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Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction

Sylvie Friant[1], Karsten D.Meier[2] and Howard Riezman[3]

Department of Biochemistry, University of Geneva, Sciences II, 30, quai E. Ansermet, CH-1211 Geneva 4, Switzerland

[1]Present address: IBCP, UMR 5086 CNRS, 7 passage du Vercors, 69367 Lyon, France
[2]Present address: Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

[3]Corresponding author
e-mail: Howard.Riezman@biochem.unige.ch

S.Friant and K.D.Meier contributed equally to this work

Serine palmitoyltransferase, the first enzyme in ceramide biosynthesis, is required for resistance to heat shock. We show that increased heat shock sensitivity in the absence of serine palmitoyltransferase activity correlates with a lack of induction of the major heat shock proteins (Hsps) at high temperature. Normal heat shock resistance can be restored, without restoration of ceramide synthesis or induction of Hsps, by overexpression of ubiquitin. This function of ubiquitin requires the proteasome. These data imply that the essential function of Hsp induction is the removal of misfolded or aggregated proteins, not their refolding. This suggests that cells stressed by heat shock do not die because of the loss of protein activity due to their denaturation, but because of the inherent toxicity of the denatured and/or aggregated proteins.

Keywords: heat stress/Hsp/sphingoid base/ubiquitin–proteasome degradation

Introduction

All organisms respond to temperature increases by induction of a conserved set of proteins, the heat shock proteins (Hsps), which protect them from damage and facilitate recovery from such heat stresses. Most of these Hsps function as molecular chaperones that prevent the accumulation of aggregated proteins or promote refolding of misfolded proteins (Hendrick and Hartl, 1993; Parsell and Lindquist, 1993; Glover and Lindquist, 1998). In eukaryotic cells, ubiquitin and certain ubiquitin-conjugating enzymes are Hsps that function in the rapid turnover of denatured proteins. The major pathway for the selective degradation of abnormal proteins in the cytosol and nucleus is the ubiquitin–proteasome pathway (Ciechanover, 1994). In the budding yeast *Saccharomyces cerevisiae*, Hsp induction is caused by increased transcription of the corresponding genes (Lindquist, 1981). Two transcriptional control systems appear to be responsible for the gene expression changes upon heat stress, one involving the heat shock factor (Hsf1p) and the other one depending on Msn2p and Msn4p transcription factors. Hsf1p binds to the heat shock promoter element (HSE) found in the promoter region of many Hsp genes. In yeast, several genes have been identified that do not contain HSEs, but whose transcription is induced by heat and other stress signals, including osmotic shock, DNA damage and oxidative stress. Msn2/4p activates these genes through the stress response element (STRE), a *cis* regulatory sequence (Ruis and Schuller, 1995).

In addition to the induction of Hsps, heat shocked yeast cells display a number of characteristic phenotypes. Cells accumulate trehalose (a thermoprotectant), acquire thermotolerance, become transiently arrested in the G_1 phase of the cell cycle and exhibit an increase in cellular levels of sphingoid bases and ceramides. Furthermore, *de novo* synthesis of sphingoid bases [phytosphingosine (PHS) and dihydrosphingosine (DHS)] is required for the yeast heat stress response (Patton *et al*., 1992; Jenkins *et al*., 1997). Sphingoid bases are potential mediators of the heat stress response, because treatment of cells with DHS activates transcription of the *TPS2* gene encoding a subunit of trehalose synthase and causes trehalose to accumulate. DHS also induces expression of a *STRE-LacZ* reporter gene, showing that the global stress response pathway can be activated by sphingoid base signals (Dickson *et al*., 1997).

To understand the role of sphingoid bases in yeast heat stress response, we used the mutant strain *lcb1-100*, which has a thermosensitive defect in *de novo* sphingolipid synthesis and fails to grow at 37°C (Zanolari *et al*., 2000). The *LCB1* gene encodes a subunit of serine palmitoyltransferase, an essential enzyme that catalyzes the first step in sphingoid base synthesis (Buede *et al*., 1991). Upon heat shock, *lcb1-100* mutant cells show no increase in sphingoid base (PHS and DHS) synthesis, no transient cell cycle arrest and no resistance to heat stress, indicating a requirement for *de novo* synthesis of sphingoid bases for the heat shock response (Chung *et al*., 2000; Jenkins and Hannun, 2001). Here, we show that overexpression of the polyubiquitin gene *UBI4* can abrogate the sphingoid base synthesis requirement for heat shock resistance and restore survival upon heat stress of the *lcb1-100* mutant strain without induction of Hsps or ceramide synthesis. This suppressor effect of *UBI4* is mediated via the ubiquitin–proteasome degradation pathway. These results suggest that the essential requirement for heat shock survival is the removal of misfolded or aggregated proteins, not their refolding and that cells stressed by heat shock do not die because of the loss of protein activity due to their denaturation, but because of the inherent toxicity of misfolded and/or aggregated proteins.

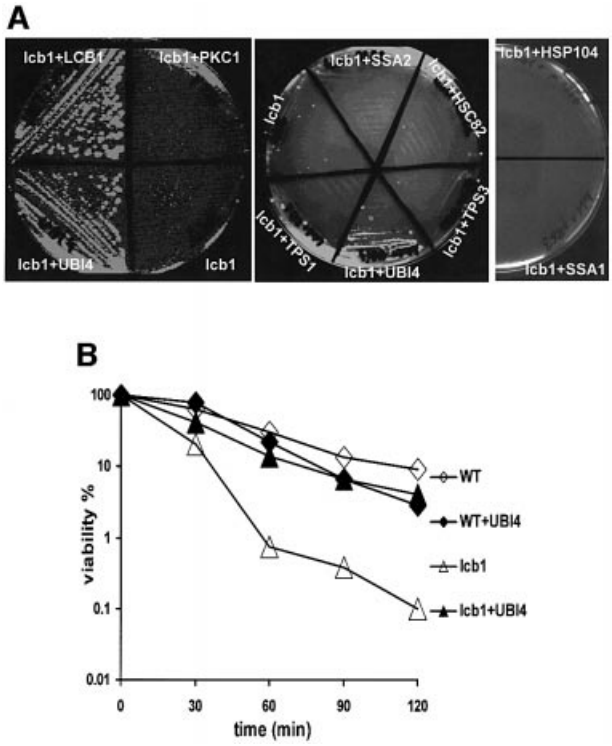

Fig. 1. (**A**) Increased ubiquitin expression suppresses the *lcb1-100* temperature-sensitive phenotype. RH3809 (*lcb1-100*) cells carrying plasmids that overexpress either *LCB1*, *UBI4*, *PKC1*, *TPS1*, *TPS3*, *HSC82* or *SSA2* were streaked onto YPUAD plates and grown at the non-permissive temperature of 37°C (left and middle panels). The RH3809 (*lcb1*) strain bearing pYSGal104 (*HSP104*) or YCp50-GAL1-SSA1 (*SSA1*) plasmids was streaked onto an SGal/Raf-ura plate and tested for growth at 37°C (right panel). (**B**) Increased ubiquitin expression restores heat resistance to *lcb1-100* cells. Mid log-phase cultures of WT, *lcb1-100* (lcb1) cells or *lcb1-100* mutant cells overexpressing *UBI4* (+UBI4) were grown in YPUAD at 24°C and an aliquot was shifted to 44°C. Samples were taken in duplicate at the times indicated, diluted into ice-cold YPUAD, and immediately plated onto YPUAD agar at 24°C to assess cell viability. Survival at 44°C was plotted on a log scale as a percentage of colony forming units relative to that found at 24°C.

Results and discussion

Overexpression of ubiquitin restores heat stress resistance to the lcb1-100 mutant

In this study we used a suppressor approach to identify proteins that are downstream effectors in the sphingoid base signaling pathway required for the heat shock response. The rationale of this study was based on the idea that overexpression of such proteins from a high copy number plasmid would result in an increased resistance of the *lcb1-100* mutant to elevated temperature. The poly-ubiquitin gene *UBI4* was isolated as a suppressor of the *lcb1-100* mutation (Figure 1A), suggesting that ubiquitin overexpression can restore the heat stress defect due to the lack of sphingoid base synthesis. This effect was specific for the heat survival defect associated with the *lcb1-100* mutation, because *UBI4* overexpression did not suppress the endocytic defect of *lcb1-100* mutant cells (data not shown) (Zanolari *et al*., 2000).

Survival at an elevated temperature was also examined. Log-phase cells of wild-type (WT), *lcb1-100* and *lcb1-100* mutant strains overexpressing the *UBI4* gene, were heat shocked at 44°C and the percentage of cells able to form colonies was determined as a function of time (Figure 1B). The *lcb1-100* mutant cells showed a clear defect in survival at high temperature when compared with WT cells. In contrast, *lcb1-100* cells with *UBI4* plasmid were 6- to 10-fold more resistant at 44°C than the parental *lcb1-100* strain (Figure 1B). Consistent with this result, we found that increased expression of a single ubiquitin gene driven from the *CUP1* promoter was also able to suppress the *lcb1-100* mutation (data not shown). Thus, the suppression of the *lcb1-100* heat shock defect results from increased ubiquitin expression.

The heat shock transcription factor Hsf1p and the stress-responsive transcription factors Msn2/4p are required for Hsp induction. To determine whether *UBI4* overexpression could also suppress the temperature-sensitive growth defect displayed by the *msn2 msn4*, *hsf1-ΔCTD*, *msn2 msn4 hsf1-ΔCTD*, *tetO-HSF1* and *msn2 msn4 tetO-HSF1* mutant strains, these strains were transformed by the YEplac181-UBI4 plasmid bearing *UBI4* and tested for growth at 37°C. None of these strains were suppressed by *UBI4* overexpression, showing that this effect was specific for the temperature-sensitive defect associated with the *lcb1-100* mutation.

The heat shock response pathway activates several genes that are under the control of the HSE and/or the STRE regulons. The *UBI4*, *HSP12*, *HSP26* and *HSP104* genes contain both stress inducible regulons (Boy-Marcotte *et al*., 1999; Simon *et al*., 1999; Amoros and Estruch, 2001). Other genes contain only the *STRE* regulon, including genes for trehalose biosynthetic enzymes *TPS1* and *TPS2*. Most of the classical heat shock protein genes are heat inducible only via Hsf1p, including the Hsp70s encoded by the *SSA1-4* genes. To determine if overexpression of other heat inducible genes could also suppress the *lcb1-100* temperature-sensitive defect, the *lcb1-100* strain was transformed with high copy number plasmids bearing *HSC82*, *TPS1, TPS2*, *TPS3*, *TSL1*, *SSA2* or *SSA4* genes (Figure 1A; data not shown for *TPS1*, *TSL1* and *SSA4* overexpression) and with a centromeric plasmid bearing the *SSA1* or *HPS104* gene under the control of the inducible *GAL1* promoter (Figure 1A). The transformants were tested for survival at 37°C. None of the genes tested was able to suppress the *lcb1-100* temperature-sensitive growth defect, even the *HSP104* gene, which like the *UBI4* gene contains both the *HSE* and *STRE* regulons in its promoter region. This result shows that in contrast to ubiquitin, overexpression of different chaperones or subunits of the trehalose synthase are not sufficient to restore survival upon heat stress in the *lcb1-100* strain. The *UBI4* gene is stress inducible and important for the survival under diverse stresses (Finley *et al*., 1987; Fraser *et al*., 1991; Jungmann *et al*., 1993; Cheng *et al*., 1994). This probably reflects the need for adequate ubiquitin levels to enable the ubiquitylation system to control the turnover of damaged proteins.

The protein kinase C–mitogen-activated protein (Pkc1–MAP) kinase pathway is inducible by elevated temperature and this activation is required for acquired thermotolerance. Indeed, activation of the MAP kinase branch of the pathway is sufficient to confer acquired thermotolerance (Kamada *et al*., 1995). It is therefore possible that

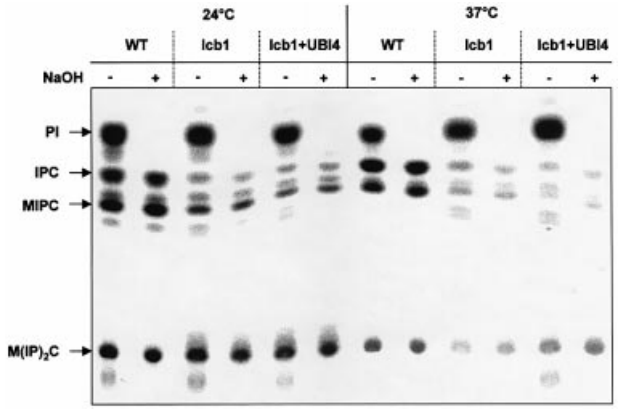

Fig. 2. Increased ubiquitin expression does not restore sphingolipid synthesis in *lcb1-100* cells. WT, *lcb1-100* mutant (lcb1) or *lcb1-100* mutant cells overexpressing *UBI4* (+UBI4) were grown in SDYE at 24°C, preshifted to 24 or 37°C and labeled with [^{3}H]*myo*inositol. Incorporation of [^{3}H]*myo*inositol into the total lipid fraction was quantified and equal c.p.m. were directly applied to TLC plates, or treated with mild base to identify sphingolipids [IPC, MIPC and M(IP)2C].

activation of the *PKC1*–MAP kinase pathway by overexpressing effectors of this pathway, could rescue the heat survival of the *lcb1-100* mutant cells. This MAP kinase signaling pathway is composed of four downstream effectors, Bck1p, Mkk1p/Mkk2p and Mpk1p (Lee and Levin, 1992; Irie *et al*., 1993; Lee *et al*., 1993), which are homologs of the MAP kinase cascade effectors in mammalian cells. The *lcb1-100* mutant was transformed with high copy number plasmids bearing *PKC1*, *BCK1*, *MKK1* or *MPK1* genes and survival at 37°C was tested. We also overproduced Pkc1p activity by transformation with a low copy number plasmid bearing a dominant, activated allele of *PKC1* (*PKC1-R398P*) (Nonaka *et al*., 1995). None of these kinases was able to suppress the heat sensitivity associated with the *lcb1-100* mutation (Figure 1A, panel lcb1+PKC1; and data not shown) despite the fact that Pkc1p overexpression can suppress the endocytic defect of *lcb1-100* cells (Friant *et al*., 2000). In yeast, the Pkh1/2p kinases that phosphorylate and activate Pkc1p, are stimulated by sphingoid bases. This sphingoid base-mediated signaling pathway is required for endocytosis (Friant *et al*., 2001). These results suggest that the lack of sphingoid base synthesis observed in the *lcb1-100* strain upon heat shock, may result in a decrease of Pkh1/2p and Pkc1p kinase activity. Therefore, we tested whether *PKH1* or *PKH2* overexpression could suppress the temperature-sensitive phenotype displayed by the *lcb1-100* mutant. Neither of the two kinases tested was able to restore growth of *lcb1-100* at 37°C, even though they are also able to suppress the endocytic defect of this strain (Friant *et al*., 2001). Taken together, these results indicate that the suppressor effect of *UBI4* is specific and is not mediated via the Pkc1p–MAP kinase pathway. These results suggest that elevated levels of ubiquitin expression can bypass the need for *de novo* sphingoid base synthesis for survival upon heat stress.

Sphingolipid synthesis is defective in the lcb1-100 cells overexpressing UBI4

The suppressor effect of the *UBI4* gene could be due to restoration of normal sphingoid base synthesis in the

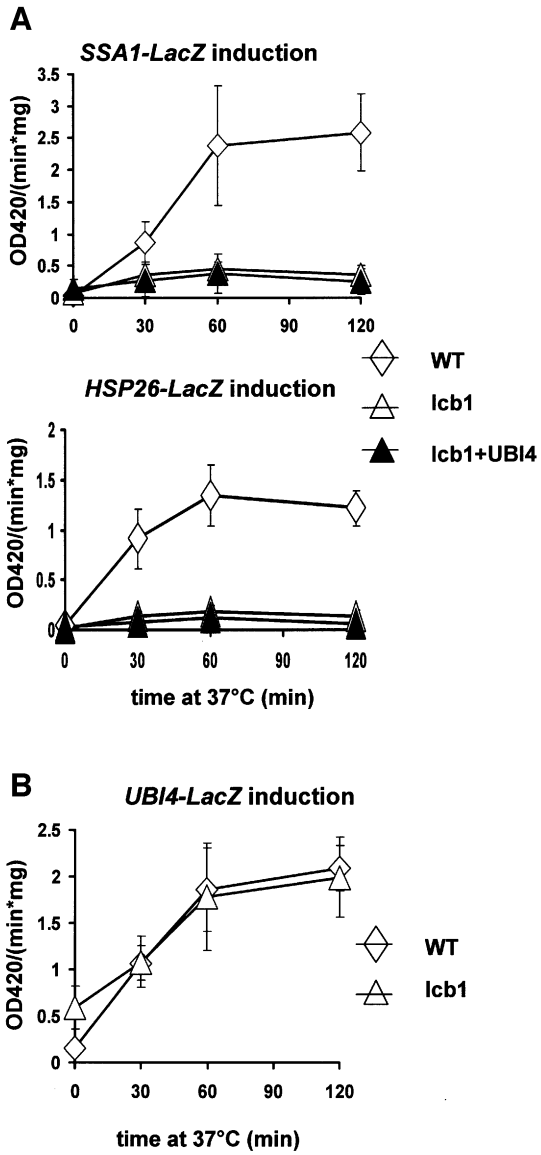

Fig. 3. (**A**) *lcb1-100* mutant cells are defective in Hsp induction. WT, *lcb1-100* mutant (lcb1) or *lcb1-100* mutant cells overexpressing *UBI4* (+UBI4) were transformed with plasmids carrying *SSA1-LacZ* or *HSP26-LacZ* reporter constructs. After growth at 24°C, transformants were shifted to 37°C for the indicated time to induce the heat shock response and β-galactosidase expression driven from these promoters was quantified. (**B**) Normal heat induction of *UBI4* in the *lcb1-100* mutant cells. WT or *lcb1-100* mutant (lcb1) cells were transformed with a plasmid carrying the *UBI4-LacZ* reporter gene and treated as described for (A).

lcb1-100 mutant strain. To investigate this possibility, sphingolipid synthesis was measured in cells overexpressing *UBI4*. WT and *lcb1-100* cells were grown at 24°C and metabolically labeled with [^{3}H]*myo*inositol at 24°C or upon heat shock at 37°C. Lipids were extracted, treated with mild base to identify sphingolipids which are base-resistant, separated by thin layer chromatography (TLC) and visualized using a PhosphorImager (Figure 2). At 24°C, both WT and mutant cells overexpressing *UBI4* or not, synthesize sphingolipids [inositolphosphoceramide (IPC), mannosylated inositolphosphoceramide (MIPC) and mannosylated di-inositolphosphoceramide ($M(IP)_2C$)], although the *lcb1-100* strains showed less

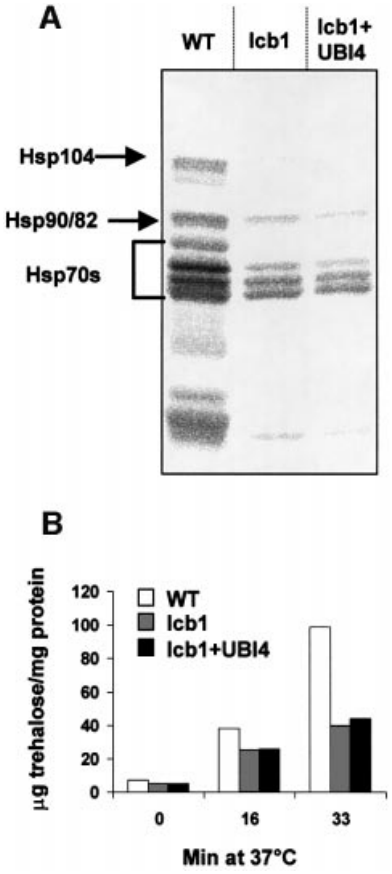

Fig. 4. Heat shock protein synthesis and sustained trehalose accumulation are defective in the *lcb1-100* cells. (**A**) Cells actively dividing at 24°C were transferred to 44°C. The production of Hsps was assessed after [^{35}S]Met/Cys labeling, followed by extraction, separation by SDS–PAGE and PhosphorImager analysis of labeled proteins. Hsp bands are indicated. (**B**) WT and *lcb1-100* (lcb1) cells bearing a plasmid without insert or *lcb1-100* cells overexpressing *UBI4* (lcb1+UBI4) were shifted from 24 to 37°C, aliquots of cells were collected at the indicated times, cell extracts were prepared and trehalose contents were determined. Similar results were obtained in two independent experiments.

sphingolipid synthesis than the WT cells. After mild heat shock (37°C), WT cells showed normal synthesis of sphingolipids. In contrast, the *lcb1-100* mutant cells showed a reduction in sphingolipid synthesis. The reduction was the same in *lcb1-100* mutant cells overexpressing *UBI4* (Figure 2). This result shows that overexpression of *UBI4* does not restore synthesis of sphingolipids in *lcb1-100* mutant cells and suggests that the requirement for heat-induced increase in sphingolipid synthesis can be overcome by *UBI4* overexpression.

Accumulation of a novel intermediate in the sphingolipid synthesis pathway upon *UBI4* overexpression could explain the restoration of the viability of the *lcb1-100* cells upon heat stress. To test this hypothesis, WT and *lcb1-100* mutant cells were labeled with [^{3}H]DHS. Addition of DHS to the *lcb1-100* mutant restores synthesis of sphingolipids at 37°C (Zanolari *et al*., 2000). Exogenously added DHS can be incorporated into phosphorylated DHS, ceramides and sphingolipids, allowing an analysis of the sphingolipid biosynthetic pathway in the *lcb1-100* strain. WT and *lcb1-100* strains overexpressing *UBI4* or not, were grown at 24°C, preincubated for 15 min at 37°C to induce the heat shock response, then [^{3}H]DHS was added and incubation was continued for 15 min. Lipids were extracted, separated by TLC and visualized using a PhosphorImager (data not shown). There was no difference in the lipid pattern between the strains bearing no plasmid and the ones overexpressing *UBI4*, showing that the suppressor effect is not due to a difference in sphingolipid synthesis.

The lcb1-100 mutant shows a defect in heat induction of Hsps

Sphingoid bases are potential mediators of the heat stress response, because treatment of cells with DHS mimics heat-induced activation of several reporter genes (Dickson *et al*., 1997). The *lcb1-100* mutant cells show very low synthesis of sphingoid bases (DHS or PHS) upon heat treatment. To determine whether the lack of sphingoid base synthesis results in loss of induction of heat shock activated genes, we tested heat induction of a reporter gene having the *HSP26* or *SSA1* gene fused in frame to the *Escherichia coli lacZ* gene in WT and *lcb1-100* mutant strains with or without a *UBI4* plasmid (Figure 3A). The WT cells showed an induction of β-galactosidase activity upon heat treatment, whereas this induction was defective in the *lcb1-100* mutant cells (lcb1) and in the *lcb1-100* cells overexpressing *UBI4* (lcb1+UBI4) for both reporter genes (Figure 3A). The *HSP26* gene promoter region contains both regulons controlling stress response whereas the *SSA1* gene is heat inducible only via Hsf1p. This result shows that activation of two different reporter genes that are under the control of the STRE and/or the HSE regulons is defective in the absence of sphingolipid synthesis and this activation is not restored upon *UBI4* overexpression.

The *UBI4* gene promoter region also contains both regulons controlling stress induction, HSE and STRE that contribute independently to heat shock regulation of the *UBI4* gene (Simon *et al*., 1999). Overexpression of the *UBI4* gene restored viability of the *lcb1-100* mutant strain upon heat stress. To determine whether the *UBI4* gene was heat inducible in the *lcb1-100* mutant, we tested heat induction of a reporter gene having the *UBI4* gene fused in frame to the *E.coli lacZ* gene in WT and *lcb1-100* mutant strains (Figure 3B). Both WT and *lcb1-100* mutant cells (lcb1) showed an induction in β-galactosidase activity upon heat treatment. This result shows that the *UBI4* gene is heat inducible in *lcb1-100* mutant cells, in contrast to *HSP26* or *SSA1* genes. Therefore, heat shock activation of *UBI4* expression is preserved in the absence of sphingoid base synthesis suggesting a difference in the mechanism of activation from the one used to induce *HSP26* or *SSA1*. *UBI4* oxerexpression could therefore restore normal heat shock resistance to the *lcb1-100* strain, because this heat shock protein is still heat inducible in the absence of sphingoid base synthesis in contrast to the other Hsps. In several genes with an essential role in stress protection, such as *HSP26*, *HSP104* or *UBI4*, Hsf1p and Msn2/4p act redundantly, assuring the expression of these genes even when one of the regulatory pathways is inactive (Amoros and Estruch, 2001). Consistent with our results, the expression of *UBI4* was not completely abolished in cells deficient for both stress pathways, suggesting the involvement of additional transcription factor(s) (Simon *et al*., 1999). Activation of this additional transcription factor(s) upon heat shock could be independent of

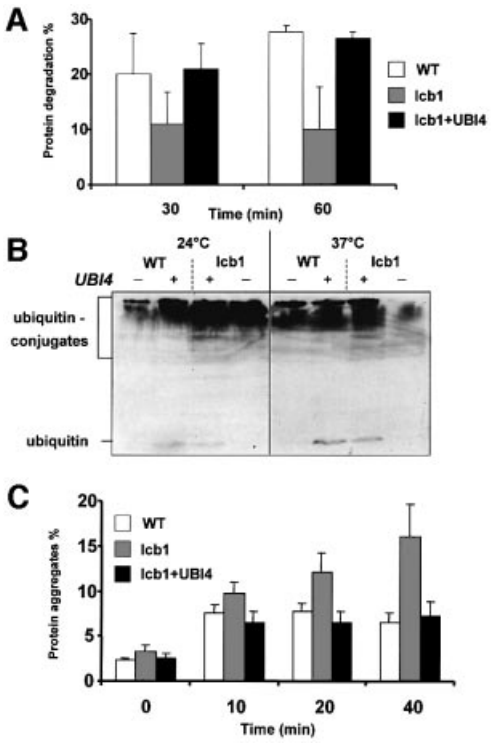

Fig. 5. Increased protein degradation and removal of protein aggregates in the *lcb1-100* strain overexpressing *UBI4*. (**A**) WT, *lcb1-100* (lcb1) and *lcb1-100* cells overexpressing *UBI4* (lcb1+UBI4) were pulse-labeled at 37°C with [^{35}S]Met/Cys and the rate of protein degradation was measured during the chase period. The data shown are mean values and standard errors obtained from four independent experiments are shown. (**B**) WT, *lcb1-100* (lcb1) cells bearing or not the *UBI4* plasmid (UBI4) were grown at 24°C, then either kept at 24°C or shifted at 37°C for 1 h and cell extracts were prepared. Equal amount of proteins were applied to a 15% SDS–PAGE gel and probed with an anti-ubiquitin antibody. Free ubiquitin and high-molecular-weight ubiquitin–protein conjugates are indicated. (**C**) The determination of the percentage of aggregated [^{35}S]Met/Cys labeled proteins was assessed in WT, *lcb1-100* (lcb1) and *lcb1-100* cells bearing the *UBI4* plasmid (lcb1+UBI4) after heat shock at 37°C for the indicated times. Protein aggregates were identified by their sedimentation at 15 000 *g* for 15 min in glycerol and non-ionic detergent at physiological salt concentrations. Total and aggregated labeled proteins were quantified by liquid scintillation counting.

sphingoid base synthesis, explaining the normal heat induction of *UBI4* in the *lcb1-100* mutant cells.

The lcb1-100 mutant is defective in Hsp synthesis and sustained trehalose accumulation upon heat shock

Activation of the heat shock response in yeast results in increased synthesis of Hsps of 100, 90 and 70 kDa, as monitored by pulse-labeling and one-dimensional SDS–PAGE (Miller *et al*., 1979). To determine whether the *lcb1-100* mutant cells exhibit a defect in Hsp synthesis upon heat stress, the general heat shock response was analyzed in the *lcb1-100* mutant cells with or without the *UBI4* plasmid and compared to WT cells. Hsp synthesis was induced by a temperature shift from 24 to 44°C. The proteins were labeled with a mix of [^{35}S]methionine and [^{35}S]cysteine 15 min after the temperature shift, because the induction of Hsps is transient, with a maximum expression at 15–20 min (Smith and Yaffe, 1991; Martinez-Pastor *et al*., 1996). Proteins were separated by SDS–PAGE and visualized using a PhosphorImager in WT, *lcb1-100* and *lcb1-100* overexpressing *UBI4* strains (Figure 4A). This analysis allows the detection of heat shock proteins Hsp104, Hsp90, Hsp82 and Hsp70s (Ssa1-4), identified according to their molecular weight. Following heat shock, the WT strain showed the expected production of Hsps, whereas the *lcb1-100* strain overexpressing *UBI4* or not, showed a strong reduction in all Hsp synthesis (Figure 4A). Hsp104p and Hsp82p were barely detected in the *lcb1* mutant strain overexpressing *UBI4* or not, whereas these two proteins were expressed in the WT cells. Hsp90p and Hsp70p proteins synthesis was greatly reduced in the *lcb1* mutant strain when compared with WT cells. Mutant *lcb1+UBI4* cells, which retain viability under these conditions similarly to WT cells (Figure 1B), were also defective in Hsp expression showing that the reason for the reduced labeling was not a reduction in cell viability.

Heat shock causes the accumulation of another thermoprotectant in yeast, the non-reducing disaccharide trehalose (Attfield, 1987). Heat stress survival of the *lcb1-100* mutant by *UBI4* overexpression could be due to increased trehalose synthesis. Trehalose accumulation upon heat shock was determined in WT cells and in *lcb1-100* mutant with or without a *UBI4* plasmid (Figure 4B). Upon incubation for 16 min at 37°C, the level of trehalose increased markedly and to a similar extent in all cells, whereas after this, only WT cells continued to accumulate trehalose significantly. The initial induction of trehalose upon temperature shift has been shown to be independent of new protein synthesis (Neves and François, 1992). This could explain why we find a similar induction of trehalose after a short incubation at 37°C. Upon longer incubations, the continued increase in trehalose would require induction of enzymes involved in trehalose production. Our results show that the *lcb1-100* cells are defective for sustained trehalose accumulation and for induction of Hsps and that these defects are not restored upon *UBI4* overexpression, even though these cells are resistant to heat stress. Therefore, the ability to synthesize trehalose or Hsps does not correlate with the ability of *UBI4* to suppress the *lcb1-100* mutation. Therefore, we predicted that the capacity of a cell to degrade misfolded and/or aggegated proteins using the proteasome–ubiquitin pathway must be the most important factor allowing resistance to heat stress in the absence of Hsps and sustained trehalose accumulation.

Heat shock-induced protein degradation and ubiquitination is restored in lcb1-100 mutant overexpressing UBI4

To test whether *UBI4* overexpression affects the rate of protein turnover in the *lcb1-100* mutant cells, protein degradation after heat shock at 37°C was determined in these cells and compared with WT cells. Cells grown at 24°C were shifted to 37°C, pulse-labeled with a mix of [^{35}S]methionine and [^{35}S]cysteine, then chased in presence of cycloheximide to prevent re-incorporation of radioactive amino acids released from proteins. At the indicated time, aliquots of cells were taken and protein degradation was determined as the percentage of incorporated radioactivity converted into TCA-soluble

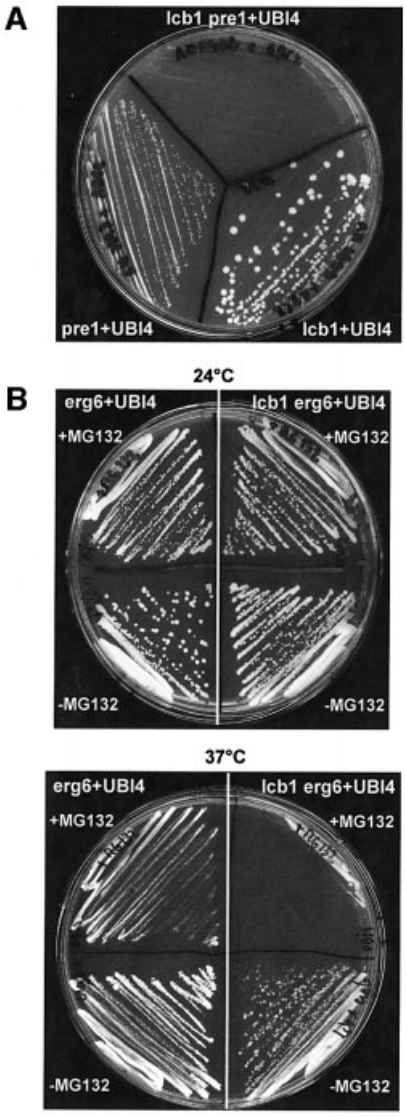

Fig. 6. The proteasome is required for *UBI4* suppression of *lcb1-100*. (**A**) The *pre1-1* strain is mutated in one subunit of the proteasome complex. Single mutant strains *lcb1-100* and *pre1-1* and the double mutant strain *lcb1-100 pre1-1* were transformed with a high copy number *UBI4* plasmid and after growth on SD selective medium at 24°C were tested for growth on YPUAD at 37°C. (**B**) *erg6* and *lcb1-100 erg6* mutant cells bearing the *UBI4* overexpression plasmid were plated onto YPUAD medium (–MG132, lower part) and on YPUAD containing the proteasome inhibitor MG132 (+MG132, upper part) and tested for growth at 24°C and 37°C.

fragments (Figure 5A). At 37°C, protein degradation in WT cells exceeded that in the *lcb1-100* mutant by ~2- to 3-fold. Overexpression of *UBI4* in the *lcb1-100* cells restored protein degradation upon heat treatment to the wild-type level. These results suggest that *UBI4* overexpression may allow the *lcb1-100* mutant cells to survive a heat stress by increasing the degradation of misfolded proteins via the ubiquitin–proteasome pathway.

The conjugation of polyubiquitin chains to short-lived or damaged proteins marks them for subsequent degradation by the proteasome. To test if this response is defective in the *lcb1-100* mutant, we compared the changes in the levels of ubiquitylated proteins after a shift to 37°C in WT and *lcb1-100* cells bearing a *UBI4* plasmid or not. Cells grown at 24°C were shifted for 1 h at 37°C to induce heat stress or kept at 24°C and the total level of ubiquitylated proteins was determined by western blotting with anti-ubiquitin antibody (Figure 5B). The *lcb1-100* mutant displayed an increase in protein ubiquitylation at 24°C compared with WT cells, showing that these mutant cells already accumulate ubiquitin conjugates at 24°C without heat stress, which is consistent with our results showing greater expression of the *UBI4-lacZ* construct in *lcb1-100* cells than in WT cells at 24°C (Figure 3B). This result suggests that even without heat shock, the *lcb1-100* mutant cells may accumulate more misfolded and/or aggregated proteins that would need to be degraded via the ubquitin–proteasome pathway. After heat shock at 37°C, WT cells and the *lcb1-100* mutant overexpressing *UBI4* displayed an increase in ubiquitylated proteins, but in the *lcb1-100* mutant cells where protein degradation was low (Figure 5A), the content of ubiquitylated proteins decreased (Figure 5B). Free ubiquitin was difficult to detect under conditions where *UBI4* was not overexpressed. Therefore, we cannot rule out that a lack of free ubiquitin is a possible cause of cell death in the *lcb1-100* mutant cells at 37°C. However, our results show that *lcb1-100* cells are able to induce the *UBI4* gene at 37°C (Figure 3B) and that protein ubiquitylation is not defective in these mutants cells. These results suggest that there probably is some free ubiquitin in the *lcb1-100* mutant cells at 37°C but that the ubiquitin level is not sufficient to respond to the quantity of accumulated, misfolded and/or aggregated proteins that need to be degraded via the ubiquitin–proteasome pathway. It was previously shown that the essential function of *UBI4* is to provide ubiquitin under conditions of stress (Finley *et al.*, 1987). Therefore, overexpression of *UBI4* in the *lcb1-100* mutant cells allows a higher synthesis of free ubiquitin upon heat stress and permits the cells to survive at 37°C via degradation of abnormal proteins presumably by the ubiquitin–proteasome pathway.

Accumulation of protein aggregates in the lcb1-100 mutant is reduced by UBI4 overexpression

To determine whether the *lcb1-100* mutant cells accumulate protein aggregates due to the lack of induction of Hsps, the percentage of aggregated proteins upon heat shock was analyzed in the *lcb1-100* mutant and compared with WT cells. Cells were pulse-labeled with a mix of [^{35}S]methionine and [^{35}S]cysteine at 24°C and then heat shocked at 37°C for the indicated time. Whole-cell extracts, containing glycerol and non-ionic detergent, of WT and *lcb1-100* cells were subjected to centrifugation to separate protein aggregates, which sedimented at 15 000 *g* and the percentage of aggregated proteins was determined (Figure 5C). The amount of aggregated proteins increased in WT cells following heat stress and then remained stable at 7%, whereas in the *lcb1-100* strain the rate of protein aggregates constantly increased after the shift to 37°C and reached 16% after 40 min incubation at 37°C. This result indicates that the *lcb1-100* mutant cells accumulate aggregated proteins upon heat shock and these aggregated proteins could be responsible for the heat sensitivity displayed by the *lcb1-100* cells. To test if *UBI4* overexpression, which restores heat shock resistance of the

lcb1-100 mutant, abrogated the accumulation of protein aggregates upon heat shock, the same experiment was performed in the *lcb1-100* cells transformed by the *UBI4* plasmid (Figure 5C). The *lcb1-100* cells overexpressing *UBI4* showed only a small accumulation of aggregated proteins similar to WT cells upon heat shock. This result shows that *UBI4* overexpression could function through the removal of denatured proteins before or after they have aggregated. If this is true, then degradation of the denatured/aggregated proteins should be required for the suppression.

The proteasome is required for UBI4-dependent suppression of lcb1-100

The proteasome is an important cellular protein degradation system that recognizes ubiquitylated proteins and functions in cellular quality control by degrading misfolded, unassembled or damaged proteins that could otherwise form potentially toxic aggregates (Ciechanover *et al*., 2000). The proteasome is a multi-enzyme complex consisting of a number of different protease subunits. In yeast cells, *PRE1* and *PRE2* genes encode two well-characterized subunits of the proteasome. The *pre1-1* mutant strain is severely deficient in cytoplasmic proteolysis, accumulates ubiquityated proteins and shows reduced growth at 37°C (Figure 6A) (Heinemeyer *et al*., 1991). To determine if the proteasome is required for *UBI4* suppression of *lcb1-100*, we constructed a double mutant strain *lcb1-100 pre1-1*; this strain is viable at 24°C, but does not grow at 37°C. We then analyzed survival at 37°C of the *lcb1-100 pre1-1* strain with or without *UBI4* overexpression. *UBI4* overexpression was not able to suppress the temperature-sensitive growth defect of the *lcb1-100 pre1-1* double mutant strain in contrast to the single *lcb1-100* mutant cells (Figure 6A). This result suggests that the heat shock resistance and survival of the *lcb1-100* cells due to *UBI4* overexpression depends on the correct function of the cytoplasmic proteasome.

The recent identification of selective proteasome inhibitors such as the peptide aldehyde MG132 allowed us to further analyze the role of the ubiquitin–proteasome pathway in the *UBI4* suppression of the *lcb1-100* heat stress defect. MG132 cannot enter wild-type yeast cells, so it is essential to use yeast strains with increased membrane permeability such as the *erg6* (*ise1*) mutant (Emter *et al*., 2002). In this mutant, MG132 blocks the rapid breakdown of proteins by the ubiquitin–proteasome pathway (Lee and Goldberg, 1998). Heat stress survival of the *erg6* and *lcb1-100 erg6* mutant strains transformed or not by the *UBI4* plasmid were analyzed by plating the different strains at 37°C on plates containing 50 μM of MG132. The *erg6* mutant strain like WT strains was able to grow at both 24 and 37°C. The double mutant strain *lcb1-100 erg6* showed a clear defect in survival at 37°C, but was resistant to this temperature when overexpressing *UBI4*, meaning that it has the same phenotype as the *lcb1-100* mutant strain and could be used to test the effect of the MG132 inhibitor (Figure 6B). The *erg6* mutant strain grew at 37°C in presence of 50 μM MG132, whereas the double mutant strain *lcb1-100 erg6* was defective for growth even with *UBI4* overexpression (Figure 6C). This result confirms that the *UBI4* suppression of the *lcb1-100* heat stress defect requires the ubiquitin–proteasome degradation pathway.

In summary, we show that Hsp induction upon heat shock is defective in cells lacking serine palmitoyl transferase activity. Both the Hsf1p- and Msn2/4p-dependent stress pathways are dependent upon serine palmitoyl transferase activity. However, the expression of *UBI4* is not completely abolished in cells deficient for these two stress pathways (Simon *et al*., 1999) or in *lcb1-100* mutant cells. These results suggest that *UBI4* expression is controlled by additional factor(s) whose activation could be independent of sphingoid base synthesis.

We have shown that the lack of Hsp induction, which presumably is the cause of a hypersensitivity to heat shock, can be overcome by increased expression of ubiquitin. The function of ubiquitin in this process requires the proteasome, because proteasome mutants and inhibitors abrogate the ability of ubiquitin to restore heat shock resistance. This suggests that the major essential function of Hsp induction at high temperature is to help refold denatured and/or aggregated proteins. Removal of these misfolded or aggregated proteins by ubiquitin-dependent proteasomal degradation is also sufficient to render cells resistant to heat shock. This shows that it is the removal of the aberrant proteins and not their refolding that is essential to recover from heat shock. This is consistent with the recent finding that aggregates formed from two non-disease-related proteins are substantially cytotoxic (Bucciantini *et al*., 2002). Our results also show that yeast cells can survive with substantially reduced levels of sphingolipid biosynthesis provided that they overexpress ubiquitin. This suggests that one of the major essential functions of the ceramide synthesis pathway is to control the expression of proteins involved in removal or refolding of denatured or aggregated cytoplasmic proteins.

Materials and methods

Plasmids and yeast strains

Previously described plasmids used in this study were pSH24 (*PKC1*), YEp195-PKH1, YEp195-PKH2 (Friant *et al*., 2000, 2001), pKN32 (gift from S.K.Lemmon), YEp352-UBI4 (gift from M.Ellison) and YEplac181-UBI4, bearing the *UBI4* gene on high-copy number plasmids, and plasmid YEp112-CUP1-Ub containing a synthetic yeast ubiquitin gene under the control of the *CUP1* promoter (gift from M.Hochstrasser) (Hochstrasser *et al*., 1991; Nelson and Lemmon, 1993; Prendergast *et al*., 1995), the YEplac195 plasmid containing *TPS1*, *TPS2*, *TPS3* and *TSL1* genes (kindly provided by J.M.Thevelein; Bell *et al*., 1998), pKAT6 (YEp24-HSC82) and pYSGal104 (pRS316-pGAL1-HSP104) (kind gifts from S.Lindquist; Nathan and Lindquist, 1995; Lindquist and Kim, 1996), YEp434-A4 (*SSA4*), YEp351-SSA2, YCp50-GAL1-SSA1, pZJHSE2-137 containing an HSE, HSE2 from *SSA1* promoter fused to *LacZ* (kind gifts from E.A.Craig (Slater and Craig, 1987), The UBI4-lacZ and the HSP26-LacZ plasmids (kindly provided by T.Schmelzle), the pUKC414 vector containing the *HSP26* promoter fused to *LacZ* (S.Christodoulou, P.Bossier, C.Stokes and M.F.Tuite, unpublished) and the UBI4-lacZ plasmid (Tanaka *et al*., 1988).

The yeast strains used in this study were RH448 (WT), RH3802 and RH3809 (*lcb1-100*) (Friant *et al*., 2000), RH3804 (*Matα lcb1-100 trp1 leu2 ura3 lys2 bar1*), RH3323 (*Mata pre1-1 his3 his4 lys2 ura3 leu2 bar1*), RH5404 (*Matα pre1-1 lcb1-100 lys2 ura3 leu2*), RH4237 (*Matα erg6::LEU2 his4 lys2 ura3 leu2 bar1*), RH4727 (*Matα erg6::LEU2 lcb1-100 his4 ura3 leu2 bar1*), and W303 derivatives (from F.Estruch) W303–1A (*Mata ade2-1 can1-100, his3-11,15, leu2-3 112, ura3-1, trp1-1*), ΔCTD (W303–1A Δ*CTD::URA3*), msn2 msn4 (W303–1A *msn2-Δ3::HIS3 msn4Δ::URA3*), msn2 msn4 ΔCTD (W303–1A *msn2-Δ3::HIS3 msn4Δ::TRP1 HSF*(*1–583*)*::URA3*), Δhsf (W303–1A tetO::HSF1::KanMX4), Δhsf msn2/4 (W303–1A *msn2-Δ3::HIS3 msn4Δ::URA3* tetO::HSF1::KanMX4) (Amoros and Estruch, 2001).

UBI4 suppression of the lcb1-100 mutation

The RH3809 (*lcb1-100*) strain carrying a temperature-sensitive allele of the *LCB1* gene was transformed with pKN32, a YEp24-based plasmid bearing *UBI4*, the polyubiquitin gene. This transformant could grow at 37°C showing that *UBI4* is a high copy suppressor of the *lcb1-100* mutation. The *lcb1-100* mutant strains RH3802, RH3804 and RH3809 were also transformed by other plasmids bearing the *UBI4* gene (YEp352-UBI4 and YEplac181-UBI4) and tested for growth at 37°C, to ensure that the suppressor effect observed was due to overexpression of *UBI4* gene. *UBI4* rescues growth at 37°C only in high copy number and is not able to suppress the viability defect associated with a *lcb1*::*URA3* strain. RH3804 strain was also transformed by YEp112-CUP1-Ub plasmid containing a synthetic yeast ubiquitin gene under the control of the CUP1 promoter and this transformant was able to grow on YPUAD plates containing $CuSO_4$ (0.1 mM final concentration) at 37°C.

Viability assay

Mid log-phase cultures of WT (RH448) and *lcb1-100* (RH3809) cells overexpressing *UBI4* or not were grown in YPUAD at 24°C and an aliquot was shifted to 44°C. Samples were taken at the times indicated in duplicate, and diluted onto ice-cold YPUAD, and immediately plated onto YPUAD agar to assess cell viability (Martinez-Pastor *et al*., 1996). Viability was expressed as a percentage of viable cells relative to the initial colony-forming units, measured at 24°C before the heat shock. The viability experiments were repeated twice, yielding similar results.

Sphingolipid analysis

[^{3}H]*myo*inositol and [^{3}H]DHS labeling of yeast cells was performed for 30 min at 24°C or 37°C after a 15 min preincubation at the corresponding temperature. The lipids were extracted, treated with base to identify sphingolipids, and analyzed by TLC and PhosphorImaging as described (Zanolari *et al*., 2000).

Liquid β-galactosidase assay

A liquid β-galactosidase assay was performed as described previously with slight modifications (Miller, 1972; Guarente, 1983). For each sample, time, OD_{420} and protein concentration, using a Bradford assay kit (Bio-Rad), was determined. The values reported are the average of three independent measurements for WT and *lcb1-100* experiments and of two independent measurements for experiments with *UBI4* overexpression respectively.

Heat shock protein labeling

Strains were grown exponentially in SD medium at 24°C, shifted for 15 min at 44°C to induce heat shock protein synthesis and pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine mix (EasyTag EXPRESS[^{35}S] mix from NEN) for 10 min, followed by 1 min chase, prior to protein extraction, proteins were resolved by SDS–PAGE analysis essentially as described previously (Miller *et al*., 1979).

Extraction and assay of trehalose

Measurement of trehalose was performed on early log phase cells (0.4–0.5 OD_{600} units/ml) grown on YPUAD at 24°C and transferred to a prewarmed large flask at 37°C for the indicated times, by a protocol similar to that described previously (Lee and Goldberg, 1998) except that trehalose extraction was for 10 min at 95°C, enzymatic trehalose digestion for 3–4 h and that total proteins were extracted using the NaOH/2-mercaptoethanol and TCA procedure (Horvath and Riezman, 1994), dissolved in 0.1 N NaOH/1% SDS, and protein was determined using the detergent compatible procedure (Pierce). Trehalose induction experiments were repeated twice with essentially identical results.

Total protein degradation

Measurement of total protein degradation was performed as described previously with the following slight changes (Lee *et al*., 1996). Cells were grown to optical density at 600 nm 0.4 to 0.6 at 24°C in SD media and 2.5 OD_{600} units of cells were pulsed with 0.2 mCi [^{35}S]methionine/[^{35}S]cysteine mix (EasyTag EXPRESS[^{35}S] mix from NEN) for 3 min as described. Chase was initiated by adding a 1/100 volume of a mixture of 0.3% methionine/cysteine in 0.3 M $(NH_4)_2SO_4$ and cycloheximide (0.5 mg/ml). At indicated time intervals after shifting the cells to 37°C, aliquots were removed and analyzed as described before (Lee *et al*., 1996). The rate of protein degradation is expressed as the percentage of incorporated radioactivity converted into acid-soluble fragments from the cells during the chase period normalized to the total amount of cells in each sample.

Ubiquitin–protein conjugates determination

Analysis of ubiquitin–protein conjugates was performed as described previously with the following slight changes (Lee *et al*., 1996). Cells were grown to 0.6–0.8 OD_{600} units/ml at 24°C in YPUAD media, 1 OD_{600} was shifted to 37°C for 1 h or kept at 24°C and cells were harvested and lysed by vortexing with glass beads in 10 mM Tris–HCl pH 7.4, 1 mM EDTA–2% SDS buffer for 3 min at 4°C. Extracts were analyzed by SDS–PAGE and western blot analysis with anti-ubiquitin antibody (Zymed Laboratories Inc.) using ECL protocols (Amersham Biosciences).

Protein aggregate analysis

Cells were grown to a density of 0.5×10^6 cells/ml in synthetic SD media containing 0.5% yeast extract and 40 mg/l of the appropriate amino acids. 1.2×10^8 cells were harvested and washed in 10 ml SD without yeast extract. 3×10^7 cells per time point in a total volume of 0.5 ml SD were labeled with 0.2 mCi [^{35}S]methionine/[^{35}S]cysteine mix (EasyTag EXPRESS[^{35}S] mix from NEN) for 10 min at 24°C. 5 µl 100× chase mix (0.3% methionine and cysteine, 0.3 M $(NH_4)_2SO_4$) were added and cells were heat shocked for the indicated time points at 37°C. Heat shock was terminated by adding NaF and NaN_3 to a final concentration of 8 mM and cooling on ice. Cells were washed with ice-cold glycerol buffer (1 mM EDTA, 150 mM KCl, 1 mM EGTA, 50 mM HEPES, 20% glycerol, 0.5% Triton X-100 pH 7.4) and resuspended in 0.2 ml ice-cold glycerol buffer containing 1 mM PMSF. Glass beads were added and lysis was performed as described (Miller *et al*., 1979). Cell debris was spun down at 3000 *g* at 4°C. 1/20 of total lysate was removed and 19/20 were centrifuged at 15 000 *g* for 15 min at 4°C. The supernatant was removed and the total or pellet fractions were analyzed by either 7.5% SDS–PAGE or liquid scintillation counting using a Packard Scintillation Counter (Packard Instrument Company, USA). The data shown represent the average of five individual experiments.

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