Analysis of protein trafficking in the yeast *Saccharomyces cerevisiae*

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1 INTRODUCTION

The presence of functionally distinct membrane enclosed organelles is an important feature of eukaryotic cells. Each organelle is characterized by a unique set of proteins. To maintain this compartmentalization, cells have evolved mechanisms to ensure efficient delivery of proteins and lipids to a given organelle.

Almost all proteins are synthesized on ribosomes in the cytosol. Newly synthesized proteins are then delivered specifically to the target cell compartment. The fate of the proteins depends on sorting signals contained in the amino acid sequence. Sorting signals direct transport from the cytosol into the nucleus, into mitochondria, peroxisomes or the endoplasmic reticulum (ER). Further transport from the ER to other destinations in the cell can also be directed by sorting signals. Proteins lacking sorting signals remain in the cytosol.

Membrane proteins are synthesized on ribosomes bound to the endoplasmic reticulum (ER) and are inserted into the ER membrane, where they can be modified by addition of N-linked carbohydrates. Proteins are then delivered via transport vesicles to the Golgi complex where they may undergo further posttranslational modifications. Newly synthesized proteins reaching the last Golgi compartment (the *trans*-Golgi network-TGN) can then be sorted into distinct pathways leading either to the cell surface or to the lysosome / vacuole (Fig. 1).

There are at least two pathways from the Golgi to the vacuole: the "CPY pathway", which involves transport through an intermediate enodosomal / prevacuolar (PVC) compartment and the "ALP pathway" which bypasses the PVC compartment. Soluble hydrolases like carboxypeptidase Y (CPY) are directed from the Golgi to the vacuole by a specific sorting signal. In the absence of such a signal these proteins are delivered to the plasma membrane (Rothman and Stevens, 1986; Marcusson *et al*., 1994). In contrast, integral membrane proteins lacking a specific signal are not delivered to the cell surface, but, instead, are transported to the vacuole ("default pathway") (Roberts *et al*., 1992; Wilcox *et al*., 1992).

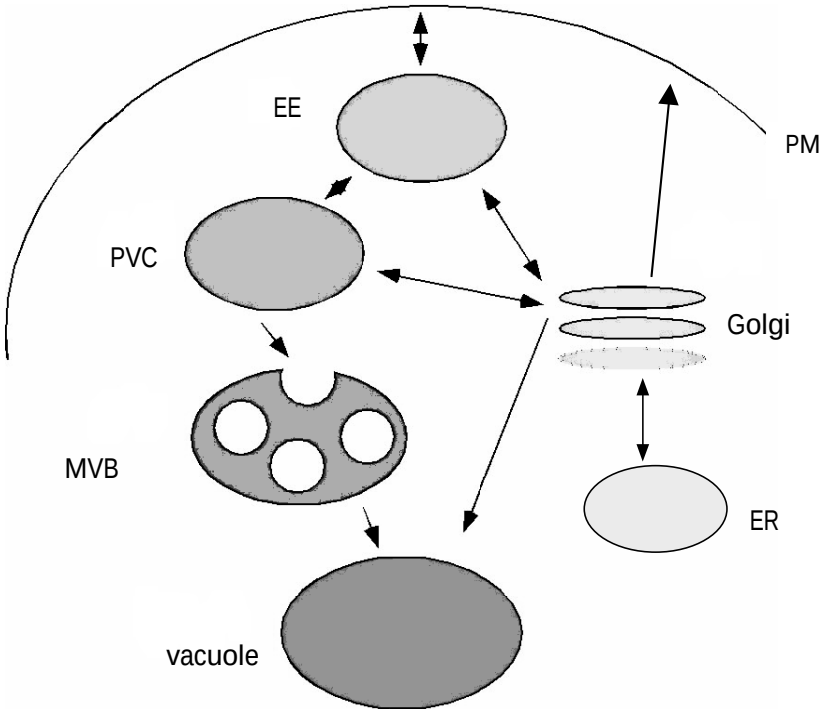


Fig. 1. Schematic representation of protein trafficking in the yeast *S. cerevisiae*. After synthesis, proteins are transported from the ER to the Golgi. The Golgi represents the first branch point in protein trafficking. Proteins can be delivered to the vacuole directly, (ALP pathway) or through an intermediate compartment (PVC) (CPY pathway), or they can be transported to the plasma membrane via secretory vesicles. Proteins can also be endocytosed from the plasma membrane and delivered to the vacuole via several endocytic compartmens, or recycled back to the plasma membrane either directly or via early or late endosomes (PVC). Arrows indicate direction of transport. PM-plasma membrane; ER-endoplasmic reticulum; EE-early endosome; PVC-prevacuolar compartment, late endosome; MVB-multivesicular body.

By genetic screens, yeast *vps* mutants were identified as defective in protein sorting to the vacuole. According to the morphology of the vacuole, the mutants were classified into six groups (class A-F) (Raymond *et al.,* 1992). The class E *vps* mutants exhibit an exaggerated prevacuolar compartment (PVC) which corresponds to the late endosome of mammalian cells. This enlarged PVC compartment observed in class E *vps* mutants is termed class E compartment. The PVC compartment also represents another branch point in protein trafficking. Here endocytic and vacuolar delivery pathways intersect.

CPY pathway

The CPY pathway was named after its prominent marker protein, the soluble vacuolar hydrolase carboxypeptidase Y (CPY), found to follow this route from the Golgi to the vacuole. Newly synthesized CPY (preproCPY) enters the ER where the signal sequence is cleaved off and where it receives N-linked core glycosylation (p1CPY). Further oligosaccharide modifications take place in the Golgi resulting in the p2 form of CPY. The mature form of CPY (mCPY) is produced by the proteolytic activity of proteases in the vacuole which cleave off the pro-region. These different forms of CPY are easily distinguished by size and are therefore used to delineate the route of CPY to the vacuole. Using mutants blocked in vacuolar delivery pathway, it was shown that CPY passes through an intermediate endosomal compartment on its way to the vacuole (prevacuolar compartment (PVC)). It was also found that CPY carried a sorting signal that is recognized by a sorting receptor (Vps10) in the Golgi. Depending on this signal, CPY is diverted from the secretory pathway into the vacuolar delivery pathway. Vps10 itself recycles between PVC and the Golgi and can therefore be used for several rounds of p2CPY transport.

ALP pathway

In contrast to CPY, the membrane protein alkaline phosphatase (ALP) bypasses the endosomal compartment on its route from the Golgi to the vacuole. The 33 residue N-terminal cytoplasmic tail of ALP is both necessary and sufficient to divert membrane proteins from the Vps-dependent CPY pathway into an alternative route to the vacuole (Cowles *et al.,* 1997; Piper *et al.,* 1997). In genetic screens, two proteins, Apl5 and Apl6 were identified that interfere with ALP sorting. These two proteins together with Apm3 and Aps3 are predicted to form the yeast AP-3 complex. The mammalian AP-3 complex is related to the AP-1 and AP-2 clathrin adaptor complexes which bind tyrosine-based sorting signals of cargo proteins and recruit them into clathrin coated pits. However, mammalian AP-3 does not copurify with clathrin-coated vesicles and may instead associate with other proteins to form a novel type of vesicle coat.

Endocytosis

Membrane proteins can be internalized from the plasma membrane into the first compartment of the endosomal system, the early endosomes. From here, endocytosed proteins can be transported to the vacuole / lysosome or recycled back to the plasma membrane. Endocytosis plays an important role in many cellular processes. It participates in the uptake of nutrients and regulates membrane dynamics and the reaction to extracellular stimuli by desensitization, down-regulation, or recycling of receptors. The removal of receptors from the plasma membrane can be a constitutive (ligand-independent) or an induced (ligand dependent) process. Endocytosis can occur by several mechanisms (Conner and Schmid, 2003). The best understood mechanism to date is clathrin-dependent endocytosis. Cargo proteins are initially observed in invaginations of the plasma membrane, known as coated pits, coated on the cytoplasmic side of the plasma membrane with polymerized clathrin. Since clathrin itself does not recruit cargo proteins, adaptors are required for incorporating appropriate cargo proteins into clathrin coated pits. In *S. cereviseae*, endocytosis of extracellular fluid was demonstrated with lucifer yellow (Riezman, 1985) and the lipophilic dye FM4-64 (Vida and Emr, 1995). Receptor mediated endocytosis can be monitored in yeast by using the mating pheromone receptors Ste2 (Jenness and Spatrick, 1986) and Ste3 (Davis *et al.,* 1993). These two receptors are internalized both constitutively and ligand-induced. In both cases they follow the same route from the cell surface to the vacuole, where they are degraded. These two modes of endocytosis are mechanistically distinct, since they can be dissected genetically (Davis *et al.,* 1993). New insights into the factors important for endocytosis were gained from studies on the Ste6 protein. Ste6 is a member of the ATP-binding cassete (ABC) transporter family, consisting of twelve putative membrane spanning segments and two ATP-binding domains (Fig. 2). It is required for the secretion of the mating pheromone a-factor, a 1.6 kDa farnesylated dodecapeptide (Kuchler *et al.,* 1989; McGrath and Varshavsky, 1989).

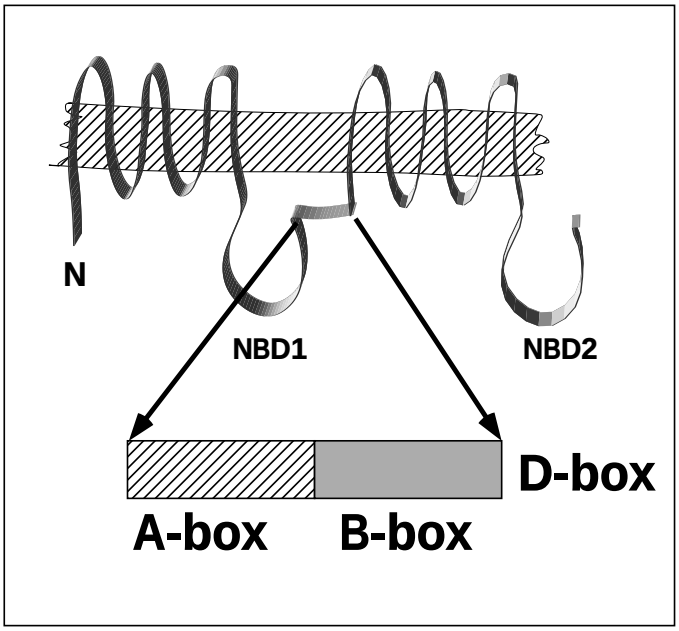


Fig. 2. Predicted membrane topology of Ste6. Structure of the linker region that connects the two homologous halves of Ste6 is indicated.

Ste6 shares substantial sequence homology with the mammalian transporters MDR and CFTR (Higgins, 1992). The similarity is underscored by the finding that mouse *mdr3* can complement a *ste6* defect in yeast (Raymond *et al.,* 1992). Although Ste6 is required for secretion of a-factor and should therefore be located at the plasma membrane, it was mostly found associated with internal membranes (Kölling and Hollenberg, 1994). In experiments with endocytosis mutants, it was found that Ste6 resides at the plasma membrane, but only transiently, due to efficient endocytosis. After internalization from the plasma membrane Ste6 is transported to the vacuole and degraded. Interestingly, Ste6 that accumulated at the plasma membrane in endocytosis mutants turned out to be ubiquitinated. For the first time this pointed to a role of ubiquitination in protein targeting to the vacuole (Kölling and Hollenberg, 1994). Subsequently, a number of other yeast cell surface proteins have also been shown to be ubiquitinated.

Ubiquitin is a highly conserved 76 amino acids polypeptide. It is covalently attached via an isopeptide bond to substrates destined for degradation by the 26S proteasome, an ATP-dependent multicatalytic proteinase complex (Ciechanover, 1994). Attachment of ubiquitin to substrates is accomplished by the action of a cascade of enzymes referred to as a ubiquitin-activating enzyme (E1), a ubiquitin-

conjugating enzyme (E2) and a ubiquitin ligase (E3). Ubiquitin ligases seem to play the primary role in determining substrate specificity. There are two major classes of E3s based on the motif used to transfer ubiquitin to the substrate; E3s with a RING domain and E3s with a HECT domain. The major distinction between the two groups is that RING E3s coordinate the transfer of ubiquitin to the substrate by bringing the E2-ubiquitin complex to the substrate, while HECT E3s form a thiol-ester bond with ubiquitin before transferring it directly to the substrate (reviewed by Pickart, 2001).

The Rsp5 protein is a member of the HECT (homology to E6-AP carboxyl terminus) E3 family of ubiquitin ligases (Huibregtse *et al.,* 1995). The founding member of this family is the human E6-AP (E6-associated protein), which associates with the human papillomavirus (HPV) E6 protein to promote ubiquitin-dependent degradation of the p53 tumor suppressor. Ubiquitin ligases of the HECT E3 family share an approximately 350 amino acids long C-terminal domain, the HECT domain. This domain contains a binding site for an E2 enzyme and a conserved catalytically active cysteine residue involved in ubiquitin transfer from E2 to a substrate protein via a thioester intermediate (Hochstrasser, 1996). In contrast to the conserved C-terminal domain, the N-terminal part of HECT E3s is variable and proposed to have a role in substrate recognition. Rsp5 belongs to the Nedd4 sub-family of HECT ubiquitin ligases. Several conserved motifs, C2 and two-four WW domains, are found in the N-terminal region of Nedd4 family members. The C2 domain was shown to interact with membrane phospholipids, inositol polyphosphates and proteins (Nalefski and Falke, 1996). The interaction is in most cases depended on or regulated by Ca^{2+}. The C2 domain could be involved in targeting Rsp5 to endosomal membranes (Dunn *et al.,* 2004). Rsp5 also contains three WW domains. WW domains are protein-protein interaction modules with affinity for short proline-rich sequences, with the consensus binding site PPxY (PY motif) (Sudol *et al.,* 1995; Einbond and Sudol, 1996). WW domains, as protein-protein interactions modules, are strong candidates for sites of Rsp5-substrate interaction. Rsp5 is an essential protein under standard growth conditions. The essential function of Rsp5 is related to the regulation of fatty acid synthesis (Hoppe *et al.,* 2000). Rsp5 is involved in regulation of the *OLE1* gene coding for the Δ9 fatty acid desaturase. Either overexpression of *OLE1*, or addition of oleic acid to the growth medium suppresses the lethality of the *RSP5* deletion (Hoppe *et al.,* 2000). Hence, providing the cells

with unsaturated fatty acids seems to be the only essential function of Rsp5 under normal growth conditions. However, the lethality of the *RSP5* deletion at elevated temperature (37°C) could not be suppressed by addition of oleic acid (Hoppe *et al.,* 2000), indicating that Rsp5 is also important for stress tolerance.

Furthermore, Rsp5 has been implicated in many other aspects of cellular physiology. Several reports suggest that Rsp5 is involved in transcriptional regulation (Huibregtse *et al.,* 1995). Consistent with a role in transcription, it was demonstrated that Rsp5 binds to the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Rpb1) (Chang *et al.,* 2000) and mediates its ubiquitination (Huibregtse *et al.,* 1997). Recent findings suggesting that Rsp5 is required for mRNA export from the nucleus further point to a global role of Rsp5 in gene expression (Rodriguez, *et al.,* 2003; Neumann *et al.,* 2003).

Besides its nuclear roles, Rsp5 was implicated in several other cytoplasmic functions. It was reported that Rsp5 is important for mitochondrial inheritance (Fisk and Yaffe, 1999). Also, Rsp5 appears to play a role in endocytosis of plasma membrane proteins. For a large nummber of proteins, inactivation of *RSP5* impairs their ubiquitination and endocytosis (Rotin *et al.,* 2000). Rsp5 seems to act at multiple steps in the endocytic pathway (Wang *et al.,* 2001; Dunn and Hicke, 2001). By ubiquitination of cargo proteins, Rsp5 promotes their internalization from the plasma membrane. In addition, Rsp5 also seems to be involved in the ubiquitination of biosynthetic cargo that enters the endocytic pathway at the level of endosomes (Katzmann *et al.,* 2004). Furthermore, Rsp5 appears to have a function at endosomes other than cargo ubiquitination. Rsp5 could affect endocytosis by interfering with the organization of the actin cytoskeleton. A possible connection between Rsp5 and the actin cytoskeleton is suggested by genetic interactions between *rsp5* and several other mutants affected in genes coding for actin cytoskeleton components, and by the demonstration of direct physical interactions between Rsp5 and components of the actin cytoskeleton (Kaminska *et al.,* 2002; Stamenova *et al.,* 2004).

As mentioned above, transport of Ste6 to the vacuole is regulated by ubiquitination (Kölling and Hollenberg, 1994). Ubiquitination and rapid turnover of Ste6 is mediated by a signal in the linker region termed D-box for destabilization box that connects the two homologous halves of Ste6 (Fig. 2) (Kölling and Losko, 1997). Ubiquitination seems to be important for sorting of Ste6 into the multivesicular

bodies (MVB) pathway (Losko *et al*., 2001). Cargo proteins destined for degradation in the lumen of the vacuole are sorted into vesicles that bud into the interior of the late endosome resulting in the formation of a multivesicular body (MVB) (Odorizzi *et al.,* 1998). After fusion of the MVBs with the vacuole, the vesicles are released into the lumen of the vacuole and proteins associated with them are degraded by vacuolar hydrolases. In the *doa4Δ* mutant, defective for a major deubiquitinating enzyme Doa4 / Ubp4, ubiquitination dependent processes are affected because the free ubiquitin levels are lowered (Swaminathan *et al.,* 1999). In this mutant, the Ste6 protein is not sorted into internal vesicles, but instead accumulates at the vacuolar membrane (Losko *et al.,* 2001). This indicates that ubiquitination is important for sorting of Ste6 into internal vesicles. Similar observations have been made with other yeast membrane proteins (Katzmann *et al.,* 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001).

Two distinct compartments, early and late endosomes, can be distinguished morphologically (Hicke *et al.,* 1997; Prescianotto-Baschong and Riezman, 2002) and biochemically (Singer and Riezman, 1990) in the yeast endocytic pathway. Internalized cell surface proteins travel through these compartments on their way to the vacuole / lysosome where they are degraded (Fig. 1). However, not all proteins share the same fate, some escape degradation and are recycled back to the cell surface. In yeast, recycling was first shown for the catalytic subunit of chitin synthase III (Chs3), an enzyme required for chitin ring formation at the incipient bud site (Ziman *et al.,* 1996). After Chs3 is internalized, it is redirected to sites of polarized growth and stored in endosome-like organelles (chitosomes) which are mobilized to the forming bud in a cell cycle-regulated manner. Recycling of another protein, the a-factor receptor, Ste3, was also demonstrated (Chen and Davis, 2000). Ste3 is constitutively endocytosed and degraded in the absence of its ligand, but in the presence of a-factor, it recycles between the plasma membrane and endosomal compartments. The recycling of another protein, the v-SNARE (soluble NSF (NEM-sensitive factor) attachment protein receptor) Snc1 which mediates docking and fusion of vesicles with the target membrane by interaction with a partner SNARE (t-SNARE) has been examined more closely (Lewis *et al.,* 2000). Snc1 travels from early endosomes via the Golgi back to the plasma membrane. Two signals are important for Snc1 recycling, a conserved cytoplasmic signal mediating internalization from the plasma membrane and a signal at or within the transmembrane domain for

delivery to the Golgi. The mechanism of docking and fusion of endosome-derived vesicles with the *trans*-Golgi has been examined in detail.

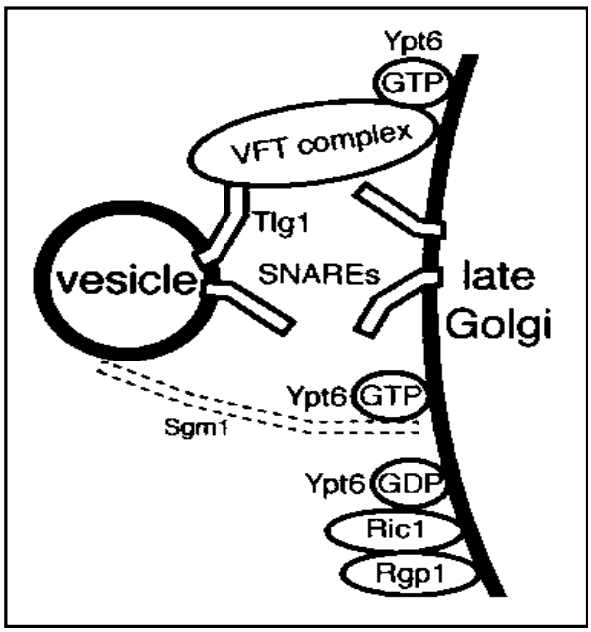


Fig. 3. Shematic representation of endosome-derived vesicle fusion with the late Golgi. The Ric1-Rgp1 complex activates Ypt6 by nucleotide exchange at the late Golgi membrane. Ypt6:GTP recruits the VFT complex, which on the other hand interacts with Tlg1. SNARE engagement and fusion follow. (taken from Siniossoglou and Pelham, 2001)

A multisubunit tethering complex, the VFT (Vps fifty-three) or GARP (Golgi-associated retrograde protein) complex, is required for the initial docking of endosomal vesicles to the Golgi membrane (Conibear and Stevens, 2000). This complex interacts with the Rab protein Ypt6 on the Golgi membrane through its subunit Vps52 and with the SNARE protein Tlg1 through its subunit Vps51 (Fig. 3) (Siniossoglou and Pelham, 2002; Conibear *et al.,* 2003). After tethering, fusion between endosomal vesicles and the Golgi is mediated by a SNARE complex consisting of Tlg1, Tlg2, Vti1 and Snc1 (Paumet *et al.,* 2001; Lewis and Pelham, 2002). After retrieval to the Golgi, recycling proteins like Snc1 are packaged again into secretory vesicles that travel along actin filaments to the bud, the site of polarized growth.

In addition to proteins internalized from the cell surface, Golgi resident proteins, like Kex2 and Ste13 (DPAP A-dipeptidylaminopeptidase A) functioning in the

processing of the mating pheromone α–factor, are also retrieved to the Golgi by endosome-derived carriers (Voos and Stevens, 1998). Another protein that shuttles between Golgi and endosomes is Vps10, the CPY sorting receptor (Conibear and Stevens, 1996). Aromatic amino acid-based retrieval signals have been identified in Kex2, Ste13 and Vps10 indicating that Golgi retrieval seems to be a signal-mediated process (Wilcox *et al.,* 1992; Nothwehr *et al.,* 1993; Cooper and Stevens, 1996). These signals are probably recognized by the retromer complex, the membrane coat complex, found to be essential for retrieval of proteins from late endosomes to the Golgi (Seaman *et al.,* 1998; Nothwehr *et al.,* 2000). The mechanisms governing the sorting from early endosomes to the Golgi are less well defined. It was suggested that sorting nexins Snx4/41/42 play a role in retrieval of Snc1 from early endosomes to the Golgi (Hettema *et al.,* 2003). Sorting of proteins from Golgi to endosomes is mediated by clathrin coats in combination with distinct adapter complexes specific for early and late endosome sorting (Black and Pelham, 2000; Costaguta *et al.,* 2001; Deloche *et al.,* 2001; Mullins and Bonifacino, 2001). Cargo destined for early endosomes appears to be packaged into clathrin coated vesicles in combination with the AP-1 adapter complex while the Gga (Golgi-localized, gamma-ear-containing, ARF-binding) proteins appear to be responsible for sorting to late endosomes.

It is known that ubiquitination plays an important role in Ste6 trafficking. However, the ubiquitin machinery involved is still not known. The E3 ubiquitin ligase Rsp5 has been implicated in ubiquitination of many membrane proteins.
Therefore, the aims of this thesis were to investigate the role of Rsp5 in Ste6 ubiquitination and trafficking and to study the influence of reduced Ste6 ubiquitination on Ste6 trafficking.
To this end, several Ste6 mutant variants with reduced ubiquitination and also different protein sorting mutants were used.

2 MATERIALS AND METHODS

2.1 Strains and Media

2.1.1 Bacterial strains

Tab. 1: *Escherichia coli (E. coli)*

Strain	Genotype	Reference
DH5αF'	F'(Φ 80 (*ΔlacZ*) M15) *Δ*(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* $r_k^-m_k^+$*supE44 thi-1 gyrA relA1*	Gibco BRL, Gaithersburg MD, USA (Woodcock, *et al*., 1989)
Xl1-Blue	F'::Tn10 $proA^+B^+$ *lacIq Δ*(*lacZ*)*M15/recA1 endA1 gyrA96* (Nalr) thi *hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac*	(Bullock *et al*., 1987)

2.1.2 Growth media for *E. coli*

Rich medium (LB): 1% Tryptone, 0,5% Yeast extract, 0,5% NaCl

For selection of cells carrying plasmids, 100 µg/ml ampicillin was added to the media after autoclaving (LB-Amp). Solid media contained 2% agar in addition.
Cells were incubated at 37°C.

2.1.3 Yeast strains

Tab. 2: *Saccharomyces cerevisiae* (*S. cerevisiae*)

Strain	Genotype	Reference or Source
#28	*MATa his1*	Ralf Kölling, Düsseldorf
#29	*MATα his1*	Ralf Kölling, Düsseldorf
JD52	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801*	Jürgen Dohmen, Köln

JD53	*MATα ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801*	Jürgen Dohmen, Köln
JD59	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δump1::HIS3*	Jürgen Döhmen, Köln
JD116	*MATa ura3-52 his 3-Δ200 leu 2-3,112 trp1-Δ63 lys2-801 doa4::LEU2*	Jürgen Dohmen, Köln
RKY592	*MATa ura3 leu2 trp1 his3 gal Δste6::HIS3 end4*	Ralf Kölling, 1997
RKY975	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δpep4:: HIS3*	Losko *et al*., 2001
RKY959	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δste6:: LEU2*	Losko *et al*., 2001
RKY1449	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δdoa4:: LEU2 Δpep4::HIS3*	Losko *et al*., 2001
RKY1511	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δvps4:: HIS3*	Kranz *et al*., 2001
RKY1634	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δsnx4::HIS3*	Ralf Kölling, Düsseldorf
RKY1659	*MATa/MATα ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801*	Ralf Kölling, Düsseldorf
RKY1875	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δvps8::HIS3*	Ralf Kölling, Düsseldorf
RKY1876	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δvps27::kanMX6*	Ralf Kölling, Düsseldorf
ZLY2	*MATα ura3 his3 leu2 trp1 ade8 ypt6-2::URA3 (ts)*	Luo and Gallwitz, 2003

Tab. 3: *S. cerevisiae* strains constructed in this thesis

Strain	Genotype
RKY1718	*MATa his3-Δ200 ura3-52 trp1-Δ63 cdc12-6*
RKY1734	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2 rsp5-1 (ts)*
RKY1924	*MATa/MATα ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801 Δrsp5::HIS3/RSP5*
RKY2048	*MATa his3-Δ200 leu2-3,112 lys2 trp1-Δ63 ura3-52 rsp5-1 Δpep4::URA3*
RKY2049	*MATα his3-Δ200 lys2 trp1-Δ63 ura3-52 rsp5-1 Δump1::HIS3*
RKY2057	*MATa ura3 his3 leu2 trp1 lys2 ypt6-2::URA3 (ts)*
RKY2074	*MATa ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801 Δvps35::HIS3*

2.1.4 Growth media for *S. cerevisiae*

Rich medium (YEPD): 1% yeast extract, 2% peptone,
2% carbon source

Synthetic minimal medium (SD): 0,67% yeast nitrogen base w/o aminoacids,
2% carbon source
Aminoacids and bases were added as followes:

Casein-Hydrolysat	1%
Ura, Ade, His, Trp, Met, Lys, Leu	30 µg/ml

As carbon source 2 % glucose or 2 % galactose were used. Solid media contained in addition 2 % agar. For selection of cells that carryed plasmids appropriate aminoacids were excluded from the growth medium.

Yeast strains were grown at 30°C, temperature sensitive strains were grown at permissive temperature (25°C), and incubated for 10 min to 3 h at non-permissive temperature (37°C).

2.2 Plasmids

Tab. 4: Plasmids used in this thesis

Plasmid	Source/Reference	Description
pFA6a-His3MX6	Longtine *et al*., 1998	Template for PCR-amplification of fragments for gene deletion with *S. pombe HIS5*-selection marker
pYKS2	Kuchler *et al*., 1993	2µ-*URA3* plasmid with c-myc tagged *STE6*. Tag is located in *Spe*I site of the first external loop
pRK69	Ralf Kölling, Düsseldorf	6,2 kb *Bgl*II/*Sal*I chromosomal *STE6* fragment cloned into the 2µ-vector YEp429 (Ma *et al*., 1987)
pRK264	Ralf Kölling, Düsseldorf	1,2kb *Pst*I *STE6* fragment carrying ΔA-box deletion (Kölling and Losko, 1997) cloned into pYKS2
pRK278	Kölling and Losko, 1997	6,2 kb *Bgl*II/*Sal*I *STE6* fragment cloned into the CEN / ARS vector YCplac33
pRK504	Ralf Kölling, Düsseldorf	*GAL1p-STE6*$_{c\text{-}myc}$ in YCplac33
pRK599	Kranz *et al*., 2001	2µ-*URA3* plasmid carrying *STE6-GFP* (S65G S72A)-fusion
pRK657	Ralf Kölling, Düsseldorf	phagemid, 11 lysine residues of *STE6* linker region were exchanged by arginine: K612R, K622R, K638R, K663R, K673R, K684R, K687R, K705R, K709R, K714R, K715R

pRK658	Ralf Kölling, Düsseldorf	1,2 kb *Pst*I *STE6* fragment of pRK278 was replaced by a corresponding fragment of pRK657 carrying 11 K→R substitutions of the *STE6* linker region
pRK659	Ralf Kölling, Düsseldorf	1,2 kb *Pst*I *STE6* fragment of pYKS2 was replaced by corresponding fragnemt of pRK657
pRK814	Ralf Kölling, Düsseldorf	3,77 kb internal *Bam*HI *STE6* fragment of pRK658 cloned into pRK69
pRK845	Ralf Kölling, Düsseldorf	5,3 kb *Sac*I / *Hind*III *STE6* fragment of pYKS2 was transferred to YEplac112
pRK886	Ralf Kölling, Düsseldorf	phagemid carrying *STE6 Y681L* exchange
YCplac33	Gietz and Sugino, 1988	CEN / ARS plasmid with *URA3* marker
YEp96	Hochstrasser *et al*., 1991	*CUP1p*-Ub on 2µ-plasmid with *TRP1* marker
YEp112	Hochstrasser *et al*., 1991	*CUP1p*-HA-Ub on 2µ-plasmid with *TRP1* marker
YEp356	Myers *et al*., 1986	plasmid for lacZ fusions with *URA3* marker
YEplac112	Gietz and Sugino, 1988	2µ-plasmid with *TRP1* marker
YEplac195	Gietz and Sugino, 1988	2µ-plasmid with *URA3* marker

Tab. 5: Plasmids constructed in this thesis

Plasmid	Description
pRK813	3,7 kb PCR fragment containig the RSP5 ORF in YCplac33
pRK873	STE6 ΔA-box$_{cmyc}$ on 2µ-plasmid with *TRP1* marker
pRK894	*HSP26p-lacZ* fusion in YEp356
pRK895	*SSA1p-lacZ* fusion in YEp356
pRK888	*STE6 F630L* in pYKS2
pRK889	*STE6 Y648L* in pYKS2
pRK890	*STE6 F656L, Y657L, Y661L* in pYKS2
pRK891	*STE6 Y681L* in pYKS2
pRK892	*STE6 Y713 L* in pYKS2
pRK907	1,2 kb *STE6 Pst*I fragment of pRK845 replaced by corresponding fragment of pRK657
pRK909	1,2 kb *STE6 Y681L Pst*I fragment of pRK891 cloned into pRK278
pRK939	3,9 kb *STE6 Y681L Bam*HI/*Hind*III fragment of pRK891 coding for the C-terminal part of Ste6 cloned into pRK278
pRK940	3,9 kb *STE6 Bam*HI/*Hind*III fragment of pYKS2 coding for the C-terminal part of Ste6 cloned into pRK278
pRK969	*UBI4p-lacZ* fusion in YEp356
pRK970	*STE6* with N-terminal c-myc tag from pYKS2 (2,1 kb *Bam*HI/*Nco*I fragment) and C-terminus from pRK278 (2,3 kb *Nco*I/*Hind*III fragment) on 2µ-plasmid with *URA3* marker
pRK974	1.3 kb *Eco*RI *GAL1p-STE6* fragment of pRK504 cloned into pRK939
pRK975	1.3 kb *Eco*RI *GAL1p-STE6* fragment of pRK504 cloned into pRK940
pRK996	1.3 kb *Eco*RI *GAL1p-STE6* fragment of pRK504 cloned into pRK909
pRK998	1,2 kb *Pst*I fragment of pRK970 was replaced by corresponding fragment of pRK886

2.3 Synthetic oligonucleotides

Tab. 6: Oligoncleotides used in this thesis

Primer	Primer sequence 5'→3'	Description
#788	CCCAAGCTTGCAGACCAGCCGCACAGT	Upstream primer for PCR amplification of *RSP5* with *Hind*III restriction site
#789	CCCGAATTCACGACAAATAACCGATAC	Downstream primer for PCR amplification of *RSP5* with *Eco*RI restriction site
#804	TATATTTAAAGTAACAGAAAGGAAAGAAAA AAGAAAAAAACGGATCCCCGGGTTAATTAA	F1 primer for *RSP5*
#805	AACGGACACCATAAATAAAAAAAAAATTGG TGCGGAATAAGAATTCGAGCTCGTTTAAAC	R1 primer for *RSP5*
#806	TAGTGCGTTTTTAGTTCG	Upstream primer for verification of *rsp5Δ*
#807	ATTTTCTTTGCTGCTTTC	Downstream primer for verification of *rsp5Δ*
#812	CCCGTCGACCAACGGACAAGGAATACG	Upstream primer for PCR amplification of *UBI4p* with *Sal*I restriction site
#813	CCCAAGCTTTCTTGACGAAAATCTGCAT	Downstream primer for PCR amplification of *UBI4p* with *Hind*III restriction site
#942	CCCGTCGACATCCGTCAATAGAATGTT	Upstream primer for PCR amplification of *HSP26p* with *Sal*I restriction site
#943	CCCAAGCTTCTGGACTGTTAAATGACAT	Downstream primer for PCR amplification of *HSP26p* with *Hind*III restriction site
#944	CCCCTCGAGATCATAAACACGGACCAA	Upstream primer for PCR amplification of *SSA1p* with *Xho*I restriction site

#945	CCCAAGCTTtACCGACAGCTTTTGACAT	Downstream primer for PCR amplification of *SSA1p* with *Hind*III restriction site
#1080	TAAAAGGAGGAGGACGAGAAAGAAGAAGCT GAAAAACACACGGATCCCCGGGTTAATTAA	F1 primer for *VPS35*
#1081	TTTATCTTGGGCATGTACGAAGAGCAAGTA CGTTATTTAAGAATTCGAGCTCGTTTAAA	R1 primer for *VPS35*
#1082	AAAAGGAGGAGGACGAGA	Upstream primer for verification of *vps35*
#1083	AAGGGAAAGCAAAGATTA	Downstream primer for verification of *vps35*

2.4 Chemicals and Enzymes

Acros	Lysine, sodium carbonate, glycine, galactose
Amersham	Amplify, proteinA-sepharose
Axis-Shield	Optiprep™
Bio-Rad	Bio-rad protein assay reagens
Braun-Melsungen	Glaspearls 0,45 µm ø
Caesar & Loretz	Glucose
Difco	Bacto agar, casaminoacids, peptone, tryptone, yeast extract, yeast nitrogen base
Fluka	Formaldehyde
Fuji	X-Ray films
GibcoBRL	Agarose, restriction endonucleases, T4-DNA-ligase, T4 polymerase-buffer
ICN	Tran^{35}S-Label™, NBT
J. T. Baker	$CaCl_2$, NaCl, NaOH, PEG4000, potassium acetate
Kodak	X-Ray films
Life Technologies	G418/Geneticine
Merck	APS, EDTA, Leucine, TEMED, adenine, Uracil, DMF, HEPES

Millipore	PVDF-membrane
New England Biolabs	Restriction endonucleases, BSA
PCR Inc. Florida	5-FOA
Qiagen	QIAprep® Spin Miniprep Kit (250), QIAquick® Gel extraction Kit (250), QIAqick® PCR purification Kit (250)
Ridel-de Haen	Acetic acid, urea, sodium azid
Roche	Alkaline phosphatase, BCIP, restriction endonucleases, Expand Long PCR System, Lumi Light Western Blotting Substrate, salmon sperm DNA
Roth	DTT, Gel 30 Acrylamide, Glycine, DMSO, tricine
Schleicher & Schuell	Nitro-cellulose-Membrane, membrane filter 45 µm
Seikagaku Kogyo Co. Ltd	Zymolyase
Serva	Glycerine, SDS, ethidium bromide, Triton X-100, Tween 20
Sigma	Ampiciline, lithiumacetate, BCIP, NBT, PonceauS, prestained molecular weight standard, sucrose, Triton X-100, BSA (IlgG-free), Tris, sorbitol, ONPG, cycloheximide, BSA (IgG-free), Fast Garnet GBS salt, N-acetyl-DL-phenylalanine ß-naphthyl ester
Thermo Hybaid	Synthetic oligonucleotides
Whatman	3 MM Paper

2.5 Antibodies

Monoclonal antibodies	
anti-ALP (diluted 1:500)	Molecular Probes
anti-c-myc (9E10) (diluted 1:1000)	BAbCO (Berkley Antibodies Co.)
anti-Dpm1 (diluted 1:500)	Molecular Probes
anti-Haemagglutinin (HA) (diluted 1:500)	BAbCO (Berkley Antibodies Co.)
anti-Ubiquitin (diluted 1:200)	Covance
Polyclonal antibodies	
anti-CPY (diluted 1:1000)	D. Wolff, Stuttgart

anti-Pep12(diluted 1:1000)	H. Pelham, London
anti-Pma1 (diluted 1:1000)	R. Kölling, Düsseldorf
anti-Ste6 (diluted 1:200)	R. Kölling, Düsseldorf
anti-Emp47 (diluted 1:5000)	S. Schröder-Köhne
anti-PGK (diluted 1:5000)	Molecular probes

Secondary antibodies	
Phosphatase conjugated anti-Mouse	dianova
Phosphatase conjugated anti-Rabbit	dianova
Peroxidase conjugated anti-Mouse	dianova
Peroxidase conjugated anti-Rabbit	dianova
FITC-conjugated anti-Mouse	dianova

2.6 Transformation

2.6.1 Transformation of *E. coli*

Transformation of *E. coli* competent cells was performed according Hanahan, 1985.

2.6.2 Transformation of *S. cerevisiae*

For transformation of yeast cells, the lithium acetate-method was used (Ito, *et al*., 1983). 5-10 ml of exponential culture was used. Cells were washed with H_2O and resuspended in 200 µl 0,1 M LiOAc in TE (10 mM Tris-Cl, pH 8,0; 1 mM EDTA pH 8,0). Then, 1 µg plasmid-DNA or 45 µl of PCR product together with 10 µl denatured salmon sperm-DNA (10mg/ml) were added to the suspension. After addition of 1,5 ml of 40 % PEG 4000 in 0,1 M LiOAc in TE cells were incubated for 45 min at 30°C or at 25°C (for temperature sensitive strains). Cells were heat-shocked for 5 min for plasmid transformations or for 30 min for transformation of PCR-fragments at 42°C. Finally, cells were centrifuged and resuspended in H_2O, and plated on selective media.

2.7. Preparation of DNA

2.7.1 Isolation of plasmid-DNA from *E. coli*

Plasmid-DNA was isolated from 1,5 ml *E. coli* stationary culture using the modified alkaline lysis method (Maniatis *et al*., 1982). For restriction analysis, 200 ng isolated plasmid DNA were digested in 20 µl.
High pure plasmid DNA for sequencing and cloning was isolated with QIAprep® Spin Miniprep Kit (250) from Qiagen according to the instruction manual.

2.7.2 Isolation of chromosomal DNA from *S. cerevisiae*

Chromosomal DNA isolated from *S. cerevisiae* was used as a template in the PCR reactions. Cells were resuspended in 10-20 µl 20 mM NaOH, and heated in the microwave oven for 30 sec. Cells were then left to sediment for 5 min, and then 2 µl of the supernatant were used in the PCR reaction.

2.8 Enzyme modification of DNA

2.8.1 DNA-Restriction

DNA was digested with 1 U of restriction enzyme per µg DNA for 2-3 h or overnight, at the temperature recommended by the manufacturer. New England Biolabs buffers were used.

2.8.2 Removal of 3' end overhangs

3'-overhangs were removed with T4-DNA Polymerase (2-5 U per µg DNA) according to the method described by Maniatis *et al*., 1982. The reaction mixture containing DNA, T4 DNA-polymerase, T4 polymerase-buffer (50 mM Tris-Cl pH 8,0; 5mM $MgCl_2$; 5 mM DTT) and 0,1 mM dNTPs was incubated for 30 min at 16°C.

2.8.3 Dephosphorylation of DNA fragments

For dephophorylation of DNA fragments, calf intestine alkaline phosphatase (CIP) was used. After digestion of the DNA, 1 U of CIP was added to the reaction mixture and incubated for 1 h at 37°C. The reaction was stopped by freezing. Further purification of DNA was done by electrophoretic separation on agarose gels (2. 10).

2.8.4 Ligation

For ligation, 40-60 ng of dephosphorylated vector and a two fold molar excess of insert DNA in a total volume of 20-30 µl was used. 1 U T4 ligase and T4 ligase buffer were added to the reaction mixture and incubated at 16°C overnight.

2.9 PCR-amplification of DNA

For the polymerase-chain-reaction (PCR), "Expand High Fidelity" polymerase (Roche) and "Pfu Ultra™ HF" polymerase (Stratagene) kits were used. The amplification was performed in a "Primus 25/96 thermocycler" (MWG Biotech). The PCR reactions were prepared in 25-50 µl volume.

2.9.1 Specific amplification of plasmid DNA

For amplification of plasmid DNA, 1,5 mM $MgCl_2$, 2 µM of appropriate primers, 200 mM dNTP-Mix, 1 ng of plasmid DNA and 1 U of enzyme were used.
The following program was used for the amplification of DNA fragments:

1.		4 min 94°C	denaturation of DNA
2.	30X	30 sec 94°C	denaturation of DNA
		1 min 55°C	annealing of the primer
		n-min 68°C	elongation (DNA-synthesis)
3.		10 min 68°C	DNA-synthesis

For elongation, 1 min per kb of expected DNA product was used.

2.9.2 Specific amplification of chromosomal DNA

For amplification of chromosomal DNA, a buffer without $MgCl_2$ was used. In addition, the reaction mixture contained 4 mM $MgCl_2$, 2 µM of each primer, 200 µM of dNTP-mix, 2 µl of DNA and 1 U of DNA polymerase.
The following program was used for amplification:

1.		4 min 94°C	denaturation of DNA
2.	35X	1 min 94°C	denaturation of DNA
		1 min 55°C	annealing of the primer
		n-min 68°C	elongation (DNA synthesis)
3.		10 min 68°C	DNA synthesis

The annealing temperature, elongation time and number of amplification cycles were adjusted to the melting temperature of the primers and the length of the expected PCR product. For Pfu Ultra DNA polymerase, 72°C was used for the DNA synthesis step.

2.10 DNA electrophoresis

DNA fragments from 0,3-10 kb were separated on 1 % agarose gels. As gel- and running-buffer 0,5 X TAE (20 mM Tris; 10 mM NaOAc; 1 mM EDTA pH 8,0) was used. Ethidium bromide was added to gel and running buffer in a final concentration of 0,5 µg/ml. DNA samples were mixed with loading buffer (0,2 % bromphenolblue in 25 % glycerol) in 10:1 ratio. DNA fragments were separated at 250 V maximum. As size and concentration standard, 1 µg of λ DNA cut with *Eco*RI and *Hind*III was used.

2.11 Isolation of DNA fragments from agarose gels

The isolation of DNA was performed with QIAquick® Gel Extraction Kit (250) from Qiagen according to the instruction manual.

2.12 Determination of DNA concentration

The DNA concentration was determined by comparing the band intensities after gel separation with DNA standards.

2.13 DNA sequencing

DNA samples were sequenced by Seq-Lab or GATC.

2.14 Preparation of protein extracts for Western analysis

Two OD_{600} units of an exponential yeast culture (OD_{600}=0,4-0,7; 2-4x10^7cells/ml) were harvested by a 500 g spin. Cells were washed in H_2O and resuspended in 100 µl lysis buffer (0,3 M sorbitol; 50 mM HEPES; 10 mM NaN_3; pH 7,5) with proteinase inhibitors (0,5 µg/ml each of aprotinin, antipain, chymostatin, leupeptin and pepstatin A and 1,6 µg benzamidine, 1 µg/ml phenanthroline, and 170 µg/ml phenylmethylsulfonyl fluoride). Cells were lysed by shaking with glass beads on a vortex mixer (vibrax) (Janke & Kunkel, Vibrax-VXR) for 5 min at 4°C. 150 µl sample buffer (4 % SDS; 20 % Glycerine; 125 mM Tris-Cl pH 6,8; 0,2 % Bromphenolblue) with 40 mM DTT were added to the lysate and then the samples were incubated for 15-30 min at 50°C. Samples were stored at –20°C until use. Samples were re-solubilized by incubation at 50°C before loading onto the SDS-polyacrylamide gels (2. 16). 30 µl of each sample corresponding to 0,2 OD_{600} units of cells were loaded on the gel.

2.15 Determination of protein concentration

The protein concentration was determined according to the method of Bradford, 1976 with the "Bio-Rad Protein Assay Dye Reagent" (Bio-Rad). 1-10 µl of cell extract dilluted with H_2O in 800 µl total volume, were mixed with 200 µl of Bio-Rad Protein Assay Dye reagent. After 10 min incubation at room temperature, the absorbance at 595 nm was measured in a spectrophotometer. A standard curve was obtained by determining the absorbance of BSA solutions of known concentrations.

2.16 LacZ assay

Cells were grown overnight in SD / CAS medium at 25°C to exponential phase. Ten OD_{600} units (5 x 10^7 cells) were harvested and resuspended in fresh medium to a density of OD_{600} = 0,5. The cells were then shifted to 37°C. At time intervals, two OD_{600} aliquots of cells were harvested and washed once in cold Z-buffer (0,1 M Na-phosphate, 10 mM KCl, 1 mM $MgSO_4$, pH 7,0). The cells were resuspended in 100 µl Z-buffer and lysed by agitation with 400 mg of glass beads for 3 min. After addition of another 100 µl Z-buffer, the cell extract was removed from the glass beads and spun for 2 min at 13,000 g to remove cell debris. A suitable amount of cleared cell extract (10-50 µl) was added to 1 ml of Z-buffer containing 1 mg/ml ONPG (2-Nitrophenyl-ß-D-galactopyranoside) and incubated at 30°C until a yellow colour developped. The reaction was stopped by addition of 0,5 ml 1M Na_2CO_3 and the absorbance was measured at 420 nm.

2.17 Metabolic labeling

Cells were grown overnight at 25°C to exponential phase in minimal medium (SD) with required nutrients. Six OD_{600} units (3 x 10^7 cells) were harvested and resuspended in fresh medium to a density of OD_{600} = 1. The cells were shifted to 37°C and after different time periods at 37°C, 1 ml aliquots of cells were labeled with 7 µCi of Tran^{35}S-label (ICN) for 10 min. Then the cells were harvested, washed once in 1 ml cold 10 mM NaN_3, to remove unincorporated label, and lysed by agitation with 400 mg glass beads for 3 min in 110 µl of lysis buffer plus protease inhibitors (2. 14). To determine the total amount of label taken up by the cells, a 10 µl aliquot was removed from the cell extract and analysed by liquid scintillation counting. To determine the amount of label incorporated into protein, another 10 µl aliquot was added to 1 ml 10 % TCA and kept on ice for 1-2 h. Then the samples were collected by vacuum filtration over glass fibre filters and washed twice with 10 ml 10 % TCA. Finally, the filters were analysed by liquid scintillation counting.

2.18 SDS-polyacrylamide gel electrophoresis (PAGE)

Proteins were separated under denaturing conditions according to Laemmli, 1970 in a discontinuous polyacrylamide gel system. A 5% stacking gel (125 mM Tris-Cl pH 6,8; 0,1 % SDS; 0,025 % APS; 0,1% TEMED; 5 % acrylamide) and a 7,5-10 % separating

gel (375mM Tris-Cl pH 8,8; 0,1 % SDS; 0,025% APS; 0,1% TEMED; 7,5-10 % acrylamide) were used. The running buffer contained 25mM Tris; 0,192 M Glycine; 0,1 % SDS; pH 8,3. Proteins were separated at 250 V (max) and a current of 35 mA (max).
For detection of ubiquitin, 16 % tricine gels prepared according to Schägger and von Jagow, 1987 were used.

2.19 Immunological detection of proteins by Western blotting

After electrophoretic separation, proteins were transferred to nitrocellulose or nylon membranes (nylon membranes for detection of ubiquitin). Transfer of proteins was done in transfer buffer (25 mM Tris; 192 mM glycine; 20 % methanol) for 3 h at 60 V or over night at 40 V. Transferred proteins were detected on the membrane by reversible staining with Ponceau S. For detection of ubiquitin, proteins were transferred for 15 min at 70 V in a transfer buffer. After transfer, the membrane was boiled in H_2O for 30 min before immunological detection of ubiquitin.
Unspecific binding was prevented by incubation of the membrane in PBS/TWEEN (10 mM Na_3PO_4 pH 7,2; 150 mM NaCl; 0,05 % TWEEN20) with 1 % dry milk. The membrane was then incubated for 90 min at room temperature with primary antibodies diluted in PBS/TWEEN with 1 % dry milk. Then the membrane was washed three times for 5 min in PBS/TWEEN and incubated with secondary antibodies (anti-Mause-IgG or anti-Rabbit-IgG, conjugated with alkaline phosphatase or peroxidase) diluted 1:5000 in PBS/TWEEN at room temperature for 60 min. The membrane was washed two more times for 5 min.
The bound antibodies were detected either via alkaline phosphatase or via peroxidase. Detection via alkaline phosphatase: the membrane was washed for 5 min one more time in substrate buffer (0,1 M Tris-Cl pH 8,8; 0,1 M NaCl; 2 mM $MgCl_2$). Antibodies were detected by a colour reaction produced by the enzyme activity. As substrates 0,1 mg/ml BCIP and 1 mg/ml NBT were used. Detection via peroxidase: antibodies were detected through peroxidase reaction using "Lumi Light Kit" (Roche) according to the instruction manual. Luminescence was detected with the "LAS" (luminescence imaging system) from Fuji.

Blots were scanned with DUOSCAN f40 scanner (AGFA). The signals were quantified with the programes "NIH Image" or "Image J".

2.20 Cell fractionation

2.20.1 Differential centrifugation

Ten OD_{600} units of cells from an exponentially growing culture were harvested, washed in H_2O, resuspended in lysis buffer with proteinase inhibitors (2. 14) and lysed by vortexing with glass beads for 5 min at 4°C. Intact cells and cell debris were removed by centrifugation at 500 g for 5 min. The cell extracts were then centrifuged at 100,000 g for 1 h to generate the P100 pellet and the S100 supernatant. Fractions were solubilized in sample buffer (2. 14) and equal portions of the fractions were assayed for the presence of proteins by Western blotting.

2.20.2 Flotation gradients

Cells were grown to logarithmic phase in YEPD. Twenty OD_{600} units of cells were harvested by centrifugation, washed with water and lysed by agitation with glassbeads in TNE buffer (50 mM Tris pH 7,4; 150 mM NaCl; 5 mM EDTA + protease inhibitors (2. 14)). After removal of cell debris and intact cells by centrifugation at 500 g for 5 min, half of the extract (125 µl) was treated with 1 % Triton X-100 for 30 min on ice to solubilize the membranes ; the other half was kept on ice without Triton. The extracts were then mixed with 250 µl of 60 % Optiprep solution (Axis-Shield). The resulting 40 % Optiprep fraction was transferred to the bottom of a centrifugation tube and overlayed with 600 µl 30 % Optiprep in TNE buffer (or TXNE buffer, i.e. TNE + 0,1 % Triton X-100 in case of Triton treated extracts) and 100 µl TNE (or TXNE) buffer. The gradients were then centrifuged at 77,000 rpm at 4°C for 2 h in a TLA 100.2 rotor in a Beckman table top ultracentrifuge. Six equal fractions were collected from the top of the gradient. Proteins were solubilized by incubation with sample buffer (2. 14) at 50°C for 30 min. Fractions were then assayed for the presence of proteins by Western blotting.

2.20.3 Sucrose density gradients

Exponentially growing cells (OD_{600}<0,8; 5 x 10^7 cells/ml) from a 100 ml culture were harvested by vacuum filtration onto nitrocellulose filters. Following filtration, cells were immediately resuspended in STED10 buffer (10 % sucrose, 10 mM Tris-Cl pH 7,6, 10 mM EDTA, 1mM DTT plus protease inhibitors (2. 14)) and lysed by vortexing with glass beads for 5 min. After addition of another 1 ml of STED10, the extract was transferred to a fresh tube and spun at 500 g for 5 min to remove cell debris. The cleared cell extract was loaded on top of a sucrose gradient. The gradient was prepared as follows. In SW40 centrifuge tube, 4 ml of STED50 (53 % sucrose), STED34 (35 % sucrose), and STED20 (20 % sucrose) were layered on top of each other.The centrifuge tubes were slowly turned into a horizontal position. After 3 h of horizontal diffusion, the gradients were again turned into an upright position, loaded with extract, and spun at 4°C for 15 h at 30,000 rpm in a Beckmann SW40 rotor; 700 µl fractions were collected from the top of the gradients and the proteins were solubilized by incubation at 50°C for 30 min in sample buffer (2. 14). Fractions were then examined for marker proteins by Western blotting.

2.21 Pulse chase experiments and immunoprecipitation

Cells were grown overnight at 25°C to exponential phase in synthetic minimal medium (SD) with required nutrients. Ten OD_{600} units (6 x 10^8 cells) were harvested and resuspended in fresh medium to a density of 2 OD_{600} units per ml. After a 15 min preincubation at 25°C, the cells were labeled with 70 µCi of Tran^{35}S-label (ICN) for 5 min at 25°C and another 10 min at 25°C / 37°C. Chase was initiated by the addition of 1/50 volume of concentrated chase solution (0,3 % cysteine, 0,4 % methionine) to the culture. Aliquots of a 1 OD_{600} unit of cells were removed at 20 min intervals (for determination of Ste6 half life) or at 45 min intervals (for determination of HA-ubiquitin half life), washed once in 1 ml of cold NaN_3, and lysed by agitation with 400 mg (± 5 mg) of glass beads for 3 min in 110 µl lysis buffer plus protease inhibitors (2.14). Proteins were solubilized with one volume of 2x sample buffer (2.14) for 30 min at 50°C. Four volumes of immunoprecipitation (IP)-dilution buffer (1,25 % Triton X-100, 6 mM EDTA, 60 mM Tris-Cl pH 7,4) were added and insoluble material was removed by centrifugation for 5 min in a table

top centrifuge. The samples were incubated overnight at 4°C with 10 µl of Ste6 antiserum or 3 µl of anti-HA antibodies and another 3 h with 50 µl of a 20 % suspension of protein A-sepharose beads (Pharmacia). The beads were washed three times with IP buffer (1 % Triton X-100, 5mM EDTA, 50 mM Tris-Cl pH 7,4) and finally resuspended in 50 µl IP buffer. Proteins were removed from the beads by incubation with sample buffer (2.14) with 100 mM DTT for 30 min at 50°C. About half of the immunoprecipitated material (50 µl) was loaded onto a 7,5 % SDS-PAGE gel for detection of Ste6 or onto a 16 % Tricine gel for detection of HA-ubiquitin. The gels were fixed for 30 min in a mixture of acetic acid (7 %) and methanol (20 %), soaked in Amplify (Amersham) for 30 min to enhance the signal, dried for 2 h at 80°C and exposed to X-ray film (Kodak or Fuji).
Autoradiograms were scanned by a DUOSCAN f40 scanner (AGFA). The signals were quantified with "NIH Image" or "Image J".

2.22 Cycloheximide chase

Cycloheximide chase was used to investigate the ubiquitin half life. Cells were grown overnight at 25°C to exponential phase (OD_{600} = 0,3-0,7) in YEPD medium. Twenty OD_{600} units of cells were harvested by centrifugation and resuspended in 40 ml of fresh medium. Half of the cells (20 ml) were further incubated at 25°C and the other half (20 ml) was shifted to 37°C. After 30 min incubation at 25°C / 37°C, cycloheximide (50 µM or 200 µM) was added to the medium to prevent new protein synthesis. Samples were collected over 2 h every 30 min. Then cell extracts were prepared (2. 14) and analysed for the presence of ubiquitin by Western blotting (2. 19).

2.23 Gal depletion experiment

Cells were grown overnight at 30°C to logarithmic phase in SD / CAS minimal medium with required nutrients supplemented with 2 % galactose / 0,2 % glucose as carbon sourse. Ten OD_{600} units of cells (6 x 10^8 cells) were harvested by centrifugation and resuspended in SD / CAS medium with required nutrients plus 5 % glucose as carbon source. Two OD_{600} units of cells were collected every 15 min

over 1 h. Then cell extracts were prepared (2. 14) and analysed for the presence of proteins by Western blotting (2. 19).

2.24 GFP fluorescence

Cells were grown overnight to exponential phase (OD_{600}=0,2-0,8; 1-4 x 10^7cells/ml) in minimal medium (SD) with required nutrients. Cells were fixed on microscope slides by mixing with low-melting agarose and examined with a Axioskop fluorescence microscope (Zeiss) using a FITC filter set. Images were aquired with a CCD camera (Axiocam, Zeiss).

2.25 Immunofluorescence

Cells were grown to exponential phase (OD_{600}=0,2-0,8; 1-4 x 10^7cells/ml) and fixed directly for 4 h with formaldehyde (final concentration 5 %). The fixed cells were spheroplasted as described by Pringle *et al*., 1989, extracted with 0,1 % Triton X-100 for 5 min and then attached to a multiwell slide treated with 0,1 % polylysine (Sigma). The cells were first incubated with anti-c-myc mouse primary antibody (9E10) (1:100 dilution in PBS + 1mg/ml BSA) for 90 min, washed four times with 1 x PBS and then incubated another 90 min with FITC-conjugated anti mouse secondary antibodies (Dianova; 1:200 dilution in PBS/BSA). Cells were washed again four times with 1 x PBS and finally, incubated for 5 min with 4,6-diamidino-2-phenylindole (DAPI) (1mg/ml in PBS). The cells were examined with a Zeiss Axioskop microscope using an FITC filter set and photographed with a CCD (charge-coupled device) camera (Axiocam, Zeiss).

2.26 Vacuole preparation

Vacuoles were isolated from 1 l of an exponentially growing cell culture (OD_{600}~0,8; 5 x 10^7cells/ml). Cells were collected by centrifugation at 4,400 g for 5 min, washed once with H_2O and resuspended in 100 ml of Zymolyase solution (1 M sorbitol, 400 U/ml Zymolyase 100T (ICN)) for spheroplasting. After incubation at 30°C for 60-90 min, spheroplasts were harvested by centrifugation at 2,200 g for 5 min and washed

twice in 1 M sorbitol. Spheroplasts were lysed by resuspension in 25 ml buffer A (10 mM MOPS pH 6,9; 0,1 mM $MgCl_2$; 12% Ficoll 400) with protease inhibitors (2.14) and homogenization on ice. Lysates were then centrifuged at 2,200 g for 10 min at 4°C to remove unlysed spheroplasts. The 2 % of the supernatant fraction was saved to assess yield and purity of isolated vacuoles. All of the subsequent manipulations were carried out on ice. The supernatant was transferred to a SW28 centrifuge tube, overlayed with 13 ml of buffer A and centrifuged at 60,000 g for 30 min in a Beckman SW28 rotor. The white wafer floating on top of the Ficoll was collected and resuspended in 6 ml buffer A. The suspension was transferred to a SW40 centrifuge tube, overlayed with 6 ml buffer B (10 mM MOPS pH 6,9; 0,5 mM $MgCl_2$; 8% Ficoll 400) and centrifuged at 60,000 g for 30 min in a Beckman SW40 rotor. The white wafer at the top of the tube was collected and solubilized in the one volume of sample buffer (2.14) with 40 mM DTT at 50°C for 30 min. Samples were then assayed for the presence of proteins by Western blotting.

2.27 Protease protection experiments

Protease protection experiments were performed with isolated vacuoles (2. 26). Three aliquots of isolated vacuoles were diluted to a final volume of 800 µl with PS100 (100 mM sorbitol; 50 mM potassium acetate, 5mM magnesium acetate; 100 mM KCl; 1 mM $CaCl_2$, 20 mM Pipes pH 6,8). Two aliquots were treated with 50 µg proteinase K for 20 min on ice. One of these aliquots had been preincubated with 1% Triton X-100 for 10 min at room temperature to solubilize membranes. One aliquot was incubated without proteinase and detergent. The reaction was stopped by a 30 min incubation on ice with 5 mM PMSF (phenylmethylsulfonylfluoride). Then, proteins were precipitated with 5 % TCA and 100 µg/ml BSA for 30 min on ice. After centrifugation at 13,000 rpm for 30 min, the TCA pellets were washed in 1 M Tris pH 8,0 and centrifuged for 10 min at 13,000 rpm. Pellets were dissolved in sample buffer (2.14) at 50°C for 30 min. Proteins were loaded onto a SDS-PAGE gel and examined by Western blotting.

2.28 Serial dilution patch mating assay

To determine the mating activity, 10-fold serial dilutions (starting with 1x 10^7cells) of exponentially growing *MATa* cells transformed with different Ste6 plasmids were spotted onto a lawn of a *MAT*α tester strain. Cells were allowed to mate over night and were then replica plated onto a selective agar plate (SD minimal medium) to select for zygotes. The plate was incubated for 3 d at 30°C.

2.29 Tetrad analysis

Haploid mating partners were mixed on a YEPD plate and allowed to mate for several hours at 25°C. Zygotes were picked with a micromanipulator on a YEPD plate and incubated at 30°C for 3 d. Grown diploid cells were grown overnight in YEPD medium, then washed once in sterile H_2O and resuspended in 2 % potassium acetate with required nutrients. After 3 d incubation at 25°C, cells were collected by centrifugation, washed once with H_2O and resuspended in 1 ml of H_2O. 25 µl of the cell suspension were incubated with 2 µl of zymolase (2,5 mg/ml) for 7 min at 37°C. Tetrads were dissected with a micromanipulator on a YEPD plate and incubated for 3 d at 25°C.

2.30 Gene deletion in *S. cerevisiae*

Gene deletions were performed essentially as described in Longtine *et al*., (1998) by one step gene replacement with PCR-generated cassettes. The deletions were verified by PCR.
For the deletion of the *PEP4* gene, a *PEP4* disruption cassette containing the *URA3* marker was used. The *PEP4* deletions were verified by a Fast Garnet test for vacuolar protease activity.

2.31 Fast Garnet test

A Fast Garnet test was used for detecting the activity of the vacuolar hydrolase carboxypeptidase Y. It was done essentially as described by Jones, 1977. Briefly, colonies were grown to fairly large size (proteases are induced rather late in logarithmic growth) on YEPD plates. Plates were then overlayed with 3 ml 0,6 %

agar mixed with 6 mg/2 ml N-acetyl-DL-phenylalanine ß-naphthyl ester (Sigma) (dissolved in dimethylformamide), prewarmed at 50°C. After the agar mix was solid, plates were overlayed with 5 ml Fast Garnet GBC salt (1 mg/ml, Sigma). After 5-10 min colonies turn red, if carboxypeptidase Y is active. The *pep4* mutants remain white.

3 RESULTS

3.1 Effect of Rsp5 on a Ste6 trafficking and turnover

3.1.1 Ste6 localization in the *rsp5-1*mutant

We were interested to find the E3 ligase, responsible for Ste6 ubiquitination. Rsp5 seemed to be a good candidate, because it has already been implicated in the ubiquitination of many other membrane proteins.

We decided to use the conditional *rsp5-1* mutant for our studies. This mutant grows fairly normal at 25°C (permissive temperature) but fails to grow at 37°C. Altered localization of Ste6 is a good indication of a trafficking defect, which would be expected, if ubiquitination of Ste6 protein is affected. Therefore, the distribution of Ste6 in the *rsp5-1* mutant on permissive and after 1 h shift to non-permissive temperature (37°C) was examined by immunofluorescence microscopy. We used a c-myc tagged variant of Ste6 expressed from plasmid pYKS2. In wildtype, at permissive temperature, Ste6 is localized to internal structures, probably corresponding to endosomes. After a 1 h shift to non-permissive temperature, no change in Ste6 distribution was obvious in wildtype. In the *rsp5-1* strain, Ste6 was observed in internal structures at permissive temperature (25°C), like in wildtype, although some staining of the vacuolar membrane could also be detected. In contrast, upon a 1 h shift to non-permissive temperature (37°C), Ste6 was mostly found in ring-like structures surrounding the vacuole in the *rsp5-1* mutant (Fig. 4). The vacuoles can be seen as light structures in the phase contrast images. The same phenotype (staining of the vacuolar membrane) was also observed with a GFP-tagged Ste6 variant in the *rsp5-1* mutant upon shift to 37°C (data not shown). This indicates that transport of Ste6 to the vacuole and its subsequent degradation was affected in the *rsp5-1* mutant cells.

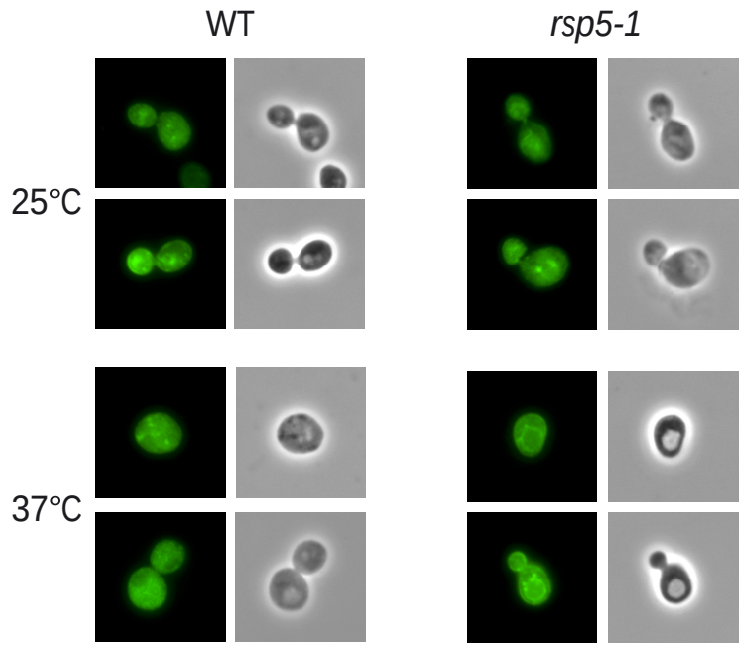


Fig. 4: Localization of Ste6 in WT and the *rsp5-1* mutant. The distribution of Ste6, expressed from a plasmid encoding c-myc-tagged Ste6 protein (pYKS2) was examined by immunofluorescence microscopy using primary antibodies against the myc epitope (9E10) and FITC-conjugated anti-mouse secondary antibodies, in cells grown at 25°C (upper panels), and after 1 h shift to 37°C (lower panels). Left panels: JD52 (WT), right panels: RKY 1734 (*rsp5-1*). Alternating: FITC fluorescence and phase contrast images.

3.1.2 Ste6 is stabilized in the *rsp5-1* mutant

Ste6 is a short-lived membrane protein. After fulfilling its role at the plasma membrane it is endocytosed and degraded in the vacuole, the yeast equivalent to the lysosome of higher eukaryots. To test if trafficking of Ste6 is indeed affected by the *rsp5-1* mutation, we examined the Ste6 turnover in this mutant after a shift to non-permissive temperature (37°C) by a pulse-chase experiment (Fig. 5). Ste6 should be stabilized, if trafficking to the vacuole is prevented. In wildtype cells at 25°C, Ste6 was quickly degraded with a half life of 22 min. Ste6 turnover was even somewhat faster in the wildtype after shift to 37°C (17 min). In contrast, in the *rsp5-1* mutant, Ste6 was already slightly stabilized at permissive temperature (25°C), with a half life of approximately 30 min. After a shift to 37°C, this stabilization of the Ste6 protein was even more pronounced. Ste6 was about 5-fold stabilized in the mutant cells compared to wildtype. Thus, as observed by immunofluorescence, Ste6 trafficking to the vacuole is blocked in the *rsp5-1* mutant cells.

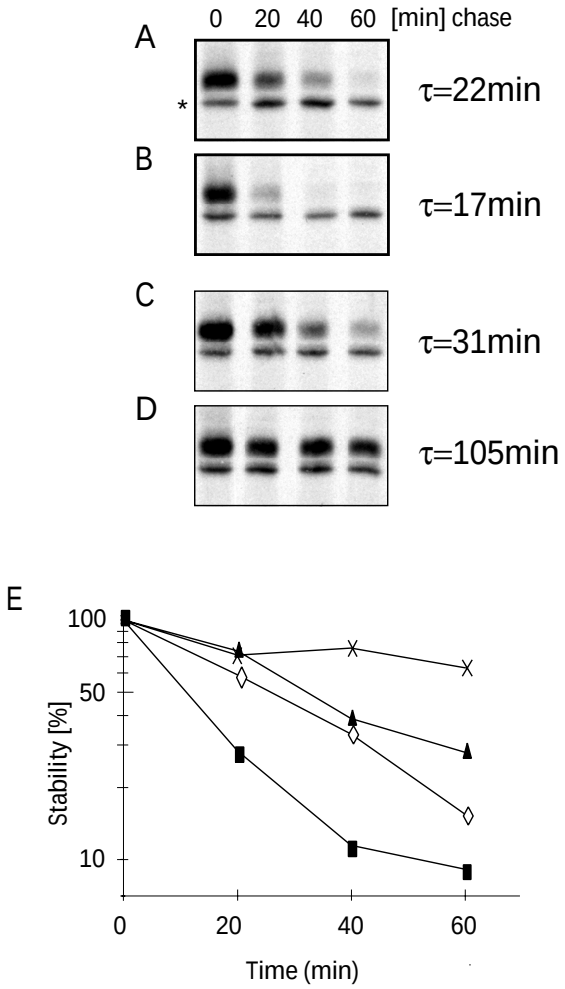


Fig. 5: Ste6 is stabilized in the *rsp5-1* mutant. The Ste6 half-life was determined by pulse-chase experiments. Cells were labeled with [^{35}S]- Trans-label (ICN) for 5 min at 25°C and then for another 10 min at 25°C (A,C) or to 37°C (B,D). Ste6 was immuno-precipitated from cell extracts prepared after different chase periods (as indicated) and inspected by autoradiography. A background band is marked with an asterisk. From top to bottom: JD52 (WT) (A,B), RKY 1734 (*rsp5-1*) (C,D). (E) Quantification of autoradio-graphy signals. X-ray films were scanned and quantified by the programme ImageJ. Intensity of the signal at t_0 was set to 100%. JD52/25°C (open diamond); JD52/37°C (filled square); RKY1734/25°C (filled trianle); RKY1734/37°C (cross), stability (%) = logarithmic scale.

3.1.3 Ste6 ubiquitination is not affected in the *rsp5-1* mutant

It is known that ubiquitination of Ste6 plays an important role in the regulation of its intracellular trafficking (Kölling and Losko, 1997; Losko *et al*., 2001). Since Rsp5 is an E3 ubiquitin ligase involved in many cellular processes, it was interesting to see, if the effects observed on Ste6 trafficking are due to a lack of Ste6 ubiquitination. To test this assumption, Ste6 was immunoprecipitated from cell extracts with anti-Ste6 antibodies. The immunoprecipitates were then examined for the presence of ubiquitin by Western blotting with anti-ubiquitin antibodies. A ubiquitin signal can only be detected, if ubiquitin is covalently attached to Ste6. Ste6 was successfully precipitated from all strains examined, as can be seen on the control blot

developped with anti-Ste6 antibodies (Fig. 6A). The amount of Ste6 in the *rsp5-1* mutant was higher than in wildtype.

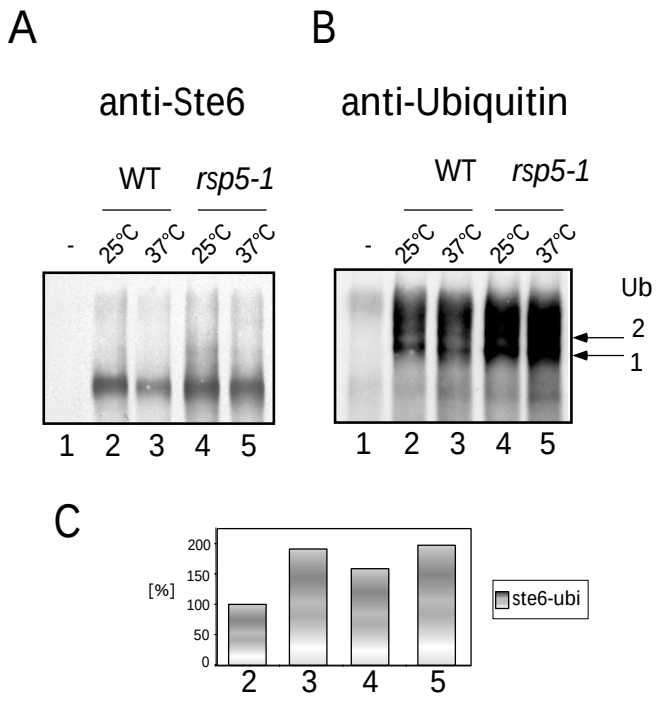


Fig. 6: Ubiquitination of Ste6 is not affected in the *rsp5-1* mutant. Ste6 was immunoprecipitated from cell extracts prepared from JD52 (WT) (lanes 2,3) and RKY 1734 (*rsp5-1*) (lanes 4,5) transformed with the plasmid pRK69 (2µ-*STE6*), grown at 25°C (lanes 2,4) and after 1 h shift to 37°C (lanes 3,5) before extract preparation. Immunoprecipitates were analyzed by Western blotting with (A) anti-Ste6 antibodies and (B) anti-ubiquitin antibodies. Mono- and di-ubiquitinated forms of Ste6 are indicated by arrows. As a negative control (-, lane 1) RKY959 (*ste6Δ*), transformed with YEplac195 (vector), was used. (C) Quantification of Western blot signals. The ubiquitin signal was normalized to the amount of precipitated Ste6 protein. Ubiquitin signal intensity of the wt strain was set to 100%.

This is consistent with the data obtained from the pulse-chase experiments. Ubiquitination of Ste6 is detected as a high-molecular "smear" on the anti-ubiquitin Western blot. Also two distinct bands could be observed underneath the "smear" which correspond to mono and di-ubiquitinated Ste6 protein. A ubiquitin signal could be detected in all strains tested (Fig. 6B). The intensity of the ubiquitin signal was normalized to the amount of precipitated Ste6 protein (Fig. 6C). Interestingly, there was no significant difference in ubiquitination of Ste6 in the *rsp5-1* mutant in

comparison to wildtype. If Rsp5 was the ubiquitin ligase for the Ste6 protein, ubiquitination of Ste6 should be reduced. Therefore, Rsp5 does not appear to be the ubiquitin ligase for Ste6. The observed effects on Ste6 turnover seem to be indirect.

3. 2 Role of Rsp5 in ubiquitin homeostasis

3.2.1 Lack of accumulation of high-molecular weight ubiquitin-conjugates upon heat-shock in the *rsp5-1* mutant

Other interesting observations support the assumption that the observed effects on Ste6 in the *rsp5-1* mutant strain are probably indirect. In the course of our studies on the role of Rsp5 in protein trafficking, we investigated the effect of the conditional *rsp5-1* mutant on the overall pattern of ubiquitinated proteins in yeast cell extracts. When wildtype cells were shifted to 37°C for 1h, an accumulation of high-molecular weight ubiquitin-conjugates in the range of 80-200 kDa was observed by Western-blotting with anti-ubiquitin antibodies (Fig. 7A). Ubiquitin-conjugates accumulated steadily with time, reaching their maximum at about 1 h after shift to 37°C and then declined to pre-shift levels at later time points (Fig. 8).

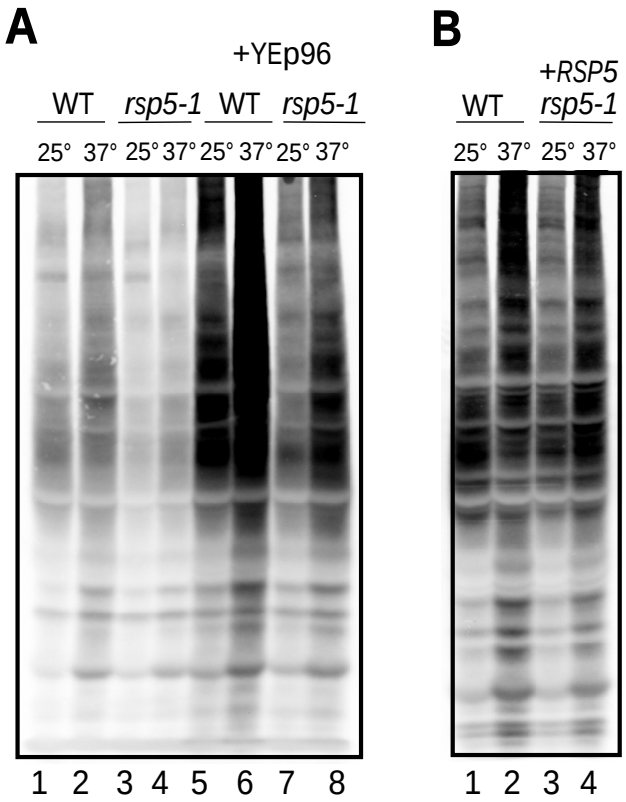


Fig. 7: Accumulation of high-molecular weight ubiquitin species after heat-shock. Cells were pregrown to exponential phase at 25°C in SD/CAS medium. Cell extracts were then prepared from strains grown at 25°C (lanes 1, 3, 5, 7), and strains shifted for 1h to 37°C before extract preparation (lanes 2, 4 , 6, 8). Equal amounts of protein were loaded in each lane. Cell extracts were inspected by Western blotting for the presence of ubiquitin with anti-ubiquitin antibodies**.** (A) JD52 (wt, lanes 1, 2, 5, 6) and RKY 1734 (*rsp5-1*, lanes 3, 4, 7, 8) transformed with YEplac112 (vector plasmid, lanes 1-4) or YEp96 (*2µUBI*, lanes 5-8). The *CUP1* promoter of YEp96 was induced for 3 h before extract preparation with 0,5 mM $CuSO_4$. (B) JD52 (wt, lanes 1, 2) transformed with the vector plasmid (YEplac112) and RKY1734 (*rsp5-1*, lanes 3, 4) transformed with pRK813 (*RSP5*).

Strikingly, this accumulation of ubiquitin-conjugates after shift to 37°C was not observed in the *rsp5-1* mutant (Fig. 7A). The same experiment was then performed with strains overexpressing ubiquitin from the YEp96 plasmid (Fig. 7A). Accumulation of ubiquitin-conjugates was partially restored in the *rsp5-1* mutant with this plasmid. The wildtype pattern could be completely recovered by transformation of the *rsp5-1* strain with a plasmid carrying the wildtype *RSP5* gene (Fig. 7B). These experiments suggest that the lack of accumulation of high-molecular weight ubiquitin-conjugates in the *rsp5-1* mutant upon heat-shock could be the result of ubiquitin deficiency.

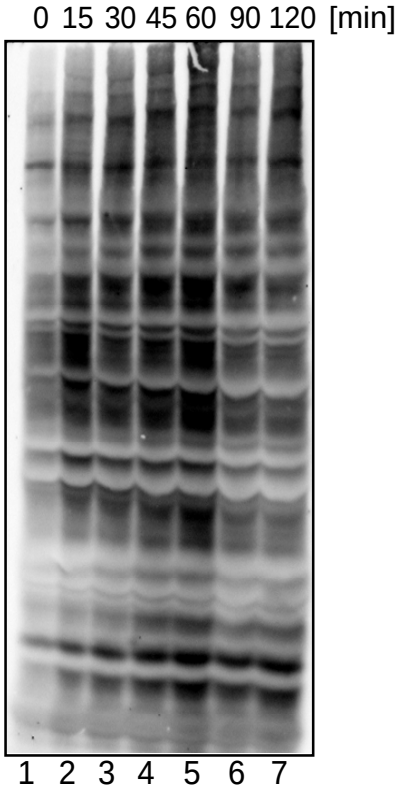


Fig. 8: Time course of ubiquitin-conjugate accumulation upon heat shock. Cell extracts were prepared from cultures of JD52 (wt) grown in YEPD medium at 25°C to exponential phase and shifted to 37°C for different time periods: (1) 0 min, (2) 15 min, (3) 30 min, (4) 45 min, (5) 60 min, (6) 90 min and (7) 120 min. Cell extracts were examined by Western blotting with anti-ubiquitin antibodies.

3.2.2 Subcellular distribution of ubiquitinated proteins

3.2.2.1 Ubiquitinated proteins can be precipitated by centrifugation

We were interested to know whether the ubiquitin-conjugates correspond to membrane proteins or to soluble proteins. Membranes can be separeated from soluble proteins by centrifugation. Nuclei are sedimented at low speed, smaller organelles like mitochondria, endosomes, vacuoles are sedimented at somewhat higher speed. Thus, proteins associated with membranes can be sedimented by centrifugation. In addition, larger protein complexes are also pelletable. Cytoplasmic proteins remain soluble and can be detected in the supernatant fraction. Cell extracts were prepared from wildtype and *rsp5-1* cells grown at 25°C or

shifted to 37°C for 1 h before extract preparation. After centrifugation at 100,000 g for 1 h, the presence of ubiquitin-conjugates was determined in the pellet (P100) and corresponding supernatant (S100) fractions in the wildtype and the *rsp5-1* mutant at 25°C and after 1 h shift to 37°C. Proteins were separated on SDS-PAGE gels and ubiquitin-conjugates were detected with anti-ubiquitin antibodies by Western blotting. As can be seen in Fig. 9A, most of the ubiquitinated proteins of the wildtype were found in the pellet fractions. The same distribution of pelletable to soluble ubiquitinated proteins was also observed with the *rsp5-1* mutant. So, the ubiquitin-conjugates observed could be membrane associated or part of a larger protein complex. In contrast, free ubiquitin was found in the soluble fractions in wildtype and also in the *rsp5-1* mutant (Fig. 9B). Plasma membrane ATPase (Pma1) was used as a marker for membrane associated proteins. Pma1 was only found in the pellet fractions (Fig. 9C).

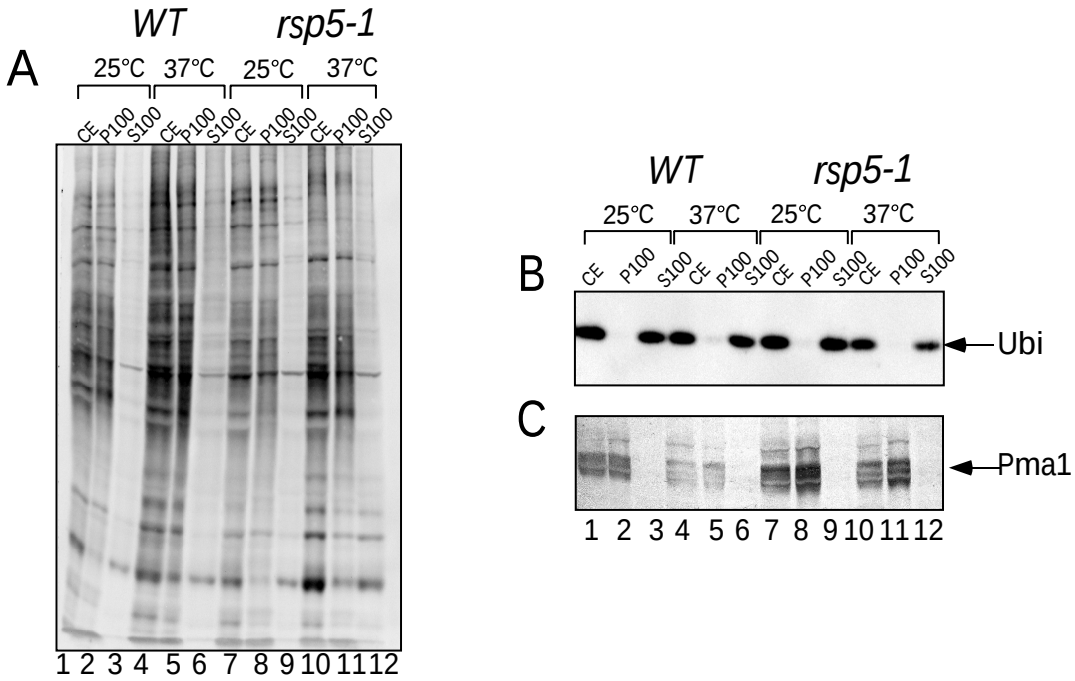


Fig. 9: Subcellular distribution of ubiquitin-conjugates. Cultures of JD52 (WT) and RKY1734 (*rsp5-1*) were grown at 25°C to exponential phase. Cell extracts (CE, lanes1, 4, 7, 10) prepared from cells grown at 25°C (lanes 1-3, 7-9) and from cells shifted to 37°C for 1 h (lanes 4-6, 10-12) before extract preparation were centrifuged at 100,000 g. Pellet (P100, lanes 2, 5, 8, 11) and supernatant fractions (S100, lanes 3, 6, 9, 12) were separated on SDS-PAGE (A, C) or Tricine gels (B) and examined by Western blotting with anti-ubiquitin (A, B) or with anti-Pma1 (C) antibodies. Free ubiquitin (Ubi) and plasma membrane ATPase (Pma1) are marked with arrows.

3.2.2.2 Ubiquitinated proteins are membrane associated

To be able to distinguish between membrane association of ubiquitinated proteins and protein aggregation, flotation experiments were performed. Since the distribution of ubiquitin conjugates was similar in both strains tested (wildtype and *rsp5-1*) flotation experiments were performed only with a wildtype strain. Cell components can be separated according to their buoyant density. Cell extracts were mixed with an opti-prep solution of high density and then overlayed with an opti-prep solution of lower density. During centrifugation, membranes together with their associated proteins float to the top of the gradient, due to their low density. Protein complexes and soluble proteins stay in the lower fractions of higher density. After centrifugation six equal fractions were collected from the top to the bottom of the gradient. The presence of ubiquitin conjugates was detected by Western blotting with anti-ubiquitin antibodies. Ubiquitin conjugates were detected mostly in the first two fractions of the gradient (Fig. 10A, B), corresponding to membrane associated proteins. To see if we are really looking at membranes, cell extracts were treated with Triton X-100 before centrifugation. Triton X-100 is a detergent used for solubilizing membranes. After Triton X-100 extraction, ubiquitinated proteins were shifted to the lower fractions of the gradient indicating that they indeed correspond to membrane associated proteins. As controls for membrane associated proteins, Pma1 (plasma membrane ATP ase) and Emp47 (cis-Golgi marker protein) were detected in the samples by Western blotting (Fig. 10C-F).

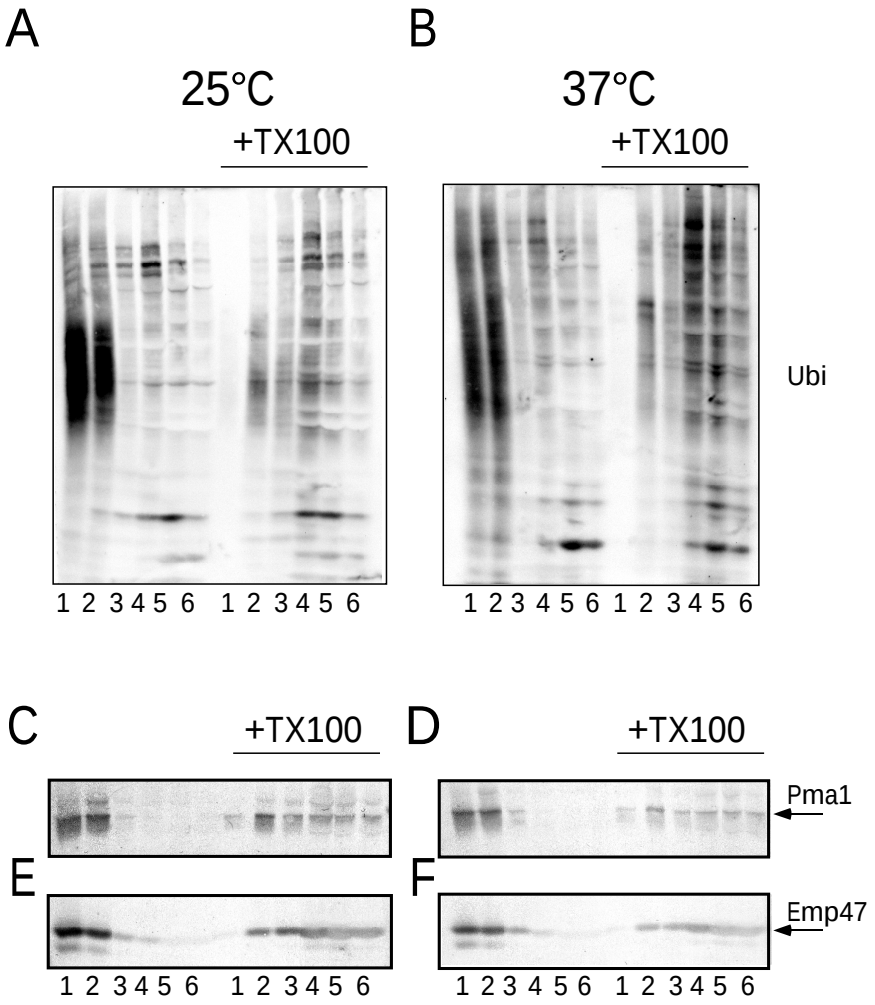
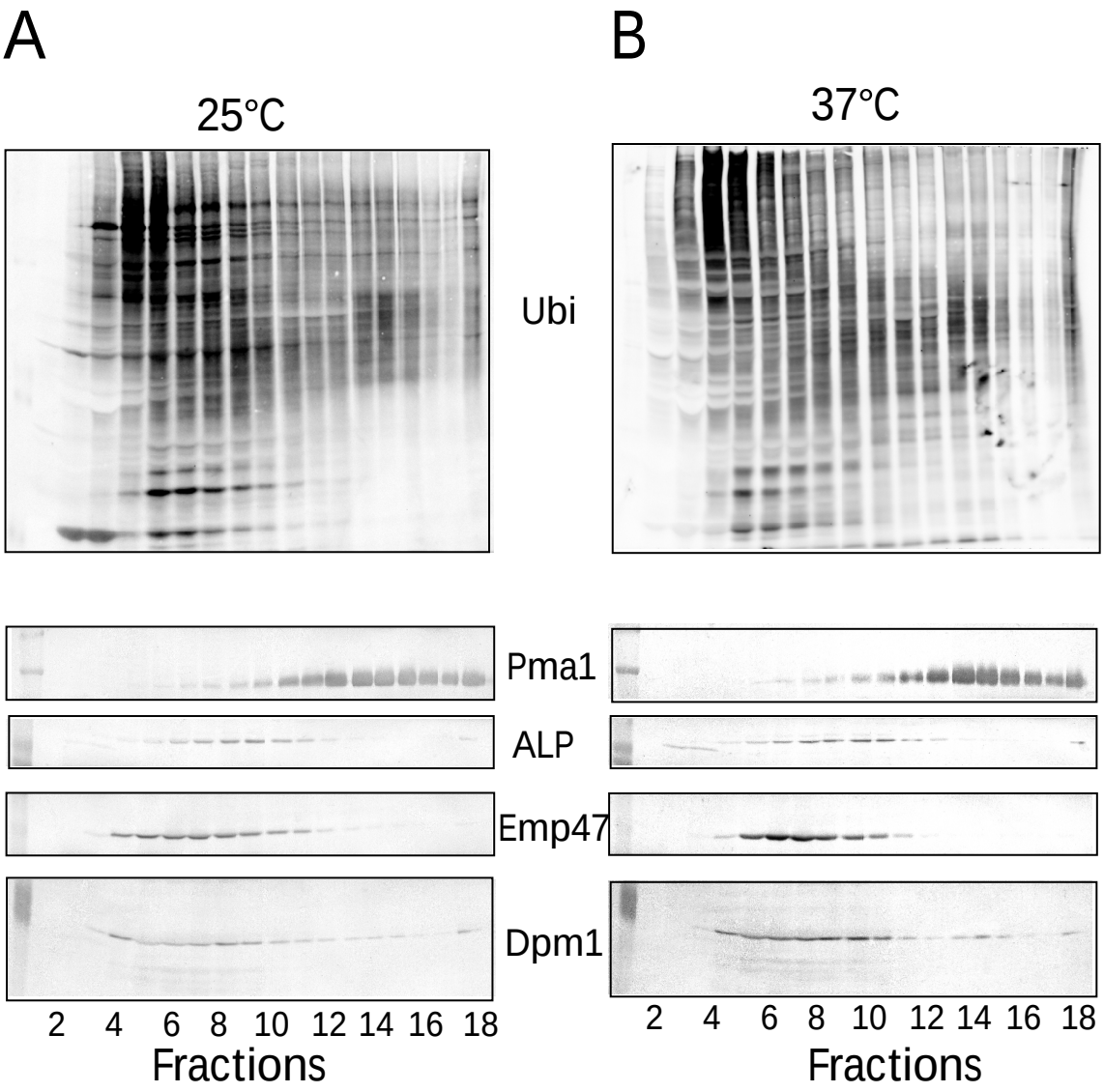


Fig. 10: Membrane association of ubiquitinated proteins. Cell extracts prepared from JD52 (wt) grown at 25°C (A, C, E) and shifted to 37°C for 1 h prior to extract preparation (B, D, F) were separated on an Optiprep™ gradient. Six fractions (lanes 1-6) collected from the top to the bottom of the gradient were examined by Western blotting with anti-ubiquitin antibodies (A, B), anti-Pma1 (C, D) and anti-Emp47 antibodies (E, F). Lanes 1, 2 are membrane associated proteins. Left: without Triton X-100, right: + 1% Triton X-100.

The ubiquitin-conjugates observed were mostly membrane associated. What membranes, or what organelles are involved? To investigate this issue more closely, sucrose density gradient experiments were performed. Cell extracts were loaded on top of a sucrose density gradient (20% at the top, 53% at the bottom of the gradient). During centrifugation, cell compartments are separated according to their size and density. With the help of marker proteins, it is possible to distinguish between different cellular compartments. Eighteen equal fractions were collected after centrifugation and examined for the presence of marker proteins by Western

blotting. A quantification of the signal intensities is depicted in Fig. 11C, D. As can be seen in Fig. 11, most of the ubiquitin-conjugates were detected in fractions 4 and 5 of the gradient. Ubiquitin-conjugates showed no co-localization with the plasma membrane marker protein Pma1 nor with the vacuolar marker protein Alp. However, some co-localization was observed between the distribution of ubiquitin-conjugates and Dpm1 (ER) and Emp47 (*cis*-Golgi). This could indicate that at least a certain fraction of ubiquitinated proteins is localized to the ER/cis-Golgi. This may be expected bearing in mind that, the ER is the place where misfolded proteins are recognized and degraded by the ERAD system.



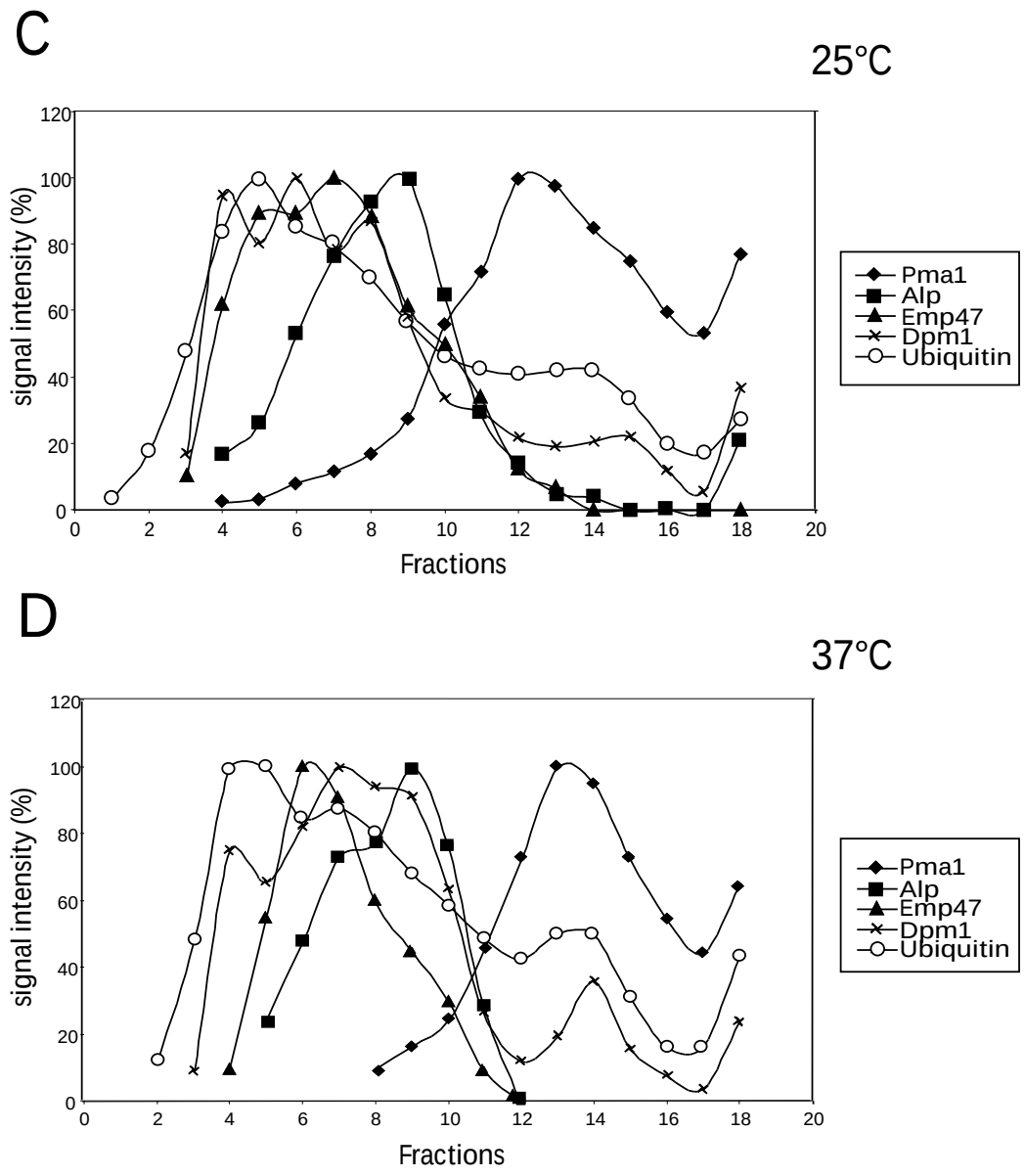


Fig. 11: Distribution of ubiquitin-conjugates in sucrose-density gradients. JD 52 (wt) cell extracts grown at 25°C (A) and shifted to 37°C for 1 h (B). The gradient fractions were analyzed by Western blotting with antibodies against (from top to bottom): ubiquitin (Ubi), plasma membrane ATP-ase (Pma1), Alkaline phosphatase (ALP, vacuole), endosomal membrane protein (Emp47, cis-Golgi), Dolicholphosphate transferase (Dpm1, ER). (C, D) Quantification of Westen-blot signals. Obtained signals were scanned, and quantified with the programe ImageJ. Strongest signal intensities were set to 100 %, ubiquitin (circles), Pma1 (diamonds), ALP (squares), Emp47 (triangles) and Dpm1 (crosses). (C) signals from experiment (A); (D) signals from experiment (B)

3.2.3 Free ubiquitin levels are reduced in the *rsp5-1* mutant upon heat-shock

The effects observed with the *rsp5-1* mutant upon shift to non-permissive temperature could be the result of ubiquitin shortage. To test this, the level of free ubiquitin was examined in the wildtype and *rsp5-1* strains. Cell extracts were separated on Tricine gels and the level of free ubiquitin was determined by Western blotting with anti-ubiquitin antibodies. At permissive temperature (25°C), free ubiquitin levels in *rsp5-1* were comparable to wildtype. On the other hand, in cells shifted to non-permissive temperature (37°C) before extract preparation, the ubiquitin level was reduced to about half the wildtype level (Fig. 12). The *doa4Δ* mutant was included for comparison. It had been reported previously that mutants defective in the deubiquitinating enzyme Doa4 show reduced levels of free ubiquitin. It is assumed that ubiquitin is degraded along with its substrate proteins in this mutant. Free ubiquitin levels in the *rsp5-1* and *doa4Δ* mutants after 1 h shift to 37°C were comparable and lower than in wildtype. As a loading control, the blots were probed against the glycolytic enzyme phosphoglycerat kinase (PGK). The intensity of the PGK bands was comparable in all lanes (Fig. 12).

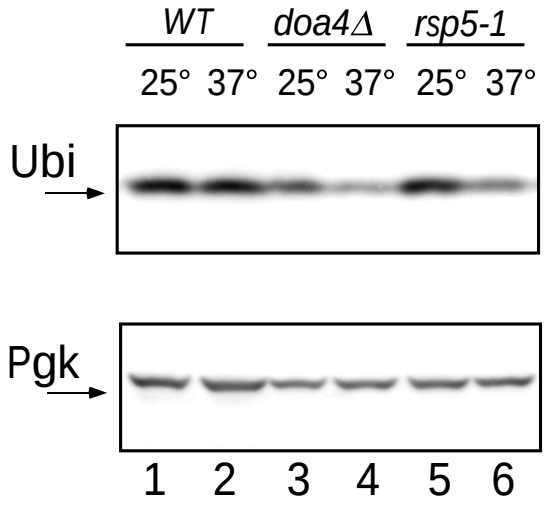


Fig 12: Free ubiquitin levels are reduced in the *rsp5-1* mutant. Yeast strains: JD52 (wt, lanes 1, 2), JD116 (*doa4Δ*, lanes 3, 4) and RKY 1734 (*rsp5-1*, lanes 5, 6) were grown in YEPD medium to exponential phase at 25°C. Cells were then shifted to 37°C for 1 h. Cell extracts prepared from cells grown at 25°C (lanes1, 3, 5) or from cells shifted to 37°C (lanes 2, 4, 6) for 1 h prior to cell extract preparation were separated on a Tricine gel and analyzed by Western blotting with anti-ubiquitin antibodies (upper panel) or with anti-PGK antibodies (lower panel).

3.2.4 The temperature-sensitive (ts) phenotype of the *rsp5-1* mutation is complemented by ubiquitin overproduction

The lack of accumulation of high-molecular weight ubiquitin-conjugates observed in the *rsp5-1* mutant upon heat-shock seems to be the result of a ubiquitin deficiency. This ubiquitin deficiency could also be linked to the temperature-sensitivity of the *rsp5-1* mutant. Therefore, it was tested if overproduction of ubiquitin from the YEp96 plasmid is able to rescue the temperature-sensitivity of the *rsp5-1* mutant. Indeed, *rsp5-1* mutant cells overexpressing ubiquitin were able to grow at restrictive temperature (Fig. 13), although at somewhat slower rate.

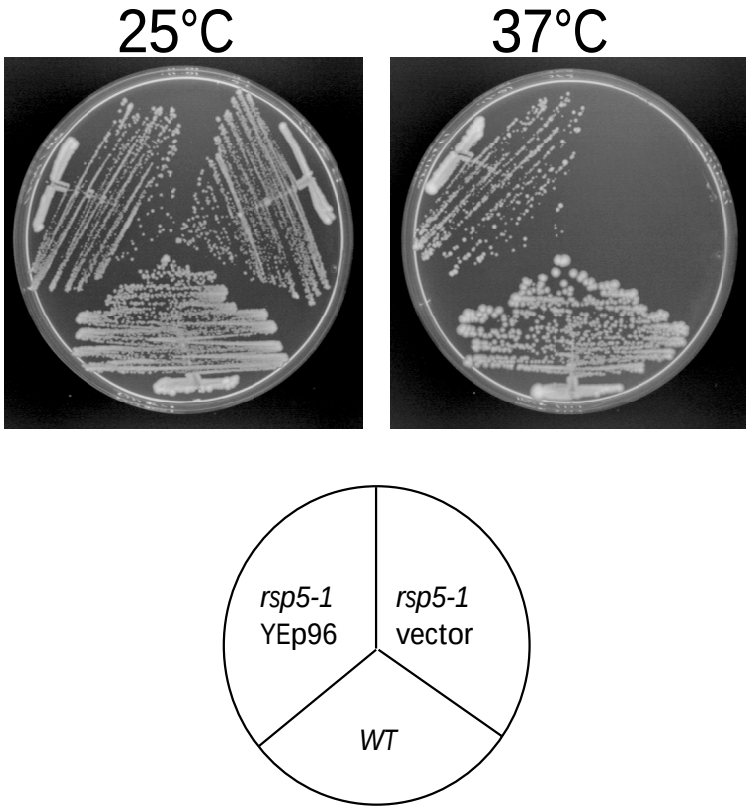


Fig. 13: Suppresion of the *rsp5-1* ts phenotype by ubiquitin overexpression. JD52 (wildtype) and RKY1734 (*rsp5-1*) transformed with YEplac112 (vector) or ubiquitin overexpression plasmid (YEp96) were incubated for 3 d at 25°C (left panel) or 37°C (right panel). Expression of ubiquitin from the *CUP1* promoter was induced by 0,5 mM $CuSO_4$.

This result is consistent with a previous report showing suppression of another *RSP5* mutation (*mdp1-1*) by overexpression of ubiquitin (Zoladek *et al*., 1997). The next question was if the complete deletion of the *RSP5* gene can also be complemented by overproduction of ubiquitin. Tetrad analysis was used to investigate this issue, since *RSP5* is an essential gene and cells cannot survive without it. A diploid yeast strain heterozygous for the *RSP5* deletion was transformed with the plasmid YEp96. After sporulation, tetrads were dissected and tested for growth on rich medium. A 2:2 segregation (viable to non-viable spores) was observed (Fig. 14).

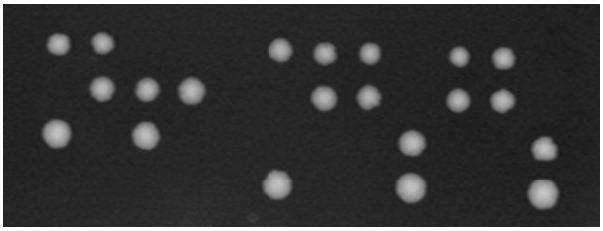


Fig. 14: Deletion of *RSP5* cannot be suppressed by overproduction of ubiquitin. Complementation of *rsp5Δ* by overproduction of ubiquitin was examined by tetrad analysis. RKY1924 (*RSP5/rsp5Δ*) was transformed with YEp96 (2µ-*CUP1p-UBI*). After sporulation, tetrads were disected and tested for growth on YEPD. To induce expression of the *CUP1* promoter 0,5 mM $CuSO_4$ was added to the growth medium.

Analysis of the genetic markers showed that all the viable spores carried the wildtype *RSP5* allele. No viable *rsp5Δ* spores were recovered. About half of the *rsp5* spores should have carried the ubiquitin plasmid and should therefore be able to grow, if ubiquitin overproduction could suppress the loss of the Rsp5 protein. Since no such spores were detected, it seems that ubiquitin overproduction cannot compensate for the loss of *RSP5*. Thus, apparently the protein encoded by the *rsp5-1* allele retains some residual function.

3.2.5 Not all of the *rsp5-1* phenotypes can be complemented by ubiquitin overproduction

We tested if other phenotypes of the *rps5-1* mutant can also be compensated by addition of extra ubiquitin. It would be interesting to see if proper Ste6 trafficking

can be restored after ubiquitin overproduction in the *rsp5-1* mutant upon heat shock. Therefore, we examined Ste6 turnover in the wildtype and the *rsp5-1* mutant upon overproduction of ubiquitin from the YEp96 plasmid under heat-shock conditions (Fig. 15). Ste6 turnover was determined by pulse chase experiments. As can be seen in figure 15, ubiquitin overproduction had no effect on Ste6 turnover in the wildtype strain (compare also: Fig. 5) neither at 25°C nor at 37°C. The same was true for the *rsp5-1* mutant. As reported in figure 5, Ste6 was stabilized in *rsp5-1.* Rapid turnover could not be restored by ubiquitin overproduction. Thus, this defect cannot be suppressed by extra ubiquitin.

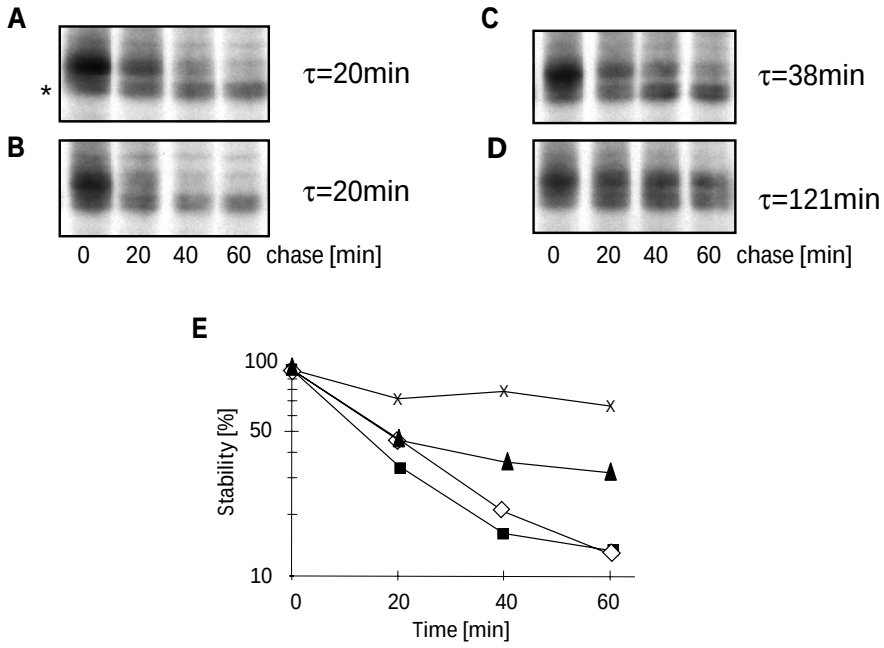


Fig. 15: The Ste6 trafficking defect in *rsp5-1* is not corrected by ubiquitin overproduction. The Ste6 half life was determined by pulse-chase experiments. JD52 (wt) (A, B) and RKY1734 (*rsp5-1*, C, D) were transformed with the ubiquitin overexpression plasmid YEp96. Cells were grown at 25°C to exponential phase and then labeled with [^{35}S]-Trans-label (ICN) for 5 min at 25°C and additional 10 min at 37°C (B, D) or 25°C (A, C). Ste6 was immunoprecipitated from cell extracts prepared after different chase periods (as indicated), and examined by autoradiography. A background band is marked with an asterisk. (A) JD52 (25°C), (B) JD52 (37°C), (C) RKY1734 (25°C), (D) RKY1734 (37°C), (E) signal intensities of experiment A (diamond), B (square), C (triangle), D (cross) plotted against chase time.

3.2.6 Altered ubiquitin homeostasis in the *rsp5-1* mutant

3.2.6.1 Is ubiquitin turnover enhanced in the *rsp5-1* mutant?

Our experiments have shown that ubiquitin is limiting in the *rsp5-1* mutant. There are two possible explanations for this finding. Either there is not enough ubiquitin produced in the cells or the turnover of ubiquitin is higher than in wildtype.
It is known that in the *doa4Δ* mutant enhanced ubiquitin turnover is responsible for the lowered ubiquitin level seen in this mutant (Swaminathan, *et al*., 1999). We were interested to know if this was also the reason for the ubiquitin deficiency observed in the *rsp5-1* mutant strain. We performed a cycloheximide chase in order to test this possibility. The cells were pre-shifted to 37°C (or further incubated at 25°C) for half an hour and then cycloheximide was added in order to block further protein synthesis. The fate of the ubiquitin already existing in the cells at the time of cycloheximide addition was followed over time. At 25°C, no significant degradation of ubiquitin was detected in any of the strains examined during the time course of the experiment (not shown). So, it appears that ubiquitin is stable in cells grown at 25°C, even in the *rsp5-1* strain. The total amount of protein did not increase during the experiment indicating that cycloheximide was effective in blocking protein synthesis (not shown). However, after shifting the cells to 37°C, faster turnover of ubiquitin was detected in the *rsp5-1* cells (Fig. 16). While ubiquitin was still stable in the wildtype, it was degraded with a half-life of about 90 min in the *rsp5-1* mutant strain. To identify the proteolytic system involved, double mutants of *rsp5-1* and proteolysis mutants were constructed. To block vacuolar proteolysis, *pep4Δ* was used and *ump1Δ* was used for impairing proteasomal degradation (Ammerer *et al*., 1986; Ramos *et al*., 1998). In both double mutants (*rsp5-1 pep4Δ, rsp5-1 ump1Δ*) ubiquitin was stable at 37°C, just like in wild type. This implies that both proteolytic systems (the vacuole and the proteasome) seem to be involved in enhanced degradation of ubiquitin in the *rsp5-1* mutant, either directly or indirectly.

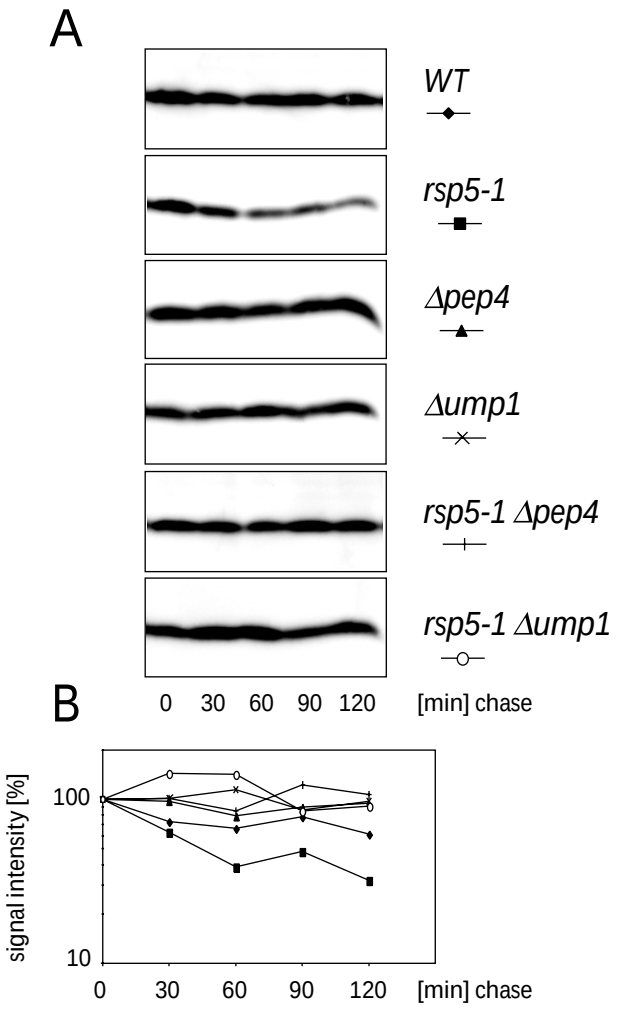


Fig. 16: Enhanced ubiquitin turnover in the *rsp5-1* strain. (A) Cells were pregrown to exponential phase at 25°C and then shifted to 37°C for 30 min. After 30 min at 37°C, cycloheximide (50 µg/ml) was added to the cultures (t_0) to block further protein synthesis. At different time periods (as indicated) after addition of cyclo-heximide ("chase"), cell extracts were prepared from constant culture volumes and examined by Western blotting with anti-ubiquitin antibodies. Strains used from top to bottom: JD52 (WT), RKY1734 (*rsp5-1*), RKY975 (*pep4Δ*), JD59 (*ump1Δ*), RKY2048 (*rsp5-1 pep4Δ*), RKY2049 (*rsp5-1 ump1Δ*). (B) Densitometric quantification of the band intensities in (A), symbols as indicated in (A), signal intensity=logarithmic scale.

While performing these experiments, it was published that ubiquitin is unstable, even in wildtype, which was inconsistent with our data (Hanna *et al*., 2003). We decided to perform pulse-chase experiments in order to double check our results. HA-tagged ubiquitin under *CUP1* promotor control on a 2µ plasmid (YEp112) was introduced into wildtype and *rsp5-1* mutant cells. Turnover of HA-ubiquitin was then followed upon shift to 37°C in the wildtype and in the *rsp5-1* mutant over 180 min. Again, we observed faster turnover of ubiquitin in the *rsp5-1* mutant (half life about 2 h) upon temperature shift (Fig. 17). But, in contrast to our previous experiments, ubiquitin was now also unstable in the wildtype upon shift to non-permissive temperature (37°C) with a half life of about 2 h. So, based on pulse chase experiments, there is no sifnificant difference in ubiquitin turnover in wildtype and

rsp5-1. The reason for this discrepancy between the cycloheximide chase experiments and pulse chase is unclear.

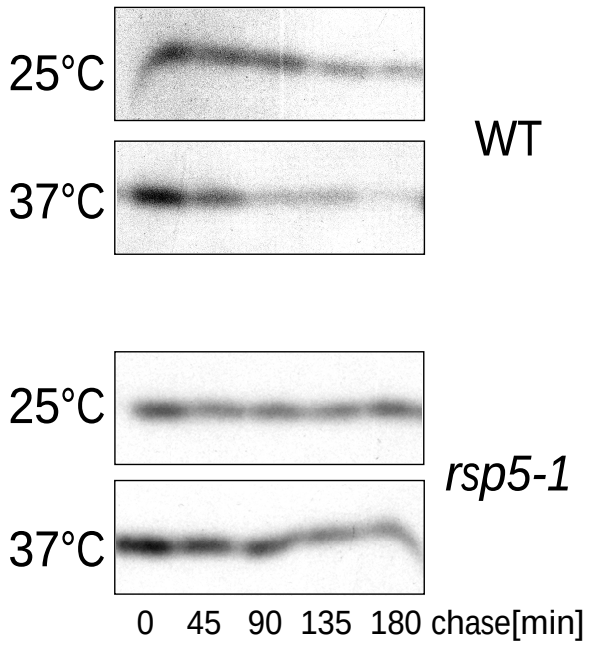


Fig. 17: Ubiquitin turnover. The half life of ubiquitin was determined by pulse chase experiment. JD52 (wt) and RKY1734 (*rsp5-1*) transformed with ubiquitin overexpression plasmid YEp112 were grown to exponential phase at 25°C. Cells were labeled with [^{35}S]-methionine for 5 min at 25°C and for another 10 min at 25°C or 37°C (as indicated). Ubiquitin was immunoprecipitated with anti-HA antibodies from cell extracts prepared after different chase periods (as indicated). Precipitated proteins were separated by Tricine gels and analyzed by autoradiography. Expression of ubiquitin from *CUP1* promoter of YEp112 plasmid was induced by addition of 50 µm $CuSO_4$ to the medium 3 h before cell labeling.

3.2.6.1.1 Vacuole preparation

The cycloheximide chase experiments suggested that ubiquitin degradation could, at least in part, take place in the vacuole. If this was true, ubiquitinated proteins should accumulate inside the vacuole when vacuolar proteolysis is blocked. We used *pep4Δ* (vacuolar protease) mutants to block degradation of proteins in the vacuole. It is published that ubiquitin is degraded faster in the *doa4Δ* mutant and that this degradation, at least in part, takes place in the vacuole (Swaminathan *et al*.,

1999). So, the *doa4Δ pep4Δ* double mutant was included as a control. Vacuoles were isolated from exponential cultures of the tested strains (*pep4Δ*; *pep4Δ doa4Δ*; *rsp5-1 pep4Δ*) grown at 25°C or shifted to 37°C for 1 h. Equal amounts of isolated vacuoles were loaded on the SDS-polyacrylamide gels (as judged from the signal intensity of the vacuolar marker protein ALP on Western blots) and the presence of ubiquitin-conjugates was detected by Western blotting with anti-ubiquitin antibodies. As seen in Fig. 18, ubiquitin conjugates could be detected in all tested strains.

Fig. 18: Detection of ubiquitin-conjugates in isolated vacuoles. Vacuoles were isolated from cells grown to exponential phase at 25°C (lanes 1, 3, 5) or after 1h shift to 37°C (lanes 2, 4, 6). Strains used from left to right: RKY975 (*pep4Δ*, lanes 1, 2), RKY1449 (*pep4Δ doa4Δ*, lanes 3, 4), RKY2048 (*pep4Δ rsp5-1*, lanes 5, 6). Equal amounts of isolated vacuoles according to the vacuole marker (ALP-alkaline phosphatase) (lower panel) were loaded onto SDS-polyacrylamide gels and examined by Western blotting with anti-ubiquitin antibodies (upper panel).

As expected, a pronounced accumulation of ubiquitinated proteins in the vacuole was observed in the *doa4Δ* mutant. In contrast, no accumulation of ubiquitinated proteins in the vacuoles could be detected in the *rsp5-1* mutant at 25°C or after 1 h shift to non-permissive temperature (37°C).

After analyzing the cell extracts for the presence of ubiquitin-conjugates, we noticed an enormous increase of conjugates in the *doa4Δ* strain compared to wildtype (data not shown). It could therefore be that the observed accumulation of ubiquitinated species in the *doa4Δ* vacuole preparation is the result of cytoplasmic contamination. To investigate this further, isolated vacuoles from wildtype and *doa4Δ* strains were treated with proteases. If ubiquitinated proteins accumulate inside the vacuole, they should be protected from degradation by external protease. After treatment of the vacuole preparations with the detergent Triton X-100, which solubilizes the vacuolar membranes, the conjugates should become susceptible for degradation by external protease. The vacuolar hydrolase CPY (carboxypeptidase Y) was included as a control. CPY is synthesized as an inactive precursor, prepro-CPY. It is translocated into the lumen of the ER where its signal sequence is removed by signal peptidase. During maturation, CPY undergoes several modifications which can be detected as p1CPY, p2CPY and mCPY forms, which differ in size and are therefore easily distinguished. Production of the mCPY form is depended on the vacuolar hydrolase Pep4. Since the vacuoles were isolated from *pep4Δ* strains, the main form detected was the p2 CPY form. CPY mobility was not affected by addition of external protease alone. However, after addition of Triton X-100, only the mature form of CPY could be detected on the Western blot (Fig. 19A). This indicates that the protease was active and that treatment with Triton X-100 was also effective in solubilizing the vacuolar membrane. Ubiquitin conjugates were detected in both strains tested. In samples treated with proteases no reduction in the ubiquitin signal could be observed. In contrast, in the samples treated with proteases together with Triton X-100 hardly any ubiquitinated proteins could be detected (Fig. 19B). This demonstrates that the detected ubiquitin-conjugates are indeed localized inside the vacuole and are not cytoplasmic contaminants.

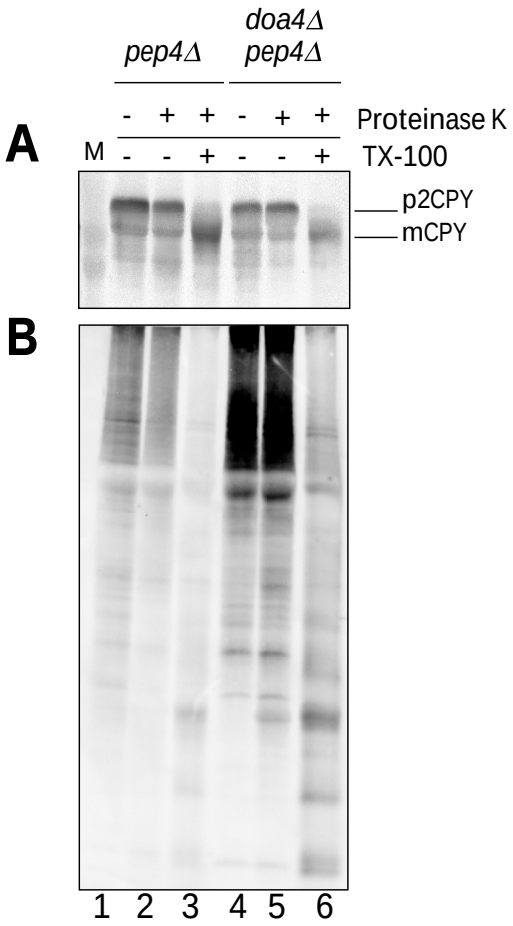


Fig. 19: Confirmation of localization of ubiquitin-conjugates in vacuoles by protease protection. Cells of the RKY975 (*pep4Δ*) (lanes 1-3) and RKY1449 (*pep4Δ doa4Δ*) (lanes 4-6) strains were grown to exponential phase at 30°C. Isolated vacuoles treated with proteinase K only (lanes 2 and 5) or with proteinase K and 1 % Triton X-100 (Tx-100) (lanes 3 and 6). The reactions were stopped and proteins were separated on SDS-polyacrylamide gel and investigated by Western blotting with anti-CPY antibodies (A) and anti-ubiquitin antibodies (B). As a control one sample each was not treated with proteinase K or Triton X-100 (lanes 1 and 4).

3.2.6.2 The heat shock response is not affected in the *rsp5-1* strain

Reduced ubiquitin synthesis could also be responsible for ubiquitin depletion in the *rsp5-1* mutant. Ubiquitin is constitutively expressed from the *UBI1*, *UBI2* and *UBI3* genes. In addition it is also expressed from the *UBI4* gene which is known to be upregulated upon stress conditions (Finley *et al*., 1987). Failure of *UBI4* to respond properly to stress conditions, like heat shock, could contribute to the observed ubiquitin deficiency in the *rsp5-1* mutant. Therefore, we examined if the *UBI4*

promoter is properly regulated upon exposure to stress conditions, like heat-shock, in the *rsp5-1* mutant. We cloned the *UBI4* promoter in front of the ß-galactosidase (*lacZ*) gene of *E.coli* on a single copy plasmid to facilitate detection of its activity. Cells were pregrown at 25°C to exponential phase and then shifted to 37°C. At different time poins after shift to 37°C, cell extracts were prepared and the LacZ activity was determined. The specific activity (U/mg protein) was somewhat lower in the *rsp5-1* mutant compared to the wildtype (Fig. 20A), but the *UBI4* promoter was nevertheless induced about 2-fold upon heat shock in both strains.

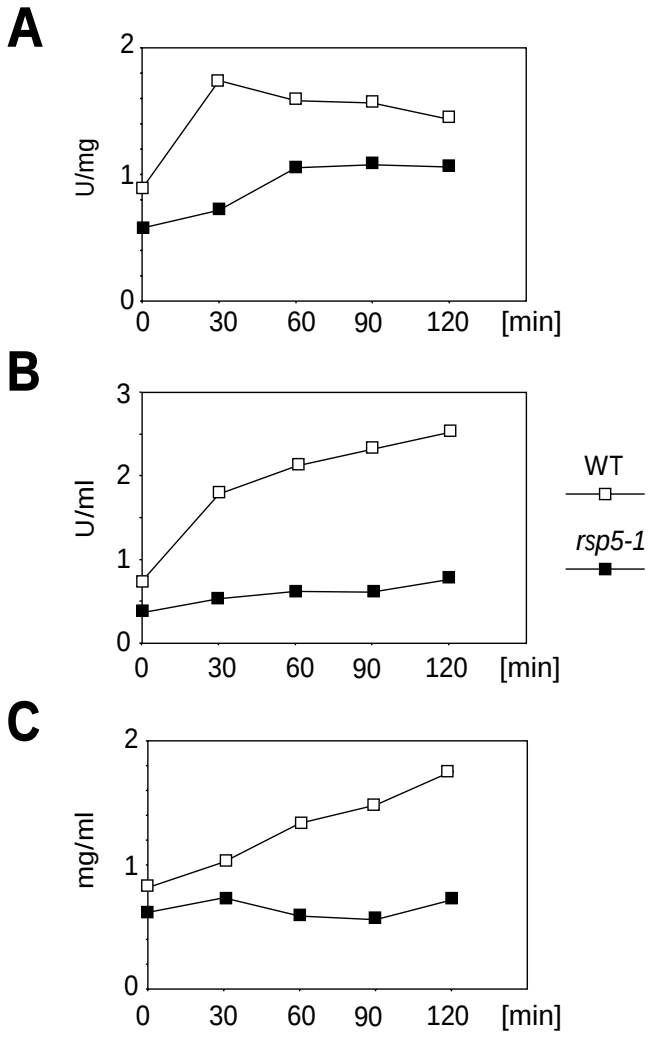


Fig. 20: Heat induction of the *UBI4* promoter. JD52 (wt, open squares) and RKY1734 (*rsp5-1*, filled squares) were transformed with the plasmid pRK969 (*UBI4p-lacZ*). Cells were pregrown at 25°C to exponential phase, and shifted to 37°C at t_0. At different time intervals after shift to 37°C, cell extracts were prepared from constant culture volumes and LacZ activity and protein concentration were determined. (A) Specific LacZ activity (U/mg), (B) volume LacZ activity (U/ml), (C) protein concentration (mg/ml).

This shows that heat-shock signaling is not affected in the *rsp5-1* strain and that the *UBI4* promoter responds normally to heat-shock conditions. Other stress inducible genes were also tested. *HSP26* contains both HSE and STRE elements like the *UBI4* gene. *SSA1* codes for Hsp70 which is heat inducible only via the Hsf protein that binds to HSE elements. For these two promoters, an increase in specific activity was observed upon heat shock, simmilar to the *UBI4* promoter (not shown).
A different picture was obtained when the volume activities (U/ml) of the *UBI4-LacZ* fusion in wildtype and *rsp5-1* mutant were compared (Fig. 20B). The LacZ activity of the *UBI4* gene increases about 4-fold in the wildtype strain during the course of the experiment. On the other hand, the activity of the *UBI4* gene in the *rsp5-1* mutant stays nearly constant. What is the reason for the discrepancy between Fig. 20A and Fig. 20B? The critical factor is the protein concentration. While the protein concentration (mg/ml extract) steadily increased in the wildtype strain, it stayed more or less constant in the *rsp5-1* strain during the observed time period (120min) (Fig. 20C). It seems that inhibition of protein synthesis could be the reason for the observed discrepancy between specific and volume activity of the *UBI4*-LacZ fusion. This was also evident from the comparison of the generation times of wildtype and *rsp5-1* mutant strains (Tab. 7).

temperature [°C]	wildtype [h]	*rsp5-1* [h]
25	3,1	3,7
30	1,8	2,1
37	2,3	8,0

Tab 7. Generation-times of wildtype and the *rsp5-1* mutant. Cells were pregrown at 25°C to early exponential phase and then shifted to 30°C or 37°C or further incubated at 25°C. The OD_{600} was measured every hour for 4-6 h and generation times were calculated from the obtained growth curves.

Generation times do not differ much between these two strains at 25°C and 30°C, but a quite huge difference is noticed at 37°C (Tab. 7). This block in protein synthesis does not seem to be a secondary effect of the *rsp5-1* mutation, since it

appears immediately after shift to 37°C. It could be that *rsp5-1* mutant cells are dying after exposure to 37°C and that this is the reason why we don't observe an increase in protein concentration. But this does not appear to be the case, since the viability of the *rsp5-1* cells was not affected during the time course of the experiment (120 min) (not shown).

3.2.6.3 Defective recovery of protein synthesis after heat-shock in the *rsp5-1* mutant

The *rsp5-1* mutant appears to be blocked in protein synthesis at 37°C. To investigate this further, the capacity to synthesize proteins at 37°C was examined by metabolic labeling. Wildtype and *rsp5-1* mutant cells were pregrown to exponential phase at 25°C in minimal medium lacking methionine. Cells were then adjusted to the same cell density, shifted to 37°C for different time periods and then labeled with [^{35}S-] methionine for 10 min. Incorporation of radioactive methionine into proteins was detected as follows: proteins were TCA-precipitated from cell extracts, collected onto glass-fiber filters and counted in a scintillation counter. An aliquot of the cell extracts before TCA precipitation was counted in the scintillation counter to determine the amount of label taken up by the cells. Incorporation of label into proteins was directly proportional to the amount of label added. This shows that the internal methionine pool is large compared to the amount of label taken up by the cells. Thus, the specific activity of intracellular methionine is directly proportional to the amount of label taken up by the cells. As a consequence, the amount of label incorporated into protein had to be normalized against the amount of label taken up by the cells. With wildtype, a drop in protein synthesis rate down to 50-70 % of the inital rate was observed, after shift to 37°C (Fig. 21). Within 40-60 min wildtype was able to recover completely from this transient protein synthesis arrest.

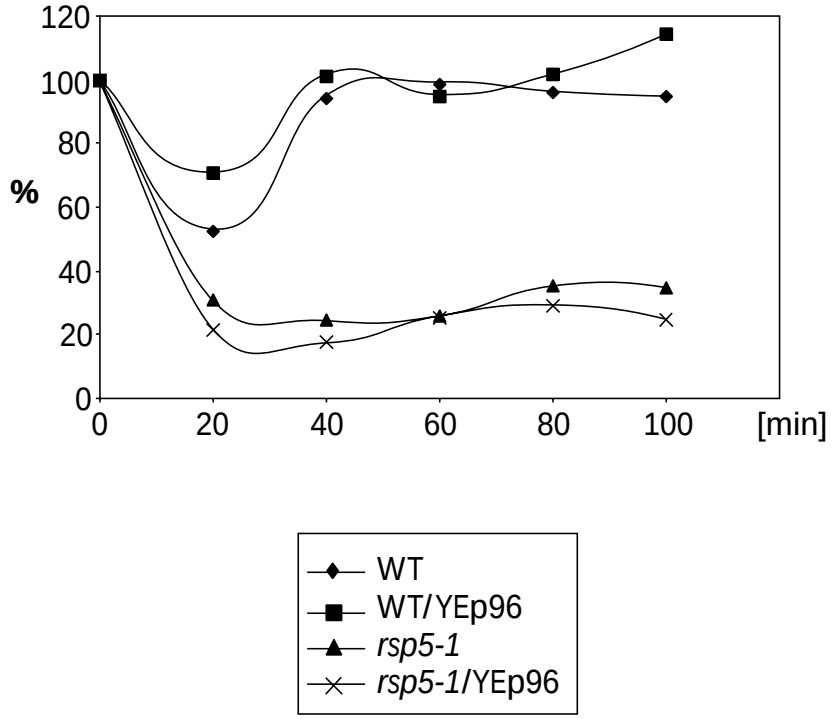


Fig. 21: Heat induced protein synthesis arrest. JD52 (wt) cells transformed with the vector YEplac112 (diamonds) or YEp96 (2µ-*UBI*) (squares) and RKY1734 (*rsp5-1*) transformed with YEplac112 (triangles) or YEp96 (crosses) were incubated in SD media at 25°C to exponential phase. Cells were then shifted to 37°C for different time periods (as indicated), and subsequently labeled for 10 min with [^{35}S]-Trans-label (ICN). The amount of label incorporated into proteins normalized to the amount of label taken up by the cells was plotted against the time of preincubation at 37°C. The protein synthesis rate at t_0 was set to 100 %.

However, another picture was obtained with the *rsp5-1* mutant. The drop in protein synthesis rate after 20 min incubation at 37°C was also detected here, but more pronounced than in wildtype (down to 10-30 % of the initial rate). What's more interesting, the cells did not recover from this protein synthesis arrest during the time course of the experiment. Overproduction of ubiquitin from YEp96 plasmid could not restore protein synthesis in the *rsp5-1* mutant cells. These data indicate that the *rsp5-1* mutant is defective in recovery from heat-induced protein synthesis arrest. These effects were observed within 20 min of shift to 37°C and represent, therefore, most likely an immediate consequence of the *rsp5-1* defect.

3.3 Control of Ste6 sorting by ubiquitination

3.3.1 The polar distribution of Ste6 ΔA-box is maintained through continuous recycling

Ubiquitination plays an important role in trafficking of the Ste6 protein (Kölling and Hollenberg, 1994). It has been shown that internalization and rapid degradation of Ste6 are mediated by a signal in the linker region, which connects the two homologous halves of the protein (Kölling and Losko, 1997). As mentioned above, wildtype Ste6 is mainly found associated with internal structures in the cell, presumably endosomes. On the other side, a Ste6 variant with a deletion in linker region (Ste6 ΔA-box) is localized at the plasma membrane. Curiously, the distribution of this variant was restricted to the plasma membrane of the newly emerging daughter cell, the bud. We were interested to find out how this polar localization is established and maintained. One possible explanation for the polar localization could be that the septin ring surrounding the bud-neck forms a diffusion barrier for the Ste6 ΔA-box protein. It is known that secretion in yeast cells is polarized, i.e. newly synthesized proteins destined for the cell surface are directed towards the growing bud. So, at first, proteins directed to the plasma membrane via the secretory pathway are asymmetrically deposited at the cell surface. However, this initial asymmetric distribution is usually quickly dissipated by lateral diffusion. So, we decided to test by immunofluorescence microscopy if the Ste6 ΔA-box distribution is affected in the septin ring mutant *cdc12-6*. Due to defective septin rings, cells express defects in cytokinesis. Although *cdc12-6* is a conditional mutant, that is, cells should not be affected at permissive temperature, they do show a mutant phenotype (elongated, distorted buds) already at permissive temperature (25°C). The mutant phenotype was even more pronounced at non-permissive temperature(37°C). However, the Ste6 ΔA-box distribution was not affected in *cdc12-6*. Ste6 was found at the bud surface even after 1 h shift to 37°C (Fig. 22). So, it seems that the septin ring does not play a role in restricting Ste6 ΔA-box to the bud cell surface. Another possibility is that Ste6 ΔA-box could be polarized kinetically by localized exocytosis and endocytic recycling, as suggested for the yeast v-SNARE protein Snc1 (Valdez-Taubas and Pelham, 2003). If this is the case for the Ste6 ΔA-box protein, it should be found inside the cell when recycling is blocked. To block recycling, the conditional mutant *ypt6-2* was used. This mutant is defective for the trans Golgi Rab protein Ypt6 (Luo and Gallwitz, 2003), which is required for the

docking of endosome derived vesicles to Golgi membranes (Siniossoglou and Pelham, 2001). So, the recycling pathway from the plasma membrane through endosomes via the Golgi back to the cell surface is interrupted in this mutant. Since *ypt6-2* is a conditional mutant, immediate consequences of Ypt6 protein inactivation can be observed by shifting the cells from permissive temperature (25°C) to non-permissive temperature (37°C). At 25°C, Ste6 ΔA-box retained its polar localization in the *ypt6-2* mutant, just like in wildtype cells (Fig. 22). However, after just 10 min exposure to non-permissive temperature (37°C), Ste6 ΔA-box was completely found in internal patch-like structures. This immediate redistribution of Ste6 ΔA-box protein from the plasma membrane to internal cell structures upon Ypt6 inactivation indicates that the polar distribution of Ste6 ΔA-box is achieved through a dynamic process, i. e. through continuous recycling.

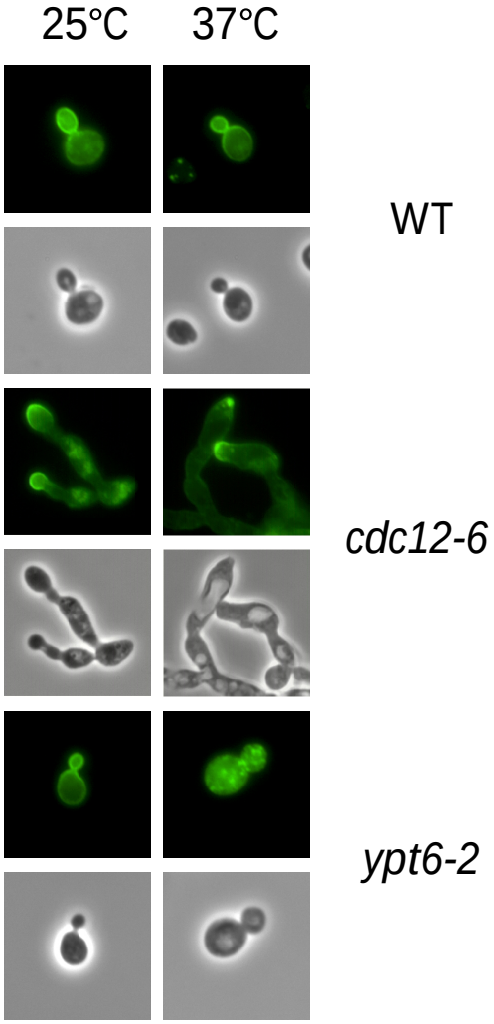


Fig. 22: Polar cell surface localization of the Ste6 ΔA-box variant. From top to bottom: JD52 (wt), RKY1718 (*cdc12-6*) and RKY2057 (*ypt6-2*) transformed with plasmids coding for a c-myc tagged Ste6 ΔA-box variant (pRK873 or pRK264). Intracellular distribution of the Ste6 ΔA-box variant was detected in cells grown at 25°C (left column) or in cells shifted to 37°C for 10 min (JD52 and RKY2057) or 60 min (RKY1718) by immunofluorescence microscopy. Anti-myc (9E10) antibodies as primary and FITC-conjugated anti-mouse as secondary antibodies were used. Alternating from top to bottom: FITC fluorescence and phase contrast images.

3.3.2 Reduced Ste6 ubiquitination leads to enhanced recycling

3.3.2.1 Ste6 ubiquitination is affected by R^{11} mutations

Ste6 ubiquitination represents a sorting signal for directing Ste6 into the vacuolar degradation pathway (Kölling and Hollenberg, 1994). The Ste6 ΔA-box variant, however, is no longer ubiquitinated (Kölling and Losko, 1997). Is this lack of ubiquitination of the Ste6 ΔA-box variant responsible for the observed enhanced recycling? Ste6 ΔA-box bears a mutation in the linker region which connects the two homologous halves of the Ste6 protein. This 100 amino acid long linker region (amino acids 607-716) can be divided into two halves according to the composition of charged amino acids. The "upstream" half (amino acids 607-661) contains predominantly acidic amino acids, and is therefore named A-box and the "downstream" half (amino acids 662-716) contains predominantly basic amino acids (B-box) (Fig. 23).

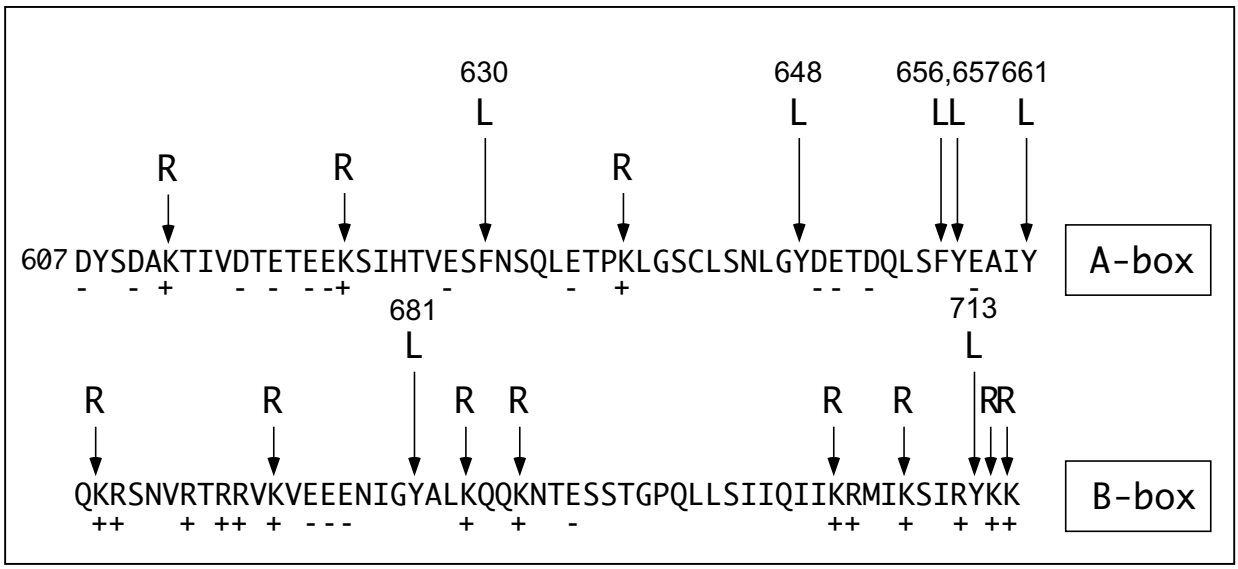


Fig. 23: Mutagenesis of the Ste6 linker-region. Based on the distribution of charged amino acids (marked with +/-) the Ste 6 linker region can be divided into A-box (predominantly acidic amino acids) and B-box (mostly basic amino acids). Changes introduced by site-directed mutagenesis are indicated with arrows: short arrows-lysin to arginine mutations, long arrows-phenylalanine/tyrosine to leucine mutations.

Since the complete A-box was deleted in the Ste6 ΔA-box variant, it could be possible that other sorting signals, in addition to the signal responsible for ubiquitination of Ste6, were removed. To rule out this possibility, Ste6 ubiquitination was selectively removed by mutating potential ubiquitin target sites in the linker region. Ubiquitin is attached to lysine residues in the substrate protein via an isopeptide bond. It seems that the ubiquitin machinery is able to use not only lysine residues of the original acceptor site, but also other lysine residues in the vicinity when the original acceptor site is no longer available (Kornitzer *et al*., 1994). Therefore, all eleven lysine residues in the linker region were mutated to arginine by site directed mutagenesis to produce the Ste6 R^{11} mutant. To test for ubiquitination of the Ste6 R^{11} variant, it was co-expressed with HA-tagged ubiquitin. Ste6 was then immunoprecipitated from cell extracts with anti-Ste6 antibodies and analysed by Western blotting with anti-HA antibodies for the presence of HA-tagged ubiquitin covalently attached to immunoprecipitated Ste6. Ubiquitin was detected as a high molecular weight "smear" on the anti-HA Western blot with the wildtype Ste6 protein (Fig. 24).

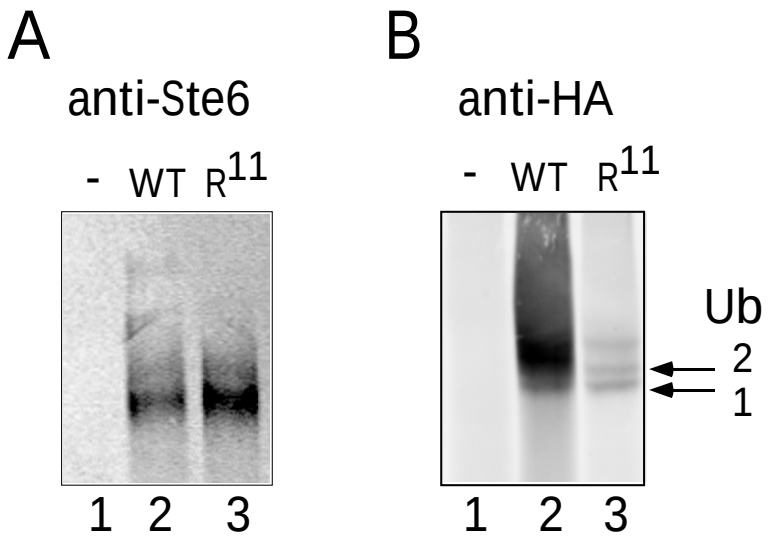


Fig. 24: Ubiquitination of Ste6 is affected by R^{11} mutations. RKY959 (*ste6Δ*) was transformed with plasmid YEp112 carrying HA-tagged ubiquitin and with (1) the vector plasmid (YEplac195), (2) pRK69 (2µ-*STE6*) or (3) pRK814 (2µ-*STE6* R^{11}). Cell extracts were prepared and Ste6 was immunoprecipitated with anti-Ste6 antibodies and analyzed by Western blotting with anti-Ste6 antibodies (A) and anti-HA antibodies(B). Mono- and di-ubiquitinated forms of Ste6 f are marked by arrows. $CuSO_4$ was added in the medium 3 h before extract preparation to induce expression of HA-ubiquitin from the *CUP1* promoter.

Also, additionally, distinct bands were visible corresponding to mono- and di-ubiquitinated Ste6, as judged from their calculated molecular weights. In contrast to wildtype, the ubiquitin signal was much weaker for the Ste6 R^{11} mutant (only 10 % of wildtype, normalized to the Ste6 signal). Moreover, the ubiquitination pattern was somewhat different in the mutant. It appeared to be shifted from higher molecular weight species to faster migrating (i. e. less ubiquitinated) species. So, although ubiquitination of the Ste6 protein could not completely be abolished, it was drastically reduced by the Ste6 R^{11} mutations.

3.3.2.2 The Ste6 protein is stabilized by R^{11} mutations

Ubiquitination serves as a sorting signal for directing Ste6 into the vacuolar degradative pathway. Hence, it is possible that the defect in ubiquitination observed with the Ste6 R^{11} protein could influence Ste6 trafficking and obstruct its delivery to the vacuole. If this is the case, Ste6 should be stabilized in this mutant. Therefore, the half life of the Ste6 R^{11} variant was determined by pulse chase experiments. As already mentioned above, Ste6 is a short lived protein with a half life of 14 min (Fig. 25). In contrast, the Ste6 R^{11} variant was about four times more stable than wildtype Ste6 (half life 56 min). Thus, reduced ubiquitination indeed leads to stabilization of Ste6.

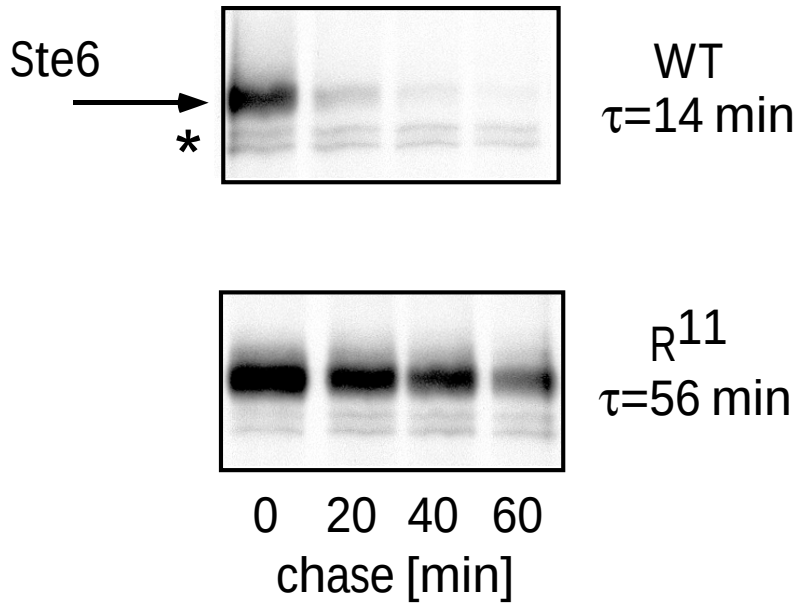


Fig. 25: Ste6 R^{11} is more stable than wildtype Ste6. Half life of Ste6 was determined by a pulse-chase experiment. RKY959 (*ste6Δ*) strain was transformed with pRK278 expressing wildtype Ste6 (upper panel) or pRK658 carrying the Ste6 R^{11} variant (lower panel). Cells were labeled with Tran-[^{35}S] label for 15 min. Ste6 was immunoprecipitated from cell extracts prepared after different chase periods (as indicated). The Ste6 band is indicated by an arrow; background bands are marked with an asterisk.

3.3.2.3 Localization of the Ste6 R^{11} variant

Since the Ste6 R^{11} mutant shows similar phenotypes as the Ste6 ΔA-box variant (reduced ubiquitination, stabilization), it was interesting to test, if its distribution also resembles that of the Ste6 ΔA-box protein. So, the localization of Ste6 R^{11} was determined by immunofluorescence microscopy. indeed, the localization resembled the distribution of the Ste6 ΔA-box variant: Ste6 R^{11} showed a polar localization like the Ste6 ΔA-box variant; it was observed mostly at the cell surface of the bud (Fig. 27). To test if the polar distribution of Ste6 R^{11} is a result of enhanced recycling, again, the conditional, temperature-sensitive *ypt6-2* mutant was used. As already mentioned, the recycling pathway from the plasma membrane via endosomes and Golgi is blocked in this mutant. The Ste6 R^{11} mutant was observed at the cell surface of the bud at permissive temperature (25°C), like in wildtype (Fig. 26). And again, like Ste6 ΔA-box, it was found associated with internal

structures upon a 10 min shift to non-permissive temperature (37°C), indicating that recycling is responsible for the polar distribution of the Ste6 R^{11} variant.

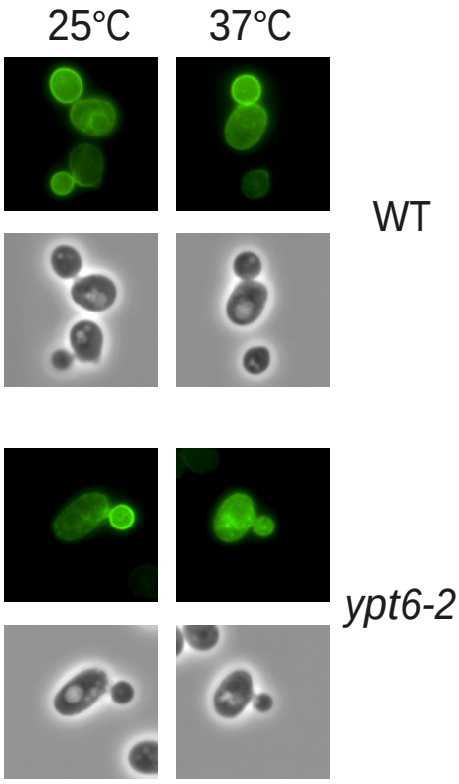


Fig. 26: Localization of the Ste6 R^{11} variant. The distribution of Ste6 R^{11} variant expressed from pRK907 was examined in JD52 (wt) and RKY2057 (*ypt6-2*) grown to exponential phase at 25°C (left column) and after 10 min shift to 37°C (right column) by immunofluorescence microscopy with anti-myc (9E10) primary antibodies and anti-mouse FITC-conjugated secondary antibodies. From top to bottom alternating: FITC fluorescence and phase contrast images.

Based on the similar phenotypes of Ste6 ΔA-box and Ste6 R^{11} variants, it appears that enhanced recycling is indeed the result of reduced ubiquitination and not the result of deletion of some other sorting signal in the linker region. Even the small differences observed between these two variants point in this direction. Namely, the distribution of the Ste6 R^{11} variant was slightly different from the distribution of the Ste6 ΔA-box. In contrast to the Ste6 ΔA-box protein, where no internal staining was detected, the Ste6 R^{11} variant showed additional internal staining of the vacuolar membrane. Hence, the residual ubiquitination determined for the Ste6 R^{11}

variant apparently enables a fraction of the protein to escape the recycling pathway and to procede further down the endocytic pathway.

3.3.3 Ste6 recycles from early endosomes

Ste6 ΔA-box and the Ste6 R^{11} variant both recycle via the Golgi. Membrane proteins endocytosed from the plasma membrane can travel back to the Golgi from early or late endosomes. To determine which pathway is used by these two Ste6 variants, localization of Ste6 ΔA-box and Ste6 R^{11} was examined by immunofluorescence microscopy in different protein sorting mutants of the endocytic pathway (Fig. 27).
To determine if the proteins travel through late endosomes on their way from the plasma membrane to the Golgi, so called class E *vps* (vacuolar protein sorting) mutants were used. In these mutants the function of the late endosomes is affected resulting in formation of a large late endosomal structure close to the vacuole, termed the "class E compartment". Hence, in class E *vps* mutants, membrane proteins that travel through late endosome are trapped in this dot-like structure near the vacuole. The *vps4Δ* mutant was used to block late endosome function. The distribution of wildtype Ste6 showed a typical class E phenotype i. e. staining of the vacuolar membrane with an associated dot-like structure. Localization of the Ste6 ΔA-box variant, however, was not affected in the *vps4Δ* mutant. It was still detected at the bud cell surface, indicating that Ste6 does not recycle through late endosomes. The Ste6 R^{11} variant in the *vps4Δ* mutant was observed at the plasma membrane of the bud, like Ste6 ΔA-box, and in addition at the vacuolar membrane (typical class E staining). This is in line with the "leaky" recycling phenotype of the Ste6 R^{11} variant already observed in the wildtype strain. The distribution of Ste6 and its two ubiquitination deficient variants (Ste6 ΔA-box and Ste6 R^{11}) was also determined in another class E *vps* mutant, *vps27Δ*. The staining was more or less identical to the one observed in the *vps4Δ* mutant. The Ste6 R^{11} variant however was different. Internal staining was hardly observed, suggesting that progression of the Ste6 R^{11} protein from early to late endosomes is affected in the *vps27Δ* mutant. This indicates that Vps27 could also act at the level of early endosomes, in addition to its function at late endosomes.
To retrieve proteins, like Vps10 and Pep12, from late endosomes to the Golgi, a multimeric protein complex, termed retromer complex is required. Therefore,

distribution of our Ste6 variants was analysed in the retromer mutant *vps35Δ*. In a *vps35Δ*, wildtype Ste6 was found in internal dot-like structures surrounding the vacuole. Like in class E *vps* mutants, polar cell surface localization of Ste6 ΔA-box and Ste6 R^{11} variant was not altered in the *vps35Δ* mutant also suggesting that late endosomes do not serve as an intermediate compartment in the recycling pathway of these variants.

In contrast to the relatively well characterised pathway from late endosomes to the Golgi, the functions required for recycling from early endosomes are poorly characterized. Snx4 (sorting nexin 4) has been implicated in recycling of Snc1 from early endosomes to the Golgi. So, we examined, if the localization of our Ste6 variants is altered in the *snx4Δ* mutant. The distribution of wildtype Ste6 and Ste6 R^{11} was not affected by the *snx4Δ* mutation. However, in contrast to its wildtype localization, where no additional internal staining was observed, Ste6 ΔA-box staining was now detected also at the vacuolar membrane, indicating that Ste6 ΔA-box recycling is partially blocked in the *snx4Δ* mutant. Considering that Snx4 is involved in recycling from early endosomes to the Golgi, this partial recycling defect serves as a hint that Ste6 recycles through early endosomes.

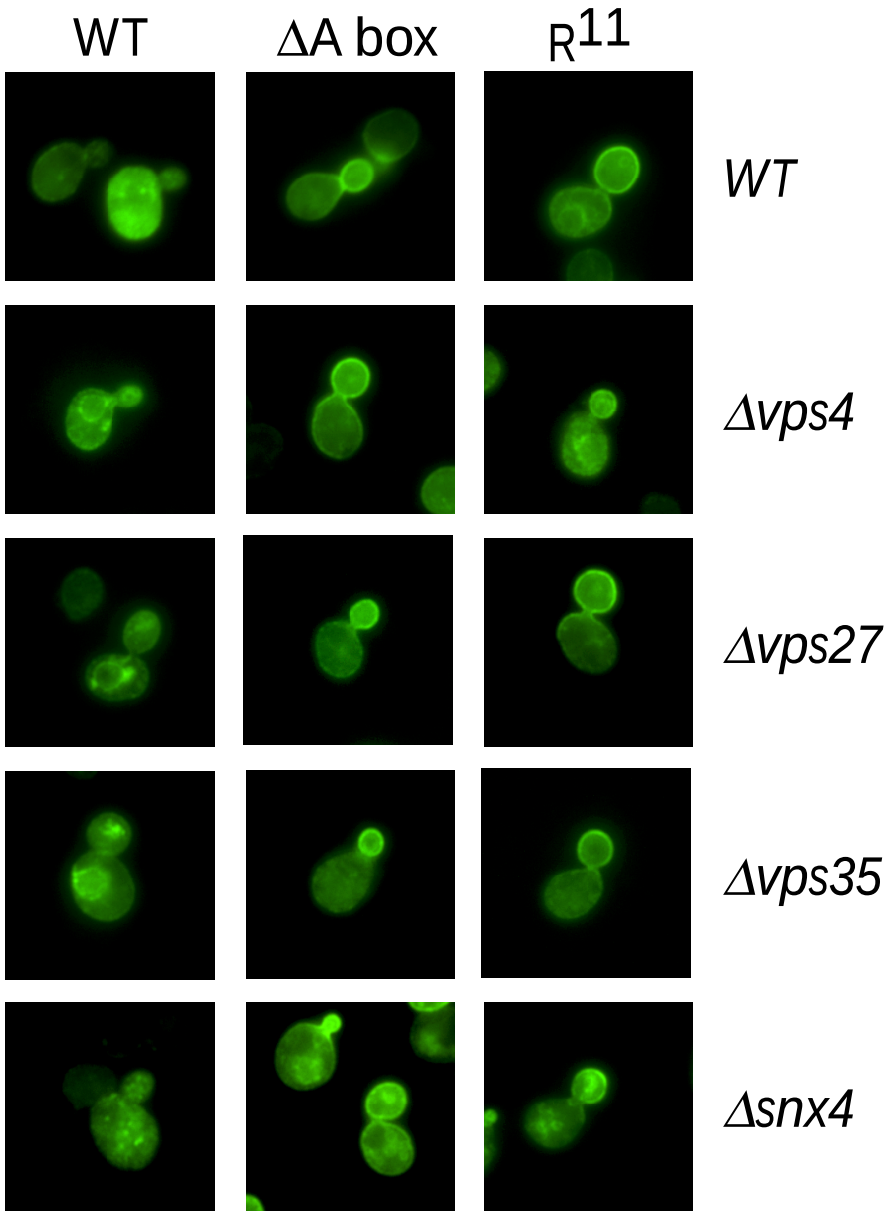


Fig. 27: Distribution of Ste6 in different protein sorting mutants. Different yeast strains were transformed with pYKS2 (WT Ste6, left row), pRK264 (Ste6 ΔA-box, middle row) and pRK659 (Ste6 $R1^{11}$, right row). Ste6 distribution was determine d by immunofluorescence with anti-myc primary antibodies (9E10), and anti-mouse FITC-conjugated secondary antibodies. Strains used from top to bottom: JD52 (wt), RKY1511 (*vps4Δ*), RKY1876 (*vps27Δ*), RKY2074 (*vps35Δ*), RKY1634 (*snx4Δ*).

3.3.4 Ste6 ubiquitination is not affected in the *doa4Δ* mutant

Doa4 is a major deubiquitinating enzyme required for sorting of proteins into MVB vesicles. Previously, it was shown that the free ubiquitin level is lowered in *doa4*

(Swamingthan *et al*., 1999 and our data), therefore, ubiquitin dependent processes could be affected in this mutant. Also, it was published that trafficking of Ste6 and several other proteins was affected in the *doaΔ* mutant (Losko *et al*., 2001; Katzmann *et al*., 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). Proteins could not be delivered into the lumen of the vacuole in this mutant. It could be that ubiquitination of cargo protein is affected in the *doa4Δ* mutant. Alternatively, ubiquitination of a component of the endocytic machinery could be affected and therefore delivery of cargo proteins to the vacuole may be blocked. To distinguish between these two alternatives, we compared Ste6 ubiquitination in wiltd type and *doa4Δ* (Fig. 28).

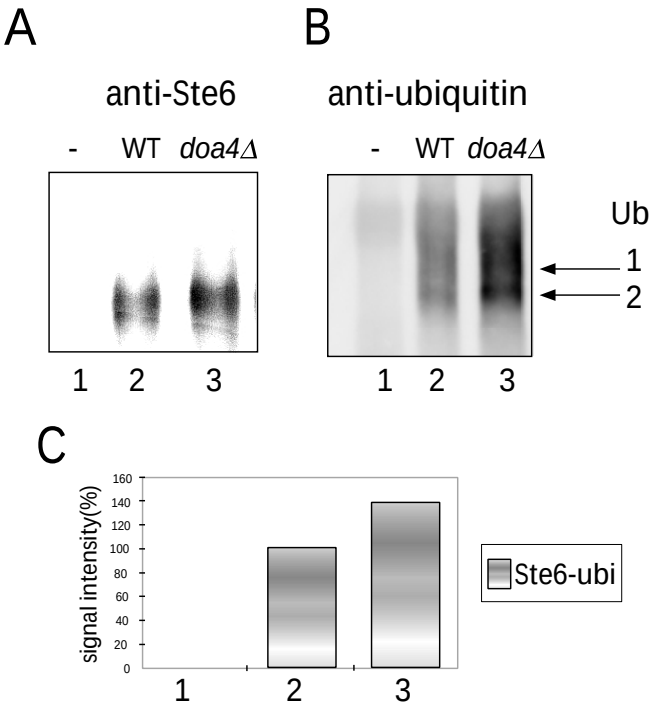


Fig. 28: Ubiquitination of Ste6 is not affected in the *doa4Δ* mutant. Ste6 was immunoprecipitated from cell extracts prepared from JD52 (WT) (lane2), and JD116 (*doa4Δ*) (lane3) transformed with the plasmid pRK69 (2µ-Ste6). Immunoprecipitates were analysed by Western blotting with (A) anti-Ste6 antibodies and (B) anti-ubiquitin antibodies. Mono- and di- ubiquitinated forms are indicated by arrows. As a negative control (lane 1) RKY959 (*ste6Δ*), trasformed with YEplac195(vector) was used. (C) Quantification of Western blot signals. Ubiquitin signal was normalized to the amount of the precipitated Ste6 protein. Ubiquitin signal intensity of the wildtype strain was set as 100%.

Ste6 was immunoprecipitated with anti-Ste6 antibodies from cell extracts prepared from wildtype and *doa4Δ* strains grown at 30°C. Immunoprecipitates were then analysed for the presence of ubiquitin with anti-ubiquitin antibodies. As mentioned above, only ubiquitin covalently attached to the Ste6 protein can be detected. As seen in figure 28, Ste6 could be precipitated in both strains tested. As seen in figure 28B the ubiquitin signal was stronger in *doa4Δ*. But at the same time, there were alsomore Ste6 protein in the immunoprecipitates in *doa4Δ* (Fig. 28A). This indicates that Ste6 is stabilized in *doa4Δ*. In line with this observation, it was reported that Ste6 is stabilized about 3-fold in the *doa4Δ* mutant (Losko *et al*., 2001). When the ubiquitin signal was normalized to the amount of Ste6 in the immunoprecipitates, no significant difference in ubiquitination of Ste6 between wildtype and the *doa4Δ* mutant was observed (Fig. 28C). Thus, ubiquitination of Ste6 does not appear to be affected in *doa4Δ*. This suggests, at least in the case of Ste6, that *doa4Δ* does not act by reducing cargo protein ubiquitination.

3.3.5 Identification of recycling signals in the Ste6 protein

3.3.5.1 Enhanced recycling of Ste6 in the *vps8Δ* strain

Recycling was observed for the Ste6 ΔA-box and Ste6 R^{11} variants. Does the wildtype Ste6 protein also recycle? To be able to detect possible recycling of Ste6, its diversion into the vacuolar degradative pathway had to be abolished. To this end, we made use of the *vps8Δ* mutant. Vps8 belongs to the so-called class D *vps* mutants. It is assumed that this class of *vps* mutants is blocked in docking and fusion of transport vesicles with late endosomes. It was shown that Vps8 interacts with Vps21 (also a class D protein), but its exact function is still unclear. So, if Ste6 is able to recycle, it should accumulate at the cell surface in the *vps8Δ* mutant, since its further progression to the vacuole is blocked in this mutant. The Ste6 distribution was, therefore, examined in the *vps8Δ* mutant cells by immunofluorescence microscopy. Indeed, Ste6 accumulated at the cell surface in a polar fashion in the *vps8Δ* mutant, like Ste6 ΔA-box in wildtype, indicating that recycling of wildtype Ste6 is enhanced in the *vps8Δ* mutant (Fig. 29).

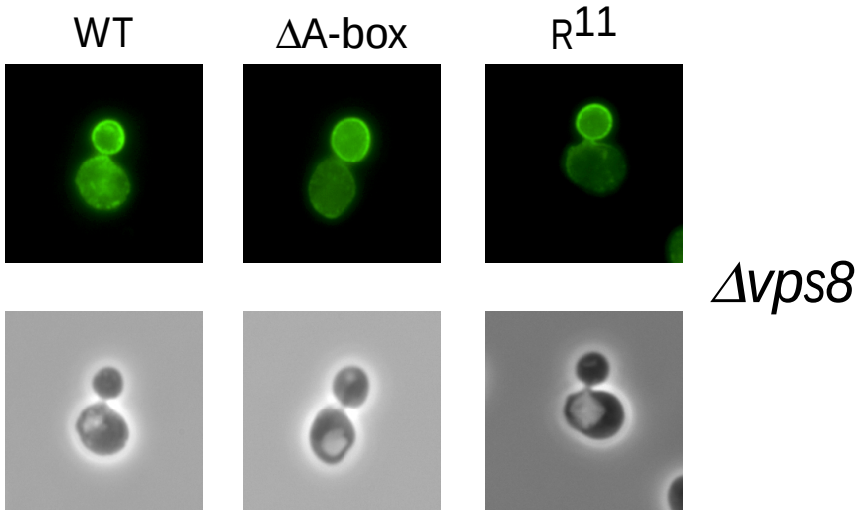


Fig. 29: Enhanced recycling of Ste6 protein in the *vps8Δ* mutant. Distribution of different Ste6 variants in RKY1875 (*vps8Δ*) was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and anti-mouse FITC-conjugated secondary antibodies. From left to right: RKY1875 strain transformed with pYKS2 (WT Ste6), pRK264 (Ste6 ΔA-box), pRK659 (Ste6 R^{11}). Upper panels-FITC fluorescence images; lower panels-phase contrast images.

The distribution of the other two Ste6 variants was also examined in this mutant. Ste6 ΔA-box and Ste6 R^{11} both showed the same polar localization like the wildtype Ste6 protein, which is also already observed in the wildtype strain. However, there was a slight difference in the distribution of the Ste6 R^{11} variant. No internal staining was observed with this variant in the *vps8Δ* mutant, suggesting that Vps8 is needed for progression of Ste6 R^{11} from early to late endosomes.

3.3.5.2 Recycling of Ste6 protein is a signal mediated process

It is possible that recycling of Ste6 is a signal mediated process. It has been reported that retrieval of other yeast proteins like Kex2, Vps10 and Ste13 from endosomes to the Golgi is mediated by aromatic amino acid-based signals. Since the linker region of Ste6 plays an important role in regulating Ste6 trafficking, we focused on this region in order to identify putative recycling signals. All tyrosine and phenylalanine residues in the linker region were mutagenized, either singly or in combination, by site-directed mutagenesis (Fig. 23). We made use of the *vps8Δ* mutant for testing

our Ste6 mutants. Namely, as a result of enhanced recycling, Ste6 shows a polar distribution at the bud cell surface in this mutant. Therefore, if recycling of Ste6 is blocked by mutations in retrieval signals, Ste6 should be found inside the cell in the *vps8Δ* mutant. Hence, five mutant proteins obtained by mutagenesis were tested for Ste6 distribution in the *vps8Δ* mutant background by immunofluorescence microscopy. Four of the five mutants examined (F630L, Y648L, F656L/Y657L/Y661L, Y713L) did not display an altered localization in the *vps8Δ* mutant compared to wildtype Ste6. However, one mutant, Y681L , behaved differently. It was no longer distributed in a polar fashion at the plasma membrane, but was instead associated with internal structures, as expected for a recycling mutant. So it seems that the tyrosine at the position 681 is part of a recycling signal for Ste6 (Fig. 30).

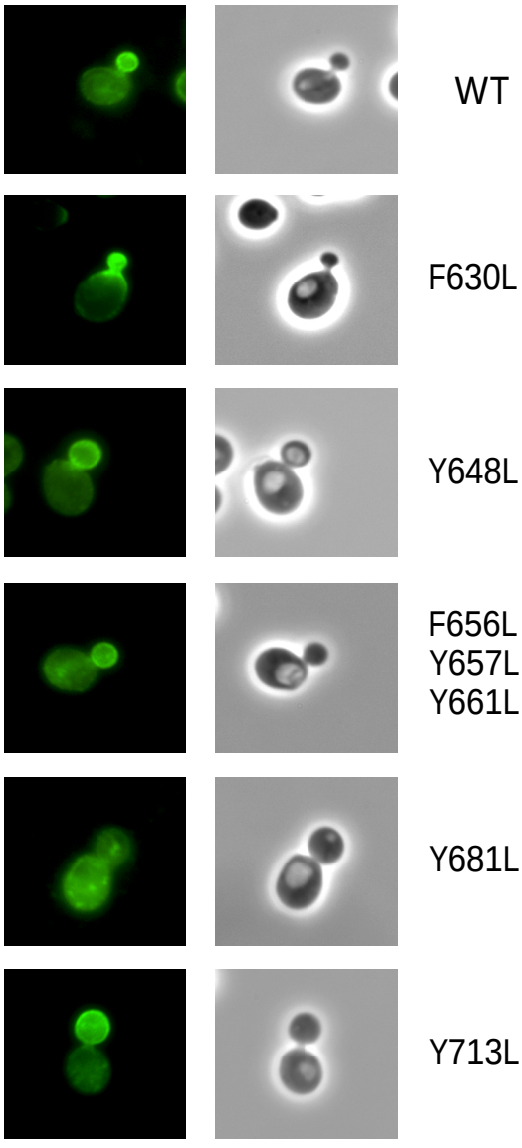


Fig. 30: Identification of putative recycling signals in Ste6. Phenylalanine / tyrosine residues of the Ste6 linker region were mutagenized to leucin (Fig. 23). The distribution of mutant Ste6 variants expressed from plasmids was examined in RKY1875 (*vps8Δ*) by immunofluorescence microscopy with anti-myc (9E10) primary antibodies and anti-mouse FITC-conjugated secondary antibodies. Left row: FITC fluorescence images; right row: phase contrast images. Plasmids used from top to bottom: pYKS2 (WT Ste6), pRK888 (Ste6 F630L), pRK889 (Ste6 Y648L), pRK890 (Ste6 F656l/Y657L/Y661L), pRK891 (Ste6 Y681L), pRK 892 (Ste6 Y713L).

Further analysis of the Ste6 Y681L variant, however, indicated that there are two redundant recycling signals in Ste6. Originally, the mutations were introduced into the plasmid pYKS2 and screened by immunofluorescence microscopy. This plasmid codes for a Ste6 variant with a slightly changed C-terminus (.. LFSRSRN instead of .. IVSNQSS). This Ste6 variant behaves in every aspect examined like wildtype Ste6 (mating activity, turnover, ubiquitination, localization). But, upon sub-cloning into other STE6 plasmids, the recycling defect was only observed in combination with the altered C-terminus (Fig. 31).

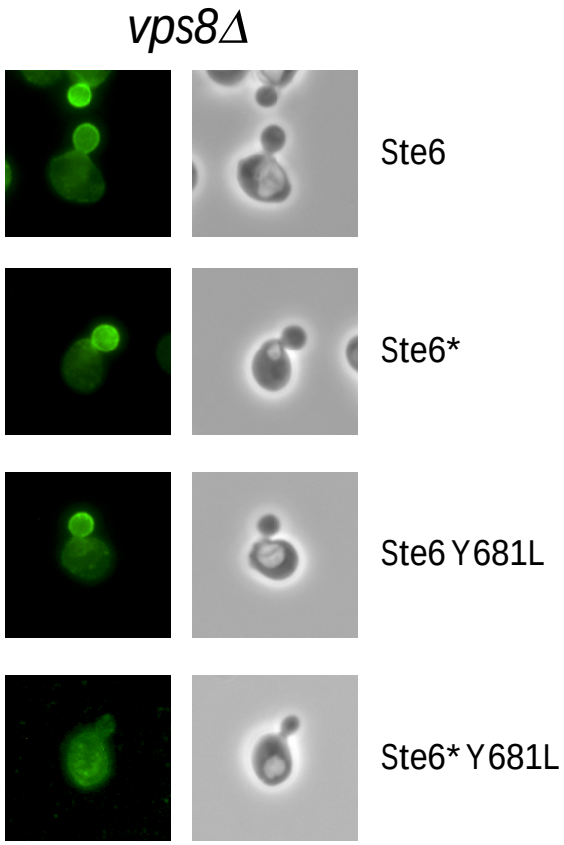


Fig. 31: Two redundant recycling signals in Ste6. The distribution of different Ste6 variants was examined in RKY1875 (*vps8Δ*) by immunofluorescence microscopy with anti-myc (9E10) primary antibodies and anti-mouse FITC-conjugated secondary antibodies. Plasmids used from top to bottom: pRK970 (WT Ste6), pYKS2 (Ste6*), pRK998 (Ste6 Y681L), pRK891 (Ste6* Y681L). Left row: FITC fluorescence images, right row: phase contrast images. (Ste6*: see text)

This suggests that there are probably two redundant recycling signals in Ste6, one in each homologous half of Ste6. In the further text, the variant with the altered C-terminus is designated Ste6* to distinguish it from wildtype Ste6.

3.3.5.3 The Y681L mutation does not affect localization of Ste6 in *vps27Δ* and *end4* strains

Loss of polar localization of the Ste6* Y681L variant could be due to misfolding of the protein and not due to a specific recycling defect. To test this possibility, Ste6* Y681L localization was examined in *end4* and *vps27Δ* mutants. Endocytosis of plasma membrane proteins is blocked in the *end4* mutant. Therefore, if Ste6* Y681L is still able to travel to the plasma membrane, it should be trapped at the plasma membrane in this mutant. *end4* is a conditional mutant. At permissive temperature (25°C), Ste6* is localized in internal dot-like structures, like in the wildtype (Fig. 32A). Upon 1 h shift to non-permissive temperature (37°C), Ste6* is found trapped at the plasma membrane (Fig. 32B). The same was observed for the Ste6* Y681L variant. It was also found at the cell surface upon a 1 h shift to non-permissive temperature (37°C) suggesting that it is able to reach the plasma membrane (Fig. 32E). Trafficking of Ste6* Y681L along the endocytic pathway was also examined. *vps27Δ* belongs to the class E vps (vacuolar protein sorting) mutants. Vps27 is thought to function at the level of late endosomes. Ste6* showed a typical class E phenotype (staining of vacuolar membrane with a dot-like structure next to it) indicating that its transport to the vacuole is blocked in this mutant (Fig. 32C). Similarly, Ste6* Y681L was observed around the vacuole and in the class E dot, representing exaggerated late endosome (Fig. 32F). Thus, Ste6* Y681L trafficking along the endocytic pathway appears to be normal. This makes it appear unlikely that the observed recycling defect is the result of misfolding of the protein.

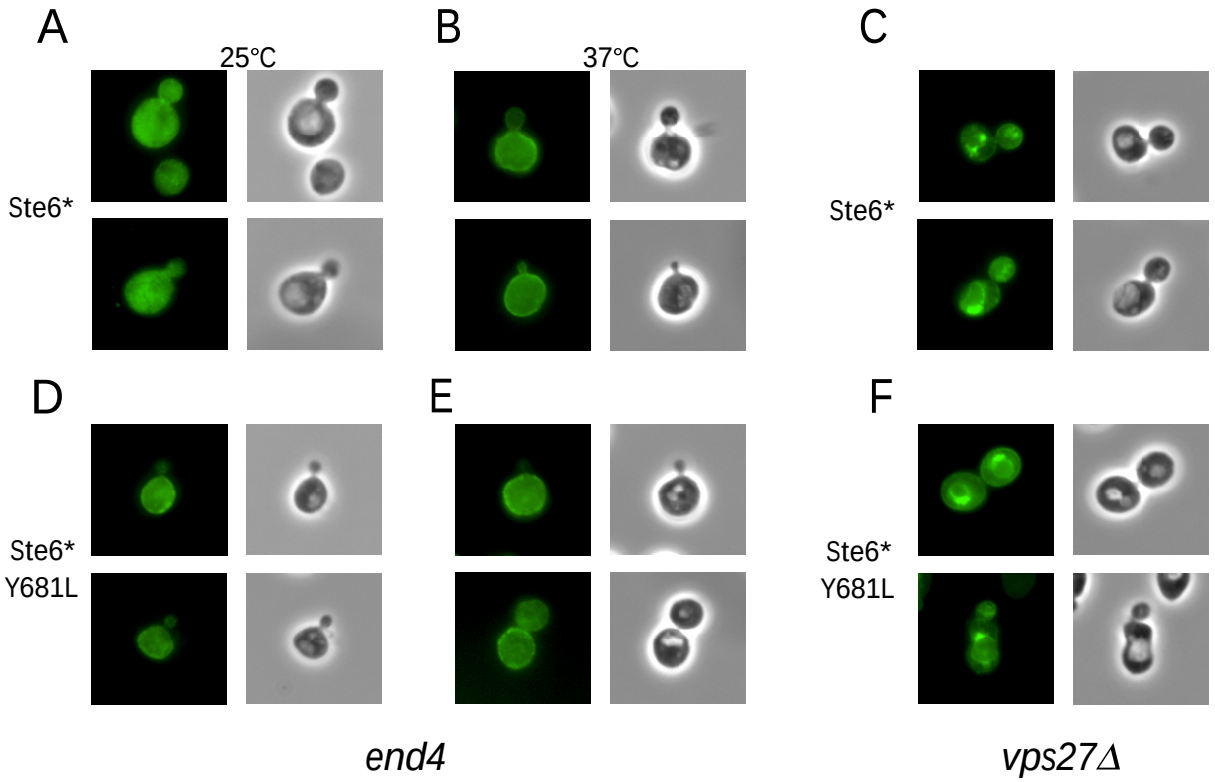


Fig. 32: Distribution of Ste6* Y681L in *end4Δ* and *vps27Δ* mutants. RKY592 (*end4 ste6Δ*) (A, B, D, E) and RKY1876 (*vps27Δ*) (C, F) were transformed with pYKS2 (Ste6*) (A, B, C) and pRK891 (Ste6* Y681L) (D, E, F). Localization of Ste6 was detected in cells grown at 25°C (A, D) and after 1h shift to 37°C (B, E) or from cells grown at 30°C (C, F) by immunofluorescence microscopy. Anti-myc (9E10) antibodies as primary and FITC-conjugated anti-mouse as secondary antibodies were used. From left to right alternating FITC fluorescence and phase contrast images.

3.3.6 Ste6 recycling is important for mating

So far, enhanced Ste6 recycling was detected only in mutant strain backgrounds or with mutant Ste6 variants. Are there any physiological situations where recycling of Ste6 could be of importance? Endocytic recycling combined with localized exocytosis provides a simple way for creating cell asymmetry. This could be convenient for situations where transient polarity is needed, like during mating. Proteins become polarized to the shmoo tip (mating projection) during mating. Mating partners fuse at their shmoo tips to form a zygote. It is possible that Ste6 is important for this fusion step. Ste6 mutants have been isolated that are specifically blocked at this step (Elia and Marsh, 1996). Therefore, it was examined whether Ste6

accumulates at shmoo tips. *MATa* cells form shmoos (pear shaped structures) upon exposure to the mating-pheromone α-factor. The Ste6 distribution was determined in α-factor treated cells by immunofluorescence microscopy (Fig. 33A). Ste6* was indeed localized at the shmoo tip upon α-factor exposure. In contrast, Ste6* Y681L was again found associated with internal structures, indicating that polarization was also lost in pheromone treated cells. Thus, if polar localization of Ste6 is important for mating, the mating activity of Ste6 should be affected by the Y681L mutation. Mating assay was used to test this possibility. In a serial dilution patch test, *MATa ste6Δ* cells transformed with different Ste6 variants on single-copy plasmids, were spotted onto a lawn of *MATα* cells and allowed to mate. Zygotes were selected by replica-plating to selective media (Fig. 33B). While no zygotes could be detected with a vector control, zygotes were observed with wildtype Ste6 down to a dilution of 10^{-3}. However, no zygotes could be detected with Ste6* Y681L, in contrast to the other Ste6 variants (Ste6* and Ste6 Y681L) where no defect in mating activity was observed. Ste6 protein was present in all strains (Fig. 33C).

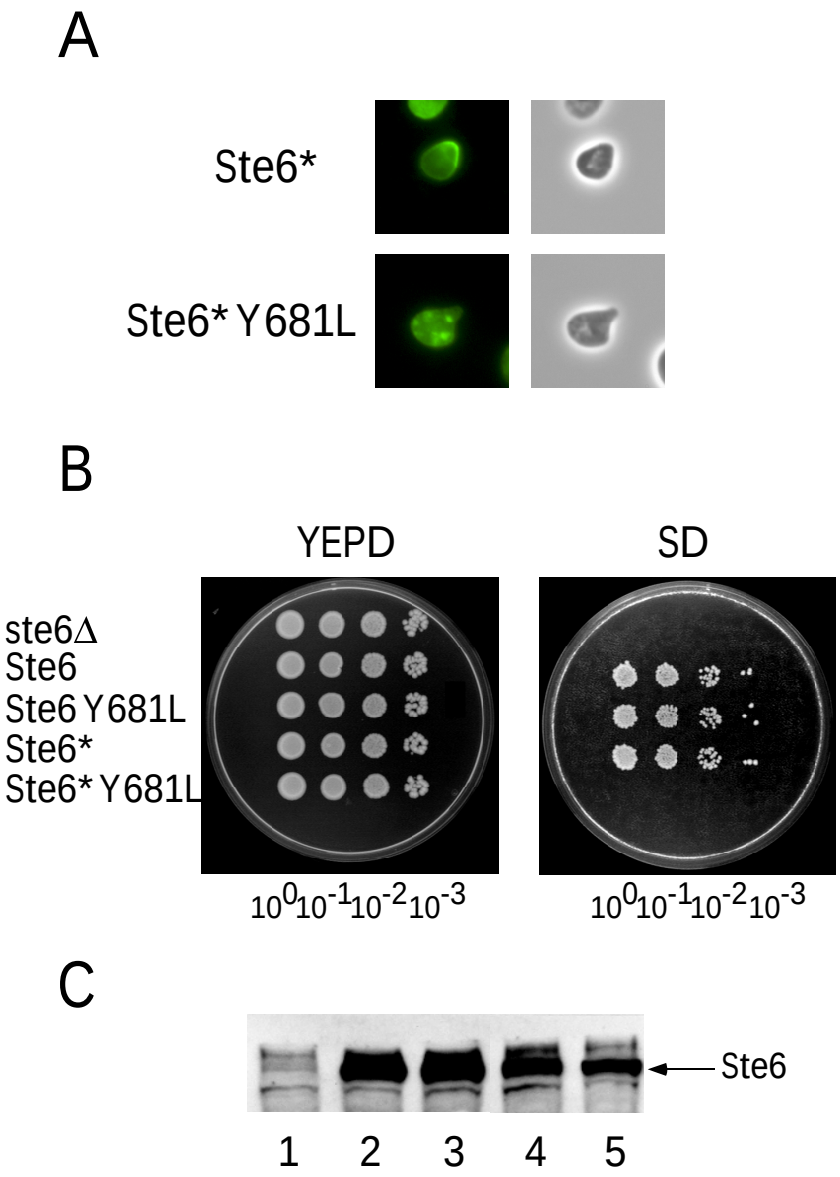


Fig. 33: Effect of the Y681L mutation on mating. (A) JD52 (wt) was transformed with pYKS2 (Ste6*, upper panel) and pRK891 (Ste6*Y681L, lower panel). The distribution of these Ste6 variants in JD52 treated for 2 h with α-factor (5µM) was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies. Left: FITC fluorescence, right: phase contrast images. (B) Serial dilution patch mating assay. RKY959 (*ste6Δ*) was transformed with single copy plasmids expressing different Ste6 variants.10-fold serial dilutions of different cultures were spotted onto a lawn of a *MATα* tester strain. Cells were allowed to mate overnight and were then replica plated onto a selective agar plate (SD minimal medium) to select for zygotes. The plate was incubated for 3 d at 30°C. The plasmids used (from top to bottom) are: YEplac195 (vector, *ste6Δ*), pRK278 (WT Ste6), pRK909 (Ste6 Y681L), pRK940 (Ste6*), pRK939 (Ste6* Y681L). (C) Cell extracts prepared from RKY959 (*ste6Δ*) transformed with single copy plasmids carrying different Ste6 variants were examined by Western blotting with anti-Ste6 antibodies. Plasmids used: (1) YEplac195 (vector), (2) pRK278 (WT Ste6), (3) pRK909 (Ste6 Y681L), (4) pRK940 (Ste6*), (5) pRK 939 (Ste6* Y681L).

The half life of the variants was also determined, by gal depletion experiments. To this end, different Ste6 variants were subcloned under the control of the *GAL1* promoter. Cells were pregrown to exponential phase in media with galactose to induce expression of Ste6 variants and were then shifted to media containing glucose to turn off expression of Ste6 from the *GAL1* promoter. Cell extracts were prepared at different time periods after shift to glucose containing medium and the fate of Ste6 protein was determined by Western blotting with anti-myc (9E10) antibodies (Fig. 34). These experiments show that Ste6 turnover is not affected by the Y681L mutation.

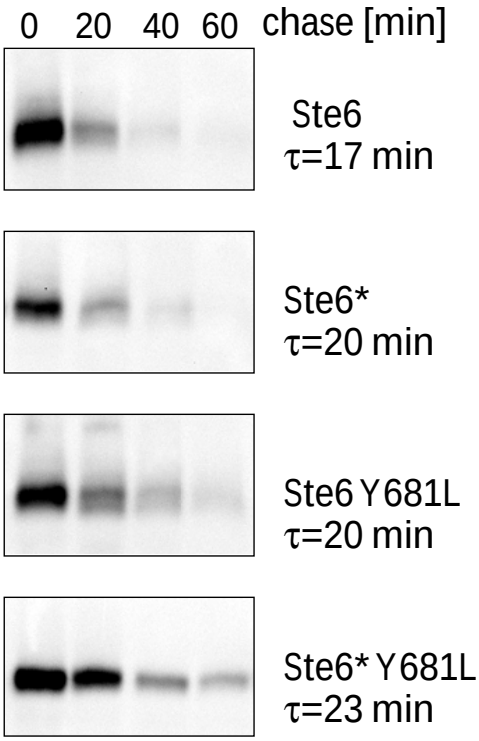


Fig. 34: Ste6 turnover is not affected by the Y681L mutation. The half life of Ste6 was determined by gal depletion experiments. Cultures of JD52 (wt) transformed with plasmids expressing different Ste6 variants under the control of the *GAL1* promoter were pregrown in media containing galactose as only carbon source. Cells were then shifted to media containing glucose as carbon source. Cell extracts were prepared at different time periods (as indicated) and separated on SDS polyacrylamide gels. Ste6 was detected by Western blotting with anti-myc antibodies (9E10). Plasmids used from top to bottom: pRK504 (wt Ste6), pRK975 (Ste6*), pRK996 (Ste6 Y681L), pRK974 (Ste6* Y681L).

4 DISCUSSION

4.1 Phenotypes of the *rsp5-1* mutant

Many membrane proteins are ubiquitinated and subsequently degraded in the vacuole / lysosome. The ubiquitin ligase Rsp5 has been implicated in the ubiquitination of most of these membrane proteins. In the case of our model protein Ste6, several phenotypes concerning Rsp5 were observed. Ste6 showed altered localization (Fig. 4), and was stabilized (Fig. 5) in the *rsp5-1* mutant after shift to non permissive temperature, indicating that Ste6 trafficking is affected in the *rsp5-1* mutant. It has been reported that ubiquitination of Ste6 is important for its delivery to the vacuole (Losko *et al*., 2001). It could be that due to inactivation of Rsp5, Ste6 ubiquitination is prevented, and that entry of Ste6 into the MVB pathway and subsequent delivery to the vacuole is blocked. This does not seem to be the case for the Ste6 protein because its ubiquitination was not affected by the *rsp5-1* mutation (Fig. 6). So, the observed effect of the *rsp5-1* mutation on the turnover of Ste6 could be due to the lack of ubiquitination of a component of the trafficking machinery rather than of the Ste6 protein itself as suggested by Dunn and Hicke (2001). What endocytic proteins could be regulated by Rsp5? A connection between actin cytoskeleton and endocytosis, and further between actin cytoskeleton and Rsp5 was reported. It was recently published that Rsp5 binds directly to Sla1 and Rvs167 (Stamenova *et al*., 2004) and that ubiquitination of Rsv167 could regulate activity of this protein complex. Sla1 and Rsv167 are yeast proteins required for receptor internalization and organization of the actin cytoskeleton. Also it was found that Rsp5 interacts genetically with another protein, Pan1 (synthetic lethality of *rsp5* / *pan1* double mutant and interaction between these two proteins in two-hybrid system) (Zoladek *et al.,* 1997). Pan1 is a yeast homologue of Eps15, a mammalian EH domain containing protein. Eps15 is a component of clathrin-coated pits and vesicles and performs an important role in endocytosis at the plasma membrane and at endosomes (Sorkina *et al*., 1999; Torrisi *et al*., 1999). Similar to Eps15, it was suggested that Pan1 containing protein complexes also participate at multiple steps in the endocytic pathway, together with yeast epsins, like Ent1, acting at both the internalization and endosomal sorting steps (Wendland, *et al*., 1999). However, Pan1 is probably not a substrate for

the Rsp5 ligase, since no ubiquitinated forms of Pan1 were detected in vivo. Furthermore, it could not be coimmunoprecipitated with Rsp5 (Kaminska *et al.,* 2002). Ent1 and the Eps15 homologue Ede1 possess putative ubiquitin interaction domains: UIMs (ubiquitin interaction motifs) were identified in Ent1 and UBA (ubiquitin-associated domain) in Ede1. Although, it was proposed that these Ub-interaction domains are important for interaction of epsins with ubiquitinated cargo proteins, it could not be ruled out that they are also important for their own ubiquitination, this possibility. It was demonstrated that UIM-containing proteins can be ubiquitinated in a UIM-dependent manner (Polo *et al*., 2002). Also, Eps15 is UIM-dependently monoubiquitinated by Nedd4 (mammalian homolog of Rsp5) upon activation of the epidermal growth factor (EGF) receptor (Polo *et al*., 2002). Thus, these proteins are potential substrates for Rsp5.

4.2. Role of Rsp5 in ubiquitin homeostasis

The observed *rsp5-1* phenotypes suggested that ubiquitin is limiting in this mutant. We noticed a different response to heat-shock in the *rsp5-1* mutant compared to wildtype. While high molecular weight ubiquitin-conjugates accumulated in the wildtype upon heat-shock, no such accumulation was observed in *rsp5-1* (Fig. 7). Interestingly, this accumulation of high molecular weight ubiquitin-conjugates could be partially restored by ubiquitin overproduction, indicating that there is not enough ubiquitin in the *rsp5-1* mutant at high temperature. It is not clear how many proteins contribute to this complex pattern of ubiquitinated proteins that forms after heat-shock. Most of them are membrane-associated proteins as judged from the centrifugation and flotation experiments (Fig. 9, 10). It is most likely that these ubiquitin-conjugates represent a large number of misfolded proteins that arise upon heat-shock and that have to be removed by ubiquitin-dependent degradation pathways. Some of these proteins could be retained in the ER by ER quality control mechanism (ERQC), and subsequently degraded in a process known as ER associated degradation (ERAD). This process involves ubiquitination of substrates and degradation by the cytosolic proteasome. Indeed, we found that at least some of the ubiquitinated proteins co-localize with the ER marker protein, Dpm1, in sucrose gradients (Fig. 11). Hitchcock *et al*. reported 211 membrane-associated proteins that were ubiquitinated to a higher degree in a *npl4-1* mutant

(defective in a component of the ERAD system), 83 of them were considered to be potential endogenous substrates of the ERAD pathway. Also, it was reported that Rsp5 could play a role at the level of the ER in assisting the Hrd1 ligase (major ERAD E3 ligase) in recognizing misfolded proteins (Haynes *et al*., 2002), which could in part explain the lack of ubiquitin-conjugates observed in the *rsp5-1* mutant after heat-shock. So, failure to remove these misfolded proteins could contribute to the temperature sensitivity of the *rsp5-1* mutant. Indeed, we could suppress the temperature sensitivity of the *rsp5-1* mutant by overexpression of ubiquitin, which serves as another hint that ubiquitin is limiting in this mutant.

There are two possible explanations why the *rsp5-1* mutant would need more ubiquitin for survival at high temperature. Either free ubiquitin levels are lower than normal, due to increased turnover or reduced synthesis of ubiquitin, or higher ubiquitin levels could compensate for a partially defective Rsp5 protein, e.g. with reduced affinity for ubiquitin. If it is just the reduced affinity for ubiquitin that causes the *rsp5-1* mutant phenotypes, then it could be assumed that all *rsp5-1* phenotypes should be suppressed by overexpression of ubiquitin. However, this was not the case. We found phenotypes that could not be suppressed by overexpression of ubiquitin, e.g. the defect in recovery from heat-induced protein synthesis arrest (see below). Also, one would not expect any change in free ubiquitin levels in the case of a partially defective Rsp5 protein. In contrast, we found that the free ubiquitin level at 37°C was lower in *rsp5-1* than in wildtype.

What is the reason for this reduced free ubiquitin levels observed in *rsp5-1* upon heat-shock? It seems that both higher turnover and reduced synthesis of ubiquitin contribute to the drop in free ubiquitin level. However, the data concerning the contribution of higher ubiquitin turnover to lowered free ubiquitin levels were contradictory. While the ubiquitin half life was consistent for the *rsp5-1* mutant in both experiments performed (cycloheximide chase and pulse chase), a difference between these two experiments was observed with wildtype. Consistent with published reports (Hanna *et al*., 2003) ubiquitin was unstable in wildtype in the pulse chase experiments. Based on these experiments, there is no significant difference in ubiquitin turnover in wildtype and *rsp5-1*. However, in the cycloheximide chase experiments, ubiquitin was stable in wildtype but unstable in *rsp5-1*. This would suggest that higher ubiquitin turnover contributes to the lowered free ubiquitin level. The reason for this discrepancy is unclear. It could be

that cycloheximide does not function properly in the wildtype strains, i. e. does not block translation completely or that it interfers with ubiquitin degradation in the wildtype strain. It has been reported that cycloheximide blocks transport through the endocytic pathway (Hicke *et al*., 1997). If a functional endocytic pathway is important for ubiquitin turnover in wildtype this could explain the observed stabilization of ubiquitin. However, since ubiquitin is unstable in *rsp5-1* even in the presence of cycloheximide, this block should not occur under these conditions.

It seems that reduced synthesis of ubiquitin contributes to the reduced free ubiquitin levels in the *rsp5-1* mutant at high temperature. We observed a transient decrease in protein synthesis upon heat-shock in wildtype cells. In contrast to wildtype, the *rsp5-1* mutant was not able to recover from this protein-synthesis arrest during the time course of the experiment. Under stress conditions, like heat-shock, cells have a higher requirement for ubiquitin, probably because of a higher load of misfolded proteins that has to be removed by ubiquitin-mediated degradation. This is accomplished by upregulation of the *UBI4* gene (Finley *et al*., 1987). Although heat-shock signaling at the *UBI4* promoter appears to be unaffected in *rsp5-1*, it could be that the amount of ubiquitin produced after heat-shock is insufficient, due to the prolonged protein synthesis arrest in the *rsp5-1* mutant.

What could be the reason for this protein synthesis arrest in the *rsp5-1* mutant? Ubiquitination by Rsp5 seems to play a role in DNA damage-induced degradation of Rpb1 (Beaudenon *at al*., 1999) the large subunit of RNA polymerase II. It was reported that RNA polymerase complexes may be stalled on DNA upon UV-irradiation or nucleotide starvation (Yang *et al*., 2003; Svejstrup, 2003). Ubiquitination of the large subunit of RNA polymerase II could facilitate displacement of stalled RNA polymerase complexes from DNA. It is therefore possible that RNA polymerase complexes are stalled on DNA also upon heat-shock. This could then explain the requirement of Rsp5 in recovery from heat-shock induced protein synthesis arrest. In addition, Rsp5 could directly control activity of RNA polymerase by ubiquitination. Furthermore, it was recently reported that *rsp5* mutants show a defect in nuclear export of mRNA (Rodrigez *et al*. 2003; Neuman *et al*. 2003). However, transcription elongation and nuclear export are tightly coupled (Strasser *et al*., 2002) and it is, therefore, difficult to resolve whether the export

defect is an immediate or indirect consequence of the *rsp5* defect. In any case, a severe defect in protein synthesis could be anticipated from these findings.

4.3 Ste6 sorting is regulated by ubiquitination

4.3.1 Polar localization of Ste6 is maintained through endocytic recycling

Ste6 ΔA-box and Ste6 R^{11} accumulate at the cell surface of the daughter cell. There are several possible explanations for this observed polar localization. The septin ring at the bud neck might form a mechanical barrier, like in the case of the Ist2 protein (Takizawa *et al.,* 2000). However, this does not seem to be the case for our Ste6 variants since the polar localization was maintained even when the septin ring was disrupted by the *cdc12-6* mutation (Fig. 22). Another mechanism for maintaining the polar localization has been postulated for the v-SNARE Snc1. Snc1 shows a similar localization like the Ste6 variants, staining of the bud cell surface, which is believed to be obtained through continuous endocytic recycling and localized exocytosis (Valdez-Taubas and Pelham, 2003). The Ste6 variants showed an immediate redistribution to internal structures upon inactivation of Ypt6, an essential component of the endocytic recycling loop (Sinissoglou *et al.,* 2000), indicating that they are also polarized by a similar mechanism. Alternatively, it was proposed that proteins are polarized to the shmoo-tip by a lipid based mechanism. According to this model, polarized membrane proteins partition into lipid rafts that are concentrated at the shmoo-tip. However, it is not clear how this lipid asymmetry should be established. Also, influence of Ypt6 is difficult to incorporate in this model. One would have to postulate that Ypt6 and thus endocytic recycling plays a central role in establishing lipid asymmetry. So, in any case, endocytic recycling would be important for protein polarization, either directly or indirectly.

4.3.2 Control of recycling by ubiquitination

Enhanced recycling was observed with the Ste6 ΔA-box and Ste6 R^{11} variants. In both variants, ubiquitination was reduced compared to wildtype. The extent of the recycling phenotype correlated with the degree of ubiquitination. The strongest

effect was observed with the Ste6 ΔA-box variant, which shows no ubiquitination. The Ste6 R^{11} variant, which displayed some residual ubiquitination, displayed somewhat "leaky" recycling phenotype, i. e. a certain fraction of the protein escaped recycling and was transported further down the endocytic pathway. In the Ste6 R^{11} variant only putative ubiquitin acceptor sites were mutated. Although, we cannot rule out that some other signal is affected by the mutations, the most likely interpretation of our data is that lack of ubiquitination is responsible for the observed recycling phenotype.

Analysis of Ste6 recycling in different mutants of the endocytic pathway suggests that the ubiquitination-deficient Ste6 variants recycle through early endosomes. Also, we have observed enhanced recycling of wildtype Ste6 in the class D *vps* mutant *vps8Δ*. How can this phenotype be explained? According to the vesicular transport model of the yeast endocytic pathway, class D functions are involved in docking and fusion of vesicles with late endosomes (Gerrard *et al.,* 2000). One would expect that vesicles derived from early endosomes can no longer fuse with late endosomes in the class D *vps* mutants. Instead, these vesicles may now fuse with the Golgi. From the Golgi, proteins could be recycled back to the plasma membrane via the polarized yeast exocytic pathway. This would imply that endocytic vesicles also have some affinity for Golgi membranes. Therefore, it could be that recycling in class D *vps* mutants is completely non-selective, i. e. all proteins contained in the membrane of early endosomes should recycle. Alternatively, class D functions could be required for maturation of early endosomes into MVBs / late endosomes, e. g. by recruiting proteins to early endosomes.

To explain how ubiquitination could interfere with Ste6 sorting, we would like to propose the following model (Fig. 35), which is largely based on informations gathered from mammalian cells about sorting events in the early endocytic pathway (Gruenberg and Stenmark, 2004; Maxfield and McGraw, 2004). The early endosome / sorting endosome constitutes a central sorting station in the early endocytic pathway. It consists of a vacuolar part from which tubular extension emanate. Most of the membrane proteins will end up in the tubular part of the sorting endosome by default, due to the high surface-area-to-volume ratio of the tubules, unless they are specifically retained in the vacuolar part of the sorting endosome ("geometry based sorting"). The tubules pinch off and develop into recycling endosomes. According to our model ubiquitinated Ste6 is retained in the

vacuolar part of the sorting endosome and directed further to the vacuole, while non-ubiquitinated Ste6 is distributed into the tubules and funneled into the recycling pathway. In mammalian cells, there is evidence that planar clathrin coats on sorting endosomes are involved in retention of cargo proteins that are destined for lysosomal degradation (Raiborg *et al.,* 2001; Sachse *et al.,* 2002). Also, ubiquitinated cargo proteins have been detected in these coat structures (Raiborg *et al.,* 2002). It is believed that sorting into the recycling pathway occurs by default (Maxfield and McGraw, 2004). In contrast, we have obtained evidence that recycling of Ste6 is mediated by redundant sorting signals. This is in line with the identification of similar signals in other yeast proteins like Kex2, Ste13 and Vps10 that are retrieved from endosomes back to the Golgi (Wilcox *et al.,* 1992; Nothwehr *et al.,* 1993; Cooper and Stevens, 1996).

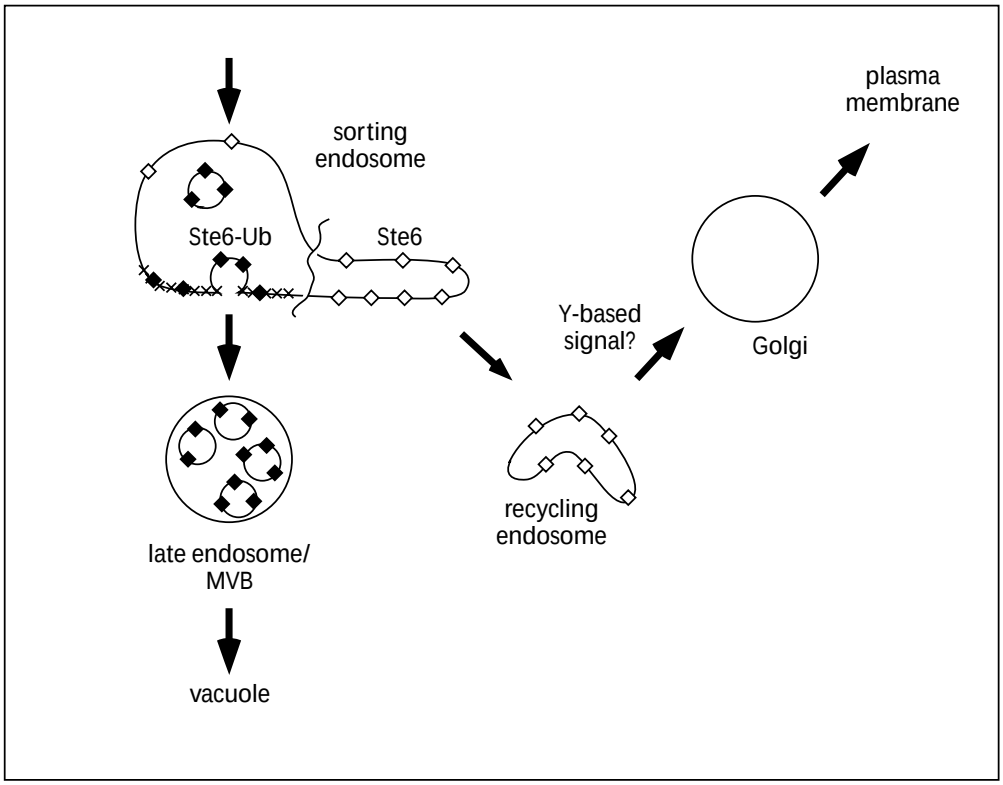


Fig. 35: A model for ubiqutination dependent sorting of Ste6 in the early endocytic pathway (modified from Maxfield and McGraw, 2004). Non-ubiquitinated Ste6 (open diamonds) is sorted into tubules that pinch off from sorting endosomes and form recycling endosomes. From there Ste6 can be transported back to the cell surface. Ubiquitinated Ste6 (filled diamonds), in contrast, is retained in the vacuolar part of the sorting endosome and directed into the multivesicular bodies (MVB) degradation pathway.

4.3.3 Difference to *doa4*Δ

After retention by the planar coat structure of the sorting endosome, ubiquitinated cargo proteins are sorted into vesicles that bud off into the interior of the endosome (Fig. 35). The sorting endosome matures into a late endosome or multivesicular body (MVB) carrying many of these internal vesicles (Gruenberg and Stenmark, 2004). After fusion of the MVBs with the lysosome / vacuole the vesicles are released into the interior of the lysosome / vacuole and the proteins associated with them are degraded. In the *doa4* mutant, the free ubiquitin level is lowered (Swamingthan *et al.,* 1999) and hence ubiquitin dependent processes are affected. In this mutant, Ste6 accumulated at the vacuolar membrane, but not at the cell surface (Losko *et al.,* 2001). Similar results were reported for other proteins (Katzmann *et al.,* 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). It seems that sorting of cargo proteins into internal vesicles is defective in the *doa4* mutant. So, after fusion of the late endosomes with the vacuole the proteins end up at the vacuolar membrane. It is possible that ubiquitination of a component of endocytic machinery instead of ubiquitination of cargo protein is affected in the *doa4Δ* mutant, since no obvious alteration in Ste6 ubiquitination was observed in the *doa4Δ* mutant compared to wildtype (Fig. 28). And while retention of proteins in the vacuolar part of sorting endosomes is not affected in *doa4Δ,* incorporation of proteins into MVB vesicles may be disrupted due to interference with the function of some other component. It was suggested that in addition to MVB cargoes, Doa4 could deubiquitinate a component of the MVB sorting machinery in order to regulate its activity (Luthala and Odorizzi, 2004). It has been suggested that the class E Vps protein Bro1 recruits Doa4 to endosomal membranes, thereby controlling the timing and location of Doa4 activity in the MVB pathway (Luthala and Odorizzi, 2004). So, Bro1-dependent coordination of Doa4 on endosomal membranes and subsequent deubiquitination of cargo proteins and possibly MVB sorting components is likely to be one of the last steps in the MVB pathway, ensuring that cargo proteins are concentrated at regions of endosomal membrane where invagination occurs. In yeast, it is not known so far whether class E Vps proteins are regulated by ubiquitination. However, in mammalian cells, several cytoplasmic proteins that control receptor down-regulation are

monoubiquitinated, including Hrs, mammalian orthologue of the yeast class E protein, Vps27 (Polo *et al*., 2002). Also, CIN85 (Cbl-interacting protein of 85 kDa) (orthologue of the yeast Sla1) and endophilin (required for the internalization step of endocytosis) (orthologue of the yeast Rsv167) are monoubiquitinated cytoplasmic components in mammalian cells that associate with endosomes (Haglund *et al*., 2000; Angers *et al*., 2004), and both proteins interact with Alix/Aip1 (Vito *et al*., 1996; Chatellard-Causse *et al*., 2002). Like Bro1 in yeast, Alix/Aip1 could recruit a UBP that deubiquitinates CIN85 and endophilin in order to regulate their function and / or localization.

So far, endocytic recycling has been demonstrated only for mutant Ste6 variants or in mutant strain backgrounds. Is wildtype Ste6 also able to recycle? There are indications that it is. First, for Ste6, a half life of about 15-20 min has been determined. Although, this looks like a pretty short half life, it is still quite longer than the half life of another endocytic protein, the α-factor receptor Ste2, which is around 5 min (Hicke and Riezman, 1996). Hence, it appears that degradation of cell surface proteins via the endocytic pathway is a very rapid process. So this relatively "long" half-life of Ste6 indicates that it could cycle a few times before degradation. What could be the function of Ste6 recycling? It may be that recycling of Ste6 is necessary to polarize Ste6 to the mating projection during mating. Polarized localization of a number of proteins to the shmoo-tip seem to be important for ordered cell fusion during mating. A Ste6 mutant with reduced activity was isolated that is specifically blocked at the fusion step (Elia and Marsh, 1996). Since this mutant provides enough a-factor activity to complete the earliar steps in the mating cascade, this suggests that the Ste6 activity is especially needed at the fusion step. So, it may be necessary to concentrate the available Ste6 protein at the shmoo-tip. Indeed, we could show that our recycling defective Ste6* Y681L mutant is no longer able to mate (Fig. 33).

5 SUMMARY

The yeast ABC-transporter Ste6 is rapidly internalized from the cell surface and transported to the vacuole. Trafficking to the vacuole is regulated by ubiquitination. However, the ubiquitin machinery involved is still unknown. Rsp5 is a HECT E3 ubiquitin ligase implicated in ubiquitination of many membrane proteins. Therefore the role of Rsp5 in Ste6 trafficking and ubiquitination was examined. We found that Ste6 trafficking was indeed affected in the *rsp5-1* mutant. The Ste6 protein was no longer delivered to the vacuole, but instead accumulated at the vacuolar membrane. However, Rsp5 was not the E3 ligase for the Ste6 protein, since Ste6 ubiquitination was not affected in the *rsp5-1* mutant, suggesting that the observed effects are indirect. Indeed, we found that free ubiquitin levels were reduced in this mutant, indicating that this could be the problem in the *rsp5-1* mutant. It appears that reduced synthesis of ubiquitin contributes to ubiquitin depletion. A transient inhibition of protein synthesis is observed in wildtype upon heat-shock. And, while wildtype cells recover quickly from this transient arrest, the *rsp5-1* cells remain arrested, suggesting that Rsp5 is important for recovery from heat-induced protein synthesis arrest.

In addition, the role of ubiquitination on Ste6 trafficking was studied. The distribution of Ste6 variants with reduced ubiquitination was examined. We found evidence that ubiquitination controls Ste6 sorting in the early endocytic pathway. While wildtype Ste6 was mainly localized to internal structures, ubiquitination deficient Ste6 variants accumulated at the cell surface in a polar fashion. However, in a recycling deficient *ypt6-2* mutant, these Ste6 variants were trapped inside the cell, indicating that their polar localization is achieved through endocytic recycling and localized exocytosis ("kinetic polarization"). Furthermore, it appears that Ste6 cycles through early endosomes, since its recycling was partially affected in the sorting nexin mutant *snx4Δ*. The identification of putative recycling signals in Ste6 suggests that recycling is a signal mediated process. Endocytic recycling and localized exocytosis could be important for Ste6 polarization during the mating process.

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7 LIST OF ABBREVIATIONS

^{35}S	radioactive Sulfer isotope
A	ampere
Ade	adenine
ALP	alkaline phosphatase
APS	ammoniumperoxydisulfate
Arg	arginine
BCIP	5-bromo-4-chloro-3-indolphosphate
BSA	bovine serum albumin
bp	base pair
CEN	centromere sequence
Ci	Curie
CPY	carboxypeptidase Y
d	days
DMF	dimethylformamide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide
DTAF	dichlorotriazinylamino-fluorescein
DTT	dithiothreitol
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin-ligase
E. coli	*Escherichia coli*
EDTA	ethylendiamintetraacetic acid-disodium salt
EE	early enosomes
e. g.	exempli gratia (=for example)
ER	endoplasmic reticulum
ERAD	ER associated protein degradation
ERQC	ER associated quality control
EtBr	ethidium bromide
GFP	green fluorescence protein
h	hour
His	Histidine

i. e.	id est (=that is)
kb	kilobase
kDa	kilo Dalton
KOAc	potassium acetate
Leu	leucine
LiOAc	lithium acetate
LAS	Luminescence imaging
Lys	lysine
µ	micro
m	mili
Met	methionine
min	minute
MVB	multi vesicular body
n	nano
NBT	4-nitrobluetetrazoliumchloride
OD_{600}	optical density at 600 nm
ONPG	2-nitrophenyl-ß-d-galactopyranosid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylenglycol
PMSF	phenylmethylsulfonylfluoride
PVC	prevacuolar compartment
rpm	rotation per minute
S. c.	*Saccharomyces cerevisiae*
SDS	sodium-dodecyl-sulfate
TEMED	N, N, N', N'-tetramethylethylendiamine
Tris	Tris(hydroxymethyl)aminomethan
Trp	tryptophane
ts	temperature sensitive
U	unit
Ura	uracil
V	volt
wt	wildtype
YNB	yeast nitrogene base

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Tamara Krsmanovic´ Düsseldorf, den 10.12.2004