Isolation and characterization of *Saccharomyces cerevisiae* mutants for vacuolar import and autophagocytosis

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I. CONTENTS

II. ABBREVIATIONS

a (or MAT**a**)	strain of mating type a
α (or MATα)	strain of mating type α
Ape1p	aminopeptidase 1
APE1 (or LAPIV)	aminopeptidase 1 gene
α-HK	α-hexokinase
c_f	final concentration
CPY	carboxypeptidase Y
FL	logarithmic growth
FS	stationary growth
g	gravity, unit of acceleration
MW	molecular weight
n.d.	no determined
no.	number
o/n	over night
OD_{600}	optical density at 600 nm
RT	room temperature
pH	hydrogen ion concentration, negative log of
ssDNA	salmon sperm DNA
$T_{1/2}$	half life time
V_f	final volume
via mutants	mutants in *vacuolar import and autophagocytosis*
v/v	volume per volume
w/v	weight per volume
wt	wild type

III. SUMMARY

The yeast vacuolar protein aminopeptidase 1 (Ape1p) is synthesised as a cytosolic precursor that is transported to the vacuole via the cytoplasm to vacuole transport (Cvt) pathway. The cytosolic protein is enclosed in a double-membrane vesicle, which is transported to and fuses with the vacuole releasing a single-membrane autophagic body into the vacuolar lumen. This is degraded and the precursor sequence of aminopeptidase 1 is removed. The Cvt pathway involves proteins that are involved also in macroautophagy. The cytosolic precursor protein and the matured vacuolar protein form homododecameric complexes. Only the matured homododecameric complex shows enzymatic activity. We developed a new genetic screen to isolate mutants in the biogenesis of the vacuolar aminopeptidase 1 based on the enzymatic activity. New mutants defective in the transport of aminopeptidase 1 to the vacuole have been isolated. Two of them have been characterised in detail. Those have defects in the dodecamer formation of the precursor form and accumulate it in pre-vacuolar vesicles. This suggests interdependence between the dodecamer formation and the formation of transport competent Cvt vesicles. The tentative identification was based on the screening of a yeast genomic library and sub-cloning of the particular genes. The effects of overexpression of some heat shock proteins, which are known to assist protein translocation processes, protein folding and assembly, indicated the participation of these factors in early events of the Cvt-pathway.

1. INTRODUCTION

1.1. Yeast *Saccharomyces cerevisiae* as a model system to study organelle biogenesis

Yeast *Saccharomyces cerevisiae* has several advantages for studying cellular biology of eukaryotes. The knowledge accumulated in the last two decades demonstrates that many of the fundamental cell biological processes are highly conserved among eukaryotic cells. Second, the entire genome of *S. cerevisiae* has been characterised genetically and physically. Third, yeast genetics enables the identification of proteins based on their cellular functions.

Haploid cells of *S. cerevisiae* exhibit either of two cellular phenotypes, the mating types **a** or **a** . These cells can reproduce vegetatively by a mitotic cell cycle. However, when cells of opposite mating type are cocultured, they exit the mitotic cell division cycle and conjugate, producing the yeast diploid (**a**/**a**) zygote. Like the haploid cells, **a**/**a** cells can reproduce by mitosis, but unlike **a** or **a** cells, **a**/**a** cells can not mate. Instead, they have the ability to undergo meiosis and sporulation when nutrients are limiting, thereby regenerating the two haploid cell types (figure 1). This allows random mutagenesis of the haploid genome and search for mutant cells expressing the desired phenotype. The genome of a mutant cell can be purified from non-related mutations by subsequent rounds of mating and meiosis. If the phenotype of the mutant cell is inherited 2:2 among the four spores, the phenotype is expressed independent of the segregation of the chromosomes and depends on a single mutation. The mutated genes are then identified by transforming of the mutant with a wild type yeast chromosomal DNA library.

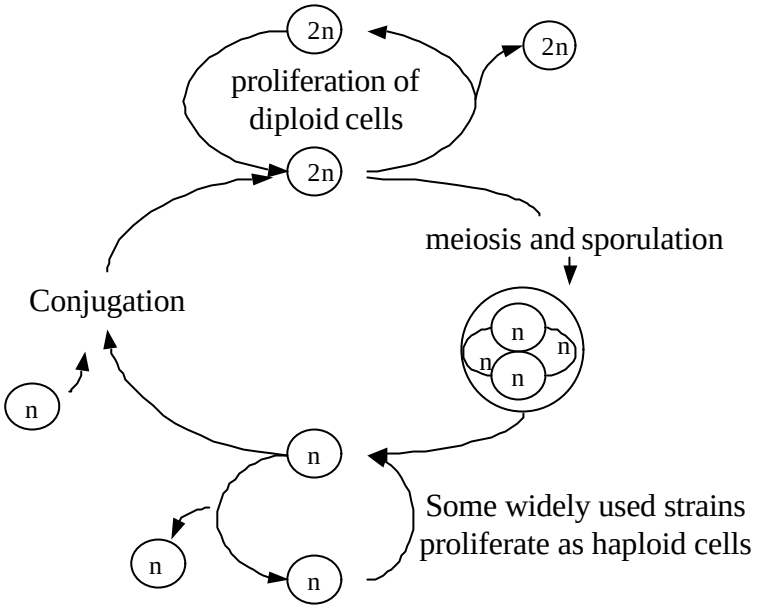


Figure 1: **The life cycle of budding yeast cells**

(according to Alberts et al., 1994)

A haploid nucleus was designed with „n" and a diploid nucleus with „2n", respectively.

1.2. The vacuole of *Saccharomyces cerevisiae*

1.2.1. Biogenesis and functions of the vacuole

Vacuoles are the largest compartments of the yeast, occupying up to 60% of the cell volume. They share many features with lysosomes in animal cells and tonoplasts in plant cells. All are organelles of low pH, rich in hydrolytic enzymes, surrounded by a single membrane. During cell growth, portions of the vacuole from the mother cell are transported into developing buds, indicating that specific cellular mechanisms exist that ensures partitioning of the organelle between dividing cells. In yeast *Saccharomyces cerevisiae,* the vacuole is involved in numerous cellular processes. The various functions of the vacuole rely on the specific and efficient delivery of its resident proteins *(Harding et al., 1995).* Vacuoles function in intracellular digestion of macromolecules. In response to changing growth conditions of yeast cells, the organelle undergoes dynamic changes. It serves as a major storage compartment for basic amino

acids, inorganic phosphate and calcium ions. Yeast mutants with impaired vacuolar function are sensitive to extremes of pH and osmotic strength, suggesting that the vacuole is required for pH and osmotic homeostasis. Protein turnover in yeast is accelerated in response to nitrogen limitation, and the liberation of amino acids from intracellular proteins requires vacuolar proteases. Vacuolar protease-deficient mutants exhibit drastically reduced viability upon nitrogen starvation. Diploids lacking vacuolar proteases fail to sporulate demonstrating the importance of vacuolar proteolysis for the survival and differentiation of the organism. *(Raymond et al., 1992; Teichert et al., 1989).*

1.2.2. Protein transport pathways to the vacuole

Subcellular compartmentalization is critical to eukaryotic cellular physiology. Accordingly, eukaryotes have evolved multiple mechanisms to deliver proteins to the various membrane surrounded subcellular compartments found in these cells. Proteins are known to enter the vacuole by five different mechanisms (figure 2). (a) The majority of the proteins are targeted to the vacuole via the secretory pathway. For example, carboxypeptidase Y (CPY) and proteinase A (PrA) are synthesized in the cytosol, enter the ER by translocation and further transit from the ER to the Golgi complex, where they are sorted into the vacuolar delivery pathway. These proteins move through the common compartments of the secretory pathway as inactive precursor proteins, but upon delivery to the vacuolar compartment, they undergo proteolytic cleavages and acquire their mature, enzymatically active form. Their propeptides contain vacuolar sorting information in addition to maintaining the hydrolases in an inactive state before their arrival in the vacuole *(Horazdovsky et al., 1995).* (b) Extracellular proteins destined for degradation enter the vacuole by endocytosis. For example, mating pheromones and their receptors are internalized and delivered to the vacuole via an endosomal intermediate *(Oda et al, 1996).* (c) Cytoplasmic proteins are taken up into the vacuole by autophagocytosis, presumably to allow recycling of critical metabolites *(Oda et al., 1996, Tsukada and Ohsumi, 1993; Thumm et al., 1994).* In order to survive starvation conditions, nonessential cytosolic proteins and organelles are sequestrated within autophagosomes, broken down and reused for essential cellular processes.

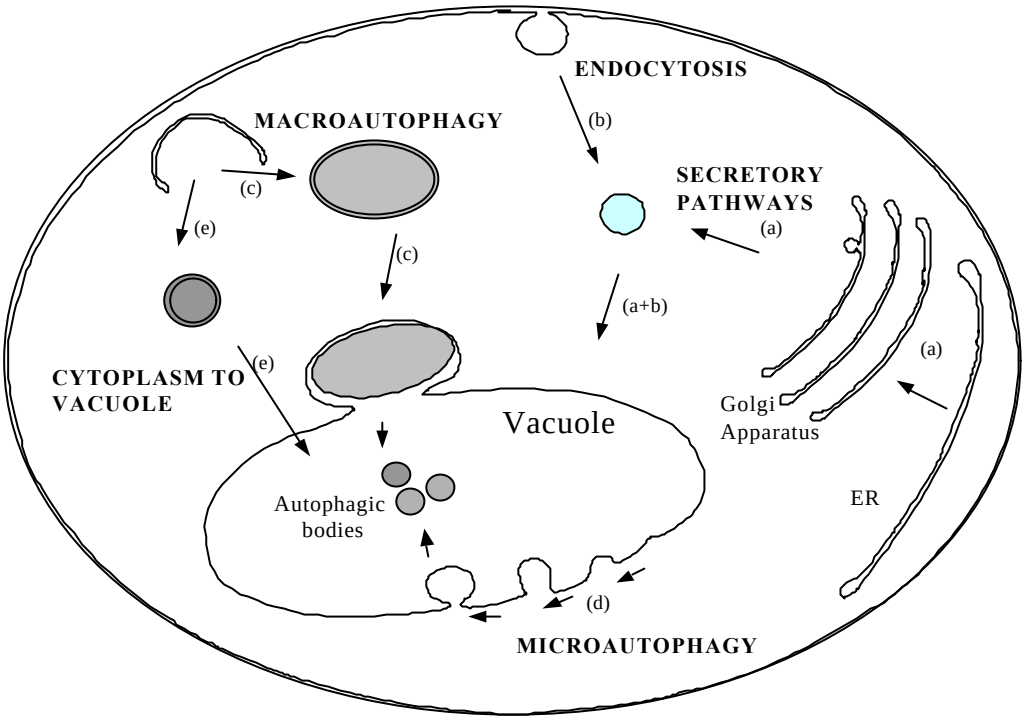


Figure 2: **Routes for protein delivery into the yeast vacuole**

(modified according to Scott and Klionsky, 1997, and Hutchins et al., 1999)

(a) the secretory pathway (soluble vacuolar glycoproteins and integral membrane proteins of the vacuolar membrane are co-translationally translocated into the ER and transit, via vesicular intermediates, to the *trans*-Golgi network, where they are sorted away from secreted proteins and further targeted to the vacuole);
(b) endocytosis (degradation pathway of extracellular proteins);
(c) macroautophagy (non-specific degradation of cytosolic components);
(d) microautophagy (specific degradation of cytosolic components);
(e) the cytoplasm to vacuole targeting pathway (Ape1p constitutive targeting pathway)

Autophagosomes are 400-900 nm diameter vesicles that could arise from the invagination of the rough ER. This process is named macroautophagy. (d) Microautophagy is a selective sequestration at the vacuolar membrane. Invaginations or finger like protrusions of the vacuolar membrane surround cellular components. For example, pexophagy is induced during adaptation to glucose of yeast cells, shifted from oleic acid or methanol. (e) Some resident proteins, in particular α-mannosidase and aminopeptidase 1, enter this organelle by an alternate mechanism directly from the cytoplasm (Cvt pathway). Cvt vesicles are about 150 nm in diameter. *(Oda et al., 1996;*

Klionsky et al., 1992; Harding et al., 1995)

Table 1: **Type of mutants that affect the vacuolar protein transport**

TYPE	MUTANTS	PHENOTYPE
pep4	deleted gene of proteinase A (*PEP4*)	lack many vacuolar hydrolytic enzymes (PrA, PrB, CPY, Ape1p)[(1)]
sec	secretory protein transport [(2)]	are temperature-conditional mutants and therefore are incapable of protein secretion under restrictive conditions
vps	vacuolar protein sorting [(3)]	missort and secrete vacuolar hydrolases as their Golgi-modified precursors; show changes in vacuolar morphology
vid	vacuolar import and degradation [(4)]	fail to degrade FBPase in response to glucose
apg *aut*	autophagy-defective [(5)] autophagy[(6)]	defective in accumulation of autophagic bodies; defective in protein degradation, in the vacuoles, induced by nitrogen starvation
cvt	cytoplasm to vacuole targeting [(7)]	were isolated as mutants defective in Ape1p import and maturation, using antiserum against pro-peptide of pApe1p; Overlap with *apg* and *aut.*
vac8	Vac8p's gene (*VAC8*) deleted [(8)]	defective in vacuolar inheritance; defective in protein transport from the cytoplasm
Dvat *vph*	Disruption of V-ATPase encoding genes [(9)]	missort soluble and membrane vacuolar proteins; defective in vacuolar acidification.

(1): Raymond et al., 1992; Hemmings et al., 1981; (2): Stevens et al., 1982;Eakle et al., 1988; (3): Bankaitis at al., 1986; Banta et al., 1988; (4): Hoffman and Chiang, 1996; (5): Tsukada and Ohsumi, 1993; (6): Thumm, et al., 1994; (7): Harding et al., 1996; Scott et al., 1996; Baba et al., 1997; (8): Wang et al., (1998); (9): Klionsky et al., 1992; Yaver et al., 1993.

1.3. Aminopeptidase 1- transport

Aminopeptidase 1 (Ape1p) is a resident vacuolar hydrolase, which catalyses the removal of leucine from the N-terminus of polypeptide chains.

Ape1p is encoded by a single copy gene, named *APE1* (previously *LAP4)*, located on chromosome XI. It is synthesized in the cytoplasm as a 61-kDa precursor (pApe1p) of 514 amino acids residues. Upon the post-translationary entry into the vacuole, pApe1p is matured by the endopeptidases PrA and PrB, yielding first a 55 kDa intermediate form and then the mature 50-kD form of 459 amino acids *(Segui-Real et*

al., 1995). The vacuolar mApe1p forms a homododecameric complex and dodecamerisation is required for its enzymatic activity. $T_{1/2}$ for processing is approximately 45 min under both vegetative and starvation conditions *(Klionsky et al., 1992; Scott et al., 1996).* pApe1p has a long processing time (45 min). It was found to be assembled into a dodecamer of approximately 732 kDa immediately after synthesis ($T_{1/2}$ 2 min) and was maintained in the oligomeric form throughout the targeting process (about 30 min) *(Kim et al., 1997)*.

The vacuolar localisation of Ape1p is independent of the secretory pathway *(Klionsky et al., 1992).* The precursor does not receive glycosyl modifications, and removal of its pro-peptide occurs in a *sec*-independent manner *(Klionsky et al., 1992, Chang and Smith, 1989).* The 45 amino acids precursor sequence forms a helix-turn-helix structure *(Martinez et al 1997; Chang and Smith, 1989).* Mutations that disturb formation of the first helix also abolish pApe1p transport *(Oda et al., 1996).* The N-terminus of pApe1p is processed prior to a C-terminal sequence-tag during the vacuolar import, suggesting that pApe1p is transported into the vacuole by a translocation event assisted by a *putative* receptor *(Segui-Real et al., 1995).* Ape1p's maturation is inhibited by low temperatures, which should not happen in a translocation event. Neither pApe1p nor the transport incompetent truncated mutants p(Δ1-16)Ape1p and p((Δ1-45)Ape1p are processed in the cytosol. This resistance to cytosolic proteases suggests that pApe1p is probably folded in the cytosol and that its translocation through the vacuolar

Table 2: **Effects of site directed mutations on pApe1p processing/targeting**
(changed accordingly to M.N.Oda et al, 1996)

Mutation	Ape1p Processing	Localisation
Δ3-5; Δ6-8	Complete block	Not determined
Δ9–11; Δ12-14; Δ15-17; Δ18-20	Complete block	Cytosolic
Δ25-27	wt	Not determined
Δ28-30; Δ31-33; Δ34-36	wt	Vacuolar
Δ37-39; Δ40-42	wt	Not determined
Ala11	Complete block	Cytosolic
Ala 34	wt	Vacuolar

membrane may require prior unfolding *(Segui-Real et al., 1995).*

Vacuolar protein degradation is enhanced under glucose starvation conditions in rich (YPD) or for nitrogen starvation in minimal (MV) media. Complementation analysis of yeast mutants defective in Ape1p maturation revealed that autophagy mutants are also defective in pApe1p transport suggesting that macroautophagy and Ape1p transport utilise many of the same molecular components. This is surprising because Ape1p targeting is known to be a selective and constitutive event, whereas macroautophagy is clearly non-selective and is induced by starvation. Ape1p import was examined by immuno-electron microscopy in both rich media and starvation conditions, to resolve the apparent discrepancy of how Ape1p could be transported both selectively and constitutively by macroautophagy, a mechanism thought to be non-selective and require starvation induction *(Baba et al., 1997).* In nutrient-rich conditions in wild-type cells cytosolic pools of pApe1p appeared clustered into specific regions of the cytosol called Cvt complexes. The clustered pApe1p appeared enwrapped in double-membrane structures called Cvt vesicles that were similar in appearance to autophagosones. The Cvt structures observed in nutrient-rich conditions were morphologically similar to the corresponding vesicular structures formed under starvation conditions with a few exceptions *(Baba et al., 1997).* The contents of Cvt vesicles stained densely and appear to be devoid of ribosomes, whereas the contents of autophagosomes are indistinguishable from bulk cytosol *(Baba et al., 1994; Baba et al., 1997).* The diameters of the two types of vesicles are different: autophagosomes were 400-900 nm in diameter, whereas the Cvt vesicles measured just about 150 nm. These data suggest that during growing conditions, vesicles are formed and those carry specific components, such as pApe1p, to the vacuole. Upon shifting to starvation, these vesicles are triggered to increase in size and gather in bulk cytosolic components in addition to their normal selective cargo.

The origin of the membrane, which may enwrap the pApe1p complex, is unknown as is the nature of the receptor, which may assist at some points in the pApe1p transport. The possibility of uptake of the monomer or oligomer pApe1p from the cytosol by a specific process, like microautophagy is not excluded. Macroautophagy as well as microautophagy and translocation may contribute to cytoplasm to vacuole transport of pApe1p.

Recently, the genes that complement several of the autophagy mutants have been cloned and sequenced. Apg5p *(Kametaka et al., 1996),* Apg13 *(Funakoshi et al., 1997)* and Aut1p *(Schlumpberger et al., 1997)* are predicted to be hydrophilic proteins of 33 kDa, 83 kDa, and 36 kDa, respectively. None of these polypeptides display significant homologies to other proteins in the database. Apg1p is predicted to be a novel serine/threonine kinase of 102 kDa *(Matsuura et al., 1997).* Through the investigation of Ape1p transport, a vacuolar t-SNARE Vam3p was identified *(Darsow et al., 1997).* This protein is required for the vacuolar delivery of vesicles originating from the endosome and is likely to be a component of the general recognition/fusion machinery at the vacuolar membrane. *cvt4* and *cvt8*, alleles of *vam4/vps39* and *vam2/vps41 (Harding et al., 1995)* , respectively, have been found to be members of a protein complex on the vacuole membrane *(Nakamura et al, 1997).* The vacuole Vp18p has been shown to be required for the delivery of both Cvt vesicles and autophagosomes to the vacuole *(Scott et al., 1997; Rieder et al., 1997).* A tubulin associated protein complex of Aut2p and Aut7p is required for vacuolar uptake of autophagosomes *(Lang et al., 1998).* Aut4p and Aut5p are essential for lysing autophagic vesicles in the vacuole *(Thumm et al., 1999).* A unique covalent-modification system was found to be essential for autophagy: the carboxy-terminal glycine residue of Apg12 (186 amino acid protein) is conjugated to a lysine at residue 149 of Apg5 (294 amino acid protein). Among the apg mutants, apg7 and apg10 were found to be unable to form an Apg5/Apg12 conjugate. The sequence of *APG7* revealed that Apg7p is a ubiquitin E1-like enzyme. The conjugation of Apg5/Apg12 could be reconstituted *in vitro* and showed dependency on ATP *(Mizushima et al, 1998).* Investigation of the function of Apg7p as an Apg12p-activating enzyme indicated that Apg12p might interact with Apg7p via a thioester bond, a mechanism already known for the ubiquitin conjugated system. Mutational analysis of Apg7p suggested that Cys^{507} of Apg7p is an active site cysteine and that the ATP-binding domain and the cysteine residue are essential for the conjugation of Apg7p with Apg12p. Cells expressing mutant Apg7p, $Apg7pG^{333A}$, or $Apg7pC^{507A}$ showed defects in autophagy and cytoplasm to vacuole targeting of pApe1p *(Tanida et al., 1999; Takahiro et al., 1999)*. Apg16 forms homo-oligomers and is a linker molecule forming an Apg12p-Apg5p-Apg16p multimeric complex *(Mizushima et al, 1999).*

2. AIMS OF THE PRESENT STUDY

The sorting and delivery pathways of pApe1p from the cytoplasm to the vacuole are controversly discussed: an incorporation into a cytoplasmatic vesicle followed by fusion with the target organelle as seen in autophagy, or a direct membrane translocation via an active transport protein or by membrane invagination as a result of binding of pApe1p to a receptor molecule.

In order to identify new factors required for transport of pApe1p, the first goal was to develop a new genetic screen in yeast *Saccharomyces cerevisiae* based on the enzymatic activity of the transported and matured dodecameric pApe1p, thus extending the screen to the final stage of pApe1p transport. EMS-mutagenesis and yeast genetics should be used to clone new genes required for cytoplasm to vacuole transport.

The second goal was to characterize these mutants biochemically, to learn more about the mechanism of pApe1p transport.

3. MATERIAL AND METHODS

3.1. Material

3.1.1. Chemicals

Acetic acid (Merck)

Acetone, p.a. (Merck)

30% Acrylamide / 0.8% Bisacrylamide in H_2O (Roth)

Agar (Sigma)

Agarose (Gibco BRL)

Ammoniumsulfate, $(NH_4)_2SO_4$ (Merck)

Ammonium persulfate (APS) (Merck)

Amino acids and nucleosides (Serva):

- Adenine, hemisulfate salt (MW=182.2)
- L-Arginine · HCl (MW=210.7)
- L-Aspartic acid*(MW=133.1)
- L-Glutamic acid (MW=147.1)
- L-Histidine · HCl (MW=209,6)
- L-Isoleucine (MW=131.2)
- L-Leucine (MW=131.2)
- L-Lysine · HCl (MW=182.7)
- L-Methionine (MW=149.2)
- L-Tyrosine (MW=181.2)
- L-Phenylalanine (MW=165.2)
- L-Serine (MW=105.1)
- L-Treonine (MW=119.1)
- L-Tryptophan (MW=204.2)
- Uracil (MW=112.1)
- L-Valine (MW=117.2)

Bacto Agar (Difco)

Bacto-Peptone (Difco)

Bromphenolblue (BioRad)

1-Butanol p.a. (Merck)

Calciumchloride, $CaCl_2$ (Merck)

Citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$ (MW=210,14) (Merk)

Chloroform p.a. (Merck)

Coomasie, Serva Blue R (Serva)

Diethyl pyrocarbonate (DEPC) (Sigma)

Dextransulfat (Pharmacia)

Dimethylsulfoxide (DMSO) (Merck)

Dithiothreitol (DTT) (Serva)

o-Dianisidine, $C_{14}H_{16}N_2O_2 \cdot 2HCl$ (Sigma)

ε-Amino-n Caproic Acid, $C_6H_{13}NO_2$ (MW=131.2) (Sigma)

Ethanol p.a. (Merck)

EDTA, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$ (MW=372.24) (Merck)

Ethidium bromide (MW= 394.32) (Serva)

Ficoll (MW = 378.5) (Sigma)

Formaldehyde (Merck)

(D+) Glucose Monohydrate (Merck)

Glycerol ($C_3H_8O_3$; 1 l =1.26 kg) (Merck)

HEPES (N-2-Hydroxyethylpiperazin-N-2-ethansulfonsäure) (Serva)

Isoamylalcohol (Merck)

Lithium Acetate ($C_2H_3O_2Li \cdot 2 H_2O$; MW= 102.0) (Sigma)

Luminol (3-aminophthalhydrazide) (Fluka)

2-Mercaptoethanol (MW=78.13) (Merck)

Methanol (Merck)

3-[N-Morpholino]propanesulfonic acid (MOPS), $C_7H_{15}NO_4S$ (MW=209.3) (Serva)

OptiPrep Density Gradient Medium (60% w/v solution of Iodixanol) (Sigma)

p-Coumaric acid (Sigma)

Perhydrol, 30% H_2O_2 (Merck)

Phenol (Merck)

Piperazine-N,N'-bis[2-etanesulfonic acid] (PIPES) (MW=378.5) (Sigma)

Polyethylenglycol (PEG 4000) (BDH Laboratory, Engl.)

Phosphoric acid, 85% H_3PO_4 (Merck)

Potassium Acetate (Merck)

2-Propanol p.a. (Merck)

Sephadex G-50 (Pharmacia)

Sodium Acetate, CH_3COONa (MW=82.03) (Merck)

Sodiumcitrat (Merck)

Sodium dodecyl sulphate (SDS), $C_{12}H_{25}O_4SNa$ (M=288.04) (Sigma)

Sodiumhydroxide (MW=40.00) (Merck)

Sodiummonohydrogenphosphate $Na_2HPO_4 \cdot 2H_2O$ (MW=177.99) (Merck)

Sodiumdihydrogenphosphate $NaH_2PO_4 \cdot H_2O$ (MW=137.99) (Merck)

Sorbitol, $C_6H_{14}O_6$ (MW=182.2)(Sigma)

Sucrose (Merck)

Sulphuric acid 95-97% (Merck)

N'N'N'N Tetramethylethylendiamine (TEMED) (Serva)

Trichloracide (TCA) (MW=163.39) (Merck)

Tris-Base (Tris) (MW=121.14) (Biomedicals, INC)

Tryptone (Difco)

Yeast extract (Difco)

Yeast Nitrogen Base w/o Amino Acids (Difco)

3.1.1.1. Antibiotics

Ampicillin trihydrate (Serva)

3.1.1.2. Enzymes

L-amino acid oxidase (type II, crude dried venom from *Bothrops atrox*; 0.64 U/mg solid) (Sigma)

β-Glucoronidase (from *Helix pomatia*; 100 U/µl) (Sigma)

Horseradish peroxidase (200 purpurogallin U/mg solid) (Sigma)

Lyticase from *Oerskovia xanthineolytica* (>7000U/mg; 0.5mg/ml) (Boehringer Mannheim)

Lysozyme (from hen's egg white; 100000 U/mg) (Merk)

Proteinase K (from *Tritirachium album*) (Boehringer, Mannheim)

Phosphatase, alkaline from calf intestine (1U/µl, S.A. 2000 U/mg) (Boehringer, Mannheim)

Restriction endonucleases (Boehringer, Gibco BRL, New England Biolabs)

RNase A (Boehringer, Mannheim*)*

Zymolyase 20T (from *Arthrobacter luteus)* (Seikagaku Corporation, Japan)

T4-DNA-Ligase (New England Biolabs)

Trypsine (from bovine pancreas; 48 U/mg) (Serva)

3.1.1.3. Detergents, Substrates and Inhibitors

Substrates:

- H-Leu-β-Naphtyl Amid (H-Leu-β-NA) (MW=256.4) (BACHEM)
- H-Leu-p-Nitroanilid (H-Leu-p-NA) (MW=256.4) (BACHEM)
- Z-Gly-Leu (BACHEM)

Detergents:

- Tween 20 (Serva)
- Tween 40 (ICN)
- Triton X-100 (Boehringer Mannheim)

Inhibitors:

- 5 - inhibitor mix, in DMSO:

  0.75 mg/ml Antipain

  0.25 mg/ml Chymostatin

  0.25 mg/ml Elastin

5 mg/ml Pepstatin

1.25 mg/ml Leupeptin

- PMSF (phenylmethyl-sulfonyl-fluoride) 100x: 200 mM in DMSO
- EDTA (Sigma)
- Natriumazide (NaN_3) (Sigma): 10% in H_2O

3.1.1.4. Standards

Standards for proteins:

- Bovine Serum Albumin Fr. V pH 7.0 (BSA)(MW=67000) (Serva)
- Ovalalbumine (MW=45)
- Thyroglobuline (MW=669)
- Rainbow™ protein molecular weight markers (Amersham Life Science)
- Prestained protein molecular weight markers (Calbiochem)
- BenchMark™ prestained protein ladder (Gibco BRL)

Standards for DNA:

1kb DNA-Ladder (Gibco BRL)

3.1.1.5. Radioactive substances

α- [^{32}P]-dCTP, 3000Ci/mmol (Ammersham)

3.1.1.6. Kits and Nucleotides for work with Proteins, DNA and RNA

RNeasy Mini Kit (Qiagen, Hilden)

Super Signal™ CL-HRP Substrate System (Pierce, Rockford/USA)

Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA)

Megaprime DNA Labelling mix

3.1.1.7.Antibodies

Table 3: **Antibodies used for biochemical characterisation of *via* mutants**

NAME	DILUTION	TYPE	SOURCE
Goat anti Rabbit-HRP	1:10000	second antibody	Dianova, Hamburg
Anti-APE1	1:1000	-first antibody -polyclonal	Dr. P.Schu, Göttingen University
Anti-CPY	1:10000	-first antibody	Dr. H.D.Schmidt, MPI Göttingen
Anti-α-HK	1:1000	-first antibody	Dr. H.D.Schmidt, MPI Göttingen
Anti-Ssa1/Ssa2	1:10000	-first antibody	Dr. E.Craig, Chicago University
Anti-αPcs60p	1:10000	-first antibody	Prof. R. Erdmann, Berlin University

3.1.2. Yeast strains

Table 4: **Yeast strains used for generation and selection of *via* mutants**

STRAINS	CHARACTERISTICS	SOURCE
SEY6211	MATa, ura3-52, his3Δ1, leu2-3,-112, trp1-289, suc2-D9, ade	Prof.Dr. S. Emr, UCSD, California, USA
II-17	MATα, ura3-52, his3Δ1, leu2-3,-112, trp1-289, lap1, lap2, lap3, lap4	Prof.Dr. D.H.Wolf, University Stuttgart
14 mutants	original EMS-mutants: MATα, Δlap1-4; pRS/LAP4, trp+	Dr.P.Schu, Göttingen University

3.1.3. Yeast DNA libraries

Table 5: **Yeast DNA libraries used for screening of *via10* and *via14***

NAME	SELECTION MARKER	SOURCE
YCP 126	**leu+**	Dr. H.D.Schmidt, MPI Göttingen
pYEP 13	**leu+**	Dr. H.D.Schmidt, MPI Göttingen
P1-P5	**ura+**	C. Sengstag, ETH Zürich

3.1.4. Bacteria

Table 6: ***E.coli*** **used for cloning experiments**

NAME	**GENOTYPE**
DH5α	F'/endA1 hsdR17 (rk – mk +) supE44 thi-1 recA1 gyrA
XL1Blue	

3.1.5. Vectors

pBlueskript KS II+ (Stratagene)

pCS19 *(Sengstag, C. (1993) Gene 124, 141-142)*

pRS *(Sikorski, R.S., and Hieter P., (1989), Genetics 19-27)*

3.1.6. Plasmids

Table 7: **Plasmids used for cloning experiments**

NAME	AUXOTROPHY MARKERS	SOURCE
p313/LAPIV p314/LAPIV	his+ trp+	Dr.P.Schu, Göttingen University
p701/ SSA2 p703/ SSA1	leu+ leu+	Dr.M.Horst, Göttingen University

p280/Vti1-2µ p283/Vti1-cen	ura+ ura+	Dr.G.Fischer von Mollard, Göttingen University

3.1.7. Buffers and stock solutions

All buffers and solutions listed were prepared in bidistilated H_2O using chemicals of highest purity available.

Ampicillin

Stock solution: 25 mg/ml of the sodium salt ampicillin in H_2O. Sterilised by filtration and store in aliquots at –20°C. Working concentration 50-100 µg/ml.

0.1 M $CaCl_2$

$CaCl_2.2H_2O$ (14.7 g) dissolved in H_2O (V_f =1 litre); autoclaved.

50 x Denhardts

1% Ficoll, 1% Polyvinylpyrolidon, 1% BSA.

1M DTT

DTT (3.09 g) dissolved in H_2O (V_f = 20 ml) and stored as aliquots at –20°C.

10 mg/ml Ethidium bromide

Ethidium bromide (0.2 g) dissolved in H_2O (V_f = 20 ml). Store at 4°C in dark. Handle with gloves and avoid inhalation!

0.5 M EDTA

$Na_2EDTA.2H_2O$ (93.05 g) dissolved in H_2O (300 ml). pH adjusted to 8.0 with 10 M NaOH (cca 25ml). Will not go into solution until about pH=7! Added H_20 to 500 ml.

20% D-Glucose

D-Glucose (100 g) dissolved in H_2O (V_f = 500 ml). Sterilised in autoclave.

100mM IPTG (isopropyl-β-D-thiogalactopyranoside)

23.8 mg IPTG in 1 ml H_2O.

NaP_i 0.1 M pH 7.4

Prepare 100 ml of NaH_2PO_4 0.5 M (6.89 g in H_2O) and 100 ml of Na_2HPO_4 0.5 M (8.89 g in H_2O). Correct the pH of one with the other one. For 50 ml NaPi 0.1 M pH 7.4 dilute 10 ml NaPi 0.5 M pH 7.4 with 40 ml H_2O.

10M NaOH

NaOH (200 g) in dissolved H_2O (V_f = 500 ml).

ssDNA stock solution

10 mg/ml in H_2O.

sonicated and denatured for 5 min at 95°C; chilled on ice.

1.2 M Sorbitol, 50 mM Tris/Cl pH 7.5, 10 mM EDTA pH 8 (STEDTA)

54.66 g	Sorbitol
5 ml	EDTA 0.5 M pH 8
12.5 ml	Tris/Cl 1M pH 7.5
till 250 ml	H_2O

0.8 M Sorbitol in TE

0.8 ml	Tris/Cl 1M pH 8
0.16 ml	EDTA 0.5 M pH 8
64 ml	Sorbitol 1M (or 11.66 g Sorbitol)
till 80 ml	with H_2O

3 M Sodium acetate

Sodium acetate.$3H_2O$ (40.8 g) dissolved in H_2O (100 ml). Adjust pH to 5.2 with 3M acetic acid.

20% SDS

SDS (20 g) dissolved in H_2O (V_f = 100 ml). Avoid breathing dust!

50 % TCA

50 g in 100 ml H_2O

1M Tris/HCl:

Tris base (121g) dissolved in H_2O (800 ml). Adjust to desired pH with concentrated HCl. Add H_2O to 1 litre.

50 x TAE

2 M Tris-Cl

0.1 M EDTA, pH 8.0 (correction with CH_3COOH)

1 x TE

10 mM Tris/Cl pH 7.5

1mM EDTA

3.2. *Sacharomyces cerevisiae* protocols

3.2.1. Yeast cultures

3.2.1.1. Media for yeast culture

YPD-Medium

1% Yeast Extract

2% Bacto-Peptone

2% D-Glucose

2% Bacto-Agar

For preparation of 1000 ml solid YPD-medium, Yeast Extract (10 g), Bacto-Peptone (20 g) and Bacto-Agar (20 g) were dissolved in 900 ml ddH_2O and sterilised by autoclaving. Afterwards, 100 ml of 20% autoclaved D-Glucose stock solution was added. For preparation of liquid YPD-medium, bacto-agar was omitted.

GNA-Medium (pre-sporulation medium)

1 % Yeast Extract

3 % Nutrient Broth

5 % D-Glucose

2 % Bacto-Agar

For preparation of GNA-Medium (500 ml) Yeast Extract (5g), Nutrient Broth (1.5 g) and Bacto-Agar (10 g) were dissolved in ddH_2O, to final volume of 375 ml and sterilised. Afterwards, 125 ml of 50% D-Glucose stock solution were added.

Acetate-Medium (sporulation medium)

0.125% Yeast Extract

1.47% Potassium Acetate

0.1% D-Glucose

2% Bacto-Agar

Acetate-Medium (500 ml) was prepared from Yeast Extract (0.625 g), Potassium Acetate (7.35 g) and Bacto-Agar (10 g) dissolved in 497.5 ml ddH_2O and sterilised. Afterwards, 2.5 ml of 20% D-Glucose stock solution were added.

Synthetic minimal medium (MV)

0.67 % Bacto-yeast nitrogen base w/o amino acids

2 % (or 0.5 %) D-Glucose

2 % Bacto-Agar

as needed Amino acids

For preparation of synthetic minimal medium (600 ml) Bacto-yeast nitrogen base w/o amino acids (4 g) and Bacto-Agar (12 g) were dissolved in ddH_2O and sterilised. D-Glucose (20% stock solutions) and amino acids mixtures were prepared separately and added as required. In table 7 are listed amino acids and their concentration on synthetic complete medium. For preparation of liquid SD-medium, bacto-agar was omitted.

YPO-Medium

0.15% Oleic acid

0.015% Tween 40

0.3% Yeast Extract

0.3% Peptone

0.7% KH_2PO_4

For preparation of liquid YPO-medium (500 ml) Yeast Extract (1.5 g), Bacto-Peptone (1.5 g), KH_2PO_4 (3.5 g) and Tween 40 (75 µl) were dissolved in ddH_2O at final volume and sterilised. Oleic acid (750 µl) was added and medium was stored protected from light.

Table 8: **Amino acids, nucleoside and their concentration in synthetic minimal medium (MV)**

Amino acids and nucleoside (*=no autoclaving)	Final concentration in SD (mg/l)	Storage of stock solution
Adenine sulphate	20	
L-Tyrosine	30	
L-Phenylalanine	50	
L-Glutamic acid	100	At room temperature
L-Aspartic acid*	100	
L-Methionine	20	
L-Arginine - HCl	20	
L-Isoleucine	30	
L-Lysine - HCl	30	
L-Valine	150	
L-Treonine*	200	At-20°C
L-Serine	375	
Uracil	20	
L-Leucine	30	
L-Tryptophan	20	
L-Histidine	20	

3.2.1.2. Inoculation and growth of yeast cultures

Yeast is non-pathogenic and therefore can be handled with few precautions. Yeast cultures were grown from a few single colonies picked from fresh selective-medium plates, at 30°C on YPD or MV media.

Usually, a pre-culture was prepared to inoculate the final culture. For pre-cultures single colonies were inoculated into 1-5 ml of the appropriate media and grown for 12-16 hours. High aeration was achieved by vigorously shaking (for example, at 200 rpm) and the culture volume was less than one third of the flask volume.

In general, the strains reached a maximum optical density OD_{600} = 2.5 in YPD. Haploid strains had a doubling time of 120 min to 210 min depending on the strain and culture media.

3.2.1.3. Yeast-strain preservation

Yeast strains have been stored for short periods of time at 4°C, on YPD medium in Petri dishes. Passages of the stock are recommended in 2 to 4 weeks intervals. Indefinitely, yeast strains are stored in 15-30% (v/v) glycerol at –80°C. To preserve yeast-strains indefinitely, either a fresh YPD-liquid culture of cells grown in appropriate media is mixed with sterile glycerol, or fresh cells grown on selective media are scraped up and suspended in the sterile YPD-glycerol solution. The caps are tightened and the vials shaken before freezing. Transferring a small portion of the frozen sample to a YPD plate can revive yeast. *(Guthrie and Fink, 1991)*

3.2.2. Tetrad analysis

3.2.2.1. Sporulation of yeast strains on plates

The products of a single meiosis in yeast are 4 haploid spores (the tetrad) hold together in a single ascus. Meiosis is induced by shifting cells to a medium without a source of

nitrogen and with acetate as the sole carbon source. Meiosis is not induced in H_2O *(Guthrie and Fink, 1991).*

To achieve sporulation cells were grown on YPD-plates for 2-3 days. Then the cells were spread on pre-sporulation media (GNA), so that one clone covered about a quarter to one half of a plate and incubated regularly about 2 days. The cells should form a thick confluent layer. Afterwards, all the cells were transferred from GNA-plates onto sporulation medium (Acetate medium) in a way that they were spread over an area of the size they were grown on the GNA-plates. The first spores could appear on sporulation medium after 3-5 days at 30°C.

3.2.2.2. Micro-manipulation and dissection of ascospores

Sporulated cultures consist of a mixture of unsporulated vegetative cells, four-spore asci, three-spore asci, etc. Dissection of asci requires the identification of four-spore asci and the relocation of each of the four ascospores to separate positions on an agar plate where they form isolated spore colonies. The procedure requires digestion of the ascus wall without dissociating the four spores from the ascus. The relocation and transfer of ascospores, zygotes, and vegetative cells were carried out, as shown in figure 3, on agar surfaces with a

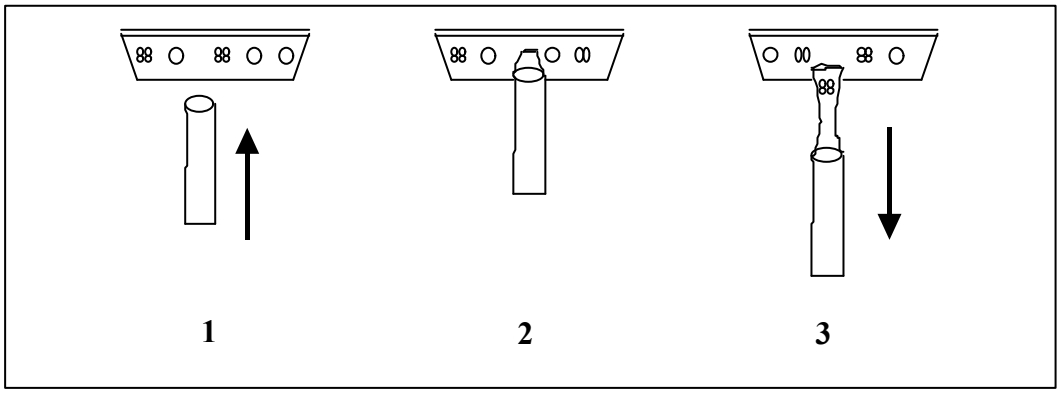


Figure 3: **Transfer of the four ascospores from an agar surface to the platform of a micro-needle**

(modified according to Guthrie and Fink, 1991)

optic glass fiber micro-needle (Singer Instruments) mounted in the path of a microscope (Zeiss) objective and a micromanipulator.

To separate spores, sporulated cells were re-suspend in 100 µl H_2O. 2µl of β-glucoronidase (100 U/µl) were added. The suspension was incubated 20 min at 30°C and then stored at 4°C up to one week. Cells were spread on one side of a YPD-plate. With a needle controlled by the micromanipulator, spores were isolated from the surrounding unsporulated cells, freed from the surrounding asci-wall and the spores were aligned as shown in figure no. 3-2 and labelled A, B,C and D.

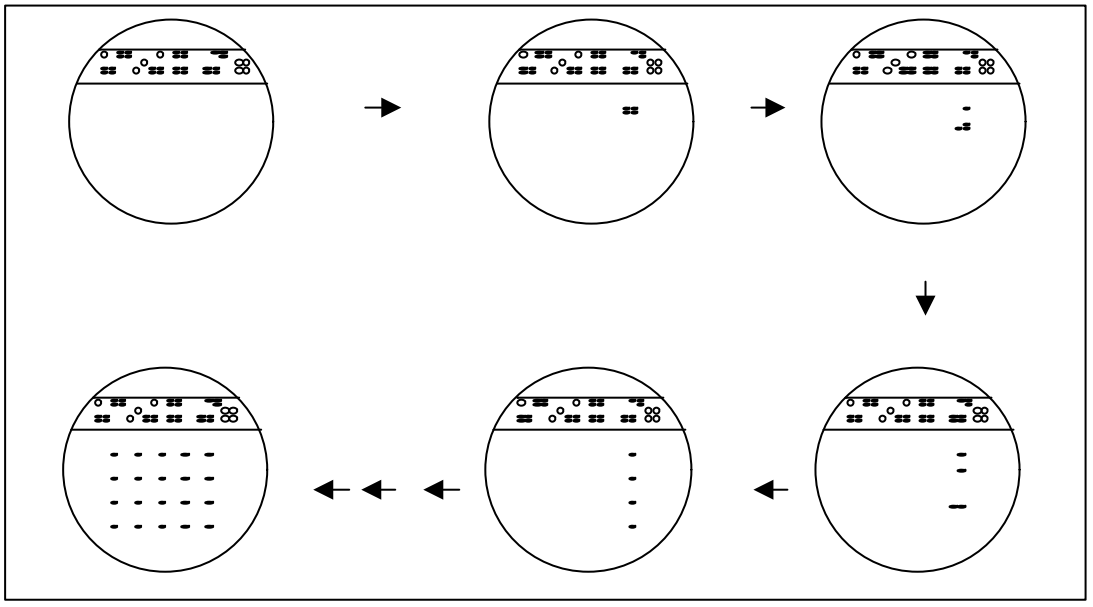


Figure 4: **The steps for sequentially separating the cluster of four ascospores on Petri dishes** *(according to Guthrie and Fink, 1991)*

3.2.2.3. Mating-type determination

Mating-type determination based on complementation of auxotrophic markers

When haploid cells of opposite mating type and carrying different markers for auxotrophy are co-cultured, they participate in a mating process. The result of mating is a diploid that carries both sets of markers for auxotrophy.

One strain of type **a**/trp+ is normally cultured on MV-Trp, and a strain of type α/his+ is normally in cultured on MV-His. When, on a YPD-plate, a few young cells of type MATa/trp+ were physically put in contact with a few young cells of type MATα/his+, after 6-12 hours, these cells should mate. A mixture of MAT**a**-type, MAT**a** –type and MAT**a**/**a** - type cells is formed. In order to separate the diploids from the haploid - "parents", the cells were plated on double selective media, MV-Trp\His.

Mating-type determination by tester-strain RC828 (MAT**a**)

When hundreds of cells are assayed for mating-type, as is the case during tetrad analysis, the best choice to distinguish between MAT**a** -cells from those being MAT**a** or MAT**a**/**a** is a method based on a pheromone test. This method is based on the fact that α-factor secreting cells induce a growth arrest in **a** cells. The tester strain RC828, MAT**a** is not able to leave the growth arrest in G1and is therefore not able to grow in the vicinity of α-factor secreting cells.

RC828 (MAT**a)** grown over night in YPD-liquid at 30°C was diluted 1:10 or 1:100 in the overlay-agar and the mixture kept liquid at 45°C and spread over YPD-plates. After the agar turned solid, strains to be tested were transferred on the overlay agar by an applicator-needle. The plates were incubated at 30°C for 1-3 days until the growth – inhibition of the tester-strain became visible. If colonies secrete pheromone, they inhibit the growth of the tester-strain in the overlay agar. This keeps the overlay- agar around the colonies transparent. Colonies that do not produce pheromone are presumably of the same mating type as the tester strain (here **a**) or **a**/α – diploids. This assignment was confirmed by determining whether they can sporulate or by mixing with α-type cells and monitoring the mating process microscopically, as described further.

Table 9: **Rule for mating-type determination based on pheromone tests**

tester-strain RC828 (MAT**a**)	Rule1:	Halo	strain tested of α-type
	Rule2:	Non-halo	strain tested of α/**a** or **a**-type; further tests are required

Overlay-Agar:

0.8% Agar

0.1M Na-Citrat, in YPD pH 4.5

The pH of 0.1M Na-Citrat in YPD (100 ml) was corrected with 0.1M Citric acid in YPD (100 ml). 0.8% Agar (w/v) was added and sterilised.

Whenever a few number of strains were assayed for mating type a method based on observations of morphology changes (figure 5) was applied. The method was used to

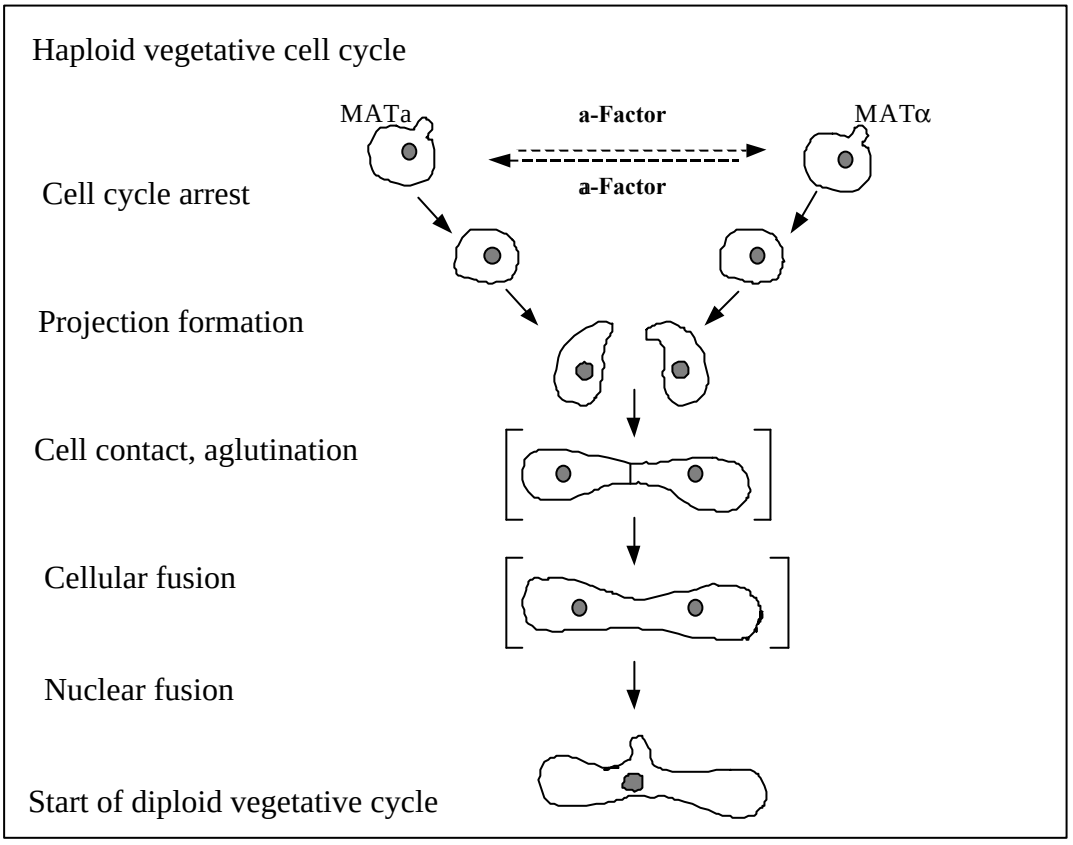


Figure 5: **The conjugation process in *S. cerevisiae***

(according to Berlin et al., 1991)

MAT**a** mixed with MATα cells, mutually stimulate each other via secreted peptide pheromones to arrest growth in G_1, induce transcription of mating-specific genes and form projections, that give the cell a pear shape, in the direction of perspective mating partners. Contacts lead to a rapid reorganisation of the cell surface and mixing of cytoplasmic contents. Nuclear fusion follows cellular fusion, and the diploid cell commences vegetative growth.

confirm the isogenity of a cell line, additional test to distinguish between **a** and **a/a** strains after pheromone tests, determination of the mating-type when the test based on complementation of nutritional requirement could not be performed.

On a YPD-plate, cells were spread with an applicator-needle as patches together with an **a**-tester strain and an α-tester strain. A third part of the plate was used for mixing the cells with each tester strain. The cells were grown for 1-2 days before mixing. After mixing, within 5-8 hours, they were analysed with an optical microscope *(20x- 40x)* for the formation of the diploid yeast zygote.

3.2.3. Ethylmethane sulphonate (EMS) - mutagenesis

EMS-mutagenesis was performed using the mutagenic agent ethylmethane sulphonate (EMS), an alkylating agent which induces high frequencies of base-pair substitution, almost exclusively at G.C sites.

A stationary yeast culture of II-17 transformed with pRS/314 LAP4 (trp+) was harvested and the cell pellet was re-suspended in 3 ml 0.1 M NaPi pH 7.4. 100µl EMS were added and the suspension was incubated for 30 min at 30°C. Cells were washed 3 times with H_2O, plated onto YPD plates and incubated for three days at 30°C.

3.2.4. Transformation of yeast cells

3.2.4.1. Yeast transformation by lithium-acetate method

The most frequently used transformation protocol for introduction of plasmid DNA into yeast cells is the lithium acetate procedure. This method is based on the fact that alkali cations make yeast competent to take up DNA. After yeast is briefly incubated in buffered lithium acetate, transforming DNA is introduced with carrier DNA. Addition of polyethylene glycol (PEG) and heat shock trigger DNA uptake. The yeasts are then plated on selective media.

Making yeast competent cells for lithium-acetate transformation

Two days before starting the experiment cultures were inoculated in 50 ml YPD-medium. The cells were grown overnight at 30°C, to OD_{600}= 0.6-1.2. If higher efficiency was desired the cells were diluted to OD_{600}= 0.3-0.4 in fresh YPD medium and grown for another 1 to 2 generations (2 to 4 hours). The cells were harvested at 3000 rpm for 5 min in Heraeus centrifuge and then washed 3-4 times with sterile H_2O. The cell pellet was re-suspended in 1.5 ml LiOAc/TE 1x and kept on ice up to 14 hours. Minimum time required to prepare competent cells is 2 hours, at 30°C.

Lithium acetate transformation

For each transformation, 200 µl suspension of yeast competent cells were mixed gently with 5 µg plasmid DNA and 100 µg ssDNA in a sterile 1.5 ml centrifuge tube. The mixture was incubated at 30°C for 15-30 min. Further, fresh prepared 40% PEG 4000 in LiOAc/TE (1.2 ml) was added to each tube. The tubes were mixed by inverting or gentle pipetting with a 1 ml tip and then incubated for 30 min at 30°C with gentle agitation or inverted occasionally. After heat shock for 5 min at 42°C and brief cooling on ice, the cells were spun down by three times centrifugation for 5 seconds each. The supernatant was removed and the cell pellet was re-suspended gently in 0.8M sorbitol-LiOAc/TE. Cell suspension (200 µl) was spread onto appropriate selection plates. The plates were incubated at 30°C for a minimum of 2-3 days.

LiOAc/TE 1x:

1 vol.	10x TE pH 7.5
1 vol.	10x LiOAc pH 7.5
8 vol.	H_2O

0.8M Sorbitol in LiOAc/ 0.1M TE (1x):

8 vol.	1M Sorbitol
1 vol.	LiOAc/ 1M TE
1 vol.	H_2O

10x LiOAc pH 7.5:

1M LiOAc pH 7.5, corrected with CH_3COOH

3.2.4.2. Yeast transformation by electroporation

When limiting quantities of transforming DNA were available, or a higher transformation efficiency was desired, method of choice to bring plasmid DNA into yeast cells was electroporation.

Electroporation of plasmid DNA into electro-competent yeast cells was done using the Stratagene Electroporator 1000.

Electrocompetent cells were prepared from 250 ml early exponentially grown cultures in YPD. The cells were harvested at 5000 rpm in JA-10 rotor of Beckman centrifuge, for 5 min, at 4°C. The pellet was washed twice with 250 ml and 125 ml ice-cold H_2O, respectively, re-suspended in 10 ml ice-cold 1M sorbitol and harvest at 5000 rpm using a JA-20 rotor of Beckman centrifuge, for 5 min at 4°C. The cells were re-suspended in 600-800 µl ice-cold 1M sorbitol and used for direct work or stored at 4°C up to one week.

Using the Electroporator 1000 for yeast transformation, field strength of 7.5 kV/cm were required to obtain maximum efficiency. DNA must be free from salt. The cells must be kept cold during the entire electroporation procedure.

Plasmid DNA (0.1 µg in less than 5 µl) was added to electrocompetent cells (65 µl) in a microcentrifuge tube. Cell - DNA mixtures were transferred to a pre-chilled cuvette and incubated 5 min on ice. The outside of the cuvette was dried, then the cuvette was inserted into cuvette holder and placed into the electroporator. After the pulse, the cuvette was removed and 1 ml of cold 1M sorbitol was added immediately. Various aliquots were plated onto selective plates containing 1M sorbitol.

3.2.5. Extraction of plasmid DNA from yeast

Extraction of plasmid yeast DNA was done either from a stationary liquid culture (2 ml) in YPD-medium or from cells of up to three SD-plates. Starting with a liquid culture, the cells were harvested at 13000 rpm, in an Eppendorf centrifuge, for 2 min. The pellet was re-suspended in lysis buffer (250 µl), with 300 mg glass beads and vortexed for 2 min. To remove proteins, phenol (250 µl) was added, mixed by vortexing for at least 2 min and centrifuged at 13000 rpm, for 5 min, at 4°C. The aqueous layer (about 200µl) was transferred into a new microcentifuge tube. If necessary steps 3 and 4 were repeated 1-2 times. Further, phenol: chloroform: isoamylalcohol (25: 24: 1, 200µl) was added and mixed as before. After centrifugation at 13000 rpm for 5 min at 4°C, the aqueous layer was transferred to a new of the supernatant was used for transforming directly 200 µl of *E. coli* (XL1Blue) - competent cells.

Whenever the quality of DNA was not sufficient, the plasmid DNA was further purified. Two methods were used: either as described for mini-preparation of plasmid DNA from *E.coli* (with a Qiagen-kit: applying DNA solution on a QIAprep spin column, washing and elution steps) or by precipitation with ethanol in the presence of 300 mM sodium acetate (1 vol. DNA solution mixed with 1/10 vol. of sodium acetate 3M. EtOH 100% added to final concentration of 70%, the mixture incubated for 30 min at –20°C. DNA was pelleted and washed with 80% ethanol).

The DNA was stored in TE at –20°.

Lysis buffer:

10 mM	Tris/Cl, pH 8
100 mM	NaCl
1mM	EDTA, pH 8
1%	SDS
2%	Triton X-100

Lysis buffer was prepared from appropriate stock solutions, as follows 1M Tris/Cl pH 8 (0.5 ml), 5M NaCl (2.5 ml), 0.5M EDTA pH 8 (0.1 ml), 20% SDS (2.5 ml), 100% Triton X-100 (1 ml), H_2O (to final volume of 50 ml), and sterilised.

3.3. *Escherichia coli* protocols

3.3.1. *E. coli* culture

3.3.1.1. Media for bacteria culture

LB-Agar:

1%	Bacto-Tryptone,
0.5%	Bacto-Yeast- Extract
0.5%	NaCl
1.5%	Agar

To prepare LB-Agar (1000ml), Bacto-Tryptone (10g), Bacto-Yeast- Extract (5g), NaCl (5g) and agar (15g) were dissolved in H_2O. The pH was adjusted to 7.4 with 1N NaOH. After sterilisation and cooling to 50°C antibiotics were added if desired.

LB (Luria-Bertani)-Medium:

1%	Bacto-Tryptone,
0.5%	Bacto-Yeast- Extract
0.5%	NaCl

To prepare LB-medium (1000ml), Bacto-Tryptone (10g), Bacto-Yeast- Extract (5g) and NaCl (5g) were dissolved in H_2O. The pH was adjusted to 7.4 with 1N NaOH and afterwards medium was autoclaved.

3.3.1.2. Inoculation and growth of bacterial cultures

Bacteria cultures were grown from a single colony picked from selective plates.

For pre-cultures single colonies were inoculated into 1-5 ml of the appropriate media and grown with vigorous shaking for 12-16 hours. Bacteria double each 30 min.

3.3.1.3. Bacteria-strains preservation

Strains were preserved for short time at 4°C, on selective medium plates (up to 4-6 weeks). Long time storage was achieved by freezing in the presence of cryo-protectants such as glycerol (15-30%) or DMSO (5-7%). Cultures were stored at –80°C. Recovery of the culture is done by removal of a few ice crystals into a fresh LB medium suitable supplemented. Repeated freezing and thawing of the stocks reduce viability.

3.3.2. Isolation of plasmid DNA from *E.coli*

3.3.2.1. Midi-preparation with Qiagen-kit

Midi-preparation with Qiagen-kit allows preparation of up to 100 µg of high- or low-copy plasmid DNA using a QIAGEN-tip-100, when it is started from maximum culture volumes of 25 ml for high-copy plasmid DNA and 100 ml low-copy plasmid DNA. The buffers used during the procedure are listed in the table 10.

A pre-culture of 2-5 ml LB medium containing the appropriate selective antibiotic was inoculated with single colony from a streaked selective plate and incubated for about 8 hours at 37°C with vigorous shaking. The pre-culture was diluted 1/500 to 1/1000 into selective LB medium and let grow at 37°C for 12-16 hours with vigorous shaking. The culture should reach a cell density of approximately 1 x 10^9 cells per ml. The bacterial cells were harvested by centrifugation at 9000 rpm for 15 min at 4 °C in a Beckman JA 10 rotor and the supernatant removed. The bacterial pellet was completely re-suspended in buffer P1 (4 ml, containing RNase A). Buffer P2 (4 ml) was added, the sample was gently mixed and incubated at room temperature for 5 min. After addition of chilled buffer P3 (4 ml), the sample was incubated on ice for 15 min. After centrifugation for 30 min at 4°C at 13000 rpm in a Beckman JA-20 rotor a clear supernatant, containing plasmid DNA was removed. The sample was loaded onto a QIAGEN-tip100 equilibrated with buffer QBT (4 ml) by gravity flow. The QIAGEN-tip was twice washed with 10 ml each buffer QC. Buffer QF (5 ml) was used to elute DNA. The DNA was precipitated by mixing with 0.7 volumes isopropanol at room-temperature and

recovered by centrifugation for 1 hour (at 4°C) at 12000 rpm in a Beckman JA20 rotor. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in a suitable volume of buffer (e.g. TE pH 8.0, or 10 mM Tris.Cl pH 8.5).

Table 10: **Composition of the buffers used for Midi-preparation of plasmid DNA from E.coli using a Qiagen-kit**

BUFFER	COMPOSITION	STORAGE
P1 (re-suspension buffer)	50 mM Tris.Cl pH 8.0 10 mM EDTA 100 µg/ml RNase A	4°C, after addition of RNaseA
P2 (lysis buffer)	200 mM NaOH, 1% SDS	room temperature
P3 (neutralisation buffer)	3 M Potassium Acetate pH 5.5	room temperature or 4°C
QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS pH 7.0 15% Isopropanol 0.15% Triton X-100	room temperature
QC (wash buffer)	1 M NaCl 50 mM MOPS, pH 7.0 15% Isopropanol	room temperature
QF (elution buffer)	1.25 M NaCl 50 mM Tris.Cl, pH 8.5 15% Isopropanol	room temperature
TE	10 mM Tris.Cl, pH 8.0 1 mM EDTA	room temperature

3.3.2.2. Mini-preparation of plasmid DNA from *E.coli*

Method with STET-buffer

Plasmid DNA preparation from E.coli started from 1.5-2 ml (mini-preparation) o/n liquid culture in LB-medium with ampicillin. The cells were harvested at 12000 rpm for 2 min in microcentrifuge and the pellet was re-suspended in STET-buffer (350 µl). STET-buffer containing 50000U/ml lysozyme (350 µl) was added, mixed and placed immediately at 95°C

for 3 min. After 5-10 min shock on ice and centrifugation at 14000 rpm for 10 min in a table-top centrifuge, the supernatant was removed and the pellet was dried at room temperature, and re-suspended in 50 µl of TE-buffer pH 8 or H_2O.

STET-buffer:

8 g	Sucrose
5 ml	Triton-X-100
5 ml	1M Tris/Cl pH 8
10 ml	0.5M EDTA pH 8
80 ml	H_2O

TE-buffer:

10 mM	Tris/Cl
0.1 mM	EDTA pH 8

Mini-preparation of plasmid DNA from E.coli using STET-buffer does not eliminate traces of RNA. If required RNA was removed by RNase treatment (20 µg/ml, 30 min, at room temperature) followed by phenol precipitation of the protein and ethanol precipitation of the DNA. DNA was re-suspended in 50-µl TE, pH 8.

Method with Qiagen-kit

Plasmid DNA purified by the QIAprep miniprep - procedure, was done as described in the protocol of the Qiagen-kit. There is expected a purification of up to 20 µg of high-copy plasmid DNA from 1-5 ml over night cultures of E.coli in LB-medium.

The QIAprep miniprep procedure is based on three steps: preparation and clearing of a bacterial lysate, a selective adsorption of plasmid DNA onto silica-gel membrane in the presence of high salt buffer, washing and elution of plasmid DNA in low-salt buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5.

The cell pellet from 2 ml liquid culture in LB-medium with ampicillin was completely

re-suspended in buffer-P1 with RNaseA (250 µl). Buffer-P2 (250 µl) was added and the sample was inverted gently. The lysis reaction was allowed to proceed for maxim 5 min when buffer N3 (350 µl) was added and the tube was gently mixed. The solution turned cloudy due to buffer N3 containing high salt concentration. After 10 min centrifugation, a compact white pellet containing co-precipitated chromosomal DANN with insoluble complexes containing salt, detergent, and protein, was formed. The resulted supernatant containing the plasmid DNA was applied to a QIAprep column, centrifuged for 30-60 sec, the flow-through was discarded and the QIAprep spin column was washed with buffer PB (0.5 ml) and buffer PE containing ethanol (0.75 ml). DNA was eluted with 50 µl of buffer EB (10mM Tris, pH 8.5) or H_2O and stored at –20°C.

3.3.3. Transformation of *E.coli* cells

3.3.3.1. Transformation of *E.coli* by $CaCl_2$ method

Preparation of *E. coli* cells competent for transformation

The use of bacteria as host cells in cloning experiments requires bacterial cells competent for DNA uptake. Usually, competent bacterial cells were prepared either fresh or in advance and stored as stock of 200 µl-aliquots at temperatures lower then –70°C.

For making competent cells by chemical method a pre-culture (0.4 ml) of *E.coli* in LB-medium without ampicillin were used to inoculate LB-medium without ampicillin (40 ml). After incubation at 37°C for about 1.5 h, time when bacteria grew exponentially (OD_{600} = 0.3), the cells were harvested by centrifugation in a JA-20 rotor at 7500 rpm, for 10 min at 4°C. The cell pellet was re-suspended in pre-cooled 40 ml of 0.1 M $CaCl_2$ for at least 45 min or over night on ice. Afterwards, the cells were harvested as before and re-suspended in 2 ml of ice-cold 0.1 M $CaCl_2$.

Transformation of *E.coli* with plasmid DNA

E.coli was transformed by adding 25-250 ng of yeast plasmid DNA to 200µl suspension of bacterial competent cells. After incubation for 30 min on ice, heat-shock at 42° C

for 3 min and chilling on ice for 1-3 min, 1 ml of LB-medium without ampicillin was added. After incubation for 1 hour at 37°C, the cells were harvested at 12000 rpm for 2 min and the supernatant volume was reduced to 250 µl. The re-suspended cells were plated out as 50 µl and 200 µl on different LB-plates with ampicillin (LB-Amp) for incubation (at 37 °C) overnight. If a Blue/White selection was desired, the cells were plated out on LB-Amp plates with 50 µl of 10% X-Gal - stock-solution in DMF and 50 µl of 100 mM (i.e. 23 mg/ml) IPTG in H_2O.

3.3.3.2. Bacteria transformation by electroporation

Making bacteria competent cells for electroporation

An *E. coli* culture (50 ml) grown over night until OD_{600} = 0.35, was transferred to a pre-chilled, sterile polypropylene tube and left on ice for 15–30 min prior centrifugation at 5000 rpm in JA-10 rotor for 15 min at 4°C. The cell pellet was washed twice with sterile water (50 ml, 25 ml) and once with 10% glycerol (20 ml). The cells were centrifuged at 6000 rpm in JA-20 rotor for 15 min at 4°C followed by re-suspending in 10% glycerol (2 ml) and aliquoting. The cells were frozen immediately at –70°C.

Electroporation of plasmid DNA into *E.coli*

In pre-chilled Eppendorf – tube, plasmid DNA (10 ng, about 1.2 µl) was mixed gently with an aliquot of electrocompetent cells (40µl) and placed on ice for 10 min. The mixture was transferred to a pre-chilled and sterile electroporation cuvette, which was further, placed into the sample chamber of the electroporator (Stratagene, Model 1000). A pulse of 1800 V was applied. The cuvette was removed and immediately added into SOC-medium (500 µl). The cell suspension was transferred to a sterile culture tube and incubated with moderate shaking at 37 °C, for 30-60 min. Aliquots of the transformation culture were plated on LB plates containing antibiotics.

SOC-medium

Yeast extract	0.5 g

Bacto-tryptone	2 g
NaCl	60 mg
KCl	19 mg
$MgSO_4$ x H_2O	200 mg

3.4. Biochemical protocols

3.4.1. Determination of protein concentration

Quantification of the amount of proteins in a solution was accomplished using colorimetric methods: the Bradford and the Lowry method.

3.4.1.1. Determination of protein concentration by Bradford method

The Bradford method *(Bradford, 1976)* depends on the quantitation of the binding of a dye, Coomasie brilliant blue, to proteins and comparing this binding to that of different amounts of a standard protein, usually BSA. The dye Coomasie Brilliant Blue G250 has the absorbation maximum at 465 nm. After binding the protein, the absorbance shifts to 595 nm.

For assay a standard protein solution (BSA) was prepared to the concentration of 1mg/ml (1mg of BSA was dissolved in 1ml H_2O). Duplicate aliquots of 0, 5, 10, 15 and 20 µl of the BSA-solutions as well as the protein solutions to be measured, were pipetted to Eppendorf-cups. H_2O was added to a final volume of 800 µl. To these, 200 µl of the dye-solution were added. After vortexing, the cups were allowed to stand 5 min at room temperature. Within 1 hours after mixing standard and samples were measured at 595 nm in plastic cuvettes of 1 ml and with 1 cm path-length. Unknown protein concentration was determined from the standard curve obtained by plotting absorbance at 595 nm versus protein concentration of aliquots of BSA-solution. The range of the determination has to be of 1-20 µg per sample; if required dilutions of unknown protein solutions were done.

3.4.1.2. Determination of protein concentration by Lowry method

The Lowry method depends on the colour obtained from the reaction of Folin-Ciocalteu phenol reagent with tyrosyl residues and comparing this value to the extinction values derived from a standard curve of a standard protein, usually BSA.

Two solutions (A and B) were prepared as described. To 5-20 µl protein extract was added H_2O to final volume of 100µl. Then there was added 1 ml of solution A and incubated 10 min at room temperature. Afterwards, 100 µl of solution B were added and incubated 10 min at room temperature. The absorbance at 660 nm was measured.

5, 10 and 15 µl BSA stock solution (1mg/ml) was used. Unknown protein concentration was determined from the standard curve obtained by plotting absorbance at 660 nm versus protein concentration of BSA-samples.

Solution A:

100 vol.	3% Na_2CO_3 in 0.1N NaOH
1 vol.	4% NaK-Tartrat
1 vol.	2% $CuSO_4.5\ H_2O$ (add drop by drop, under stirring!)

Solution B:

1 vol.	Folin-reagent
2 vol.	H_2O

3.4.2. Precipitation of proteins

3.4.2.1. Methanol precipitation

To 1 volume of protein solution, 2 volumes of methanol and 2/3 volume of chloroform were added. After a centrifugation at 12000 rpm, for 2 min, the upper phase was discarded. 1ml methanol was added to the tube and mixed again. A new centrifugation at 12000 rpm for

2 min, was performed and the supernatant was completely discarded. The protein pellet was re-suspended in sample buffer or stored at –20°C.

3.4.2.2. TCA precipitation

To one volume of protein solution TCA yielding to final concentration of 10% was added and mixed. After 30 min on ice, the samples were centrifuged for 10 min at 13000 rpm. The resulted pellet was washed with 4°C - cold acetone, dried in a speed vacuum (model Speed Vac SVC 100H from Savant Instruments) and re-suspended in sample buffer.

Alternatively, protein was precipitated by 20% TCA 80% acetone.

3.4.3. SDS -polyacrylamide gel electrophoresis

One-dimensional gel electrophoresis of the proteins can provide information about the molecular size and purity of proteins, as well as the number and molecular size of subunits.

3.4.3.1. Sample preparation

The proteins loaded on SDS-PAGE were either protein solutions, or protein pellets. Sample preparation is required prior loading proteins on SDS-PAGE.

The sample preparation was performed differentially: either protein solution was diluted 1:1 with 2x protein solubilizer, or the protein pellet was re-suspended in 1x protein solubilizer buffer. The protein solubilizer contained SDS as a denaturing reagent and ß-ME or DTT to reduce disulphide bonds. To bring proteins in solution, vortexing of 1-2 min per sample was usually enough and exceptionally sonication was performed. The probes were boiled for 5-10 min at 95 °C. A centrifugation step at 13000 rpm in a table top-centrifuge at room temperature for 3-5 min was performed and the supernatant was loaded in the pockets

of stacking gel. The protein-molecular weight standard was diluted in 1x SDS protein solubilizer buffer according to supplier's instructions and used as molecular mass marker.

Two protein solubilizer buffers were used (type A and type B). Usually, protein solubilizer-buffer-type A was used for solubilization of the proteins pellet form resulted from methanol precipitation and protein solubilizer-buffer-type B was used for solubilization of the proteins in pellet form resulted from TCA precipitation and for dilution of the protein in solution.

1x Protein Solubilizer Buffer - type A

100 mM	Tris/HCl pH 6.8
0.1 %	Bromphenolblue (BFB)
15 %	Sucrose
10 %	SDS
10 mM	DTT

Three stock solutions were prepared: solution 1, 2x (200 mM Tris/HCl pH 6.8, 0.2% BFB, 30% Sucrose; store at –20°C), solution 2, 2x (20% SDS) and solution 3, 100x (1M DTT). A working solution 1x was obtaining by mixing 1:1 solution 1 with solution 2; solution 3 was added always fresh to final concentrations.

2x Protein Solubilizer Buffer - type B

20 mM	DTT or ß-ME
2 %	SDS
250 mM	Tris/HCl, pH 6.8
20 %	Glycerine

To prepare 1 ml of 2x Protein Solubilizer - type B, 3.1 mg DTT and 20 mg SDS were dissolved in ddH_2O to 0.3 ml. To this 0.5 ml of 0.5 M Tris /HCl pH 6.8 and 0.2 ml Glycerine were added. Just before use, 20 µl of 1M DTT were added to 980 ml of 2x Protein Solubilizer Buffer-type B without DTT.

3.4.3.2. Laemmli gel system: denaturing discontinuous gel electrophoresis

By one-dimensional gel electrophoresis under denaturing conditions (0.1% SDS) proteins are separated according to their molecular mass. Proteins migrate toward the anode through pores in the polyacrylamide gel matrix. The pore size decreases with higher acrylamide concentrations.

The polyacrylamide gel was casted as a separating gel topped by a stacking gel and secured in a vertical electrophoresis apparatus, under temperature control.

The stacking gel allows the concentration of the proteins before entry in the separation gel. Lengths of 11-12 cm size of 1mm thickness and 10% concentration of the separation gels were sufficient for separation of proteins up to 100 MW with a good resolution.

To make a separating gel, the glass-plates were cleaned with H_2O and detergent, rinsed with technical ethanol (or methanol) and assembled as a sandwich which then was locked in the casting stand. Separating gel solution (as described in the table 11), were prepared and poured between the glass plates. Above it 1 ml butanol (H_2O saturated) was added to achieve a uniform polymerisation of the gel. After polymerisation of the separating gel, the 2-butanol was removed, rinsed twice with H_2O, and the stacking gel prepared as described in table 12 and poured on top of the separating gel. Immediately after the gel was poured, a comb of required size and teeth-number was introduced to make the pockets for sample application.

Tables 11: **Recipes for polyacrylamide separating gels**

SEPARATING GEL	CONCENTRATION			
	5%	7.5%	10%	12.5%
30% Acrylamide, 0.8% Bisacrylamide	5.25 ml	7.9 ml	10.5 ml	13.1 ml
1,5 M Tris/HCl pH 8.8, 0.4% SDS (w/v)	7.5 ml			
10% APS	250 µl			
TEMED	25µl			
H_2O	16.98 ml	14.33 ml	11.73 ml	9.12 ml

Tables 12: **Recipe for stacking gel**

STACKING GEL	CONCENTRATION
	4%
30% Acrylamide, 0.8% Bisacrylamide	1.3 ml
0.5M Tris/HCl pH 6.8, 0.4% SDS (w/v)	2.5 ml
10% APS	100 µl
TEMED	10 µl
H_2O	6.1 µl

1,5 M Tris/HCl pH 8.8, 0.4% SDS (w/v)

91g Tris-base were dissolved in 300 ml H_2O. The pH was corrected to 8.8 with 1N HCl and the volume was completed with H_2O till 500 ml. 2 g SDS were added at the end. The solution was stored at 4°C.

0.5 M Tris/HCl pH 6.8, 0.4% SDS (w/v)

6.05 g Tris-base were dissolved in 40 ml H_2O. The pH was corrected to 6.8 with 1N HCl and the volume was completed with H_2O till 100 ml. 0.4 g SDS were added at the end. The solution was stored at 4°C.

The proteins were run in an anode – cathode buffers containing system. The SDS-PAGE separations were performed under constant current at 50 mA 4°C for about three hours. A SDS-Electrophorese System (model SE-600 from Hoeffer Scientific Instr.) and the electrophoresis power supply (model 494, ISCO) were used

Anode Buffer for SDS-PAGE (pH=6.8):

50 mM Tris/HCl

192 mM Glycine

Cathode Buffer for SDS-PAGE:

1%	SDS (w/v)
0.001%	Bromphenolblue
	in Anode Buffer

3.4.4. Staining proteins in gel

Two methods were used to detect the location of the proteins in gels: Coomasie blue staining and Silver staining.

3.4.4.1. Coomasie blue staining

The detection limit by Coomasie Blue staining is of 0.3 - 1 µg per protein band and depends on non-specific binding of the dye, Coomasie Brilliant Blue, to proteins.

Coomasie Blue Staining and distaining of the proteins were performed using a "direct colloidal Coomasie-Staining" *(Wiltfang et al., 1991).* Stained gels were dried between celophan-folia in gel dryer type G1200 (Fröbel)

Hydrated gels were stained for about 12 hours, in a colloidal suspension of 0.1% (w/v) Coomasie brilliant blue G 250 in 10% (w/v) $(NH_4)_2SO_4$, 2% (v/v) H_3PO_4 and 20% (v/v) CH_3OH.

A working colloidal suspension was made by adding 20 ml CH_3OH to 80 ml of stock colloidal suspension. The stock colloidal suspension was prepared by dissolving, in that order, in about 800 ml H_2O, 100 g $(NH_4)_2SO_4$ and 20 g 85% H_3PO_4. The volume was corrected to 980 ml with H_2O. At the end, 20 ml of 5% solution Coomasie Brilliant Blue (in 10% CH_3COOH, 50% CH_3OH) were added under stirring.

To remove the dye-particles attached to the surface, gels were rinsed twice with 25% (v/v) CH_3OH.

3.4.4.2. Silver staining

Detection of protein bands in a gel by silver staining depends on binding of silver to various chemical groups (e.g. sulfhydryl and carboxyl moieties) in proteins. The detection limit is 2 to 5 ng per protein band.

To obtain Silver staining gels were fixed in two solutions: first, for 1-12 h, at 4°C, with 30% Ethanol, 10% Acetic acid, and second, for 1 h, with 30% Ethanol, 0.5M NaAcetat 0.5% Glutaraldehyde 20 mM $Na_2S_2O_3$. After three washing steps with cold H_2O for 15 min each, the gels were stained with silver nitrate solution for 1 h, then developed for 3-10 min, with developing solution and stopped (30 min) with 0.05 M Glycine.

Silvernitrate solution for staining

0.1 g $AgNO_3$ was dissolved in 80 ml H_2O. 54 µl of 37% Formaldehyde was added and the final volume was completed with H_2O to 100 ml.

Solution for developing

2.5 g Na_2CO_3 was dissolved in 100 ml H_2O. 54 µl of 37% Formaldehyde was added just before use.

3.4.5. Western blotting

3.4.5.1. Protein transfer with Semi-Dry systems

Following separation by SDS-PAGE, the proteins (antigens) were transferred to a support - membrane (nitro-cellulose or PVDF membrane) in a semi-dry blot system. The assembly of immunoblot sandwich is shown on the figure 6.

Before building up the immunoblot sandwich, the gel, chromatographic paper sheets (type Schleicher & Schüll, GB002) and the nitro-cellulose membrane (Sartorius, BA 85, 045 µm) were cut to the exact size. Then, the nitro-cellulose membrane was soaked in H_2O.

Similar, 4 sheets of blotting paper each were soaked in anode-buffer and in cathode-buffer, respectively. The anode buffer - sheets were placed one by one on the bottom of the stack followed by nitro-cellulose membrane and then the gel. The cathode buffer - papers were applied without air bubbles, and excess liquid was wiped off. The upper part (cathode) of the apparatus was placed on the stack and a weight of approximately 750g was put on top. A constant current of 1mA/cm^2 was applied for protein transfer for 60-80 min.

If a PVDF membrane was used in state of nitro-cellulose an activation of the membrane was required. This was done as follows: the membrane was let floating on the surface of methanol, rinse for 5 min in distilled H_2O and immerse in transfer buffer (cathode buffer) for 10min. PVDF-membrane must be kept wet at all times.

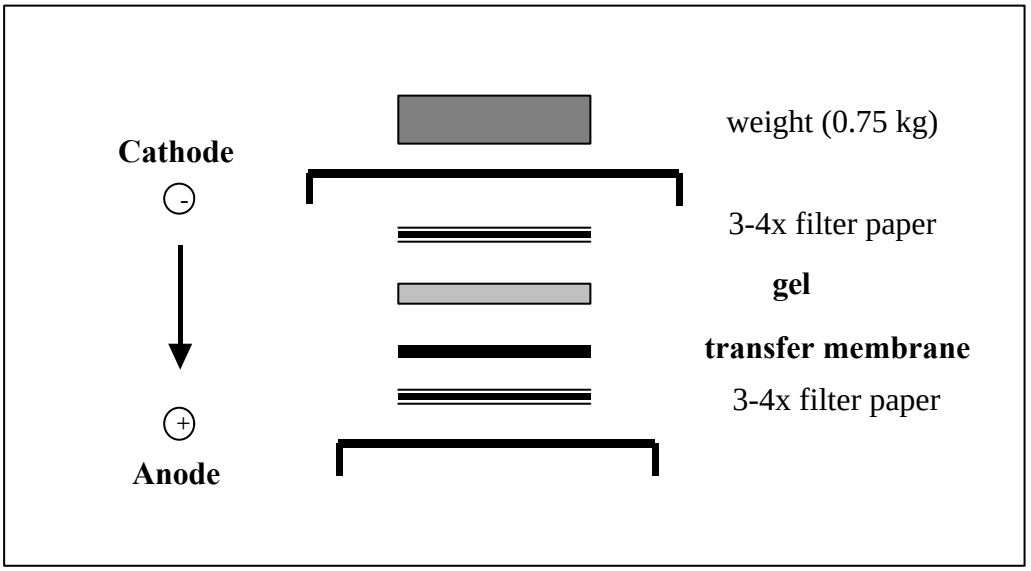


Figure 6: **Scheme of Semi-dry blot sandwich**

The arrow shows the electro-transfer direction of the proteins.

Cathode buffer for Western-Blot Semidry, pH 9:

40 mM ε-Amino-n-caproic	10.5 g
20 mM Tris/Cl	4.85 g
20% Methanol	400 ml
H_2O	till 2 l

Anode - buffer for Western-Blot Semidry, pH 7.4:

75 mM Tris/Cl	18.2 g
20% Methanol	400 ml
H_2O	till 2 l

3.4.5.2. Protein detection on membranes

3.4.5.2.1. Ponceau staining

The transfer of proteins from a SDS - polyacrylamide gel was monitored either by staining the gels after blotting (see above) or on blot transfer membranes with Ponceau S solution.

Following protein transfer to nitro-cellulose or PVDF, the membrane was placed in Ponceau S solution 5 min at room temperature. For de-staining, the membrane was placed in H_2O till the background became white (2-10 min).

3.4.5.2.2. Antibody detection

Immobilised proteins are probed with specific antibodies to identify and quantitate antigens present.

The membrane is immersed in a blocking buffer to cover all protein-binding sites with a non-reactive protein or detergent. Blocking buffer was TBS-Tween 20 (1x) with 5% milk powder. The incubation was done on a shaker, either o/n, at 4°C, or for 1 h 30 min, at 37°C. The membrane is placed in a solution containing the antibody directed against the antigen (primary antibody). For this purpose, the membranes were incubated in TBS-Tween 20(1x) with 5% milk powder in the presence an appropriate dilution of antibodies, as described in the table no. 3-1. For best results as well as because the need of reusing the same buffer with antibody against Aminopeptidase 1, the incubation was performed over night at 4°C. The optimum was obtained for a dilution of 1:1000. All other antibodies were used at optimum

dilution for minimum 1h 30 min at room temperature. If necessary the incubation was performed also o/n at 4°C. To remove the non-specific binding two washing steps with TBS-Tween 20 (1x), for 10 min were done before the blot was exposed to an enzyme-antibody conjugate directed against the primary antibody. For immunoprobing with directly conjugated secondary antibody, the membranes were incubated with goat/anti-rabbit IgG coupled horseradish peroxidase (HRP) as second antibody in TBS-Tween 20(1x) with 5% milk powder, dilution 1:10000. Complexes protein–antibody were visualised by treatment with developing reagent after 3 washing steps of 2x 10 min 1x 5 min with TBS-Tween 20 (1x).

TBS-Tween-20 (1x):

10 mM	Tris/Cl pH7.4
150 mM	NaCl
0.1%	Tween 20

To prepare 10x concentrated TBS - Tween 20 1.21 g Tris-base and 8.77 g NaCl in were dissolved 80 ml bdH_2O. The pH was corrected to 7.4 with HCl. 1 ml Tween 20 and bdH_2O were added yielding a final volume of 100 ml. A working solution was obtained by diluting 1:10 this solution in H_2O.

3.4.5.2.3. Visualisation with luminescent substrates

Two methods were used for visualisation of the proteins on the blotting membrane: Pierce-kit and luminol / p-coumaric acid system. Both methods are based on using chemiluminescent substrates.

Chemiluminesce is a chemical reaction that results in light emission. The chemical reaction that occurs between horseradish peroxidase (HRP) and luminol in the presence of hydrogen peroxide produces a weak light. With the addition of an enhancer, the light that is produced intensifies and the light emission duration increases. The maximum light emission wavelength is 425 nm and the initial flash of light occurs instantaneously after the reaction of the substrate with the enzyme.

Visualisation with luminol / p-coumaric acid system

When using luminol / p-coumaric acid system: after the final wash, the blot was immersed in a freshly prepared substrate solution for 1 min. There is no need to do this in the dark; the chemiluminiscence is quite stable in daylight. The excess of liquid was drained from the blot by pressing it between two filter papers. It was very important to drain the blot properly; the system is so sensitive that all extra liquid will result in background! The blot was wrapped in cellophane and exposed.

Substrate solutions:

Solution 1(10 ml):	100 µl	250 mM luminol
	44 µl	90 mM p-coumaric acid
	1 ml	1M Tris-HCl pH 8.5
	8.85 ml	H_2O
Solution 2(10ml):	6 µl	H_2O_2, 30%
	1 ml	1M Tris-HCl pH 8.5
	9 ml	H_2O

Luminol

0.44 g into 10ml DMSO, aliquot into 100 µl portions for single use. Store –20°C.

p-coumaric acid

0.15 g into 10 ml DMSO, aliquot into 44 µl portions for single use. Store –20°C.

Visualisation with Pierce-kit

Another possibility of visualising the imunocomplexes was the incubation of the blot membrane in a working solution of SuperSignal Substrate that is available commercially. A Pierce-kit, named SuperSignal Substrate, Western Blotting, contains two solutions, Luminol/Enhancer Solution and Stable Peroxide Solution. These must be stored at 4°C.

To prepare a working solution, equal parts of Luminol/Enhancer Solution and Stable Peroxide Solution must be mixed prior use. Recommended volume is 0.125 ml/cm^2 of blot surface. The incubation time is of 5-10 min and there is no need to prepare the solution in dark. To reduce background, the excess of liquid from the blot must be drained out. Afterwards, the blot is wrapped in cellophane and exposed.

The exposure was done in a dark room either to X-ray films or using a CSC camera, depending on the time required for exposure. Usually, for up to 5 min exposure time a CSC camera was used. For longer exposures (like 15 min to overnight), the choice was X-ray films. A film cassette insured a tight fit between membrane and film.

3.4.5.2.4. Protein size determination

Protein size determination using prestained markers

For SDS-PAGE and Western blot analysis, prestained markers for high molecular weight range have been used: Rainbow^{TM} coloured protein molecular weight markers (from Ammersham Life), Prestained protein molecular weight markers (Calbiochem), BenchMark^{TM} prestained protein ladder (from Gibco BRL).

Prestained markers are mixtures of pure proteins of known molecular weight. They are loaded into SDS-PAGE and further transferred to the blot membrane as is done with the protein samples. The stain makes them visible during electrophoresis and therefore their movement can be monitored even after the marker dye front has migrated off the gel, aiding in optimal resolution of high molecular weight polypeptides. After immunoblotting prestained markers give information about the transfer of the proteins. Because the covalently coupled stain causes a slight change in electrophoretic mobility, prestained protein molecular weight markers provide only approximate size information.

Protein size determination using purified proteins of high molecular weight

Finding the molecular weight of proteins of higher molecular weight then 200 was solved by performed glycerol gradients. Proteins of known molecular weight, like ovalalbumine (43 kDa), BSA (68 kDa), tyroglobuline (669 kDa), were separated on step

glycerol gradients, collect a number of equal fractions and load on SDS-PAGE. The gels were stained with coomasie staining and identified the fraction corresponding to a certain molecular weight. Standard curves were obtained by plotting log MW versus fraction number where protein has been found abundant. The molecular weight of unknown proteins, separed under the same conditions but transferred to membrane and visualised using methods described, was determined based on the abundance on the fractions, from the standard curve.

3.4.5.3. Stripping and reusing western – blot membranes

Two methods of striping off the immuno-detection systems were used.

Stripping with NaOH consists in three incubation steps: first, in H_2O for 5 min, then in 0.2N NaOH for 5 to 30 min, and last, for 5-10 min in H_2O.

Stripping with stripping buffer: the blot was incubated with a stripping buffer (2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β mercaptoethanol) for 30 min at 50-70°C and washed with washing buffer (TBS-Tween 1x) 2 times (10 min each).

Re-probing membranes after stripping was done beginning with blocking reagent as described in western blot procedures.

3.4.6. Preparation of yeast cell extracts

The procedure of preparing yeast cell extract for SDS-PAGE consists in preparing spheroblasts, and then destroying their surrounding membrane by mechanic treatment and temperature shock.

For this purpose exponentially or stationary growing cultures, cultivated at 30°C in YPD or MV-media were used. Spheroblasts were prepared from a starting amount of cells equivalent to 20 OD_{600}.

Cells were harvested at 3000 rpm, for 5 min, in a Heraeus – centrifuge and the supernatant was discarded. The cell-pellet was re-suspend in 1ml/10 OD_{600} in 0.1M Tris/SO_4 pH 9.4, 10mM DTT (freshly added) and incubated over 20 min at 30°C. Afterwards, the

cells were spun down at 1500 rpm for 5 min in Heraeus – centrifuge, the supernatant was discarded and the cell-pellet re-suspended in 2ml/10 OD_{600} of 1.2 M Sorbitol, 50 mM Tris/Cl pH 7.5, 10 mM EDTA. For lysis of the cell wall, lyticase (2.5 µl /10 OD_{600} cells; i.e. 0.125 µg per OD_{600} cells) was added and incubated with shaking for 30 min, at 30°C. Spheroblast formation was monitored at 600 nm after hypo-osmotic shock of the cells. Spheroblasts were spun down at 1500 rpm for 5 min in Heraeus – centrifuge and re-suspended in 700 µl of 0.1M NaP_i pH 7.4 with inhibitors (70 µl NaN_3 10%, 3.5 µl 0.2M PMSF, 0.7 µl 5-inhibitor mix). From now on the extract must be kept on ice.

Spheroblasts, transferred in a new Epp-cup containing 300 µl cold glass beads and were broken by vigorously mixing (vortex) for three times each 2 min followed by cooling for at least 1 min on ice. Glass – beads, unbroken cells and nuclei were spun down at 500g. The supernatant was transfer into a new Epp-cup and protein concentration was determined.

Proteins were stored at –20°C.

3.4.7. Determination of enzymatic activities

3.4.7.1. Specific enzymatic activitiy

The specific activity of Ape1p and CPY were measured in cell extracts prepared similar as for western blot analysis. Two modifications of the original protocol were introduced: first, the extract was prepared free of protease inhibitors and second, it represents the supernatant fraction from an extra centrifugation step (5 min, 13000 rpm, in tabletop centrifuge).

Protease assays were based on discontinuous spectrophotometric recording, in thermoregulated cuvettes, at 405 nm. The measurements began immediately adding the enzyme solution to reaction mixture containing substrate, buffer and H_2O. Due to the instability of the substrate, an unspecific activity was measured. Velocity of reaction was calculated from the rate of change of absorbance with respect to time, using the difference between molar

absorptivity of the substrate and reaction product. Specific activity was expressed in mU x $(mg)^{-1}$.

Formula for calculation of specific activity

$$mU \times (ml)^{-1} = (\Delta E \times 1000 \times V_t) \times (V_s \times \varepsilon \times \Delta t)^{-1}$$

$$mU \times (mg)^{-1} = mU \times (ml)^{-1} \times mg\ (ml)^{-1}$$

3.4.7.1.1. Ape1p-activity assay

The mApe1p - activity was measured by incubating protein extract with H-Leu-pNA, as a substrate, at 30°C. $ZnCl_2$ (c_f = 1 mM) was added as activator of Ape1p. The absorbance at 405 nm was measured at different time points: 0, 15, 30 and 45 min. The extinction coefficient (ε) of free pNA at 405 nm is 10.5 $M \times (cm)^{-1}$.

Ape1p- assay-mixture (1.5 ml):

900 µl	buffer (75 mM Tris/Cl pH 7.5, 3 mM EDTA)
100 µl	10 mM $ZnCl_2$ in buffer
75 µl	20 mM H-Leu-pNA (i.e. 5.27 mg/ml in DMSO)
20 µl	protein extract (or NaP_i pH 7.4, for control)
405 µl	H_2O

3.4.7.1.2. CPY-activity assay

The measurement of CPY-activity was done by incubating protein solution with substrate mixture at 25°C. Prior reaction, protein solution was prepared by treating protein extract with EDTA (c_f = 5 mM) and SDS (c_f = 0.25%) and incubation at 25°C, for 10 min. The absorbance at 405 nm was measured immediately after mixing (point zero) and at different other time points: 0, 15, 30 and 45 min. The extinction coefficient of the oxidation product of o-dianisidine at 405 nm is 7.25 $M \times (cm)^{-1}$.

CPY- assay-mixture (1.1 ml):

500 µl	solution 1
500 µl	solution 3
50 µl	solution 2
50 µl	Protein extract (or NaP_i, for control)

Solution 1 (in 0.1 M NaP_i pH 7.4):

0.25 mg/ml	L-amino acid oxidase
0.40 mg/ml	Horseradish Peroxidase

Solution 2 (in H_2O):

2 mg/ml	o-Dianisidine

Solution 3 (in 0.1 M NaP_i pH 7.4):

20 mM	Z-Gly-Leu (i.e. 6.4 mg/ml)

3.4.7.2. Ape1p activity of yeast colonies

Ape1p activity of yeast colonies was determined by an Ape1p -'overlay' assay.

is based on enzymatic activity of the mature amino-peptidase 1 (mApe1p). To test for presence of mApe1p in *vivo*, an overlay agar, containing leucine-ß-naphthyl-amide (H-Leu-β-NA) as substrate, was prepared. Chloroform permeabilizes cells on the surface of colonies. Aminopeptidase 1 catalyses cleavage of H-Leu-β-NA and ß-naphthylamide (ß-NA) is released. Due to the product fluorogenic group, a strong fluorescence if illuminated at 365nm is observed. Caution: ß-naphthylamide is a carcinogen.

Ape1p -'overlay' was performed with fresh cells, as single – colony streaks, grown on selective media-plates (MV-plates). Chromatographic-paper-filters (Ø 80 mm, Schleicher and

Schuell) were labelled with a pencil, transferred onto the MV-plates and let stand until the entire filter turned wet. The replica – filters were taken off from the plates, transferred onto YPD-plates with the colony-site up and incubated over night. The filters were taken from the plates and placed with the colony-site down into $CHCl_3$ for 20-30 sec. Excess of $CHCl_3$ was sucked off and the filters were dried on paper-sheets. The filters were placed in Petri dishes and covered with assay-agar.

The assay-agar mixture was prepared by mixing the buffer and the melted agarose in a Falcon tube thoroughly. The substrate-solution (always prepared fresh!) was added and vortexed. Immediately, per one plate, 5 ml of agar solution was poured over the colonies. Plates were incubated at room temperature for 15-30 min. Then, the activity of Ape1p was detected with an UV-lamp of 365nm.

Assay-agar mixture:		
	For 1 sample (V_f 5 ml):	
50 mM Tris/Cl pH 7.5	3.33 ml	75mM Tris/Cl pH 7.5 3mM EDTA
2 mM EDTA		
0.6 % agarose	1.42 ml	2% agarose
10 mM H-Leu-β-NA	0.25 ml	200 mM H-Leu-β-NA in CH_3OH

3.4.8. Protease protection experiments

3.4.8.1. Protease protection assay

Protease protection assays were performed using 70 OD cells, grown to OD_{600} 0.7-1.1. Cell extract containing intact vesicles and vacuoles was prepared similar as for western blot analysis until spheroblasts were obtained. Maximum efficiency of spheroblast formation was obtained with 0.2 mg/50 OD_{600} cells zymolyase 20 T, in 1ml/6 OD_{600} cells of 0.6 M Sorbitol, 50 mM KP_i pH 7.5, 10 mM DTT, 0.2% Glucose in YP-medium. Incubation was done for 15 min, at 30°C, with shaking at 200rpm and 15 min, at 30°C, standing but mixed gently each 5 min.

Further, spheroblasts were carefully lysed (by differentially lysis) leaving intact subcellular vesicles. To check for intact vesicles, lysed material was centrifuged (15 min at 8000 rpm, 4°C in Eppendorf-centrifuge). Pellet and supernatant were analysed in western blot with specific markers (carboxypeptidase Y (CPY) – vacuolar, α-Hexokinase (α-HK) - cytosol). Pellet should contain intact vesicles and supernatant should contain cytosol and eventually the contents of broken vesicles.

Differential lysis of the spheroblasts suspension in SP – buffer (V_f = 720 µl / 50 OD cells) was done by adding DEAE-Dextran (V_f = 45µg / 50 OD cells) and incubating successively for 1 min at 4 °C, non-shaking; 5 min at 30°C, shaking (70 rpm); kept at 4 °C after incubation.

SP – buffer:

200 mM Sorbitol, 20 mM Pipes pH 6.8

Protease protection was tested as follows: lysed material is incubated at 4°C for 30 min without adding protease, in presence of tyrosine or proteinase K (each 50 µg/ml) and in presence of either protease and 0.2% Triton X-100. Incubation is terminated by adding PMSF to c_f = 1 mM and incubation for 10 min at 4°C, and afterwards adding 1 volume 20% TCA 80% acetone. Processing of pApe1p can be analysed after SDS-PAGE by western – blotting.

Subfractionation of lysed spheroblasts was performed by ultracentrifugation, using a TLA100.3 rotor, at 100000g for 45 min 4°C.

In total lyzed spheroblasts (corresponding to 70 OD cells) were used for subfractionation and protease protection experiment of the subfractions. The spheroblasts were subjected to differential lysis, using 60-90 µg DEAE-Dextran/100 OD cells. From cell homogenate, a part (corresponding to 10 OD cells) was centrifugated on 100 µl 10% ficoll (in order to avoid breaking the vesicles during ultracentifugation) after mixing with 0.72 µl stock solution PMSF (in order to avoid unspecific processing during procedure). Three fractions are collected: pellet (P), ficoll (F) and supernatant (S) and analysed in western blot. Another part of lyzed spheroblasts (corresponding to 60 OD cells) was mixed with 300 µl

10% ficoll and centrifugated. Ficoll (350 µl) and supernatant (700 µl) fractions are subjected to protease protection assay, as described.

3.4.9. Glycerol gradient fractionation

Glycerol gradient centrifugation was performed to analyse homo-oligomerization. Yeast crude extract used for Glycerol Gradients was prepared as described for western blot analysis, from 40 OD cells in logarithmic or stationary growth phase. To reduce volume, spheroblasts pellet was re-suspended in 700 µl of 0.1 mM NaPi pH 7.4 plus protease inhibitors, as follows: 70 µl NaN_3 10%, 7 µl PMSF, 1.4 µl 1000x 5-inhibitor mix. The extract was used fresh.

Calibration of the glycerol gradient was done with known proteins: thyroglobuline (MW 669), BSA (MW 65), ovalalbumine (MW 45). Due to their molecular weight they migrate in different fractions of the gradient.

Glycerol gradients preparation

Preparation of step - glycerol gradients was done using 20% – 50% of glycerol in 20 mM K-PIPES pH 6.8 (1x).

Table 13: **Recipe of preparing solution (40%, 30% and 20%) for glycerol gradient**

STOCK SOLUTION	20%	30%	40%
Glycerol 50%	1760µl	2640µl	3520µl
K-Pipes 1x	2640µl	1760µl	880µl
V_f	4400µl	4400µl	4400µl

Setting Glycerol Gradients

The Glycerol Gradient tube has to be filled up completely, in order from the bottom to the top, with:

Glycerol 50%	525 µl
Glycerol 40%	525 µl
Glycerol 30%	525 µl
Glycerol 20%	525 µl
Protein extract (1.2-1.5 mg)	400 µl
V_f	2500 µl

The gradients without protein extract could be prepared in advance and stored at -80°C.

Centrifugation of the Glycerol Gradients

Centrifugation of the Glycerol Gradients was done in a tabletop ultracentrifuge with a rotor TLS-55 at 55000 rpm, at 15 °C, for 4 h.

Collection of the fractions and preparation of the samples for SDS-PAGE

After separation by centrifugation, 10 fractions, of 250 µl each were collected and TCA-precipitated as described. After 2-5x washings with cold acetone (4 °C), the protein pellet was re-suspended in 80 µl sample buffer + DTT (1x). Samples were run in 10% SDS-PAGE. As a control, 70 µg of the same yeast crude extract, solubilised in 70-90 µl sample buffer + DTT (1x) was run on the gel.

3.4.10. Isolation of pApe1p - containing vesicles in OptiPrep gradients

Isolation of pApe1p - containing vesicles was done in OptiPrep Gradients. 250 OD fresh cell culture with OD_{600} start 0.9-1.1 was used.

Spheroblasting protocol was used with modifications, as follows. With the exception of the incubation steps all other steps were carried out at 4°C. Cells were re-suspended in spheroblasting buffer (1.2M Sorbitol, 50 mM Tris/Cl pH 7.5), 1ml/60 OD. Spheroblasts were obtained using Zymolyase 20 T to c_f = 0.5 mg/50 OD. Spheroblasts are collected by 6 min centrifugation (2000 rpm, 4°C) in JA-20, and were lysed with 4 ml H_2O, on ice for 5 min.

Homogenate was separated from non-lysed spheroblasts by centrifugation at 2500 rpm 4°C, two times 2 and 3 min, respectively.

From the protein extract, an aliquot was saved for TCA precipitation, as a control of crude extract. 2 ml crude extract were mixed carefully (use 1ml-cut tips and shaker 4°C) with 3 ml of 60% OptiPrep solution (the final concentration was 37%).

Set of OptiPrep gradients

Flotation gradients were set as follows:

5 ml	37% OptiPrep in protein extract,
2 ml	30% OptiPrep in 10 mM Pipes pH 6.8
2ml	25% OptiPrep in 10 mM Pipes pH 6.8
2ml	19% OptiPrep in 10 mM Pipes pH 6.8.
1.5-2 ml	10 mM Pipes pH 6.8

Centrifugation is done at 25000 rpm, for 6 hours, 4°C in L8-M Ultracentrifuge/ Beckman, with SW-40 ultracentrifuge rotor.

Collection and analysis of the fractions for OptiPrep Gradients

The 0% fraction, 0-19% interface, 19-25% interface, 25-30% interface, 30-37% interface, 37% fraction were collected and TCA precipitated. Western blot analysis was done with Ape1p, CPY and α-HK antibodies.

3.5. Manipulation of nucleic acids

3.5.1. Quantification of nucleic acids

The concentration of nucleic acids may be determined spectrophotometrically or by comparison with a control sample of known concentration in an agarose gel. DNA can not be distinguished from RNA by spectrophotometry since both exhibit a maximum absorbance at a

wavelength of 260 nm. One OD unit corresponds to 40 µg/ml of RNA and one OD unit corresponds to 50 µg/ml of double stranded DNA. It is recommended to use a dilution of the RNA/DNA sample that will give an OD value between 0.1-0.5. Contamination of nucleic acids with proteins is demonstrated by measuring the OD at 280 nm, since proteins typically have a maximum absorbance at this wavelength. A 260/280 absorbance ratio of 1.8-2.0 is appropriate for pure RNA/DNA. Contaminating protein will result in lower values of the 260/280 ratio.

3.5.2. DNA manipulation

3.5.2.1. Electrophoresis of DNA in agarose gel

Medium- and large - size DNA molecules were separated on agarose gels using an horizontal electrophoresis apparatus. Depending on the fragment-size, the concentration of agarose-varied from 0.5% to 1.5% (w/v) (table 14). The necessary amount of agarose was brought in 1 x TAE, boiled in microwave, cooled to approximately 55°C prior adding ethidium bromide (c_f = 0,5 µg/ml) and poured into the mould and a well-forming comb was immediately positioned. After completely setting (at RT, for 15-30 min), the comb was removed and the gel placed in the electrophoresis apparatus containing buffer (1 x TAE) sufficient to cover (1-3 mm) the gel. DNA-probes containing 1% (v/v) sample buffer (LP IV or Ficoll-Marker) were

Table 14: **Agarose concentration required to separate nucleic acids of various sizes** *(according to Andrews, 1991)*

AGAROSE (%)	LINEAR NUCLEIC ACID SIZE (KB)
0.5	0.7-45
0.8	0.4-20
1.0	0.3-10
1.2	0.2- 8
1.5	0.2 -6

loaded in the wells of the gel and run (3-4 V/cm) at RT. To determine the sizes of nucleic acid molecules, a standard (DNA-ladder, 10µg per lane) was added.

Due to ethidium bromide (intercalating dye, binds to double-stranded DNA or RNA) added to the buffer used for making up the gel, the progress of DNA separation was followed at any stage by illuminating the gel with UV radiation. For this purpose a UV-Hand lamp (312 nm) or a transluminator (300nm) were used.

DNA - sample buffer:

LP IV:	0.25%	Bromphenolblue
	40%	Saccharose in 1x TAE
Ficoll-Marker:	0.05%	Bromphenolblue
	0.05%	Xylencyanol
	15%	Ficoll

DNA - standard ("DNA-ladder"): a mixture of oligonucleodides that comprise fragments of defined size (75 kb – 12.216 kb).

3.5.2.2. DNA modifications

3.5.2.2.1. Digestion of DNA with restriction endonucleases

The activity of restriction endonucleases is declared in units (U). One unit of restriction endonuclease activity is defined as the amount of enzyme required to completely digest 1 µg of substrate DNA in a total reaction volume of 50 µl in one hour using the NEBuffer.

Restriction enzyme digestions were performed by adding the reagents into capped microcentrifuge tubes and mixed gently with the pipette tip. Incubations were performed at the appropriate incubation temperature (37°C or 25°C) as indicated on the technical data card, for the desired length of time (1.5 - 3 hours). For analysis of reaction efficiency, an aliquot was

subjected to agarose gel - electrophoresis. Whenever desired quantitative digestion, this was performed under the same conditions but doubling the incubation time.

Reaction mixture for DNA – digestion (20-100 µl):

	DNA
2x U	restriction endonuclease
10% (v/v)	reaction buffer 10 x
till V_f	H_2O

As reaction buffer was used the New England Biolabs (NEB) buffer system. These buffers were provided from the manufacturer (as 10 x concentrated) together with the enzymes. The NEB system consists beside enzyme-specific buffer of four basis-buffers, as follows (given final concentration):

NEB 1: 50 mM Tris/HCl (pH 7), 10 mM $MgCl_2$, 1 mM DTT
NEB 2: 50 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl
NEB 3: 50 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl
NEB 4: 50 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 50 mM KOAc

3.5.2.2.2. Preparation of plasmid-vectors for subcloning DNA - fragments

Plasmids were used as cloning vectors. They were digested at one locus either by a single restriction enzyme or by two at a multi-cloning site to achieve insertion of target DNA in a defined orientation. Digestion products were separated on agarose gel electrophoresis and DNA recovered using Qiagen – kit for DNA – gel extraction.

Whenever desired, the digested vector was treated with alkaline phosphatase for removing the 5'-phosphates from the digested plasmids. The aim was prevention from self ligation of the vector and therefore reducing the background of colonies, which do not contain

recombinant molecules in the experiment. Calf intestinal phosphatase (CIP) was provided from Boehringer Mannheim. For incubation procedure the use of 1U per 1-100 pmoles DNA is recommended. One unit of CIP was defined as the enzyme activity which hydrolyses 1 µmol of 4-nitrophenyl phosphate in 1 min at 37°C under assay conditions.

Reaction mixture for DNA - dephosphorylation:

50 µl (8 µg)	DNA
6µl	CIP (1U/µl)
6µl	dephosphorylation - buffer 10 x

Dephosphorylation buffer 10x: 0.5 M Tris/HCl, 1mM EDTA, pH 8.5

DNA - dephosphorylation using calf intestinal phosphatase (CIP) was carried out at 37°C for 60 min. The reaction was stopped by heating the mixture at 65°C for 10 min in presence of 5 mM EDTA, before purifying the phosphatase-treated DNA by QIAquick PCR purification.

3.5.2.2.3. DNA-gel extraction with Qiagen-kit

The recovery of the after electrophoretic separation was achieved using QIAquick Gel Extraction - Kit (Qiagen). This system combines spin – column technology with the selective proprieties of a "silica" – gel membrane.

The desired DNA – fragment was excised from the agarose gel under UV-light (UV – handlampe, 312 nm) and weighed. The gel slice (1 volume) to which was added QG – buffer (3 volumes) was incubated at 50°C, until agarose was completely dissolved (about 10 min). If the colour of the mixture changed from yellow 3M NaOAc pH 5 (10 µl) were added and mixed. If extraction of DNA fragments larger then 4 kb was desired, 100% isopropanol (1 volume) was added to the sample and mixed. From this mixture, a maximum amount of 400

mg - gel slice was applied per a QIAquick centrifugated for 1 min at 10000 g. Traces of agarose were removed with QG – buffer (500 µl) and centrifugation. Salt traces were removed by washing with PE – buffer (750 µl) and centrifugation. An additional centrifugation (1 min, at 10000 g) was performed to remove residual ethanol from PE – buffer. DNA was eluted with 50 µl EB – buffer or H_2O (pH 7 – 8.5) and stored at –20°C. Since EDTA may inhibit subsequent enzymatic reaction, TE was not used as elution buffer.

EB - buffer: 10mM Tris/HCl, pH 8.5

3.5.2.2.4. Construction of recombinant DNA molecules

An appropriate digested target and vector DNA were linked together in a recombinant molecule prior to transfer to *E.coli* in a DNA-ligation reaction.

DNA ligase links fragments of DNA, containing blunt-end and cohesive-end termini, to each other in a covalent manner. It catalyses the formation of a phosphodiester bound between juxtaposed 5' phosphate and 3' hydroxyltermini in duplex DNA. Recommended total DNA concentration in a plasmid cloning experiment is of maximum 1 µg/ml *(Gannon and Powell, 1991)*. The cohesive end - ligation was performed for a mixture contained 1 : 3 = vector: DNA fragment. The molar ratio was increased for bunt – ligation.

For ligation reactions T4 - DNA Ligase (400,000 U/ml, provided from New England Biolabs) was used. One NEB unit of T4 DNA ligase was defined as the amount of enzyme required to give 50% ligation of Hind III fragments of Lambda DNA in 30 minutes at 16°C in 20µl assay mixture and 5'DNA termini concentration of 0.12µM (300µg/ml).

The ligation mixture was incubated at 16°C over night. Optional, T4 DNA ligase was inactivated at 65°C for 10 min and ligation products were transformed into bacteria. The correct and complete ligation was analysed by gel electrophoresis with unligated material as a marker. Recombinant DNA was stored at –20°C.

DNA – ligation mixture:

1 µl	T4 - DNA Ligase
1 µl	T4 - DNA Ligase – buffer 1x
x µl	DNA fragment
y µl	vector
till 10-20 µl	H_2O

Ligase – buffer (1x):

50 mM Tris/HCl pH 7.5, 10 mM MgCl, 10 mM DTT, 1 mM ATP, 25µg/ml BSA

3.5.2.2.5. Radioactively labelling a DNA-probe

Labelling DNA with ^{32}P

Radioactively labelling a DNA-probe for northern analysis was performed for APE1 gene which was isolated from a plasmid, p313/LAP.

To about 25 ng DNA add H_2O to a final volume of 45 µl, incubated at 95°C for 2-5 min and then cool down on ice. Transfer all to a cup of lyophilised labelling - premix (containing dATP, dTTP, dGTP, buffer, Klenow Polymerase and hexameric primers) and re-suspend carefully. Add 2.5 – 5 µl of α-^{32}P-dCTP and incubate for 10-15 min at 37°C.

Probe DNA purification

Labelled DNA probes were purified to determine the specificity of the labelling reaction. Purification procedure was based on filtration through a Sephadex G.50 column. The product was eluted with TE (or H_2O).

First, a Sephadex G.50 spin column in TE buffer was prepared: glass wool placed in a blue-pipette tip and Sephadex G.50 in TE was added. The tip was centrifuged at 3500 rpm, for 2 min in Haeraus centrifuge. Then, the buffer was discarded and into the column, the sample plus 100 µl TE was loaded and centrifuged (under the same conditions). The sample was collected and 1/100 of it was monitored measuring Cerenkov reaction (efficiency of 52%).

3.5.2.2.6. QIAquick PCR purification of PCR-products

QIAquick PCR purification kit from Qiagen is designed to purify single- and double-stranded DNA fragments (ranging from 100 bp to 10 kb) from PCR and other enzymatic reactions. This kit was used for cleaning up DNA from alkaline phosphatase reaction. PB – buffer (5 volumes) was mixed with products mixture of alkaline phospatase reaction (1 volume), loaded to the QIAquick column and centrifuged (10000 g, 1 min). Washing with PE – buffer (750µl) and an additional centrifugation to remove residual ethanol (10000 g, 1 min) were performed. DNA was eluted with 50µl EB- buffer and used direct for *E.coli* transformation or stored at –20°C.

3.5.2.3. DNA-sequencing

3.5.2.3.1. Cycle Sequencing with Dye – Terminator

Plasmid DNA subjected to DNA sequencing was purified using mini-prep Qiagen kit, quantified spectrophotometrically and amplified with a 25 cycles polymerase chain reaction (PCR) in a thermoblock (Mastercycler gradient, Eppendorf.).

For PCR reaction dsDNA was mixed with primers (forward or reverse primers), a premix (that contains DNA polymerase, dNTP- mixture, ddNTP-mixture and buffer) and H_2O.

PCR reaction mixture:

0.25 – 0.5µg	double strains DNA
2µl	30 mg/µl primers
4µl	premix buffer
till 20 µl	H_2O

For purification of amplified DNA from residual reaction components, PCR product (20 µl) was mixed by vortexing with NAACO (2µl of 0.3M) and ethanol (50µl of 100%),

stand on ice for 10 min, and centrifuged at 13000 rpm, 4°C for 20 min. The supernatant was discarded and DNA pellet washed with 70% ethanol (250 µl) and dried on Speed vacuum. DNA was re-suspended in 2.8 µl formamid / EDTA (5:1).

3.5.2.3.1. DNA-Sequence analysis

Amplified DNA fragments for DNA-Sequence analysis were sent to a service laboratory where were solved on polyacrylamide gels and monitored by computer software. The DNA - sequence was analysed using yeast data bank available on the Internet.

3.5.3. RNA analysis

3.5.3.1. Total yeast RNA extraction using RNAeasy kit

Total yeast RNA was extracted using RNAeasy kit, which was purchased from Qiagen. Before and during RNA-extraction RNase-free environment was maintained conforming to supplier advice: working in aseptic conditions and wear of gloves while handling reagents and RNA samples to prevent RNase contamination; using sterile, disposable plastic ware RNase-free. Non-disposable plastic ware, glassware and electrophoresis tanks were treated to inactivate RNases with 0.1 M NaOH (12 hours at room temperature) followed by RNase-free H_2O. RNase-free H_2O was obtained by treating deionizated H_2O with 0.1% DEPC and autoclaving (to remove residual DEPC). All solutions were prepared using H_2O treated with DEPC.

Total yeast RNA extraction was done from 5 OD cells (equivalent to 5 $x10^7$ cells) in early log-phase growth. The principle of RNA extraction is summarised in figure no. 3-5. Total yeast RNA extraction was performed after RNeasy Mini Handbook 03/97, Qiagen. All steps were done at room temperature.

Buffers for yeast RNA extraction:

Buffer Y1

1M	Sorbitol
0.1M	EDTA pH7.4

Before use, 0.1% ß-ME and 50U Lyticase/Zymolase were added per 10^7 cells.

Buffer RPE was supplied as a concentrate. Before using, 4 volumes of ethanol (96-100%) were added to obtain a working solution.

Buffer RLT was checked for precipitate formation. If necessary, it was worm to re-dissolve. Before use, 10µl ß-ME per 1 ml of buffer RLT were added to obtain a working solution.

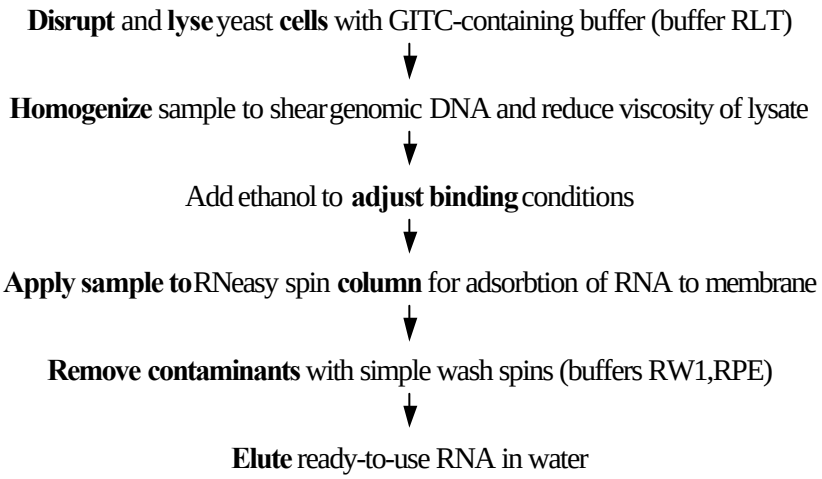


Figure 7: **Scheme of RNeasy Mini Procedure**

Cells were harvested for 15 min at 1000x g in Heraeus centrifuge at 4°C and the supernatant completely removed. This was done to avoid inhibition of cell wall digestion in next step. Cell pellet was re-suspend in 2 ml enzyme-containing Buffer Y1 and incubated for 10-30 min 30°C with gentle shaking to prepare spheroblasts. Complete spheroblasting was

essential for efficient lysis. Spheroblasts were pelleted by centrifugation for 5 min at 300x g. Lysis of the spheroblasts was performed in 350µl buffer RLT and vigorously vortexing. If necessary, the lysate was centrifugated for 2 min at 3000 rpm in Heraeus centrifuge to collect insoluble material. The supernatant was transferred to a microfuge tube. 1 volume of 70 % ethanol was added and mixed well. The sample (usually 700µl) including any precipitate which might have formed, was applied to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifugated for 15 sec at 8000x g. More washing steps were performed. First washing was done with 700µl Buffer RW1 onto the RNeasy column by centrifugation (15 sec at 8000x g). Second washing was done with 500 µl buffer RPE onto the RNeasy column by an identical centrifugation. For last washing, 500 µl Buffer RPE were applied onto the RNeasy column. This was centrifugated for 2 min at maximum speed to dry the RNeasy membrane. RNA was eluted with 30-50 µl of RNase-free H_2O by centrifugation for 1 min at 8000x g.

Total RNA was stored at –70°C in H_2O.

3.5.3.2. Separation of RNA in formaldehyde-agarose

3.5.3.2.1. RNA sample

RNA sample (4 volumes) was diluted with 5x RNA loading buffer (1 volume), incubated for 3-5 min at 65°C, chilled on ice, and loaded onto equilibrated FA gel.

5x RNA Loading Buffer (stabile three months at 4°C)

16 µl	saturated bromophenol blue
80 µl	500 mM EDTA, pH 8.0
720 µl	37% formaldehyde
2 ml	100% glycerol
3084 µl	formamid
4 ml	10x FA gel buffer
till 10 ml	RNase-free H_2O

3.5.3.2.2. Formaldehyde – agarose (FA) gel electrophoresis

Formaldehyde – agarose (FA) gels were prepared from agarose melted in microwave, $_2$O bath, mixed with 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of ethidium bromide (10mg/ml) and poured into gel support. Prior running, the gel was equilibrate in 1xFA gel running buffer for at least 30 min. Formaldehyde – Agarose (FA) Gel Electrophoresis was done at 5-7 V per cm with 1x FA gel-running buffer.

10x FA Gel Buffer (pH 7.0):

200 mM	MOPS
50 mM	Sodium acetate
10 mM	EDTA

MOPS (20.93 g), sodium acetate (0.205 g) and EDTA (0.186 g) were dissolved in 500 ml RN-ase free H_2O. The pH was corrected with NaOH.

1.2% FA Gel

For 100 ml:	1.2 g	Agarose
	10ml	10xFA Gel Buffer

1x FA Gel Running Buffer:

100 ml	10x FA Gel Buffer
20 ml	37% Formaldehyde
880 ml	RNase-free H_2O

3.5.3.3. Northern blot analysis

3.5.3.3.1. RNA blotting. Assemble the Northern blot sandwich

RNA can be transferred by capillary action, vacuum blotting and electro-blotting.

Using the classic capillary transfer method, transfer time of about 12 hours was required to achieve high transfer efficiency.

Blot storage

Blots were used immediately or stored at room temperature.

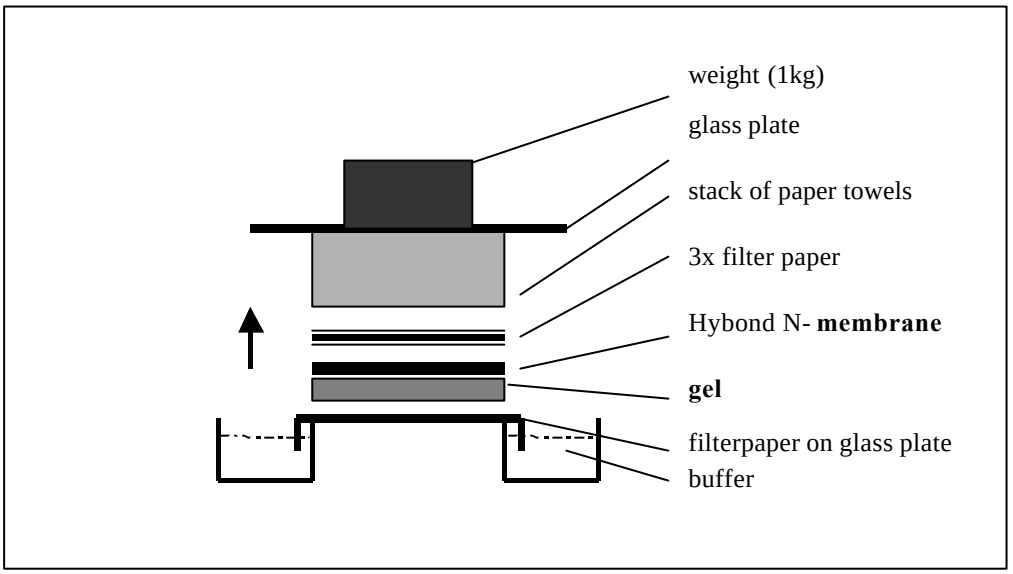


Figure 8: **Schema of capillary transfer of RNA by Northern blot.**

The arrow shows the direction of liquid-stream

3.5.3.3.2. Fixation of RNA onto membrane

Baking, alkali and UV irradiation are procedures that may be used function of the type of the membrane.

RNA was immobilised on a nylon hybridisation transfer membrane (Hybond^{TM}-N) using the UV cross-linking technique. RNA-molecules were immobilised by covalent binding between light-activated nucleotides and C = C groups on the membrane surface. Because the binding of the molecule is UV dose dependent and over exposure of the blot may result in

complete binding of the molecule and decreased hybridisation capacity, the blot was exposed to UV light only 10-15 sec. Membranes were dried thoroughly, at 65°C for 20 min.

3.5.3.4. Hybridisation of RNA

3.5.3.4.1. Membrane hybridisation with a labelled-DNA probe

DNA, RNA and synthetic oligonucleotides can all be used as probes in membrane hybridisation experiments.

Shortly, a membrane on which RNA has been blotted was incubated with a hybridisation solution containing the labelled-DNA probe (see Radioactively labelling a DNA-probe). To reduce non-specific binding of the probe the membrane was washed after hybridisation at controlled temperature, salt concentrations and detergents. Well-matched hybrids could be washed at high stringency conditions (0.1x SSC); whereas poorly matched hybrids were washed in lower stringency conditions (2-6x SSC). 1-2 µCi / ml radioactively labelled-DNA probe in hybridisation-mix solution (H-S) were used. The membrane was rolled, with the face on which have been blotted RNA to the inner centre, into a 50 ml Falcon-tube. 2-5 ml H-S sspDNA and labelled-DNA probe were added. The mixture was prepared as follow: in an Eppendorf cup, to 1 ml of H-S add 100 µg ssp DNA (see Appendix) This was incubated at 95 °C until labelled-DNA probe was added and further incubated to 95°C for 3 min. Afterwards, all was added to H-S into the Falcon tube containing the membrane and hybridise over night, on a shaker at controlled temperature of 42°C.

Second day the washing of the membrane was performed in 2x steps. First, with a 2x SSC, 0.1% SDS, at room temperature for 15 min or more. The membrane should have not more than 25 cpm radioactivity before the second washing which has to be done at 65°C, with 0.2x SCC, 0.1% SDS, for 15 min.

<u>20 x SSC - buffer, pH 7.0:</u>

3M NaCl

0.3 M $Na_3Citrate \times 2\ H_2O$

For 1 l SSC - buffer 20x, NaCl (175.3 g) and Na_3Citrate x 2 H_2O (88.2 g) were dissolved in 800 ml H_2O. The pH was corrected to 7.0 with 1M HCl and H_2O was added to final volume.

Hybridisation-mix solution (H-S):

72 ml Formamid
36 ml 20x SSC
1.5 ml 1M Tris/HCl, pH 7.5
1.5 g SDS
3 ml 50x Denhardts
30 ml 50% Dextransulfat

First, 15 g Dextransulfat were added to ddH_2O within a final volume of 30 ml and were solved at 80°C. Subsequently, there were added the other substances. At the end, ddH_2O was added to a final volume of 150 ml.

3.5.3.4.2. Detection techniques of RNA-DNA complexes

RNA bands complementary to radioactively marked DNA probe were visualised by membrane-exposure to a phosphoimager screen with Phosphoimager, IPR 1000 (Fuji BAS 1000). The analysis was performed with computers' programs like ImageReader and ImageGouge.

4. RESULTS

4.1. Isolation of Ape1p transport mutants

Aminopeptidase 1 (Ape1p) is synthesized in the cytoplasm as an enzymatically inactive precursor protein, pApe1p, which is proteolytically processed when reaching the vacuolar lumen yielding the mature form (mApe1p). This processing and a dodecamerisation of the monomer are required for Ape1p enzymatic activity *(Metz et al., 1977)*

4.1.1. Screening strategy for mutant isolation

Isolation of pApe1p transport mutants was based on non-specific and aleatoric DNA mutagenesis of a wild type strain and selection of yeast mutants defective in a particular transport step. The mutant gene will be isolated by complementation cloning (figure 9).

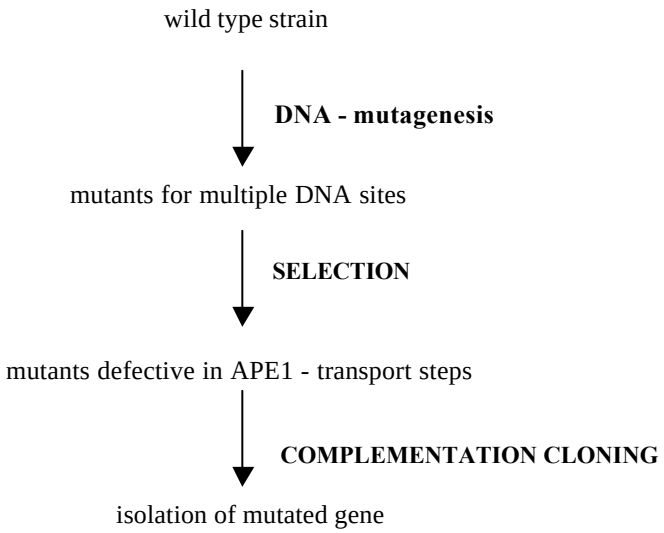


Figure 9: **Screening strategy for mutant isolation**

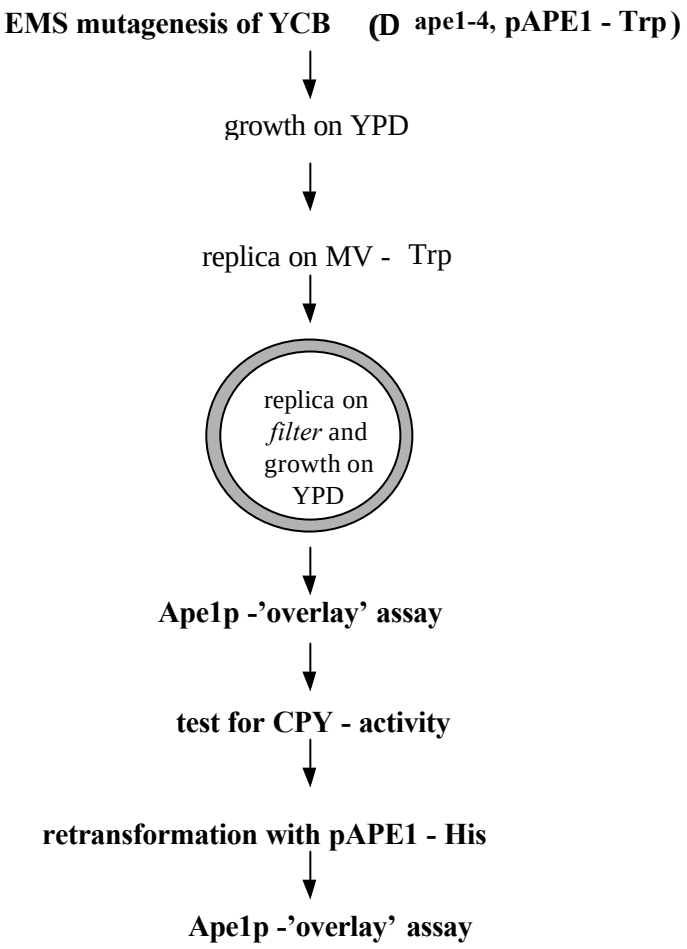
4.1.2. Isolation of mutants deficient in Ape1p activity

A wild type strain (II.17) lacking four aminopeptidase activities was transformed with the APE1 gene (LAP IV) under the control of its endogenous promoter isolated from a genomic DNA library on a single-copy plasmid (p314/LAP, trp+). The new strain (YCB 18: Δape1, Δape2, Δape3, Δape4, pAPE1-Trp) showed wild type levels of Ape1p expression and activity, indicating that processes like translation, transport and maturation of aminopeptidase 1 occurred normally. The enzymatic activity of Ape1p in these strains was quantified using Ape1p-overlay activity test for yeast colonies. Transformation of II.17 with two independent replicating single-copy plasmids carrying the APE1 gene demonstrated that the 'overlay' activity allows detecting alterations of 25% of the wild type enzymatic activity. The level of activity was set to 0 for II.17 and +4 for YCB 18.

YCB 18 was EMS – mutagenized and clones devoid of Ape1p activity were isolated. In figure 10, the isolation of Ape1p – transport mutants is summarised on a flow chart (panel A) and the Ape1p 'overlay' assay used for their isolation as shown on panel B.

The EMS – mutagenesis was carried out by incubating YCB 18 - cell suspension in 0.1 NaPi pH 7.4 with 33% (v/v) EMS for 30 min at 30°C. Mutagenized cells were plated on YPD- medium plates as single colonies. Since the chromosomal APE1 gene was disrupted, the mutants isolated could carry mutations in genes responsible for Ape1p processing, i.e. transcription, translation, transport, maturation as well as of the APE1 gene introduced on the Trp-plasmid. Mutagenized cells, which grew on YPD, were replica plated on selective minimal medium containing all supplements except tryptophan (MV-Trp) to select for the ectoptic APE1 gene. From these plates replica were made on chromatography papers, which were placed on rich media plates (YPD) to avoid upregulation of unspecific autophagocytosis. After incubation of the filters overnight at 30 °C, colonies were screened for loss of Ape1p activity by the 'overlay' assay. Clones devoid of Ape1p activity were isolated.

Panel A



Panel B

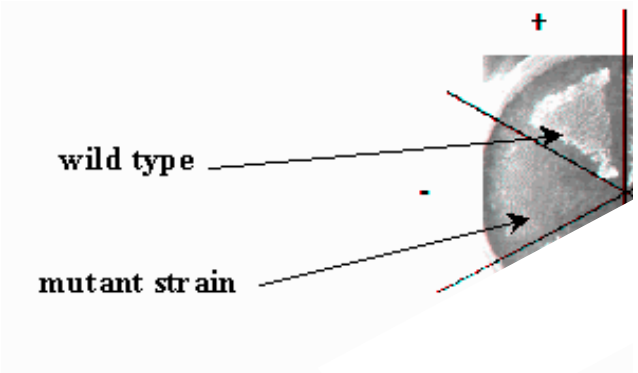


Figure 10: **Flow chart of Ape1p – transport mutant isolation (panel A)**
(details in text)

Ape1p overlay activity assay (panel B)

Image recorded at 365 nm using CCD camera. Strains with Ape1p enzymatic activity emit fluorescent light and colony streaks become visible. Strains shown: the original mutant (without Ape1p - activity) and wild type (with Ape1p - activity).

Ape1p is activated by the same endopeptidases, proteinase A (PrA) and proteinase B (PrB), as two other vacuolar enzymes carboxypeptidase Y (CPY, soluble protein) and carboxypeptidase S (CPS, membrane anchored protein). Therefore, clones lacking Ape1p activities were further assayed for an intact secretory pathway and the presence of the activating vacuolar enzymes, PrA and PrB by testing for CPY and CPS enzymatic activities in a micro-titer plate assay *(P.Schu, unpublished*). This allowed identifying and excluding mutants defective in vacuole biogenesis. In order to test for the mutagenesis and expression of the APE1 gene introduced by Trp-plasmid, mutants were re-transformed by a second APE1 gene on a single - copy plasmid (p313/LAP, his+), after they had lost the trp-plasmid.

In the table 15, the statistics for the isolation of pApe1p-transport mutants are presented. The number of cells which survived EMS – mutagenesis, was set to 100%. From these only 60% still contained a functional Trp-plasmid. 0.6% of the colonies carried mutations which affected the expression of Ape1p enzymatic activity. Only 18% of these had an intact vacuole. After retransformation 14 mutants, which represent 0.07% of the original colonies, were selected for analysis by yeast genetics.

Table 15: **Statistics for the isolation of pApe1p - transport mutants by Ape1p activity**

<table>
<tr><th>Colonies on YPD</th><th>Colonies on selection-medium</th><th>Clones without activity</th><th>Clones positive for CPY –CPS activities</th><th>Clones without any activity</th><th>Clones with reduced activity</th></tr>
<tr><td>135000</td><td>80000</td><td>850</td><td>150</td><td>93</td><td>57</td></tr>
<tr><td rowspan="2">100%</td><td rowspan="2">60%</td><td rowspan="2">0.6%</td><td rowspan="2">0.1%</td><td>0.07%</td><td>0.04</td></tr>
<tr><td>after re-transfor-mation 14 mutants</td><td>?</td></tr>
</table>

4.2. Selection of mutants deficient in Ape1p transport

Since the mutations were induced aleatoric, and therefore have been expected to affect multiple genes, an approach based on non-specific recombination of the genes was taken. Successively mutant-strains were mated with wt-strains, diploids were isolated and tetrads dissected (figures 11, 12). The isolated spores were assayed for mating type, Ape1p activity and expression of Ape1p.

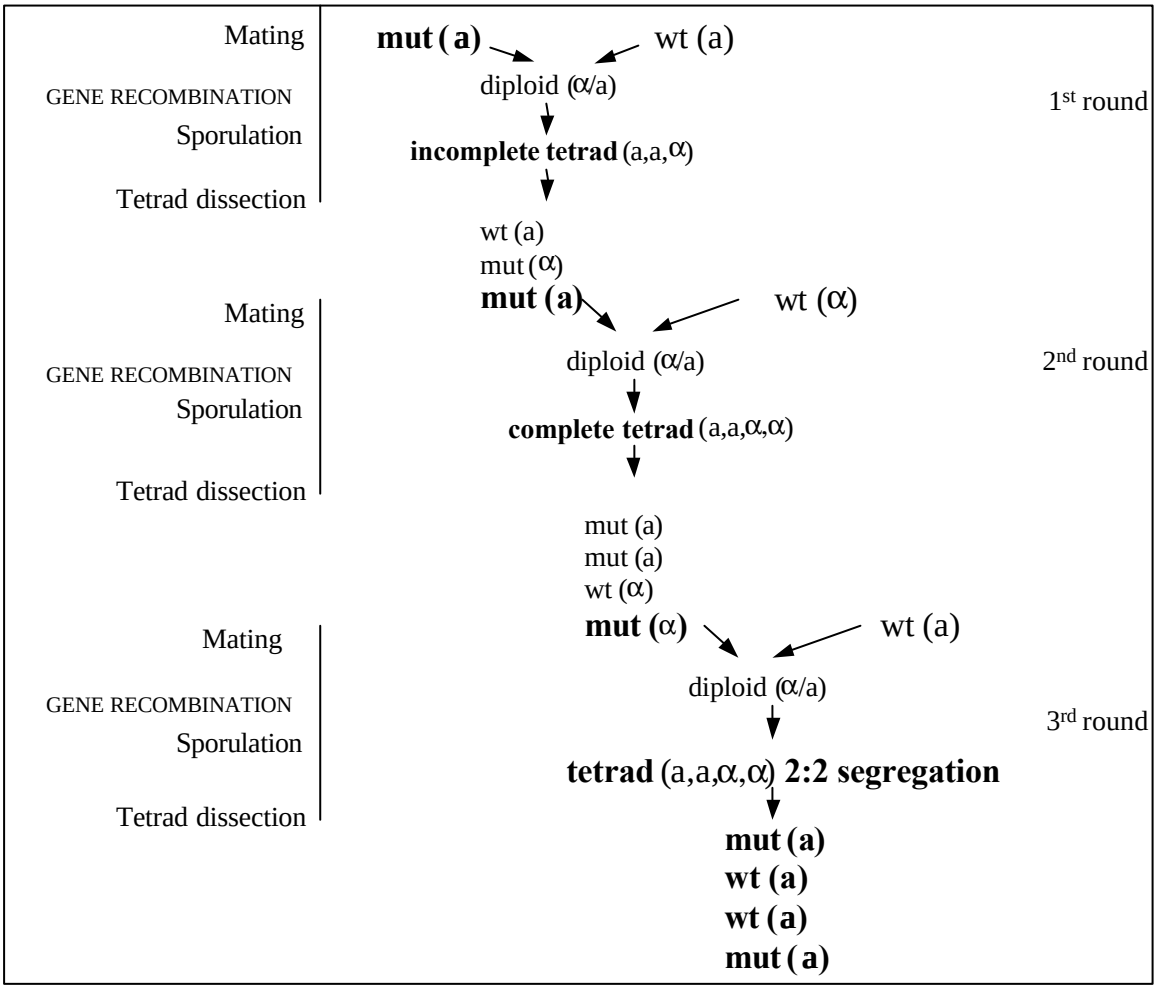


Figure 11: **Successive gene recombination for isolation of Ape1p – transport mutants**

The EMS-original mutants were all MATα. Successively mutant-strains were mated with wt-strains, diploids were isolated and tetrads dissected. The cycle for repairing mutated DNA, based on gene recombination was repeated until 2:2 segregation of the mating type and phenotype concerning Ape1p activity was obtained.

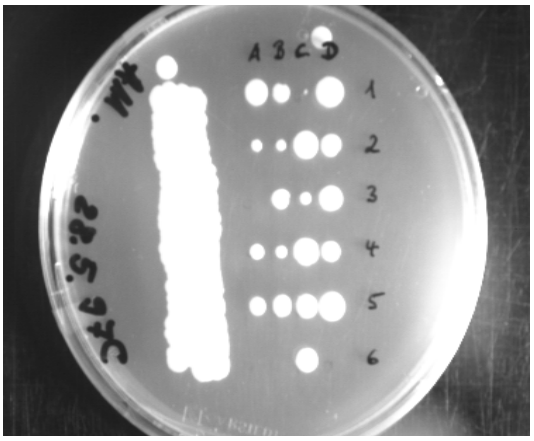


Figure 12: **Image of tetrad dissection on YPD – plate**

Sporulated cells were re-suspended in 100 µl H_2O; 200 U of β–glucoronidase was added and the suspension was incubated 20 min at 37°C. With a needle controlled by a micromanipulator ascii were separed from unsporulated cells and spores were isolated and aligned (on positions A, B, C, D) on a YPD plate. Each set of 4 spores corresponds to a tetrad (named 1, 2, 3, etc). The segregation of growing phenotype can be seen in tetrads named 2, 4 and 5. Growth defects can be seen in the tetrads named 1, 3 and 6.

To ensure intragenic recombination, in each round of mating, the mutant spore of a different mating type than the parent mutant was used for subsequent tetrad analysis. Therefore, two wt strains were required. The genesis of the two wild types (wt), C6A (**a**) and C6C (α), used for gene recombination is described further.

Isogenic strains of opposite mating type, deficient in the 4 leucineaminopeptidases were generated by mating II.17 with SEY6211. Sporulation was induced by acetate, and spores (MAT**a** and MATα) were randomly selected *(P. Schu, unpublished)*. The APE1 - gene on two different plasmids (p313/LAP, his+, p314/LAP, trp+) was expressed in these spores. Transformants, named PSY13/LAP and PSY3/LAP, were mated. After tetrad dissection spores were tested for leucine-aminopeptidase activities using the overlay – assay (figure 13).

Leucine-aminopeptidase deficient strains did not show any phenotype related to vacuolar functions, as growth under nitrogen limitation and sporulation. C6A (MAT**a**, lap+4) and C6C (MATα, lap+4) were used for *via* – mutants selection.

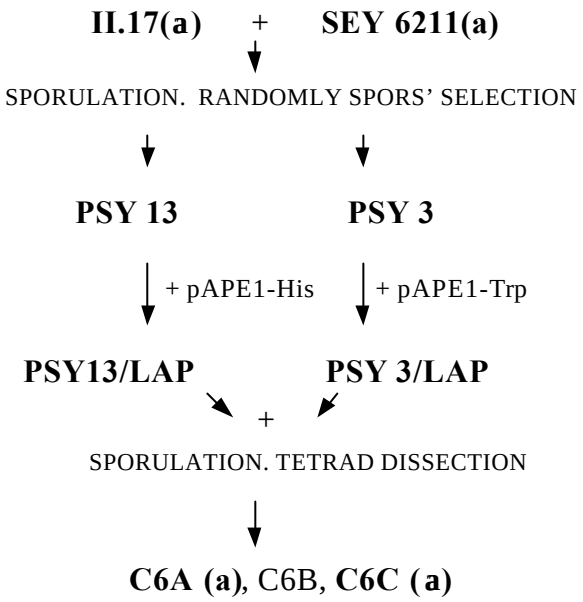


Figure 13: **Genesis of C6A (a) and C6C (a)**

(details in text)

When C6A was mated with C6C, complete tetrads were achieved and all four isolated spores (v1, v2, v3, and v4) had lost the his-plasmid. Once pAPE1-His was introduced they show wt - level for Ape1p expression and activity. Spore v1 was used as negative control for characterisation of *via* – mutants.

Since a mutant (Trp+) was mated with a wild type (His+) diploids selection was based on complementation of nutritional requirements. Meiosis was induced by plating diploid cells on medium without nitrogen and with 1.47 % acetate as carbon source. Separation of the spores from ascus was performed using a micromanipulator (Zeiss) for tetrad dissection. At first round of tetrad dissection, only incomplete tetrads (3n) were isolated. This fact shows that the mutagenesis might have affected also genes involved in cell differentiation. Up to 4 successive rounds of crossing with wt, sporulation and tetrad dissection were performed. In the last round of dissection the phenotype of the four spores was 2:2 with respect to mating type and Ape1p activity. The four spores were shown to carry either one of the plasmids, (Trp+) or (His+), or both.

From the isolated 14 EMS – mutants, two did not form diploids (EMS-mutants:

1.87, 12.126) and other three did not sporulate (EMS-mutants: 11.15, 12.126, 17.314) after crossing with wild type. One mutant (10.75) turned all four spores to wt – phenotype after first round of sporulation, indicating accumulation of synergistic mutations. 8 mutants were analysed further. Six of them reached the isolation stage of complete tetrad and showed 2:2 segregation for Ape1p – activity. With the exception of *via14*, one to three spores from all other tetrads lost the His-plasmid during selection. Therefore those spores had to be re-transformed with p313/LAP plasmid. Due to the screening and selection strategy, the isolated mutants were named ***via*** **-** mutants, for Vacuolar Import and Autophagocytosis. They were generated from the EMS-mutants, named: 7.511

Table 16: **Genesis of *via3, 10* and *14* – mutants. Mating type, phenotype and origin**

Mutant	Number of Tetrads	Dissected Spores	Mating Type	Phenotype: Ape1p- 'overlay' test	Origin: EMS-mutant
via3	one	3 III A	α	lap-	20L14 (α, trp+, lap-)
		3 III B	a	lap-	
		3 III C	a	lap+4	
		3 III D	α	lap+4	
	one	3 IV A	a	lap+4	
		3 IV B	α	lap+4	
		3 IV C	a	lap+1	
		3 IV D	α	lap+1	
via10	two	10 II A	a	lap+4	1.112 (α, trp+, lap-)
		10 II B	α	lap+1	
		10 II C	a	lap+4	
		10 II D	α	lap+1	
		10 II A'	a	n.d.	
		10 II B'	α	n.d.	
		10 II C'	a	n.d.	
		10 II D'	α	n.d.	
via14	one	14 II A	a	lap-	9.38 (α, trp+, lap-)
		14 II B	a	lap+4	
		14 II C	α	lap+4	
		14 II D	α	lap-	
	one	14 III A	a	lap-	
		14 III B	α	lap+4	
		14 III C	a	lap-	
		14 III D	α	lap+4	

(*via1*), 24.37 (*via2*), 20L14 (*via3*), 21.412 (*via7*), 1.112 (*via10*), 9.38 (*via14*). So far, 3 of those were further characterised: *via3, via10* and *via14*.

4.2.1. Specific activities of vacuolar enzymes

During the screening procedure enzymatic activities were determined by plate assays to test large numbers of colonies. Specific activities of vacuolar enzymes (Ape1p and CPY) in *via10* and *via14* mutants were determined to verify the data collected by plate assays.

Crude protein extracts were prepared from cells grown in YPD medium to mid-log phase and stationary phase by similar procedure as for western blot analysis omitting protease inhibitors and including at the end, an additional centrifugation of 5 min, at 13000 rpm. CPY and Ape1p - enzymatic activities were calculated from initial velocity of the reaction over the first 15 minutes. Not more then 150 µg of total extract per assay was applied.

The Ape1p – activity was assayed with H-Leu-pNA as substrate and $ZnCl_2$ was present to stabilise mApe1. Enzymatic activity was followed for 30-45 min at 30°C. *via14* and *via10* have showed 0-20 % of the wild type activity in stationary growth conditions (table 16) where enzymatic activity of mApe1p reaches its maximum - as it was measured in overlays assay. The Ape1p – activity in exponentially growing cultures was reduced on average by only 30% (1.6 mU/mg) compared to stationary growth conditions (2.08 mU/mg) in wild type (C6C). It was noted, that in protein extract from stationary growing cells, *via14* appeared to have 15% more activity than *via10,* which contrasts the data from the overlay tests (table 15). This result may be explained by efficient stabilisation of mApe1p by Zn^{2+} present in the assay mixture or that mApe1 in *via14* is more sensitive to the $CHCl_3$ used to permeabilize the cells in the overlay assay.

CPY activity was measured in cell extracts where it forms a complex with a cytoplasmic inhibitor. CPY has to be activated prior incubating with substrate leading to variations of data values from experiment to experiment. However, under exponentially growth conditions (table 18) *via10* showed 70% of the wild type CPY- activity and exceeded with 45% that of wild type in stationary growth conditions. *via14* showed 80 -

85% of wild type CPY activity under exponentially growth conditions and exceeded with 25% the activity of wild type. From these results we could conclude that in *via* - mutants accumulation of the precursor form of aminopeptidase 1 is not due to a defective vacuolar processing machinery.

Table 17: **Ape1p specific activity in *via* - mutants in stationary growth conditions**

STRAIN	CONTROL	*via14*	*via10*
Ape1p specific activity under stationary growth conditions (mU/mg)	2.38 ±0.15	0.89 ±0.4	0.45 ±0.15

Table 18: **CPY specific activity in *via*-mutants under logarithmic and stationary growth conditions**

STRAIN	CONTROL	*via14*	*via10*
CPY – specific activity under logarithmic growth conditions (mU/mg)	0.61	0.50	0.42
CPY – activity under stationary growth conditions (mU/mg)	0.47	0.58	0.67

4.3. Characterization of *via* - mutants

4.3.1. *via* - Mutants accumulate pApe1p

via - Mutants were isolated based on the absence of Ape1p enzymatic activity. Expression and processing of Ape1p were analysed by SDS-PAGE and western blot. Protein extractions were performed from yeast cells growing on YPD – medium to mid-log phase to avoid induction of bulk autophagocytosis. A defect in the transport of pApe1p to the vacuole, should result in a defect in maturation and accumulation of the precursor form.

Crude extracts were prepared from the strains in the presence of protease inhibitors to block *in vitro* pApe1p processing. Proteins were separated by SDS-PAGE, blotted onto nitro-cellulose membranes and precursor and mature Ape1p were detected using a rabbit anti-Ape1p antiserum.

Wild type cells showed 30% pApe1 and 70% mApe1 under logarithmic growth conditions (OD_{600} = 0.8 – 1.2) in rich media (YPD) while less then 10% pApe1p can be detected in stationary cultures (OD = 2.2 – 2.5) (figure 15).

In *via10* up to 70% of Ape1p accumulated as pApe1p, while in *via14* up to 80% of Ape1p was found as pApe1p under logarithmic growth conditions (figures 14, 15). Under stationary growth conditions pApe1p levels were reduced in *via10* and *via14* by 5-10%.

The same ratio of precursor protein to mature under logarithmic and stationary growth was found in *via3* (figure 16), however expression level is drastically reduced (see chapter 4.3.3). These results indicate that in *via*-mutants autophagocytosis can be up-regulated under starvation conditions. This reduction corresponds to the change seen in wild type cells, but it can not compensate the defects observed under logarithmic growth conditions.

In figure 14 is shown the accumulation of pApe1p in *via10* – mutants, under the logarithmic growth conditions, in YPD. Wild type and *via10* spores, which were positive spores for Ape1p activity (10 II A, 10 II D) do not accumulate pApe1p.

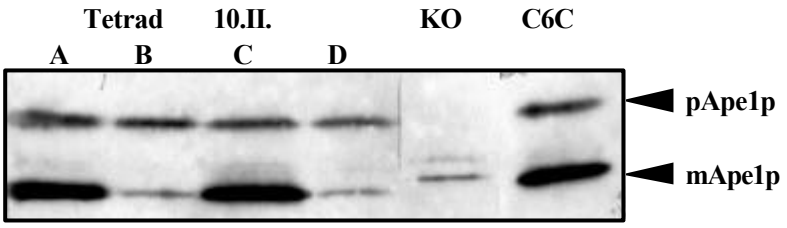


Figure 14: **Western Blot analysis of the *via* 10-tetrad**

Cell cultures were grown until exponentially growth phase in YPD. The positive spores in Ape1p overlay assay (10 II A and 10 II D) and wild type strain convert up to 80% Ape1p to mature form while negative spores accumulate mainly pApe1p. KO designates a control strain having APE1 disrupted.

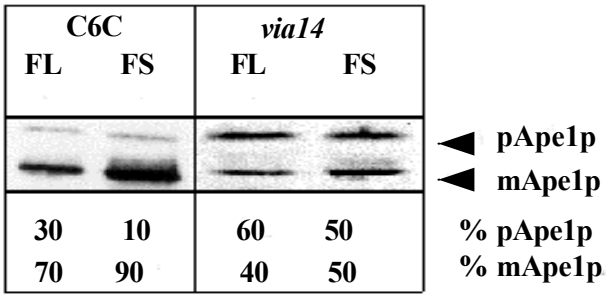


Figure 15: **Western Blot analysis of the *via14* mutant and wild type (C6C) in logarithmic and stationary cultures**

Protein extract (65µg applied per each lane) was separated in 10% SDS-PAGE, blotted on nitro-cellulose membrane and developed with 1:1000 antibody against Ape1p in 5% milk in TBS-Tween 1x. On the figure, FL corresponds to protein extract in exponentially growth phase in YPD and FS to stationary growth phase.

Figure 16: **Processing and expression level of pApe1p in *via3* mutants and wild type (C6C) under logarithmic and stationary growing (Western Blot analysis)**

Protein extract (90µg applied per each lane) was separated in 10% SDS-PAGE and

blotted on nitro-cellulose membrane. The blot was developed with 1:1000 antibody against Ape1p in 5% milk in TBS-Tween 1x.

4.3.2. *via* – Mutants define two complementation groups

To avoid working with mutants of the same genes, complementation tests were performed for *via3*, *via10* and *via14*. A complementation test consists in the introduction of two mutant chromosomes from cells with similar phenotype, simultaneously into the same cell and looking for the complementation of the genes. Lack of complementation for two mutations is indicative of allelism.

Gene complementation was assayed using pApe1p - overlay test for diploids generated by mating of *via*- mutants with each other and with wt cells (figure 17).

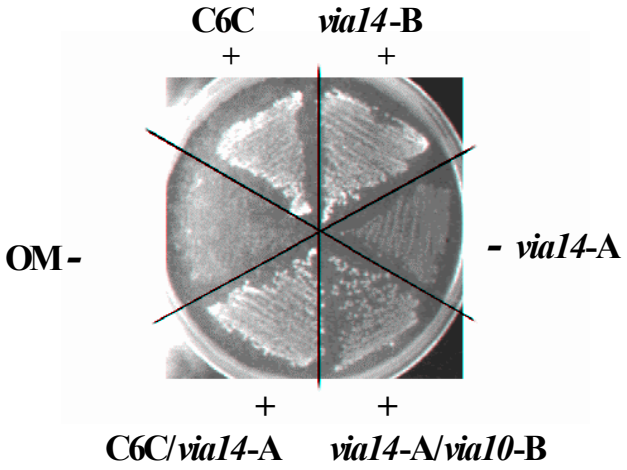


Figure 17: **Complementation test based on Ape1p-overlay activity**

The Ape1p- overlay activity assay of a mutant (*via14*-A) and two diploids (*via14* A/*via 10*-B, *via14*-A/wt). To achieve contrast, yeast cells were replica plated on black paper. The picture was taken at 365 nm using a CCD camera. Strains with Ape1p enzymatic activity emit fluorescent light and colony streaks become visible. With OM was marked the original mutant isolated from the screen. *via14*-B spore is positive and *via14*-A negative for Ape1p activity. C6C is the wt strain. The Ape1p activity recorded for the diploid of wt/*via14* and *via10/via14* are similar to C6C.

Since all *via*-mutants have the same marker for auxotrophy, His+, the selection for diploids was based on morphology. Diploid cells are larger then haploid cells and so are colonies of diploids. After mating cells were streaked on YPD plates to form single colonies. Cells from the largest colonies were tested for diploidy by testing their ability to mate with **a** or α cells. Lack of mating indicates diploid formation. In table 19, the Ape1p activity of the isolated diploids is summarised. Mating of two Ape1p negative *via14* spores did not restore Ape1p activity as expected. The diploids resulting from mating *via14* with *via10* had enzymatic activity similar to wild type demonstrating that different genes are mutated. Crossing of *via3* with *via14* did not lead to diploid formation. Mating of *via3* with *via10* did not show gene complementation. This indicates that *via3* and *via10* belong to the same complementation group. Alternatively, *via3* could carry a dominant negative allele. The Ape1p-activity recorded for the diploid of wt and *via*-mutant was always similar to wt. Therefore, two classes of pApe1p transport mutants have been identified: *via10* and *via14*.

Table 19: **Complementation tests for *via3, via10 and via14***

Complementation tests	Ape 1 – overlay assay of diploid
via14 x *via14:* 14.II.D x 14.III.A	(negative)
via14 x *via10:* 14.III.A x 10.II.B	+3/+4
via14 x *via3:* 14.III.A x 3.III.A	no diploids
via10 x *via3:* 10.II.B x 3.III.B	+1 / +2
via14 x *wt:* 14.III.C x C6C	+4
via10 x *wt:* 10.II.B x C6A	+3
via3 x *wt:* 3.III.A x C6A	+3

4.3.3. Evidence that *via3* is a transcription - mutant

It is known that vacuolar proteolysis is important for the survival and differentiation of the organism. Diploids lacking vacuolar proteases fail to sporulate *(Teichert, et al., 1989)*.

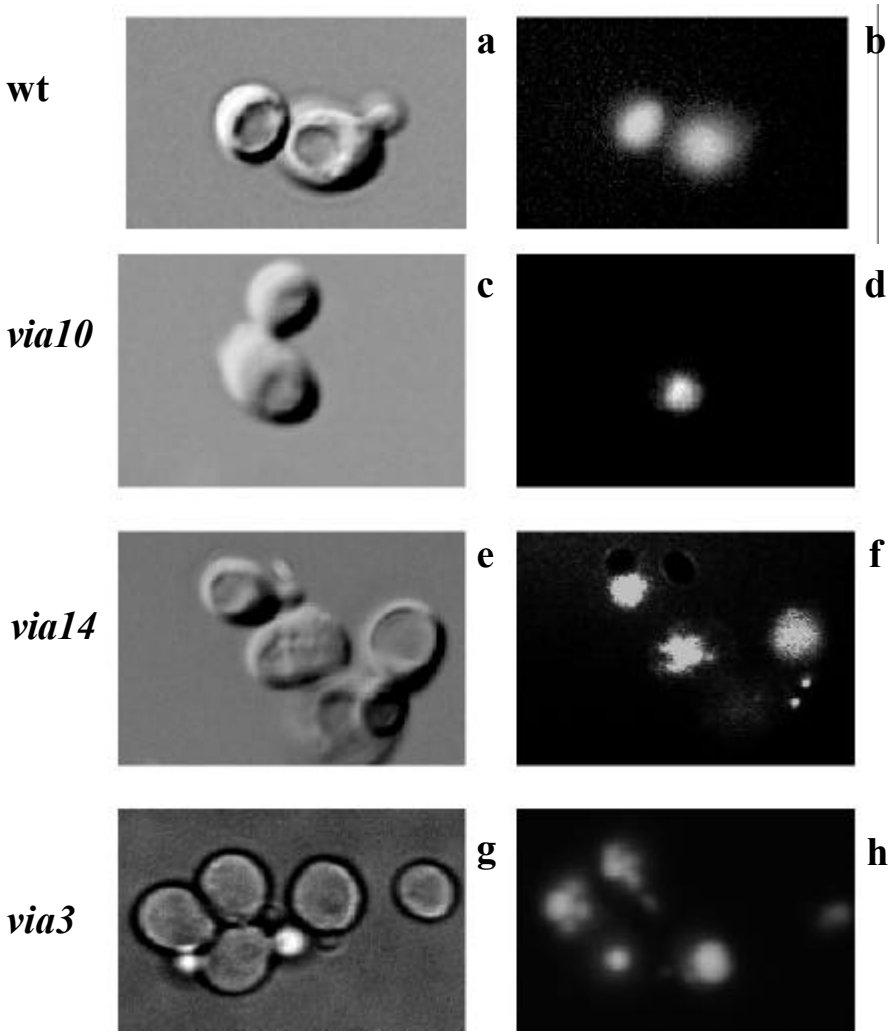


Figure 18: **Vacuolar morphology among the *via*-mutants**

Nomarski optical images (in the left column: a, c, e) and the corresponding fluorescence signal due to vacuole staining with CL_2CFDA (in the right column: b, d, f, h). Cells, which were grown until mid-log in YPD, re-suspended in Na^+-Citrate (50mM pH 4) with CL_2CFDA (1µl of 10mM in DMSO) were incubated at 30°C 10 min, harvested and re-suspended in 0.1 M NaPi pH 7.5, 2% glucose. Visualisation was performed using a Zeiss Axiovert 100 equipped with a PlanNeofluar 63x/1.25.

As shown on table 18, *via3* mutants could be mated with *via10*, but not with *via14*. No or very low efficiency of diploid formation indicates severe physiological defects in *via3*. Staining of vacuoles with a fluorescent dye which accumulates in acidic compartments revealed a large number of small acidic compartments in *via*3, whereas wild type cells and *via10* and *14* contained only one or two vacuoles (figure 18).

In western blot analysis, CPY expression level was equal in all four *via3* spores (figure 19) while APE1 expression varied from wt-level for positive spores and very low for mutant – spores (figure 16). This indicates that the defect is specific for pApe1p transport pathway. Reduced expression levels could be caused by increased instability of the protein or by reduced transcription/translation rates.

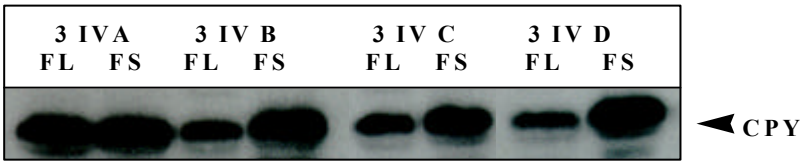


Figure 19: **CPY expression level among *via3* spores**

Protein extract (65µg applied per each lane) was separated in 10% SDS-PAGE, blot on the nitro-cellulose membrane and developed with 1:10000 antibody against CPY in 5% milk in TBS-Tween 1x.

To investigate if *via3* is a transcription mutant, the relative abundance of APE1-mRNA of negative and positive spores of the *via3*-tetrad and wild type was determined. RNA extracted from early exponentially growing cultures in YPD-medium, was subjected to Northern blot analysis. The entire APE1 – gene was used as a DNA-probe. APE1- probe was isolated from p313/LAPIV plasmid with XhoI endonuclease and gel extraction by Qiagen. The labelling reaction was performed with ^{32}P-dCTP. 2µCi labelled probe ($4.4x10^6$ cpm) in 3 ml hybridisation solution was used for blot hybridisation. Visualisation was performed using a Phosphoimager model Fuji IPR 1000 and Image Reader and Image Gauge software. The fluorescent image of the RNA gel demonstrates that equal amounts of RNA have been loaded on the gel (figure 20). APE1 gene has an ORF of 1544 bp and together

with upstream sequences until the TATA box measures about 1800 bp. In the figure 21, the intensity of the upper band (a non – related RNA signal) does not vary with the genotype and which therefore can serve as internal control for the amount of RNA loaded on the gel. The RNA – band migrating above the 28S rRNA corresponds to the APE1-mRNA. APE1-mRNA in *via3*– mutants (3IVC and 3IVD) is significant lower then those in wild type (C6C) as well as in *via3* – positive spores (3IVA and 3IVB). Smaller RNA species whose signal intensities are also decreased in *via*3 are probably degradation products of APE1-mRNA.

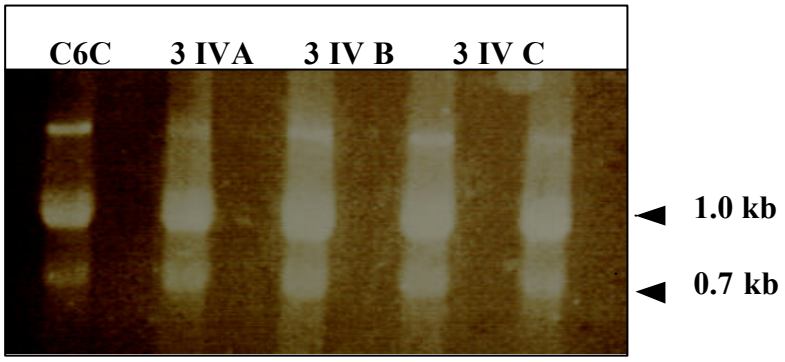


Figure 20: **Fluorescent image of RNA gel**

On the figure is indicated the 28S rRNA (equivalent to 1kb DNA) and the 18S rRNA (equivalent to 0.7 kb DNA).

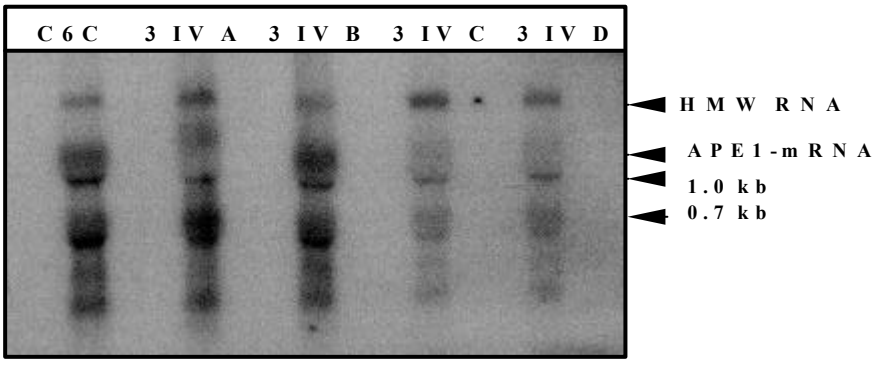


Figure 21: **Northern blot analysis of *via* 3 spores**

Equal amount of RNA (10µg) was applied per lane (see figure 20). APE1- probe (1.8 kb) was isolated from p313/LAPIV plasmid with XhoI endonuclease and gel extraction by Qiagen. The labelling reaction was performed with ^{32}P-dCTP. 2µCi labelled probe ($4.4x10^6$ cpm) in 3 ml hybridisation solution was used for blot hybridisation. Any decrease in mRNA levels in *via3* mutants versus wild type cells should reflect changes specific to mutation of gene transcription. HMW is a non-related RNA signal of high molecular weight.

This result demonstrates that transcription of APE1 is reduced in *via3* or that factors stabilising APE1-mRNA are mutated in *via3*. However by hybridisation with non-related genes, as actin or glyceraldehyde 3-phosphate dehydrogenase, has to be performed to verify the result.

4.4. Analysis of *via10* and *via14* mutants

As was concluded in chapter 4.3.2., two classes of pApe1p transport mutants have been identified, *via10* and *via14*, and they were further analysed.

4.4.1. Viability under nutrient starvation

Mutants of autophagy and of the Cvt pathway for pApe1p transport show reduced viability under nitrogen starvation. In stationary cultures of any media vacuolar protein degradation is induced. Specific activities of vacuolar enzymes and the transport of proteins to be degraded to the vacuole are increased. If vacuolar function is impaired cells are unable to respond to the starvation stress and have a reduced viability. To investigate whether *via*-mutants have a similar phenotype, growth curves were recorded.

The growth of *via10* and *via14* cells was monitored in rich (YPD) and in nitrogen limited media (MV) and the results are shown on figure 22. Both mutants grew as fast as the wt strain and reached the same cell densities in rich media, but the *via14* mutant showed a prolonged lag-phase after inoculating the media from a stationary culture (24 h) in rich media (YPD). Under nitrogen limiting conditions (MV-His) neither of the *via*-mutants reached the cell densities of the wt strain. *via10* cells grew in the early log-phase as fast as the wt strain. *via14* cells displayed a prolonged lag – phase also under these conditions, grew slower then the wt and did not reach the cell density of the *via10* mutant. Differences in cell numbers however are small, so the number of dead cells in the cultures were measured by tryphan-blue staining. Both mutant cultures contained 20% dead cells as compared to 6% in the wt culture during

mid-log phase at glucose concentrations between 1.5-0.5% and up to 40% compared to 15% in stationary cultures at 0.25% glucose as measured by trypan-blue staining (table 20).

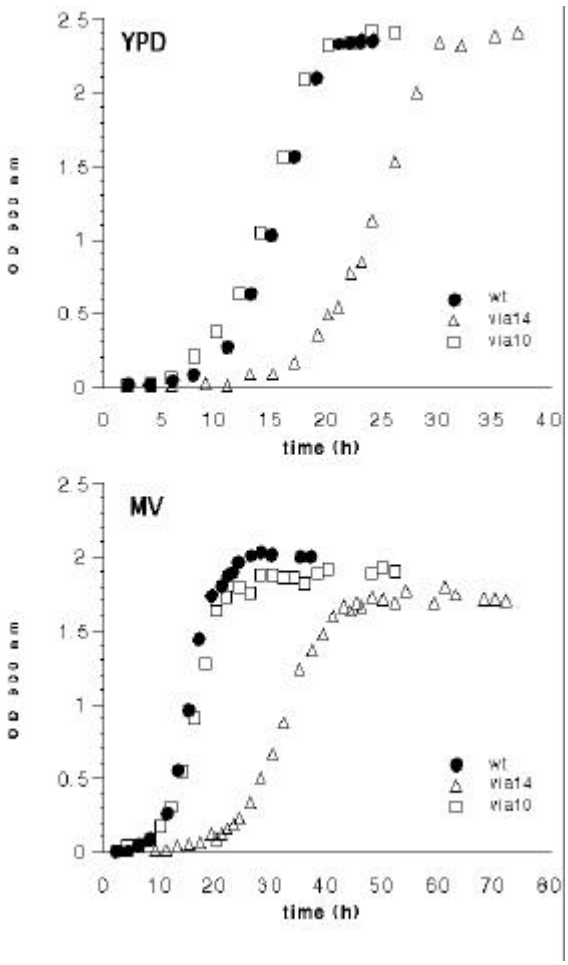


Figure 22: **Growth of wt, *via10* and *via14* cells in rich medium (YPD) and in nitrogen limited medium (MV) at 30°C**

Liquid cultures were inoculated 1:10000 from stationary 24 h YPD cultures.

Table 20: **Viability of *via*-mutants and wild-type**

Glucose concentration (%) in MV	wt	*via10*	*via14*
1-2	n.d. (start)	14% (start)	14% (start)
0.5	7% (30h)	21% (50h)	21% (77h)
>0.25	15% (50h)	32% (78h)	38% (98h)

Numbers give the amount of dead cells in percent. The culture time is given in parenthesis.

4.4.2. Vesicular accumulation of pApe1p

The transport of pApe1p to the vacuole can be subdivided into 5 distinct steps:

(1) pApe1p dodecamerization in the cytoplasm,

(2) formation of the *cvt* vesicle,

(3) transport of the *cvt* vesicle to the vacuole,

(4) fusion of the *cvt* vesicle membrane with the vacuolar membrane,

(5) vacuolar degradation of the *cvt* vesicle inner membrane and proteolytic maturation of pApe1p.

To analyze whether in *via10* and *via14* pApe1p accumulates in the cytosol or in a membrane enclosed compartment protease protection experiments were performed. The vesicular localization of pApe1p in *via*-mutants was analyzed (figure 23) by testing the susceptibility of the precursor protein to trypsin or proteinase K in the presence or absence of detergent. Cells in logarithmic growth stage were converted to spheroblasts and lysed with DEAE-Dextran. Lysis of the spheroblasts and presence of intact vesicles after the lysis were tested by centrifugation of the lysate at 5000 g and western-blot

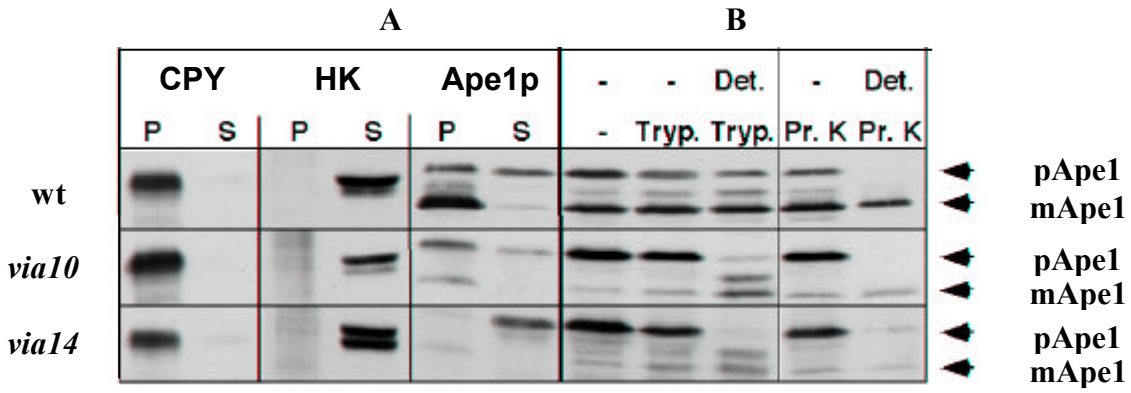


Figure 23: **Subfractionation of pApe1p and mApe1p by 5000 g centrifugation (A) Protease protection assay (B)**

Incubations for protease protection were performed in the absence (-) or presence of Triton X-100 as detergent (Det.) and in the absence (-) or presence of trypsin (Tryp) or proteinase K (Pr.K). Equivalent of 10 OD_{600} cells were loaded on each lane

analysis of pellet and supernatant fractions for the vacuolar carboxypeptidase Y (CPY) and cytosolic α-hexokinase (αHK). mApe1p and the majority of pApe1p were found in the pellet fractions in the wild type (C6C), which is in agreement with previous results *(Scott at al., 1997)*. The same distribution of Ape1p proteins was found in the *via10* mutant. However in *via14* pApe1p was found in the supernatant.

The lysate were incubated for 30 min at 4 °C without proteinase added, with added trypsin or proteinase K and with or without Triton X-100. In the absence of detergent the majority of pApe1p was found to be protease protected in *via10* and *via14* cells as in wild type cells. Difference concerning protease sensitivity of pApe1p was observed between the wild type (wt) and the *via*-mutants when the experiment was performed in the presence of detergent and using trypsin instead of proteinase K. Membrane protected pApe1p from wt cells was resistant to proteolysis by trypsin, but was degraded by proteinase K (figure 23). pApe1p accumulating in *via10* and *via14* cells was processed by trypsin to proteins of the size of the intermediate and mature Ape1p forms. Proteolysis of pApe1p in both *via* mutants by proteinase K led to complete degradation of pApe1p. From this result it was concluded that pApe1p accumulates in a membrane-enclosed compartment in *via10* and *via14*, and that pApe1p is not in a native conformation in both *via* mutants.

Prevacuolar accumulation of pApe1p

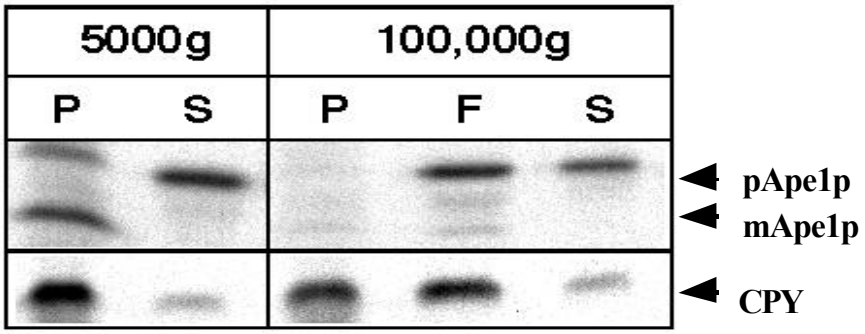


Figure 24: **pApe1p localisation in *via14* cells after fractionation of cell lysate**

Fractionation was performed by centrifugation at 5000 x g and 100000 x g on a 10% ficoll cushion. Equivalent of 10 OD_{600} cells were loaded on each lane. The symbols on the figure represent: the pellet fraction (P), the ficoll fraction (F) and the supernatant fraction (S). Fractionation of the vacuolar CPY served as control.

In the *via14* mutant pApe1p accumulates in pre-vacuolar vesicles, which had a lower density then wild type *cvt* vesicles and were found in a 5000 g supernatant fraction in 1.2 M sorbitol. *via 14* vesicles were subjected to a 100000 g centrifugation (45 min, at 4 °C) onto a 10% ficoll cushion to exclude the possibility that only conformational changes of the protein are responsible for the detergent requirement for proteolytic degradation. 80% of pApe1p was found at the 10% ficoll fraction, 20% were in the supernatant and no pApe1p was found in the pellet (figure 24). 50% of pApe1p in the ficoll fraction was protease protected while those in the supernatant were not protected.

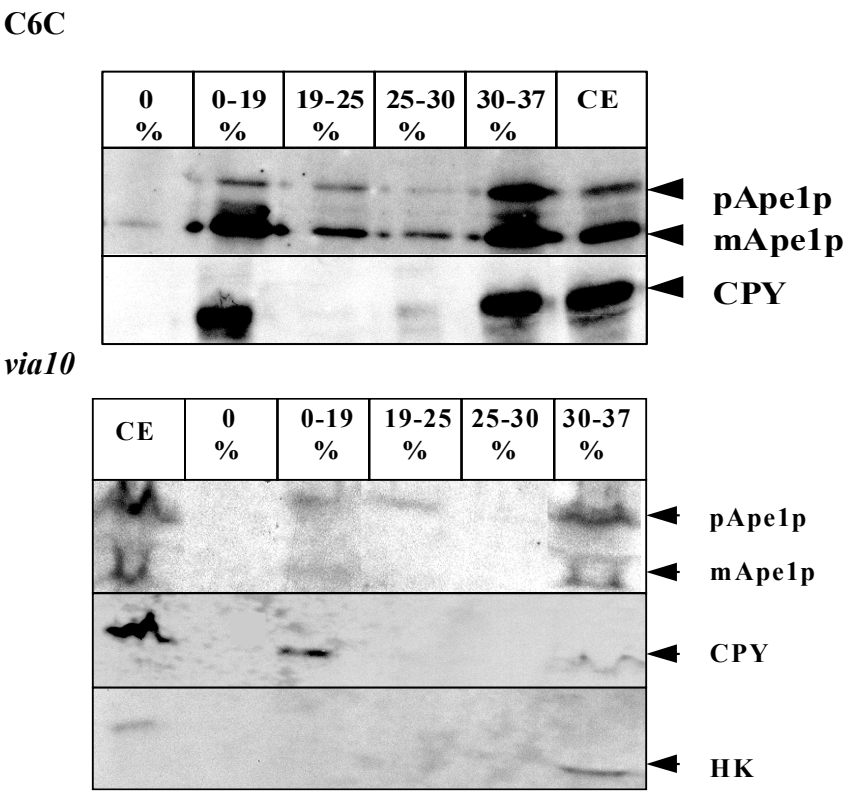


Figure 25: **Flotation gradient of wild type (C6C) and *via 10***

On the figure are shown the fractions of the Optiprep gradient and the protein crude extract (CE) in homogenate aplied on the gradient. The equivalent of 250 OD_{600} cells in logarithmic growth were converted to spheroblasts with zymolyase 20T (c_f 0.5 mg/50OD). Lysed spheroblasts were mixed with spheroblasts with zymolyase 20T (c_f 0.5 mg/50OD). Lysed spheroblasts were mixed with Optiprep medium to a final concentration of 37% and applied on the bottom of a multi-step gradient (concentration as indicated). Separation was monitored by western 25000 rpm in a SW-40 rotor.

In *via10* pApe1p accumulated in a membrane enclosed compartment, which co-fractionated with vacuoles in the protease protection experiment. To test whether pApe1p accumulates within vacuoles or in a prevacuolar compartment *via10* speroblasts (lysed with) were subjected to flotation gradients in Optiprep. No pellet was formed during the centrifugation; lysed spheroblasts remained in the high density fraction. The distribution of precursor and mature pApe1p were analysed by western blot. As intern control, the western blot was developed with vacuolar carboxypeptidase Y (CPY) and cytosolic α-hexokinase (α HK). In extracts from wild type (C6C) vacuoles and mApe1p was found at the 0-19% interface, while *cvt* vesicles pApe1p were in the 30-37% interface. The uncompleted lysis of the spheroblasts, which remained in the high density fraction explain the amount of mApe1p and CPY in fraction 30-37% (figure 25). In *via10* pApe1p was found mainly in 30-37% density fraction and the vacuoles, as in C6C at the 0-19% interface. This result shows that pApe1p accumulates in *via10* in a pre-vacuolar compartment.

4.4.2. *via 10* and *via 14* mutants are deficient in dodecamer formation of pApe1p

As shown by *Kim et al. (1997*), the pApe1p monomer assembles into a homododecameric complex in the cytosol. The dodecameric complex of mApe1p is required for its enzymatic activity *(Metz and Marx, 1977).* The protease protection experiments demonstrated an alterated conformation of the pApe1p in the *via*-mutants. Therefore oligomerization of Ape1p was analysed in the *via* mutants by glycerol density centrifugation.

Crude protein extracts were prepared from cells grown in YPD to OD_{600} = 0.8-1.2 by breaking spheroblasts with glass beads in the presence of protease inhibitors. 10 fractions of the glycerol gradient were collected and proteins were separated by 10% SDS-PAGE. Ape1p was detected by western blot analysis and signal intensities were quantified (wincam software). Ovalbumin (45 kDa), bovine serum albumin (65 kDa) and thyroglobulin (669k Da) were used to follow separation on the gradient. Distribution of mApe1p and pApe1p over the gradient were quantified separately to demonstrate the alterations in the distribution of the two proteins. In wt cells 90% of Ape1p accumulated

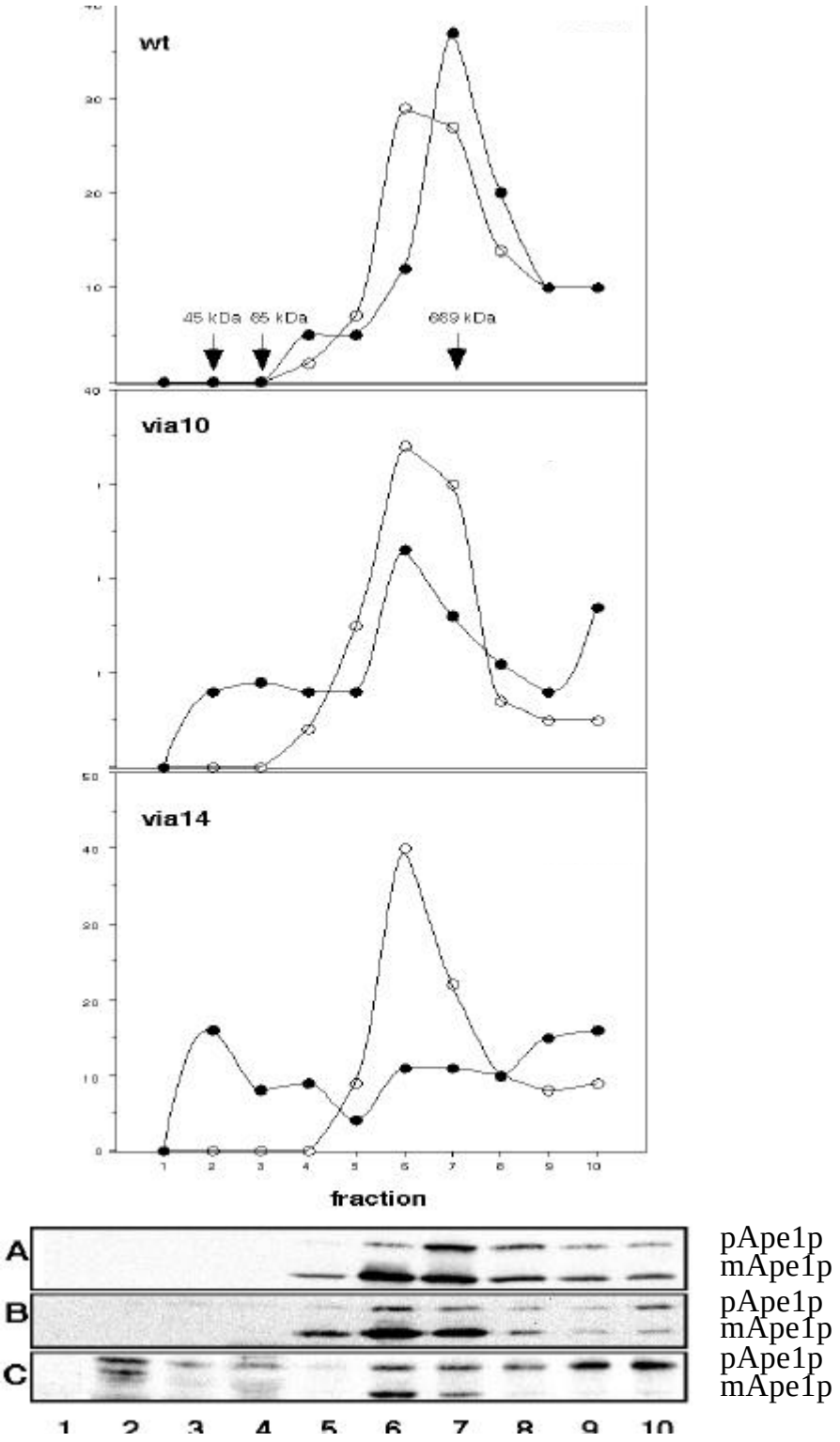


Figure 26: **pApe1p and mApe1p oligomers separated on a glycerol gradient**

On the graphics mApe1p/pApe1p distribution [%] of the fraction number; pApe1p was represented by (°) and mApe1p by (•). The multiple-step glycerol gradient (2.5 ml) was set up by loading 525 µl of each glycerol solutions (50, 40, 30 and 20%) and protein extract (1.5 mg). 10 fraction (each of 250µl) were collected after centrifugation for 4 h at 55000 rpm in a TLS-55

ultracentrifuge rotor and subjected to western blot analysis with antibody against Ape1p.

as dodecamers of pApe1p and mApe1p in fractions 5, 6 and 7 and 10% of each, pApe1p as well as mApe1p, were found in larger complexes in fraction 10 of the gradient (figure 26-A). In *via10* about 10% of total pApe1p are found in fractions 3 and 4 and 20 % of pApe1p are found in fraction 10 demonstrating defects in pApe1p - complex assembly and formation of high molecular weight aggregates (figure 26-B). In *via14* mutant (figure 26-C) 90% of the vacuolar mApe1p form stable dodecameric complexes as in *via10* and wt. pApe1p accumulats in a non-native state in *via14* and is almost evenly distributed over the entire gradient. Slight enrichment was observed in fractions 2 and 9, 10 of the gradient.

4.4.4. Overexpression of Ssa 1 and Ssa 2 in *via10* and *via14* mutants

Heat shock proteins have been shown to assist protein translocation across membranes, protein folding and protein complex assembly. It has been shown by C. Satyanarayana (unpublished) that the hsp70 - heat shock proteins Ssa1 and Ssa2 are involved in the transport of pApe1p from the cytoplasm into the vacuole. Therefore we tested whether the defects in the *via10* and *via14* – mutants can be corrected by Ssa1p and Ssa2p. Both strains were transformed with each of the genes on a single copy plasmid containing the gene under its own promotor. Transformants were selected on MV-HIS, LEU plates and screened by Ape1p-'overlay' assay for the complementation of the Ape1p activity defect. No effect was observed. Western blot analysis of the transformants were performed with antibodies against Ape1p and Ssa1/2. In *via10* transformed with SSA1 or SSA2 and in *via14* transformed with SSA2 pApe1p processing was upregulated by overexpression of Ssa1 and Ssa2 (figure 27). No effect was found in *via14* transformed with SSA1.

Whether Ssa 1 or Ssa 2 may assist in oligomerization of pApe1p, protein extracts were analysed by glycerol gradient experiments. Preliminary data (figure 28) have not shown any effect on dodecamerization in *via10* transformed with Ssa1 or Ssa2 (panel A, B) and also not in *via14* transformed with Ssa1 (panel C). Smaller pApe1p

complexes were still found in the gradient (fractions 2 - 4, hardly seen on figure 28) and also larger aggregates at the bottom fraction of the gradientare still formed (clearly seen on fractions 8-10, figure 28). No clear data about Ssa2 involvement in transport of pApe1p in *via14* transformed with Ssa2 were obtained. Further experiments are required.

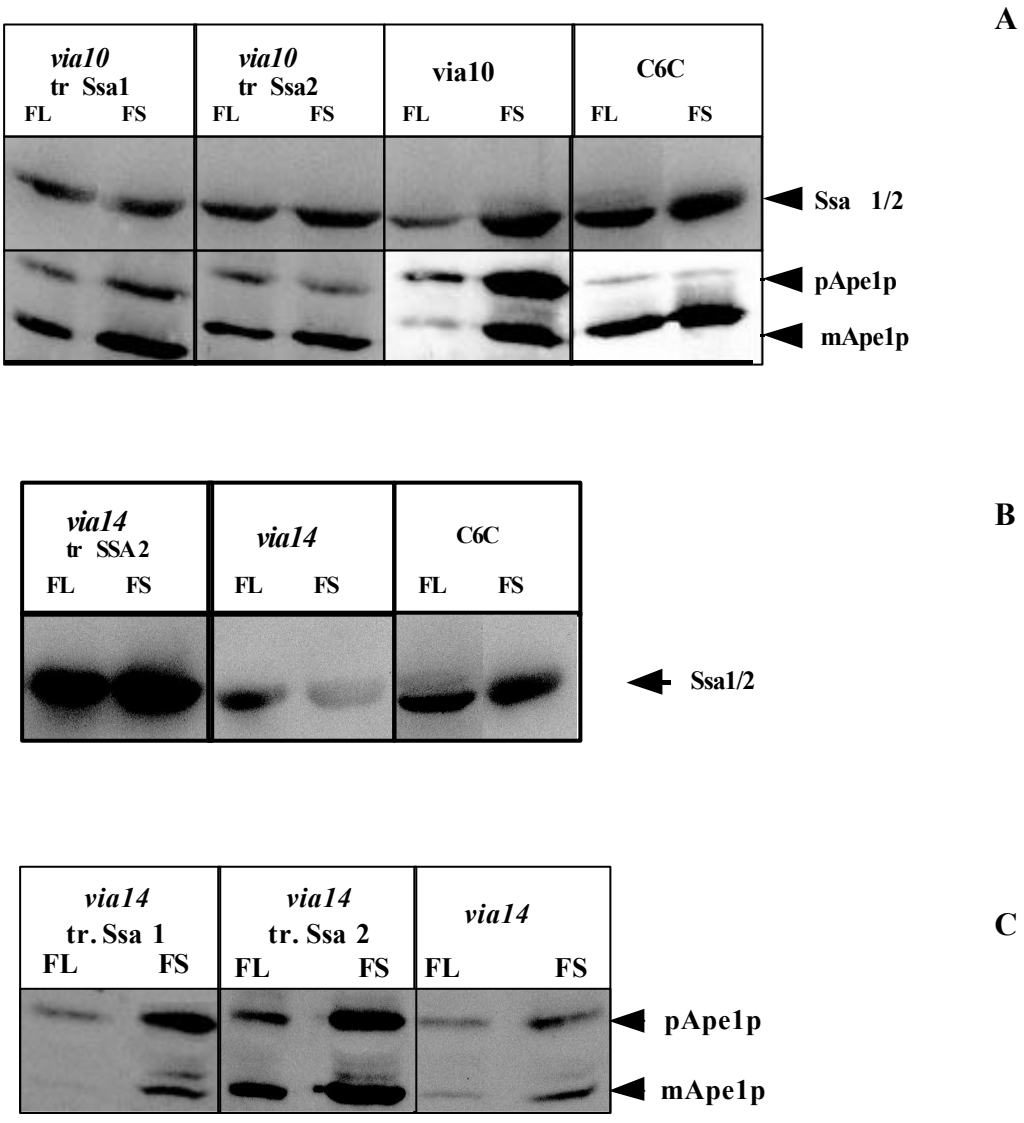


Figure 27: **Western blot analysis of *via*-mutants transformed with SSA1 and SSA2**

via10 tr. Ssa1, *via10* tr. Ssa2, *via14* tr. Ssa1 and *via14* tr. Ssa2 were named the *via* mutants in which Ssa1/2 have been expressed and analized. In log-phase (FL) and stationary phase (FS) Ssa1 and Ssa2 induce Ape1p maturation in *via10* (panel A). In *via14* only Ssa2 assist pApe1p maturation (C). The weak signal for Ssa1/2 in *via14* is probably due to weak fluorescence signal and an artefact of western blot development (panel B, *via14*).

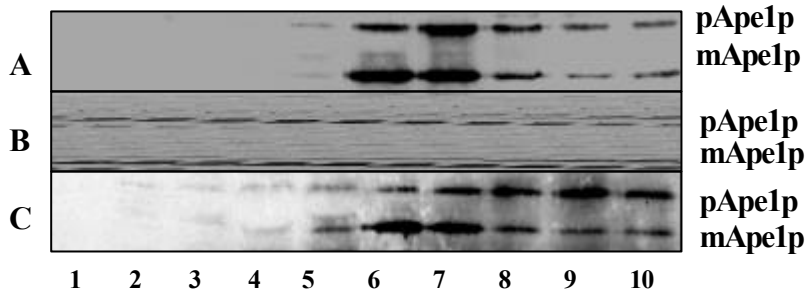


Figure 28: **Glycerol gradients of *via* – transformants with Ssa1 or Ssa2**

panel A: *via10* tranformed with SSA1; panel B: *via10* tranformed with SSA2; panel C: *via14* tranformed with SSA1; pApe1p agregates at the bottom of the gradient in panel A, B, C. The work was performed as described to figure 26.

4.4.5. Library screen for *VIA10* and *VIA 14* genes

Library screen for *VIA10* genes

A genomic DNA library of *S. cerevisiae* (fractions named P1- P5, from 13 in total) on the centromeric plasmid pCS19 was transformed in *via10*. From 4800 colonies tested by APE1-overlay assay 22 colonies with increased activity were identified. The library plasmids were isolated from five of transformants and expressed in *E.coli*. So far, two different library plasmids were identified. They were named CA2 and CA4. To prove the correction of the defect in the positive clones identified by library screening of *via10*, *via10* was re-transformed with CA2 and CA4. Transformants showed restoration to 60-70% of wild type Ape1p activity. Therefore, these genes are suppressors of *via10*.

The 2.8 kb/Pst1 fragment and 5.5 kb/Pst I fragment of the CA2 library plasmid (P11.10) and the 10 kb/Pst1 fragment of CA4 library plasmid (P10.5) were subcloned into pBluescript II SK vector. The 2.8 kb/Pst1 fragment was found to share 99% identity to S. cerevisiae chromosome IV – cosmid 8119. They will be further characterised by subcloning and sequencing.

Library screen for *VIA*14 genes

via14 (14IIA) was transformed with two yeast genomic plasmid libraries (YCP126 and pYEP13) and transformants were selected on MV-HIS, Leu plates. The transformants were then screened by Ape1p-'overlay' assay. In the case of transforming 14 II A with YCP 126 library, 164 colonies were tested and 3 positive colonies were identified. In the case of transforming 14IIA with pYEP13 library, from 111 colonies tested 3 positive colonies were identified. Also these do not restore Ape1p activity to wt levels and are therefore suppressors of *via14* rather then the *VIA14* gene. These library plasmids will be further analysed.

5. DISCUSSION

A resident vacuolar protein, aminopeptidase 1 (Ape1p) is transported to the vacuole by a pathway independent of the secretory pathway. It has a long processing time (45 min) and it‘s maturation involves vacuolar protaeses. Sorting of pApe1p to the vacuole is mediated by its bipartite N-terminal extension. The active form of Ape1p is a homododecamer of 600 kDa.

A translocation event at the vesicular / vacuolar membrane or an incorporation into a cytoplasmic vesicle (as in macroautophagy) or a specific uptake at the vacuolar membrane (as in microautophagy) were suggested as possible mechanisms of pApe1p import into the vacuole.

The screen employed by D.J.Klionsky was designed to identify mutants in pApe1p transport (*cvt* mutants) by western – blot detection of mutant colonies accumulating the pApe1p precursor peptide. The autophagy mutants (*aut*-, *apg*-) were selected for defect in the accumulation of autophagy bodies by a screen based on observations by light microscopy of the EMS mutagenized cells *(Thumm, 1994; Tsukada and Ohsumi, 1993).*

Biochemical and morphological studies using *cvt-, aut*- and *apg*- mutants showed that pApe1p is transported to the vacuole directly from the cytoplasm via a vesicle mediated mechanism termed the cytoplasm to vacuole targeting (Cvt) pathway. It was suggested that the precursor form of aminopeptidase 1 (pApe1p) rapidly dodecamerizes in the cytosol and is then enwrapped by a double membrane. The formed vesicles are transported to and fuse with the vacuolar membrane like an autophagosome and pApe1p can be detected in single – membrane vesicles within the vacuole of mutants defective in the degradation of these membranes. During vegetative growth, pApe1p utilises the Cvt pathway, while under starvation conditions pApe1p is transported by macroautophagy.

5.1. A new genetic screen to analyse vacuolar import and autophagocytosis mutants of aminopeptidase 1

Our screen was designed to detect mutants in pApe1p transport but based on the enzymatic activity of the processed and dodecameric Ape1p thus extending the screen to the end of the trafficking pathway to the vacuole. With this new screen we isolated mutants which display defects in pApe1p transport which have not been reported for other mutants isolated so far. They were named *via*-mutants from vacuolar import and autophagocytosis. Enzymatic activity of mature and dodecameric mApe1p were assayed by a culture – plate ‚overlay' activity assay. Confirmation of lack of Ape1p - activity in *via* mutants was verified by measuring the specific enzymatic activity. This revealed that *via* mutants have reduced activities, but that they are higher than expected from the colony 'overlay' – activity assay. The major difference between the two assay-procedures is that for specific activity measurements Zn^{2+} is included, which is required for full enzymatic activity, and that the Leu-pNA is a more specific substrate for Ape1p, then the Leu-βNA used in the overlay assay.

pApe1p is processed by the endopeptidases PrA and PrB, which reach the vacuole along the secretory pathway. To test for intact pApe1p processing conditions in the *via*-mutants, specific activity of the vacuolar CPY was determined (see 4.2.1.). CPY reaches the vacuole also by secretory pathway and is also processed by proteases PrA and PrB. This and the staining of the vacuole with a fluorescent dye revealed an intact vacuole in *via10* and *via14* mutants (see 4.3.3. and figure 18).

Two complementation groups were formed so far: *via10* and *via14.* (see 4.3.2.) Both *via*–mutants accumulate pApe1p, in membrane enclosed compartments demonstrating defects in pApe1p transport. While this compartment in *via10* was indistinguishable from the wild type vesicles, the *via14* vesicle displayed reduced density compared to those in wild type cells and are extremely fragile, demonstrating a severe defect in Cvt vesicle biogenesis.

While in wild type cells all pApe1p and mApe1p are in dodecameric complexes, assembly of pApe1 into dodecameric complexes is disturbed in both *via*-mutants. Besides the formation of aggregates with molecular size higher than the dodecameric 770 kDa in both mutants, smaller complexes of pApe1p were found in both mutants and are more abundant in *via14.* The mature Ape1p in the mutants instead was found to be

dodecameric, which is in agreement with the specyfic enzymatic activities found in the mutants. However the increased sensitivity of the pApe1p to denaturation conditions indicates that the dodecamer is not properly assembled or stabilized.

In the model for the Cvt pathway (*Scott and Klionsky, 1996)* pApe1p rapidly dodecamerizes in the cytoplasm and is then enwrapped by a double membrane. These vesicles are transported to and fuse with the vacuolar membrane like an autophagosome and pApe1p can be detected in single – membrane vesicles in the vacuole in mutants defective in the degradation of these membranes. In the *via*- mutants, formation of the surrounding membrane takes place, but pApe1p is not properly assembled; smaller complexes and also aggregates are formed. This phenotype was not previously described in the *cvt* and *apg, aug* mutants analysed so far (figure 29).

Processing of pApe1p in the *via*-mutants by trypsin in the presence of detergent leads to the formation of intermediate sized proteins of the same molecular mass as the intermediate and mature forms of the protein generated by the vacuolar endopeptidases proteinase A and proteinase B in *vivo*. The same experiment performed in the absence of detergent did not lead to the pApe1p processing. This indicates that the signal sequence is protease protected. The increased sensitivity to proteases and the defect in dodecamerisation in the *via* cells indicates that Cvt vesicles in wild type cells contain factors which stabilize the dodecamer.

5.2. Model of pApe1p transport based on *via10* and *via14* phenotype

In the model for the Cvt pathway, pApe1p rapidly dodecamerizes in the cytoplasm with a $T_{1/2}$ = 2 min. The dodecamer is then enwrapped by a double-membrane to form the electron dense cvt vesicle. Formation of the Cvt vesicle and ist vacuolar transport requires additional 43 min (figure 29 - a). This is extremely slow compared to the $T_{1/2}$ = 5 min for the CPY transport through the secretory pathway. The *via*-mutants reveal that Cvt vesicle formation and pApe1p dodecamerization are linked processes.

We do not know whether in *via*-mutants smaller pApe1p complexes are enwrapped by a membrane or whether dodecamers initially form in the cytoplasm,

become enwrapped, but are less stable in the vesicles (Figure 29: b). Klionsky reported that Cvt vesicles are small and contain a dense core of pApe1p dodecamers which

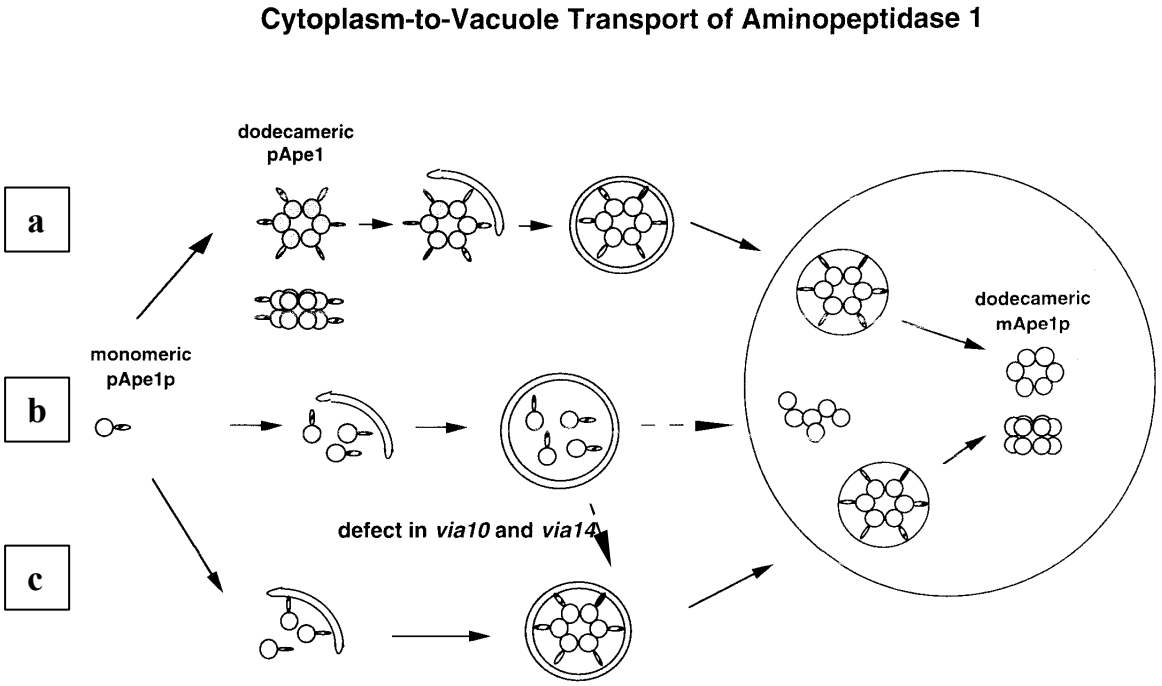


Figure 29: **Model of pApe1p transport based on *via10* and *via 14* phenotype**

a: Cvt pathway;
b: mutant phenotype in *via10* and *via14*
c: modification of the model for the Cvt pathway based on *via10* and *via14* phenotypes

would not leave space for dissociation and re-association reactions. *via10* vesicles have the same density as wt vesicles, but pApe1 dodecamerisation is affected. Furthermore, we observe in both mutants enhanced formation of large pApe1p aggregates, indicating an uncoordinated complex formation and not absence of complex formation. In addition vacuolar dodecameric mApe1p displays increased sensitivity to denaturing conditions (see 4.4.2), demonstrating that these complexes are not in a native state. This can also explain the reduced amount of mApe1p in the mutants. The proper oligomerisation is disturbed in both *via* mutants. The accumulation of pApe1p in prevacuolar vesicles

demonstrates that complex formation as well as vesicular transport are disturbed, indicating that formation of transport vesicles and oligomerisation are linked processes (figure 29: c). This could be caused for example by chaperones normally present in those vesicles, which interact with pApe1p as well as proteins required for vacuolar transport of Cvt vesicles. This interpretation is supported by the observation that Ssa1/2 can stimulate vacuolar transport, but this does not restore Ape1p enzymatic activity (see 4.4.4.).

5.3. Specificity of *via*-mutants

Ape1p was shown to use two pathways for vacuolar import: constitutively, the cytoplasm to vacuole pathway, and the, predicted as being non-selective, autophagy pathway when high protein turnover is necessary for the cell to adapt to a new state. Majority of mutants in the autophagy and Cvt pathways display the same phenotypes on pApe1p transport *(Harding et al., Scott et al., 1996).* Inhibition of peroxisome degradation in *cvt* and *apg* mutants indicates that these pathways also overlap with that of peroxisomal degradation pathway (selective autophagocytosis or microautophagy) to the vacuole *(Hutchins et al., 1999*). To test the specificity of the defect in pApe1p transport in *via* mutants studies are in progress for the analysis of pexophagy. I followed in *via* mutants the αPcs60p induced expression by starvation in rich and selective medium (YPD, MV) as well as in medium containing oleic acid (YPO) and its degradation upon shift into fresh rich and selective medium (YPD, MV). Preliminary results suggest that pexophagy is not affected in *via10.* This supports the view that this new genetic screen is usefull to isolate mutants with specific defects in pApe1p transport.

At the moment , the identification of the *VIA10* and *VIA14* genes by a library cloning approach is under the way. However, since many mutants are generated and the complete yeast genome sequences are available, an approach based on proteom analysis *(Patterson and Aebersold, 1995; Shevchenko et al, 1996)* might hurry the identification of *VIA10* and *VIA14* gene products. This approach would have the advantage of shortening the way of identifying the proteins involved in pApe1p transport. Implication

of an identified protein has to be verified by generating a knock-out of the encoding gene.

6. CONCLUDING REMARKS

A new genetic screen for pApe1p transport mutants was performed to isolate new transport mutants.

Characterization of the transport defects in two *via* mutants reveals that pApe1p dodecamer formation and Cvt vesicle formation are linked processes. This is a new and unexpected finding, because rapid formation of the stable pApe1p dodecamer was observed in the cytosol of wt cells and Cvt vesicles contain dense core pApe1p complexes.

The strategy to use the enzymatic activity of dodecameric mApe1p to search for new pApe1p transport mutants has turned out to be useful for the isolation of specific pApe1p transport mutants.

7. REFERENCES

ALBERTS, B., BRAY, D., LEWIS,J., RAFF, M., ROBERTS, K. AND WATSON, J.D. (1994)
Molecular biology of the cell,
3rd ed, Garland Publishing, Inc.

ANDREW, A.T. (1994)
Electrophoresis of nucleic acids, in Essential Molecular Biology, a practical approach, vol I, ed. Brown, T.A., Oxford University Press, USA, pg 89.

AUSUBEL, F.M., BRENT, R,, KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A., STRUHL, K (1994-1998)
Current protocols in molecular biology, vol. 1, 3
John Willey & Sons. Inc

BABA, M, OHSUMI, M., SCOTT, S.V., KLIONSKY, D.J. AND OHSUMI, Y. (1997)
Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome
J.Cell Biol. 139, 1687-1695.

BANKAITIS, V.A., JOHNSON L.M. AND EMR, S.D. (1986)
Isolation of yeast mutants defective in protein targeting to the vacuole
Proc.Natl.Acad.Sci.USA 83, 9075-9079.

BANTA, L.M., ROBINSON, J.S., KLIONSKY, D.J. AND EMR, S.D. (1988)
Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting
J.Cell Biol. 107, 1369-1383.

BERLIN, V., BRILL, J.A., TRUEHEART J., BOEKE, J.D. AND FINK, G.R.
Genetic screens and selections for cell and nuclear fusion mutants, pg. 774-776,

in Guthrie, C. and Fink, G.R. Methods in enzymology, 194, Guide to yeast genetics and molecular biology, Academic Press Inc, 1991, USA .

BRADFORD, M.M. (1976)

A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding.

Anal. Biochem. 72, 248-254.

CHANG, Y.H. AND SMITH, J.A. (1989)

Molecular cloning and sequencing of genomic DNA encoding aminopeptidase I from *Saccharomyces cerevisiae*

J.Biol.Chem. 264, 6979-6983.

DARSOW, T, RIEDER, S.E., EMR, S.D. (1997)

A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole

J.Cell Biol. 138, 517-529.

EAKLE, K.A., BERNSTEIN, M., AND EMR, S.D. (1988)

Characterization of a component of the yeast Secretion machinery: identification of the *SEC18* gene product

Molecular and cellular Biology 8, 4098-4109.

GANNON, F. AND POWELL, R. (1991)

Construction of recombinant molecules, pg. 155-157, in Essential Molecular Biology, A practical approach, vol. I, Ed. T.A. Brown, Oxford University Press, USA.

GOFFEAU, A., BARREL, B.G., BUSSEY, H., DAVIS, R.W., DUJON, B., FELDMANN, H., GALIBERT, F., HOHEISEL, J.D., JACQ, C., JOHNSTON, M., LOUIS, E.J., MEWES, H.,W., MURAKAMI, Y., PHILIPPSEN, P., TETTELIN, H. AND OLIVER S.G. (1996)

Life with 6000 genes
Science 274, 546-567

GUTHRIE, C. AND FINK, G.R. (1991)
Methods in enzymology, 194, Guide to yeast genetics and molecular biology
Academic Press Inc, 1991, USA.

HEMMINGS, B.A., ZUBENKO, G.S., HASILIK, A. AND JONES, E.W. (1981)
Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*
Proc.Natl.Acad.Sci.USA 78, 435-439.

HARDING,T.M., MORANO, K.A., SCOTT,S.V. AND KLIONSKY, D.J. (1995)
Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway
J.Cell Biol. 131, 591-602.

HARDING,T.M., HEFNER-GRAVINK, A., THUMM, M. AND KLIONSKY, D.J. (1996)
Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway
J.Biol.Chem. 271,17621-17624.

HOFFMAN M. AND CHIANG H.-L. (1996)
Isolation of degradation-deficient mutants defective in the targeting of fructose-1,6-bisphosphatase into the vacuole for degradation in *Saccaromyces cerevisiae*
Genetics 143, 1555-1566.

HORAZDOVSKY, B.F., DEWALD D.B. AND EMR S.D. (1995)
Protein transport to the yeast vacuole
Curr.Opin.Cell Biol. 7, 544-551.

HUTCHINS, M.U., VEENHUIS, M., KLIONSKY, D.J. (1999)
Peroxisome degradation in Saccharomyces cerevisiae is dependent on

machinery of macroautophagy and the Cvt pathway
J. Cell Science 112, 4079-4087.

HUANG, P.H. AND CHIANG, H.L. (1997)
Identification of novel vesicles in the cytosol to vacuole protein degradation pathway
J.Cell Biol. 136, 803-810.

KIM,J., SCOTT, S.V., ODA, M.N. AND KLIONSKY, D.J. (1997)
Transport of a large oligomeric protein by cytoplasm to vacuole protein targeting pathway
J.Cell Biol. 137, 609-618.

KIM, J., DALTON, V.M., EGGERTON, K.P., SCOTT, S.V., AND KLIONSKY, D.J. (1999)
Apg7p/Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways,
Mol.Biol.Cell 10, 1337-1351.

KLIONSKY, D.J., BANTA, L.M. AND EMR, S.D. (1988)
Intracellular sorting and processing of yeast Vacuolar Hydrolase: Proteinase A peptide contains vacuolar targeting information
Mol.Cell.Biol. 8, 2105-2116.

KLIONSKY, D.J. AND EMR, S.D. (1989)
Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase
EMBO J. 8, 2241-2250.

KLIONSKY, D.J. AND EMR, S.D. (1990)
A new class of lysosomal/vacuolar protein sorting signals
J.Biol.Chem., 265, 5349-5352.

KLIONSKY, D.J., CUEVA, R. AND YAVER, V. (1992)

Aminopeptidase I of *Saccharomyces cerevisiae* is localized to the vacuole independent of the secretory pathway
J.Cell Biol.119, 287-299.

KLIONSKY, D.J., NELSON, H. AND NELSON, N. (1992)
Compartment acidification is required for efficient sorting of proteins to the vacuole in *Saccaromyces cerevisiae*
J.Biol.Chem. 267, 3416-3422.

KLIONSKY D. (1998)
Nonclassical protein sorting to the yeast vacuole
J.Biol.Chem. 18, 10807-10810.

LANG, T., SCHAEFFELER, E., BERNREUTHER, D., BREDSCHNEIDER, M., WOLF, D.H. AND THUMM, M. (1998)
Aut2p and Aut7p, two novel microtuble associated proteins are essential for delivery of autophagic vesicles to the vacuole
EMBO J. 17, 3597-3607.

LAMMELI, U.K. (1970)
Cleavage of structural proteins during assembly of the head of bacteriophage To
Nature 227, 680-685.

METZ, G., MARX, R. AND RÖHM, K. (1977)
The quaternary structure of yeast aminopeptidase I. Molecular forms and subunit size. Z. Naturforsch. 32c, 929-937.

MIZUSHIMA N., NODA, T., YOSHIMORI, T., TANAKA, Y., ISHII, T., GEORGE, M.D., KLIONSKY, D.J., OHSUMI, M. AND OHSUMI, Y. (1998)
A protein conjugation system essential for autophagy
Nature 395, 395-398.

MIZUSHIMA N., NODA, T. AND OHSUMI, Y. (1999)

Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway
EMBO J. 18, 3888-3896.

ODA, M.N., SCOTT, S.V., HEFNER-GRAVINK, A., CAFFARELLI, A.D., AND KLIONSKY, D.J. (1996)
Identification of a cytoplasm to vacuole targeting determinant in aminopeptidase I
J.Cell Biol. 132, 999-1010.

PATTERSON, S.D., AEBERSOLD R. (1995)
Mass spectrometric approaches for the identification of gel-separed proteins
Electrophoresis, 1791-1814

RAYMOND, C.K., ROBERTS, C.J., MOORE, K.E., HOWALD I. AND STEVENS,T.H. (1992)
Biogenesis of the vacuole in *Saccharomyces cerevisiae*
International Review of Cytology 139, 59-120.

RIEDER, S.E. AND EMR, S.D. (1997)
A novel RING finger protein complex essential for late step in protein transport to the yeast vacuole
Cell 8, 2307-2327.

ROBINSON, J.S., KLIONSKY, D.J., BANTA L.M. AND EMR S.D. (1988)
Protein sorting in *Saccaromyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases
Mol.Cell.Biol. 8, 4936-4948.^

SAMBROOK, J., FRITSCH, E, MANIATIS, T (1989)
Molecular cloning. A laboratory manual, 2nd ed., CSH Lab. Press.

SCOTT, S.V. AND KLIONSKY, D.J. (1995)

In vitro reconstitution of cytoplasm to vacuole protein targeting in yeast
J.Cell Biol. 131, 1727-1735.

SCOTT, S.V., HEFNER-GRAVINK, A.,.MORANO, K.A, NODA,T., OHSUMI, Y. AND KLIONSKY, D.J. (1996)
Cytoplasm-to-vacuole targeting and autophagy emploi the same machinery to deliver to the yeast vacuole,
Proc.Nacl.Acid.93, pg. 12304-12308.

SCOTT, S.V. AND KLIONSKY, D.J. (1997)
Nonclassical protein sorting
Trends in Cell Biology 7, 225-229.

SCOTT, S.V. AND KLIONSKY, D.J. (1998)
Delivery of proteins and organelles to the vacuole from cytoplasm
Curr.Opin.Cell Biol. 10, 523-529.

SCOTT, S.V., BABA, M., OHSHUMI,Y. AND KLIONSKY, D.J. (1997)
Aminopeptidase I is targeted to the vacuole by a nonclassical vesicular mechanism
J.Cell Biol. 138, 37-44

SEGUI-REAL, B., MARTINEZ, M. AND SANDOVAL, I.V. (1995)
Yeast aminopeptidase I is post-translationally sorted from the cytosol to the vacuole by a mechanism mediated by its bipartite N-terminal extension,
EMBO J. 14, 5476-5484

SHEVCHENKO, A, JENSEN, O.N., PODTELEJNIKOV, V., SAGLIOCCO, F., WILM, M., VORM, O., MORTENSEN, P., SHEVCHENKO, A, BOUCHERIE, H AND MANN, M (1996)
Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels
Proc. Natl. Acad. Sci. USA

SIKORSKI, R.S. AND HIETER, P. (1989)

A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae

Genetics 122, 19-27.

STEVENS, T., ESMON, B. AND SCHEKMAN R. (1982)

Early stages in the yeast secretory pathway are required for transport of Carboxipeptidase Y to the vacuole

Cell 30, 439-448

TANIDA, I., MIZUSHIMA N., KIYOOKA, M., OHSUMI, M., UENO, T, OHSUMI, Y. AND KOMINAMI (1999)

Apg7p/Cvtp: A novel protein – activating enzyme essential for autophagy

Mol. Biol. Cell 10, 1367-1379

TEICHERT, U., MECHLER, B., MÜLLER, H. AND WOLF, D.H. (1989)

Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival

J.Biol.Chem. 264, 16037-16045

TAKESHIGE, K., BABA, M., TSUBOI, S., NODA, T. AND OHSUMI,Y. (1992)

Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction

J.Cell Biol. 119, 301-311

THUMM, M., EGNER, R., KOCH, B., SCHLUMPBERGER, M., STRAUB, M., VEENHUIS, M. AND WOLF, D.H. (1994)

Isolation and autophagocytosis mutants of *Saccharomyces cerevisiae*

FEBS Lett. 349, 275-280

THUMM, M. (1999)

Molecular Mechanism of autophagy in *S.cerevisiae*

Poster to „Transport of proteins and membranes in eukaryotic cells“, Göttingen.

TSUKADA, M AND OHSUMI,Y. (1993)

Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*

FEBS 333, 169-174

YAVER, D.S., NELSON, H., NELSON, N. AND KLIONSKY, D.J.(1993)

Vacuolar ATPase mutants accumulate precursor proteins I a pre-vacuolar compartment

J. Biol. Chem. 268, 10564-10572

YUAN, W., STROMHAUG, P.E. AND DUNN, W.A., JR. (1999)

Glucose-induced autophagy in Pichia pastoris requires a unique E1-like protein, Mol.Biol.Cell 10, 1353-1366

WANG, Y.X., CATLETT, N.L. AND WEISMAN, L.S. (1998)

Vac8p, a vacuolar protein with armadillo repeats, functions in both vacuole inheritance and protein targeting from the cytoplasm to vacuole

J.Cell Biol. 140, 1063-1074

WILTFANG J., AROLD, N., NEUHOFF V. (1991)

A new multiphasic buffer system for sodium dodecile polyacryamide gel electrophoresis of proteins and peptides with molecular masses 100000-100, and their detection with picomolar sensitivity

Electrophoresis 12 (5): 352-366.

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Lebenslauf

Am 14. März 1972 wurde ich , Luminita-Cornelia Andrei, als zweites von drei Kindern der Elisabeta Andrei, geb. Paunescu, und des Pavel Andrei in Galati/ Rumänien geboren.

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