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YET1, YET2 and YET3 of Saccharomyces cerevisiae Encode BAP31 Homologs with Partially Overlapping Functions

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Abstract: BAP31 is a polytopic integral membrane protein of the endoplasmic reticulum in mammalian cells. It has been shown to participate in various cellular functions such as protein transport and apoptosis. *Saccharomyces cerevisiae* genome contains three open reading frames *YKL065C*, *YMR040W* and *YDL072C*, which encode BAP31 homologs. We named these *YET1* (for Yeast ER Transmembrane protein), *YET2* and *YET3*, respectively. Similar to mammalian BAP31, Yet1p was shown to be an integral membrane protein localized to the endoplasmic reticulum. *YET* genes were not essential for viability, but disruption of *YET1* increased and *YET3* decreased the cell growth in liquid cultures. In the *yet1Δ yet3Δ* and *yet2Δ yet3Δ* double disruptant cells the growth was restored to the level observed in the wild type cells. The results suggest that Yet proteins have partially specialized, but overlapping functions. Furthermore, *yet3Δ* cells showed a defect in invertase secretion. The possible role of the yeast BAP31 homologs in the ER export is discussed.

Key words: Endoplasmic reticulum, *YET*, *Saccharomyces cerevisiae*, protein transport, invertase secretion, yeast cell growth

INTRODUCTION

Mammalian BAP31 is an integral membrane protein located in the endoplasmic reticulum (ER). It has three putative transmembrane domains (TMDs) and its C-terminus is oriented to the cytosol (Ng *et al.*, 1997). The cytosolic region of BAP31 is mainly formed of heptad repeats, which may facilitate homo/oligomeric formation of coiled-coil structures. The C-terminal end of the protein contains a conserved KKXX sequence motif known to interact with coat protein I (COPI) complex (Letourneur *et al.*, 1994).

BAP31 was originally identified as a 31 kD membrane protein, which interacts with membrane-IgD in mouse B-cells (Kim *et al.*, 1994; Adachi *et al.*, 1996) and since then it has been implicated in diverse cellular functions: protein transport, quality control and apoptosis. Recent studies have shown that BAP31 interacts with the MHC class I complex (Spiliotis *et al.*, 2000; Paquet *et al.*, 2004). It binds to both MHC class I heavy chain and tapasin in the class I complex and it appears to participate either directly or indirectly in the recruitment of the class I

molecules to the ER exit sites (Paquet *et al.*, 2004). Removal of BAP31 from cells by using siRNA technique causes a delay in the ER to Golgi transport of MHC class I molecules (Paquet *et al.*, 2004). In the absence of BAP31 tetraspanins CD9 and CD81 are not transported to the plasma membrane (Stojanovic *et al.*, 2005). BAP31 may also have a role in the ER quality control process of a specific subset of proteins. It has been shown to mediate ER retention of mutant cystic fibrosis transmembrane conductance regulator (CFTR). When expression of BAP31 is inhibited with antisense technique, a mutant form of CFTR is released from the ER and transported to the plasma membrane (Lambert *et al.*, 2001). In addition to operating in protein transport, BAP31 regulates the intrinsic apoptotic pathway. It appears to function in the ER-mitochondria communication in apoptotic cells. Human BAP31 is a substrate for caspase cleavage at two sites (Ng *et al.*, 1997; Granville *et al.*, 1998; Ng and Shore 1998; Määttä *et al.*, 2000). The caspase cleavage product p20 causes cell death by inducing calcium signaling from the ER, which promotes mitochondrial fission and release of cytochrome c

(Breckenridge *et al.*, 2003; Simmen *et al.*, 2005). On the contrary, caspase resistant mutant of BAP31 is anti-apoptotic. During apoptotic stimulus it prevents the mitochondrial fission and the release of cytochrome *c* from mitochondria (Nguyen *et al.*, 2000; Breckenridge *et al.*, 2003; Simmen *et al.*, 2005).

Although knowledge on the functions of mammalian BAP31 is increasing, there are no studies on the function of the BAP31 homologs in other species. The database searches indicate that homologs of the BAP31 are found in several eukaryotic species (<http://www.freidok.uni-freiburg.de/volltexte/287/>). There are at least 12 true or hypothetical proteins in eight different organisms: human, mouse, fruit fly, nematode, baker's yeast, fission yeast, zebra fish and thale cress. In *Saccharomyces cerevisiae* (baker's yeast) there are three genes coding for BAP31 homologs. *S. cerevisiae* *YKL065C* gene has been designated *YET1* and the corresponding protein Yet1p (for Yeast ER Transmembrane Protein). The two other homologous open reading frames (ORFs), *YMR040W* and *YDL072C*, have been denoted *YET2* and *YET3*, respectively. We present here biochemical characterization of Yet1p and show that the absence of either Yet1p or Yet3p affects cell growth. Furthermore, *yet3Δ* cells were found defective in invertase secretion.

MATERIALS AND METHODS

Strains, media and culture conditions: The *Saccharomyces cerevisiae* strains are presented in Table 1. The SKY strains are haploid derivatives of diploid strains SKY28 (H2612 x H1282), SKY29 (H2612 x H1290) and SKY30 (H2612 x H1292) obtained by tetrad dissection. Yeast transformation was done as described before (Toikkanen *et al.*, 1996). Yeast cells were cultivated in either YPD or SCD medium (Sherman *et al.*, 1989). For plasmid selection SCD lacking uracil or leucine was used. The carbon source was 2% glucose except when Yet1p was overproduced from *GALI* promoter in the presence of 2% galactose. The growth of *YET* disruptants at different temperatures (38°C, 37°C, 36°C, 35°C and 34°C) or on various carbon sources (glucose, glycerol) was tested as patches on plates and was monitored for three successive days. For growth curve cultivations, 10 mL precultures were grown for two days at 30°C, 240 rpm. The cell growth was monitored by measuring the Optical Density (OD) of the cultures at 600 nm. Cultivations were started from the preculture so that early logarithmic growth phase (approximately OD₆₀₀ 0.2-0.3) was obtained after overnight cultivation at 30°C, 240 rpm.

Plasmids and DNA methods: *YET1* ORF was cloned by PCR using oligos 5'-AAG GTA CCG CGA TGA GTT TAT ACT TTA CGA C-3' and 5'-AAG AAT TCT TAG

TTT CCT TTC TTG GAA GCT TC-3' and either yeast genomic library in pHR70 (a kind gift from Hans Ronne, Uppsala University, Sweden) or yeast cDNA library (McKnight and McConaughy, 1983) as a template. The PCR fragment was digested with *KpnI/EcoRI* and cloned into similarly opened pZerO-1 (Invitrogen) to create pZerO-YET1. The gene was sequenced with the dideoxy method (Sanger *et al.*, 1977) from both strands of six independent clones, of which three were amplified from cDNA library and three from genomic library. To simplify subsequent cloning pGEM-YET1 was constructed by transferring *YET1* as a *KpnI/EcoRI* fragment from pZerO-YET1 into pGEM-3Zf(+) vector (Promega). pYES2-YET1 plasmid for inducible Yet1p overproduction was constructed by ligating a *KpnI/EcoRI* digested *YET1* gene from pGEM-YET1 into pYES2 vector (Invitrogen). For constitutive Yet1p overproduction *YET1* gene was first transferred into pSP73 vector (Promega) as a *KpnI/EcoRV* fragment from pZerO-YET1. This construct was further digested with *XbaI/PstI* and *YET1* gene containing fragment was ligated into pVT102L vector (Vernet *et al.*, 1987), opened with the same restriction enzymes, to create YEpYET1. pGEX2T-YET1_{tail} encoding a GST fusion protein containing the last 81 amino acids from the C-terminus of Yet1p and an N-terminal GST tag was constructed as follows: the C-terminal tail of Yet1p was amplified from pGEM-YET1 with oligos 5'-AAG GAT CCA AGA GAC TGG TGA AAT ACC-3' and 5'-AAG AAT TCT TAG TTT CCT TTC TTG GAA GCT TC-3', digested with *BamHI/EcoRI* and ligated into pGEX-2T (Amersham Pharmacia Biotech). The same PCR product was cloned also to pRSET-A vector (Invitrogen) to obtain pRSET-YET1_{tail}, a plasmid expressing the Yet1p tail with an N-terminal histidine tag. *YET2* ORF was amplified by PCR from the yeast genomic library with oligos 5'-AAG GAT CCA TGG GTG TTT ATT TGG CAG TAC TC-3' and 5'-AAG AAT TCT TAA AAT TTC TTC TTC TGC AAT TCC-3'. The PCR product was digested with *BamHI/EcoRI* and transferred into pGEM-3Zf(+) vector to create pGEM-YET2.

Disruption cassettes were generated as follows: for disruption of *YET1* pGEM-YET1 was digested with *BamHI*, blunt-ended with Klenow, digested with *BpmI* and treated with T4 DNA polymerase to fill in the cohesive end and the blunt-ended *URA3* was inserted. The resulting *YET1* disruption cassette contained the *URA3* flanked by the first and the last 87 nucleotides of *YET1* coding region. For *YET2* gene deletion a kanamycin resistance module kanMX4 was amplified from pFA6-kanMX4 (Wach *et al.*, 1994) with oligos 5'-GCT CTA GAG GTG TTT ATT TGG CAG TAC TCT TTT CGT TAC TGG TCA TCG TAC GCT GCA GGT CGA CGG AT-3' and 5'-GCT CTA GAT TAA AAT TTC TTC TTC TGC AAT TCC TCT AAA TAT TTT TGC CAT CGA

Table 1: Yeast strains used in this study

Strain	Genotype	Source
BY23	<i>Matα/Matα leu2/leu2 ura3/ura3 Gal⁺/Gal⁺</i>	P. Novick
NY179	<i>Matα leu2-3,112 ura3-52</i>	P. Novick
CKY263	<i>Matα leu2-3,112 ura3-52</i>	C. Kaiser
H1217	<i>Matα leu2-3,112 ura3-52</i>	S. Keränen
W303-1A	<i>Matα ace 2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	J. Thorer
H1282	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3</i>	This study
H1283	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3</i>	This study
H1284	<i>Matα/Matα leu2/leu2 ura3/ura3 yet1Δ::URA3/YET1 Gal⁺/Gal⁺</i>	This study
H1285	<i>Matα/Matα leu2/leu2 ura3/ura3 yet1Δ::URA3/YET1 Gal⁺/Gal⁺</i>	This study
H1290	<i>Matα leu2-3,112 ura3-52 yet2Δ::KanMX4</i>	This study
H1291	<i>Matα leu2-3,112 ura3-52 yet2Δ::KanMX4</i>	This study
H1292	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet2Δ::KanMX4</i>	This study
H1293	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet2Δ::KanMX4</i>	This study
H2612	<i>Matα leu2-3,112 ura3-52 yet3Δ::LEU2</i>	This study
H2614	<i>Matα leu2-3,112 ura3-52 yet3Δ::LEU2</i>	This study
SKY28-1A	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet3Δ::LEU2</i>	This study
SKY28-12A	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet3Δ::LEU2</i>	This study
SKY29-11B	<i>Matα leu2-3,112 ura3-52 yet2Δ::KanMX4 yet3Δ::LEU2</i>	This study
SKY29-1D	<i>Matα leu2-3,112 ura3-52 yet2Δ::KanMX4 yet3Δ::LEU2</i>	This study
SKY30-11A	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet2Δ::KanMX4 yet3Δ::LEU2</i>	This study
SKY30-14A	<i>Matα leu2-3,112 ura3-52 yet1Δ::URA3 yet2Δ::KanMX4 yet3Δ::LEU2</i>	This study

TGA ATT CGA GCT CG-3'. 5'-ends of both oligos had homologous regions immediately upstream and downstream of *YET2* ORF. *YET3* ORF was deleted by replacement with a *LEU2*. Oligos 5'-CAC ATT ATA CTC ATA ACC ATA GAA TTC ACA GAA TTG AAT CCC CAA CAA TTA CAT CAA AAT CC-3' and 5'-TAC ACA CTT CTG TGC AGA AGA AAA GGA ATA AAA CGT CAA AAG TTT CGT CTA CCC TAT GAA CA-3' were designed so that they had 20 base pairs homologous to upstream and downstream sequences of *LEU2* gene and 40 base pairs homologous to sequences flanking *YET3* ORF.

Antibodies and protein methods: Antibodies were raised against the hydrophilic tail of Yet1p. Expression and purification of GST-Yet1p_{tail} fusion protein was done according to the manufacturer (Amersham Pharmacia Biotech) from *E. coli* DH5 α cells transformed with pGEX2T-YET1_{tail}. For immunization of a New Zealand white rabbit the Yet1p_{tail}-portion was released with thrombin and used as an antigen. Antigen preparation and immunization were done according to Harlow and Lane (1988). Yet1p antibodies were affinity-purified using CnBr-activated Sepharose 4B column where His₆-Yet1p_{tail} fusion protein was coupled according to manufacturer's instructions (Amersham Pharmacia Biotech). Affinity-purified Yet1p antibody was used as a 1:100 dilution. Preparation of antibodies against Seb1p (used as 1:500 dilution) and Sso2p (1:20000) have been described (Jäntti *et al.*, 1994; Toikkanen *et al.*, 1996). Antibodies against Hsp150 (1:400; Paunola *et al.*, 1998), CPY (1:100; Russo *et al.*, 1992) and Gas1p (1:100; a kind gift from Kai Simons, Max Planck Institute, Dresden, Germany) were used in immunoprecipitations and

Kar2p specific antibodies (1:5000; a kind gift from Mark Rose, Princeton University, USA) in indirect immunofluorescence. Pig anti-rabbit IgG conjugated with TRITC (1:1000) was purchased from DAKO A/S (Glostrup, Denmark) and goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) from Promega. Proteins were separated in SDS-PAGE with 10-15% acrylamide gels (Laemmli, 1970). For Western analysis, proteins were blotted onto Hybond ECL-membranes (Amersham Pharmacia Biotech) and visualized with ECL detection system (Amersham Pharmacia Biotech).

Membrane association and subcellular fractionation:

Yeast membranes were isolated from wild type strain NY179 as described (Franzoso *et al.*, 1991). Membrane association assay was modified from Hardwick and Pelham (1992). Hundred microliter of yeast membranes (1.5 mg protein) were mixed with the same volume of either lysate buffer (200 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 μ g mL⁻¹ pepstatin, 1 μ g mL⁻¹ leupeptin) (1), lysate buffer containing either 0.1% (2) or 1% Triton X-100 (3), 0.1 M Na₂CO₃, pH 11.5 in water (4), 1 M KCl in water (5), or 1 M urea in lysate buffer (6). Samples were incubated on ice for 30 min and pelleted 100 000 \times g for 1 h in an ultracentrifuge (Beckman Instruments Inc.) at 4°C. Membrane pellets and acetone-precipitated supernatants were dissolved in 100 μ L of Laemmli sample buffer and proteins in 10 μ L sample of each were analyzed by SDS-PAGE and Western blotting. Yet1p was detected with affinity purified Yet1p specific antibody. Subcellular fractionation of yeast membranes was modified from (Antebi and Fink, 1992; Schröder *et al.*, 1995). One milliliter of yeast membranes was layered on top of a step-wise 22-60% sucrose gradient in 10 mM Hepes, pH

7.5, 1 mM MgCl₂ and centrifuged 178 000×g for 2.5 h in a SW-41 rotor of Beckman L-70 ultracentrifuge at 4°C. One milliliter fractions were collected from the top of the gradient. Twenty microliter aliquots of fractions were subjected to SDS-PAGE and Western analysis using antibodies against Yet1p, Seb1p and Sso2p.

Other methods: For Northern blotting the total RNA from the strain NY179 was isolated with RNeasy Total RNA kit (Qiagen), electrophoresed in agarose gel containing 2.2 M formaldehyde (Sambrook *et al.*, 1989) and blotted onto Hybond C nitrocellulose filter (Amersham Pharmacia Biotech). [α -³²P]dATP (Amersham Pharmacia Biotech) labeled full length ORF sequences of *YET1* and *YET2* were used as probes. *In vitro* translations were done with TNT[®] Coupled Reticulolysate System kit (Promega) with or without canine pancreatic microsomes according to the manufacturer, and β -lactamase and α factor were used as the control proteins. Indirect immunofluorescence was done according to Redding *et al.* (1991). Yeast lysates were prepared as in Jazwinski (1990) except that 1% Triton X-100 was used instead of 5% Brij 58. Invertase activity was determined as described in Makarow (1988) except that SC medium instead of YEP was used and the cells were cultivated at 30°C. Pulse-chase analysis was according to Paunola *et al.* (1998) except that the chase mixture contained 1 mg mL⁻¹ methionine and 1 mg mL⁻¹ cysteine in 15% yeast extract.

RESULTS AND DISCUSSION

Yet proteins are BAP31 homologs: Many proteins, which perform the core biological functions, are conserved from yeast to mammals. Since baker's yeast provides a good model to study functions of eukaryotic proteins, we characterized the yeast homologs of BAP31. *Saccharomyces cerevisiae* genes *YKL065C* and *YMR040W* were cloned and designated *YET1* and *YET2*, respectively. The *YKL065C/YET1* gene was isolated from both a genomic and a cDNA library and identical sequences were obtained from both of them indicating that there are no introns in the gene. It encodes a protein of 206 amino acids, the size of which corresponds well to the detected mRNA (data not shown). However, the sequencing revealed a G205A nucleotide change (causing a Val46Met substitution) in the gene amplified from both libraries in comparison to the ORF sequence of *YKL065C*. This is probably due to different strain background used for DNA libraries in our study and in the *Saccharomyces cerevisiae* genome sequencing project. *YMR040W/YET2*, which encodes a hypothetical protein of 160 amino acids, was cloned from the genomic library. In contrast to *YET1*, the mRNA corresponding to *YET2* was not detected in a Northern analysis of our strains (data not shown) and

therefore we decided not to proceed further with the biochemical characterization of the hypothetical protein Yet2p *per se*. However, the gene was included in the disruption studies. The third yeast ORF identified in the homology search, *YDL072C*, encodes a protein of 203 amino acids. This gene was named *YET3* and the corresponding protein Yet3p. A global localization analysis of yeast proteins has shown that it localizes in the ER although it does not contain the KKXX-motif (Huh *et al.*, 2003).

Of these three yeast proteins, Yet1p and Yet2p are more closely related and share 56% identity at the amino acid level. In contrast, Yet3p appears to be more distantly related and shows 31% and 21% identity at the amino acid level to Yet1p and Yet2p, respectively. The overall sequence identity between human BAP31 and these yeast homologs was somewhat lower than the one observed between BAP31 and the other homologs. The yeast proteins were 22-30% identical and 43-60% similar to BAP31 sequence, Yet1p being the most conserved of them, while the other homologs showed in average 53% identity and 70% similarity to BAP31. The predicted domain structure of BAP31 and its *Saccharomyces cerevisiae* homologues was found to be similar (Fig. 1): (1) a polypeptide chain of 160 to 246 amino acids, varying mainly in the length of the hydrophilic tail, (2) three TMDs located approximately within the first 120 amino acids, (3) the hydrophilic tails form coiled-coil structures with high probability, (4) a conserved short peptide motif QRNxYxxG in the same position in relation to their TMDs, (5) a glutamate residue at position 15 in the first predicted TMD and (6) a C-terminal KKXX-motif except in Yet3p. Based on these data we conclude that these proteins are *S. cerevisiae* homologs of BAP31.

Yet1p is an integral membrane protein of the yeast ER: For biochemical characterization of the *YET1* gene product, antibodies were raised against the C-terminal region (amino acids 126-206) of Yet1p. The specificity of the antibody was confirmed by Western analysis of proteins in cell lysates prepared from wild type yeast, Yet1p overproduction strain and *yet1* disruptant strain (Fig. 2). The calculated molecular weight of Yet1p is 23.4 kD and a band of approximately 24 kD was detected both from the wild type cells (Fig. 2, lane 1) and the cells overproducing Yet1p from a multicopy plasmid (Fig. 2, lane 2) and this band was missing from the *yet1* disruptant strain (Fig. 2, lane 3). In addition, a nonspecific band migrating above 24 kD was detected in all the cell lysates and smaller bands (14.2-16.9 kD) appeared when Yet1p was overproduced (Fig. 2, lane 2). Whether these smaller bands represent specific cleavage products is currently not known.

A

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BAP31 1  MSLQWIAVATFITYAEVVFVLLLCIPFISPKRMQKIFKSRLVELLVSYGNTFFVVLIVILV
Yet 1p 1  MSLYFTTLFLLLTIVVWVLFVLPFPHRRRGLFSTYNQLTAKQIKIIEITGCLVGI
Yet 2p 1  MGVYLAFLFSLLVIEAALFIVLPLPCRVRVLYIYVSIISTNKKFRIVVGMIFVGL
Yet 3p 1  MSLYITLVFATLVVEIFMFSIALPPIPSRYRRPITLLLLKPFKSTVQVAIKCVLGFLL

BAP31 61  LILVIDAVREIRKYDDVTEKVNLCNPPGMEH--FHMK--LFRAGRNLTYAGFSLLLSFL
Yet 1p 61  LFI DSWKRSQIRVSLYHNDNSGSI GSSAVTP--ICALASRAYNQRNVIYI SGFIIYF-SIC
Yet 2p 61  LFI DSWKRSQIRVSTYRNQKINPYIIN-SVTP--VDALASRAYNQRNVIYI SGFIIYF-YIC
Yet 3p 61  LFI DCTNRVYSIDKELQLSSASGNNGAIACDRTEVLSRKFACRNVIYIIGITLFLTFVV

BAP31 117 RRLVTLISQQATLLASNEAFKKQAES--ASEAMKKYVEENFCLIKGARVVDGKLDVGNAE
Yet 1p 118 PPLVMSLVKRIIVKYQGLINEQEKQLNKPSSNKKKSNEADSTKIQEELRKKKESITF--
Yet 2p 117 LITVMSLRRIVEVN-----DKMKAQDDIKKELRKKCKYEE--
Yet 3p 121 VRTFGLVIELTMKDIYR-ASPP-----VASSDVKKNSVTAEAAQCSGASKDD--

BAP31 175 VKLEENRSLKADLCCKLDELASTKQLEKKAENQVLAMRKQSEGLTKEYDRLL EEHAKLG
Yet 1p 175 -GLCKQVKNLEK--YFDKKNOPGNVAAAEASKKQ-----
Yet 2p 154 -ELCKKIF-----
Yet 3p 169 -HGDEKNFELKIKLQDIDDEIARLKEKSESLQEEIN-----

BAP31 235 AAVDGPMDKKEE
Yet 1p -----
Yet 2p -----
Yet 3p -----
    
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B

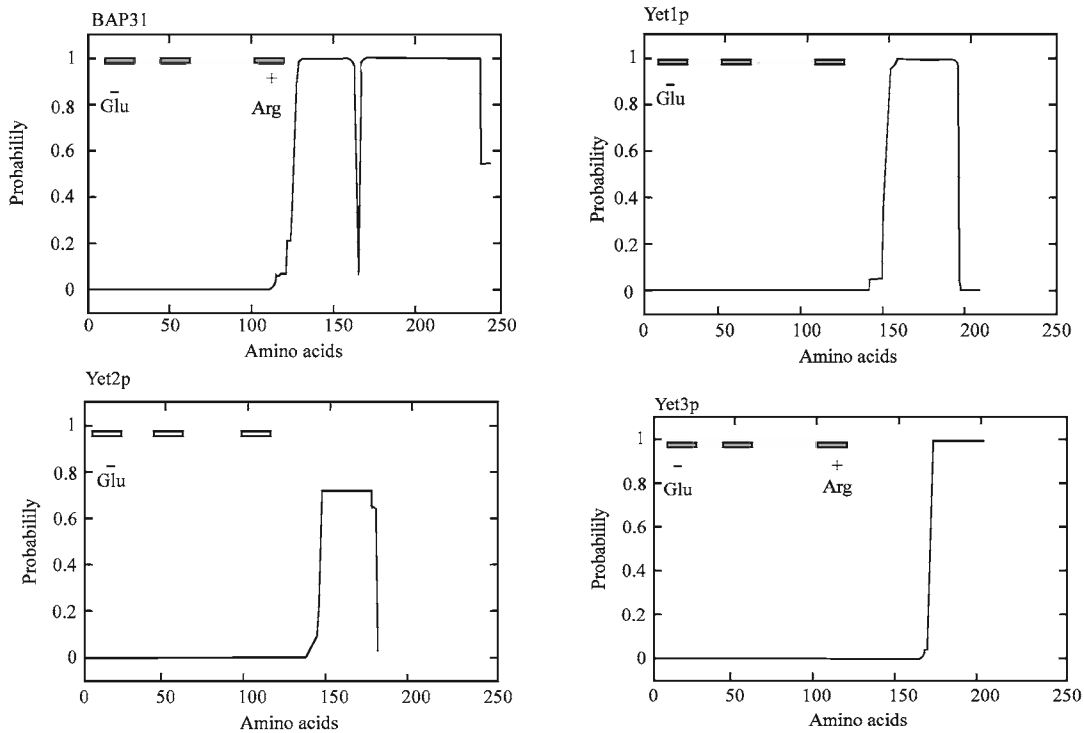


Fig. 1: Comparison of amino acid sequences of human BAP31 and its *S. cerevisiae* homologs. (A) ClustalW alignment of human BAP31 and the three yeast Yet proteins. The identical amino acids are marked in black boxes and the similar ones in gray boxes. (B) Predictions of coiled-coil structures are presented as probabilities on the vertical axes. The number of amino acids of the proteins are shown on the horizontal axes. Predicted TMDs are presented as rectangles and the presence of charged amino acids within TMDs are indicated. The sequences were analyzed with programs provided by ch.EMBnet.org

BAP31 was previously shown to be an integral ER membrane protein without a cleavable signal peptide (Annaert *et al.*, 1997; Ng *et al.*, 1997). To test the membrane association of Yet1p, yeast membranes were isolated. Centrifugation of the samples demonstrated that Yet1p sediments with isolated yeast membranes (Fig. 3).

To test the quality of association of Yet1p with the membranes, they were solubilized with 0.1% or 1% Triton X-100 prior to centrifugation. Yet1p was partially dissociated from the membranes in the presence of 0.1% Triton X-100 and only app. 50% of Yet1p was released even with 1% Triton X-100 (Fig. 3). Further,

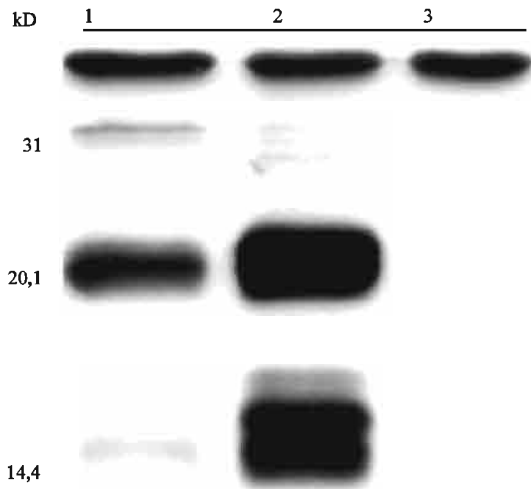


Fig. 2: Recognition of Yet1p with polyclonal antibodies. Western analysis of proteins in yeast cell lysates with affinity-purified antibodies, which were raised against amino acids 126-206 of Yet1p. Lane 1: wild type strain (W303-1A), lane 2: W303-1A transformed with pYES2-YET1 to overproduce Yet1p from the *GAL1* promoter, lane 3: *yet1Δ* strain (H1282). The sizes of the molecular weight marker proteins are indicated on the left

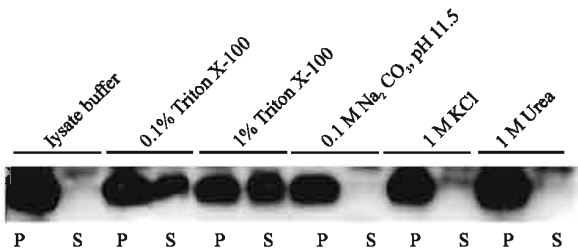


Fig. 3: Yet1p is an integral membrane protein. Yeast membranes isolated from a wild type strain NY179 were treated either with lysate buffer, lysate buffer containing 0.1% or 1% Triton X-100, 0.1 M Na_2CO_3 , pH 11.5, 1 M KCl, or 1 M urea in lysate buffer and pelleted by centrifugation. Western blot analysis with polyclonal antibodies was used to detect Yet1p in membrane pellets (P) and respective supernatant (S) samples

treatment with 0.1 M sodium carbonate (pH 11.5), 1 M potassium chloride, or 1 M urea did not detach Yet1p from the sedimented membranes (Fig. 3). In conclusion, these results indicate that Yet1p is an integral membrane protein.

Similarly to BAP31, Yet1p does not have a predicted signal peptide cleavage site at its N-terminus. The possible processing of Yet1p was analyzed by *in vitro*

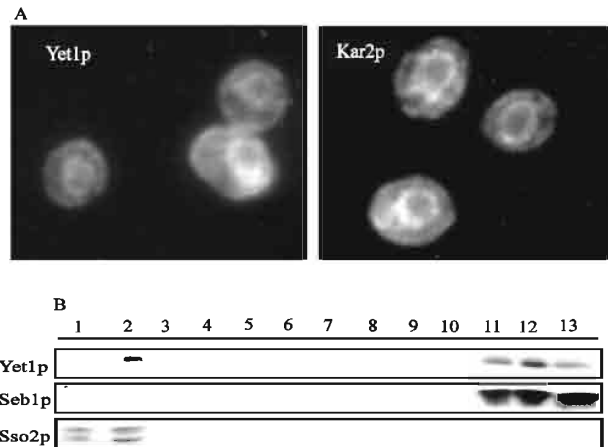


Fig. 4: Yet1p is localized to the ER. (A) Yet1p shows similar immunofluorescence staining pattern as the ER protein Kar2p. (B) Sucrose gradient analysis of Yet1p, Seb1p and Sso2p localization in membranes of wild type yeast (NY179). Parallel Western blots of the sucrose gradient fractions were analyzed with antibodies against affinity purified Yet1p, Seb1p and Sso2p. Numbering of the fractions is from the top (1st fraction) to the pellet (13th fraction)

translation of the protein. According to the SDS-PAGE analysis protein products of identical size (app. 25 kD) were generated both in the presence and absence of microsomes (data not shown), which indicates that there is no cleavable signal peptide in Yet1p.

BAP31 is localized to the ER (Annaert *et al.*, 1997; Ng *et al.*, 1997) and the KKXX-motif in the C-terminal tail of Yet1p suggested that also Yet1p could be an ER resident protein. However, *e.g.*, the yeast protein Emp47p has been shown to reside in the Golgi at steady state although it is a type I transmembrane protein and carries a KKXX-motif at its C-terminus (Schröder *et al.*, 1995). The localization of Yet1p was studied by indirect immunofluorescence microscopy. Exponentially growing wild type and Yet1p overproduction cells were harvested, fixed with paraformaldehyde and processed for immunofluorescence microscopy. The staining of Yet1p in wild type cells was rather faint, but in the Yet1p overproduction strain the antibody clearly stained a rim around the nucleus as well as stretches beneath the plasma membrane (Fig. 4A). A similar staining pattern was observed with antibodies against an ER luminal chaperone Kar2p (Normington *et al.*, 1989; Rose *et al.*, 1989).

The ER localization of Yet1p was further studied with subcellular fractionation. Isolated yeast membranes were

separated in a step-wise 22-60% sucrose gradient and Western blot analysis was used to detect Yet1p together with marker proteins in 13 gradient fractions (13th fraction was the pellet). Typically to the proteins of the rough ER membranes, the bulk of Yet1p cosedimented in the bottom fractions (Fig. 4B, lanes 11-13) with Seb1p, a subunit of the ER translocation complex (Toikkanen *et al.*, 1996) and the plasma membrane syntaxin Sso2p (Aalto *et al.*, 1993) was detectable only in the lighter fractions (Fig. 4B, lanes 1-2). A signal was detected with Yet1p antibody also in fraction 2 (Fig. 4B, lane 2). This may represent nonspecific staining (Fig. 2) although a presence of a pool of Yet1p in lighter membranes cannot be excluded.

Previously, ER localization of C-terminally GFP-tagged Yet1p has been observed in a global yeast protein analysis (Huh *et al.*, 2003). It is interesting that although the GFP-tagging inactivates function of KKXX-motif in Yet1p, the protein remains localized in the ER. This would indicate that Yet1p possesses in addition to KKXX another ER localization signal as has been previously suggested for truncated BAP31 (Annaert *et al.*, 1997).

Disruption of *YET1* or *YET3* affects cell growth: Next, the possible effects of disruption of *YET* genes on cell phenotype were studied. The chromosomal *YET1* gene was disrupted from both a diploid and a haploid yeast strain by replacing 80% of the coding sequence with *URA3*. The disruption of the gene was verified by PCR (data not shown) and the absence of the encoded protein in the haploid strain by Western analysis (Fig. 2, lane 3). Both disrupted strains were viable and showed no detectable phenotype different from a wild type strain when their abilities of utilization of various carbon sources or growth at different temperatures were tested with a plate assay (data not shown).

YET2 and *YET3* genes were disrupted from haploid yeast strains by replacing the complete ORFs with a kanMX4 module (Wach *et al.*, 1994) and *LEU2* gene, respectively. Previously, unaltered growth on plates for haploid *yet3Δ* cells was observed in a systematic gene deletion study (Blasco and Sanz, 2000). In our strain background neither *yet2Δ* nor *yet3Δ* caused a detectable phenotype on growth at any of the temperatures or carbon sources tested. Therefore, we proceeded to make crosses between the single or double disruptant strains to gain various disruption combinations (*yet1Δ yet2Δ*, *yet1Δ yet3Δ*, *yet2Δ yet3Δ* and *yet1Δ yet2Δ yet3Δ*) for further analysis. Again, on plates they showed no difference in growth in comparison to single disruptants and the parental wild type strains.

A more quantitative analysis of the cell growth was obtained when the growth rates of the strains containing

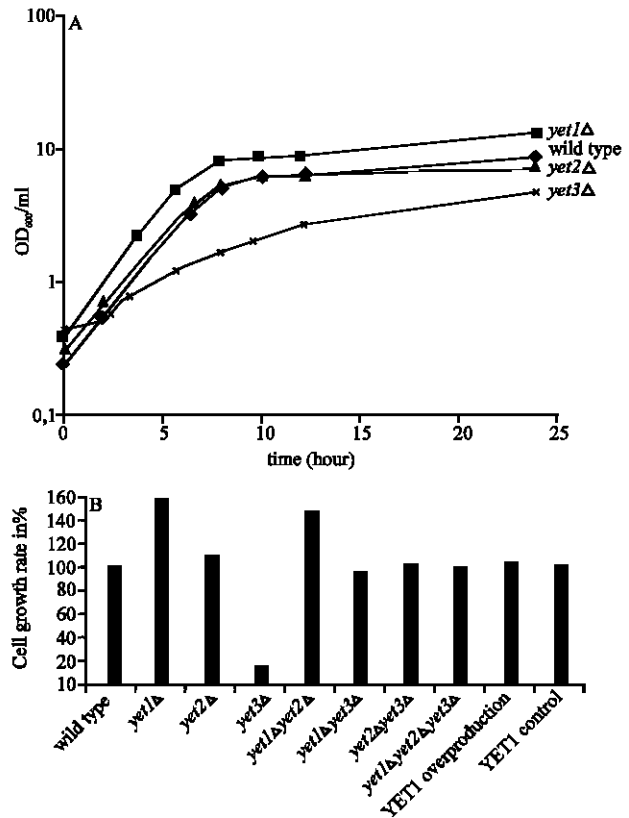


Fig. 5: Disruption of *YET1* increases and *YET3* decreases cell growth in liquid medium. (A) Growth of single *yet* disruptants and wild type strains in shake flask cultivations at 30°C. Mean values of the optical density (OD) measured from two cultivations of two independent disruptant clones and those of two respective isogenic wild type strains (NY179 and H1217), are presented. (B) Comparison of the growth rates of the strains at the exponential growth phase. The growth rates of the wild type strains and the control strain for Yet1p overproduction were set to 100% (actual values were 0.22 h⁻¹ and 0.15 h⁻¹, respectively) and the growth rates of disruptants and *YET1* overexpression strain were compared to them in percents, respectively. Strains were grown in SCD, except Yet1p overproduction and its control strain in SCD -Leu. The studied strains were: NY179 and H1217 (wild type), H1282 and H1283 (*yet1Δ*), H1290 and H1291 (*yet2Δ*), H2612 and H2614 (*yet3Δ*), H1292 and H1293 (*yet1Δ yet2Δ*), SKY28-1A and SKY28-12A (*yet1Δ yet3Δ*), SKY29-11B and SKY29-1D (*yet2Δ yet3Δ*) and SKY30-11A and SKY30-14A (*yet1Δ yet2Δ yet3Δ*). The Yet1p overproduction strain was NY179 transformed with YEpYET1 and the respective control strain that transformed with empty vector pVT102L. Two independent clones of each were analyzed

Table 2: Disruption of *YET3* decreases the amount of secreted invertase activity. The intracellular and secreted invertase activities from wild type (H1217) and *yet3Δ* (H2612) cells were determined at indicated time points post-derepression of invertase production

	Time (min)	0	15	30	45	60	90	120
Intracellular activity	Wild type	0.051	0.064	0.071	0.074	0.076	0.089	0.085
	<i>yet3Δ</i>	0.061	0.075	0.073	0.082	0.071	0.088	0.079
Secreted activity	Wild type	0.022	0.053	0.066	0.144	0.218	0.350	0.353
	<i>yet3Δ</i>	0.026	0.031	0.055	0.053	0.074	0.166	0.168

Mean values of three independent experiments are presented (as relative invertase activity/amount of cells corresponding to OD₆₀₀ 1)

yet1Δ, *yet2Δ*, or *yet3Δ* single disruptions and strains containing disruptions of two or all three *YET* genes were studied with shake flask cultivations in SCD medium at 30°C. The cultivations of these strains were started at the same cell density and the growth was monitored by measuring the optical density of the cultures. A haploid *yet2* disruptant strain grew similarly to its parental wild type strain (Fig. 5A). However, disruption of either *YET1* or *YET3* alone affected the ability of yeast cells to grow in liquid cultivation. Surprisingly, these effects were of opposite nature: disruption of *YET1* enabled the cells to grow slightly faster than the wild type cells whereas the growth of *yet3Δ* strain was retarded in comparison to the parental strain (Fig. 5A). Thus, these results suggest that it is not sufficient to use only plate assays in search for phenotypes of nonessential yeast genes.

The growth rates were measured at the logarithmic growth phase (Fig. 5A) and the growth of each disruptant strain was compared to its wild type parental strain, whose growth rate was set to 100% (Fig. 5B). During this time period the growth rate of *yet1Δ* strain was 1.5 fold higher than that of the parental strain. Instead, the growth rate of *yet3Δ* strain was significantly lower, less than 20% of the corresponding wild type strain. Interestingly, when these two disruptions were combined in the *yet1Δ yet3Δ* strain, they compensated each other's effects and the strain grew similarly to the wild type strain. Simultaneous disruption of *YET2* had hardly any effect on the enhanced growth observed in the absence of Yet1p. On the other hand, the growth retardation due to *yet3Δ* was suppressed in combination with *YET2* disruption and was not evident in the triple disruptant strain. Therefore, the fact that simultaneous deletion of *YET2* rescued the growth compromising effect caused by *yet3Δ* suggests that YMR040W/*YET2* is not a silent locus. It is likely to encode a protein, which may function as a negative regulator since the growth was restored to the wild type level in *yet2Δ yet3Δ* cells. Finally, although disruption of *YET1* caused an increase in cell growth rate, overproduction of Yet1p did not alter it markedly (Fig. 5B). Thus, the members of BAP31-like proteins in yeast may have at least partially overlapping functions, which become apparent when studying growth of *yet* disruptants in liquid cultures.

In *Saccharomyces cerevisiae* there are several other examples of genes that encode highly homologous proteins with partially overlapping and partially specialized functions. Such are for instance the *SEB* genes encoding ER translocation factors (Panzner *et al.*, 1995; Finke *et al.*, 1996; Toikkanen *et al.*, 1996, 2003) and the *SSO* genes encoding the plasma membrane syntaxins (Aalto *et al.*, 1993; Jäntti *et al.*, 2002). Furthermore, specialized functions have been observed also for mammalian BAP31 homologs. For example, removal of BAP31 but not BAP29 causes a delay in the ER to Golgi transport of MHC class I molecules (Paquet *et al.*, 2004).

Lack of Yet3p decreases the level of secreted invertase:

The increased growth rate of the yeast cells lacking Yet1p and the slow growth in the absence of Yet3p could be due to alterations in the secretory process. Therefore we analyzed by pulse-chase experiments the intracellular transport of a soluble secretory glycoprotein Hsp150 (Russo *et al.*, 1992), a vacuolar protease CPY (Stevens *et al.*, 1982) and a GPI-anchored plasma membrane protein Gas1p (Nuoffer *et al.*, 1991). However, in comparison to the parental strains, significant differences were not detected in the maturation kinetics of Hsp150 and CPY in *yet1Δ* and *yet3Δ* cells or in the maturation kinetics of Gas1p in *yet3Δ* strain (data not shown). Thus, despite the altered growth rates of the cells lacking either Yet1p or Yet3p, the intracellular transport of none of the tested proteins appeared to be affected.

Next, we compared transport of invertase to the cell wall of *yet3Δ* and its parental strain (Table 2). The cells were grown to early logarithmic phase in SCD and then shifted to the SC medium containing 0.1% glucose to derepress transcription of the *SUC2* gene. Accumulation of intracellular and extracellular invertase activities was followed up to two hours. The level of intracellular activity remained at equal level throughout the experiment in both wild type and *yet3Δ* cells. Also the secreted invertase activity was maintained at the similar level during the first 30 min after the derepression. However, upon prolonged incubation the secreted invertase activity in *yet3Δ* cells was considerably reduced. It was less than half of that observed in the wild type cells (Table 2). Since

invertase did not accumulate inside the *yet3Δ* cells, it is possible that either these cells do not synthesize as much secreted invertase as the wild type cells or, perhaps, a portion of it is degraded. A third option would be that invertase had leaked to the culture medium of *yet3Δ* cells, which might happen if the cell walls were defective. However, that possibility was ruled out by the findings that in comparison to the wild type cells neither the growth of *yet3Δ* cells in the presence of calcofluor was impaired nor was invertase detected in their culture supernatant in higher amounts (data not shown). Thus, we favor the idea that the observed delay in secretion of invertase may be due to slower or compromised maturation of the protein at the ER. Also, a portion of immature protein could be degraded, which would further decrease the secreted invertase activity.

Previous studies have demonstrated that there are specific molecular mechanisms, which are required for the export of a subset of proteins from the yeast ER. Shr3p has been shown to facilitate folding of amino acid permeases and thereby to promote their ER exit (Gilstring *et al.*, 1999). A recent study on interactions of yeast integral membrane proteins suggests that Shr3p may function as a more general chaperone for transporter proteins (Miller *et al.*, 2005). Furthermore, several integral membrane proteins, which appear to facilitate ER export of distinct cognate substrates, have been identified in the yeast ER (Gilstring *et al.*, 1999; Kota and Ljungdahl, 2005). Mammalian BAP31 is likely to participate in regulation of protein transport at the level of the ER. The absence of mammalian BAP31 causes a delay in the ER to Golgi transport of MHC class I molecules due to defective recruitment to the ER exit sites (Paquet *et al.*, 2004). A functional BAP31 is also needed for the ER export of tetraspanins CD9 and CD81 (Stojanovic *et al.*, 2005). On the other hand, a membrane protein complex containing BAP31 has been shown to mediate the ER retention of membrane-bound IgD (Schaniel *et al.*, 2003). It is tempting to speculate that like BAP31 also its *S. cerevisiae* homologs act directly or indirectly in the early steps of the transport of a specific subset of proteins. This would be in accordance with the result that a general defect in intracellular transport was not detected in *yet* disruptant cells. Yeast BAP31-like proteins could function either as chaperones or cargo receptors at some step in the ER assembly line of exported proteins. A recent analysis with split-ubiquitin technique suggests that Yet1p interacts with several integral membrane proteins of the ER, which participate in posttranslational modifications of proteins (Miller *et al.*, 2005). Many factors are required to maintain fidelity of export of proteins from the ER due to the

existence of subcompartments and various exit routes at the yeast ER (Kamhi-Nesher *et al.*, 2001; Huyer *et al.*, 2004; Watanabe and Riezman, 2004; Jüschke *et al.*, 2005). The yeast homologs of BAP31 described in this work could be part of such machinery, which ensures correct maturation and exit of cargo proteins from the ER. The *yet* disruptant strains will provide tools for more detailed studies on functions of BAP31-like proteins.

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