYS1* or *YPT6*, respectively, were not analyzed.

5 Discussion

Ypt6p, the homologue of mammalian Rab6 protein, is dispensable for *Saccharomyces cerevisiae*, but cells become heat-sensitive upon loss of *YPT6* gene function. In an earlier study, four multicopy suppressors of the *ypt6* mutant temperature-sensitivity were screened and named *SYS1*, *SYS2*, *SYS3* and *SYS5* (Tsukada and Gallwitz, 1996). The characterization of these proteins may allow new insights into the functional interplay of the Ypt6 protein. This Ph.D. project mainly involved examining Sys1 protein.

5.1 Sys1p is a tetra-spanning membrane protein localized to *trans*-Golgi and/or endosomal compartments

The sequence of Sys1p predicts a protein with a molecular mass of 23.7 kDa with four putative membrane-spanning domains. It was efficiently pelleted from cell homogenates by centrifugation and could not be extracted from these pellets with sodium chloride or urea, but could be solubilized by detergent, clearly indicating membrane insertion. Sequence similarities to synaptogyrin indicated that the termini of Sys1p are likely to be cytoplasmic (Tsukada and Gallwitz, 1996). This was demonstrated in the present study by limited protease digestion of isolated organelles. The C-terminus was completely digested by proteinase K with no protected fragments being detectable. As a control, the Golgi membrane protein Emp47p was subjected to the same procedure and found to be substantially protease resistant when probed with an antibody to an intralumenal domain. Based on these results and the hydropathy profiles, Sys1p is likely to be a tetra-spanning membrane protein with the termini on the cytoplasmic site of the membrane.

To investigate the localization of Sys1p, subcellular fractionation studies and fluorescence microscopy were employed. Cell homogenates were fractionated by equilibrium sucrose-density gradient centrifugation, and the distribution of Sys1p was compared to that of marker proteins of distinct organelles, such as the ER, the *cis*- and the *trans*-Golgi. Under these conditions, Sys1p was separated from the ER, the *cis*-Golgi and the vacuole, with most of it being found in fractions containing the *trans*-Golgi marker protein Kex2p. *In vivo*, C-terminal tagging of Sys1p with GFP,

expressed at endogenous levels in the absence of the corresponding untagged Sys1 protein, gave a signal too weak to detect by fluorescence microscopy. To monitor its distribution, Sys1p was tagged, at the amino terminus, with GFP and expressed from a strong promoter. The GFP chimera remained functional as judged by its *ypt6* suppressor activity. It also co-localized with wildtype Sys1p on sucrose gradients. Visualization of the GFP-tagged Sys1p revealed a punctate pattern generally seen with Golgi and endosomal proteins. Since the resulting fluorescent signal remained still hard to image satisfactorily, co-localization experiments turned out to be difficult. Also the deficiency of antibodies for typical marker proteins of the *trans*-Golgi and/or early endosomes inhibited a clear-cut localization of Sys1p. Thus, further studies are needed to pinpoint Sys1 protein's correct localization, but the studies presented here strongly suggest that it might be primarily associated with *trans*-Golgi or endosomal membranes.

5.1.1 Sys1p belongs to a group of proteins showing the same membrane topology

Despite some similarities to synaptogyrin, Sys1p is a novel protein with no significant sequence homology to other known proteins either in yeast or in other eukaryotes. It represents a new addition to the growing list of proteins that contain four transmembrane domains, with both termini facing the cytosol, and that reside on intracellular late Golgi or post-Golgi compartments. This topology is the same as that of members of at least five mammalian protein families: the physins (synaptophysin, synaptoporin, pantophysin, and mitsugumin), the gyrins (synaptogyrin and cellugyrin) (Stenius et *al.*, 1995), the SCAMPs (Singleton et *al.*, 1997; Hubbard et *al.*, 2000), the connexins (Lampe and Lau, 2000) and the tetraspanin superfamily (Maeker et *al.*, 1997). Of these five families, the physins have clearly received most attention. The best known member of this group is synaptophysin that is known to bind to the *v*-SNARE synaptobrevin, suggesting a role in the control of the exocytotic fusion machine (Calakos and Scheller, 1994). Synaptophysin is thought to recruit synaptobrevin following disassembly of the SNARE complex after vesicle fusion. This seems to assure that the extremely reactive synaptobrevin is prevented from entering the SNARE complex in an uncontrolled manner and released, when required for another round of rapid exocytosis (Becher et *al.*, 1999). A similar role for Sys1p would suggest that this protein acts as a suppressor of the *ypt6* deletion phenotype

by providing a reserve pool of respective *v*-SNAREs for endosome-to-Golgi transport that can be readily recruited during periods of high need. Consistent with this potential role of Sys1p, also high expression of the Sed5p-interacting *v*-SNAREs Gos1p and Ykt6p can suppress the *ypt6* deletion phenotype (Bensen et *al.*, 2001). High expression of Sys1p could have the same function by providing a rapidly available pool of Gos1p and Ykt6p and also by recruiting both proteins back to the endosomes after disassembly of the SNARE complex for another round of vesicle fusion. Support for such a role is also provided by the observation that the single deletion of Sys1p, as well as that of synaptophysin, does not inhibit the respective vesicle fusion events (Tsukada and Gallwitz, 1996; McMahon et *al.*, 1996). Both are not a prerequisite for correct vesicle fusion but are needed if a malfunction, for example the *ypt6* deletion, has to be overruled. The other members of the synaptophysin family are thought to be involved in a similar tuning of neurotransmitter release (Janz and Südhof, 1998). Pantophysin has been localized to cytoplasmic vesicles in cultured human cell lines that participate in intracellular traffic between various membrane compartments. In the same vesicles, the *v*-SNARE cellubrevin and SCAMP proteins were also identified (Haass et *al.*, 1996).

Secretory carrier membrane proteins (SCAMPs) are ubiquitous components of recycling vesicles that shuttle between the plasma membrane, endosomes, and the *trans*-Golgi complex. Recent data suggest that SCAMPs participate in endocytosis via a mechanism, which may involve the recruitment of clathrin coats to the plasma membrane and to the *trans*-Golgi network by direct interaction of their cytoplasmic N-terminal domain with intersectin 1 and γ-synergin (Fernández-Chácon et *al.*, 2000). Connexin proteins reside on the plasma membrane of vertebrate cells and possess four membrane-spanning domains, two extracellular loops and two cytoplasmic tails. These connexins form intercellular channels, so called Gap junctions, that allow the intercellular exchange of cytoplasmic components directly without the interference of extracellular elements (Lampe and Lau, 2000). Also the twenty-six known tetraspanins display the same topology. Their function is not precisely known, but data from biochemical studies or knock-out mice suggest that they play a major role in membrane biology. For both, connexins and tetraspanins, no functional relationship to Sys1p could be easily envisaged.

The structural organization of Sys1p, however, sets it apart from the physins, connexins and tetraspanins at several levels. First, the transmembrane spans are

spaced very differently. In Sys1p, comparable to SCAMPs and gyrins, they are quite closely bunched with interconnecting segments, which are all shorter than 23 residues. Tetraspanins have one, and physins and connexins each have two ectodomain loops connecting the transmembrane spans that are more than 33 residues long. Although the single endodomain loop between transmembrane spans 2 and 3 in tetraspanins, gyrins and SCAMPs is short, like in Sys1p, the loop in connexins and physins is very extended. Second, Sys1p, as SCAMPs and gyrins, is not glycosylated and does not contain disulfid bonds in ectodomains. Tetraspanins and physins are predicted to contain both of these features (Johnston and Südhof, 1990; Maekler, 1997), and both ectodomain loops of connexins contain multiple cysteins, which would be compatible with the formation of disulfide bonds.

By these criteria, the membrane topology of Sys1p resembles that of SCAMP and gyrin proteins. In addition, all three do not form disulfide bonds within loops between transmembrane domains, as members of the other protein families do, and they are not glycosylated. Sys1p and SCAMPs, both have ectodomain loops of 7 and 22 residues and they operate in late or post-Golgi transport. Furthermore, both are incorporated into a vesicle coat by direct protein interactions, Sys1p in the COPII coat and the SCAMPs in the clathrin coat.

The structural organization of the yeast proteins Sft2p and Got1p also resembles that of Sys1p to some extent. Sft2p and Got1p have the same membrane topology as Sys1p, and like Sys1p, their transmembrane spans are concentrated within a region containing less than 125 residues of the entire sequence (Conchon et *al.*, 1999). Furthermore, the length and composition of the transmembrane spans of Sys1p, Sft2p and Got1p are fairly similar, as the two transmembrane domains residing on the C-terminal side are shorter than those on the N-terminal side and rich in phenylalanine residues. Both, Got1p and Sft2p are located predominantly in the Golgi apparatus, but whereas Got1p shows substantial colocalization with Sed5p in early cisternae, Sft2p is found, like Sys1p, mainly in later cisternae (Wooding and Pelham, 1998). Like Sed5p, Got1p is required for a step that follows vesicle tethering of ER-derived vesicles at the *cis*-Golgi. Sft2p performs a function in vesicular transport analogous to that of Got1p, but acts primarily in later Golgi compartments. Its suggested role would be to facilitate fusion of endosome-derived vesicles with the Golgi (Conchon et *al.*, 1999). Quite strikingly, a similar role has been proposed for Sys1p (Tsukada and Gallwitz, 1996).

Despite the speculations about the precise functional interplay of these proteins, it becomes evident that tetra-spanning proteins as Sys1p, Got1p and Sft2p in yeast and as physins and gyrins in mammalian cells fulfill defined functions in vesicular protein transport, most probably in vesicle docking events. All these proteins are not essential for cell viability, but are able to suppress the mutant phenotype of proteins involved in vesicle docking. Also the mammalian SCAMP proteins are thought to be involved in transport vesicle docking with their target membrane (Hubbard et *al.*, 2000). The model of involvement of the tetra-spanning proteins in vesicle fusion serves as a basis for several structural and functional predictions that need to be tested in future studies, particularly with respect to interactions with the membrane fusion machinery.

5.1.2 Both Sys1p and Ypt6p, act in the same pathway

Sys1 protein was previously identified as a suppressor of Ypt6 GTPase-dependent endosome-to-Golgi trafficking defects (Tsukada and Gallwitz, 1996). The deletion of *SYS1* in a wildtype strain did not alter cell growth, but its disruption in a *ypt6* deletion strain led to a slow-down of cell-growth and to an aggravation of the CPY missorting defect observed in single *ypt6* null mutants (Tsukada and Gallwitz, 1996). In the double mutant, also secretion of α-factor and recycling of Kex2p from endosomes to Golgi appeared to be inhibited (Tsukada and Gallwitz, 1996). The synthetic negative effects on cell growth and protein sorting are a clear indication for Ypt6p and Sys1p acting in the same transport step. However, if Sys1p is important for the Ypt6p-regulated pathway, one would expect cells lacking Sys1p to have phenotypes similar to those of *ypt6* mutants. Such a similarity could be detected in the present study by another assay used to examine endosome-to-Golgi traffic. In this assay the distribution of a plasma membrane protein, Snc1p, that normally recycles using the same route was examined (Lewis et *al.*, 2000). Snc1p is a *v*-SNARE that mediates fusion of Golgi-derived vesicles with the plasma membrane. It is found mostly on the cell surface, but recycles through endosomes continually. When retrieval from endosomes is blocked, for example in a *tlg1* mutant, GFP-Snc1p accumulates in late endosomes or is directed to the vacuole (Conchon et *al.*, 1999). In the present study the distribution of GFP-Snc1p in *ypt6* and *sys1* mutants was examined in order to see whether this SNARE behaved similarly in these strains. Strikingly, both mutants showed no GFP-Snc1p at the cell surface. Instead, it was

present in punctate structures or small intracellular circles corresponding to the vacuole. These results indicate that both, Ypt6p and Sys1p are involved in the retrieval of GFP-Snc1p from endosomes to the Golgi (This study; Galan and Haguenauer-Taspis, 2001; Siniossoglou et *al*., 2000)

5.1.3 Ydl173w binds Sys1p, but shows a different intracellular localization pattern

Using the two-hybrid screen, a novel yeast protein was identified that can interact with the carboxyl-terminal 53 amino acids of the Sys1p cytoplasmic domain. The sequence of the 295 amino acid long Ydl173w excludes it to be an integral membrane protein or to have an obvious organelle targeting sequence. As Ydl173w was found here to be easily pelletable with membrane fractions, it might be located on the cytosolic site of the membrane where it would have access to the Sys1p cytoplasmic domain *in vivo*. However, by sucrose gradient centrifugation of cell lysates, Ydl173w co-fractionated with ER marker proteins. Since Ypt6p and Sys1p have been shown to localize to *trans*-Golgi or endosomal compartments, Ydl173w does not appear to play a role in the same functional context as Ypt6p and Sys1p.

5.1.4 A short segment in the C-terminal cytoplasmic tail of Sys1p is important for its biological activity

To identify functionally important regions within the Sys1 protein, series of *sys1* mutant alleles were constructed that encode proteins with deleted termini. As shown in this work, the deletion of the complete N-terminus and of 32 amino acids from the C-terminus did not affect the ability of Sys1p to suppress the growth defect of *ypt6* mutants. However, the deletion of only 18 residues adjacent to the last transmembrane domain abolished the suppressor function, suggesting that this short tail segment of the C-terminus is important for the biological activity of Sys1p. To screen for interacting proteins that could link Sys1p to the membrane fusion machinery, GST fused to the 52 amino acid long C-terminal tail of the protein, including the functional domain, was immobilized and used as an affinity matrix with *Saccharomyces cerevisiae* proteins solubilized in the presence of 1% CHAPS. The unexpected binding of the C-terminus of Sys1p to the COPII heterodimer Sec23p-Sec24p suggested that truncation of the complete C-terminal domain disrupts a forward trafficking signal rather than a functional domain relevant for the biological activity. To discriminate between both possibilities, the 20 amino acids adjacent to

the most C-terminally located transmembrane domain and the residual 32 C-terminal amino acids were separately fused to GST, and both GST-fusions were tested for

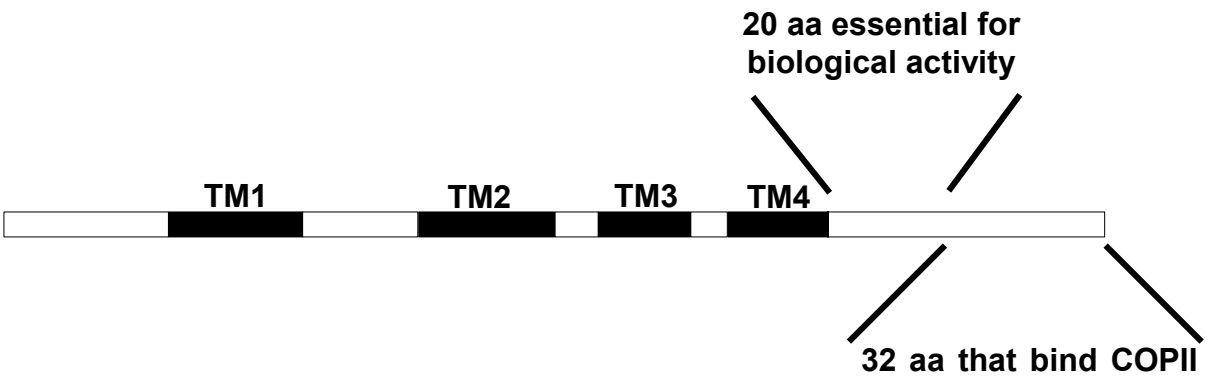


Figure 41. A short tail segment of 20 amino acids immediately adjacent to the most C-terminally located transmembrane domain is important for the biological activity of Sys1p whereas a more distal sequence serves for COPII interaction. TM, transmembrane domain.

interaction with Sec23p-Sec24p. The domain that before has been described to be relevant for the biological activity of the protein did not bind to the COPII coat proteins, whereas the C-terminal 32 amino acids showed a tight interaction with Sec23p-Sec24p. Loss of binding to the COPII coat, therefore, does not appear to abolish the suppressor function of Sys1p, as the deletion of 32 amino acids from the C-terminus did not affect the ability of Sys1p to suppress, at high intracellular concentrations, the growth defect of *ypt6* mutants. So, the 20 amino acid long segment, whose deletion results in a loss of suppressor function, may be sufficient for the biological activity of Sys1p or needed for the correct insertion into the bilayer as it ends right next to the last transmembrane domain.

Nevertheless, a link between the functional domain of Sys1p and vesicle tethering or fusion in the Ypt6p-mediated transport pathway remains open. Since the functional domain of Sys1p may depend on the neighboring transmembrane stretch, it is possible that the hydrophilic C-terminus alone does not show the expected link. By affinity chromatography, no other proteins than Sec23p and Sec24p were found to bind efficiently and specifically to the Sys1p tail sequence. The fusion of the C-terminus to the DNA-binding domain of *GAL4* activates transcription on its own and, therefore, did not allow a meaningful two-hybrid library screen. However, affinity studies under less stringent conditions might still allow the identification of tail-interacting proteins important for Sys1p function.

5.2 An acidic sequence in the C-terminus of Sys1p directly interacts with Sec23p-Sec24p and facilitates its ER export

The unanticipated finding that the hydrophilic tail of Sys1p interacts with the cytoplasmic Sec23p-Sec24p complex led to the assumption that the tail sequence served an ER export function. Since the export of membrane proteins from the ER is a selective rather than a default process, the selectivity may be accomplished by sequence information within the cytoplasmic domain of Sys1p that leads to preferential coat protein recruitment. To date, only a few such sequence motifs for promoting ER export have been described. It has been reported recently that both cytoplasmic sequences of yeast Emp24p and Erv25p contain a di-aromatic motif that binds directly to Sar1p GTPase and Sec13p-Sec31p subunits of the COPII coat and promotes export from the ER (Belden and Barlowe, 2001). Besides the di-aromatic motif, a second targeting determinant composed of a Leu-Val dipeptide at the extreme C-terminus supports ER export of Emp24p (Nakamura et *al.*, 1998). Also the mammalian protein ERGIC-53 is incorporated into COPII vesicles with the help of a di-phenylalanine motif (FF) together with a second motif composed of two glutamines (QQ). Although both sequences, the (FF)- and the (QQ)-motif, are present also in the C-terminal tail of Sys1p, the present study showed that they do not mediate binding to Sec23p-Sec24p. Rather a di-acidic motif, Asp-Leu-Glu (DXE, where X represents any residue) was demonstrated to efficiently interact with the COPII coat. Substitution of the acidic residues with alanines results in a complete loss of binding to Sec23p-Sec24p. The di-acidic motif was first described in the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) in baby hamster kidney cells to be required for efficient recruitment to COPII vesicles (Nishimura and Balch, 1997). As described for VSV-G, the mutation of the Sys1 protein's (DXE)-motif led to a slightly reduced extent of ER export, and in comparison with wildtype cells approximately 30 percent of the protein were retained in the ER. Fusing of the C-terminal tail of Sys1p to the ER resident membrane protein Wbp1p resulted in an efficient export of the chimeric protein from the ER, whereas a chimera with a mutated di-acidic motif stayed completely in the ER. Although the exact intracellular location of the Wbp1-Sys1 fusion protein could not be determined, on gradients it co-localized almost perfectly with Sys1p-containing membrane fractions. This was not due to deleting the di-lysine ER retrieval signal in the C-terminus of Wbp1p, because previous studies had shown that truncation of the C-terminus of Wbp1p alone does not affect its intracellular

localization (Gaynor et *al.*, 1994). The positive result of directing an ER localized protein to the Golgi by fusing it with the Sys1p tail excludes less efficient folding as a possible reason for ER retention of mutant proteins with a defective di-acidic motif. Studies of VSV-G transport in mammalian cells had shown that transport defects arising from mutations of the (DXE)-motif predominantly affect the rate of export. Due to the lack of Sys1p glycosylation, a comparison of ER export kinetics of newly synthesized wildtype and (DXE)-mutated Sys1p could not be followed.

In order to confirm that yeast uses the (DXE)-motif as a common ER exit signal for membrane cargo, other proteins besides Sys1p were screened for Sec23p-Sec24p binding. The amino acid permease Bap2p and the peptide transporter Ptr2p bound the COPII heterodimer, although less efficiently than Sys1p. The permeases Tat1p and Tat2p, despite having a di-acidic sequence in their C-termini, showed no interaction. The fact that not any (DXE) tripeptide situated in the hydrophilic tail of a transmembrane protein mediates COPII binding reinforces that the sequence environment of a di-acidic code is essential for functionality. The functionality of the di-acidic motif in VSV-G has been reported to depend on an upstream tyrosine residue (Sevier et *al.*, 2000). However, a tyrosine residue N-terminal of the acidic residues is absent from the Sys1p tail. The importance of the (DXE)-flanking residues was recently highlighted by a study of the transport of inwardly rectifying potassium channels Kir1.1 and Kir2.1 in mammalian cells (Ma et *al.*, 2001). Both proteins contain distinct ER-to-Golgi forward-trafficking signals around a di-acidic motif. Kir2.1, like VSV-G, contains an upstream tyrosine residue, whereas Kir1.1 has an ER export signal without the aromatic residue. Consistent with this, the yeast amino acid permease Bap2p possesses an upstream tyrosine residue, the peptide transporter Ptr2p does not. In conclusion, the flanking sequences are not limited to Tyr-based residues as previously suggested. But the di-acidic tripeptide itself is more flexible in its amino acid composition than assumed before. Although six of the so far proven di-acidic sequences are composed of an aspartic acid and a glutamic acid residue separated by a variable non-polar amino acid (DXE), the sequences mediating ER export of the potassium channel proteins differ in their primary structure (Figure 42). Kir1.1 has the inverted sequence (EXD), whereas the di-acidic signal of Kir2.1 is constituted of two glutamic acid residues separated by an asparagine (EXE). Regarding the composition of the acidic sequences and the flanking residues,

VSV-G	RQI**Y**T**D**I**E**MN	(Virus)	(Nishimura and Balch, 1997)
LAP	**Y**RHVA**D**G**E**DH	(Human)	(Nishimura and Balch, 1997)
PRA1	QIEPA**D**G**E**EL	(Human)	(Abdul-Ghani et *al.*, 2001)
Kir1.1	NFVLS**E**V**D**ET	(Frog)	(Ma et *al.*, 2001)
Kir2.1	NSFC**YE**N**E**VA	(Frog)	(Stockklausner et *al.*, 2001)
Sys1p	PIQLK**D**L**E**SQ	(Yeast)	(Votsmeier and Gallwitz, 2001)
Bap2p	HRRI**YD**P**E**LM	(Yeast)	(Votsmeier and Gallwitz, 2001)
Ptr2p	APKAN**D**I**E**IL	(Yeast)	(Votsmeier and Gallwitz, 2001)

Figure 42. Alignment of the (DXE)- and related (D/EXE/D)-motifs found in the cytoplasmic domain of transmembrane proteins that have been shown to mediate export from the ER. Abbreviations: VSV-G, vesicular stomatitis virus glycoprotein; LAP, lysosomal acid phosphatase; PRA, prenylated Rab acceptor; Kir, potassium inward rectifier; Bap, branched-chain amino acid permease; Ptr, peptide transporter.

variation of forward-trafficking signals presents one potential mechanism for differential regulation of ER export of different cargo proteins.

It has been proposed, that the Sar1 GTPase, bound to GTP, is required to allow interaction between membrane cargo proteins and the COPII coat (Aridor et *al.*, 1999). However, the *in vitro* binding experiments performed in the present study did not reveal Sar1p binding along with Sec23p/Sec24p, even by sensitive immunoblot analysis. Under the stringent conditions chosen, all of the other bands found on the silver stainings correspond either to breakdown fragments of the GST-Sys1 tail fusion protein or to abundant cytosolic proteins, such as heat shock proteins that are known to bind unspecifically. Thus, the efficient interaction of the bacterially produced GST-Sys1 tail fusion protein with the COPII heterodimer Sec23p-Sec24p does not appear to depend on Sar1p•GTP, and one can no doubt draw the conclusion that the Sys1p tail sequence binds directly to the Sec23p-Sec24p COPII subcomplex in a (DXE)-motif dependent fashion. These findings are entirely consistent with a recent study showing that Erv25 protein, which shuttles between the ER and the Golgi, binds directly to the COPII subcomplex Sec13p-Sec31p dependent on a di-aromatic motif (YF), but independent of Sar1p (Belden and Barlowe, 2001). Both results, the direct COPII coat interaction of Sys1p and of Erv25p, contradict the observation that

the Sec23p-Sec24p complex by itself fails to bind to the (DXE)-motif of the VSV-G tail (Aridor et *al.*, 2001).

In similar affinity binding studies as those performed in this study, COPII binding to the *v*-SNARE Bet1p was not detectable with either Sec23p or Sec24p alone but only with the complex of these two proteins (Springer and Schekman, 1998). In contrast, direct and specific interaction with Sec24p of the Golgi *t*-SNARE Sed5p, which cycles through the ER, was proven by means of affinity chromatography and two-hybrid analysis (Peng et *al*., 1999). To address the question which component of the COPII heterodimer interacts with the (DXE)-motif, both Sec23p and Sec24p, fused to GST were bound separately on glutathione-Sepharose and were incubated with bacterially produced MBP-Sys1 or His_6-Sys1 tail fusion proteins. Neither GST-Sec23p nor GST-Sec24p was able to recruit the Sys1 tail fusions to the beads. According to these results, Sys1p and the (DXE)-sequence in general appears to interact only with the heterodimeric Sec23p-Sec24p complex.

As a model, COPII coat formation is initiated by the small G-protein Sar1p•GTP, which binds directly to the lipid surface and, possibly, to the cytosolic sequence of some cargo proteins, for example to the di-phenylalanine motif of Emp24p (Belden and Barlowe, 2001). The two heterodimers Sec23p-Sec24p and Sec13p-Sec31p then bind sequentially (Lederkremer et *al.*, 2001). Once they are attached to the lipid surface of the ER membrane they actively recruit membrane cargo proteins through direct interaction mediated by signal sequences. The di-acidic motif in the cytosolic sequence of Sys1p, Bap2p and Ptr2p mediates direct interaction to Sec23p-Sec24p and directs these proteins into COPII vesicles. The main purpose of this interaction is to help collect cargo during vesicle formation and facilitate its export from the ER as shown in the present study.

5.2.1 Endoplasmic reticulum storage diseases

In a family of disorders of protein trafficking known as endoplasmic reticulum storage diseases (Kim and Arvan, 1998), even a single amino acid change can lead to failure of protein export from the ER. For example, cystic fibrosis is caused by point mutations in the chloride conductance channel (CFTR) that leads to retention and degradation of the protein in the ER (Peters et *al.*, 2000). Whereas most known disorders of this family are due to failure in folding or degradation in the ER, a new group of ER storage diseases begins to be uncovered in which abnormalities in

cargo selection result in decreased levels of functional protein. For example, the above mentioned mannose-binding lectin ERGIC-53 cycles through the early secretory pathway and serves as a cargo receptor for incorporation of specific cargo proteins into COPII vesicles. Mutation of the anterograde transport (FF)-signal leads to a secretion block of coagulation factors V and VIII and development of the bleeding disorder (Moussalli et *al.*, 1999). The direct involvement of the di-acidic motif in cargo recruitment in ER-to-Golgi transport suggests that a mutation in this motif can also lead to secondary effects that cause diseases. The biological activity of such mutants in anterograde transport signals would be preserved, but the protein transport would be deficient. The primary clinical difference between these mutants and mutants without a potential enzymatic activity is that they may be amenable to therapies designed to down-regulate the quality control of ER export so that potentially functional molecules can escape the ER and reach their intended intracellular destination. The deciphering of the di-acidic signal and the characterization of its involvement in cargo export from the ER could be the foundation for future studies about transport diseases and their molecular causes.

5.2.2 Relevance of selective protein export from the ER for gene therapy

Gene therapy is a promising approach to provide proteins that require rapid and regulated delivery. However, in most cases, safe and effective therapy will require that genes be equipped with a regulatory system that permits natural concentrations and kinetics of protein expression to be reproduced. Recombinant proteins that regulate fast processes must be produced on a time scale of minutes. For example, insulin needs to be delivered in a brief pulse (Polonsky et *al.*, 1988). Endogenous insulin is stored in secretory granules in specialized cells until their release is signaled. For such proteins to be delivered effectively by a gene therapy approach direct control of secretion will likely be required. Remarkably, the endoplasmic reticulum can also serve as a practical storage container for genetically engineered proteins such as insulin or growth hormone (Rivera et *al.*, 2000). With this approach, storage and secretion of biologically active proteins should be possible in any type of cell. The deciphering of regulatory mechanisms of ER export will no doubt improve the ability to generate designer cargo target proteins and to control their secretion. Mimicking secretory granules through the ER and the better understanding of events

that direct ER export, make gene therapy a viable method for delivery of polypeptides that require rapid and regulated distribution.

5.3 The putative cis-Golgi membrane-association of Sys3p/Imh1p reinforces an involvement of Ypt6p in transport to the *cis*-Golgi

Two alternative pathways for the Ypt6p-mediated transport have already been introduced. Both models support the hypothesis that Ypt6p acts in a retrograde pathway from endosomes, but it remains unclear whether the pathway leads as suggested by the first model to the *trans*-Golgi or according to the second model to the *cis*-Golgi (Tsukada and Gallwitz, 1996; Bensen et *al.*, 2000). The first model is based on vacuolar enzyme transport defects in the *ypt6* mutant, whereas the second model implicates the *cis*-Golgi *t*-SNARE Sed5p in the Ypt6p-dependent pathway (For details see section 1.4.3). After activation Ypt6p•GTP is thought to recruit the VFT tethering complex resulting in SNARE engagement and vesicle fusion at the *trans*-Golgi compartments (Siniossoglou and Pelham, 2001). Comparably, it is tempting to speculate that Ypt6p performs at *cis*-Golgi membranes a relative function for Sys3p/Imh1p, which shows some similarity with the *cis*-Golgi tethering factor Uso1p (Tsukada and Gallwitz, 1996). Indeed, Sys3p/Imh1p contains a GRIP domain, which has been shown to target proteins to the Golgi and to bind to the mammalian homologue of Ypt6p on blots (Barr, 1999; Kjer-Nielsenet *al.*, 1999; Munro and Nichols, 1999). In this study it was shown that Sys3p/Imh1p co-localizes with *cis*-Golgi marker proteins on sucrose gradients reinforcing a function of the protein on *cis*-Golgi membranes. However, direct interaction between Ypt6p and Sys3p/Imh1p could neither be shown by two-hybrid analysis nor by coprecipitation (Preliminary result of this study; Will and Gallwitz, unpublished results; Siniossoglou et *al.*, 2000).

5.4 Sys2p/Sro9p acts most probably in mRNA translation and rather indirectly in Ypt6p-mediated transport steps

Together with the other Sys proteins, Sys2p/Sro9p was screened as a multicopy suppressor of the temperature-sensitive growth phenotype and of the missorting of carboxypeptidase Y in *ypt6* mutants. Gene disruption of *SYS2*/*SRO9* did not result in significant growth defects, whereas its deletion in the *ypt6* null mutant enhances defects in vacuolar protein sorting and in cell growth. However, enhanced expression of Sys2p/Sro9p was not as effective as the other multicopy suppressors

in reinstating failures of the *ypt6* null mutant (Tsukada and Gallwitz, 1996; Luo and Gallwitz, unpublished observation). Beyond intracellular transport mutants, high-copy *SYS2*/*SRO9* also suppresses the cold sensitivity of several mutations that affect pre-mRNA-splicing (Sobel and Wolin, 1999). Moreover, it has been demonstrated that Sys2p/Sro9p binds by its highly conserved La-motif to RNA, preferentially with translating ribosomes (Sobel and Wolin, 1999).

Consistent with the results obtained in this study, Sys2p/Sro9p has been described to be pelletable, and the sedimentation is unaffected by the addition of triton (Sobel and Wolin, 1999). Deviating from the results obtained here, Sys2p/Sro9p has been reported to co-localize in sucrose gradients with protein markers from polysomes and not with those from *cis*-Golgi membranes. (Sobel and Wolin, 1999).

Summarizing, Sys2p may reside on multiprotein complexes that associate to polyribosomes or on *cis*-Golgi membranes. Because *SYS2/SRO9* is a high-copy suppressor of mutations in several processes, the actual function of this protein is unclear (Kaiser et *al.*, 1997; Imai et *al.*, 1996; Kagami et *al.*, 1997). Since it shares with authentic La proteins the motif that is important for RNA binding and cells lacking *SYS2/SRO9* are less sensitive to certain inhibitors of translation, it is most probable that the protein has a role in mRNA translation. A subtle defect in protein synthesis could also aggravate mutations in other pathways, possibly explaining the genetic interaction with *YPT6* and other genes.

6 Summary

This Ph.D. project involved examining the yeast membrane protein Sys1p that was initially identified as a multicopy suppressor of the loss of Ypt6p, a GTPase regulating retrograde transport from endosomes back to the Golgi. Here, it was demonstrated that the loss of Sys1p causes accumulation in endosomes of Snc1p, a plasma membrane *v*-SNARE known to recycle through the Golgi via endosomes. This suggests that Sys1p may participate directly in the same process as Ypt6p. Sys1p was found to reside on post-ER organelles (Golgi and /or endosomes) and to span the membrane four times with its termini oriented to the cytoplasm. Successive deletion of the cytoplasmic termini revealed that the removal of only 20 amino acids adjacent to the last transmembrane region rendered the protein unable to act as suppressor of *ypt6* mutants and hence to fulfill its biological function. By affinity chromatography, Sec23p/Sec24p, a heterodimeric subcomplex of the COPII coat of ER-derived transport vesicles, was found to efficiently bind to the 53 amino acid-long, hydrophilic tail of Sys1p. The sequence binding to Sec23p/Sec24p was not required for the biological function of Sys1p. Several amino acid substitutions led to the identification of a di-acidic motif (Asp-X-Glu, DXE) within the Sys1p tail as being absolutely necessary for Sec23p/Sec24p binding *in vitro*. Binding of the COPII subcomplex to the Sys1p tail was apparently independent of the Sar1 GTPase. The C-terminal tails of the amino acid permease Bap2p and the peptide transporter Ptr2p, both containing a (DXE)-sequence, also interacted with Sec23p/Sec24p, but less efficiently than Sys1p. Similar to the situation with some membrane proteins traversing the secretory pathway in mammalian cells, the (DXE)-motif of the Sys1p tail could be shown to facilitate ER export of Sys1p in yeast cells. Fusing the Sys1p hydrophilic tail to the C-terminus of the ER membrane protein Wbp1p caused export of the fusion protein out of the ER as long as the (DXE)-sequence was left intact.

This is the first demonstration that an (DXE)-motif can serve as an ER exit signal in the well studied yeast system. In addition, it provides the first evidence in any system for a direct interaction between a (DXE)-signal sequence and the COPII coat.

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8 Abbreviations

aa	amino acid
APS	ammoniumpersulfate
ARF	ADP-ribosylation factor
3-AT	3 amino 1,2,4 triazol
ATCC	American Type Culture Collection
CEN	centromere elements
COPI/II	coatamer protein complex I/II
CPY	carboxypeptidase Y
dH_2O	deionized H_2O
dsDNA	double strand DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
5-FOA	5-fluoroorotic acid
FPLC	fast protein liquid chromatography
g	gram
g	gravity
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GMP	guanosine monophosphate
GPI	glycosyl phosphatidyl inositol
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour(s)
HA	influenza virus hemagglutinin epitope
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
Ig	immunoglobulin
IPTG	isopropylthio-ß-D-galactoside
kDa	kilo Dalton
KOAc	potassium acetate
l	litre
LiOAc	lithium acetate
m	meter
M	molar (mol x l^{-1})
mA	milli Amper
MBP	maltose binding protein
2-ME	β–mercaptoethanol
mg	milligram (10^{-3}g)
µg	microrgram (10^{-6}g)
MgOAc	magnesium acetate
min	minute(s)
ml	millilitre (10^{-3} l)
µl	microlitre (10^{-6} l)
µm	micrometer (10^{-6} m)
mM	millimolar (10^{-3} M)
µM	micromolar (10^{-6} M)
MYC	c-myc epitope
NaOAc	sodium acetate
nm	nanometer (10^{-9}m)
OD	optical density
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
RPM	rotation per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SMM	semi-minimal medium
SNARE	SNAP receptor
ssDNA	single strand DNA

TBS	tris buffered saline
TCA	thrichloroacetic acid
TEMED	1,2-bis-(dimethylamino)-ethane
TGN	*trans*-Golgi network
TRAPP	transport protein particle
Tris	tris(hydroxymethyl)aminomethane
YEPG	yeast extract, peptone, glucose
VAMP	vesicle associated membrane protein
v/v	volume/volume
w/o	without
wt	wildtype
w/v	weight/volume
Ø	diameter

Amino acid abbreviations:

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

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