FEBS Letters 577 (2004) 422–426 FEBS 28966

A genome-wide screen identifies Yos9p as essential for ER-associated degradation of glycoproteins

Bettina A. Buschhorn[1], Zlatka Kostova[1,2], Balasubrahmanyam Medicherla[1], Dieter H. Wolf[*]

Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany

Received 26 August 2004; revised 7 October 2004; accepted 14 October 2004

Available online 27 October 2004

Edited by Felix Wieland

Abstract We undertook a growth-based screen exploiting the degradation of CTL*, a chimeric membrane-bound ERAD substrate derived from soluble lumenal CPY*. We screened the *Saccharomyces cerevisiae* genomic deletion library containing ~5000 viable strains for mutants defective in endoplasmic reticulum (ER) protein quality control and degradation (ERAD). Among the new gene products we identified Yos9p, an ER-localized protein previously involved in the processing of GPI anchored proteins. We show that deficiency in Yos9p affects the degradation only of glycosylated ERAD substrates. Degradation of non-glycosylated substrates is not affected in cells lacking Yos9p. We propose that Yos9p is a lectin or lectin-like protein involved in the quality control of N-glycosylated proteins. It may act sequentially or in concert with the ERAD lectin Htm1p/Mnl1p (EDEM) to prevent secretion of malfolded glycosylated proteins and deliver them to the cytosolic ubiquitin-proteasome machinery for elimination.

Keywords: Yos9p; Endoplasmic reticulum; Quality control; Protein degradation; ERAD; Glycoprotein

1. Introduction

Secretory proteins enter the endoplasmic reticulum (ER) through a translocation channel in an unfolded state. Before delivery to their site of action, proteins are modified and folded to acquire their functional conformation [1,2]. Major ER modifications include N-linked glycosylation, disulfide bond formation and glycosyl phosphatidyl inositol (GPI)-anchoring [3,4]. Non-properly folded or orphan proteins are recognized as such in the ER, retrograde transported back to the cytosol and degraded by the ubiquitin-proteasome system [5–8]. The folding process in the ER is controlled by a retention-based quality control system consisting of ER-resident chaperones, protein disulfide isomerases (PDI), and lectins. This system differentiates between properly folded proteins and incompletely folded, potentially cell damaging conformers and decides upon delivery of proteins to their site of action or retrograde transport to the cytoplasm for degradation [3,5,9]. N-linked carbohydrate chains play an essential role in ER-based quality control of secretory proteins. Following co-translational addition of Glc3Man9GlcNAc2 oligosaccharides to proteins, N-glycans are matured by stepwise removal of the two terminal glucose residues by alpha-glucosidases I and II. In mammalian cells, the resulting Glc1Man9GlcNAc2 structure interacts with the lectins calnexin and calreticulin, which also bind PDI and participate in the folding of the protein. Cleavage of the terminal glucose residue by α-glucosidase II interrupts the lectin interaction allowing properly folded proteins to leave the ER. Incompletely folded proteins are, instead, recognized by UDP-glucose:glycoprotein glucosyltransferase, which adds back a single glucose residue, thereby allowing a new round of lectin binding and assisted folding. Proteins unable to acquire their native conformation following rounds of deglucosylation-folding-reglucosylation become targets of ER α-mannosidase I, which releases a mannose residue from the inner branch of the N-glycan, giving rise to Man8GlcNAc2. It is postulated that proteins containing this oligosaccharide structure are recognized by another lectin, EDEM in mammalian cells, Htm1/Mnl1p in yeast, which prevents secretion of the misfolded protein and initiates the retargeting for retrograde transport into the cytosol [3,5,9]. Although yeast lacks the re-glucosylating UDP-glucose:glycoprotein glucosyltransferase, the basic machinery for carbohydrate trimming and recognition of malfolded glycoproteins is present and functions like in mammalian cells [10–14].

We have undertaken a genome-wide screen for yeast mutants defective in ER-quality control and associated protein degradation (ERAD). Using this screen, we recently identified Dsk2p and Rad23p as proteins delivering the polyubiquitinated substrate from the trimeric Cdc48-Ufd1-Npl4p complex to the proteasome [15]. Among the newly discovered proteins was Yos9p, previously involved in ER-to-Golgi transport of GPI-anchored proteins [16]. Yos9p is a lumenal, membrane-associated ER protein with homology to human OS-9 which is found in all tissues and amplified in osteosarcomas [17,18]. Yos9p has a mannose-6-phosphate receptor homology domain of unknown function [18–20]. Here, we show that Yos9p is required for the ER-associated degradation of N-glycosylated proteins.

* Corresponding author. Fax: +49-711-685-4392.
E-mail address: dieter.wolf@ibc.uni-stuttgart.de (D.H. Wolf).

[1] These authors, in alphabetical order, contributed equally to this work.
[2] Present address: Center for Cancer Research, NCI, NIH, Frederick, Maryland 21701, USA.

Abbreviations: CPY, carboxypeptidase Y; CPY*, mutated carboxypeptidase Y; CTG*, mutated carboxypeptidase Y transmembrane domain – GFP; CTL*, mutated carboxypeptidase Y transmembrane domain – Leu2p; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; GPI, glycosyl phosphatidyl inositol; MRH, mannose-6-phosphate receptor homology; PDI, protein disulfide isomerase; UPR, unfolded protein response

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.10.039

2. Material and methods

2.1. Yeast strains and plasmids

Molecular biological and genetic techniques were carried out using standard methods [21,22]. *S. cerevisiae* strains used in this study are based on W303ΔC (*MATa*, *ade2-1*, *can1-100*, *his3-11 15*, *leu2-3 112*, *trp1-1*, *ura3-1*, *prc1*Δ::*LEU2*) [23]. Cells were grown at 30 °C or 38 °C (strains carrying the temperature sensitive *sec61-2* allele) in synthetic complete media. Generation of strain YZK105 (Δ*prc1*Δ*htm1*) is described by Z. Kostova and D.H. Wolf, submitted. Strain YBB1 was generated from strain YZK105 according to Longtine [24] using plasmid pFA6a-His3MX6 and the primer set 5′ yos9del (GATCTTCACATATATCGTTATCATCCCCTTTCTTCCCTGTTTCACGGATCCCCGGGTTAATTAA) and 3′ yos9del (CAGCTGCTACGTTTGTTACCTCCGATTCAGTATTCCCTGTCGAATTCGAGCTCGTTTAAAC).

Strains YXL009 (Δ*prc1*Δ*der3*/*hrd1*) and YBB9 (Δ*prc*1Δ*yos*9) were obtained by crossing and tetrad dissection of W303ΔC to YWO0433 (Δ*der3*Δ*hrd3*) and YBB1 to W303-1B [25], respectively.

The *prc1-1* insert from bMK150 [23] was subcloned into pRS316 [26] to express CPY*. Construction of the glycosylation mutant CPY*0000 and of pRS316Gal4-Sec61-2-Leu2p is described by Z. Kostova and D.H. Wolf, submitted. Plasmid pRS316 expressing Gal4-CTL* was described by Medicherla et al. [15] and pTV3 expressing *Sec61-2::3HA* by Caldwell et al. [27]. For complementation assay, Yos9p was expressed from pYOS9 based on plasmid pRS313.

2.2. Pulse-chase analysis and immunoprecipitation

Pulse-chase experiments were carried out essentially as described by Taxis et al. [28]. To follow Sec61-2-HAp degradation, 10 OD cells/time point were used and cultures were shifted to 38 °C for 10 min before labeling. Microsomal membranes were prepared as described in [29]. Polyclonal CPY [30] and monoclonal HA (Convance) antibodies were used for immunoprecipitation. Samples were separated by SDS–PAGE on 8% gels and analyzed using PhosphoImager and ImageQuaNT™ software.

3. Results

We carried out a genome-wide screen using the EUROSCARF yeast library consisting of about 5000 *Saccharomyces cerevisiae* strains, each deleted for a single non-essential gene [15], to look for new components involved in protein quality control and ER-associated degradation. As a ''sensor'' for defective ER quality control or degradation, we used the modular substrate CTL*. CTL* consists of CPY* in the ER lumen connected to the Leu2 protein (3-isopropylmalate-dehydrogenase) in the cytoplasm via a transmembrane domain (Fig. 1A). Due to recognition of malfolded CPY* in the ER lumen, the hybrid CTL* protein is retrotranslocated to the cytosol and readily degraded in wild type cells resulting in a ''no growth'' phenotype in leucine-auxotrophic cells incubated in leucine-deficient growth medium. In contrast, leucine-deficient cells can grow on media lacking leucine when a component of the ER quality control or degradation system is missing: impaired degradation of CTL* results in complementation of the leucine deficiency. Most of the ERAD-components known to date, as well as a number of new potential ERAD players, were recovered from this screen [15]. One of the strains expressing CTL* and capable of growth in the absence of exogenous leucine supplementation carried a deletion in YDR057W, encoding the Yos9 protein (Fig. 1B). CTL* is a derivative of CTG*, a N-glycosylated protein carrying four carbohydrate chains [31]. To investigate whether Yos9p is specifically involved in quality control of glycosylated proteins, we examined the growth of Δ*yos9* cells expressing Leu2p fused to the malfolded non-glycosylated ER protein Sec61-2p [29] (Fig. 1A). Δ*yos9* cells, as well as cells defective in the lectin Htm1p/Mnl1p, expressing Sec61-2-Leu2p are unable to grow at 38 °C (Fig. 1C), the restrictive temperature that induces Sec61-2p unfolding [29]. This confirms that Sec61-2-Leu2p is degraded in Δyos9 and Δ*htm1*Δ*mnl1* cells as in wild type. To substantiate the finding that Yos9p is a component of the quality control system for malfolded ER glycoproteins, we performed pulse-chase measurements with soluble CPY*, membrane-bound CTG*, and unglycosylated Sec61-2-HAp as ERAD substrates [31,32]. We found that both CPY* (Fig. 2A) and CTG* (Fig. 2B) degradation is considerably slowed down in cells deficient in Yos9p. However, there was no observable difference in turnover of Sec61-2-HAp between Δ*yos9* and wild type cells (Fig. 2C). As expected [29,33], degradation of Sec61-2-HAp is substantially inhibited in cells lacking the ubiquitin-protein

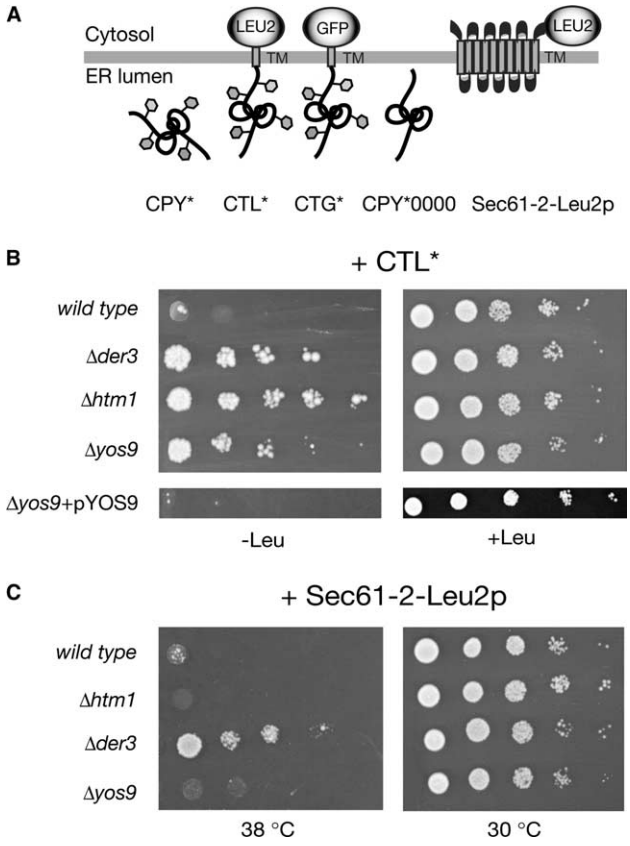
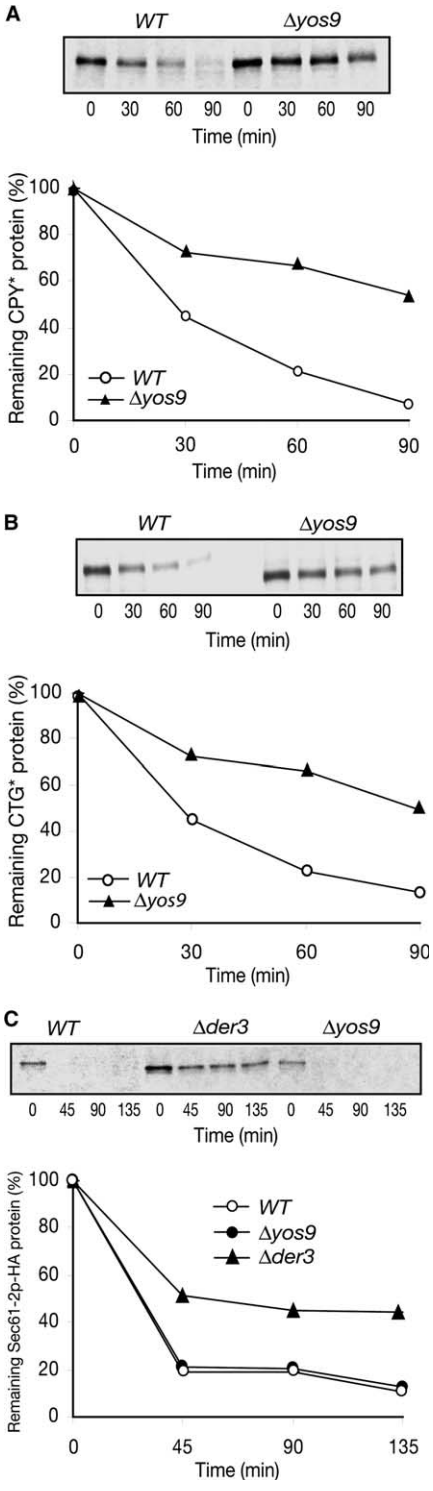


Fig. 1. Model ERAD substrates used in this study and identification of Yos9p as a putative ERAD component involved in the degradation of glycoproteins. (A) Schematic representation of the ERAD substrates CPY*, CTL*, CTG*, CPY*0000 and Sec61-2-Leu2p. (B) Isogenic wild type and mutant cells were plated in serial dilutions on CM medium with and without leucine. Uracil was also omitted to select for cells carrying the URA3-based CTL* plasmid. Plates were incubated for 2–4 days. The leucine auxotrophic WT strain expressing CTL* cannot grow in the absence of leucine. However, the leucine deficiency is complemented by CTL* in the ERAD defective strains Δ*der3* and Δ*htm1*, and in a strain carrying a deletion of *YOS9*. Wild type phenotype is observed when Yos9p is expressed from plasmid pYOS9 in Δ*yos9* cells. (C) Wild type and mutant cells expressing the conditional non-glycosylated ERAD substrate Sec61-2-Leu2p were plated as described in (**B**) and incubated at 30 °C (control) and 38 °C for 4–5 days. Lethality due to leucine deficiency is complemented only in the Δ*der3* strain, indicating that Yos9p, just like Htm1p, does not participate in the degradation of non-glycosylated substrates.

ligase Der3p/Hrd1p (Fig. 2C). We investigated the contribution that Yos9p and Htm1p/Mnl1p make to the degradation of glycosylated CPY*. The stabilization caused by the absence of Yos9p is slightly, but consistently, greater (~10%) than that caused by deletion of Htm1p/Mnl1p. However, CPY* degradation is not affected further in the double deletion Δ*yos9*Δ*htm1*, indicating that their effects are not additive (Fig. 3). Finally, we examined the degradation of CPY*0000 (formerly d4CPY*), a CPY* species lacking all four N-glycans ([11]; Z. Kostova and D.H. Wolf, submitted). Degradation of this fully unglycosylated CPY* is considerably delayed in wild type cells (Fig. 4). Deletion of neither Htm1p/Mnl1p nor Yos9p results in additional stabilization of CPY*0000, indicating a requirement for carbohydrate chains on the malfolded protein for Htm1p/Mnl1p or Yos9p action (Fig. 4).



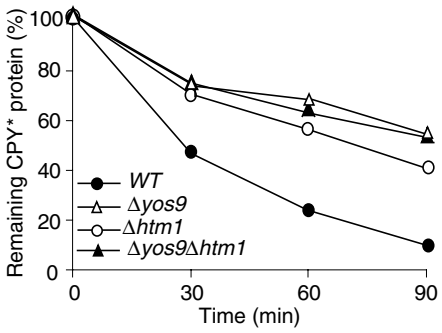


Fig. 3. Yos9p and Htm1p do not exhibit an additive effect in CPY* degradation. Degradation of CPY* in wild type (WT), Δ*yos9*, Δ*htm1*, and Δ*htm1*Δ*yos9* strains of the W303 background was quantified by pulse-chase analysis. Absence of Htm1p leads to a stabilization of ~35% following a 90 min chase. Deletion of Yos9p alone or, both of Htm1p and Yos9p results in a slightly higher stabilization (~45%) of CPY* during the same time interval. Data represent the average of three independent experiments with an average deviation of less than 4% for each time point.

4. Discussion

Yos9p is a protein of unknown function that appears to be the yeast homolog of the ubiquitous human protein, OS-9. The function of OS-9 is not well characterized, however, its chromosomal locus lies within a region frequently amplified in human sarcomas [17]. The highly conserved rat and mouse homolog is a peripheral ER protein exposed to the cytoplasm and associates transiently with the metalloendoproteinase meprin β, possibly mediating its ER-to-Golgi transport [34]. To date, the only study directly addressing Yos9p function has been undertaken in yeast in relation to ER-to-Golgi transport of GPI-anchored proteins [16]. Interestingly, the yeast protein possesses a carboxy-terminal HDEL ER-retention motif. In fact, localization studies show that Yos9p is a lumenal glycoprotein tightly associated with the ER membrane. Yos9p can be immunoprecipitated with GPI-anchored Gas1p and Mkc7p. The maturation rate of Gas1p was found to be directly

Fig. 2. Degradation of CPY* and CTG* but not of unglycosylated Sec61-2-HAp is inhibited in the absence of Yos9p. Isogenic wild type (WT) and *yos9* cells each expressing plasmid encoded CPY* (A) or CTG* (B) were labeled with [^{35}S]methionine and chased for the indicated times. Cell extracts were prepared and subjected to immunoprecipitation with CPY antibodies. Data represent the average of three independent experiments with an average deviation of less than 4% for each time point. (C) Wild type (WT), Δ*der3*, and Δ*yos9* strains of the W303 background expressing plasmid encoded Sec61-2-HAp were shifted to 38 °C for 10 min prior to labeling, to induce unfolding of the substrate. Samples were chased for the indicated times and membrane fractions were prepared. Sec61-2-HAp was immunoprecipitated with HA-antibodies. Data represent the average of four independent experiments with an average deviation of ~5% for each time point.

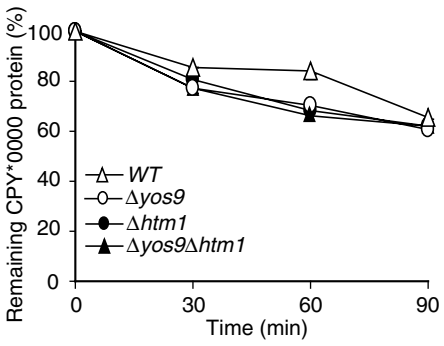


Fig. 4. Deletion of *YOS9* does not affect the degradation of unglycosylated CPY*. Pulse-chases analysis reveals no difference in the degradation kinetics of CPY*0000 expressed in the isogenic strains Δ*yos9*, Δ*htm1*,Δ*htm1*Δ*yos9* and wild type. Data represent the average of three independent experiments with an average deviation of less than 5% for each time point.

proportional to the level of Yos9p present, suggesting a direct involvement of Yos9p in GPI-anchored protein processing [16].

In this study, we show that Yos9p is necessary for efficient degradation of the glycosylated ERAD substrates CPY* and CTG*, but plays no role in the degradation of unglycosylated Sec61-2-HAp (Fig. 2). Moreover, unglycosylated CPY*0000, whose degradation is already impaired in wild type cells, is not further affected due to loss of Yos9p (Fig. 4), underscoring the link between glycosylation and Yos9p function. Like human OS-9, yeast Yos9p contains a mannose-6-phosphate receptor homology (MRH) domain, which may play a general role in N-glycan recognition [20]. A similar domain is found in the β-subunits of *H. sapiens*, *S. pombe* and *S. cerevisiae* glucosidase II [20], an enzyme required for ER quality control [4]. We propose that Yos9p is a lectin with a general role in the ER-quality control of misfolded N-glycosylated proteins. In fact, like Htm1p/Mnl1p [12,13], Yos9p is not required for transport of properly folded glycoproteins such as CPY and invertase, nor does its absence lead to the unfolded protein response (UPR) [16].

To date, with the exception of calnexin/calreticulin, the only other ER-lectin directly associated with ER-quality control is Htm1/Mnl1p (EDEM in mammals) [12,13,35–37]. Htm1/Mnl1p is believed to recognize the $Man_8GlcNAc_2$ degradation signal of improperly folded ER glycoproteins. Comparison of the degradation kinetics of CPY*0000 in wild type, Δ*htm1*, Δ*yos9*, and Δ*htm1*Δ*yos9* strains indicates that, just like Htm1p (Z. Kostova and D.H. Wolf, in preparation), Yos9p function depends on the presence of glycan chains on the malfolded substrate (Fig. 4). We also find that the action of Yos9p and Htm1/Mnl1p in ERAD is not additive (Fig. 3). Loss of Yos9p has a slight, but consistently stronger effect on CPY* degradation than does the deletion of Htm1/Mnl1p, although we cannot exclude that this difference may fall within the range of experimental error (Fig. 3). One possibility is that Htm1p and Yos9p target the substrate simultaneously (with or without a predetermined order), but recognize different glycan-based determinants, therefore act independent of each other. Alternatively, Htm1p may be necessary for full Yos9p function, or vice versa (depending on whether the 10% difference is statistically significant or not). It may also be speculated that Yos9p is part of the delivery process of the glycosylated substrate to the retrotranslocation machinery.

The still undefined involvement of Yos9p in GPI anchor processing does not exclude its participation in ER quality control of malfolded proteins. It is plausible that Yos9p may be a dual-function ER-quality control component, like calnexin and PDI [3], with a general lectin-chaperone role for glycosylated malfolded substrates and a more direct role in the processing or even quality control of GPI-anchored proteins. Disposal of proteins that fail to become GPI-anchored either due to the presence of a faulty GPI-anchoring signal or due to problems in the GPI-anchor assembly pathway seems to occur via intracellular cytoplasmic degradation with ERAD features [38,39]. Proteins destined to acquire a GPI-anchor may appear as malfolded to the ER quality control machinery till they become attached to the GPI-anchor. Yos9p may be the component involved in quality control of GPI-anchor addition. Recognition and function may lie within the glycans (either on Yos9p or the substrate, or both) or the mannose-6-phosphate receptor homology (MRH) domain or a, still, unknown determinant. Studies addressing the multiple aspects of Yos9p action will broaden our understanding, both of ERAD and GPI-anchor addition, and reveal the link between these processes.

Acknowledgements: We thank A.A. Cooper and L. Xiao for plasmids and strains. The help of E. Tosta, S.-H. Park and A. Schäfer with preparation of the manuscript is gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

References

[1] Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996) Annu. Rev. Biochem. 65, 271–303.
[2] Haigh, N.G. and Johnson, A.E. (2002) in: Protein targeting, transport and translocation (Dalbey, R.E. and von Heijne, G., Eds.), pp. 74–106, Academic Press, London-New York.
[3] Ellgaard, L. and Helenius, A. (2003) Nat. Rev. Mol. Cell Biol. 4, 181–191.
[4] Helenius, A. and Aebi, M. (2001) Science 291, 2364–2369.
[5] Kostova, Z. and Wolf, D.H. (2003) Embo J. 22, 2309–2317.
[6] Plemper, R.K. and Wolf, D.H. (1999) Trends Biochem. Sci. 24, 266–270.
[7] Brodsky, J.L. and McCracken, A.A. (1999) Semin. Cell Dev. Biol. 10, 507–513.
[8] Sommer, T. and Wolf, D.H. (1997) Faseb J. 11, 1227–1233.
[9] Ellgaard, L., Molinari, M. and Helenius, A. (1999) Science 286, 1882–1888.
[10] Hitt, R. and Wolf, D.H. (2004) FEMS Yeast Res. 4, 815–820.
[11] Knop, M., Hauser, N. and Wolf, D.H. (1996) Yeast 12, 1229–1238.
[12] Nakatsukasa, K., Nishikawa, S., Hosokawa, N., Nagata, K. and Endo, T. (2001) J. Biol. Chem. 276, 8635–8638.
[13] Jakob, C.A. et al. (2001) EMBO Rep. 2, 423–430.
[14] Jakob, C.A., Burda, P., Roth, J. and Aebi, M. (1998) J. Cell Biol. 142, 1223–1233.
[15] Medicherla, B., Kostova, Z., Schaefer, A. and Wolf, D.H. (2004) EMBO Rep. 5, 692–697.
[16] Friedmann, E., Salzberg, Y., Weinberger, A., Shaltiel, S. and Gerst, J.E. (2002) J. Biol. Chem. 277, 35274–35281.
[17] Su, Y.A., Hutter, C.M., Trent, J.M. and Meltzer, P.S. (1996) Mol. Carcinog. 15, 270–275.
[18] Kimura, Y., Nakazawa, M. and Yamada, M. (1998) J. Biochem. (Tokyo) 123, 876–882.
[19] Schrag, J.D., Procopio, D.O., Cygler, M., Thomas, D.Y. and Bergeron, J.J. (2003) Trends Biochem. Sci. 28, 49–57.

[20] Munro, S. (2001) Curr. Biol. 11, R499–501.
[21] Ausubel, F.M., Kingston, R.E., Seidman, F.G., Struhl, K., Moore, D.D., Brent, R. and Smith, F.A. (1992) (Greene, Ed.) Greene, New York.
[22] Guthrie, C. and Fink, G.R. (1991) in: Methods in Enzymology, Vol. 194, San Diego.
[23] Plemper, R.K., Deak, P.M., Otto, R.T. and Wolf, D.H. (1999) FEBS Lett. 443, 241–245.
[24] Longtine, M.S., McKenzie3rd, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J.R. (1998) Yeast 14, 953–961.
[25] Chiang, H.L. and Schekman, R. (1991) Nature 350, 313–318.
[26] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
[27] Caldwell, S.R., Hill, K.J. and Cooper, A.A. (2001) J. Biol. Chem. 276, 23296–23303.
[28] Taxis, C., Vogel, F. and Wolf, D.H. (2002) Mol. Biol. Cell 13, 1806–1818.
[29] Biederer, T., Volkwein, C. and Sommer, T. (1996) Embo J. 15, 2069–2076.
[30] Finger, A., Knop, M. and Wolf, D.H. (1993) Eur. J. Biochem. 218, 565–574.
[31] Taxis, C., Hitt, R., Park, S.H., Deak, P.M., Kostova, Z. and Wolf, D.H. (2003) J. Biol. Chem. 278, 35903–35913.
[32] Hiller, M.M., Finger, A., Schweiger, M. and Wolf, D.H. (1996) Science 273, 1725–1728.
[33] Bordallo, J., Plemper, R.K., Finger, A. and Wolf, D.H. (1998) Mol. Biol. Cell 9, 209–222.
[34] Litovchick, L., Friedmann, E. and Shaltiel, S. (2002) J. Biol. Chem. 277, 34413–34423.
[35] Wang, T. and Hebert, D.N. (2003) Nat. Struct. Biol. 10, 319–321.
[36] Molinari, M., Calanca, V., Galli, C., Lucca, P. and Paganetti, P. (2003) Science 299, 1397–1400.
[37] Oda, Y., Hosokawa, N., Wada, I. and Nagata, K. (2003) Science 299, 1394–1397.
[38] Ali, B.R., Claxton, S. and Field, M.C. (2000) FEBS Lett. 483, 32–36.
[39] Wilbourn, B., Nesbeth, D.N., Wainwright, L.J. and Field, M.C. (1998) Biochem. J. 332, 111–118.