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Research Article

Screening for suppressors of temperature sensitivity in a yeast mutant defective in vacuolar protein degradation

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Abstract

Vps33p is a member of the Sec1/munc18-like protein (SM protein) family involved in vesicular protein transport to the yeast vacuole. It is part of a high molecular weight complex which is required for homotypic vacuole fusion, and participates in Golgi-to-endosome and endosome-to-vacuole transport steps. Deletions in the *vps33* gene result in severe vacuolar protein sorting and vacuolar morphology defects. We used a temperature sensitive (ts) *vps33* deletion strain in a high copy plasmid suppressor approach to identify genes possibly acting along the same vesicular trafficking pathway(s) as Vps33p. While only the original *VPS33* gene could restore the vacuolar enzyme sorting and vacuolar morphology defects, several suppressors of temperature sensitivity were found. Sequence analysis identified the ubiquitin-ligase Ufd4p as the only open reading frame (ORF) of two suppressor plasmids. Further suppressor candidates included the ubiquitin-processing protease Ubp10p and genes whose products are involved in more general stress responses. The result of this screening supports the emerging concept of a crosstalk between vesicular trafficking pathways and the ubiquitin/proteasome system of protein degradation.

Key words: VPS33, UFD4, lysosomal trafficking, ubiquitin, endosome.

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Introduction

The vacuole of baker's yeast *Saccharomyces cerevisiae* is the functional equivalent of the lysosome of higher eukaryotic cells. Apart from playing roles in the storage of metabolites and in regulation of ion homeostasis, its main function is the degradation of macromolecules (Klionsky *et al*., 1990). The vacuole harbors a variety of hydrolases, which, after biosynthesis in an inactive precursor form, enter the lumen of the endoplasmic reticulum (ER) and are transported in vesicles to the Golgi apparatus. In a late Golgi-compartment, they are separated from secretory proteins in an active sorting process and finally reach the vacuole trafficking through endosomal / prevacuolar compartments (Götte and Lazar, 1999). Proteolytic activation of the proenzymes takes place in the acidic environment of the vacuolar lumen (van den Hazel *et al*., 1996).

Mutations in over 50 genes lead to maturation and secretion defects of vacuolar enzymes and to changes in vacuolar morphology and function. Among these, the VPS (**v**acuolar **p**rotein **s**orting) mutants, which were isolated based on the secretion of vacuolar hydrolases, constitute the largest group (Robinson *et al*., 1988; Rothman *et al*., 1989). Mutations in the VPS genes partially overlap with mutants displaying a **v**acuolar **a**berrant **m**orphology (VAM) (Wada *et al*., 1992). Based on biochemical and morphological criteria, the *vps* mutants were grouped into 5 classes, named A-E (Raymond *et al*., 1992). The four class C mutants, which are identical to the class I *vam* mutants, display the most severe vacuolar protein-sorting and vacuolar morphology defects. A highly fragmented, barely visible vacuole, and maturation and/or sorting defects of the vacuolar enzymes carboxypeptidase Y (CPY), proteinase A (PrA), proteinase B (PrB), and alkaline phosphatase (ALP) are characteristic for these mutants. A complex consisting of the class C Vps proteins, as well as Vam6p and Vam4p, stimulates nucleotide exchange on the Ypt7p GTPase, and is required for homotypic vacuole fusion (Seals *et al*., 2000; Wurmser *et al*., 2000). It has been proposed that the class C Vps complex associates with the activated syntaxin-related Vam3-protein to mediate the assembly of trans-SNARE complexes during both vesicle docking/fusion and vacuole-to-vacuole fusion (Sato *et al*., 2000). Genetic and biochemical data suggest that the class C Vps complex is required for membrane docking and fusion at both the Golgi-to-endosome and endosome-to-vacuole transport steps (Peterson and Emr, 2001).

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Recently, the involvement of ubiquitin in targeting of proteins to the endocytic and vacuolar protein-sorting pathways has become the focus of intense research (Amerik *et al*., 2000; Reggiori and Pelham, 2001; Katzmann *et al*., 2001). The 76aa-residue protein ubiquitin is covalently conjugated to proteins marked for ATP-dependent degradation by the 26S proteasome, a multi-subunit protease complex (Voges *et al*., 1999). Ubiquitin-activating and ubiquitin-conjugating enzymes, as well as ubiquitin-ligases, are involved in this process. The regulated protein degradation by this ubiquitin-proteasome system plays a role in processes as diverse as stress responses, the cell cycle, and differentiation (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2000). Ubiquitin-ligases are found at multiple sites along the endocytic pathway in yeast (Wang *et al*., 2001; Shin *et al*., 2001), and ubiquitin has been shown to target certain transmembrane proteins to invaginating endosomal vesicles and multivesicular bodies (Reggiori and Pelham, 2001, 2002).

We used a multicopy plasmid suppressor screening, a proven tool in studying vesicular trafficking mutants (Dascher *et al*., 1991; Götte and Gallwitz, 1997), to identify new genes possibly involved in a similar vesicular trafficking step as the class C vps protein Vps33p (Banta *et al*., 1990; Wada *et al*., 1990). The Vps33p ATPase belongs to the group of Sec1/Munc18-related SM proteins and is part of the class C Vps complex involved in homotypic vacuole fusion (Seals *et al*., 2000; Wurmser *et al*., 2000; Sato *et al*., 2000). *vps33* mutants are characterized by maturation defects of the vacuolar proteins CPY, PrA and vgp72, by the lack of a visible vacuole, and by a growth defect on lysine-rich media (Wada *et al*., 1990), as well as by defects in methionine metabolism (Jaquemin-Faure *et al*., 1994). Apart from candidate genes known to be involved in general stress responses, we identified a gene of the ubiquitin pathway and a second candidate gene acting along the same pathway as suppressors of temperature sensitivity of *vps33*. The results of this screening provide further support to the emerging concept of a crosstalk of the ubiquitin and endocytic pathways.

Materials and Methods

Materials

Except stated otherwise, all reagents were from Sigma, Deisenhofen, Germany. Restriction endonucleases and other enzymes for DNA manipulation were from New England Biolabs, Schwalbach, Germany. Zymolyase 100-T was from Seikagaku, Kogyo, Tokyo, Japan. Tran^{35}S-label was from Amersham-Buchler, Meckenheim, Germany. The vector pBluescript II KS + was from Stratagene, Heidelberg, Germany.

Strains, growth of cells and construction of plasmids

The following yeast strains were used in this study: SEY6210: *Mat a suc2-Δ9 ura3-52 leu2-3,113 his3- Δ 200 trp1- Δ 901 lys2-801* (Robinson *et al*., 1988), PS42-1A *Mat a ura3 leu2 trp1 lys2 suc2- Δ 9 pep4::HIS3* (Boehm *et al.*, 1994), YMG1: PS42-1A *vps33::URA3* (this study), YMG2: SEY6210 *vps33::URA3* (this study). *E. coli* and DNA manipulations were performed according to standard procedures (Sambrook *et al*., 1989). Yeast manipulations were performed according to standard procedures (Sherman *et al*., 1986). Gene disruption by homologous recombination was performed as described previously (Rothstein, 1983). Yeast strains were grown in 1% yeast extract (Gibco, Eggenstein, Germany), 2% peptone 140 (Gibco, Eggenstein), 2% glucose (YEPD), or in synthetic glucose medium (SD) supplemented as necessary (Rose *et al*., 1990). Solid media were prepared by adding 2% agar (Gibco, Eggenstein). Temperature sensitivity of *vps33*-mutants was assayed as described previously (Götte and Gallwitz, 1997). In brief, 5 µL of a logarithmically growing culture of YMG1-transformants were applied to SD-LEU agar plates in serial 1:10 dilutions and incubated at the permissive (25 °C) and nonpermissive (37 °C) temperatures, respectively, for 3 days. Lithium acetate transformation of yeast cells was performed as described by Ito *et al.* (1983).

Radiolabelling and immunoprecipitation

Pulse-chase experiments were performed as described previously by Götte and Stadtbäumer (2002), using the anti CPY-antiserum described by Benli *et al.* (1996). In brief, single clones of YMG2 transformands were cultured until they reached the logarithmic growth phase in liquid medium. Cells were converted to spheroblasts using Zymolyase 100-T and metabolically labeled for 15 min with Tran^{35}S-label at 30 °C. CPY was immunoprecipitated using a specific antiserum either from the medium (external fraction: e) or from the spheroplast lysate (intracellular fraction: i) immediately after labelling (0 min chase) or after 30 min of chase with nonradioactive medium (30 min). After purification of protein A sepharose, the immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as described previously (Laemmli, 1970; Burnette, 1981). For steady-state Western blotting, yeast extracts were prepared by alkaline lysis followed by TCA precipitation as described (Benli *et al*., 1996). Antisera were provided by M. Benli (CPY, ALP, Benli *et al*., 1996) and Y. Anraku (PrA). Secondary horseradish-peroxidase-labeled anti-mouse or anti-rabbit IgG antibodies were from Amersham Buchler, Braunschweig, Germany. The ECL-system (Amersham Buchler, Braunschweig) was used for signal detection after

Western blotting, followed by exposure on X-ray-film (Kodak, Rochester, NY). Antibodies were used at the following dilutions: anti-ALP: 1:2,000; anti-PrA: undiluted mAb, secondary antibodies: 1:10,000.

Vital staining

Staining of yeast cells with the fluorescent dye CDCFDA was done exactly as described by Roberts *et al.* (1991). Cells were observed with a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany) and photographed using Kodak T-Max400 film at t = 15-30 s for fluorescence and t = 0.75-1.5 s for Nomarski pictures.

Results and discussion

Construction of a temperature-sensitive vps33 mutant strain

A probe for *VPS33* comprising codons 424-640 was amplified from genomic yeast DNA with specific PCR primers and used for colony hybridization after labeling with ^{32}P. Screening of 19,000 colonies of a yeast genomic library (Dascher *et al*., 1991) in *E.coli* yielded two clones which hybridized under stringent conditions. Southern-blotting and DNA-sequencing confirmed that one clone contained full-length *VPS33*. The *VPS33* gene was subcloned into the SmaI site of pBluescriptIIKS+ as a blunt-ended ApaLI/AvrII-fragment (Figure 1a). The flanking polylinker sites BamHI and SalI were utilized to subclone *VPS33* into the shuttle vectors pRS315 (CEN, LEU) and pRS325 (2µ, LEU; Sikorski and Hieter, 1989). In order to obtain *vps33* deletion strains, the yeast strains SEY6210 and PS42-1A were transformed with a linearized EcoRI/BamHI-Fragment of the vector pMG19 (Figure 1b) and selected on SD-Ura agar plates. *VPS33* codons 78-367 were replaced in vector pMG19 by a 1.1 kB HindIII-Fragment of the yeast *URA3* gene. The correct integration of the construct was verified by a PCR reaction using genomic DNA of the respective strains as template (Figure 1c).

While both YMG1 (PS42-1A background) and YMG2 (SEY6210 background) mutant strains exhibited the highly fragmented vacuolar morphology of *vps33* deletion strains (Banta *et al*., 1990; Wada *et al*., 1990) (see also Figure 2c), only YMG1 (*vps33/pep4*) exhibited a pronounced temperature sensitivity at 37 °C (see Figure 2a). Therefore, this strain was chosen for a search for temperature sensitivity suppressors. *pep4* strains have reduced vacuolar hydrolase activity due to the key role of proteinase A, which activates several vacuolar enzymes by proteolytic cleavage of their precursor forms (van den Hazel *et al*., 1996; Westphal *et al*., 1996). For the suppressor screening, a yeast genomic library in vector Yep13 (Dascher *et al*., 1991) was transformed into YMG1, followed by growth at 25 °C for 24 h and a shift to 37 °C for 48 h. A control plate incubated at 25 °C allowed to calculate the transformation

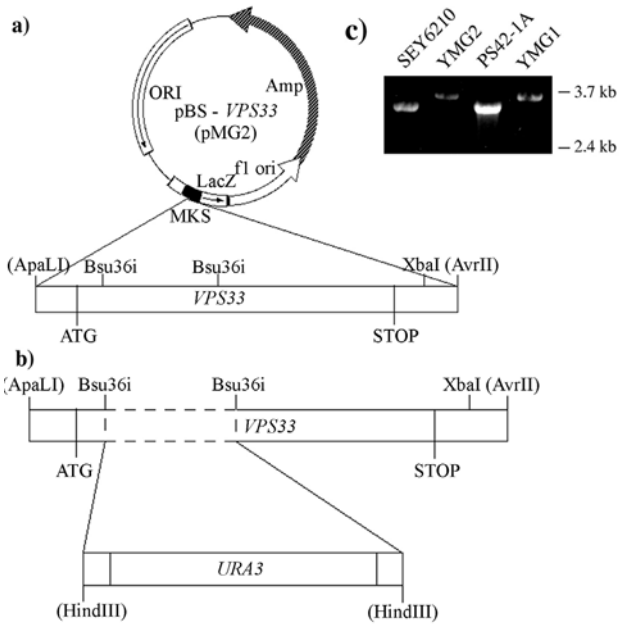

Figure 1 - Cloning of the *VPS33* gene and deletion of *VPS33* in the wild-type yeast strains SEY6210 and PS-42-1A. a) schematic drawing of the cloning strategy for *VPS33* (see text for details). MKS = multicloning site; ATG = translation initiation codon; STOP = stopcodon; Amp = β-lactamase gene; ori = origin of replication. b) schematic drawing of the pMG19 insert used for gene disruption of *VPS33*. pMG19 was created by cloning the blunt-ended HindIII fragment of the yeast *URA3* gene into the blunt-ended Bsu36i sites of vector pMG2 (see Figure 1a). c) verification of *VPS33* gene disruption by PCR. Genomic DNA was prepared from yeast strains SEY6210, PS42-1A, YMG1, and YMG2, and used as a template for a PCR reaction with *VPS33*-specific primers. The locations of the primers were 683 bp upstream of the START-codon and 520 bp downstream to the STOP-codon, resulting in the amplification of a 3.3 kB fragment in wild-type cells and a 3.6 kB fragment in *vps33* deletion mutants.

efficiency to 8,400 colonies, corresponding to 3.3 x 10^7 screened basepairs of the yeast genome. 33 transformants were capable of growing at 37 °C on the original screening plates. Seventeen of these colonies were able to grow well in liquid culture at the nonpermissive temperature. The plasmids (pS1 to pS17) were isolated from these cultures, retransformed into and amplified in *E.coli* and used for a retransformation into *vps33* mutant strains YMG1 (*vps33/pep4*) and YMG2 (*vps33/PEP4*). The sequence of the 5'- and 3' ends of the plasmid inserts was determined by DNA sequencing. In addition to the plasmids identified in the screening, 2µ plasmids containing the genes encoding the small transport GTPases Ypt6p and Ypt7p, as well as the yeast syntaxins Pep12p and Vam3p, were tested for suppressor activity. Like Vps33p, these proteins are involved in vesicular trafficking to the vacuole (for discussion, see Götte *et al*., 2000; Götte and Stadtbäumer, 2002). While strain YMG1 was used to confirm the suppression of temperature sensitivity, strain YMG2 was used to test for complementation of the vesicular trafficking and vacuolar morphology defects of *vps33* deletion mutants. The suppressor plasmids could be divided into three categories

(I-III) (Figure 2b), which differed in the degree of suppression of *vps33* mutant phenotypes.

Suppression of the growth defect at 37 °C

The suppression of the growth defect was determined in a drop test using different dilutions of yeast cultures of a defined OD_{600} (see Figure 2a and Götte and Gallwitz, 1997). After retransformation, the *PEP12* and *YPT6* genes, as well as 4 putative suppressor plasmids of the first round of screening, were not capable of supporting growth of *vps33* mutants at the nonpermissive temperature (category I inserts). In contrast, plasmids containing the *VPS33* gene allowed for growth at 37 °C, as expected. Restriction analysis and DNA-sequencing revealed the presence of *VPS33* in 5 suppressor plasmids (category III inserts, see Table 1). The genes encoded by the plasmid inserts of category II had an intermediate effect: they allowed for growth of *vps33* cells at the nonpermissive temperature, however, the cells did not grow as well as YMG1 cells transformed with *VPS33* (Figure 2a). Since the temperature sensitivity of *vps*-mutants can be seen as a secondary effect of the respective mutations, we tested for suppression of more specific phenotypes of the *vps33* deletion, using the YMG2 strain.

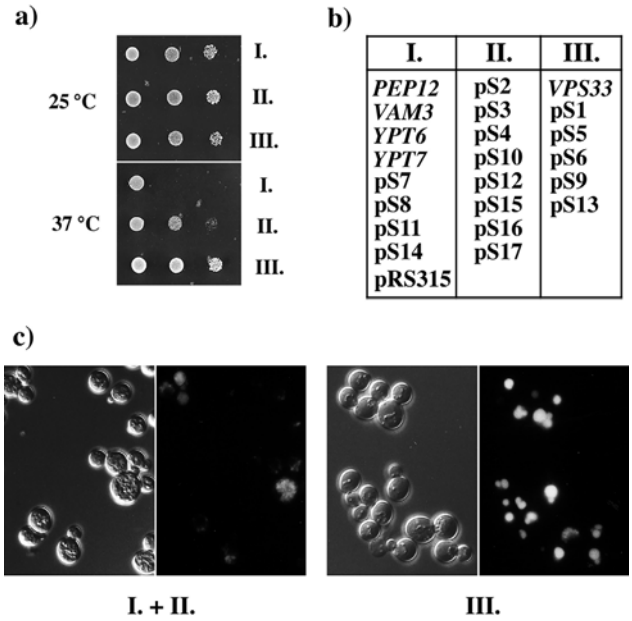

Figure 2 - Suppression of temperature sensitivity for growth and vacuole fragmentation in *vps33* mutants. *VPS33* mutant strains YMG1 and YMG2 were transformed with the plasmids indicated in 2b). Three classes of suppressors (I-III), which displayed similar degrees of suppression, were identified. a) Suppression of the ts-growth defect of *vps33* deletion strain YMG1. Agar plates containing serial dilutions of logarithmically growing YMG1-transformants were incubated at the permissive (25 °C) and nonpermissive (37 °C) temperatures, respectively, for 3 days. Plasmids of category I (cf. b) did not support growth at 37 °C after retransformation. Plasmids of category II allowed for growth, whereas plasmids of category III supported growth similar to permissive conditions. c) Suppression of vacuole fragmentation of the *vps33* mutant strain YMG2. The vacuoles of YMG2 were stained with the fluorescent dye CDCFDA and viewed with Nomarski-DIC optics (left panels) or fluorescence optics (right panels), respectively. Class I and class II plasmids could not suppress vacuole fragmentation, whereas class III plasmids restored wild-type vacuole morphology. Suppressor categories I-III are represented by control vectors pRS315 (cat. I, part a), pS3 (cat. II), and pS9 (cat. III, *VPS33*), respectively.

Test for suppression of the aberrant vacuolar morphology of vps33 mutants

The most prominent morphological aberration of *vps33* mutants is the absence of a typical vacuole (Banta *et al*., 1990; Wada *et al*., 1990). Even after vacuole staining with fluorescent dyes, only a few fluorescent spots can be observed in *vps33* cells (Kitamoto *et al*., 1988). Vacuole staining of YMG2-transformants with the fluorescent dye CDCFDA revealed that only plasmids containing the *VPS33* gene (category III) were capable of restoring the vacuole morphology to a wild-type appearance (Figure 2c). The other ts-suppressor- plasmids, as well as 2μ plasmids containing the *VAM3* and *YPT7* genes, were unable to restore wild-type morphology. In Nomarski differential interference contrast (DIC) pictures, the vacuole showed a highly fragmented appearance and only little staining with CDCFDA in fluorescence microscopy (Figure 2c).

Test for suppression of vacuolar enzyme maturation and sorting defects in vps33 mutants

VPS33 deletion mutants display severe sorting defects of the vacuolar enzymes carboxypeptidase Y (CPY) and proteinase A (PrA), as well as maturation defects of these enzymes and of the enzyme alkaline phosphatase (ALP) (Banta *et al*., 1990; Wada *et al*., 1990). We tested for suppression of these defects by the ts-suppressor plasmids using CPY-, ALP- and PrA-antisera in Western blots of

Table 1 - Sequence analysis of category II suppressors for temperature sensitivity of *vps33* strain YMG1 (cf Mewes *et al.* 1997, Cherry *et al*. 1998).

Suppressor	Chromosome	Location (bp)	Open reading frames
pS2	IV	613036-618580	YDR084c, AFR1, SSS1, RRP1
pS3, pS17	XI	420627-424892	UFD4 (C-terminus)
pS4	X	470010-471904	BNA1, YJR024c
pS10	XIV	289050-293350	UBP10, YNL184c, MRPL19
pS12, pS15	V	508369-512841	CHD1 (C-terminus), PAB1
S16	XV	178730-184167	IRA2 (C-terminus), REX4, YOL079w, YOL078w (N-terminus)

whole cell extracts of YMG2 transformants. In a part of the transformants, a detailed analysis of the maturation kinetics of CPY was performed in pulse-chase immunoprecipitation experiments (Figure 3). Only ts-suppressor plasmids containing the *VPS33* gene were capable of suppressing the maturation- and sorting defects of the respective enzymes. Therefore, the ts-suppressor plasmids were not suppressors of the vesicular protein transport defects of the *vps33* mutants, but exerted the suppression of temperature sensitivity through an alternative mechanism (see discussion below).

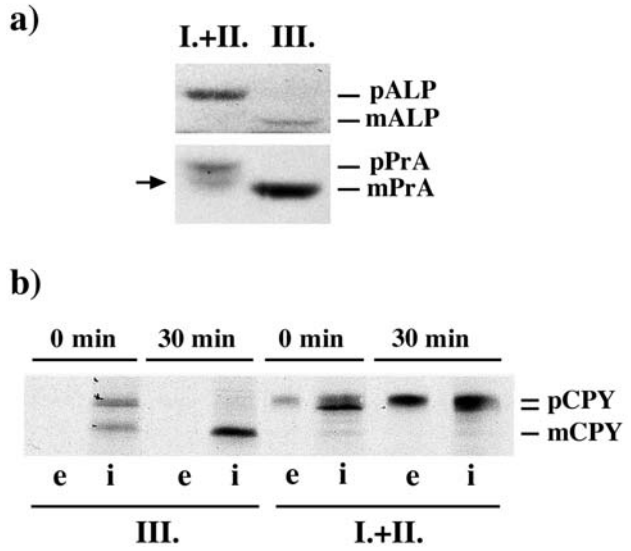

Figure 3 - Influence of suppressor plasmids on the processing of vacuolar enzymes in the *vps33* mutant strain YMG2. a) Processing of vacuolar enzymes in YMG2-transformants under steady-state conditions. Whole cell extracts of YMG2 cells transformed with the plasmids indicated in Figure 2b were subjected to SDS-PAGE and Western blotting. The blot membranes were probed with anti-ALP (upper panel) or anti-PrA antibodies, followed by incubation with POD-coupled secondary antibodies and enhanced chemoluminescence detection. Only category III plasmids allowed for a maturation of the vacuolar enzymes. m = mature form of enzymes; p = precursor form of enzymes; arrow = extracellular, autocatalytically processed form of PrA (van den Hazel *et al*., 1996). b) Sorting and kinetics of carboxypeptidase Y (CPY) maturation (cf. Benli *et al*., 1996). The vps33 mutant strain YMG2 was transformed with the plasmids indicated in Figure 2b, and spheroplasts of single clones were metabolically labeled with Tran^{35}S-label. CPY was immunoprecipitated using a specific antiserum either from the medium (external fraction: e) or from the spheroplast lysate (intracellular fraction: i) immediately after labeling (0 min chase) or after 30 min of chase with nonradioactive medium (30 min). Mature and precursor forms of CPY were detected by autoradiography following SDS-PAGE. Suppressor plasmid pS9, containing the *VPS33* gene is shown representing category III inserts. Suppressor plasmid pS2 containing ORFs YDR083w-YDR087c is shown representing category I and II inserts. Only category III inserts were capable of suppressing the enzyme maturation/sorting defects of the *vps33* mutant strain YMG2, resulting in the appearance of the mature form of CPY after a 30 min chase, and the absence of CPY precursor forms in the extracellular fraction. m = mature form of CPY; p = precursor forms of CPY. (upper band: Golgi-modified form; lower band: ER-modified form); i = intracellular CPY; e = secreted CPY.

Analysis of suppressor plasmids

The suppressor plasmids of categories I-III were subjected to restriction enzyme analysis and partial DNA sequencing of the Yep13-inserts. Plasmids pS7, pS8, pS11, and pS14 (category I) were not capable of ts-suppression after retransformation into YMG1 and thus not analyzed further. The sequence information of the remaining plasmid inserts was compared to the genome of *Saccharomyces cerevisiae* using the MIPS and SGD databases (Mewes *et al*., 1997; Cherry *et al*., 1998). Plasmids pS1, pS5, pS6, pS9, and pS13 (category III) contained the original *VPS33* gene. The sequence information on the ts-suppressor plasmids of category II is given in Table 1.

Although the *vps33* deletion was created in a *pep4* background in the YMG1 strain, the *PEP4* gene was not found as a suppressor in this screening, indicating either its absence in the plasmid library or a negative effect of *PEP4* overexpression on growth at an elevated temperature. Sequence analysis of the category II inserts did not reveal any candidate genes belonging to protein families well-known for involvement in vesicular trafficking, like the Ypt/Rab or SNARE proteins (Götte and Fischer von Mollard, 1998; Götte *et al*., 2000). This is in accordance with the lack of complementing activity regarding the vesicular trafficking phenotype of the *vps33* mutant. Two pairs of suppressor plasmids contained the same inserts and were isolated twice (see Table 1).

High expression of the ubiquitin ligase Ufd4p suppresses temperature sensitivity of the YMG1 strain

The insert of plasmids **pS3** and **pS17** contained a fragment of chromosome XI ranging from bp 420627 to 424892. This chromosomal region harbors the major part of the *UFD4* gene (YKL010c), encoding a ubiquitin-protein ligase (Johnson *et al*., 1995; Xie and Varshavsky, 2000) as the only open reading frame (ORF) on the plasmid. The suppressor insert encodes the Ufd4 protein starting at amino acid 214. The resulting protein contains both HECT domains, which are responsible for the ubiquitin ligase activity of Ufd4p (Johnson *et al*., 1995). The respective ATG codon is in frame with the ORF, and the 5' end contains a TATA sequence. The Ufd4 protein is physically associated with the 26S proteasome (Xie and Varshavsky, 2000), and was recently found in association with the ubiquitin-conjugating enzyme Ubc4p and the medium subunit of the clathrin-associated complex, Apm1p, in a mass spectrometry study of yeast protein complexes (Ho *et al*., 2002). Proteolysis regulation by the ubiquitin pathway is part of a cellular stress response (Hershko and Ciechanover, 1998). However, recent findings by several laboratories have established a link between the ubiquitin/proteasome pathway and vesicular trafficking along the endocytic pathway:

Ubiquitin can act as an endocytosis signal in yeast (Rotin *et al*., 2000; Dupre and Haguenauer-Tsapis, 2001;

Polo *et al*., 2002). Proteasome inhibitor studies in transfected mammalian cells had already suggested that the ubiquitin-proteasome pathway is involved in an endosomal sorting step of selected membrane proteins to lysosomes (van Kerkhof *et al*., 2001). Yeast ubiquitin ligases localize to multiple sites along the endocytic pathway (Wang *et al*., 2001; Shin *et al*., 2001), and ubiquitin acts as a sorting signal for a subclass of transmembrane proteins into invaginating endosomal vesicles and multivesicular bodies (Reggiori and Pelham, 2001, 2002): Carboxypeptidase S and other membrane proteins containing polar residues exposed to the lipid bilayer are sorted to multivesicular bodies and the yeast vacuole depending on the ubiquitin ligase activity of the Golgi-resident Tul1 protein. The Vps33 protein is known to be involved in both vesicular trafficking steps. Ufd4p could be a further member of the growing group of ubiquitin ligases involved in marking proteins for targeting to vacuolar/endosomal compartments and in quality control along vesicular trafficking pathways. Its identification in a complex with Apm1p (Ho *et al*., 2002) might indicate that, like Apm1p, Ufd4p could be involved in protein sorting at a late Golgi compartment (Stepp *et al*., 1995) (Figure 4).

Sequence analysis of candidate ORFs encoded by suppressor plasmids

Sequence analysis of the remaining suppressor plasmids revealed that, in contrast to **pS3** and **pS17**, these plasmids contained more than one ORF.

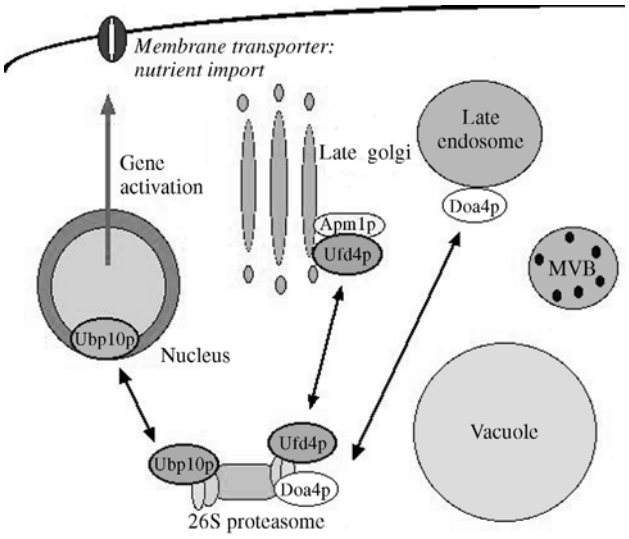

Figure 4 - Possible roles of the ubiquitin pathway enzymes Ufd4p and Ubp10p in vacuolar/endosomal trafficking and protein degradation (see text). Both Ufd4p and Ubp10p promote protein degradation via the proteasome. The ubiquitin ligase Ufd4p might act along the endocytic/Vps pathway, possibly by association with Apm1p. Like Doa4p, the deubiquitinating enzyme Ubp10p could reversibly associate with a compartment along the endocytic route. Alternatively, nuclear Ubp10p/Dot4p could activate genes involved in nutrient utilization. This process, as well as facilitated vacuolar or proteasomal protein degradation, would alleviate the stress conditions of the *vps33* mutant strain YMG1 at the nonpermissive temperature. MVB = multivesicular body.

The insert of suppressor plasmid **pS10** comprises bp 289050-293350 of chromosome XIV. Three complete ORFs are found in this chromosomal region: YNL084c is a hypothetical ORF of unknown function, which does not contain any well-characterized functional domains. YNL084c null mutants are viable (Winzeler *et al*., 1999). The neighboring ORF YNL185c encodes the Mrpl19 protein, a mitochondrial ribosomal protein of the large subunit (Graack and Wittmann-Liebold, 1998). The most likely candidate for the suppressor activity of pS10 is encoded by *UBP10/DOT4* (Kahana and Gottschling, 1999; Kahana, 2001), the gene for a ubiquitin-processing protease. Ubp proteins allow for a recycling of ubiquitin, thus promoting protein degradation via the ubiquitin/proteasome pathway (Voges *et al*., 1999). Kahana (2001) has demonstrated that Dot4p regulates nutrient uptake, suggesting that Dot4p overexpression might lead to an upregulation of genes involved in nutrient transport processes. Most interestingly, the *vps33* mutation can act as an extragenic suppressor of the *doa4-1* mutation (Amerik *et al*., 2000). *DOA4* encodes another deubiquitinating enzyme, which reversibly localizes with the late endosome. It is required for rapid degradation of ubiquitin-proteasome pathway substrates. Apart from *vps33*, several class E *VPS* genes were identified in the screen for extragenic suppressors of *doa4-1* (Amerik *et al.* 2000). Class E Vps proteins are also required for ubiquitin-dependent sorting into the multivesicular body (Katzman *et al*., 2001). As a deubiquitinating enzyme, Ubp10p/Dot4p would fall into the same functional category as Doa4p and could thus play a comparable role, maybe at a different trafficking step (Figure 4).

The insert of suppressor plasmids **pS12** and **pS15** contained bp 508369-512841 of yeast chromosome V. It comprises the 3' end of the *CHD1* gene (YER164w) encoding a transcriptional regulator and the complete ORF of the poly-A-binding protein *PAB1* (YER165w). *PAB1* physically interacts with the hsp70 homologue Ssa, and is strongly upregulated under heat-shock conditions (Gasch *et al*., 2000; Horton *et al*., 2001). Pab1p binds to the translation intitiation complex and influences mRNA stability (Vilela *et al*., 2000; Morrissey *et al*., 1999; Mangus *et al*., 1998). Most interestingly, *PAB1* has recently been identified as a multicopy suppressor for a temperature-sensitive *tif5-1A* mutant yeast strain at an elevated temperature (Valentini *et al*., 2002): In this mutant of the eukaryotic translation initiation factor eIF5A, defects in degradation of short-lived mRNAs are noted, and the authors suggest that iIF5A may also be involved in ribosomal synthesis and in the WSC/PKC1 signaling pathway for cell wall integrity.

The suppressor plasmid **pS2** contained 4 ORFs spanning bp 613036-618580 of chromosome IV: YDR084c is an ORF of unknown function lacking well-known structural motifs, and the deletion of YDR084c does not influence viability (Winzeler *et al*., 1999). The neighboring *AFR1* (YDR085c) gene product regulates alpha-factor re-

ceptor signaling and induction of morphogenesis during conjugation (Konopka, 1993; Davis *et al.*, 1998). *AFR1* mRNA is highly induced upon a temperature shift to 37 °C under several varying conditions (Gasch *et al*., 2000). Interestingly, Gga1p, a gene product involved in trafficking from the Golgi apparatus to the vacuole (Hirst *et al*., 2000), showed a very similar stress response pattern in the study by Gasch *et al*. (2000). However, the significance of this finding is not clear. The high induction of *AFR1* under heat shock conditions makes it a candidate for the suppressor activity of the pS2 insert. In addition, the *SSS1* (YDR086c) gene was found on the pS2 insert. The suppressor of *sec61*, Sss1p, is involved in the transfer of secretory precursors through the ER membrane (Esnault *et al*., 1993). Overexpression of *SSS1* restores translocation in *sec61* mutants, while depletion of Sss1p results in accumulation of secretory and membrane proteins devoid of post-translational modification. One could speculate that a more efficient transport of proteins into the ER might compensate for some of the stress conditions exerted under heat shock conditions in *vps33* mutants. Thus, both *AFR1* and *SSS1* are candidates for the suppressor activity of pS2. The *RRP1* gene (YDR087c) is the final ORF found on the pS2 insert. Its gene product is involved in the processing of rRNA precursors into mature rRNAs (Fabian and Hopper, 1987), and is not a likely suppressor candidate.

pS4 comprises bp 470010-471904 of chromosome X, including the major part of ORF YJR024c, truncated at the 3' end, and *BNA1/HAD1* (YJR025c). While the function of YJR024c is not yet clear, *BNA1* has been shown to encode 3-hydroxyanthranilic acid dioxygenase (Kucharzyk *et al*., 1998). *BNA1* is upregulated under heat stress conditions (Gasch *et al*., 2000), and its deletion results in upregulation of a large number of genes involved in carbon metabolite utilization and transport, the cell cycle, and amino acid metabolism (Bernstein *et al*., 2000). Bna1p is part of a feedback loop modulating the histone deacytylase Rpd3p, and seems to play a role in telomeric silencing (Sandmeier *et al*., 2002). Thus, changes in Bna1p levels will influence the expression of a large number of genes, which defies any easy interpretation of our findings.

Suppressor plasmid **pS16** contains an insert ranging from bp 178730 to bp 184167 of chromosome XV. This insert contains the 3' end of the gene encoding the RAS-GTPase activating protein Ira2p (Tanaka *et al*., 1990). The complete ORF YOL080c, encoding *REX4*, a member of the RNA 3'-5' exonuclease family (Moser *et al*., 1997), is also present on the suppressor insert. YOL079w, a hypothetical ORF encoded on the Crick strand, overlaps with YOL080c on the Watson strand of chromosome XV. Only a C-terminally truncated fragment of the ORF YOL078w is present on the suppressor plasmid pS16. Deletion of this ORF of unknown function leads to lethality (Winzeler *et al*., 1999). YOL078w mRNA is significantly induced under restricted temperature shift conditions (temperature shift from 29 °C to 33 °C for 30 min; Gasch *et al*., 2000). The translated amino acid sequence displays weak homology to the SAP kinase interacting protein 1 of *Schizosaccharomyces pombe* (Wilkinson *et al*., 1999), however, the significance of this finding is not yet clear. At present, it is difficult to determine which mechanism might underlie the ts-suppressor activity of pS16 on the *vps33* mutant strain YMG1.

Concluding remarks

In summary, two groups of suppressor candidates arise: One group includes candidate genes which are part of a more general heat shock/stress response (*PAB1, AFR1, SSS1, BNA1*). The respective gene products are known to be upregulated under heat shock conditions, or to induce a secondary upregulation of a whole group of other gene products. Further experiments are needed to identify the ORFs which harbor the suppressor activity. At present knowledge, none of the gene products identified appears to be involved in vesicular trafficking to the yeast vacuole.

The second group of ts-suppressors consists of the ubiquitin ligase Ufd4p and a candidate gene, *UBP10*, which also acts along the ubiquitin/proteasome pathway. The isolation of genes encoding ubiquitin-pathway enzymes supports recent observations indicating a functional link between the ubiquitin system and vacuolar protein sorting/endocytosis. Under the restrictive conditions exerted in the *vps33* suppressor screening, high expression of Ufd4p or Ubp10p could lead to a more efficient utilization of alternative trafficking routes to degradative compartments. In addition, an increase in protein degradation in the proteasome can be envisaged. Both effects would compensate for impaired vacuolar protein degradation in the *vps33* mutant.

Our results suggest that Ufd4p and possibly Ubp10p are two new candidates for the growing family of ubiquitin-processing enzymes involved in trafficking along the endocytic/Vps pathway.

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