

Identification of a new factor essential for vacuolar aminopeptidase I activity

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**vorgelegt von
Pasupuleti Naga Rekha
aus Hyderabad (Indien)**

D7

Referent: Prof. Dr. Kurt von Figura

Korreferent: Prof. Dr. Gerhard Gottschalk

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To

Mom, Dad and Sis....

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Abbreviations

aa	Amino acid
Amp	Ampicillin
APE1(or LAPIV)	Aminopeptidase I gene
Ape1p	Aminopeptidase 1
APS	Ammoniumperoxodisulfate
ATP	Adenosine 5'-triphosphate
BSA	Bovine Serum Albumin
cDNA	complementary DNA
ct	Control
ddH ₂ O	double distilled water
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleicacid
dNTPs	Deoxynucleosidetriphosphate (dATP, dGTP, dCTP, dTTP)
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamintetraacetate-Disodium salt
ER	Endoplasmic Reticulum
EtOH	Ethanol
Fig	Figure
GFP	Green fluorescent protein
GST	Glutathione-S-Transferase
h	Hour
HEPES	N-2-Hydroxyethylpiperazin-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatogryphy
HRP	Horseradish-Peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl- α -D-Thiogalactopyranoside

kb	Kilobase
kDa	Kilodalton
l	Liter
LB	Luria Bertani
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MOPS	Morpholinoethane sulfonic acid
nm	Nanometer
OD	Optical density
PAGE	Poly-acrylamide gel electrophoresis
pApe1p	Precursor aminopeptidase I
PCR	Polymerase chain reaction
pH	Negative logarithm of H^+ concentration
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room Temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium Dodecyl Sulfate
sec	Seconds
SNARE	Soluble N-ethylmaleimide Attachment protein Receptor
TAE	Tris-Acetate-EDTA-Buffer
TBS	Tris buffer saline
TCA	Trichloroacetic acid
TE	Tris-EDTA-Buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit

UV	Ultraviolet
V	Volt
v/v	(volume/volume)
Vti1a	Vps10p interacting
w/v	(weight/volume)
wt	Wild type
YPD	Yeast Peptone Dextrose
μ	Micro

1 Introduction

1.1 The yeast vacuole

The vacuole in yeast is the analogous organelle to lysosomes in mammalian cells. It has an acidic pH and is rich in hydrolases. The yeast vacuole is the biggest organelle occupying up to 60% of the cell volume. During cell division portions of the vacuole are targeted to the growing bud.

Vacuole is integrally involved in a wide array of physiological processes which include:

- pH and osmoregulation: yeasts mutants with impaired vacuolar functions have reduced viability in media of extreme pH and osmotic strength.
- Protein metabolism: During periods of nitrogen starvation, amino acid supplies are maintained by protein degradation that takes place in the vacuole. Proteinase A and proteinase B are two vacuolar resident endopeptidases responsible for the activation of exopeptidases in the vacuole.
- Diploids lacking vacuolar protease activities fail to sporulate indicating the importance of vacuolar proteolysis for the survival and differentiation of the organism (Raymond *et al*, 1992; Teichert *et al*, 1989). The vacuole serves as the store house for basic amino acids, small ions and polyphosphates. Hydrolase-deficient yeast cells do not survive long periods of starvation.

Thus the vacuole is involved in various and diverse processes, which necessitate the presence of a specific group of hydrolases and transmembrane proteins.

1.2 Delivery of proteins to the vacuole

Vacuolar hydrolases are compartmentalized in the organelle to prevent non-specific cellular damage. There are four pathways by which proteins are known to enter the vacuole. These are the secretory pathway, endocytosis, autophagocytosis and the cytosol-to-vacuole-targeting or Cvt pathway. Vacuolar resident proteins are either transported by the secretory or the Cvt pathway and proteins destined for degradation are transported by autophagocytosis and endocytosis. For example, mating pheromones and their receptors are endocytosed and delivered to the vacuole and degraded in a PEP4 (proteinase A) dependent manner (Sanger and

Reizman, 1990; Dulic and Reizman, 1989; Konopka *et al.*, 1986). The other pathways are discussed in detail in the following chapters.

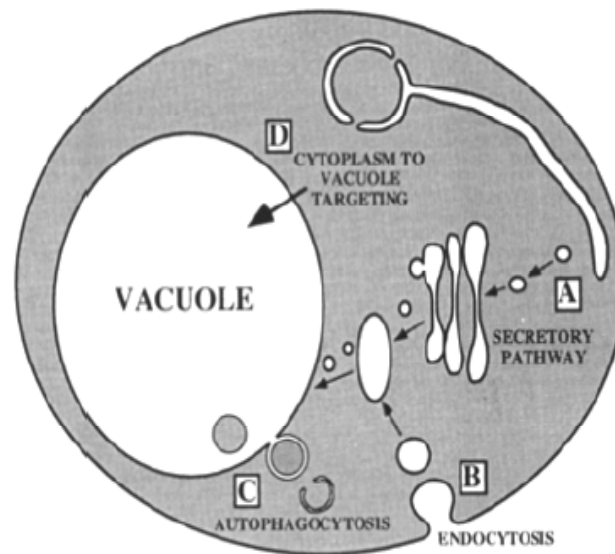


Fig 1: Four routes for the delivery of proteins into the yeast vacuole (Harding *et al.*, 1995).

1.2.1 Secretory pathway

The secretory pathway is utilized in eukaryotic cells to sort and transport proteins from their site of synthesis to several intracellular organelles or plasmamembrane and the periplasma. Most of the vacuolar resident proteins are known to enter the vacuole by this pathway. Proteins are synthesized on ER-bound ribosomes and translocated into the lumen of the ER. There exists an amino terminal cleavable or a non-cleavable internal hydrophobic signal which targets the protein to the ER membrane (Rapoport *et al.*, 1996 Ann. Rev. Biochem). Cleavage of the signal sequence releases the proteins into the lumen of the ER. N-linked carbohydrate addition occurs co-translationally. Proteins are then delivered via transport vesicles to the Golgi complex, where they undergo further post-translational modifications of the carbohydrate side chains before being sorted in a late Golgi compartment, the *trans*-golgi network (TGN) away from proteins to be secreted.

In yeast, vacuolar proteins such as carboxypeptidase Y (CPY), proteinase A and proteinase B transit the early stages of the secretory pathway before being sorted in a late Golgi compartment.

CPY is a serine peptidase which has been studied extensively and is used as a marker protein to follow the vacuolar transport along the secretory pathway. CPY is synthesized as a prepro-precursor molecule and translocated into the ER lumen, where its signal sequence is cleaved off to form pro-CPY (Stevens *et al.*, 1982, Blachy-Dyson & Stevens 1987, Johnson *et al* 1987). pro-CPY receives N-linked core glycosylation in the ER, which results in a 67 kDa form known as p1-CPY. In the Golgi complex, extension of the core oligosaccharides results in an increase in the apparent molecular weight of CPY to 69 kDa; this form is known as p2-CPY. Finally, p2-CPY is proteolytically processed in a proteinase A and proteinase B dependent manner (Mechler *et al.*, 1987) to generate the 61 kDa enzymatically active mature form, referred to as m-CPY. It is made up of a single subunit of 61 kDa, out of which 10 kDa is carbohydrate in the form of N-linked oligosaccharides (Hasilik and Tanner, 1978a). Overproduction of CPY results in missorting and secretion. The secreted CPY is in its Golgi-p2-CPY form. Missorting due to overproduction demonstrates the presence of a saturable limiting component, such as a receptor molecule that facilitates its recognition and/or sorting to the vacuole. The sorting receptor Vps10p is responsible for the vacuolar delivery of CPY. Vps10p binds p2-CPY in the late Golgi sorting compartment, and the receptor-ligand complex is delivered to a prevacuolar/endosomal compartment. In the endosome, CPY dissociates from the receptor and the receptor is recycled back to the sorting compartment for another round of protein transport. p2-CPY then moves on to the vacuole, where it is processed to its mature form.

1.2.2 Autophagy and Cytoplasm - to - vacuole - targeting (Cvt) pathway

Autophagy is a vacuolar trafficking pathway that targets cytosolic proteins and compartments to the vacuole for degradation. The word “autophagy” is derived from Greek ‘self’ (auto) and ‘to eat’ (phagy) *i.e.* ‘to eat oneself’. It is the primary intracellular catabolic mechanism for degrading and recycling long-lived proteins and organelles of the yeast cell. It occurs as a cellular response to both extracellular stress conditions as nutrient starvation and intracellular stress conditions as accumulation of damaged organelles and proteins. Two types of autophagy have been identified, microautophagy and macroautophagy that involve rearrangement of subcellular membranes to sequester cargo for delivery to the vacuole, where the sequestered material is degraded and recycled.

Microautophagy involves the engulfment of cytoplasm directly by the vacuolar membrane, by invagination, protrusion and/or septation of the vacuolar limiting membrane. In contrast, macroautophagy involves the formation of cytosolic double-membrane vesicles, distinct from the vacuoles called autophagosomes, into which portions of the cytoplasm are sequestered. Fusion of an autophagosome with the vacuole results in the delivery of a single membrane autophagic body into the lumen of the vacuole, where the autophagic bodies are degraded. In yeast, autophagy occurs primarily by macroautophagy, based on electron microscopic studies of nitrogen-starved cells (Baba *et al.*, 1994).

Autophagy in yeast was studied by three distinct genetic screens. Apg deficient mutants were selected based on the ability of the mutants to grow in nitrogen starvation media (Tsukada and Ohsumi, 1993). Aut deficient mutants were selected based on the failure to degrade fatty acid synthase (Thumm *et al.*, 1994).

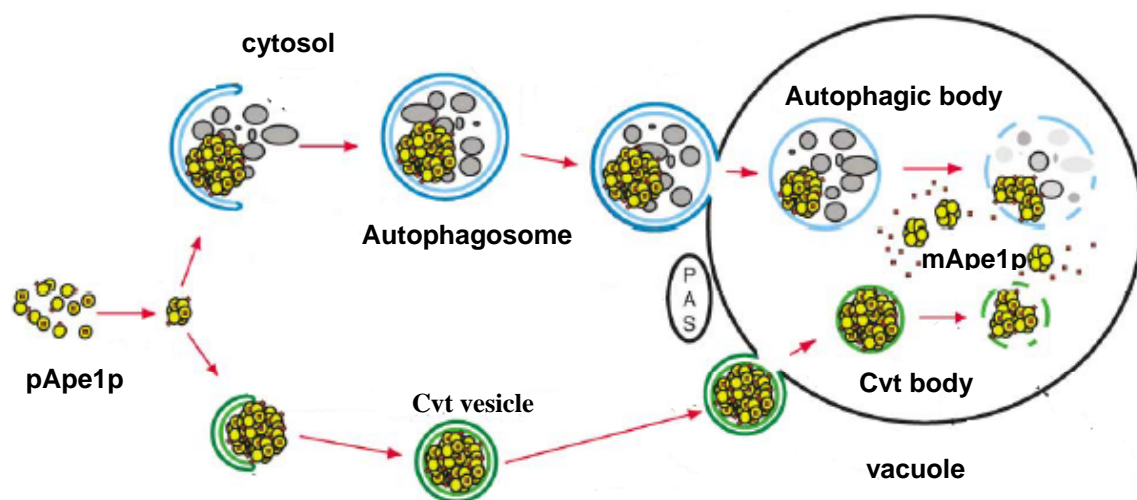
A screen for the mutants the accumulating the cytoplasmic precursor form of aminopeptidase I yielded Cvt mutants which largely overlapped with mutants of the autophagic pathway (Harding *et al.*, 1995, 1996).

Recently the nomenclature of the involved proteins/genes was unified to Atg for autophagy to avoid the confusion with different names of the Apg, Aut and Cvt mutants.

Genetic screens to follow peroxisome degradation and glucose-induced autophagy did not reveal unique genes for these pathways so far.

The cytoplasm-to-vacuole-targeting (Cvt) pathway is an example of a specific type of autophagy; proteins that are destined to become resident vacuolar hydrolases as aminopeptidase I and α -mannosidase are specifically packaged into cytoplasmic vesicles called cvt vesicles and delivered to the vacuole. This pathway is active under nutrient-rich conditions. The Cvt pathway converts into autophagy under nutrient starvation conditions. Aminopeptidase I is then found in autophagosomes.

Starvation: Autophagy



Nutrient rich conditions: Cvt pathway

Fig 2: Autophagy and Cvt pathway are active under different physiological conditions (Levine and Klionsky, 2004).

A number of differences distinguish the two modes of transport, such as Ape1p transport is a specific biosynthetic event that is constitutive, occurring even under nutrient-rich conditions (Klionsky *et al.*, 1992; Scott *et al.*, 1996). Bulk autophagy is non-selective, degradative, and only detectable during starvation for nutrients such as nitrogen or carbon (Takeshige *et al.*, 1992). Also pApe1p has a half-time for vacuolar delivery of 30-45 min (Klionsky *et al.*, 1992), while the half time for delivery of cytosolic proteins by macroautophagy is at least 12 h when induced by nitrogen starvation (Scott *et al.*, 1996). Morphologically, cvt vesicles seen in rapidly growing cells are smaller than their autophagosome counterparts. Cvt vesicles are approximately 150 nm in diameter while autophagosomes are approximately 300-900

nm (Takeshige, *et al.*,1992). Finally, the Cvt pathway is specific (excluding bulk cytoplasm) and saturable, while autophagy is non-specific and is not saturable.

Despite these differences, the morphology of autophagy and the Cvt pathways are very similar. Most of the molecular machinery overlaps between the two pathways, except that the Cvt pathway uses components that are required for the specific packaging of cargo. Autophagosomal receptors e.g. for peroxisomes have not been identified.

The major events of autophagy and Cvt pathways are induction (only for autophagy), cargo selection (only in case of Cvt pathway) and packaging, vesicle nucleation, vesicle expansion, retrieval of components, vesicle docking and fusion and finally vesicle breakdown.

1.2.2.1 Induction

The degradative autophagic pathway is induced upon starvation through a mechanism that involves the protein kinase Tor, a phosphatidylinositol (PI)-related serine/threonine kinase. Tor acts as the nutrient-sensing element that regulates autophagy (Noda T and Ohsumi Y., 1998). In response to starvation, Tor kinase is inhibited which results in the induction of autophagosome formation.

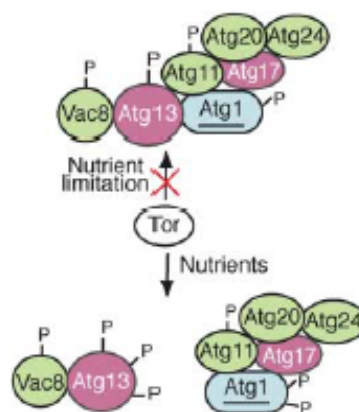


Fig 3: Regulation of induction by Tor kinase (Levine and Klionsky, 2004).

The Tor-mediated inductive signal for autophagy appears to be transduced through the phosphoprotein Atg13 and the serine/threonine kinase Atg1 (Kamada *et al.*;

2000). Both of these proteins are required for Cvt vesicle and autophagosome formation (Matsuura *et al*; 1997). Inhibition of Tor causes partial dephosphorylation of Atg13. This hypophosphorylated form of Atg13 has a higher affinity for Atg1 kinase. *In vitro* assays demonstrate that Atg1 kinase activity is essential for both Cvt and autophagy. Other proteins that interact with Atg1 complex are shown in figure 3.

1.2.2.2 Cargo packaging

The Cvt pathway is a cargo specific autophagic process that uses additional components that are involved in the recognition and packaging of dodecamers of pApe1p cargo, which then bind to the Atg19 that has characteristics of both a receptor and an adaptor protein for Cvt cargo, the aminopeptidase I and the α -mannosidase I. Atg19 is localized to punctuate structures or near the vacuole. Atg19 is a peripheral membrane protein that binds to the the cargo and travels to the vacuole with the cargo (Scott *et al.*, 2001). Subsequent interaction of Atg19 with Atg11 brings the cargo into contact with the ubiquitin-like protein Atg8 and allows its inclusion into the sequestering vesicle that is formed from the preautophagosomal structure (PAS).

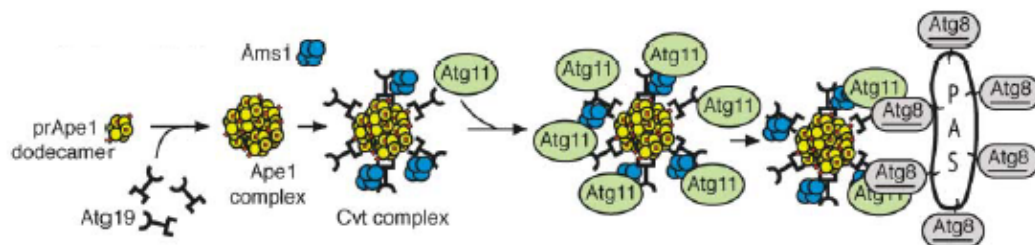


Fig 4: Cargo selection and packaging (Levine and Klionsky, 2004).

1.2.2.3 Vesicle nucleation

Unlike vesicle formation throughout the endomembrane system involving ER, Golgi complex etc, autophagic vesicles do not appear to bud off from pre-existing organelles. Rather, the vesicle is thought to form *de novo*. However, the source of vesicle membrane is unknown. Phosphoinositol kinases (PI) may play a fundamental role in vesicle nucleation (Kihara *et al* 2001).

The PI 3-kinase complex I, including Vps34, Vps15 serine/threonine kinase together with Atg6/Vps30 and Atg14, functions in vesicle nucleation in both autophagy and Cvt pathway.

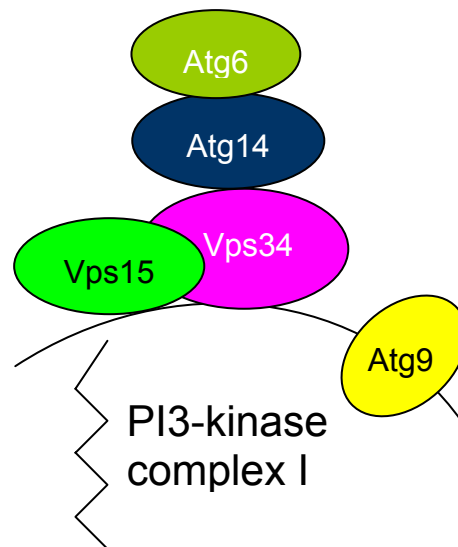


Fig 5: Phosphoinositol-3 kinase Vps34 plays a role in vesicle nucleation (Levine and Klionsky, 2004).

1.2.2.4 Vesicle expansion and completion

Majority of proteins involved in autophagy and Cvt pathway act at the step of vesicle formation. The mechanism by which the double membrane enwraps and sequesters cytoplasmic components is not known. Recent studies have identified several genes as being part of a novel conjugation system required for autophagy and Cvt vesicle formation. Atg8 (ubiquitin-like protein), undergoes two post-translational processing events resulting in conjugation with phosphatidyl-ethanolamine (PE) and recruitment to the PAS membrane. The Atg7 protein is homologous to E1 ubiquitin activating enzymes and is required for the activation of Atg8 as well as Atg12, a second ubiquitin-like protein. Atg12 is covalently linked via its carboxy-terminal glycine to an internal lysine residue of Atg5. A novel coiled-coil protein Atg16 has been shown to physically bind the Atg12-Atg5 conjugate and oligomerization of Atg16 allows the formation of a multimeric complex. The conjugation process also requires Atg4, Atg3 and Atg10.

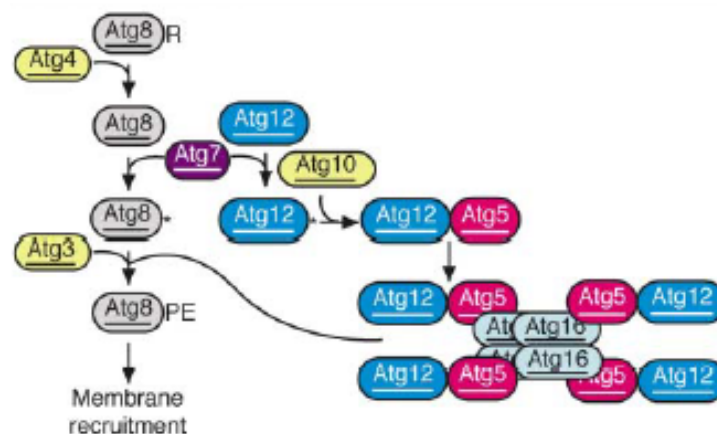


Fig 6: Conjugation of Atg8 to PE and Atg12 to Atg5 is required for the vesicle formation (Levine and Klionsky, 2004).

1.2.2.5 Retrieval

Retrograde trafficking processes are used to retrieve certain components for reutilization. Atg8 is the only structural protein known to remain with the completed autophagosome or Cvt vesicle. Other proteins that are thought to act at the stage of vesicle formation are not associated with the autophagosome suggesting that they are retrieved at some point prior to, or up to, vesicle completion. The integral membrane protein Atg9 is removed through a specific retrieval process involving Atg2 and Atg18.

1.2.2.6 Vesicle targeting, docking and fusion

The components required for the fusion of Cvt vesicles and autophagosomes with the vacuole are common to all the pathways that terminate at the vacuole. Ccz1 and Mon1, have been shown to function at the tethering or docking stage that brings the vesicles into proximity with the vesicles before fusion (Wang and Klionsky, 2003).

1.2.2.7 Vesicle breakdown

The double membranous vesicle loaded with the cytoplasmic cargo fuses with vacuolar membrane releasing the single membrane autophagic body into the lumen of the vacuole. The single membrane autophagic body is broken down to degrade the cytoplasmic components. The vesicle breakdown is known to depend on the acidic pH of the vacuolar lumen, and proteinase B. Atg15 is a putative lipase that reaches the vacuole through the secretory, multivesicular body pathway and is required for the breakdown of Cvt and autophagic bodies (Teter *et al.*, 2001).

1.3 Aminopeptidase I

There are four identified aminopeptidases in *S.cerevisiae* that hydrolyze preferentially leucine substrates (Trumbly and Bradley, 1983). Of these, only animopeptidase I (previously called LAPIV) appears to be localized to the vacuole based on subcellular fractionation studies (Matile *et al.*, 1971; Frey and Röhm, 1978).

Ape1p catalyzes the removal of leucine from the N-terminus of polypeptide chains. Aminopeptidase I is encoded by the APE1 gene located on chromosome XI. It has been shown that Ape1p is synthesized as an inactive zymogen that is processed in a PEP4-dependent manner (Chang and Smith, 1989, Trumbly and Bradley, 1983; Cueva *et al.*, 1989) like all other soluble vacuolar proteins that transit through the secretory pathway.

The APE1 gene encodes a protein of 514 amino acids containing four potential sites for N-linked glycosylation (Cueva *et al.*, 1989). The active enzyme is a multimeric protein having a molecular mass of approximately 600 kDa. The mature Ape1p monomer has a molecular mass of 50-51 kDa based on its migration pattern on SDS-PAGE (Metz and Röhm, 1976; Chang and Smith, 1989). The molecular mass based on the amino acid composition is predicted to be a protein of approximately 45 kDa (Metz and Röhm, 1976). The maturation of Ape1p from its precursor form has been shown to be a 2 step process. The 61 kDa cytoplasmic pApe1p is proteolytically matured to a 55 kDa intermediate form and 50 kDa vacuolar mApe1p form by the sequential action of the two vacuolar proteinases PrA and PrB (Segui-Real *et al.*, 1995). Ape1p does not follow the secretory pathway which was concluded from the following observations.

- The N-terminus of pApe1p does not have the typical structure of a ER-targeting sequence. Using various temperature sensitive *sec* mutants, defective in the secretory pathway, it was shown that Ape1p does not enter the secretory pathway.
- Using tunicamycin, which blocks the addition of N-linked oligosaccharides to proteins, and Concanavalin A, to which both N-linked and O-linked oligosaccharides bind, it was shown that aminopeptidase 1 is not glycosylated, even though it has four potential glycosylation sites (Klionsky *et al.*, 1992).
- Ape1p has a half time of 45 min for vacuolar delivery, compared to a half time of approximately 6 min for CPY, proteinase A or alkaline phosphatase (ALP) (Klionsky and Emr, 1989; Klionsky *et al.*, 1988; Hasilik and Tanner, 1978).
- Overproduction of CPY and PrA leads to their secretion, but over expressed Ape1p is not secreted (Klionsky *et al.*, 1992).

These observations led to the conclusion, that Ape1p is transported directly from the cytosol into the vacuole along the cytosol to vacuole targeting pathway.

The precursor form of Ape1p contains an amino-terminal 45 amino acid propeptide. The propeptide lacks a consensus signal sequence. Ape1p propeptide is not necessary for folding of the mature portion of Ape1p polypeptide, but instead plays a direct role in the entry of Ape1p into the vacuole. The predicted secondary structure for the pApe1p propeptide is composed of an amphipathic α -helix followed by a β -turn and another α -helix forming a helix-turn-helix structure. Using NMR spectroscopy it has been shown that mutations, which destabilize the first α -helix inhibit the transport of pApe1p (Martinez *et al.*, 1997). pApe1p targeting is sensitive to changes in periodicity and content of the predicted amphipathic α -helix, but is insensitive to similar changes in the second helix (Oda *et al.*, 1996).

It has been shown that the prepro-part of Ape1p is necessary and sufficient to target the protein into the vacuole. This was shown by tagging GFP to the prepro-sequence of pApe1p. This construct was targeted into the vacuole and the reporter protein was released. They have also shown that the separated pre- and pro-parts do not target GFP to the vacuole (Martinez *et al.*, 1999).

The following evidences suggest that the transport of pApe1p is a vesicular event.

- Analysis of *cvt* mutants indicate that a substantial overlap between genes involved in pApe1p transport and those required for autophagy. In addition, phenotypic examination of *cvt* and autophagy mutants revealed that the majority of these mutants are both autophagy defective and accumulate pApe1p precursor forms (Harding *et al.*, 1996; Scott *et al.*, 1996).
- Several studies indicated that Ape1p enters the vacuole as a dodecamer. Pulse chase experiments suggested that oligomerisation is an early event in transport (Kim *et al.*, 1997). The half time for transport of Ape1p into the vacuole is about 45 min while the half time of oligomerisation is about 2 min. Mutants in *cvt* pathway or propeptide deletion mutants are shown to be defective only in the import step and not in the oligomerisation. The import into the vacuole appears to be the rate limiting step (Kim *et al.*, 1997). Transport of a large oligomer into the vacuole appears to be similar to the transport of large pre-assembled oligomeric complexes into the peroxisomal lumen (Rachubinski and Subramani, 1995; Subramani, 1993). It has been suggested that the oligomeric precursor Ape1p is enwrapped by a double membrane (Baba *et al.*, 1997, Scott *et al.*, 1997) and this *cvt* vesicle transports Ape1p into the vacuole.

1.4 *via* mutants

Based on the enzymatic activity of the dodecameric complex, a new genetic screen was developed for analyzing the biogenesis pathway of aminopeptidase 1 and two complementation groups were characterized termed as *via* for vacuolar import and autophagocytosis (Andrei *et al.*, 2000). Enzymatic activity of mature and dodecameric mApe1p was measured by Ape1p overlay assay using leucine- β -naphthylamide as substrate. *via* mutants have reduced activity and show reduced levels of Ape1p expression compared to the wt.

Two complementation groups were identified namely *via10* and *via14*. Both *via* mutants accumulate pApe1p in membrane enclosed compartments demonstrating defects in pApe1p transport. While this compartment in *via10* was similar to the wt vesicles, the *via14* vesicles were extremely fragile and displayed reduced density when compared to wt cells, thus demonstrating a severe defect in Cvt vesicle biogenesis.

In wt cells pApe1p and mApe1p of Ape1p, form stable dodecameric complexes whereas in both *via* mutants, the assembly of pApe1p into dodecamers is disturbed. pApe1p in *via* mutants does not form stable dodecameric complexes, demonstrating defects in complex assembly and formation of high molecular weight aggregates. Vacuolar mApe1p is found in dodecameric complexes in *via* mutants, but these are not enzymatically active indicating that cytoplasmic oligomerization is essential for the formation of active enzyme complexes. This supports the Cvt pathway and makes translocation mechanism across the vacuolar membrane unlikely.

According to the model for the Cvt pathway (Scott and Klionsky, 1996) pApe1p rapidly dodecamerizes in the cytoplasm and is then enwrapped by a double membrane. In *via* mutants, formation of the surrounding membrane takes place, but pApe1p is not properly assembled; smaller complexes and aggregates are formed. This phenotype was not described previously in the *cvt* and autophagy mutants studies so far.

Protease protection experiments reveal that pApe1p accumulates in Cvt vesicles in *via* mutants. Processing of pApe1p is delayed, but can be up-regulated by inducing autophagy. However this does not restore enzymatic activity. This explains a specific defect in the Cvt pathway and that dodecamerization of pApe1p is linked to the Cvt pathway. The accumulation of pApe1p in Cvt vesicles demonstrates that complex

formation and vesicular transport is disturbed, indicating that formation of transport vesicles and dodecamerization are linked processes. Further more it was discovered that the cytoplasmic pApe1p is enzymatically active unlike the peptidases transported along the secretory pathway.

Coupling of pApe1p dodecamerization and compartmentalization into transport vesicle, is thus a mechanism to protect cytosolic proteins from Ape1p activity.

1.5 Aim of the study

A new genetic screen was developed for analyzing the biogenetic pathway of aminopeptidase I based on the enzymatic activity of the dodecameric complex which led to the isolation of *via* mutants.

The aim of this project was to identify the *via* genes as well as single-copy suppressors of the *via* mutant phenotypes. Characterization of the corresponding gene products should give us insights into the mechanisms involved in the formation of enzymatically active dodecameric Ape1p complexes and the Cvt pathway.

2 Materials and Methods

2.1 Materials

2.1.1 Equipments

Electroporator 1000	Stratagene, USA
Gene quant II, RNA/DNA calculator	Pharmacia Biotech, UK
Intelligent Dark Box II, LAS-1000+	Fuji, Japan
Phosphoimager Fujix BAS1000	Fuji, Japan
Ultra turrax T8	IKA Labortechnik, Staufen
SMART System for HPLC	Amersham Pharmacia Biotech
Mastercycler gradient	Eppendorf, Hamburg

2.1.2 Materials for molecular biology and biochemical experiments

Autoclave bags	Sarstedt, Nümbrecht
Bottle top filters	Sarstedt Inc., Newton, USA
Centrifuge tubes:	
JA-10 Polypropylene tube	Nalgene, München
JA-20 Polypropylene tube	Nalgene, München
Culture flasks for bacteria	Schott, Mainz
Glass pipettes	Schütt, Göttingen
Microscope slides	Menzel-Glaser
Needles for syringes	B.Braun Melsungen AG, Melsungen
Parafilm	American National Can™ Chicago
Pasteur pipettes	Schütt, Göttingen
Pipette tips	Sarstedt, Nümbrecht
Plastic tubes 10, 15 and 50 ml	Sarstedt, Nümbrecht
Polycarbonate ultracentrifuge tubes 1,5ml	Beckman, München
Superdex 200 column	Amersham Pharmacia Biotech Uppsala, Sweden.

2.1.3 Materials for Western Blotting

Nitrocellulose membrane 0.2 µM	Schleicher and Schüll, Dassel
Whatman GB002 paper	Schleicher and Schüll, Dassel
Whatman GB003 paper extra thick	Schleicher and Schüll, Dassel
X-ray films XAR-5	Kodak, Stuttgart
Supersignal Chemiluminiscent Substrate	Pierce, Illinois/ USA
Luminol and p-coumaric acid	Sigma

2.1.4 Chemicals

All chemicals were bought from	Boehringer / Roche, Mannheim; Merck, Darmstadt; Roth, Karlsruhe; Serva, Heidelberg; Sigma, Deisenhofen, Gibco BRL
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All the chemicals were of analysis grade.

2.1.5 Oligonucleotides for PCR

Name	Oligonucleotide	Purpose
PSYDR1 5'	CGG ATA GCC TCT GGT TAG GT	Amplification of SUVIA
PSYDR2 3'	TTG AGC CGG GGC ATA CAG GT	Amplification of SUVIA
PSYB1 5'	TTT ATT AAA TTT GGA TCC TTC AAT	Introduction of BamHI site in SUVIA
PSYB2 3'	ATT GAA GGA TCC AAA TTT AAT AAA	Introduction of BamHI site in SUVIA
PSYH1 5'	CCT TAT CCC TTC AAG CTT CGG TTC GAA AGG	HindIII site in SUVIA
PSYH2 3'	CCT TTC GAA CCG AAT CTT GAA GGG ATA AGG	HindIII site in SUVIA

10YHR1 5'	AAG CTA TTC ATG GTA CCC GCT GC	Amplification of YHR054C
10YHR2 3'	GGT AAT GGG ATC GGC TTA CTA ATT	Amplification of YHR054C
10YJL1 5'	AGA CAA ATG TTA CAG AGT ATA GTC	Amplification of YJL171C
10YJL2 3'	CAA TGA CTT AAA ATC TCA AAA C	Amplification of YJL171C
14YER1 5'	GTG GGG TAT ACT ACG GAG CAT GG	Amplification of YER187W
14YER2 3'	GTC AACAGT TCT TAA TTT CGG GTC	Amplification of YER187W
14YGL117W	CAA TAT CTT ACA ATG CAG CCA ATT TC	Amplification of YGL117W
14YGL117W	GTC AAA TTA TCA TAT AAC CCT TCT ATG	Amplification of YGL117W
PNACT1 5'	CAC GCT TAC TGC TTT TTT CTT CCC	Amplification of actin
PNACT2 3'	GAG AGC TAC ATA CAT AAA CAT ACG C	Amplification of actin
PNYHRKO1	AAA GCT ATT CAT GGT ACC CGC TGC TGA AAA CCT ATC TCC GAC GGA TCC CCG GGT TAA TTA A	Deletion of YHR054C
PNYHRKO2	GCA CGT ATA GCG CCC AAA TGT CGG TAA TGG GAT CGG CTT AGA ATT CGA GCT CGT TTA AAC	Deletion of YHR054C

2.1.6 Enzymes and Substrates

Lysozyme	Merck
Restriction endonucleases	New England biolabs
Zymolyase 20T	Seikagaku Corporation
H-Leu- β -Naphthyl Amide (H-Leu- β -NA)	BACHEM

2.1.7 Proteins, Protease inhibitors and Protein standards

Antipain	Sigma
Bovine Serum Albumin (BSA)	Serva
Chymostatin	Sigma
Elastin	Sigma
Glutathione-sepharose	Amersham-Pharmacia
Iodoacetamide	Sigma
Leupeptin	Biomol
Protein A sepharose	Amersham-Pharmacia
Protein G agarose	Calbiochem
Pepstatin A	Biomol
Phenylmethylsulfonylfluoride (PMSF)	Serva
Prestained protein molecular weight markers	Biorad

2.1.8 Vectors and DNA standards

pCR2.1	Invitrogen
pBluescript	Stratagene
pGEX-2T	Pharmacia Biotech
pYX	Dr. G.Fischer Von Mollard
pRS	Sikorski and Hieter, 1989

2.1.9 Radioactive substances

α -[³²P]-dCTP, 3000 Ci/mmol

Hartmann Analytic, Braunschweig

2.1.10 Kits for DNA, RNA and Protein

Bio-Rad Protein Assay

Bio-Rad

HiSpeed plasmid midi kit

Qiagen

HiSpeed plasmid mini kit

Qiagen

Megaprime DNA Labelling mix

Amersham-Pharmacia

RNeasy midi kit

Qiagen

QIAquick Gel extraction kit

Qiagen

2.1.11 Antibodies

Primary Antibody	Immunized Species	Dilution for western blot	Reference
anti-aminopeptidase-I	Rabbit	1:2000	Andrei et al.,2000
anti-GFP	Mouse	1:3000	MBL
anti-GST	Goat	1:3000	MBL
anti-HA	Mouse	1:1000	Dr.Fischer von Mollard

Secondary antibodies

Goat anti-rabbit Horseradish peroxidase conjugate

Goat anti-mouse Horseradish peroxidase conjugate

Rabbit anti-goat Horseradish peroxidase conjugate

All secondary antibodies were diluted 1:10000

All secondary antibodies were purchased from Dianova, Hamburg.

2.1.12 Yeast Strains

Strain	Genotype	Source
SEY6211	MAT _a , ura3-52, his3 Δ 1, leu2-3,-112, trp1-289, suc2-D9, ade	Prof. S. Emr
II-17	MAT _{α} , ura3-52, his3 Δ 1, leu2-3,-112, trp1-289, suc2-D9, ade	Prof.D.H.Wolf
C6C	SEY6211 X C6C	
BY4741	MAT _{α} , his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0, YDR360w::kanMX4	EUROSCRAF
<i>suvia1Δ</i>	C6C X BY4741	This study

2.1.13 Bacterial Strains

Strain	Genotype	Source
XL1-Blue	recA1,endA1, gyrA96, thi-1, hsdR17,supE44, relA1 lac, [F',proAB, lacI ^q Z Δ M15,Tn10(Ter) ^r] ^c Heidelberg	Stratagene

2.1.14 Media for *S. cerevisiae* cells

YPD Medium	20 g Peptone
	10 g Yeast extract
	20 g Dextrose

To prepare 1000 ml of YPD medium, Peptone (20 g) and Yeast extract (10 g) were dissolved in 900 ml of ddH₂O and autoclaved. After autoclaving, 100 ml of 20% autoclaved Dextrose (20 g) stock solution was added.

YPD Agar Plates

2% of Bacto-agar (20 g), 20 g of peptone and 10 g of yeast extract were dissolved in 900 ml of ddH₂O and autoclaved. After autoclaving 100 ml of 20% sterile Dextrose (20 g) stock solution was added and the mixture was poured in 10 cm petriplates.

Presporulation Medium

2% Peptone

1% Yeast Extract

5% Glucose

Starvation Medium

5 g Sodium Acetate

10 g Potassium Chloride

15 g Bacto-agar. Dissolved in
1000 ml of water and autoclaved.

Synthetic minimal medium (SD)

6.7 g yeast nitrogen base w/o amino
acids

20 g Dextrose

To prepare 1000 ml of synthetic minimal medium, yeast nitrogen base w/o amino acids and supplements mixture were mixed and autoclaved. After autoclaving, 100 ml of 20% autoclaved Dextrose (20 g) stock solution was added and poured in 10 cm petriplates.

Supplements and their concentration used are listed in the table below

Supplements and the concentration used :

Supplements	Final Concentration(mg/l)	Stock per 100ml
Adenine sulphate	20	200 mg
L-Tyrosine	30	300 mg
L-Phenylalanine	50	500 mg
L-Glutamic acid	100	1000 mg

Working concentration and stored aliquots at -20 °C.
50 µg/ml

2.1.17 Stock solutions and buffers

1 M DTT 3.08 g Dissolved in 20 ml of 0.01 M
sodium acetate pH was 5.2. Filter
sterilized and aliquots stored
at -20 °C.

0.5 M EDTA 181.1 g Ethylene Diamine Tetra Acetic
acid (sodium salt) dissolved in
800ml of water, pH was set to
8.0 with NaOH. Volume was
made up to 100 ml and autoclaved.

Ethidium Bromide 0.2 g Dissolved in 20 ml of water,
stored at 4 °C.

0.1 M IPTG 23.8 mg Dissolved in 1 ml of water.
Sterile, filtered and aliquots stored at -
20 °C.

10 x PBS 80 g Sodium chloride
1.6 g Disodium hydrogen phosphate
dissolved in 800 ml water. Volume
was made up to 1000 ml.

Protease Inhibitor mix 1.25 mg/ml Leupeptin
0.75 mg/ml Antipain
0.25 mg/ml Chymostatin
0.25 mg/ml Elastin

		5.0 mg/ml Pepstatin dissolved in DMSO, aliquots stored at -20 °C.
<u>3M Sodium acetate</u>		Dissolved in water and adjusted the pH to 5.2 with glacial acetic acid sterilized by autoclaving..
<u>2.5 M Sorbitol</u>	227.7 g	Dissolved in water. Volume was made upto 500 ml, sterilized by autoclaving.
<u>50 x TAE</u>	242 g 57.1 g	Tris base glacial acetic acid 100 ml of 0.5 M EDTA (pH 8.0) Dissolved in water and the final volume was made upto one litre.
<u>TBS (1x)</u>	150 mM 10 mM	Sodium chloride Tris/ HCl pH 7.4
<u>TE</u>	10 mM 1 mM	Tris/ HCl pH 7.5 EDTA
<u>1 M Tris/ HCl</u>	121.1g	Tris-Base dissolved in 800 ml water. pH was set to the required value with conc. HCl. Volume was made upto 1000 ml and autoclaved.
<u>1 M Tris / SO4</u>	121.1g	Tris-base dissolved in 800 ml of

water, pH was set to 9.4.

2.2 Methods

2.2.1 MOLECULAR BIOLOGY

2.2.1.1 Protocols for *E.coli*

Preparation of competent *E. coli*

Buffers

TFB I	30 mM Calcium acetate, pH 5.8 100 mM Rubidium chloride 10 mM Calcium chloride 50 mM Manganese chloride 15% Glycerol (w/v)
TFB II	10 mM MOPS, pH 6.5 75 mM Calcium chloride 10 mM Rubidium chloride 15% (w/v) Glycerol

2-3 ml bacterial pre-culture was grown in LB medium at 37 °C, overnight. 1 ml of the pre-culture was inoculated into LB medium (99 ml) and grown at 37 °C to an OD₆₀₀ of 0.4-0.6. Cells were pelleted at 3000 rpm for 5 min at 4 °C, resuspended in 0.2 culture volume of TFB I and incubated on ice for 5 min. Cells were centrifuged at 3000 rpm for 5 min at 4 °C, the pellet was resuspended in 0.04 culture volume of TFB II and incubated on ice for 30 min. Aliquots of 50 µl were frozen at -80 °C.

Transformation of competent *E.coli*

50-100 ng of DNA was added to each 50 µl aliquot of competent cells and incubated on ice for 30 min. Cells were subjected to heat shock by incubating at 42 °C for 2 min and incubated on ice for 1-2 min. 0.9 ml of LB medium was added to the cells followed by incubation at 37 °C in the shaker for 1 hour. Cells were plated on LB-agar plates containing appropriate antibiotic and incubated overnight at 37 °C. For blue-white selection, cells were plated on plates containing IPTG (Isopropyl-β-D-

thiogalactopyranoside; 40 µl of 100 mM solution in water) and X-Gal (40 µl of 4% solution in dimethylformamide).

Preparation of electrocompetent *E.coli*

10 % (v/v) Glycerol: 1 ml (1.26 g) of glycerol in 10 ml sterile water

A single *E. coli* colony was inoculated into 10 ml of LB media and allowed to grow overnight at 37 °C in a shaking incubator. This pre-culture was inoculated in 1 l LB media and allowed to grow to an OD₆₀₀ of 0.5-0.7 at 37 °C. Cells were pre-chilled on ice for 15 min and then pelleted at 5000 rpm for 15 min at 4 °C. Pellet was resuspended in 1 l of ice cold water and centrifuged as described above. This washing was repeated one more time. Pellet was resuspended in 20 ml ice cold sterile 10% glycerol and centrifuged at 6000 rpm for 15 min at 4 °C. Pellet was resuspended in 2 ml ice cold, 10% glycerol, aliquots of 40 µl were stored at -80°C.

Transformation of the electrocompetent *E.coli*

SOC medium	0.5% Yeast extract
	2% Bacto-tryptone
	10 mM Sodium chloride
	2.5 mM Potassium chloride
	10 mM Magnesium chloride

For each electroporation 40 µl of the electrocompetent cells were used. 0.5 µg of DNA was added to the cells and the contents were transferred into a pre-chilled, sterile electroporation cuvette. The cuvette was placed in the electroporator and pulse was given. SOC medium was added to the cells and they were allowed to recover in sterile tubes for 30 min at 37 °C in a shaker incubator. Cells were plated on LB plates containing appropriate antibiotic and incubated overnight at 37 °C.

Glycerol stocks of bacterial strains

Bacterial cultures were grown overnight at 37 °C in a shaking incubator. 0.3 ml of sterile 100% glycerol was taken in freezing vials to which 700 µl of the overnight culture was added. The contents were gently mixed and stored at -80 °C.

Mini preparation of plasmid DNA from *E.coli*

Plasmid DNA was isolated from *E.coli* cultures using the kit provided by Qiagen. Isolation was done as follows according to the instructions of the manufacturer.

Buffer P1 50 mM Tris/HCl pH 8.0
 10 mM EDTA
 100 µg/ml RNase A

Buffer P2 0.2 M NaOH
 1% SDS

Buffer N3 3 M Potassium acetate pH 5.5

A single *E.coli* colony was inoculated into 2 ml of LB medium containing 100 µg/ml of antibiotic and grown overnight at 37 °C in a shaking incubator. Cells were pelleted in a table-top centrifuge at 10,000 rpm for 10 min. Cell pellet was resuspended in buffer P1 (250 µl) and buffer P2 (250 µl) was added, mixed gently by inverting the tube 3-4 times. To this, buffer N3 (350 µl) was added gently, mixed and centrifuged for 10 min at 13000 rpm in a table-top eppendorf centrifuge. The supernatant was applied onto a QIAprep spin column and centrifuged for 1 min at 13000 rpm. Flow through was discarded, the column was washed with buffer PE (0.75 µl) and centrifuged again for 1 min. Flow through was discarded and the column was centrifuged for an additional 1 min to remove any residual wash buffer. The column was placed in a clean eppendorf tube and 50 µl of sterile water was added directly to the centre of the column. The column was let to stand for 1 min and DNA was eluted by centrifuging at 13000 rpm for 1 min.

Midi preparation of plasmid DNA from *E.coli*

Composition of buffer P1, P2 and P3 used in this preparation are same as described for mini DNA preparation.

QBT 750 mM Sodium chloride
 50 mM MOPS pH 7.0
 15% Ethanol
 0.5% Triton X-100

QC: 1 mM Sodium chloride
 50 mM MOPS pH 7.0
 15% Ethanol

QF: 1.25 M Sodium chloride
 50 mM Tris/HCl pH
 15% Ethanol

E. coli was inoculated into 50 ml LB media containing 100 µg/ml ampicillin. The culture was grown at 37 °C overnight, in a shaking incubator. Cells were pelleted in a Beckman JA10 rotor at 6000 rpm for 15 min. Pellet was resuspended in 6 ml of buffer P1. 6 ml of buffer P2 was added and gently mixed. This was incubated at room temperature for 5 min. During the period of incubation, the QIA filter cartridge was prepared, 6 ml of buffer P3 (prechilled) was added, mixed gently, and the lysate was poured into the barrel of the QIAfilter cartridge and incubated for 10 min. A HiSpeed Midi Tip was equilibrated by applying 4 ml of buffer QBT and the column was allowed to empty by gravity flow. Plunger was gently inserted into the cartridge and the cell lysate was filtered into the equilibrated HiSpeed Tip. The tip was washed with 20 ml of buffer QC. DNA was eluted with 5 ml of buffer QF. Eluted DNA was precipitated by adding 3.5 ml of isopropanol. QIAprecipitator was attached to the 20 ml syringe and the DNA was filtered through the precipitator using constant pressure. The precipitator was then attached to 5 ml syringe and 1 ml of sterile water was added. DNA was eluted into a new collecting tube. This step was repeated again with the same eluate to get higher concentration of DNA.

2.2.1.2 Protocols for yeast

Transformation of yeast cells- Chemical Method

10 x TE buffer	0.1 M Tris/HCl 0.01 M EDTA, pH 7.5
10 x LiOAc	1 M Lithium acetate, pH 7.5 (pH was adjusted using dilute acetic acid)
Salmon sperm DNA 10mg/ml	Dissolved in water.Sonicated and denatured for 5 min at 95°C. Chilled on ice.

Cells were grown in YPD media to an $OD_{600} = 0.6 - 1.2$ and pelleted at 3000 rpm for 5 min at 4 °C. Cells were washed four times in sterile water.

Cell pellet from a 200 ml culture was resuspended in 1.5 ml LiOAc/TE (1x) and incubated on ice for 2 hours. To 100 µl of the competent cells 1-5 µg of plasmid DNA and 100 µg of salmon sperm DNA was added and incubated at 30 °C for 30 min. 1.2 ml of 40 % PEG 4000 in 1x LiOAc/TE was added to each of the tubes and carefully mixed by pipetting. Cells were incubated at 30 °C for 30 min with intermittent mixing. Cells were heat shocked at 42 °C for 5 min and then allowed to cool on ice. Cells were pelleted by centrifugation at 6500 rpm for 30 sec. The cell pellet was resuspended in 1 ml of 1x TE buffer. 0.2 ml of cells were spread over the selection plate and incubated at 30 °C for 3-4 days.

Glycerol stocks of yeast cells

Yeast cells were spread onto YPD plates and the cells were allowed to grow at 30 °C. 3 ml of sterile 15% glycerol was taken in a vial. Half of the lawn of cells on the

plate were scraped of using a small sterile spatula and resuspended into the glycerol. The vials were then placed directly at -70°C .

Isolation of genomic DNA from Yeast

Spheroplasting buffer	1.2 M Sorbitol
	50 mM Tris/HCl pH 7.5
	10 mM EDTA
	10 mM DTT
	0.5 mg/ml Zymolyase
Lysis Buffer	2% SDS
	0.1 M Tris/HCL pH 8.0
	10 mM EDTA pH 8.0

A 200 ml of liquid stationary culture of Yeast in YPD was used for the isolation of genomic DNA. Cells were pelleted down and washed with water. Pellet was resuspended in 8 ml Spheroplasting buffer in JA-20 tube and incubated for 30-40 min at 37°C and was mixed time to time. 10 ml of Lysis buffer was added, vortexed shortly and kept at 65°C for 5-10 min. 10 ml of 5 M Potassium acetate was added and centrifuged for 15 min at 12000 rpm at 4°C . Supernatant was collected into a new JA-20 tube and the pellet was discarded. 200 μl of Ammonium acetate per 300 μl of supernatant and 1 μl of isopropanol (cold) per 300 μl of supernatant was added. This was incubated overnight at -20°C .

Next day, the experiment was continued by centrifuging at 12000 rpm at 4°C .

Pellet was washed with 70% Ethanol (cold) and dried at 37°C . Pellet was resuspended in 15 ml TE buffer and incubated together with 150 μl RNase (10 mg/ml) for 1 hour at 37°C . 300 μl of 5 M NaCl, 150 μl of Pronase (20 mg/ml) was added and incubated for 30 min at 37°C . DNA was extracted with cold Phenol (twice in 1:1) and with Chloroform at 13000 rpm for 5 min. DNA was precipitated as described in the later section.

Isolation of plasmid DNA from Yeast

Lysis Buffer	1% SDS
	10 mM Tris/HCL pH 8.0
	1 mM EDTA pH 8.0
	100 mM NaCl
	2% Triton X100

2 ml liquid culture of Yeast in SD medium was used for the isolation of plasmid DNA. Cells were taken in a microcentrifuge tube and centrifuged for 30 sec at 13000 rpm. Pellet was resuspended in 200 μ l of Lysis buffer. 100 μ l of glass beads and 200 μ l of Phenol-Chloroform was added to remove the proteins. This was vortexed for 2 min and centrifuged for 5 min at 13000 rpm at 4 °C. The upper phase was carefully transferred into a new microfuge tube without taking any phenol. To this, 20 μ l of 3 M Sodium acetate pH 5.2 and 100% of Ethanol was added and centrifuged for 10 min at 13000 rpm at 4 °C. The pellet was washed with 70% Ethanol (cold) and centrifuged again for 1 min. Pellet was dried at 37 °C. DNA pellet was resuspended in sterile water.

Mating and Sporulation

Yeast can exist stably in either haploid or diploid states. A haploid can be of two mating types, either MAT α or MATa. The result of mating is a diploid cell α/a which is formed by the fusion of an α cells and an a cell. A diploid cell grows indefinitely, but under starving conditions, it undergoes meiosis forming 4 haploid spores enclosed in a structure called ascus. Meiosis is induced by shifting cells to a medium without nitrogen source with acetate as the sole carbon source.

MAT α and MATa were mated on YPD plate by streaking few cells of each strain. Mating was observed after 6 hrs under the microscope. Diploid cells were streaked on YPD plates. To achieve sporulation, diploid cells were grown in presporulation media for 2 days at 30 °C. Cells were harvested and washed with water (2X). Cells were spread on acetate plates and incubated at 30 °C for 3 days. Cells form a thick layer. Cells were scraped out and collected in Tween80 water.

Cells were vigorously vortexed for 30 sec with glass beads and paraffin oil and centrifuged at 200x g for 5 min (3x). Cells were spread with 15% gelatin on selection plates.

2.2.1.3 Protocols for DNA / RNA

Precipitation of DNA with ethanol

Two volumes of 100% ethanol was added to the DNA. This was incubated at -70 °C for 20 min or 10 min on dry ice or overnight at -20 °C. DNA was pelleted at 13000 rpm for 10 min. The pellet was washed with 70% ethanol and pelleted by centrifuging for 5 min at 13000 rpm. The DNA pellet was dried at 37 °C and was resuspended in sterile water.

Determining the concentration of DNA

DNA concentration was determined using a spectrophotometer at 260 nm. DNA was diluted in water and the absorbance was measured at 260 nm.

Absorbance or optical density (OD) of 1 at 260 nm corresponds to ~50 µg/ml of double stranded DNA or ~40 µg/ml of single stranded DNA and RNA or ~20 µg /ml of oligonucleotides. The ratio of the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. Any contamination with proteins or phenol would yield values less than mentioned above.

Restriction endonuclease digestion of DNA

The activity of restriction enzyme is measured in Units (U).

One unit of restriction enzyme is the amount of enzyme required to completely digest 1 µg substrate DNA in 1 h.

Plasmid DNA	0.5 - 1 µg
10 x buffer	2 µl
Restriction enzyme	1-2 U
BSA	1 µg/µl (added where essential)

Water was added to a final volume of 20 μ l. Reaction mix was incubated at 37 °C for 2 hours.

*Incubation temperatures were set as recommended by the manufacturer (New England Bio Labs Beverly U.S.A.) which varies from enzyme to enzyme. Reaction mix was analyzed on an agarose gel (0.8 to 2% depending on the size of the DNA of interest)

The buffers and enzymes used were all from New England Biolabs.

The composition of the buffers is as follows:

NEB 1: 10 mM Bis Tris Propane/HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT

NEB 2: 10 mM Tris/HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT

NEB 3: 50 mM Tris/HCl (pH 7.9), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT

NEB 4: 20 mM Tris/acetate (pH 7.9), 10 mM Magnesium acetate, 1 mM DTT,
50 mM Potassium acetate

Agarose gel electrophoresis of DNA

The size and purity of DNA is analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is, the larger the DNA, the lower is the concentration of agarose.

Agarose concentration (%)	DNA size (kb)
0.7	20 - 1
0.9	7 – 0.5
1.2	6 – 0.4
1.5	4 – 0.2
2.0	3 – 0.1

Gel loading buffer (10x)

0.25% (w/v) Bromophenol blue

40% Saccharose in 1x TAE

Agarose was weighed and dissolved in 1x TAE by boiling in microwave oven. The agarose solution was allowed to cool till about 60 °C and ethidium bromide was added to a final concentration of 0.5 µg/ml. This was poured into the agarose gel cassette and allowed to polymerize completely. The sample DNA was mixed with gel loading buffer and loaded into the lane. The gel electrophoresis was carried out at 100 V. Ethidium bromide is a fluorescent dye which contains a planar group that intercalates between the stacked bases of the DNA. The fixed position of this group and its close proximity to the bases causes the dye to bind to the DNA to display an increased fluorescent yield compared to that of the dye in free solution. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red orange region of the visible spectrum. Hence DNA can be visualized under a UV transilluminator. The gel was photographed using a gel documentation system.

Cloning and DNA ligation

DNA ligase links fragments of DNA, containing blunt end or cohesive-end termini, to each other in a covalent manner. It catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA.

A plasmid vector DNA and desired fragment DNA was digested with appropriate restriction endonucleases. Both the vector and the desired fragment were purified from agarose gel using Quiagen gel extraction kit. Ligation was performed using T4-DNA Ligase. Ligation mixture was incubated at 16 °C overnight and then used for *E.coli* transformation.

Ligation Mixture (10 µl)

Vector	10-20 ng
Desired fragment	3 times the vector concentration
T4 DNA ligase buffer (10X)	1 µl

T4 DNA ligase 1 μ l

Phosphatase treatment of digested plasmid DNA

To prevent the religation of plasmids that were digested with single restriction endonuclease, they were treated with alkaline phosphates to remove the 5' phosphate from the digested plasmid. To the digested plasmid, in a reaction volume of 50 μ l, 2 μ l of alkaline phosphatase (Boehringer) was added (if digestion was done in NEB buffer 1.5 μ l of 10x NEB buffer N3 was added) and the reaction mix was incubated for 1 h at 37 °C. Reaction was stopped by the addition of 0.5 μ l of 5 mM EDTA, followed by heating at 75 °C for 20 min. Plasmid was purified from agarose gel using Quigen gel extraction kit.

Polymerase Chain Reaction (PCR)

PCR is a rapid method for enzymatic amplification of specific DNA fragments.

It includes 3 steps, which are necessary for DNA amplification.

1. Template DNA denaturation to get single stranded DNA.
2. Annealing (binding of oligonucleotide primer to single strands).
3. Extension (synthesis of DNA, strating from bound primers).

Example:

1. 95 °C 30 sec
2. 50 °C 30 sec (T_m of primers)
3. 72 °C 1 min (Length of the template)

30 Cycles

PCR Mix

10X Taq buffer (100 mM Tris/HCl pH 9.0; 500 mM KCl)	5 μ l
2.5 mM dNTPs (Amersham, Stock 100 mM)	1 μ l
Oligonucleotide primer 1 (5 pmol/ml)	4 μ l
Oligonucleotide primer 2 (5 pmol/ml)	4 μ l
Template DNA	500 ng
Taq polymerase	1 μ l
Final volume made upto 50 μ l	

DNA Sequencing PCR:

Sequencing premix	2 μ l
Primer	10 pmol (1 μ l)
DNA template	200-500 ng (plasmid DNA)
Final volume made upto 10 μ l.	

Seuqncing PCR:

96 °C	10 sec
50 °C	5 sec
60 °C	4min
30 cycles	

Sequencing products were precipitated at room temperature by addition of 1/5 vol of 3 M NaOAc pH 5.2 and 5 vol of 95% ethanol, vortexing and incubating for 10 min. DNA was pelleted by centrifugation at full speed for 20 min, washed once with 70% ethanol, dried and resuspended in 25-50 μ l HPLC-H₂O.

Site directed mutagenesis

Site directed mutagenesis is used to make point mutations, delete or insert single or multiple amino acids and introduction of restriction sites into cloned DNA of interest. This is a specialized PCR protocol. Pair of PCR primers were designed to amplify plasmid completely so that both at 5' end and complementary 3'end, a mutation is introduced.

PCR is done as described in the preovious section. PCR product is treated with DpnI. The DpnI endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation containing synthesized DNA. 1 μ l of DpnI was added to the PCR product and incubated for 2 hours at 37 °C which was then used for transformation in *E.coli*.

RNA Analysis**Isolation of total yeast RNA using RNAeasy kit**

Total yeast RNA was extracted using RNAeasy kit from Quiagen. Before and during RNA extraction RNase-free environment was maintained by wearing gloves while handling reagents and RNA samples to prevent RNase contamination. Plasticware,

glassware and electrophoresis tank were treated to inactivate RNases with 0.1 M NaOH (overnight) followed by RNase-free water. RNase-free water was obtained by treating deionized water with 0.1% DEPC and autoclaving. All solutions were prepared using DEPC water.

Buffer Y1

1 M Sorbitol

0.1 M EDTA pH 7.4

Before use, 0.1% β -ME and 50 U Zymolyase was added per ml of buffer Y1

Buffer RPE

BufferRLT

Before use, 10 μ l of β -ME per ml of buffer RLT was added.

Total yeast RNA isolation was done using 40 OD cells in early logarithmic phase.

All steps and centrifugations were done at RT.

Yeast cells were harvested by centrifuging at 500 g for 5 min at 4 °C. Cell pellet was resuspended in freshly prepared buffer Y1 containing zymolyase and incubated at for 30 min at 30 °C to generate spheroplasts. 1 ml of buffer Y1 was used for per 10^8 yeast cells. Centrifugation was done for 5 min at 500 g to pellet spheroplasts and supernatant was discarded. Spheroplasts were disrupted by adding 2 ml of buffer RLT. Cells were homogenized by passing the lysate at least 5-10 times through a 20 gauge needle fitted to an RNase-free syringe. Cell lysate was centrifuged for 5 min at 3000 g. Supernatant was transferred into a new 15 ml tube by pipetting and this was used for further steps. 1 volume (4 ml) of 70% ethanol was added to the homogenized lysate and was thoroughly mixed by shaking vigorously. Sample was applied to an RNeasy column placed in a 15 ml tube and centrifuged for 5 min at 3000 g. 4 ml of buffer RW1 was added to the column and centrifuged again to wash the column. Flow through was discarded at each step. 2.5 ml of buffer RPE was added and centrifuged for 2 min at 3000 g. This step was repeated again to dry the RNeasy silica gel-membrane. RNeasy column was transferred into a new collection tube and RNA was eluted with 250 μ l of RNase-free water by centrifuging for 3 min at 3000 g. To obtain a higher yield, the elution step was repeated using the first eluate.

Northern Blot

All instruments, containers and work place used were cleaned extensively in advance to prevent RNase contamination, as described in Quiagen RNeasy Kit manual.

10X MOPS buffer	200 mM MOPS, 50 mM NaOAC, 10 mM EDTA, pH 7.0
1X MOPS running gel buffer	1x MOPS buffer. 0.74% formaldehyde (FA)
5X RNA loading dye	4 mM EDTA, pH 8.0, 2.7% FA, 20% glycerol, 31% formamide, 4x MOPS buffer, bromophenol blue
20X SSC buffer	3 M NaCl, 0.34 M Na-Citrate, pH 7.0
Salmon sperm DNA (SSP)	10 mg/ml
TE buffer	10 mM Tris/HCL, pH 7.5, 1mM EDTA
α - ³² P-dCTP	5 μ Ci/ μ l

Agarose gel electrophoresis of RNA

Agarose gel buffer	
MOPS (10X)	15 ml
DEPC water	150 ml
Agarose	1.2%

Electrophoresis buffer (MOPS 1X)

MOPS	200 mM
Sodium acetate	50 mM
EDTA	10 mM

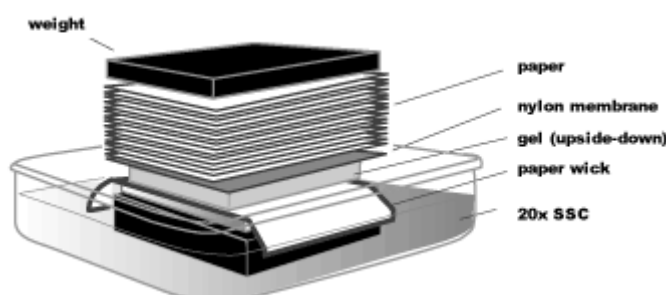
10X stock solution was made in DEPC water and pH was adjusted using NaOH.

Gel loading buffer	
Bromophenol blue	1X
DEPC water	4X
Formamide	4X
Formaldehyde	2X
MOPS 10X	2X

Solutions were prepared according to the Qiagen RNeasy Kit Manual. For the agarose gel, agarose was boiled in 1x MOPS buffer and left to cool until about 50 °C. Formaldehyde to a concentration of 0.7% and ethidium bromide was added before pouring the gel. RNA samples were mixed with 5x dye, incubated at 65 °C for 5 min and kept on ice briefly before loading. The gel was run at 30-40 mA from 4-7 h. To prepare for the transfer, the gel was briefly washed with water and equilibrated in 20x SSC buffer.

RNA transfer to nylon membrane: After cutting the gel to the required size using a scale and taking a picture including the scale, the gel is transferred back in the 20x SSC. For overnight transfer of RNA to the nylon membrane the setup is as follows:

After overnight transfer, the membrane was dried at room temperature, exposed to UV light for 15 sec to crosslink the RNA to the membrane and incubated at 60 °C for 1 h. The membrane was rolled and kept in falcon tube.



Radioactive labelling of DNA fragment: Approximately 25 ng of DNA fragment was diluted to 45 µl in TE buffer. After 5 min at 95 °C and 5 min on ice, the solution was transferred to a cup containing rediprime random DNA labelling kit lyophilisate, on ice. 30-50 µCi of ³²P-dCTP was added, depending on the calibration date of the radioactivity, and the lyophilisate was resuspended by pipetting. The labelling reaction mix was incubated at 37 °C for 15 min. Qiaspin nucleotide removal kit was used to purify the labelled DNA and 1/100 of the eluate was measured in a scintillation counter using Cerenkov radiation. 2-15 µCi was used for one membrane.

Hybridization: To prepare the membrane for hybridization, salt was removed by washing the membrane twice with water for 5 min. Prehybridisation was done in 3 ml Rapid-hyb hybridization buffer for at least 1 h at 65 °C. After labelling the probe, SSP was boiled for 10-15 min. 10 µl of SSP/ ml of hybridization buffer was added to the radioactive DNA solution, the mix was boiled for 5 min and added dropwise to the hybridization buffer. The tube was double-sealed in a plastic bag and incubated overnight at 65 °C in a rolling hybridization oven.

The membrane was washed with 0.1% SDS in 2x SSC for 15 min at room temperature and with 0.1% SDS in 0.2x SSC for 15 min at 65 °C. Further washes were done eventually after the first exposure to a phosphorimager screen, depending on the signal/background relationship.

Stripping of the membrane: The membrane was placed in boiling 0.1% SDS/0.2x SSC solution and allowed to cool. The remaining signal was detected after overnight exposure, if any signal was detected, the procedure was repeated 3 times.

2.2.2 BIOCHEMICAL METHODS

2.2.2.1 Analysis of proteins

Determination of protein concentration using the BIORAD reagent

Bovine Serum Albumin (BSA) stock solution 1 mg/ml

Concentration range: 5-20 µg / µl

A standard curve was made using BSA in the range of 5-20 µg. 2 µl of the sample was used for the protein estimation. The volume was made up to 800 µl with water. 200 µl of the BIORAD reagent was added and the samples were incubated for 3-5 min at room temperature, optical density was measured at 595 nm in the Spectrophotometer.

Precipitation of proteins using Trichloro acetic acid (TCA)

Trichloroacetic acid (TCA) solution

(100%) 100 g of TCA crystals was weighed and the volume was made up to 100 ml with water

2% Sodium acetate in ethanol 2 g of Sodium was dissolved in small amount of water and volume was made up to 100 ml with ethanol

The proteins were precipitated by adding TCA to a final concentration of 10% and incubated on ice in the cold room overnight. The precipitated samples were centrifuged at 13000 rpm, for 10 min at 4 °C. The supernatant was discarded and the pellets were washed 500 µl of 2% sodium acetate solution in ethanol. The samples were again centrifuged as above, supernatant was discarded and the pellets were air-dried for 10-15 min at RT. The pellets were resuspended in 1x SDS-PAGE buffer, boiled and loaded on SDS-PAGE for further analysis.

Solubilisation of proteins

2 x Laemmli buffer 125 mM Tris/HCl, pH 6.8
4% SDS
0.00 4% Bromophenol blue
20% (w/v) Glycerol
20 mM DTT

Proteins were resuspended in 1x Laemmli buffer. The resuspended proteins were boiled at 95 °C for 5 min, cooled on ice for 1 min, centrifuged at 13000 rpm for 2 min and resolved by SDS-PAGE.

Gel Electrophoresis

Gel running buffers

Anode buffer (5x) 50 mM Tris
192 mM Glycine

Volume was made up with double distilled water.

Cathode buffer (1x) 0.1% SDS in anode buffer

Volume was made up with double distilled water.

Separating gel buffer (4x) 1.5 M Tris/HCl pH 8.8

0.4% SDS

0.4% APS

Volume was made up with double distilled water.

Stacking gel buffer (4x) 0.5 M Tris/HCl pH 6.8

0.4% (w/v) SDS

Acrylamide Solution 30% (w/v) Acrylamide

0.8% (w/v) Bisacrylamide

Ammonium per sulphate 10% (w/v)

Recrystallised ammonium peroxodisulphate (APS) was dissolved in double distilled water. This was prepared fresh every week.

Preparation of acrylamide solution for SDS-PAGE: (for one minigel-8 ml)

All volumes are in ml unless indicated otherwise.

Gel (%)	5	10	12.5	15	3 ml Stacking Gel (4.5%)
Acrylamide/ Bis-acrylamide	1.33	2.67	3.3	4	0.45
4 x resolving gel buffer	2	2	2	2	4x stacking gel buffer 0.45
Water	4.58	3.2	2.6	1.9	1.77
Temed	8 µl	8 µl	8 µl	8 µl	3 µl
10% APS	80 µl	80 µl	80 µl	80 µl	30 µl

SDS-PAGE is a biochemical technique where proteins are separated based on their molecular weight. The gel is discontinuous; it has a stacking gel which is present on top of the resolving gel. SDS-PAGE can be used to separate proteins based on their native size (native SDS-PAGE) or their subunit size (reducing SDS-PAGE). Two clean glass plates were fixed together with a spacer of appropriate thickness (0.75-1.5 mm) using holders or clips. The resolving gel was poured and isobutanol was layered on top of it. The gel was allowed to polymerize for about 30 min and isobutanol was thoroughly washed off using double distilled water. The stacking gel

was poured on top of the resolving gel and a comb, of appropriate size and appropriate number of wells, was placed in the stacking gel and left undisturbed for about 30 min. After polymerisation of the stacking gel, the combs were removed and the wells were cleaned with double distilled water to wash off any unpolymerized acrylamide. Boiled and denatured protein samples were loaded into the wells, the gel was placed in a tank with electrophoresis buffer and run at 100 -125 V for 2 h.

Staining of Polyacrylamide gels

1. Coomassie blue staining

Coomassie blue stain	0.5% (w/v) Coomassie blue 50% (v/v) Methanol 10% (v/v) Acetic acid in double distilled water
Destaining solution	50% Methanol and 10% glacial acetic acid dissolved in double distilled water.

The gel was stained in Coomassie blue solution at RT for 1-2 hours and destained using the destaining solution overnight. After complete destaining the gel was washed with double distilled water for 15 min and then dried in a gel drier.

2. Silver Staining

Fixative	30% Ethanol 10% Acetic acid
Sodium thiosulphate solution	0.5 M Sodium acetate 30% Ethanol 0.5% Glutaraldehyde 0.2% Sodium thiosulphate
Silver nitrate stain	0.1% Silver nitrate

0.02% Formaldehyde (37%)

Developer 2.5% Sodium carbonate
 0.01% Formaldehyde (37%)

Stop solution 0.05 M Glycine

All the solutions were made in double distilled water with final volume of 250 ml. for one gel.

The gel was first incubated in Fixative for 90 min at RT and then in Sodium thiosulphate solution for 1hour at RT or overnight at 4 °C. After 3 washes with dd water for 15 min each, the gel was stained with Silver nitrate solution for 1hour at RT and washed with water for 2 min. The gel was washed for 1 min with half of the Developer which was then discarded, and again washed for 1-4 min with rest of the solution. Developing was stopped with Stop solution.

2.2.2.2 Western blot analysis (semi dry method)

Semidry blot buffers

Cathode buffer 40 mM ϵ -Ammonium caproic acid
 20 mM Tris/HCl
 20% Methanol

Dissolved in water, pH was set to 9.0 with free Tris base and volume was made up with water.

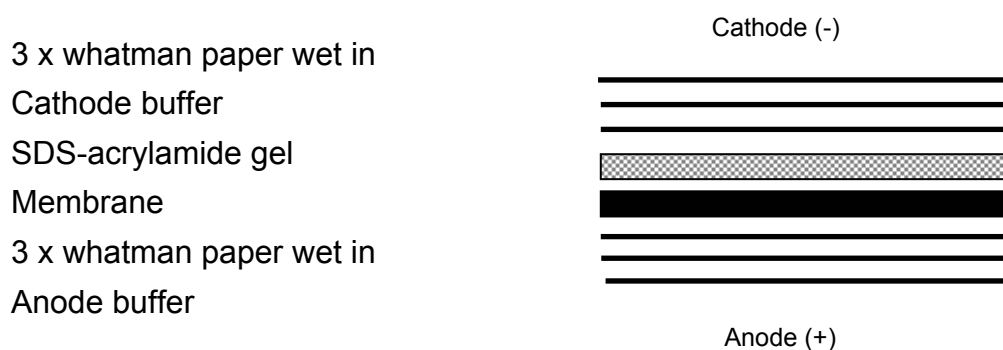
Anode buffer 75 mM Tris/HCl
 20 % Methanol

Dissolved in water, pH set to 7.4 with HCl and volume made up with water.

TBS -Tween TBS containing 0.1% Tween-20
Blocking buffer TBST containing 5% milk powder

Proteins separated by SDS-PAGE were transferred onto nitrocellulose or PVDF membranes for western blot analysis by chemiluminescence. 6 sheets of whatman paper were cut according to the size of the gel. Three sheets were immersed in the semidry anode buffer and placed in the chamber. A glass pipette was rolled on it to remove any air bubbles. Then the membrane dipped in the cathode buffer was

placed on top of it and again the air bubbles were removed. The gel was also soaked in the cathode buffer before placing on the membrane. Another three whatman paper sheets dipped in cathode buffer were placed on top of the gel. The glass pipette was again rolled on the set up to remove air bubbles. The proteins were transferred onto the membrane using current at 1 mA/cm^2 for 60 - 90 min.



After the transfer, membrane was incubated in TBST containing 5% milk powder for 1 h at room temperature to block all non-specific interaction sites on the membrane. After blocking, primary antibody diluted in blocking buffer at the concentrations as mentioned in 2.1.11, was added onto the membrane and incubated overnight in the cold room on a rocker. The non-specifically bound antibody was washed off by incubating the blot on a rocker with blocking buffer, changing the buffer once every 10 min for three times. The membrane was then incubated with the secondary antibody for 1 h at room temperature and the membrane was washed 4 times, 10 min each, with TBST. The proteins were detected by chemiluminescence.

Detection techniques

a. Chemiluminescent Substrate from PIERCE

Enhancer and peroxide solutions were mixed in a 1:1 ratio and incubated at room temperature for 5 min. The substrate was washed off and signals were detected using a CCD camera or an X-ray film.

b. Luminol Detection system

Luminol 0.44 g was dissolved in 10 ml of DMSO and aliquots were stored at -20°C

p-coumaric acid 0.15 g dissolved in 10 ml DMSO, aliquots stored

	at -20°C
Solution 1 (10 ml)	100 µl 250 mM Luminol 44 µl 90 mM p-coumaric acid 1 ml 1 M Tris/HCl pH 8.5 8.9 ml Water
Solution 2 (10 ml)	1 ml 1 M Tris/HCl pH 8.5 6 µl hydrogen peroxide (from 30% stock solution) 9 ml water

Solution 1 and solution 2 were mixed just before adding onto the blot.

Solutions were added onto the blot and incubated at room temperature for 1 min. The substrate was washed off and the membrane was wrapped in a polythene sheet and signals were detected using a CCD camera or an X-ray film.

Stripping the antibody from the membrane

The membrane was washed twice with water for 5 min each to wash off the chemiluminescent substrate. The membrane was stripped off the antibodies by washing with 0.2 N NaOH for 10 min on a rocker. The membrane was again washed for 5 min with water, and 5 min with TBST, incubated in blocking buffer followed by antibody of interest for next round of chemiluminescence.

2.2.2.3 Purification of GST-tagged recombinant protein

Buffer A	10% sucrose
Buffer B	25 mM Tris/HCl pH 7.5, 0.5 mM EDTA, 1mMDTT
Buffer C	1% Triton X-100 in buffer B
Buffer D	50 mM Tris/HCl pH 8.0, 25 mM glutathione, adjusted to pH 8.0 with NaOH (prepared freshly).

A bacterial clone expressing the GST-fusion protein under the control of an inducible promoter was grown in 20 ml LB as a preculture overnight. The culture was diluted 1:20 in fresh LB medium and grown for 1 h up to an $OD_{600} = 0.5-0.8$. Protein expression was induced by adding 0.1 mM IPTG to the culture, which was further

grown for 3-5 h at 30 °C. The cells were harvested at 6000 rpm in a JA 10 rotor and washed once with cold PBS. All the following steps were performed on ice. The pellet was resuspended in 4 ml buffer A, transferred to a 50 ml falcon tube and mixed with 4 ml buffer A containing 2 mg/ml lysozyme. After 15 min of incubation, the volume was made up to 20 ml with buffer A. The suspension was sonicated twice for 30 sec to reduce the viscosity of the homogenate, it was treated with 10 mg/ml RNase and 5 mg/ml DNase for 15 min. The lysate was finally supplemented with Triton X-100 to a concentration of 1% to increase the solubility of its components. The insoluble fraction was pelleted by centrifugation at 12000 rpm in a JA 20 rotor and the supernatant transferred into a new tube. 260 µl of glutathione-coupled sepharose beads (75% suspension), corresponding to a bead volume of 200 µl, were centrifuged at 1000 rpm for 5 min in a tabletop centrifuge and washed twice with 2 ml of each buffer B and C. The beads were incubated with the soluble lysate fraction on a spinning wheel at 4°C overnight. Next day, the sepharose was washed twice with buffer C and twice with buffer B. While the buffers A-C contain DTT at pH 6-7.5 to prevent oxidization of glutathione, elution is facilitated at pH 8 without the addition of DTT. GST-fusion protein was eluted from the beads by adding 200 µl of buffer D and incubating the sample for 30 min at 4 °C. Elution was repeated 4 times

2.2.2.4 Protein extracts from *S.cerevisiae*

Spheroplasting

Tris /SO₄ DTT buffer: 0.1 M Tris /SO₄ pH 9.4
 10 mM DTT

Spheroplasting buffer: 1.2 M Sorbitol
 50 mM Tris/ HCl pH 7.5
 10 mM EDTA

Yeast cells are surrounded by a firm cell wall. The cell wall has to be removed before performing biochemical experiments with the cells. After the cell wall is removed the cells are called spheroplasts and the process is called spheroplasting.

The cell pellet was resuspended in Tris/SO₄ DTT buffer at a final concentration of 10 OD₆₀₀/ml and incubated at 30 °C for 15 min with slow shaking. Cells were pelleted at 3000 rpm for 5 min. The pellet was resuspended in spheroplasting buffer at a concentration of 6 OD₆₀₀/ml. An aliquot of the resuspended cells was retained on ice to determine the spheroplasting (NS) efficiency. 0.5 mg/50 OD₆₀₀ of Zymolyase 20T was added and the cells were incubated at 30 °C for 30 min with slow shaking. An aliquot of the cells after spheroplasting was also taken (S). The two samples were diluted with water and the optical density was measured at 600 nm. The O.D. value obtained for the spheroplasted sample (S) indicates the amount of non spheroplasted cells. Using this the percentage of spheroplasting or the spheroplasting efficiency can be determined.

Calculation of spheroplasting efficiency:

O.D. of cells in spheroplasting buffer - O.D of cells after spheroplasting

Cells which are spheroplasted .

Percentage of spheroplasting obtained/ Spheroplasting efficiency

$$= \frac{\text{Cells which are spheroplasted}}{\text{O.D. of cells in spheroplasting buffer (NS)}} \times 100$$

Preparation of yeast cell lysates

a. Preparation of cell lysates using glass beads

Glass beads: Glass beads were immersed in conc. HCl overnight.

The beads were then washed in distilled water till the pH reached 7.0. These beads were autoclaved and stored at 4 °C.

Yeast cells were grown at 30 °C in a shaker incubator in either minimal media or YPD. Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted and pelleted at 2000 rpm for 6 min. Spheroplasts were resuspended in 700 µl of 0.1 M

sodium phosphate buffer. To this protease inhibitor mix was added to a final concentration of 1x. The spheroplasts were incubated in the cold room on a rotating wheel for 10 min. Spheroplasts were lysed using glass beads by vortexing at maximum speed for 6 min with intermittent cooling on ice. The unlysed cells and glass beads were pelleted by centrifuging at maximum speed for 3 min. The supernatant, cell lysate, was collected and the protein content was estimated.

b. Preparation of cell lysates using DEAE dextran

Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted as described above. Spheroplasts were pelleted at 2000 rpm for 2 min. Spheroplasts were resuspended in 700 μ l of 0.1 M sodium phosphate buffer. DEAE dextran was added to a final concentration of 50 μ g / 100 OD₆₀₀ (concentration of DEAE dextran had to be standardized for each strain). Spheroplasts were incubated on ice for 1 min and then at 30 °C for 5 min. Cells were centrifuged at 2000 rpm for 5 min to get the S5 fraction (homogenate).

2.2.2.5 Subcellular fractionation

Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted as described above. Spheroplasts were pelleted at 2000 rpm for 2 min. Spheroplasts were resuspended in 700 μ l of 0.1 M sodium phosphate buffer in presence of pretease inhibitors. Cells were lysed with DEAE dextran. 500 μ l of S5 fraction was centrifuged at 13000 rpm for 10 min to obtain the S13 and P13 fractions. S13 fraction was then centrifuged at 50000 rpm for 20 min to obtain the S200 (cytosolic proteins) and the P200 fraction (membrane proteins). P13 and P200 fractions were resuspended in 500 μ l of phosphate buffer with protease inhibitors added.

2.2.2.6 Glycerol Density Gradients

(reference: Kim *et al* 1997)

Glycerol solutions

20 %	2.52 g glycerol in 10 ml 20 mM PIPES, pH 6.8
30 %	3.78 g glycerol in 10 ml 20 mM PIPES, pH 6.8

40 %	5.04 g glycerol in 10 ml 20 mM PIPES, pH 6.8
50 %	6.30 g glycerol in 10 ml 20 mM PIPES, pH 6.8

Cells were grown to a mid log phase at 30 °C in a shaking incubator. Cells were pelleted at 5000 rpm for 5 min. Cells were spheroplasted using the protocol described in section 2.2.2.4. Spheroplasts were then lysed using glass beads. The protein content of the cell lysate was determined using the BIORAD method. 20-50 % glycerol gradients were made using 450 µl of each of the solutions. The gradients were allowed to linearize for 4 hours in the cold room. 1200 µg of protein was loaded onto the gradient. The gradients were centrifuged at 55000 rpm (260000 g) for 4 hours at 15°C using the Beckman TLS55 rotor. 10 fractions were collected from each of the gradients and TCA precipitated. The proteins were solubilised in Laemmli buffer and analysed by SDS PAGE followed by western blot.

2.2.2.7 Gel filtration

Gel filtration is a chromatographic technique which separates the proteins based on their relative size. Chromatography is performed using the porous beads as chromatographic support. A column of gel particles is in equilibrium with a suitable mobile phase for the molecules to be separated. Large molecules that are completely excluded from the pores elute first. Smaller molecules are distributed between the mobile phase inside and outside the molecular sieve and then pass through the column at a slower rate, hence elute later. The porosity of the gel particles and the size of the molecules to be separated determine the quality of separation.

Superdex 75 PC 3.2/30 and Superdex 200 column were used for the experiments. The Superdex column consists of dextran and crosslinked agarose. Superdex 75 has an exclusion limit of 100 kDa and Superdex 200, an exclusion limit of 1300 kDa.

The column was installed in the SMART system and equilibrated with the buffer (in which protein extracts were made) with at least one column volume (2.4 ml). All the buffers used for the column were made with HPLC water, filtered and degassed. The column was calibrated using a protein standard solution containing Transferrin, BSA, Albumin, Carbonic anhydrase and Cytochrome C.

Cells were made into spheroplasts and homogenized with potter. S200 fraction was collected. 100 μ g of protein was loaded onto the column at a flow rate of 40 μ l/min. Elution fractions of 50 ml were collected 20 min after the load.

2.2.2.8 Ape1p activity by overlay assay

Ape1p enzymatic activity of yeast colonies was determined by Ape1p overlay assay. To test the activity of the enzyme, an overlay agar containing leucine- β -naphthylamide (H-Leu- β -NA) as substrate was prepared. Chloroform was used to permeabilize cells. Aminopeptidase I catalyses the cleavage of H-Leu- β -NA and β -naphthylamide is released (β -NA) which is fluorogenic. Hence fluorescence is observed at 365 nm.

Assay-agar mixture

Tris/HCl	50 mM pH 7.5
EDTA	2 mM
Agarose	0.6%
H-Leu- β -NA	10 mM

Fresh cells are used for the assay. Colony streaks were made on YPD plates and incubated O.N. at 30 °C. Chromatographic paper filters (80mm, Schleicher and Schuell) were placed on the YPD 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 side up and incubated overnight at 30 °C. Next day, the filters were taken from the plates and placed in CHCl_3 with colony side downward for 15-20 sec. Excess CHCl_3 was removed by drying the filters on paper towels. These were then placed in Petri plates and overlaid with assay-agar mixture. The mixture was prepared by mixing the buffer and agarose in a falcon tube. Substrate solution was prepared and added immediately to the assay mixture which was poured onto the petri plates containing the filters. Plates were incubated for 10-15 min at RT. Activity of Ape1p was detected at 365 nm using an UV-lamp.

2.2.2.9 Immunofluorescence microscopy

Fixative: 1 g of paraformaldehyde is dissolved in 25 ml of water by heating above 60 °C. 187.5 µl of 6 N NaOH is added and then 0.34 g of potassium hydrogen phosphate is added. The pH of the solution is 6.5.

TE β buffer (5 ml)

Tris/HCl pH 8.0	200 mM
EDTA	20 mM
β -mercaptoethanol	1%

Spheroplasting buffer (5 ml)

Sorbitol	1.2 M
Potassium phosphate pH 7.3	50 mM
Magnesium chloride	1 mM

Sorbitol-Azide (5 ml)

Sorbitol	1.2 M
Sodium azide	5 mM

Sorbitol-SDS (10 ml)

10% w/v SDS	2%
Sorbitol	1.2 M
Sodiumazide	5 mM

PBS-BSA-Azide (50 ml)

BSA	5 mg/ml
Sodiumazide	5 mM
10X PBS	1X

On day I the cells were streaked on SD plate and incubated overnight at 30 °C. On day II, 5ml of SD medium was inoculated with cells and grown overnight at 30 °C. Next day, cells equal to 2.5 OD 600 were harvested and 10 ml of YPD medium was inoculated and incubated for 4 hrs at 30 °C. Cells were then fixed by adding 1-1.2 ml of 37% formaldehyde (final concentration 3.7%), incubated for 30 min in the shaker.

Cells were harvested and resuspended in 2 ml fixative and incubated overnight at room temperature on the rocker. Fixed cells were harvested and suspended in 1 ml TE β for 10 min. Spheroplasts were obtained by incubating with zymolyase at 30 °C with gentle shaking. While cells were spheroplasting, slides (8-well multitest slides) were prepared. 20 μ l of 1 mg/ml polyL-lysine in water was added to each well and allowed to sit for 1 min. Each well was washed with 25 μ l of water (6 times). Aspirated and allowed to air dry. Spheroplasted cells pelleted at 6500 rpm, 30 sec and washed once with 1 ml of 1.2 M sorbitol containing 5 mM sodium azide and repelleted. Cells were resuspended in 1.2 M sorbitol containing 10% SDS and incubated for 2 min. cells were pelleted at 6500 rpm, 30 sec and washed twice with 1 ml of 1.2 M sorbitol. Cells were resuspended in appropriate volume of 1.2 M sorbitol depending on the size of the pellet. 40 μ l of cells per well were added and allowed to settle for 10 min. Remaining cells were aspirated and each well was washed 3 times with 20-25 μ l of PBS-BSA. The slides were allowed to sit in humid chamber for 15 min at room temperature. Wells were aspirated and primary antibody was added and incubated for 1h at room temperature. Cells were washed with 20-25 μ l of PBS-BSA-azide 6 times. Secondary antibody was added and incubated for 1h. Cells were washed 6 times with PBS-BSA-azide. 10 μ l of DAPI (1 mg/ml) was added, cells were allowed to sit for 10 min and washed with PBS-BSA-azide 3 times. 8 μ l of mounting media was added in each well, slides were covered with coverslip and allowed to settle. After 5 min, ends of the coverslips were sealed with nail polish. Slides were viewed under a confocal laser-scanning microscope. DAPI staining was examined at 395 nm.

3 Results

In order to clone the *via*-genes and *via* mutant suppressors, both the mutants were transformed with two yeast DNA libraries. One library was provided by H.D. Schmidt (MPI for biophysical chemistry, Göttingen) and the other by C. Sengstag (ETH Zürich). In the course of the cloning attempts it was noticed that the low transformation frequency of *via* mutants was further reduced by the increasing plasmid size. Cloning attempts with both the libraries were not successful.

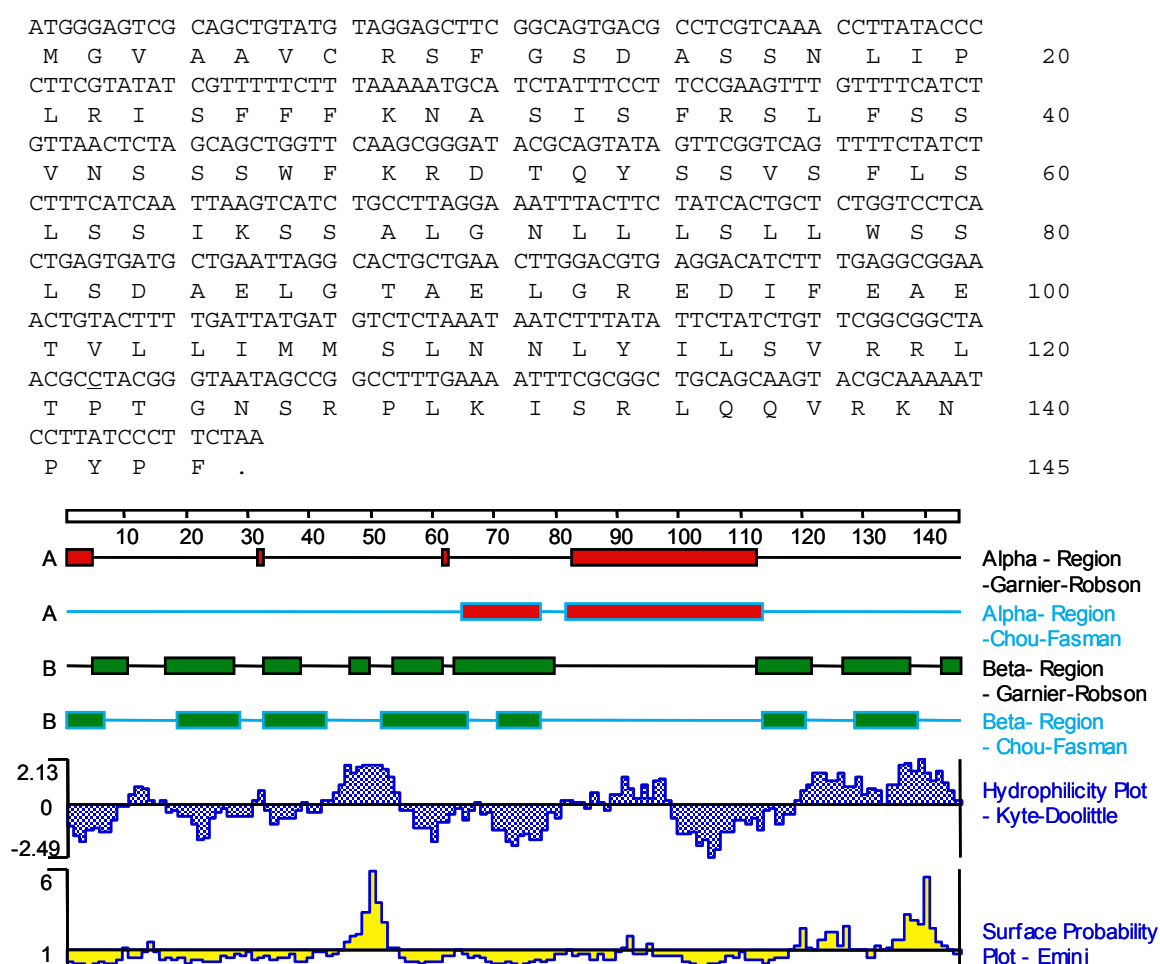


Fig 7: DNA and peptide sequence of YDR360W as isolated from the wt strain.

Therefore a DNA library was constructed using the isogenic wild-type strain and the small pRS 316 vector.

DNA fragments varying from 2-5 kb were isolated and subcloned to limit the size of the resulting library plasmids. This isogenic library was used to transform *via* mutants using the Li/Acetate method (2.2.1.2). Colonies were replica plated on YPD medium to suppress the degradative autophagy pathway and screened for Ape1p enzymatic activity by the Ape1p overlay assay with Leu- β -naphthylamid as substrate (2.2.2.8). Colonies showing maximum activity were recovered and retested for Ape1p enzymatic activity. A plasmid was identified carrying a 1.5 kb fragment on chromosome IV with the ORF YDR360w. Sequencing of ORF YDR360w isolated from the wt strain revealed a larger reading frame than the sequence given in the yeast genome data library. The ORFs isolated from *via10* and *via14* mutants were identical to the ORF from the wt strain. The ORF sequence from wt had an additional 'C' (Fig 7), causing a frameshift and an ORF encoding 145 instead of 129 aa. Secondary structure analysis predicts N-terminal β -sheets and an α -helical domain between amino acids 83-112.

To test whether the expression of YDR360w is reduced, a northern blot experiment was performed. Total RNA was isolated from the wt, *via 10 via14* strain and the deletion strain of YDR360w, which was obtained from the EUROSCRAF (Frankfurt.M.) which has collection of yeast knock out strains. Northern blot experiment was done as described in materials and methods (2.2.1.3). 10 μ g of RNA from each were loaded and hybridized with ³²P-d CTP labeled YDR360w. It was observed that the expression of mRNA was comparable in wt and *via* mutant strains and no signal was observed in *ydr360w Δ* strain. This experiment demonstrates that the transcription of YDR360w is not reduced in the *via* mutants and is normal (Fig 8).

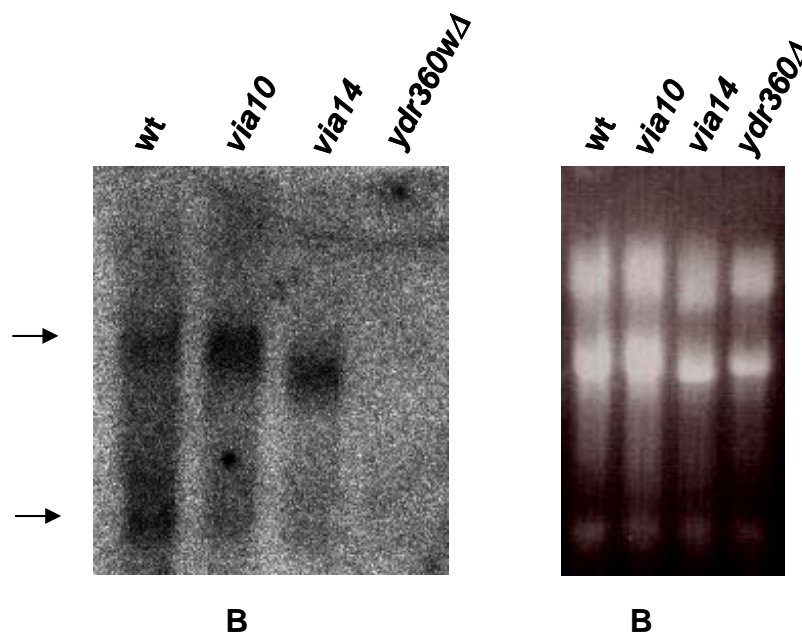


Fig 8: Expression of YDR360w gene at mRNA level in wt and *via* mutants. A: Northern blot showing the expression of mRNA was normal in wt and *via* mutants for YDR360w gene. No signal for YDR360w was observed in *ydr360wΔ* strain. B: Agarose gel showing that comparable amounts of RNA loaded (upper band). Arrows indicate the position of rRNA. 10 μ g of RNA was loaded in each lane from wt, *via10*, *via14* and *ydr360wΔ* strain.

3.1.1 *SUVIA1* is a suppressor of *via* mutants

The ORF YDR360w was amplified by PCR from the genomic DNA of the parental wt strain and *via* mutants. The ORF was cloned into *cen* (single-copy) pYX141 and 2 μ (multi-copy) pYX242 yeast expression vectors using EcoRI and HindIII restriction sites. The *via* mutants were transformed with YDR360w plasmid carrying Leu as the auxotrophic marker. Since Ape1p is expressed from a His plasmid, transformants were selected on plates lacking His and Leu. The colonies obtained after transformation were analyzed for Ape1p enzymatic activity by overlay assay.

via10 has 20% of the wt Ape1p enzymatic activity and *via14* does not show any enzymatic activity. When the ORF was expressed in the *via* mutants, the mutants with both *cen* and 2 μ Suvia1p plasmid had Ape1p enzymatic activity comparable to the wt cells (Fig 9).

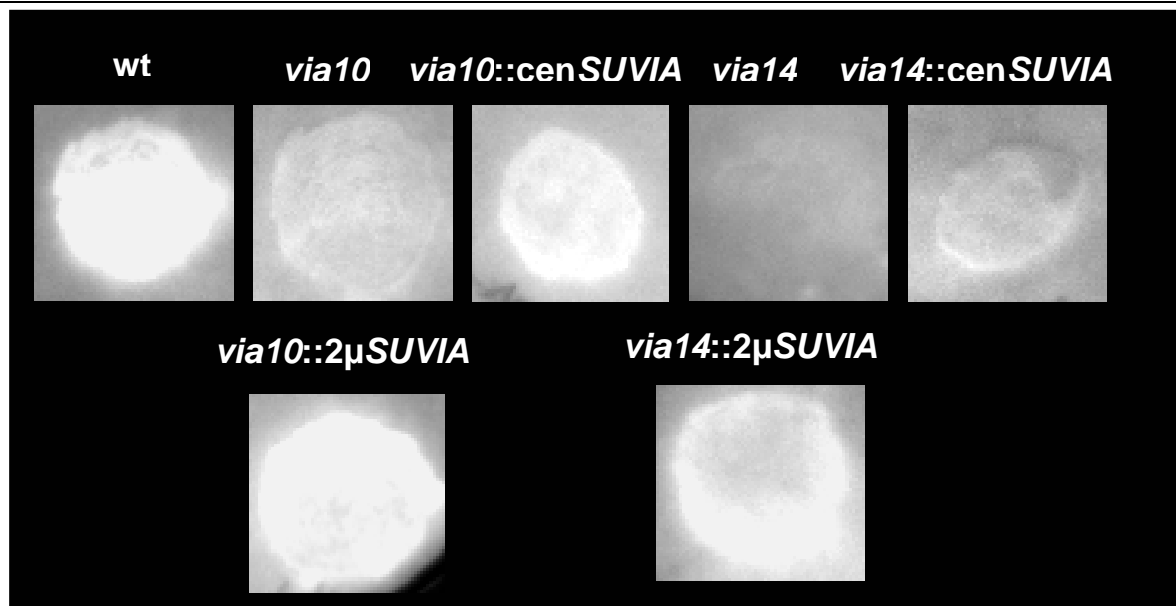


Fig 9: Ape1p-overlay assay for testing the Ape1p enzymatic activity. The image was recorded at 365nm using a CCD chemiluminescence camera. Strains with Ape1p enzymatic activity emit fluorescent light and colonies become visible.

Because YDR360w isolated from *via10* and *via14* did not show any mutations, YDR360w should be a suppressor of *via10* and *via14*. Since ORF YRD360W suppresses the defect in the *via* mutants, and is not mutated itself, it is termed as *SUVIA1* “suppressor of via”.

3.1.2 Ape1p expression and processing is enhanced by Suvia1p

Suvia1p expression could lead to higher pApe1p expression due to the formation of native and stable dodecamers and also enhanced vacuolar transport of pApe1p. To investigate this, protein extracts were made from cells grown at 30°C in nutrient-rich medium (YPD) until logarithmic phase between OD₆₀₀ = 0.7-1.2 (when Cvt pathway is active) and stationary phase OD₆₀₀ above 2.0 (when autophagy is active). Cells were converted into spheroplasts and lysed with glass beads (2.2.2.4). Crude cell extracts were prepared in the presence of protease inhibitors to analyze Ape1p expression and processing in Suvia1p transformed *via* mutants. Proteins were detected on SDS-PAGE and blotted onto nitrocellulose membrane. Ape1p was detected using a rabbit anti-Ape1p antiserum. Blots were also developed with mouse anti-CPY antiserum as a control for the quantification of Ape1p signals (Fig 10).

The signal intensities for precursor aminopeptidase (pApe1p) and mature form (mApe1p) were quantified, using the signals for vacuolar carboxypeptidase Y which reaches the vacuole through the secretory pathway. The expression level and processing of Ape1p is depicted in the graph.

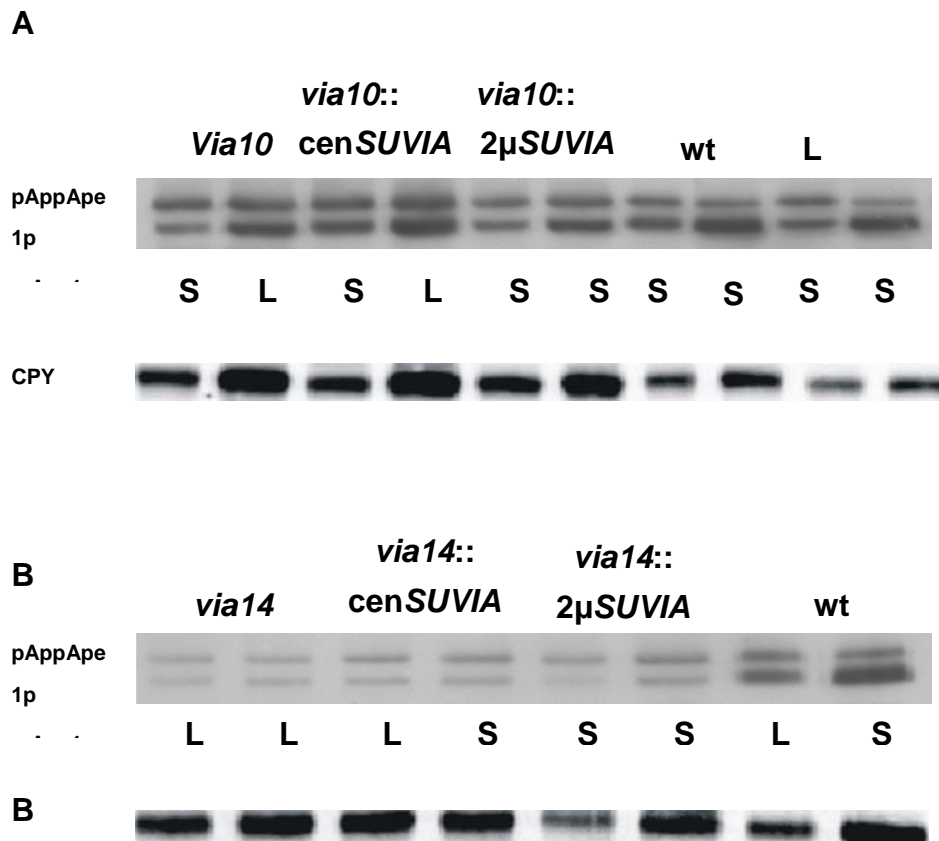


Fig 10: Western blot analysis of *via* mutants transformed with *Suvia1p*. Protein extracts were made from logarithmically (L) and stationary (S) cultures grown in rich media. A. Protein extracts from *via10* and *Suvia1p* transformants. B. Protein extracts from *via14* and *Suvia1p* transformants. 10μg of protein from wt and 20μg of protein from the other strains were loaded. CPY served as internal control.

In *via* mutants, the pApe1p form is more abundant than in the wt demonstrating a defect in Cvt pathway. Both mutants express low levels of Ape1p as the protein is present in a non-native conformation, which leads to enhanced proteolytic cleavage. When *Suvia1p* is expressed in *via* mutants, the expression level of Ape1p is two-fold.

Suvia1p not only improves the expression levels of Ape1p in *via* mutants, but also has an effect on processing and thus vacuolar transport of Ape1p in the mutants. Under logarithmic phase, when the Cvt pathway is active, wt cells have 30% of Ape1p as pApe1p, whereas *via10* accumulates 60-70% of Ape1p as precursor form. When Suvia1p is expressed in the *via* mutants from *cen* or 2μ plasmids, the delay in processing is reduced to 45% in *via* mutants (Fig 11A).

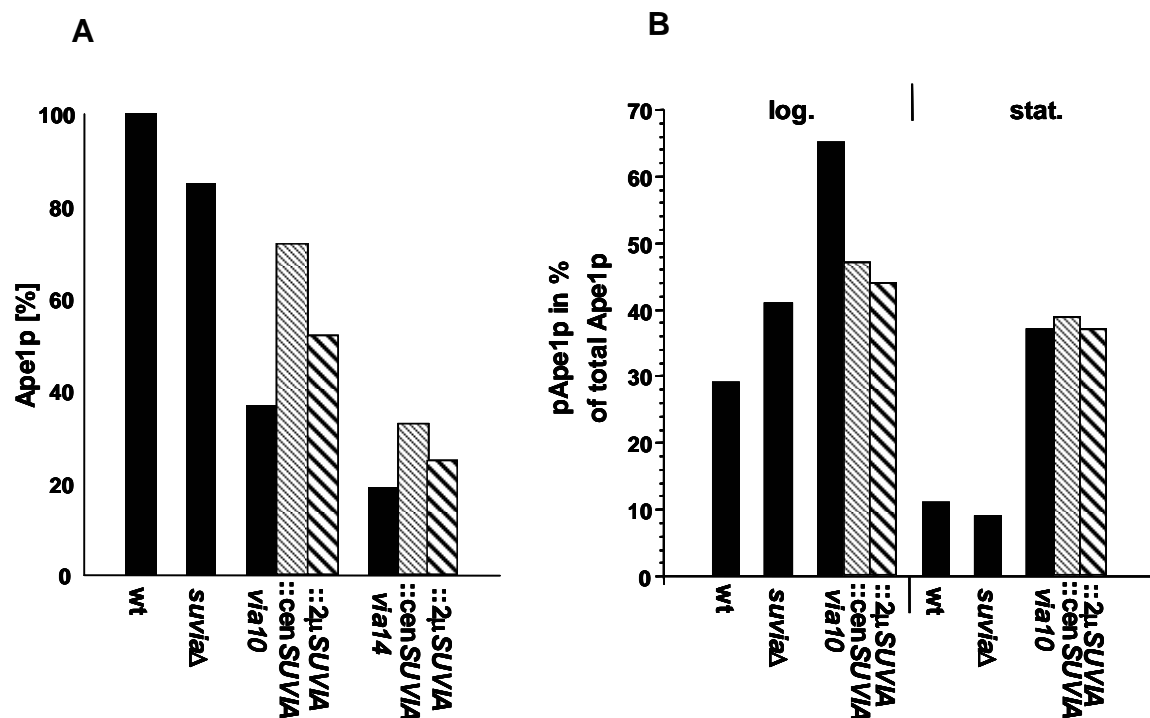


Fig 11: Graphical representation of Ape1p expression and processing in Suvia1p expressing *via* mutants. A) Expression levels of Ape1p increases two folds in *via* mutants.

B) Suvia1p expression enhances pApe1p processing along the Cvt pathway (log), but not along the autophagic pathway (stationary).

In stationary phase, where autophagy is induced by glucose starvation, wt shows only 10% of pApe1p, and pApe1p levels are reduced in *via10* and *via14* mutants and mApe1p is detected even in *via14*. Suvia1p has no effect in the processing of Ape1p when there is upregulation of autophagy under starvation conditions. Thus upon Suvia1p expression, *via10* show increased levels of Ape1p and processing of pApe1p is enhanced during the vegetative growth phase.

Suvia1p expression in *via14*, having the most severe phenotype, does not stimulate pApe1p transport.

3.1.3 Suvia1p enhances dodecamerization of pApe1p

The pApe1p monomer assembles into a homododecameric complex in the cytoplasm with a half time of 2 min and the dodecameric complex of mApe1p is required for its enzymatic activity. *via10* and *via14* mutants do not form stable dodecamers of pApe1p. They are defective in complex assembly, form high molecular weight aggregates and Ape1p is rapidly degraded due to enhanced sensitivity to proteolysis (Andrei *et al.*, 2001). To analyze the pApe1p complex assembly in *via* mutants expressing Suvia1p, Ape1p oligomers were separated on a glycerol density gradient (2.2.2.6). Crude protein extracts were prepared from cells grown in YPD to OD₆₀₀ = 0.7-1.2 by breaking spheroplasts with glass beads in the presence of protease inhibitors. 10 soluble fractions of the glycerol gradient were collected and proteins were separated on 10% SDS-PAGE. Ape1p was detected by western blot analysis and signal intensities were quantified and are shown in the graph (Fig 12).

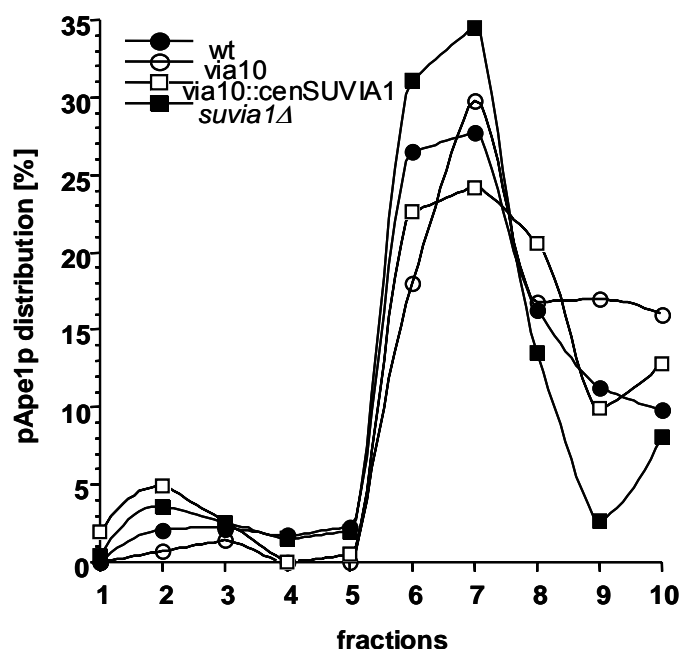


Fig 12: Oligomers of pApe1p and mApe1p separated on a glycerol gradient. Gradients were run for wt, *via10*, *suvia1Δ* and *via10::cenSUVIA1*. 10 fractions were collected from each and analyzed by western blot and quantified. Numbers give the percentage of total pApe1p in the fractions.

In wt cells, 90% of Ape1p accumulated as dodecamers of pApe1p in fractions 6 and 7. In *via10*, dodecamers are seen in fractions 6 and 7, but the fractions 9 and 10 contain ~40% of pApe1p demonstrating a defect in complex assembly and formation of high molecular weight aggregates. In *via10::cenSUVIA1*, only 15% of pApe1p is found in fractions 9 and 10 demonstrating enhanced formation of dodecameric complexes as indicated by the enzymatic assay. In *suvia1Δ*, the amount of pApe1p in fractions 9 and 10 is not increased compared to the wt and pApe1p is present as dodecamers. *via14::cenSUVIA1* was not tested for dodecamerization of pApe1p because the suppression phenotype was as in *via10::cenSUVIA1*. This demonstrates that Suvia1p is not essential for pApe1p dodecamerization even though it supports formation of native dodecamers in *via10*.

3.1.4 Suvia1p is required for Ape1p enzymatic activity

Since Suvia1p is not required for the dodecamerization of pApe1p, we wanted to check, if Ape1p is enzymatically active in the absence of Suvia1p. wt and *suvia1Δ* strains were mated and random sporulation (2.2.1.2) was done to generate spores lacking leucine aminopeptidase activity. Ape1p was then transformed and Ape1p activity test was done by the overlay assay. Ape1p did not show any enzymatic activity (Fig13) indicating that dodecamers are not in a native conformation. Thus Suvia1p is required for the formation of native dodecamers of Ape1p.

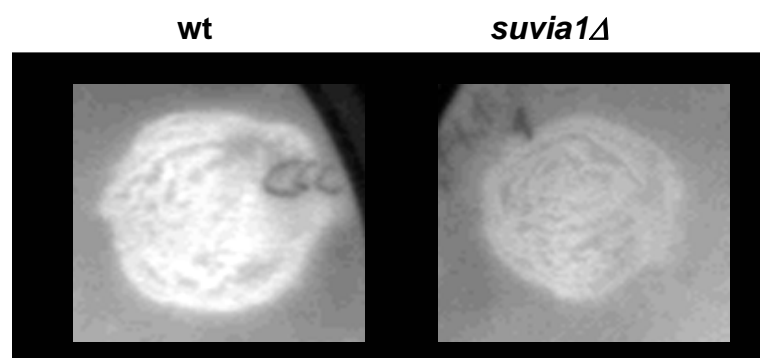


Fig 13: Ape1p is not enzymatically active in *suvia1Δ* cells. Ape1p enzymatic activity was analyzed by overlay assay. *suvia1Δ* cells lack Ape1p activity when compared to the wt cells.

3.2 Characterization of Suvia1p

To further investigate and characterize Suvia1p, we wanted to generate antibodies against Suvia1p. Suvia1p was expressed in *E.coli* as a His₆ and GST-tagged protein, but the fusion proteins either did not bind to the affinity matrix or formed insoluble inclusion bodies. A small fraction of GST-Suvia was soluble and could be purified, but rabbits did not produce anti Suvia1p antibodies. Therefore, Suvia1p was tagged with the hemagglutinin tag (HA) at the C- terminus.

3.2.1 HA-tagged Suvia1p complements Suvia1p-deficiency

Suvia1p was cloned in pYX yeast expression vector to add the HA tag at the C-terminus using EcoRI and HindIII sites. We checked if Suvia1p-HA transformed in *suvia1Δ* cells is biologically active by testing for Ape1p enzymatic activity.

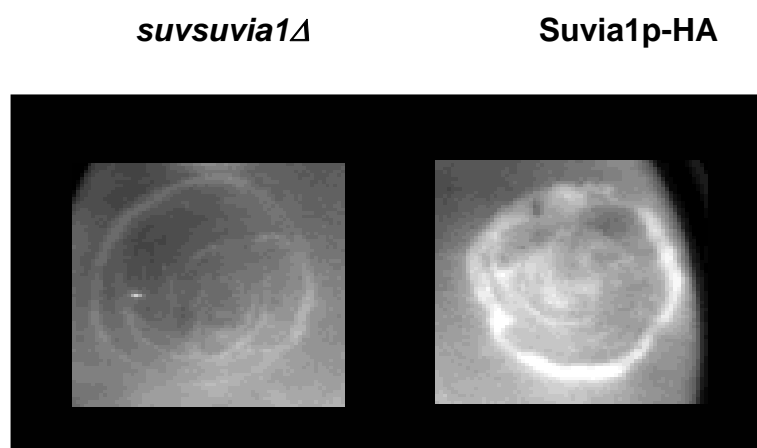


Fig 14: Suvia1p-HA complements Suvia1p deficiency: Suvia1p was transformed in *suvia1Δ* cells and Ape1p overlay assay was done. It was observed that HA-tagged Suvia1p complemented Ape1p activity in Suvia1p deficient cells.

Suvia1p-HA was able to complement Suvia1p deficiency demonstrating biological activity of the fusion protein.

Suvia1p-HA clones were picked and crude cell extracts were made from the cultures grown until logarithmic phase. Spheroplasts were lysed with glass beads and proteins were separated on SDS-PAGE. Western blot was done and HA tagged Suvia1p was detected using anti-HA monoclonal mouse antiserum.

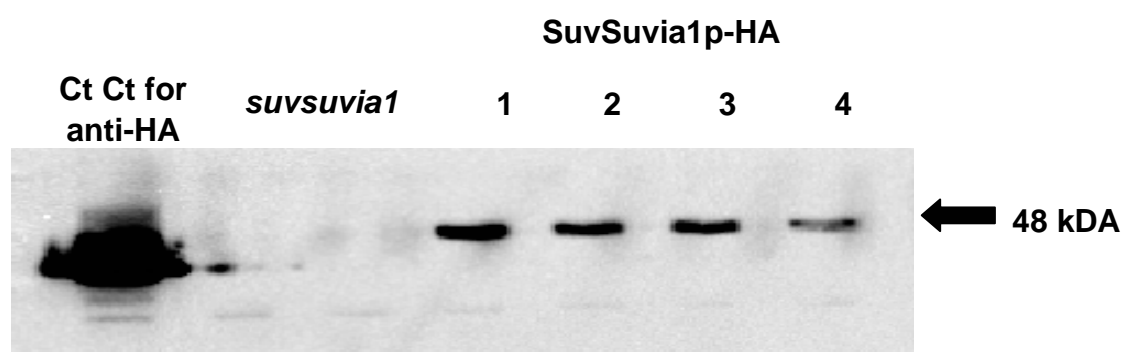


Fig 15: Western blot analysis of positive Suvia1p-HA clones. A positive control for HA tag (Use1p-HA provided by Dr. G. Fischer Von Mollard) was loaded in the first lane. *suvia1Δ* does not show any signal and Suvia1p-HA clones 1-4 show signal for anti-HA at 48 kDa (approximately).

Suvia1p is predicted to be a 16 kDa protein based on the ORF. On SDS-PAGE Suvia1p-HA migrates at about 48 kDa (Fig 15). This could be caused by incomplete denaturation or by a post-translational modification like phospho-rylation or covalent binding to a protein(s). Therefore we did gel filtration experiments (2.2.2.7) to confirm the molecular mass of Suvia1p-HA.

To determine the exact molecular weight of Suvia1p, gel filtration experiments were done in the presence of 6 M urea. Suvia1p-HA cells were converted into spheroplasts in presence of 6 M urea and homogenized with a potter. S200 fraction was prepared where Suvia1p is present described in the following section (3.2.2). This S200 fraction was prepared and 50 μ g of protein was loaded onto a Superdex 75 PC 3.2/3.0 column at a flow rate of 40 μ l/min. The column was calibrated with molecular weight standard proteins as given in materials and methods. Suvia1p-HA was detected in the fractions by western blot using anti-HA mouse monoclonal antiserum.

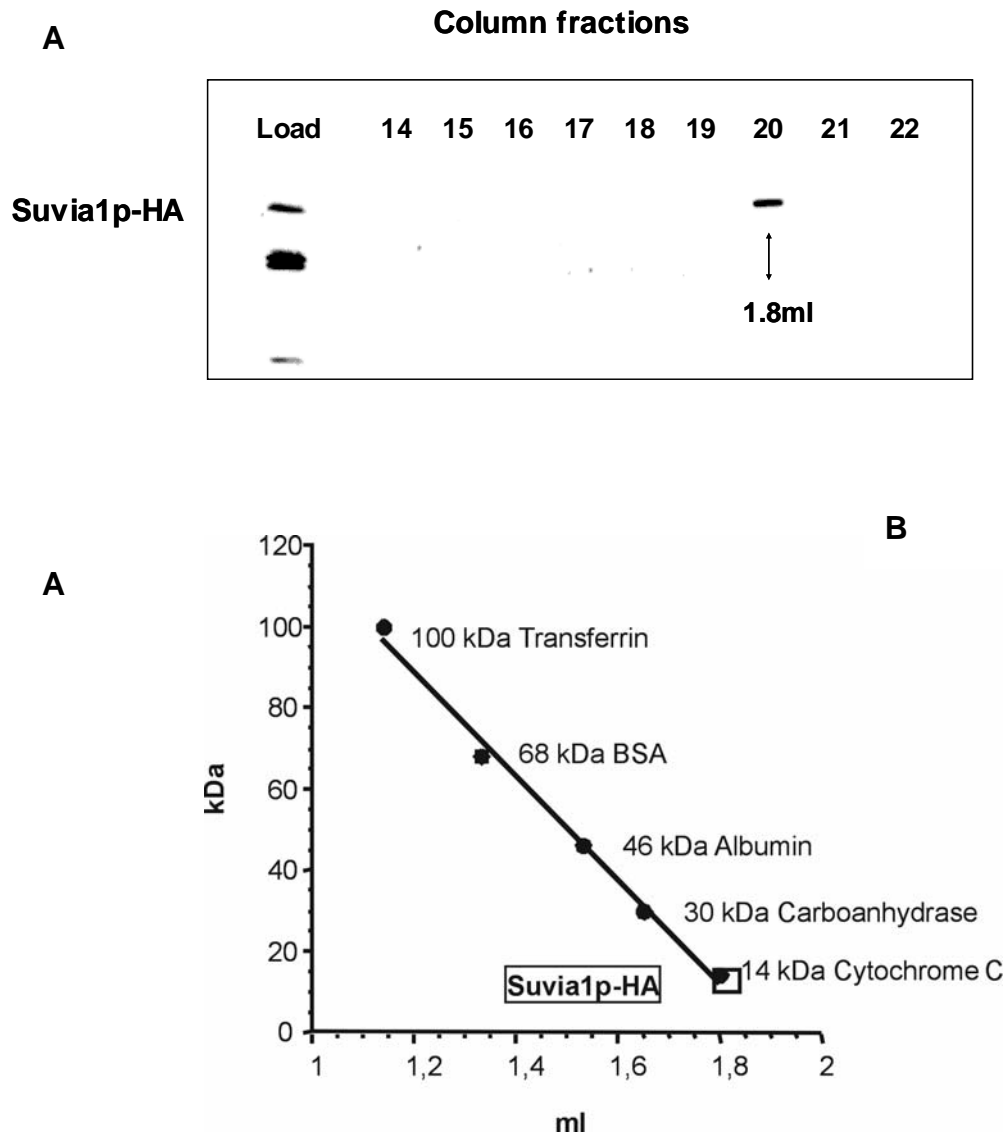


Fig 16: Gel filtration of Suvia1p-HA in Superdex 75 column: A. Western blot showing that Suvia1p-HA was detected by anti-HA antiserum in column fractions 20. B. Suvia1p-HA was eluted at 1.8 ml which corresponds to a molecular size of ~14 kDa. Open box indicates fraction/molecular mass of Suvia1p-HA. 50 μ g of protein was loaded as reference in lane1.

Suvia1p-HA was detected in fractions which corresponded to a molecular mass of about 14 kDa (Fig 16). Thus in presence of 6M urea Suvia1p was able to denature to its monomeric size of 16 kDa.

3.2.2 Suvia1p is a cytosolic protein

To analyze the subcellular localization of Suvia1p, Suvia1p-HA expressing cells were fractionated and Suvia1p-HA distribution was followed by western blots for the HA-tag. Cells were grown upto logarithmic growth phase and converted into spheroplasts. Spheroplasts were lysed with DEAE-dextran in presence of protease inhibitors at 30°C for 5 min. The lysate was centrifuged at 500 g for 5 min to obtain the S5 fraction. The S5 fraction was centrifuged at 13000 rpm for 10 min to get the S13 and P13 fractions. S13 fraction was further centrifuged at 200,000 g for 20 min to obtain S200 and P200 fractions (2.2.2.5). Aliquots of the fractions were loaded on 10% SDS-PAGE, followed by western blot to detect the proteins. Suvia1p-HA was detected by anti-HA monoclonal mouse antiserum.

Ct *suvia1*Δ S5 P13 S200 P200



Fig 17: Subcellular fractionation of Suvia1p-HA. Suvia1p-HA was seen in S200 fraction. First lane is the positive control for anti-HA anti serum. Suvia1p is seen in S200 fraction.

Suvia1p-HA was exclusively detected in S200 fraction indicating that Suvia1p is a cytoplasmic protein.

3.2.3 Suvia1p appears to be part of 200 kDa complex

Next spheroplasts were made in the absence of 6 M urea and homogenized with a potter. Experiment was done in the same way as described for Superdex 75 column, except that spheroplasts were made in the absence of 6 M urea and sample was loaded on Superdex 200 column. The S200 fraction was prepared and 50 µg of protein was loaded onto a Superdex 200 column at a flow rate of 40 µl/min.

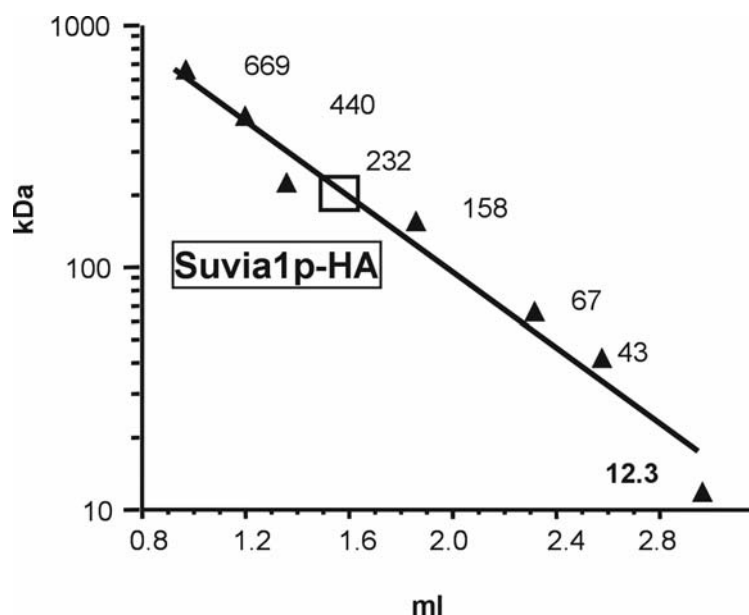
B**Column fractions****B**

Fig 18: Gel filtration of Suvia1p-HA in Superdex 200 column: A) Western blot showing that Suvia1p-HA was detected by anti-HA antiserum in column fractions 15-17. B) Suvia1p-HA was eluted at 1.6 ml which corresponds to a molecular size of ~200 kDa. Open box indicates fraction/molecular mass of Suvia1p-HA. 50 μ g of protein was loaded as reference in lane1.

Suvia1pHA was detected in the fractions by western blot using anti-HA mouse monoclonal antiserum. Suvia1p-HA was exclusively detected in the fractions correspondings to a molecular weight of about 200 kDa (Fig 18). Hence, Suvia1p is part of 200 kDa complex.

3.2.4 Immunofluorescence microscopy of Suvia-1p

To analyze subcellular distribution of Suvia1p in more detail, indirect immunofluorescence microscopy was done with Suvia1p-HA expressing cells. Cells grown until stationary phase ($OD_{600} = 2.5$) were used for the experiment (2.2.1.9). Cells were fixed in paraformaldehyde overnight. Cells were converted into spheroplasts, which were then fixed on the slides.

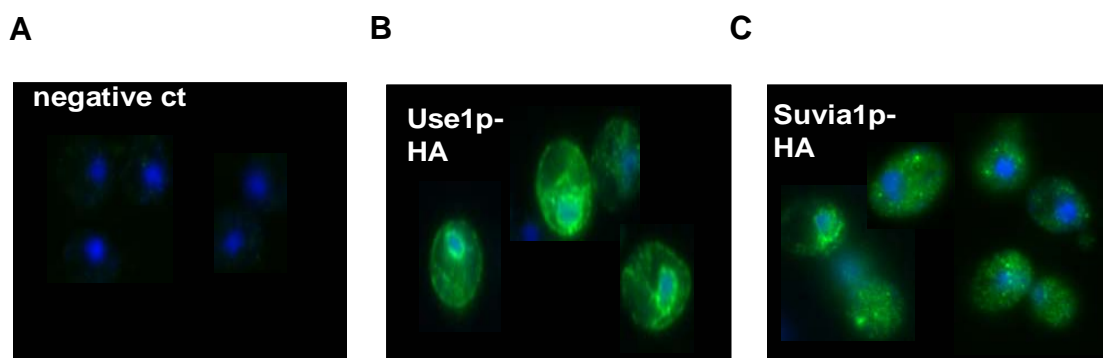


Fig 19: Localization of Suvia1p-HA. A: *svia1Δ* as negative control. B: Use1p is the positive control for anti HA. C: Suvia1p-HA shows staining in the cytoplasm as dots.

The cells were stained with antibodies against HA. Suvia1pHA is localized in the cytoplasm as dots and concentrated as small structures. In *svia1Δ*, no staining is observed. The positive control Use1p-HA is localized to the ER and hence staining is seen around the nucleus.

3.2.5 Suvia1p and Atg8p do not colocalize

Since Suvia1p showed cytoplasmic concentration and because it appears to be involved in the formation of native Ape1p dodecamers, we thought it might be part of the pre-autophagosomal complex. Atg8p (Aut7p) is known to be a protein to localize to the pre-autophagosomal structure and is required for Cvt transport vesicle formation. Immunofluorescence microscopy of Suvia1p-HA and Atg8-GFP (construct provided by Prof. Ohsumi) was done to study the colocalization of the two protein.



Fig 20: Immunofluorescence microscopy of Suvia1p and Atg8p. A: Aut7p-GFP is seen in green B: Suvia1p-HA in red and C: is the merge of (a) and (b). Arrows indicate Suvia1p-HA in red and Atg8p in green.

Suvia1p-HA cells were transformed with Aut7p-GFP. Cells of $OD_{600} = 2.5$ were used for the experiment. Cells were fixed in paraformaldehyde overnight, converted into spheroplasts which were then fixed on the slides. The cells were stained with antibodies against anti-HA for Suvia1p-HA.

Suvia1p and Aut7p are localized to different cytosolic structures, which are however, in close proximity. Some structures appear yellow (arrow shown in yellow), indicating co-localization of Suvia1p and Atg8p (Aut7p) to the same structure, but sometimes they appear as two distinct spots next to each other. It could be possible that Suvia1p contain Atg8p at some point but all Atg8p do not have Suvia1p. However co-immunoprecipitation experiments failed to demonstrate co-localization (data not shown). The data demonstrate that the Suvia1p complex and the pre-autophagosomal structure are in close proximity, and that both complexes might even get in contact with each other.

3.3 Interaction of Suvia1p with Aminopeptidase I

Suvia1p is required for the formation of native Ape1p dodecamers. Therefore, it was tested whether an interaction of Suvia1p-Ape1p could be demonstrated *in vitro*. GST pull-down experiments were used to study the interaction of Suvia1p with Ape1p (2.2.2.3).

Suvia1p was cloned into the vector pGEX-2T to express the fusion protein. Glutathione-S-transferase (GST) was fused at the N-terminus via a linker of 10 aa. GST-Suvia1p fusion protein was expressed in *E.coli* and isolated by binding to glutathione (GSH)-coupled Sepharose.

The molecular weight of Suvia1p and GST is 15 kDa and 27 kDa respectively. The molecular weight of GST-Suvia1p fusion protein will be 42 kDa. Western blot was done for the fractions in which the fusion protein was eluted and detected using anti-GST goat antiserum.

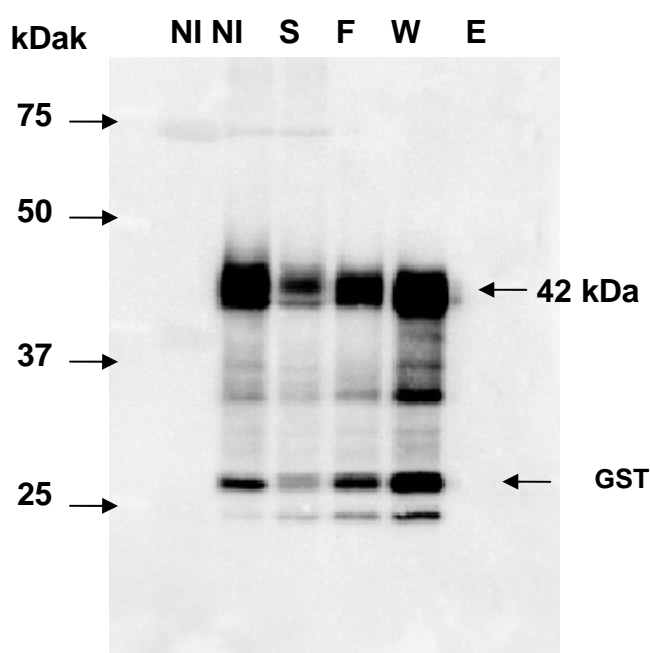


Fig 21: Western blot showing GST-Suvia1p fusion protein. NI is the non-induced fraction with no GST-fusion protein. S is the supernatant fraction, F is the flow through after the extract was incubated with the GSH Sepharose beads, W is the wash fraction and E is the eluate, where the fusion protein at 42 kDa is observed in addition to free GST protein at 27 kDa.

A signal at 42 kDa was seen, which corresponds to the molecular weight of the GST-Suvia1p fusion protein (Fig 21).

To test, if GST-bound Suvia1p could pull down Ape1p, GST-Suvia1p was bound to sepharose beads. Protein extracts from wt and *suvia1Δ* cells were prepared after spheroplasting the cells. Cells were lysed with DEAE dextran (90 μ g/100 OD₆₀₀) in the presence of protease inhibitors. 100 μ g of protein extracts were loaded on 50 μ l of GST-Suvia1p and GST-beads (as control) and incubated overnight at 4 °C on spinning wheel at a very slow speed. Next day beads were washed and proteins were eluted with buffer containing glutathione. All the fractions were loaded on 10% SDS-PAGE and analyzed by western blot. Ape1p was detected using a rabbit anti-Ape1p antiserum.

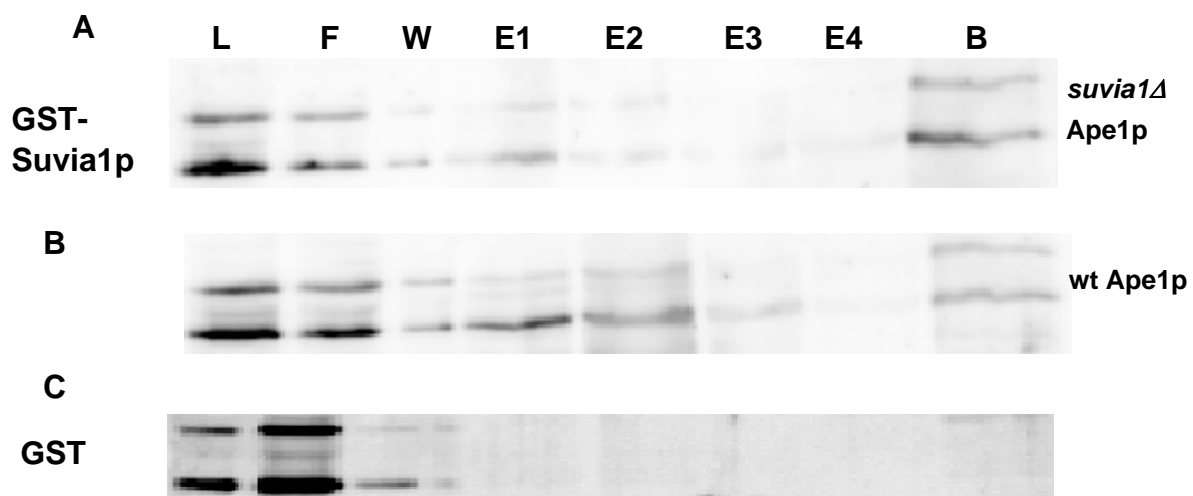


Fig 22: Interaction of Suvia1p with Ape1p from *suvia1Δ* cells. A) Protein extracts from *suvia1Δ* cells loaded on GST-Suvia1p beads. B) Protein extracts from wt cells loaded on GST-Suvia1p beads. Protein extracts loaded on GST beads which served as a negative control. No signals of Ape1p was observed.

The signals were quantified and it was observed that of the total protein from *suvia1Δ* cells that was loaded onto the GST-Suvia1p beads, only 15% of Ape1p from *suvia1Δ* was bound to the beads. Bound Ape1p from *suvia1Δ* were not efficiently eluted (Fig 22A). A large fraction of Ape1p was irreversibly bound to Sepharose beads. Only 6% could be eluted from the beads. The amount of protein that was bound and eluted is shown in (Fig 23).

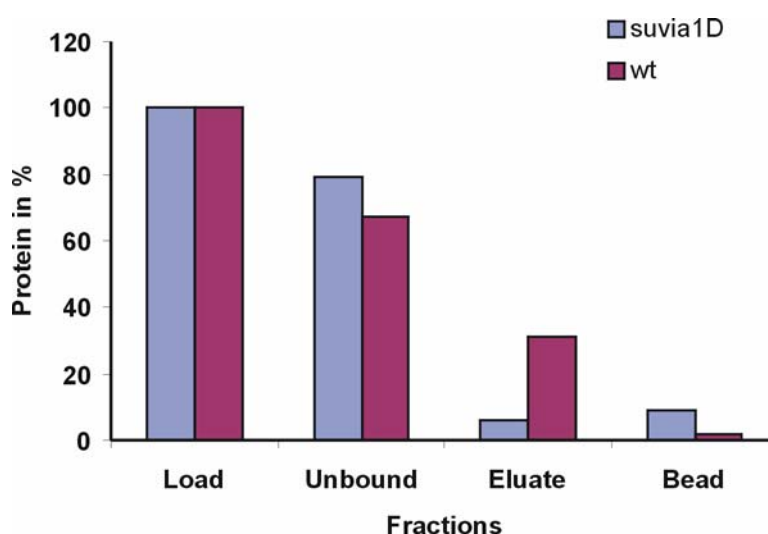


Fig 23: Signal intensities of Ape1p that was bound to the GST-Suvia1p beads. 79% of the protein remain unbound, 5.8% was eluted and 9% remained bound to the beads. (N=3)

In case of protein extracts prepared from wt cells, 30% of the applied Ape1p was bound to the bead, out of which 33% of the protein was seen in the elution fractions and ~2% of Ape1p remained bound to GST-Suvia1p beads (Fig 23). GST beads were used as control. No Ape1p should be eluted from GST beads as Suvia1p was not bound to the beads. When protein was loaded on to GST beads no Ape1p was observed in the eluate fractions (Fig 22C).

The data given here is the average of 3 different experiments. These experiments suggest that there exists a weak interaction between Suvia1p and Ape1p. There seems to be no specific binding to either pApep or mApe1p from wt or *suvia1Δ* cells. Suvia1p-Ape1p interaction was also tested by yeast-two-hybrid (Y2H) experiments.

The Y2H system identifies DNA sequences encoding for proteins that are physically associated with a given protein *in vivo*. The MATCHMAKER Two-Hybrid Systems were used for the construction and expression of the hybrid (fusion) proteins.

Full-length Suvia1p was cloned in GAL4 Activation Domain (AD) cloning vector pACT2 which has Leu as selection marker and full-length Ape1p was cloned in GAL4 DNA-Binding Domain (DNA-BD) cloning vector pAS2-1 which has Trp as selection marker. Both full-length proteins were expressed in the yeast tester strain AH109. AH109 is auxotrophic for leucine and tryptophan and also has four reporter genes (HIS3, ADE2, MEL1, and lacZ). Colonies were tested for adenine and α -galactosidase which is extracellularly secreted and gives a blue colour around every positive yeast colony.

No growth was observed in –Ade plates even after 5 days at 30 °C. Colonies were also tested for α -galactosidase, but no blue colour was observed.

3.4 Analysis of *via10* and *via14* genes

3.4.1 Microarray studies

Because cloning of *via10* and *via14* genes by complementation was not successful, a DNA array analysis was done. Mutations in promoter sequences could lead to reduced mRNA expression levels and also mutations in the ORF could lead to mRNAs with reduced stability.

Microarray studies were performed to identify genes, where mRNA transcription was down-regulated. Total RNA was isolated from the wt and both mutant strains (2.2.1.3). cDNA-labelling experiments were done in collaboration with Dr. Andrease Polten (Agilent technologies), who introduced new yeast arrays.

Array	Cy3	Cy5
1	WT	<i>via10</i>
2	<i>via10</i>	<i>via14</i>
3	<i>via14</i>	WT

Fig 24: Different sets of slides that were hybridized with labeled cDNAs from wt and *via* mutants. The cDNA were labeled with Cy3 and Cy5. The experiment was done in 4 sets to obtain the best result.

Microarray slides with 60mers corresponding to the 3'end of the 6253 annotated genes from *S.cerevisiae* were hybridized with labeled cDNAs from the isogenic wt and the 2 *via* mutants.

Combinations were used for hybridization: wt / *via10*, *via10* / *via14* and *via14* / wt and each of these were labeled with fluorescent Cy3 and Cy5 respectively. Four sets of each combination were made for the analysis (Fig 24).

Mathematical calculations and data analysis of microarray studies were done by Dr. Jobst Landgrebe (Microarray facility, Medical faculty).

Comparisons were done between *via10*/wt, *via14*/wt and *via10*/*via14* (Fig 25).

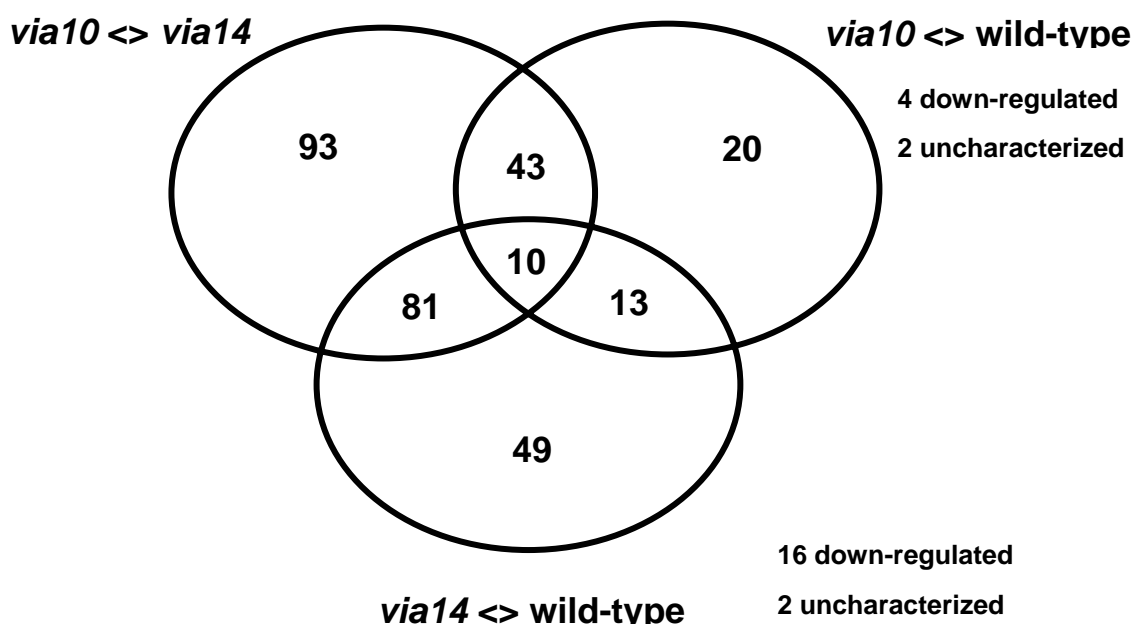


Fig 25: Comparison of the differentially expressed genes. *via* mutants were compared with the wt as well as with each other. The numbers represent the number of genes that were differentially expressed in the comparisons. No genes were differentially expressed in all three.

93, 20 and 49 are the total number of genes that were differentially expressed, either up-regulated or down-regulated when comparisons were made between *via10/via14*, *via10/wt* and *via14/wt* respectively. 43, 81 and 13 represent the numbers of genes that were differentially expressed and found common in the comparisons made between *via10/via14* and *via10/wt*, *via10/via14* and *via14/wt*, and *via10/wt* and *via14/wt*. 10 genes were found to be either up-regulated or down-regulated in all the three comparisons.

Interestingly when *via10/wt* were compared, out of 20, 4 genes were down-regulated, out of which 2 were uncharacterized. Similarly, when *via14/wt* were compared, out of 49, 16 genes down-regulated, out of which 2 were uncharacterized. No Atg or related genes were found to be differentially expressed in the *via* mutants. The four uncharacterized genes do not belong to any described category. Since our interest remained in analyzing the genes that were down-regulated, experiments were done to investigate the 4 candidate genes further.

3.4.2 Analysis of down-regulated genes in *via* mutants

The four candidate genes that were down-regulated are YHR054c and YJL171c from *via10*, YER187w and YGL117w from *via14*. These four candidates were amplified from the genomic DNA of the wt, *via10* and *via14* strains by PCR. The amplified PCR products were cloned into pCR 2.1 vector and the ORF was sequenced to check, if there were any mutations in the ORFs. The table below indicates the genes that were down-regulated and their size.

Genes down-regulated in <i>via</i> mutants	ORF	Size in base pairs
<i>via10</i>	YHR054c	1065
	YJL171c	1191
<i>via14</i>	YER187w	426
	YGL117w	798

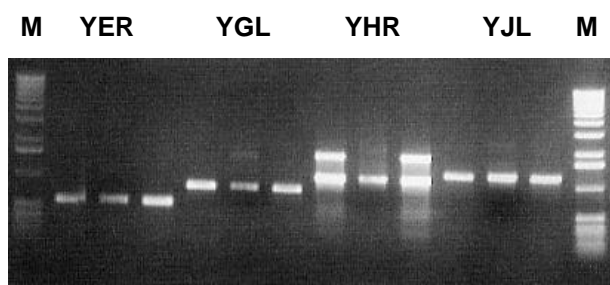


Fig 26: Agarose gel showing the amplified genes by PCR. The four genes that were down-regulated in *via* mutants were amplified from the genomic DNA of wt and *via* mutants. 10µl of each sample was loaded in the order of wt, *via10* and *via14*.

Mutations were found in the ORFs of the candidate genes amplified from *via* mutants. There was a single mutation at G42D in YHR054c amplified from *via10* and 3 mutations at G42D, K28R and N87D in *via14*.

No mutations were found in YJL171c ORF amplified from both the mutants. Single mutation was found at M1T in YER187w amplified from *via14*. In case of YGL117w, amplified from *via14* a D45G mutation was found.

Next microarray data were verified by northern blot experiments for the two genes down-regulated in *via10* (Fig 27) and in *via 14* (Fig 28). Total RNA was isolated from wt, *via10* and *via14* strains and hybridized with radioactively labeled DNA probes (2.2.1.3). Actin was used as a control to standardize the hybridization signals of the *via* mutant RNAs.

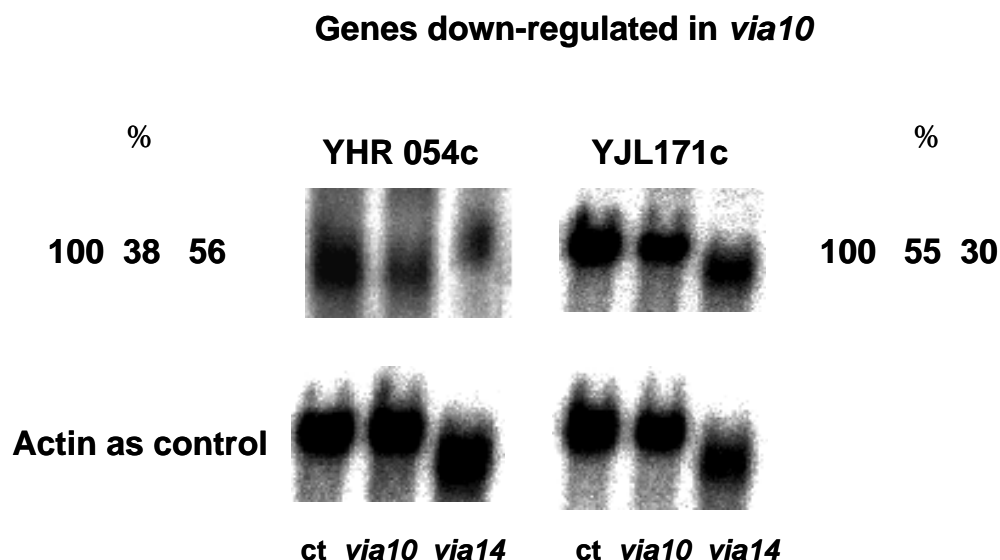


Fig 27: Northern blot showing reduced expression of down-regulated genes in *via10* at mRNA level. In *via10*, YHR045c and YJL171c genes are down-regulated by 38% and 55% respectively. In *via14*, YHR054c and YJL171c genes were down-regulated by 56% and 30% respectively. Actin was used as control to normalize the *via*-gene signals. Numbers indicate the percentage by which the genes were down-regulated. 10µg of RNA was loaded for each sample.

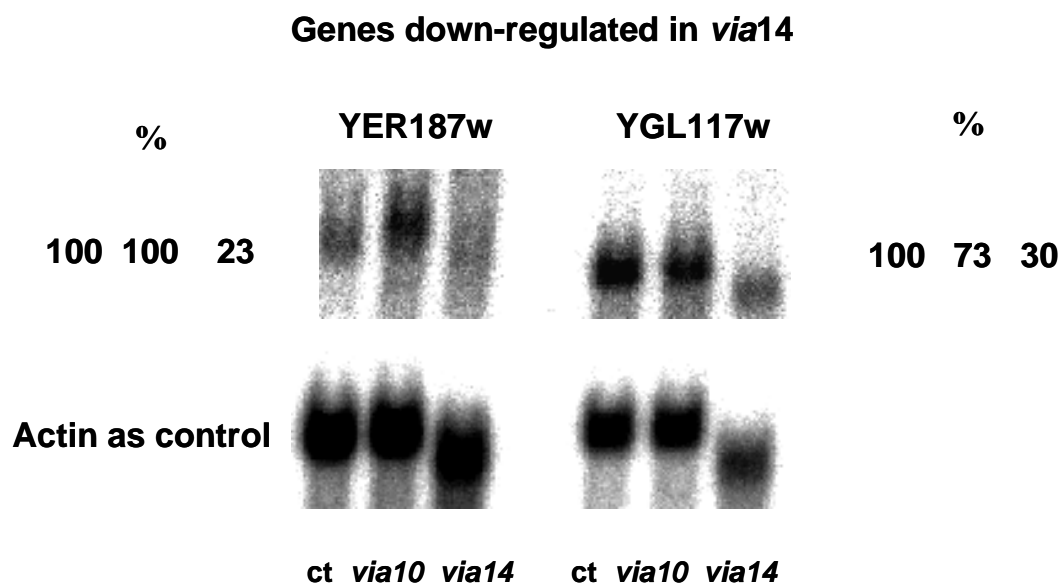


Fig 28: Northern blot showing reduced expression of down-regulated genes at mRNA level. A: In *via14*, YER187w and YGL117w genes are down-regulated by 23% and 30% respectively. In *via10*, YGL117w is down-regulated by 73%. Actin was used as control to normalize the *via*-gene signals. Numbers indicate the percentage by which the genes were down-regulated. 10µg of RNA was loaded for each sample.

The microarray data could be verified. However, YHR054c and YJL171c were found to be down-regulated in both mutants. The experiments were done twice and the results were similar.

3.4.3 Rescue of Ape1p activity in the *via* mutants

The four genes YHR054c, YJL171c YER187w and YGL117w, amplified from the wt were cloned into pRS vectors. These were then transformed into *via10* and *via14* mutants to check if the Ape1p enzymatic activity is restored.

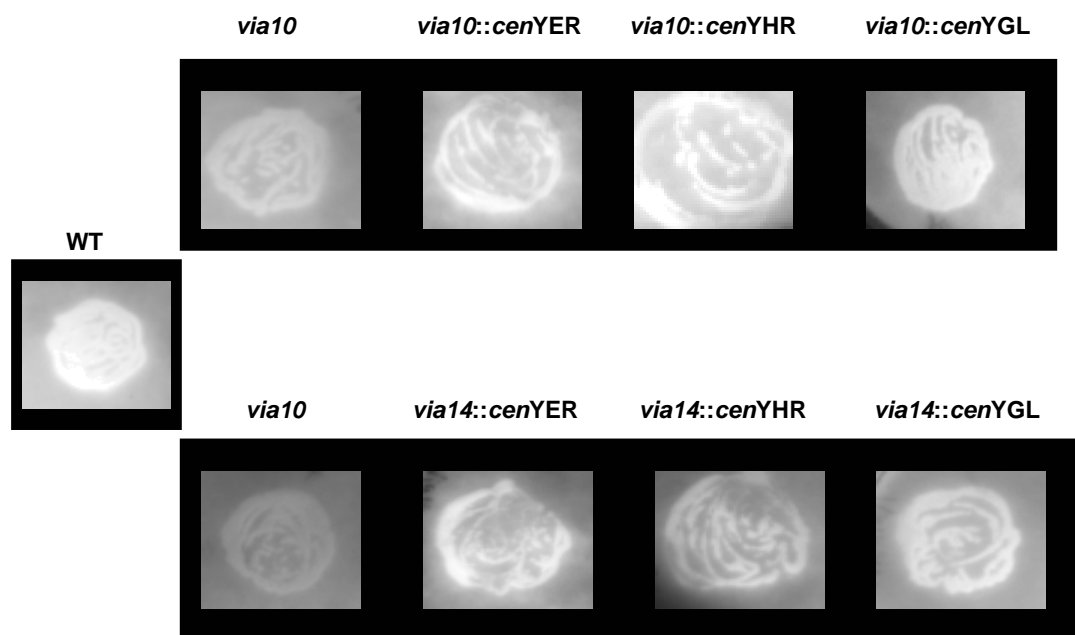


Fig 29: Ape1p overlay assay of Ape1p enzymatic activity. *via* mutants restore the ape1p activity when transformed with candidate gene.

Overlay assay of wt, *via10*, *via14* and *via* mutants transformed with the candidate genes was done. It was observed that Ape1p activity is rescued in *via* mutants upon the expression of the candidate genes (Fig 29).

YJL171C could not be cloned in pRS vectors yet, hence no activity was tested

Preliminary data on Ape1p expression levels and vacuolar transport indicate that the 3 genes tested so far, show different suppression/complementation phenotypes in *via10* and *via14* mutants. More quantitative experiments have to be done to evaluate these data.

4 Discussion

The *Saccharomyces cerevisiae* vacuolar aminopeptidase I oligomerizes into a homododecameric complex, which is transported from the cytoplasm to the vacuole by the Cvt pathway under nutrient-rich conditions and by autophagy during starvation. Ape1p is one of four leucine aminopeptidases found in *S. cerevisiae* and is the only one to be localized in the vacuole (Frey *et al.*, 1978; Matile *et al.*, 1971). Ape1p contains an amphipathic α -helix, which is necessary and sufficient to target the protein into the vacuole.

The 61 kDa precursor Ape1p homododecamerizes in the cytoplasm and upon transport to the vacuole, the 61 kDa pApe1p is converted into a 55 kDa intermediate form by proteinase A and further processed to a 50 kDa mature form by the action of proteinase B (Segui-Real *et al.*, 1995).

Various groups used genetic screens to study cytoplasm to vacuole transport pathways. Mutants were named *cvt* (Harding *et al.*, 1995, 1996), *aut* (Thumm *et al.*, 1994) and *apg* (Tsukada and Ohsumi, 1993), but recently a unified nomenclature was chosen and all are now classified as Atg genes. These studies revealed that pApe1p is transported to the vacuole directly from the cytoplasm to the vacuole via a vesicle mediated mechanism called the Cvt pathway or under starvation conditions by autophagy. pApe1p oligomerizes into a homododecamer in the cytoplasm and is enwrapped in a double-membrane vesicle which is then transported to and fuses with the vacuolar membrane like an autophagosome. pApe1p is detected in single-membrane vesicles, so called autophagic bodies within the vacuole of mutants that are defective in the degradation of these vesicles.

Cvt mutants were identified due to accumulation of pApe1p by western blot colony screening. Autophagy mutants were selected based on the accumulation of autophagic bodies observed by light microscopy (Tsukada and Ohsumi, 1993, Thumm *et al.*, 1994).

A new genetic screen was developed for Ape1p to analyze vacuolar import based on the enzymatic activity of the homododecameric complex of Ape1p (Andrei *et al.*, 2001). This screen led to the isolation of two complementation groups *via10* and

via14. In the *via* mutants, the cytoplasmic pApe1p precursor accumulates in cytoplasmic vesicles and the homooligomers are not in a native conformation as demonstrated by their sensitivity to proteolysis and their reduced leucine-aminopeptidase activity. Both mutants were defective in transport vesicle formation and oligomerization of Ape1p to an enzymatically active form indicating a functional interdependence of a coordinated oligomerization and transport vesicle formation. In the course of these studies it was discovered that pApe1p is already enzymatically active unlike other precursor forms of vacuolar proteases. Thus the pathway appears to couple formation of the active enzyme and compartmentalization in to a transport vesicle. This is a mechanism to protect the proteins from the proteolytic activity of cytoplasmic pApe1p.

In this study, we identified a previously uncharacterized 16 kDa protein as a single-copy gene suppressor of the *via* mutant phenotype and named it Suvia1p for suppressor of *via*.

Screening based on the enzymatic activity of Ape1p by overlay assay led to the isolation of a suppressor gene, YDR360w. YDR360w was listed as a putative ORF in the yeast genome database. YDR360w ORFs isolated from the wt and *via* mutants were identical. Northern blots confirmed that there was no difference observed in the mRNA expression level of wt and *via* mutant strains. The ORF in our yeast strain is longer than the ORF submitted in the yeast genome database, because of an additional 'C' at position 365 and hence the encoded protein is 144 amino acids long instead of 129 (Fig. 8). Suvia1p does not belong to any characterized protein family. Homologies were found to the helical domain at the C-terminus of the protein in bacteria, yeast and mammals: The yeast anthranilate synthase component II, the mammalian menin I and the AI-2-binding periplasmic protein LuxP. LuxP is part of a *Vibrio harveyi* extracellular signaling system, autoinducer-2, which binds boron and senses cell density leading to induction of luminescence. Its partner is LuxQ, a membrane embedded histidine protein kinase with a response regulator domain. Mature LuxP is 245 aa long and the crystal structure of LuxP from *Vibrio harveyi* shows indeed a α -helical domain in the region of homology (147-164 aa) to Suvia1p. Menin I (615 aa) or endocrine neoplasia type I protein is expressed in embryogenesis and in multiple tissues. Menin I is responsible for a heritable predisposition to

endocrine tumours in man and binds to a number of proteins e.g. replication protein A, JunD, nuclear factor- κ B etc. Syntaxin18 is a SNARE protein involved in transport vesicle tethering and fusion. Its helical domain forms a tetrameric complex with the helices of 3 other SNARE proteins (Fasshauer, *et al.*, 1998, Sutton *et al.*, 1998). Homologies were also found to *A. thaliana* proteins and interestingly all of these are unclassified, putative proteins. Helices are often protein-protein interaction domains and the sequence homologies to proteins which are part of protein complexes indicate that this helix domain in Suvia1p is also required for protein-protein interactions. This was supported by the fact that tagging Suvia1p with YFP rendered it inactive, whereas the smaller HA tag had no negative effect on the Suvia1p function.

In *via* mutants, pApe1p is degraded rapidly due to its non-native conformation. Upon Suvia1p expression in *via* mutants, the expression of Ape1p is almost doubled (Fig.11A). Suvia1p enhances the processing of pApe1p and thus vacuolar transport of Ape1p in *via* mutants (Fig.11B). Suvia1p deficiency renders Ape1p inactive, but it does not cause sensitivity to proteolysis or delayed vacuolar transport. Therefore, Suvia1p induces the native conformation of Ape1p in the *via* mutants, which makes it more stable against proteolysis. Stimulation of transport appears to be induced only by the formation of more native pApe1p as proposed from the *via* mutant phenotypes, because Suvia1p deficiency does not delay transport. Ape1p dodecamerization is not reduced in Suvia1p-deficient cells indicating that the conformational changes in Ape1p are subtle compared to those in the *via* mutants.

To investigate Suvia1p functions we tried to generate specific antisera. Several attempts were made, but all failed. Since no antibody against Suvia1p could be generated, Suvia1p was tagged to hemagglutinin at the C-terminus and this Suvia1p-HA was used for further investigation and characterization of Suvia1p. Suvia1p-HA was biologically active as it complemented Suvia1p deficiency (Fig14). Suvia1p-HA was tested with HA antibody by western blot.

Suvia1p has a molecular mass of 16 kDa as predicted from its ORF YDR360W. But on SDS-PAGE it shows a different migration pattern and migrates at about 48 kDa (Fig15). We assumed that this variation in the molecular mass of Suvia1p-HA is

possible, if Suvia1p-HA forms a trimeric complex or due to incomplete denaturation of the protein by SDS or by some post-translational modification.

Gel filtration experiments were done using a Superdex 75 column in the presence of 6 M urea. Under these conditions, Suvia1p-HA was observed in the eluate fractions corresponding to a molecular mass of ~14 kDa (Fig17). Suvia1p-HA migration behaviour on SDS-PAGE is therefore likely due to a trimeric complex of Suvia1p-HA and not due to a post-translational modification of the protein.

Subcellular fractionation studies revealed that Suvia1p is a cytosolic protein. Suvia1p-HA was only found in the supernatant 200,000 x g cytosolic fraction (Fig18). This subcellular localization was further confirmed by indirect immunofluorescence microscopy experiments. Suvia1p-HA was observed to be concentrated in small structures, but fluorescence was also observed throughout the cytoplasm (Fig19).

The earliest structure in Cvt vesicle formation known is the so called pre-autophagosomal structure or PAS. GST-tagged Atg proteins Atg1, Atg2, Atg5, Atg8 and Atg16 were shown to assemble in the pre-autophagosomal structure (PAS) in the vicinity of the vacuole (Suzuki *et al.*, 2001). Analysis of *apg* mutants revealed that the formation of both a phosphatidyl-ethanolamine-conjugated Atg8 and Atg12p-Atg5p conjugate is essential for the localization of Atg8 to the PAS (Suzuki *et al.*, 2001). Because Suvia1p appears to be involved in the early events of pApe1p vesicular transport, we wanted to test whether Suvia1p is associated with the PAS. Atg8 (Aut7) is used as a marker protein for the PAS and is required for Cvt vesicle formation (Suzuki *et al.*, 2001). Immunofluorescence microscopy for Suvia1p-HA and Atg8p-GFP localization revealed that Suvia1p and Aut7p were in close proximity. In some cases the two proteins even appeared to be colocalized (Fig 20C). However, coimmunoprecipitation experiments failed to demonstrate an interaction of the two proteins to the same complex. Therefore the resolution of the microscopic images is probably too low to resolve the two closely localized structures.

Purification of Suvia1p and analysis of the complex composition should be extended by gel filtration experiments followed by mass spectroscopy to untangle the questions, if it forms a complex with itself by oligomerization or forms a complex with other proteins.

Suvia1p-Ape1p interactions by GST-pull down experiments revealed that there exists a weak interaction between Suvia1p and Ape1p *in vitro*. Ape1p from *suvia1Δ* cells was not eluted from the beads as efficiently as Ape1p from wt cells. A large fraction of Ape1p from *suvia1Δ* cells was irreversibly bound to the GST-Sepharose beads (Fig 23). The inefficient elution of Ape1p from *suvia1Δ* cells indicates that the non-native Ape1p tends to form aggregates *in vitro* impairing its binding to Suvia1p as well as elution from the beads.

via mutants have a reduced transformation rate, which is further reduced by increasing plasmid size. Therefore a DNA library was constructed with smaller genomic DNA inserts than are normally used. This library enabled us to isolate *SUVIA1*, but unfortunately the *via* genes were not cloned. Therefore we tried to exploit the DNA array technology to identify the *via* genes. It could very well be that mutations in the ORF and mutations in the promoter regions of the *via* genes could lead to reduced stability or lower expression levels of the mRNAs respectively. Microarray data revealed that 162 genes were differentially expressed, either up-regulated or down-regulated, when the *via10* and *via14* mutants were compared with each other and with the wt (Fig 27). When *via10*/wt were compared, 2 out of 4 down-regulated genes were uncharacterized. Similarly, when *via14*/wt was compared, 16 genes were down-regulated, out of which 2 were uncharacterized. No Atg or related genes were found to be differentially expressed in the *via* mutants.

YHR054c and YJL171c were down-regulated in *via10*, YER187w and YGL117w were down-regulated in *via14*. These four genes were amplified from the genomic DNA of the wt, *via10* and *via14* strains by PCR. The amplified PCR products were cloned and sequenced to check, if there were any mutations in the ORFs. The expression levels of mRNA in these genes were further analyzed by northern blots. When these genes were transformed in *via* mutants, to check if the Ape1p activity was rescued in these mutants by overlay assay, it was observed that the Ape1p enzymatic activity in the *via* mutants were comparable to the wt (Fig 31). Ape1p expression levels were also tested by western blots and it was found that Ape1p expression is enhanced by the candidate genes in *via14*. However, in *via10* the expression level of Ape1p remained the same (Fig 32).

Further investigations have to be done on these 4 uncharacterized genes to learn the significance and role of the respective proteins. Expression and processing of Ape1p in the absence of these genes should provide us with more information about the impact of the uncharacterized proteins on Ape1p assembly and transport.

5 Conclusions

Unlike other vacuolar proteins, vacuolar aminopeptidase I is known to be transported to the vacuole by cytoplasmic double membrane vesicles by the Cvt pathway. The precursor form, pApe1p oligomerizes in the cytosol into a dodecamer and is enclosed in a double membrane to form a cvt vesicle, which is then 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 Ape1p is removed. The cytosolic precursor protein and the matured vacuolar protein form homododecameric complexes and only this complex has enzymatic activity.

In this project, my aim was to identify the *via* genes by complementation cloning. Using this approach, a single-copy suppressor of the *via* mutant phenotype has been isolated encoded by the putative ORF YDR360w. It encodes a 16 kDa protein, which does not belong to any described protein family. We named it Suvia1p for 'suppressor of *via*'.

Suvia1p is a cytosolic protein and found to be part of a 200 kDa cytoplasmic complex localized in close proximity to the pre-autophagosomal complex.

In both *via* mutants, pApe1p is more sensitive to degradation and accumulates in cytoplasmic Cvt vesicles. Ape1p protein levels and Ape1p enzymatic activity is increased in both *via* mutants due to Suvia1p expression from a single copy plasmid. This demonstrates reduced sensitivity to proteases indicating proper pApe1p folding and complex assembly. Thus Suvia1p supports the formation of native dodecamers of pApe1p. Suvia1p also enhances the processing of pApe1p and its vacuolar transport in the *via10* supporting the model that pApe1p dodecamerization into the enzymatically active form is linked to Cvt vesicle formation. However, Suvia1p does not stimulate vacuolar transport of Ape1p under conditions of autophagy. Suvia1p is not required for the dodecamerization of Ape1p. There exists a weak interaction between Ape1p and Suvia1p *in vitro*. Thus we interpret from our studies that Suvia1p is involved in the formation of native dodecamers of pApe1p.

In an attempt to identify the *via* genes by DNA microarray, 162 genes were found to be differentially expressed, out of which 2 were down-regulated in *via10* and *via14*

respectively. These are uncharacterized proteins and also does not belong to any classified family. Mutations were found in the ORFs of these genes isolated from *via10* and *via14* and also mRNA expression levels were significantly reduced. When the ORFs were expressed in the *via* mutants, Ape1p enzymatic activity was comparable to the wt. Ape1p expression levels was also improved in case of *via 14* but had no significant effect in *via10*. Further experiments have to be done to learn more about the unknown proteins and their effect on pApe1p folding and complex assembly.

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Lebenslauf:

Name:	Pasupuleti Naga Rekha
Date of Birth:	20.10.1977
Geburtsort:	Hyderabad, India
1993 – 1995	Pre-university education, Kendriya Vidyalaya Picket, Secunderabad, India
1995-1998	Bachelor of Science in Botany, Zoology and Chemistry, Osmania University College for Women, Hyderabad, India.
1998-2000	Master of Science in Biochemistry, University of Hyderabad, India.
June 2001-present	Joined the “Zentrum Biochemie und Molekulare Zell Biologie” (Biochemie) to pursue doctoral studies under the supervision of PD. Dr. Peter Schu to work on the research project “ Identification of a new factor essential for vacuolar aminopeptidase I activity ”