

Research article

**THE EFFECT OF CALNEXIN DELETION ON THE EXPRESSION  
 LEVEL OF BINDING PROTEIN (BiP) UNDER HEAT STRESS  
 CONDITIONS IN *Saccharomyces cerevisiae***

HUILI ZHANG<sup>1</sup>, BINGJIE HU<sup>1</sup>, YANYAN JI<sup>1</sup>, AKIO KATO<sup>2</sup>  
 and YOUTAO SONG<sup>1\*</sup>

<sup>1</sup>Department of Life Science, Liaoning University, Shenyang 110036, China,

<sup>2</sup>Department of Biological Chemistry, Yamaguchi University,  
 Yamaguchi 753-8515, Japan

**Abstract:** In order to investigate the effect of calnexin deletion on the induction of the main ER molecular chaperone BiP, we cultured the wild-type and calnexin-disrupted *Saccharomyces cerevisiae* strains under normal and stressed conditions. The growth rate of the calnexin-disrupted yeast was almost the same as that of the wild-type yeast under those conditions. However, the induced level of BiP mRNA in the ER was evidently higher in calnexin-disrupted *S. cerevisiae* than in the wild-type at 37°C, but was almost the same in the two strains under normal conditions. The Western blot analysis results for BiP protein expression in the ER showed a parallel in the mRNA levels in the two strains. It is suggested that under heat stress conditions, the induction of BiP in the ER might recover part of the function of calnexin in calnexin-disrupted yeast, and result in the same growth rate as in wild-type yeast.

**Key words:** Calnexin, Molecular chaperone, BiP, Heat stress

## INTRODUCTION

Calnexin, a membrane protein of the endoplasmic reticulum (ER), is generally thought to function as a molecular chaperone which associates transiently with numerous newly synthesized glycoproteins during their maturation in the

\* Author for correspondence: e-mail: [ysong@lnu.edu.cn](mailto:ysong@lnu.edu.cn), tel: +86-24-62202280, fax: +86-24-86864476

Abbreviations used: ER – endoplasmic reticulum; PAGE – polyacrylamide gel electrophoresis; PDI – protein disulfide isomerase; RT-PCR – reverse transcriptase-polymerase chain reaction; UPR – unfolding protein response

ER [1-3]. In addition to functioning in folding and assembly, calnexin has been proposed to be a component of the ER quality control system that assists in the initial folding of nascent proteins and retains misfolded intermediates through their oligosaccharide moieties until these substrates fold properly or until the misfolded proteins are degraded [2-7]. Cne1p, the homologue of calnexin in the yeast *Saccharomyces cerevisiae*, is about 23% identical to that in mammalian cells, but it lacks a cytoplasmic tail and has no calcium-binding capacity [8]. Moreover, *S. cerevisiae* has no UDP-Glc:glycoprotein glucosyltransferase (GT), which is the key element in the quality control of glycoprotein folding in mammalian cells [9,10]. Therefore, although the proposal was made in previous papers that the calnexin in *S. cerevisiae* acts in the folding and quality control of glycoproteins [11], it is unclear whether the model proposed for the quality control of glycoprotein folding in mammalian cells is applicable to *S. cerevisiae*. Interestingly, the disruption of the calnexin gene in *S. cerevisiae* did not have gross effects on the levels of cell growth, even though the disruption of the gene was lethal for *Schizosaccharomyces pombe* or mammalian cells [12].

In our previous study, we investigated the levels of cell growth of the calnexin-disrupted strain under the internal stress conditions of overexpressing non-glycosylated or glycosylated lysozymes in the ER. There was no evident difference between the growth curve of the calnexin-disrupted strain and that of the wild-type strain [13]. These results indicated that calnexin was not always essential in *S. cerevisiae*. It is also suggested that some other chaperones may act with the function of glycoproteins in *S. cerevisiae*, as backups for calnexin. Protein disulfide isomerase (PDI) and binding protein (BiP) are two major molecular chaperones which play significant roles in the protein-folding process in the ER. It appears that the deletion of calnexin results in the induction of the unfolded protein response (UPR), and increases the levels of PDI and BiP to fold the unfolded glycosylated lysozymes (internal stress) [13]. We found that the induction of PDI in the ER could recover part of the function of calnexin in calnexin-disrupted yeast under heat stress conditions [14]. BiP, unlike PDI, which directly interacts with calnexin, was also found to cooperate with calnexin in the ATP-dependent refolding of glycoprotein and non-glycosylated substrates [15]. It had been established that in the case of some glycoproteins, calnexin and BiP could act sequentially [16]. It seems that calnexin and PDI work together to help fold nascent polypeptides with glycans locating towards the N-terminus of a protein, whereas PDI and BiP may engage in proteins that lack glycans or have sugar towards the C-terminus [17]. Interestingly, Fourn *et al.* found that BiP and calnexin had opposite effects on the folding behavior of human thyroperoxidase (hTPO) in transferred Chinese hamster ovary cells, and therefore that the action of specific molecular chaperones may crucially determine the fate of glycoproteins [18]. Recently, using the model of *C. elegans*, Lee *et al.* found that the BiP chaperone system may compensate for the deficiency of the calreticulin/calnexin chaperone system [19]. However, the relationship between BiP and calnexin in yeast is still elusive.

In this study, we investigated the induced mRNA level of BiP in calnexin-disrupted yeast under external heat stress conditions to attempt to obtain some insights into why calnexin deletion does not lead to gross effects on the levels of cell growth under normal and stressed conditions. Moreover, we used Western blot analysis to further analyze the protein expression level of BiP in the ER. This paper also compares the concentration of the molecular chaperone BiP in the ER when the wild-type and calnexin-disrupted *S. cerevisiae* strains are cultured under normal and stressed conditions. These observations may shed light on the relationship between calnexin and other molecular chaperones in the ER in *S. cerevisiae*.

## MATERIALS AND METHODS

### Materials

The DNA sequencing kit, competitive DNA construction kit, competitive RNA transcription kit and mRNA selective PCR kit were purchased from Takara Shuzo Co., Ltd. (Japan). Synthetic oligonucleotides were purchased from Kurabo Co., Ltd. (Japan). The RNeasy mini kit was purchased from Qiagen K.K. (Japan). The DNA sequencing was carried out using a Thermo Sequenase Core sequencing kit from Amersham (Japan). The peroxidase-conjugated rabbit anti-rat IgG was purchased from Sigma and the peroxidase-conjugated goat anti-rabbit IgG was purchased from BioSource International Inc. (Camarillo, CA, USA). All the other chemicals were of analytical grade for biochemical use.

### Strains and growth media

The *S. cerevisiae* haploid strains W303-1b (*Mat a ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15*) and W303-1b  $\Delta(cne1::Leu2)$  were provided by Dr. Parlati, McGill University, Canada. The *S. cerevisiae* strain AH22 (*MAT a, Leu2, His4, Cir<sup>+/+</sup>*) was provided by Dr. I. Kumagai, Tohoku University. For the growth of the *S. cerevisiae* W303-1b strain, YPD (1% yeast extract, 2% peptone, 2% glucose) was routinely used.

### Disruption of the *S. cerevisiae* AH22 Cne1 gene

The genomic DNA of *S. cerevisiae* W303-1b with disrupted calnexin was extracted and then amplified by PCR with primers from the *CNE1* gene. The PCR product was transformed into the *Leu2<sup>-</sup>* yeast strain AH22. Transformants were selected on BMM (Burkholder minimum medium) minus the leucine plates. Disruption of the *Cne1* gene was confirmed by PCR with genomic DNA and by RT-PCR with total mRNA, using the same primers. For further genetic analysis, diploids were sporulated and tetrad dissection was performed by standard procedures, and the presence of the disruption in the parent cells and spores was confirmed by RT-PCR.

### **Analysis of mRNA via the competitive reverse transcriptase-polymerase chain reaction**

The analysis of BiP expression was performed using the competitive reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Siebert and Larrick [20]. Competitive RT-PCR is a method developed to overcome difficulties with basic RT-PCR. In it, the plateau phase of normal RT-PCR indicates almost the same amount of amplified products with sufficient cycles of PCR, regardless of the initial amount of the templates. A BiP-specific primer pair (5'-GCCCGCTGTAGAAGTAAGTG-3' and 5'-CTCTTGGTGCTGGTGAATG-3') yielding RT-PCR products of 1099 bp was prepared based on the BiP cDNA sequences of *S. cerevisiae* [21, 22]. The internal standard RNA was constructed as follows. A 913-bp homologous (for BiP) competitor mRNA fragment (a competitor that has the same nucleotide sequences as the target RNA but contains a deletion of about 200 bp), competing for the same set of primers, was obtained as described using a competitive DNA construction kit and a competitive RNA transcription kit (Takara, Japan).

Yeast transformants were cultured at 30°C in 50 ml of selective medium (JMM) to the mid-log phase ( $OD_{600} = 1.5$ ) and then harvested by centrifugation at 1,000 g for 5 min at 4°C. Total yeast RNA was isolated using RNeasy Mini Kits (Qiagen K.K., Japan). 500 ng of total RNA and  $2 \times 10^7$  copies of competitive mRNA were co-converted into first-strand cDNA using the antisense specific primers. Subsequently, equal portions of cDNA (a mixture of the target and a competitor) were co-amplified by PCR with the BiP-specific primer pair. The products were then resolved on a 1.5% agarose gel stained with ethidium bromide. Because of the competition, the ratio of the amounts of the two amplified products reflects the ratio between the target mRNA and the RNA competitor. The amount of target mRNA is directly proportional to the log ( $A_t / A_c$ ), where  $A_t$  is the amount of amplified product from the target mRNA, and  $A_c$  is the amount of amplified product from the competitor RNA. The densities of the target and competitor bands in the gel after RT-PCR were analyzed using a Molecular Imager (Bio-Rad, Japan). The entire gel was digitized and the relevant band scanned. The lane scanning width was 4 mm (80-90% of the total lane width). This scanning width was adjusted slightly in some lanes to compensate for artifacts. The DNA levels corresponded to density absorbance units ( $OD \times mm$ ).

### **Analysis of the induction of the molecular chaperone via Western blot analysis**

Cell lysates were prepared from a total of 2.0  $A_{600}$  units of exponentially growing cells that were pelleted and washed once with 10 mM  $NaN_3$ , before the cell pellet was frozen at -20°C and thawed on ice. A 20  $\mu$ l aliquot of sample buffer (80 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 100 mM dithiothreitol and 10% glycerol) was then added, and the samples were incubated at 95°C for 2 min. A total of 0.12 g of glass beads (Sigma G-9268) were added, the cells were disrupted by agitation on a vortex mixer for 1 min, and 80  $\mu$ l of sample buffer was

added before the aliquots were reheated and applied to SDS-polyacrylamide gels. After the transfer of the electrophoresed proteins to nitrocellulose membranes, antibodies against yeast BiP (1:2,000, Affinity BioReagents, Inc. MA3-001) were used to probe the cell lysates and HRP-conjugated rabbit anti-rat IgG (1:10,000, for BiP) to detect the primary antibodies. The loading and transfer control (load) on the PVDF membrane was stained with Amido Black. The resulting blots were washed, and then exposed and processed by ECL analysis. The densities of the BiP and loading bands in the gel were analyzed using a Molecular Imager (Dolphin gel analysis, USA). The entire gel was digitized and the relevant bands scanned. The protein levels correspond to the integration optical density.

## RESULTS AND DISCUSSION

### Calnexin gene deletion has no effect on the growth of *S. cerevisiae* under temperature stress conditions

The effect of calnexin gene deletion on the growth of *S. cerevisiae* W303-1b was investigated in our previous study. The growth rate of the calnexin-deleted strain was almost the same as that of the wild-type strain [23]. Here, we further investigated the generation time of calnexin-deleted *S. cerevisiae* W303-1b under conditions of temperature stress (16°C, 24°C, 30°C, 37°C and 42°C). As shown in Fig. 1 and Tab. 1, we observed that the calnexin-deleted and wild-type strains grew less well under the stressed conditions than under normal conditions. However, and interestingly, there was no evident difference between the growth rates of the colonies of calnexin-deleted and wild-type strains at any given

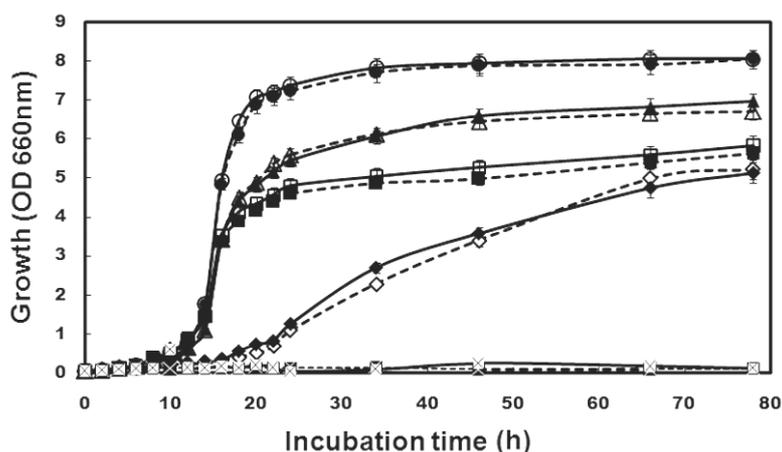


Fig. 1. The effect of calnexin deletion on the generation time of *S. cerevisiae* W303-1B under heat stress conditions. A wild strain W303-1b (open marker and solid line) and a calnexin-disrupted strain (solid marker and dash line) were incubated at 16°C (diamond), 25°C (triangle), 30°C (circle), 37°C (square) and 42°C (cruciform) in YPD medium for the period indicated. The generation time was calculated by following the turbidity at OD 660 nm. The vertical bars indicate the standard deviations ( $n = 3$ ).

temperature stress condition. A similar growth manner was obtained for another strain, *S. cerevisiae* AH22 (data not shown). We again noted that the calnexin-deleted and wild-type strains had similar growth rates regardless of the temperature stress conditions.

Tab. 1. The effect of calnexin deletion on *S. cerevisiae* under different conditions.

Strains	Conditions	Relative induced mRNA levels of BiP	Generation time (h) <sup>b</sup>
W303-1b wild-type	Normal (control)	1.00	1.61
W303-1b wild-type	37°C	3.03	4.32
W303-1b wild-type	LzWT (stable)	2.13 <sup>a</sup>	1.72
W303-1b wild-type	LzG49N (stable)	4.10 <sup>a</sup>	ND
W303-1b wild-type	LzK13D(unstable)	3.96 <sup>a</sup>	ND
W303-1b wild-type	LzK13D/G49N(unstable, glycoprotein)	4.13 <sup>a</sup>	1.78
W303-1b $\Delta$ CNE	Normal	1.02	1.65
W303-1b $\Delta$ CNE	37°C	4.35	4.22
W303-1b $\Delta$ CNE	LzWT (stable)	2.13 <sup>a</sup>	1.71
W303-1b $\Delta$ CNE	LzG49N (stable)	5.00 <sup>a</sup>	ND
W303-1b $\Delta$ CNE	LzK13D(unstable)	3.93 <sup>a</sup>	ND
W303-1b $\Delta$ CNE	LzK13D/G49N(unstable, glycoprotein)	7.00 <sup>a</sup>	1.75

The values are the means of three measurements. <sup>a</sup>Revised data from our previous study [13]. <sup>b</sup>Growth rates were measured in liquid YPD at 30°C, except for those for the heat stress conditions (at 37°C). ND – not determined; Lz – lysozyme; wt – wild type; K13D, G49N, K13D/G49N – point mutations on lysozyme

### The induced mRNA levels of BiP in calnexin-disrupted *S. cerevisiae* under heat stress conditions

BiP, a member of the Hsp70 protein family found in the lumen of the ER, is known to be associated with a variety of folding and assembling intermediates of cellular and viral membrane proteins [24]. It also acts as chaperone, and is a part of a quality-control system for the correct folding of the proteins in the same subcellular compartment [25]. Therefore, it seems likely that the deletion of calnexin induces the unfolded protein response (UPR) and increases the level of BiP to fold the unfolded glycoprotein [10]. To investigate whether the molecular chaperones in the ER are induced as a result of calnexin-disruption, we measured the mRNA concentration of BiP in wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. The level of BiP induction is shown in Fig. 2. The yeast cells showed a similar amount of BiP (Fig. 2, lanes 1 and 2) in the wild-type and calnexin-disrupted strains under normal conditions, from which we speculated that calnexin deletion did not induce the formation of the ER molecular chaperone BiP in *S. cerevisiae* under normal conditions. By comparison, significant differences in the inductions of BiP (Fig. 2, lanes 3 and 4) were observed when the wild-type

and calnexin-disrupted *S. cerevisiae* were cultured under heat stress conditions. Interestingly, BiP was induced at a higher concentration in the calnexin-disrupted strain than in the wild strain. The quantitative analysis of the RT-PCR patterns was further measured with a densitometer. The graphs in Fig. 2b show the ratios of the density of the target band (above) to that of the competitor band (below) in calnexin-disrupted (black column) and wild-type *S. cerevisiae* (white column). However, it was difficult to obtain the difference in the induced molecular chaperone BiP between calnexin-deleted and wild-type strains under the cold stress of 16°C using the competitive reverse transcriptase-polymerase chain reaction.

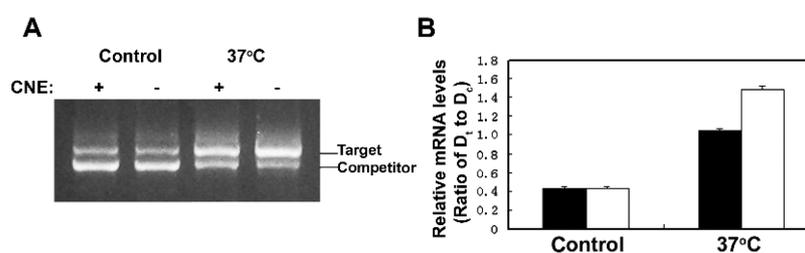


Fig. 2. A quantitative analysis of BiP mRNA levels when the wild-type and calnexin-disrupted *S. cerevisiae* were grown under normal and stressed conditions. A – The electrophoresis of RT-PCR products separated in 1.5% agarose gel was stained with ethidium bromide. The 1099-bp products (upper) from the yeast RNA and the 913-bp products (below) from the BiP competitor RNA are indicated. B – In order to quantitatively analyze the mRNA levels, the concentration of the RT-PCR patterns was measured with a densitometer. The graph shows the ratios of the density of the target band (upper) to that of the competitor band (below) in calnexin-disrupted *S. cerevisiae* (white column) and wild-type *S. cerevisiae* (black column). D<sub>t</sub>: density of the band from the target mRNA; D<sub>c</sub>: density of the band from the competitor RNA. The vertical bars indicate the standard deviations (n = 3).

### The BiP protein expression level parallels its mRNA level in both wild-type and calnexin-disrupted *S. cerevisiae*

To identify the effect of calnexin deletion on BiP expression at the translational level, we next performed SDS-PAGE and Western blot to detect BiP proteins in the cell lysates. These were prepared from a total of 2.0 A<sub>600</sub> units of exponentially growing cells. The cell pellets were disrupted with glass beads by agitation on a vortex mixer and used for SDS-PAGE. After transferring the electrophoresed proteins to nitrocellulose membranes, antibodies against yeast BiP were used to probe the cell lysates and HRP-conjugated rabbit anti-rat IgG (for BiP) to detect the primary antibodies. The resulting blots were washed, and then exposed and processed by ECL analysis (Fig. 3). The yeast cells showed a similar protein concentration of BiP in the wild-type and calnexin-disrupted strains under normal conditions (Fig. 3, lanes 1 and 2), while the protein levels of BiP in the ER were

evidently increased in the calnexin-disrupted *S. cerevisiae* under the heat stress conditions (Fig. 3, lane 3 and 4). Therefore, the data from the Western blot for proteins showed similar results to that for the mRNAs. This indicates that the BiP protein expression level parallels its mRNA level in both strains.

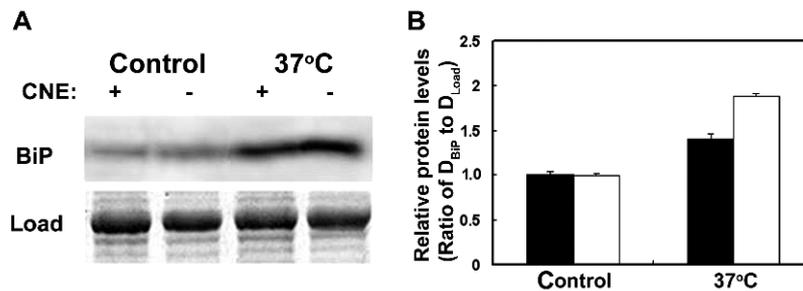


Fig. 3. A Western blot analysis of the BiP expression level when the wild-type and calnexin-disrupted *S. cerevisiae* were grown under normal and stressed conditions. A – The whole cell lysates of wild-type (CNE<sup>+</sup>) and calnexin-disrupted (CNE<sup>-</sup>) yeasts under the conditions indicated at the top were subject to Western blot analysis probing for BiP as indicated. The bottom section shows portions of the membranes stained with Amido Black as loading and transfer controls (input). B – The concentrations of the bands measured with a densitometer. The graph shows the ratios of the density of the BiP band (above) to that of the load band (below) in calnexin-disrupted (white column) and wild-type *S. cerevisiae* (black column). The vertical bars indicate the standard deviations (n = 3).

These results indicate that the high induction level of BiP occurred only in the calnexin-disrupted strain under heat stress conditions *in vivo*. The significant increases in the induction of BiP in the calnexin-disrupted yeast suggested that calnexin was involved in the folding of glycoproteins and that the increased BiP seemed likely to fold unfolded glycoproteins instead of calnexin in calnexin-deleted yeast under the stressed conditions. This finding in yeast is quite similar to that for *C. elegans*, as reported by Lee *et al.* The deficiency of the CRT/CNX chaperone system may trigger an unfolded protein response and the BiP chaperone system may compensate for the deficiency of the calreticulin/calnexin chaperone system [19]. Furthermore, in our previous study, we obtained evidence of the quality control of calnexin by comparing the secreted amounts of the wild-type and various mutant glycosylated lysozymes in both wild-type and calnexin-disrupted yeast strains. The calnexin-disrupted *S. cerevisiae* exhibited a great increase in the secretion of unstable glycosylated lysozyme mutants, despite the low level of secretion by the wild type strain. In addition, we also found that high-level induction of BiP only occurred for those glycosylated proteins overexpressed in the calnexin-disrupted strains [13]. Combined with the results that we obtained in this study, it seems that both the external and internal ER stresses (overexpressing glycoproteins in the ER) could lead to an increase in the induced level of the ER molecular chaperone BiP in calnexin-disrupted

*S. cerevisiae* (Tab. 1). The significant increases in the expression of BiP in the calnexin-disrupted yeast suggest that Cne1p is involved in the folding of glycoproteins cooperating with PDI, and its failure or removal increases the efficiency of BiP, which then folds the unfolded glycoproteins instead of Cne1p in the calnexin-deleted yeast. Follow-up experiments are needed to validate our predictions so as to establish an experimentally useful phenotype for yeast, and could include using a UPRE element in front of a selectable gene where the real strengths of yeast genetics can be used. This attempt must shed light on the cooperation mechanism of ER molecular chaperones.

**Acknowledgments.** We would like to thank Dr. Hongyu Shang (Princeton University, USA) and Dr. Hiroyuki Azakami (Yamaguchi University, Japan) for their helpful comments and critical review of the manuscript. This study was supported by grant 20072055 from the Natural Science Foundation of Liaoning Province of China, grant 1063305-1-00 from the Shenyang Science and Technology Bureau, and grant 20060358 from the Education Department of Liaoning Province, China.

## REFERENCES

1. Ou, W.J., Cameron, P.H., Thomas, D.Y. and Bergeron. J.J.M. Association of folding intermediates of glycoproteins with calnexin during protein maturation. **Nature** 364 (1993) 771-776.
2. Bergeron, J.J.M., Brenner, M.B., Thomas, D.Y. and Williams, D.B. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. **Trends Biochem. Sci.** 19 (1994) 124-128.
3. Letourneur, O., Sechi, S., Willete-Brown, J., Robertson, M.W. and Kinet J.P. Glycosylation of human truncated Fc epsilon RI alpha chain is necessary for efficient folding in the endoplasmic reticulum. **J. Biol. Chem.** 270 (1995) 8249-8256.
4. Degen, E., Cohen-Doyle, M.F. and Williams, D.B. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both beta 2-microglobulin and peptide. **J. Exp. Med.** 175 (1992) 1653-1661.
5. Hammond, C., Braakman, I. and Helenius, A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. **Proc. Natl. Acad. Sci. USA** 91 (1994) 913-917.
6. Jackson, M.R., Cohen-Doyle, M.F., Peterson, P, A. and Williams, D.B. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). **Science** 263 (1994) 384-387.
7. Ware, F.E., Vassilakos, A., Peterson, P.A., Jackson, M.R., Lehrman, M.A. and Williams, D.B. The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. **J. Biol. Chem.** 270 (1995) 4697-4704.

8. Parlati, F., Dominguez, M., Bergeron, J.M. and Thomas, D.Y. *Saccharomyces cerevisiae* CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus. **J. Biol. Chem.** 270 (1995) 244-253.
9. Jakob, C.A., Burda, P. S., te Heesen, S., Aebi, M. and Roth, J. Genetic tailoring of N-linked oligosaccharides: the role of glucose residues in glycoprotein processing of *Saccharomyces cerevisiae* *in vivo*. **Glycobiology** 8 (1998) 155-164.
10. Mori, K., Ogawa, N., Kawahara, T., Yanagi, H. and Yura, T. Palindrome with spacer of one nucleotide is characteristic of the cis-acting unfolded protein response element in *Saccharomyces cerevisiae*. **J. Biol. Chem.** 273 (1998) 9912-9920.
11. Shahinian, S., Dijkgraaf, G.J.P., Sdicu, A.M., Thomas, D.Y., Jakob, C.A., Aebi, M., and Bussey, H. Involvement of Protein N-Glycosyl Chain Glucosylation and Processing in the Biosynthesis of Cell Wall-1,6-Glucan of *Saccharomyces cerevisiae*. **Genetics** 149 (1998) 843-856.
12. Denzel, A., Molinari, M., Trigueros, C., Martin, J.E., Velmurgan, S., Brown, S., Stamp, G. and Owen, M.J. Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. **Mol. Cell Biol.** 22 (2002) 7398-7404.
13. Song, Y., Sata, J., Saito, A., Usui, M., Azakami, H. and Akio, K. Effects of calnexin deletion in *Saccharomyces cerevisiae* on the secretion of glycosylated lysozymes. **J. Biochem.** 130 (2001) 757-764.
14. Zhang, H., He, J., Ji, Y., Kato, A. and Song, Y. The effect of calnexin deletion on the expression level of PDI in *Saccharomyces cerevisiae* under heat stress conditions. **Cell Mol. Biol. Lett.** 13 (2007) 38-48.
15. Stronge, V.S., Saito, Y., Ihara, Y. and Williams, D.B. Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates. **J. Biol. Chem.** 276 (2001) 39779-39787.
16. Choukhi, A.L., Ung, S., Wychowski, C. and Dubuisson, J. Involvement of endoplasmic reticulum chaperones in the folding of hepatitis c virus glycoproteins. **J. Virol.** 72 (1998) 3851-3858.
17. Lith, M.V., Karala, A.R., Bown, D., Gatehouse, J.A., Ruddock, L.W., Saunders, PTK, and Benham. A.M. A developmentally regulated chaperone complex for the endoplasmic reticulum of male haploid germ cells. **Mol. Biol. Cell.** 18 (2007) 2795-2804.
18. Fourn, V.L., Fernandez, S.S., Ferrand, M. and Franc, J.L. Competition between calnexin and BiP in the endoplasmic reticulum can lead to the folding or degradation of human thyroperoxidase. **Biochemistry** 45 (2006) 7380-7388.

19. Lee, W., Kim, K.R., Singaravelu, G., Park, B-J., Kim, D.H., Ahnn, J. and Yoo, Y.J. Alternative chaperone machinery may compensate for calreticulin/calnexin deficiency in *Caenorhabditis elegans*. **Proteomics** 6 (2006) 1329-1339.
20. Siebert, P.D. and Larrick, J.W. Competitive PCR. **Nature** 359 (1992) 557-558.
21. Rose, M.D., Misra, L.M. and Vogel, J.P. KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. **Cell** 57 (1989) 1211-1221.
22. Lamantia, M., Miura, T., Tachikawa, H., Kaplan, H.A., Lennarz, W.J. and Mizunaga, T. Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. **Proc. Natl. Acad. Sci. USA** 88 (1991) 4453-4457.
23. Arima, H., Kinoshita, T., Ibrahim H.R., Azakami, H. and Kato, A. Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of N-glycosylation signal and the disruption of calnexin gene in *Saccharomyces cerevisiae*. **FEBS Lett.** 440 (1998) 89-92.
24. Brodsky, J.L. and Schekman, R. The biology of heat shock proteins and molecular chaperones. **Cold Spring Harbor Laboratory Press**, Plainview, N.Y. (Marimoto RI, Tissieres A and Georgopoulos C), (1994) pp. 85-109.
25. MinHee, K.K. and EunDuck, P.K. Differential interaction of molecular chaperones with procollagen I and type IV collagen in corneal endothelial cells. **Mol. Vis.** 8 (2002) 1-9.