2.2. Yeast strains

The wild-type yeast strain used in this study was W303 (MATa *ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1*). During the screening, W303 with a VCP expression plasmid was mutagenized with ethyl methanesulfonate (EMS; Sigma–Aldrich Co. LLC, St. Louis, MO, USA) and plated on glucose at 25 °C. The viability of EMS-treated cells was 49% compared to that of non-treated cells. Among approximately 40 000 EMS-treated cells, colonies that grew on galactose but not glucose at 37 °C were selected. We obtained one strain from the screening, and the strain was crossed with W303 three times to generate the 1–30 strain (*gpi10-2*) to eliminate extraneous mutations. During the screening, we also tried to generate strains that grew on glucose but not on galactose at 37 °C, but we were unable to generate such strains.

2.3. Plasmid construction

A list of the plasmids used in this study is provided in Supplementary Table 1. A plasmid expressing VCP under the *GAL1* promoter (M123) was created as follows: the EcoRI-BamHI fragment of pRS316 was ligated to the EcoRI-BamHI fragment of the *GAL1* promoter to create V039. The pCMX-VCP-GFP plasmid was cut with Asp718 and BamHI and blunted and ligated with a BamHI fragment of V039, which had been blunted. The plasmids expressing VCP or Cdc48 under the *GPD* promoter have been described previously [6]. The pRS316-GPI10(WT) and pRS316-UBI4(WT) plasmids were constructed as described below. The DNA of *GPI10* and *UBI4* were amplified by polymerase chain reaction (PCR) from wild-type genomic DNA and then cloned into pRS316. pRS316-GPI10(*gpi10-2*) was constructed by cloning the amplified *gpi10-2* from the genomic DNA of 1–30 into pRS316. The pR12-28 and pR12-19 plasmids were obtained from the YEP13-based yeast genomic library, which was a gift from Dr. K. Matsumoto.

The pCZY1 plasmid, which contained a *lacZ* reporter gene that was driven by the *CYC1* core promoter and that was fused with the unfolded protein response (UPR) element (UPRE) to monitor UPR activity [7], was a gift from Dr. Y. Kimata.

2.4. Growth assay of yeast

For the spot assay, the cell densities were adjusted to OD600 = 1.0 and diluted serially by 10-fold dilutions. Each diluted sample was spotted on the indicated plates and incubated for 2 or 3 days at the indicated temperatures.

2.5. β-Galactosidase assay for UPR response

The β-galactosidase activity for the UPR response was determined according to the protocol of Kimata et al. [8].

3. Results

3.1. Screening of the temperature-sensitive mutants with growth defects that were rescued by VCP overexpression

To find novel VCP/Cdc48 functions and eventually find proteins that are functionally related to VCP/Cdc48, we generated yeast temperature-sensitive (ts) mutants that had growth defects that were suppressed by the expression of human/mouse VCP (Fig. 1A). Briefly, wild-type cells with a plasmid expressing VCP under the control of the *GAL1* promoter, which functions in galactose media but not in glucose media, was treated with EMS and the colonies were replicated. Each replica was plated onto glucose or galactose media. Colonies that grew in galactose but not in glucose

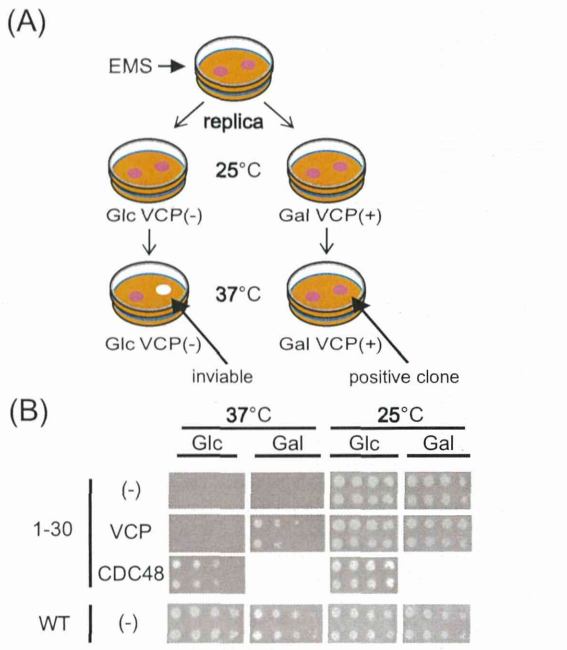

Fig. 1. Isolation of the temperature-sensitive (ts) mutant with ts growth defects that were rescued by VCP and Cdc48. (A) Screening strategy. Wild-type cells with a plasmid expressing VCP under a galactose-inducible promoter (GAL1p) were mutagenized with ethyl methanesulfonate. Ts mutants were screened on glucose and for suppressing ts on galactose at 37 °C. (B) Isolation of the ts strain 1–30. Cells with or without exogenously expressed VCP and Cdc48 were diluted, spotted on synthetic casamino medium (SC)-ura or SGal-ura plates, and grown at the indicated temperatures for 3 days.

media at 37 °C were selected. From about 40 000 EMS-treated cells, we obtained one strain (referred to as 1–30), which was able to grow only in the presence of VCP expression at 37 °C (Fig. 1B). In addition to VCP, the overexpression of yeast Cdc48 also rescued the growth defect of strain 1–30 at 37 °C (Fig. 1B), which suggested that some conserved function(s) of the VCP/Cdc48 families were responsible for the suppression.

To identify the mutated gene in the 1–30 strain, we screened yeast genomic multicopy libraries for plasmids that would complement its ts phenotype. One plasmid (pR12-28) allowed the 1–30 strain to grow at 37 °C (data not shown). A deletion analysis of pR12-28 identified the suppression activity in a region containing the entire *GPI10* open reading frame and its flanking regions (Fig. 2A). Another plasmid pR12-19 rescued the ts phenotype of 1–30 but not as efficiently as pR12-28 did (data not shown). A deletion analysis of pR12-19 identified a region containing *UBI4*, which is a polyubiquitin gene, and its flanking region for the partial ts-suppressing activity. Moreover, we found that the expression of *UBI4* by a multicopy plasmid [pRS426-UBI4(WT)] but not by a single-copy plasmid [pRS316-UBI4(WT)] was required for the efficient suppression of the temperature sensitivity of the 1–30 mutant (Fig. 2B).

3.2. Identification of the mutation in GPI10 in the 1–30 strain

The efficient suppressive activity that occurred by the introduction of *GPI10* in a single-copy plasmid suggested that the mutated gene in strain 1–30 was *GPI10*. Therefore, we amplified *GPI10* and its flanking region by polymerase chain reaction (PCR) from 1–30 genomic DNA and then tested for ts suppression in a single-copy plasmid. Indeed, it failed to rescue the growth defect of strain 1–30 at 37 °C (Fig. 2A, bottom). In contrast, *UBI4* and its flanking

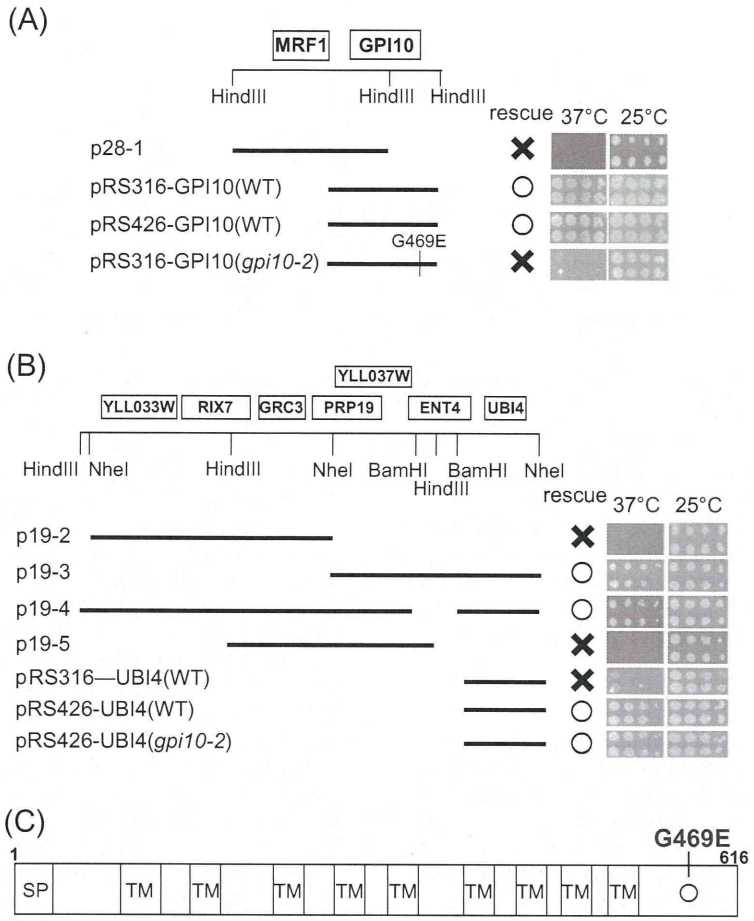

Fig. 2. Identification of the mutation in *GPI10* in strain 1–30. (A) Mapping of the suppressing gene for the 1–30 ts mutant to identify *GPI10*. Plasmids were checked for their ability to suppress the ts growth of 1–30. pRS316-GPI10(WT) and pRS426-GPI10(WT) contained *GPI10* DNA fragments that were obtained by polymerase chain reaction (PCR) as wild-type genomic DNA, and pRS316-GPI10 (*gpi10-2*) contained a PCR-amplified *GPI10* DNA fragment from 1–30 cells. Cells with a plasmid were diluted, spotted on YPD plates, and grown at the indicated temperatures for 3 days. Two independent transformed cells are shown in two columns. (B) Mapping of the suppressing gene to identify *UBI4*. Plasmids pRS316-UBI4(WT) and pRS426-UBI4(WT) contained *UBI4* DNA that was obtained by PCR with wild-type genomic DNA, and pRS426-UBI4 (*gpi10-2*) contained PCR-amplified *UBI4* DNA fragment from 1–30 cells. (C) Localization of the G469E point mutation in the Gpi10 protein. SP indicates a predicted signal peptide, and TM indicates a predicted transmembrane region.

region, which were obtained from 1–30 genomic DNA, rescued the growth defect of strain 1–30 at 37 °C in a multi-copy plasmid [Fig. 2B, pRS426-UBI4(*gpi10-2*)]. These results indicated that strain 1–30 contained the responsible mutation in *GPI10* but not in *UBI4*.

Glycosylphosphatidylinositol (GPI) anchor proteins are membrane integral proteins that function at the cell surface [9,10]. GPI anchors are glycolipids that consist of phosphatidylinositol, glucosamine, mannose, and ethanolaminephosphate, and they are made through many successive enzymatic steps in the ER. After the final step, the resultant GPI-anchor is added to substrate proteins to make GPI-anchor proteins. During the process of GPI-anchor synthesis in yeast, Gpi10, which is a mannosyl transferase, transfers the third mannose to a GPI intermediate containing already two mannoses linked to glucosamine-phosphatidyl inositol, which is an intermediate of the GPI anchor [9]. *GPI10* is essential and encodes a protein with nine transmembrane domains in the ER membrane with a C-terminus region toward the cytosol [11].

By sequencing the *GPI10* gene from the 1–30 mutant, we identified a single nucleotide change from the 1406th guanine to adenine in the *GPI10* gene. This mutation changed the 469th amino acid in Gpi10 from Gly to Glu at and was predicted to be located in the C-terminal cytoplasmic tail of Gpi10 (Fig. 2C) [12]. Introducing the mutation into the *GPI10* gene of wild-type cells conferred a ts phenotype (data not shown). Therefore, we concluded that the mutated gene in strain 1–30 was *GPI10*. The mutated allele in *GPI10* is referred to as *gpi10-2* hereafter because a mutant for *GPI10* (*gpi10-1*) has been previously reported [13].

3.3. Sensitivities of cell-wall stressors in gpi10-2 mutant

Because defects in GPI-anchor proteins lead to a loss of integrity of the cell wall [14,15], we examined the effects of cell-wall stressors on the growth of the *gpi10-2* mutant. As expected, the *gpi10-2* mutant showed enhanced sensitivities to the two cell-wall stressors that were tested, sodium dodecyl sulfate and caffeine,

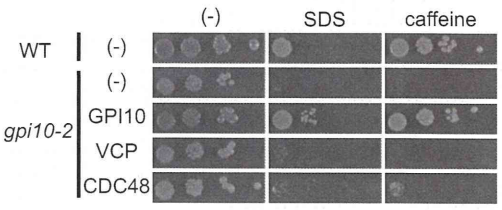

Fig. 3. Sensitivities against cell-wall stressors in the *gpi10-2* mutant. The effect of cell-wall stressors. Wild-type and *gpi10-2* cells with or without exogenously expressed VCP or Cdc48 were diluted, spotted on SD-uracil(−) and SD uracil(−) containing 0.006% SDS or 3 mM caffeine, and incubated at 33 °C for 2 days.

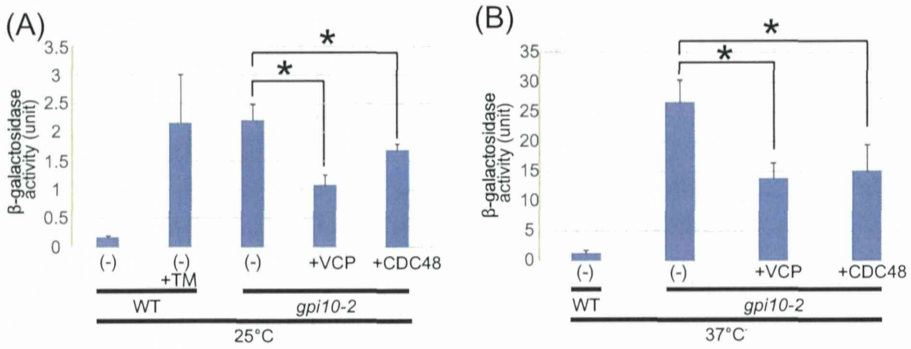

Fig. 4. Increased unfolded protein response (UPR) in the *gpi10-2* mutant. (A) β-gal activities at 25 °C. Cells with or without exogenously expressed VCP or Cdc48 were measured for β-galactosidase activity. Tunicamycin (TM) was added at 2 μg/mL for 2 h. The data are presented as the mean ± standard error of the mean (S.E.) values of four independent experiments. *$P < 0.05$. (B) The β-gal activities after 2 h at 37 °C.

compared to those of wild-type cells, which suggested that GPI anchoring was defective in the *gpi10-2* mutant (Fig. 3).

We examined whether VCP or Cdc48 expression rescued the cell wall-related sensitivities of *gpi10-2*. Unexpectedly, we did not see the recovery of growth defects against the cell-wall stressors by VCP overexpression and saw a very small, if any, rescue by Cdc48 overexpression in the *gpi10-2* mutant (Fig. 3).

3.4. Increased UPR in the gpi10-2 mutant

The UPR is induced by the accumulation of unfolded proteins in the ER [16]. Because the induction of the UPR was reported in deletion mutants of *BST1* and *LAS21*, which are both involved in GPI-anchor production [17], we tested whether the *gpi10-2* mutation would cause the UPR. In the UPR, Ire1, which is a transmembrane protein with endoribonuclease activity, is one of the core mediators of the intracellular UPR signal. Ire1 functions in the splicing of *HAC1* mRNA to produce the translatable mRNA for Hac1, which is a transcription factor that induces ER chaperones. Hac1 directly binds to the UPRE for the expression of ER chaperones. Therefore, we checked the activation of UPR by the induction of a *lacZ* reporter under the control of UPRE (Fig. 4). Treatment with tunicamycin, which is a well-known inducer of UPR, clearly induced the β-galactosidase activity up to 12.8-fold in wild-type cells at 25 °C (Fig. 4A). We observed that, without any inducer, β-galactosidase activity was constitutively activated in the *gpi10-2* mutant even at the permissive temperature of 25 °C; the activity was about 13-fold higher in the *gpi10-2* mutant compared to that in the wild type. Shifting to a high temperature induced UPR in both the wild-type and *gpi10-2* mutant, β-galactosidase activity reached much higher levels in the *gpi10-2* mutant than in wild-type cells (Fig. 4B). We observed that exogenous VCP and Cdc48 overexpression decreased the β-galactosidase activity partly but not completely. These results suggested that the *gpi10-2* mutation induced UPR and that the overexpression of VCP/Cdc48 partially reduced the increased levels of UPR.

4. Discussion

In this study, we isolated a ts mutant with a mutation of *GPI10*, *gpi10-2*, which had temperature-sensitive growth defects that were rescued by VCP or Cdc48 overexpression. The *gpi10-2* mutant contained a single nucleotide change that resulted in a single amino-acid change of Gly469Glu in Gpi10 and that was located in the C-terminus region of Gpi10. The function of the C-terminus region of Gpi10 is unknown.

Currently, the mechanism of how VCP/Cdc48 expression rescued the ts phenotype of *gpi10-2*, is not clear. It is noteworthy that ubiquitin overexpression has been shown to rescue the ts mutant of *LCB1*, which encodes a serine palmitoyltransferase, the first enzyme in ceramide biosynthesis; this restores protein degradation, and reduces aggregates of the *lcb1* mutant [18]. Moreover, the maturation of GPI-anchored proteins is delayed in the *lcb1* mutant [19]. Given that VCP/Cdc48, together with some of its cofactors, functions in ubiquitin–proteasome-mediated degradation, such as ER-associated protein degradation (ERAD), it is possible that ERAD might be induced by the overexpression of VCP/Cdc48 to degrade misfolded proteins due to the lack of GPI anchors in ER, which might be the underlying cause of the ts phenotype. Consistent with this notion, *UBI4* expression also rescued the ts phenotype of *gpi10-2*, albeit partially. However, this hypothesis may be oversimplistic. We have not observed any apparent exacerbation of the growth defects, at both 25 °C and 33 °C, in double mutants of *gpi10-2* combined with either *cdc48-3*, Δ*hrd1*, Δ*doa10*, Δ*ubx2*, Δ*ubx3*, Δ*ubx4*, Δ*ubx5*, Δ*ubx6*, Δ*ubx7*, Δ*npl4*, Δ*shp1*, *ufd1-2*, or Δ*ufd3* (data not shown). At the least, these observations appear to preclude the involvement of ERAD in the ts phenotypes of *gpi10-2*. Therefore, we do not know, at present, where and how VCP/Cdc48 works to rescue the ts phenotype of *gpi10-2*.

Even with the full recovery of the growth ability of *gpi10-2* by the expression of VCP or Cdc48, this condition could only partially reduce the increased UPR, and it was not able to rescue its increased sensitivities to cell-wall stressors. Both defective phenotypes were probably due to the defective formation of GPI-anchor proteins, which was expected from the defectiveness of the mannosyl transferase activity in the *gpi10-2* mutant. These observations suggested that the mannosyl transferase activity was defective in the *gpi10-2* mutant at 37 °C and that VCP expression did not rescue this defect at 37 °C. Notwithstanding, VCP expression rescued the growth defects of the *gpi10-2* mutant at 37 °C, indicating the possibilities that Gpi10 has uncovered cell growth-related functions that differ from mannosyl transferase activity and that these functions are rescued by VCP expression. These possibilities remain to be clarified.

Acknowledgements

We thank Drs. K. Matsumoto, K. Kohno, Y. Kimata, P. Orlean, A. Conzelmann, Y. Yoshida, and Y. Saeki for materials and advice, and the members of the Kakizuka and Tanaka labs for helpful discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.01.017.

References

[1] Meyer, H., Bug, M. and Bremer, S. (2012) Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. 14, 117–123.

[2] Stolz, A., Hilt, W., Buchberger, A. and Wolf, D.H. (2011) Cdc48: a power machine in protein degradation. Trends Biochem. Sci. 36, 515–523.

[3] Kimonis, V.E., Kovach, M.J., Waggoner, B., Leal, S., Salam, A., Rimer, L., Davis, K., Khardori, R. and Gelber, D. (2000) Clinical and molecular studies in a unique family with autosomal dominant limb-girdle muscular dystrophy and Paget disease of bone. Genet. Med. 2, 232–241.

[4] Watts, G.D., Wymer, J., Kovach, M.J., Mehta, S.G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M.P. and Kimonis, V.E. (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. Nat. Genet. 36, 377–381.

[5] Johnson, J.O., Mandrioli, J., Benatar, M., Abramzon, Y., Van Deerlin, V.M., Trojanowski, J.Q., Gibbs, J.R., Brunetti, M., Gronka, S., Wuu, J., Ding, J., McCluskey, L., Martinez-Lage, M., Falcone, D., Hernandez, D.G., Arepalli, S., Chong, S., Schymick, J.C., Rothstein, J., Landi, F., Wang, Y.D., Calvo, A., Mora, G., Sabatelli, M., Monsurro, M.R., Battistini, S., Salvi, F., Spataro, R., Sola, P., Borghero, G., Galassi, G., Scholz, S.W., Taylor, J.P., Restagno, G., Chio, A. and Traynor, B.J. (2010) Exome sequencing reveals VCP mutations as a cause of familial ALS. Neuron 68, 857–864.

[6] Takata, T., Kimura, Y., Ohnuma, Y., Kawawaki, J., Kakiyama, Y., Tanaka, K. and Kakizuka, A. (2012) Rescue of growth defects of yeast cdc48 mutants by pathogenic IBMPFD-VCPs. J. Struct. Biol. 179, 93–103.

[7] Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M.J. and Sambrook, J.F. (1992) A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins. EMBO J. 11, 2583–2593.

[8] Kimata, Y., Kimata, Y.I., Shimizu, Y., Abe, H., Farcasanu, I.C., Takeuchi, M., Rose, M.D. and Kohno, K. (2003) Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol. Biol. Cell 14, 2559–2569.

[9] Orlean, P. (2012) Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. Genetics 192, 775–818.

[10] Pittet, M. and Conzelmann, A. (2007) Biosynthesis and function of GPI proteins in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 1771, 405–420.

[11] Sutterlin, C., Escribano, M.V., Gerold, P., Maeda, Y., Mazon, M.J., Kinoshita, T., Schwarz, R.T. and Riezman, H. (1998) *Saccharomyces cerevisiae* GPI10, the functional homologue of human PIG-B, is required for glycosylphosphatidylinositol-anchor synthesis. Biochem. J. 332 (Pt 1), 153–159.

[12] Oriol, R., Martinez-Duncker, I., Chantret, I., Mollicone, R. and Codogno, P. (2002) Common origin and evolution of glycosyltransferases using Dol-P-monosaccharides as donor substrate. Mol. Biol. Evol. 19, 1451–1463.

[13] Canivenc-Gansel, E., Imhof, I., Reggiori, F., Burda, P., Conzelmann, A. and Benachour, A. (1998) GPI anchor biosynthesis in yeast: phosphoethanolamine is attached to the alpha1,4-linked mannose of the complete precursor glycophospholipid. Glycobiology 8, 761–770.

[14] Ram, A.F., Wolters, A., Ten Hoopen, R. and Klis, F.M. (1994) A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. Yeast 10, 1019–1030.

[15] Ram, A.F., Brekelmans, S.S., Oehlen, L.J. and Klis, F.M. (1995) Identification of two cell cycle regulated genes affecting the beta 1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. FEBS Lett. 358, 165–170.

[16] Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101, 451–454.

[17] Promlek, T., Ishiwata-Kimata, Y., Shido, M., Sakuramoto, M., Kohno, K. and Kimata, Y. (2011) Membrane aberrancy and unfolded proteins activate the endoplasmic reticulum stress sensor Ire1 in different ways. Mol. Biol. Cell 22, 3520–3532.

[18] Friant, S., Meier, K.D. and Riezman, H. (2003) Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction. EMBO J. 22, 3783–3791.

[19] Sutterlin, C., Doering, T.L., Schimmoller, F., Schroder, S. and Riezman, H. (1997) Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeast. J. Cell Sci. 110 (Pt. 21), 2703–2714.