# Mechanisms of Sphingolipid Functions during Heat Stress in *Saccharomyces cerevisiae*

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Prof. Dr. Hans-Jakob Wirz, Dekan

Wir müssen etwas Wichtigeres tun. Wir müssen ihn kurz und klein zusammenschlagen, den Sausinn, damit wir die notwendige Arbeit tun können. Das ist was viel was Schwereres, die notwendige Arbeit ist: die Wahrheit schreiben von allem, die keinen Big Sinn nicht hat, aber notwendig ist, notwendig ist das einfache wahre Abschreiben der Welt.

Rainald Goetz, Subito, 1983

# SUMMARY

Sphingolipids are important membrane constituents in all eukaryotic cells. Ongoing sphingolipid synthesis is required for a variety of cellular processes including response to heat shock. During heat stress, *de novo* sphingolipid synthesis is upregulated and mutants defective in the biosynthesis of sphingolipids show a significantly greater loss of viability. To dissect the mechanisms of sphingolipid functions during heat stress we made use of the *Saccharomyces cerevisiae lcb1-100* mutant. This mutant is conditionally impaired in serine palmitoyltransferase activity, the first committed step in sphingolipid biosynthesis. *lcb1-100* cells are highly sensitive to heat shock and die at elevated temperatures.

In this study we could show that the increased sensitivity to heat stress in *lcb1-100* cells correlated with a lack of induction of the major heat shock proteins at high temperature. This defect could be restored by the overexpression of ubiquitin, a situation that increased turnover of proteins and prevented accumulation of protein aggregates in *lcb1-100* cells. These data showed that the essential function of heat shock protein induction is the removal of misfolded or aggregated proteins. Furthermore it suggested that heat stressed cells 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.

In addition we tried to explain the lack of induction of heat shock proteins in *lcb1-100* cells. We could show that transcription and nuclear export of heat shock protein mRNA was not affected in these cells. Under heat stress conditions, *lcb1-100* cells exhibit a strong decrease in protein synthesis and polysome analyses demonstrated a defect in translation initiation. Furthermore we demonstrate that efficient translation under these conditions relies upon the synthesis of sphingoid base. Deletion of the eIF4E binding protein Eap1p partially restores translation initiation and the synthesis of heat shock proteins in *lcb-100* cells. Thus, sphingoid bases signal to the cap-dependent translation initiation apparatus to enhance heat shock protein synthesis. In addition, ubiquitin overexpression in the *lcb1-100* mutant allowed recovery of translation, but not at the initial phase where heat shock proteins are made. Therefore the recovery process seems to be dependent upon the function of the heat shock proteins that are made during the initial phase of heat stress.

Altogether we have uncovered a new function for sphingoid bases and provide an explanation for the sphingoid base synthesis requirement for survival during heat stress.

# CONTENTS

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# PREFACE

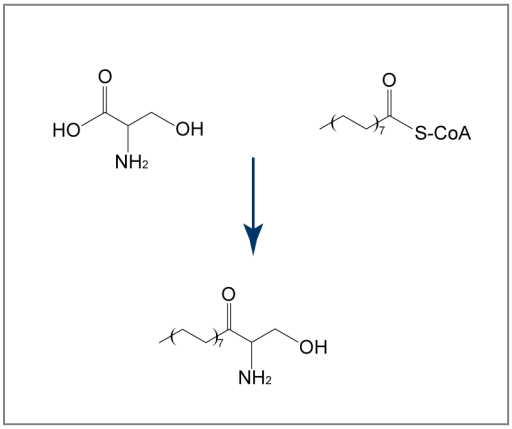
This thesis is basically divided into four main chapters. In the first chapter, the introduction, information for the subsequent three chapters can be found. This introduction, however, is not exhaustive concerning the growing field of sphingolipid synthesis, including all the metabolites, their broad range of functions, and heat stress in all organisms analyzed. Still all relevant work and also new and exciting findings in this specific fields are included.

The two following chapters represent two major findings that were made during this study. The first important finding was that cells which are deficient for sphingolipid synthesis are unable to produce heat shock proteins. These cells nevertheless are able to survive a heat stress provided they have enough ubiquitin. This proved that the major task of heat shock proteins is the removal of unfolded proteins. The experimental part in this work was done in close collaboration with Sylvie Friant who also made the initial observation. This study was published in 2003 in EMBO Journal.

The second finding was that cells, deficient in sphingoid base synthesis, have a defect in translation initiation. The analysis of this defect uncovered a novel lipid-mediated regulation of translation initiation and in addition gave raise to some new functions for eIF4E-binding proteins. This study was carried out with the help of Olivier Deloche, Kentaro Kajiwara and Koichi Funato and yielded a manuscript prepared for submission to Journal of Biology.

In the last chapter these new results were brought together and are discussed in a broader context. Although this part is hypothetical at least it should display the upcoming concepts in current research about sphingolipid synthesis, metabolism and their functions in yeast. Sphingolipid synthesis during heat stress: To maintain and change the balance.

# CHAPTER I



# Introduction

## HEAT STRESS

Cells constantly sense and respond to their changing external environment. In order to sense such alterations, signals must be generated and transmitted. One of the most dramatic and acute changes is a rapid increase in temperature. Heat damages cells in a variety of ways, most critically by disrupting the integrity of membranes and by causing proteins to denature and aggregate (Parsell and Lindquist, 1994). To survive heat, cells developed several mechanisms to cope with heat stress by generating and transmitting signals. These mechanisms affect a variety of cellular processes such as transcription, translation, folding and degradation of proteins and the cell cycle (Figure 1-1 and below).

At the onset of heat stress cells change their transcriptional profile dramatically (Gasch et al., 2000; Murray et al., 2004). In the budding yeast *Saccharomyces cerevisiae* several transcriptional control systems appear to be responsible for the changes in mRNA levels upon heat stress. One system involves the heat shock factor HSF. In yeast, HSF is encoded by a single essential gene, *HSF1*. Hsf1p binds to heat shock elements (HSE's) found in the promoter region of many heat shock protein genes (Amin et al., 1988; Wu, 1995). HSE's consist of a 5bp DNA consensus element nGAAn and about 50 genes were shown to be dependent of activation by Hsf1p (Boy-Marcotte et al., 1999). HSF proteins of various organisms share common structural motifs including the helix-turn-helix DNA-binding domain and are functionally conserved (Pirkkala et al., 2001; Wu, 1995). In yeast, Hsf1p is constitutively present as a DNA bound trimer and activates transcription upon heat stress (Jakobsen and Pelham, 1988; Sorger and Pelham, 1987). Biochemical evidence suggests that some heat shock proteins (HSP) can physically interact with HSF's (Nadeau et al., 1993; Shih et al., 2000; Zou et al., 1998). This proposed a model in which the cellular level of HSPs directly or indirectly regulates HSF activity. However this seems to be unlikely since an increase in basal expression of HSPs does not affect the Hsf1p activation temperature (Hjorth-Sorensen et al., 2001). Moreover it was shown that heat stress inducibly phosphorylates Hsf1p – but the precise role of this phosphorylation event is still under debate (Hoj and Jakobsen, 1994; Sorger, 1990). The signal which reflects and transmits the physiological state of the cell to Hsf1p remains an open question.

Another transcriptional control system depends on the Msn2p and Msn4p transcription factors. Genes which are transcriptionally not only induced by heat but also by other stresses like osmotic shock, DNA damage, oxidative stress and glucose starvation were shown to contain stress response elements (STRE's) in their promoter (Kobayashi and McEntee, 1990; Kobayashi and McEntee, 1993). Msn2p and Msn4p are two highly homologous $C_2H_2$ zinc finger proteins that bind to the STRE (Martinez-Pastor et al., 1996). The core consensus sequence of an STRE was

determined to be CCCCT (Marchler et al., 1993) and the yeast genome contains as many as 186 potentially STRE regulated genes (Moskvina et al., 1998; Treger et al., 1998). Mutants deleted for *MSN2* and *MSN4* are highly sensitive to various stresses (Martinez-Pastor et al., 1996). In contrast to Hsf1p, activation of Msn2p and Msn4p is better understood. Msn2p and Msn4p are localized throughout the cytoplasm in unstressed cells and after stress treatment they accumulate in the nucleus (Gorner et al., 1998). Heat activation of an STRE reporter was enhanced by a *ras2* mutation and reduced in a *BCY1* deletion (Marchler et al., 1993), suggesting a role for the PKA pathway in the regulation of Msn2p and Msn4p. Indeed, mutations in the potential PKA modification sites of Msn2p lead to constitutive nuclear localization of this protein (Gorner et al., 1998). However, the PKA pathway appears only to transmit the glucose sensing signal to Msn2p and Msn4p because heat stress does not affect the PKA-responsive site (Gorner et al., 2002). The fact that, in contrast to Hsf1p, Msn2p and Msn4p are also regulated by other stresses enhanced understanding of the regulation of these transcription factors. For instance treatment of cells with rapamycin, a potent inhibitor of TORC1 (reviewed in Crespo and Hall, 2002; Loewith et al., 2002), was shown to induce the nuclear accumulation of Msn2p (Beck and Hall, 1999), regulated by the PP2A type protein phosphatase (Santhanam et al., 2004). This nuclear accumulation is only transient and thought to occur oscillatory, depending on the strength of the applied stress (Jacquet et al., 2003).

Whereas activation of Hsf1p and Msn2/4p lead to an increase in transcription of a specific set of genes also transcriptional down-regulation of a distinct class of genes was observed. Already in the 70's it was shown that heat stress leads to a transient decrease in the synthesis of ribosomal proteins (Gorenstein and Warner, 1976) which was caused by a transient inhibition of transcription of ribosomal protein genes (Kim and Warner, 1983). Also the production of full-size 35S pre-rRNA was shown to decline for a short period upon a temperature up-shift (Warner and Udem, 1972). The repression of ribosomal protein genes was observed for multiple stress responses (Warner, 1999) and is thought to be regulated by the transcription factor Rap1p (Li et al., 1999; Moehle and Hinnebusch, 1991). There is some evidence that Rap1p interacts with Fhl1p on ribosomal protein gene promoters (Lee et al., 2002; Shore, 1994; Wade et al., 2004) and Fhl1p itself was found to be under the control of the RAS-PKA and the TOR pathway (Martin et al., 2004; Schawalder et al., 2004). The exact mechanism of regulation of theses factors during heat stress still needs to be understood.

Several different studies then showed the regulation of gene transcription during heat stress with the use of high density DNA microarrays (Lashkari et al., 1997). These studies were able to prove the specific upregulation of genes during heat stress and also showed that heat stress leads to a rapid but temporary repression of ribosomal gene transcription in yeast (Causton et al., 2001; Eisen et al., 1998; Hahn et al., 2004). In contrast to yeast cells, the overlap in the responses of

cultured human cells to different stresses was rather poor (Gasch et al., 2000). Heat stress, however, leads to an increase of many heat shock proteins also in cultured HeLa and primary lung fibroblast cells (Murray et al., 2004).

Following transcription, the corresponding mRNA's are exported from the nucleus (Stutz and Rosbash, 1998). mRNA export was also suspected to occur through different pathways under normal and under heat stress conditions. Studies of mRNA export during heat shock led to the hypothesis that non heat shock mRNA's accumulate within the nucleus whereas as mRNA's encoding HSPs are efficiently exported (Saavedra et al., 1996). In addition, a nucleoporin like protein was found that was required for export of heat shock specific mRNA's during heat stress (Saavedra et al., 1997). More recent results could show, that heat shock and non heat shock mRNA's are exported via similar pathways and that mRNA export under normal and heat stress conditions seems to subjected to competition among different mRNA molecules for a limiting amount of common transport factors (Vainberg et al., 2000).

After export from the nucleus, heat shock specific mRNA's are translated in the cytoplasm (McCarthy, 1998). The process of translation during heat stress is also regulated. Incubation of mammalian cells at elevated temperatures decreases the fraction of active, polysomal ribosomes from being more than 60% to less than 30%, showing a decrease in translation initiation. Return to lower temperature then leads to an increase in protein synthesis, consistent with the recovery from such stress conditions (Burdon, 1987; Duncan and Hershey, 1989). Important to note is that during the inhibition of total protein synthesis, heat shock proteins can still be made (Duncan and Hershey, 1989). Similar mechanisms were found in yeast, although Uesono *et al.* reported that down-regulation of total protein synthesis during the initial phase of heat stress was not detected (Uesono and Toh, 2002; and below). The exact mechanism by which cells down-regulate total protein synthesis is unclear. One explanation might be that the translation initiation factor eIF2$\alpha$ gets transiently phosphorylated and inactivated during heat stress, reducing translation initiation on most mRNA's (Deloche et al., 2004; and below).

After translation, many of the heat shock mRNA's give rise to a set of functional heat shock proteins. This proteins are then responsible for the synthesis of trehalose, a sugar which acts as a thermoprotectant (Singer and Lindquist, 1998b) against misfolding of proteins and for the degradation of unfolded and aggregated proteins (Imai et al., 2003; Riezman, 2004).

Trehalose is synthesized by the trehalose phosphate complex, consisting of at least three distinct, copurifying components: Tps1p and Tps2p with Tps3 or Tsl1p (De Virgilio et al., 1993; Vuorio et al., 1993). An increase in trehalose levels is thought to be regulated by the synthesis of

additional trehalose phosphate complex members, of which *TPS1* and *TPS2* contain STRE sequences in their promoter and are transcriptionally upregulated upon heat stress. An increase in trehalose levels can also be accomplished by a rise in intracellular levels of the substrates (Neves and Francois, 1992). Trehalose was initially thought to stabilize membranes during heat stress (Crowe et al., 1992). More recent work however suggests that trehalose protects cells from heat by stabilizing proteins at high temperatures and by preventing aggregation of denatured proteins (Singer and Lindquist, 1998a).

Aside from synthesizing trehalose, heat shock proteins function as molecular chaperones. These prevent protein aggregation and refold denatured proteins to their native confirmation. For example, Hsp70, a DnaK homolog, was shown to bind extended hydrophobic amino acid sequences that are normally sequestered in the core of the protein. These regions often tend to stick together and to promote the formation of protein aggregates (Parsell and Lindquist, 1994). Hsp104, one of the major stress response proteins, was shown to mediate the resolubilization of polypeptides that have already begun to aggregate (Parsell et al., 1994). Later, Hsp104 in concert with Hsp70 and Hsp40, a DnaJ homolog, were then described as a chaperone system that is able to dissolve and refold aggregated proteins (Glover and Lindquist, 1998). Not only refolding but also degradation of unfolded and aggregated proteins occurs during heat stress since not every non-native protein can be re-/ folded successfully.

Those proteins that are not accurately folded or refolded are tagged with ubiquitin and directed to the proteasome which is responsible for the selective elimination of abnormal and denatured proteins by degradation (Imai et al., 2003; Seufert and Jentsch, 1990). Ubiquitin is activated in an ATP dependent process by which a single ubiquitin molecule is loaded with the help of an E1 ubiquitin-activating enzyme onto an E2 ubiquitin-conjugating enzyme. The E3 ubiquitin protein ligase then recognizes the target and transfers the ubiquitin moiety from E2 to the target (Hochstrasser, 1996; Pickart, 2001). The acceptor protein usually undergoes polyubiquitination, resulting in chains in which the carboxy-terminus of each ubiquitin is linked to either lysine at position 29, 48 or 63 of the preceding ubiquitin. Especially during heat stress, linkage to lysine 63 was shown to be important since replacement of this lysine with a residue that can not be ubiquinitated decreased survival at high temperature (Arnason and Ellison, 1994). Linkage at lysine 63 residue was shown to be perfomed by the two ubiquitin-conjugating enzymes Ubc4p and Ubc5p. Moreover, deletion of *UBC4* and *UBC5* significantly increased sensitivity to heat stress (Arnason and Ellison, 1994). This was in agreement with a previous result, showing that deletion of the poly-ubiquitin encoding gene UBI4 (Finley et al., 1987) increased sensitivity to diverse stresses, demonstrating once more the importance of this pathway under heat stress conditions. The multi-ubiquitin chain is generally believed to improve targeting to the proteasome (Pickart, 1997;

van Nocker et al., 1996). Recently it was reported that not only ubiquitin but also ubiquitin-conjugating enzymes interact with the proteasome (Tongaonkar et al., 2000). A new model proposes that unfolded proteins are recognized by Hsp90 or Hsp70 and upon inability to refold the protein these complex is bound by a specific E3 ubiquitin ligase, mediating ubiquitination and destruction in the proteasome (Hohfeld et al., 2001; Meacham et al., 2001).

In order to give the cell enough time to adjust to the new environmental conditions the progress in cell cycle is transiently arrested in the $G_0/G_1$ phase during heat stress (Shin et al., 1987), resulting in a decrease in budding after one hour of heat stress (Johnston and Singer, 1980). A decrease in $G_1$ cyclins induces a $G_0/G_1$ arrest and the decrease in budding during heat stress was shown to be blocked by expression of a hyperstable *CLN3* allele (Rowley et al., 1993). After thermotolerance has been achieved, the second phase of the heat stress response is characterized by a resumption of normal growth at the elevated temperature. This phase is marked by a HSP70 dependent process of trehalose degradation (Hottiger et al., 1992; Singer and Lindquist, 1998b) and resumption of normal cell cycle (Johnston and Singer, 1980). The exact molecular mechanism that causes this cell cycle response is not yet understood.

Little is known about the way through which heat activates the stress response. It was proposed that heat increases the amount of reactive oxygen species within cells (Jacquet et al., 2003), which leads to the activation of the RAS-PKA pathway. Also, certain membrane proteins were thought to act as a sensor for heat stress. The *WSC* proteins localize to the plasma membrane and deletion of all three *WSC* genes was shown to increase heat stress sensitivity, similar to mutants in the *PKC1* pathway. Increased activity of the MAP kinase *SLT2* was impaired in *wsc*Δ strains and in addition, overxpression of *WSC* genes suppressed the heat shock sensitivity of a hyperactivated RAS allele (Verna et al., 1997). A following study showed that Wsc1p regulates the actin cytoskeleton via activation of Rho1p. Activated Rho1p then activates the downstream *Pkc1p* pathway and the regulation of the de- and repolarization of the actin cytoskeleton during heat stress (Delley and Hall, 1999).

Still it remains to be determined what acts as a sensor of heat stress and causes the dramatic changes within a cell. Although several signaling pathways like the RAS-PKA and the WSC-PCK pathway are likely to be involved in the regulation of this response, the exact trigger, the cause, of this responses is not yet known. A potential trigger could be the synthesis of sphingolipids. In response to heat stress, cells induce the *de novo* synthesis of free sphingoid bases, ceramides and sphingolipids (Dickson et al., 1997a; Jenkins et al., 1997; Wells et al., 1998). Induction of

sphingolipid synthesis during heat is thought to participate in many, if not all, responses during heat stress and therefore moved into the centre of interest (see below).

## SPHINGOLIPID METABOLISM

Sphingolipids and their metabolites are abundant components of many membranes in eukaryotic cells (Hechtberger et al., 1994; Patton and Lester, 1991). The first and rate-limiting early step in mammalian and *Saccharomyces cerevisiae* sphingolipid synthesis is similar and involves condensation of L-serine and palmitoyl-CoA by the serine-palmitoyltransferase (SPT) (Figure 1-2). The SPT was located to the endoplasmic reticulum in mammals (Yasuda et al., 2003) and yeast cells (Han et al., 2004). At least two genes, *LCB1* and *LCB2* are necessary for SPT activity in all higher eukaryotes (Hanada, 2003) and their proteins were shown to interact tightly (Gable et al., 2000). Deletion of either of these genes is lethal but can be rescued by supply of exogenous sphingoid bases in yeast (Pinto et al., 1992a). In addition yeast contains a third gene which is required for optimal SPT activity during heat stress, *TSC3*. Tsc3p may bind palmitoyl-CoA and deliver it to the SPT, thus increasing its activity (Gable et al., 2000; Monaghan et al., 2002). Condensation of L-serine with palmitoyl-CoA then generates the sphingoid base 3-ketodihydrosphingosine (3-KDS).

3-KDS is reduced into dihyrosphingosine (DHS), also called sphinganine. This reaction is performed by the Tsc10p enzyme and requires NADPH (Pinto et al., 1992b). Like the SPT subunits, Tsc10p is essential for growth (Beeler et al., 1998). In all organisms studied so far, DHS is rapidly metabolised (Dickson and Lester, 2002). In yeast, DHS can be modified in three ways: hydroxylation, phosphorylation or condensation with a very long chain fatty acid to make ceramide. This in contrast to mammalian cells where mainly fusion occurs.

Hydroxylation of DHS at the 4 position yields the primary sphingoid base in yeast, phytosphingosine (PHS, also called 4-hydroxysphinganine) (Grilley et al., 1998). This reaction is catalyzed by Sur2p, which can also hydroxylate DHS-derived ceramide to produce PHS-ceramide (Grilley and Takemoto, 2000; Haak et al., 1997) (see below and Figure 1-2). Sur2p is an integral membrane protein and localized in the ER. Interestingly, deletion of *SUR2* is not essential for growth indicating redundancy between the two sphingoid bases (Cliften et al., 1996).

DHS and PHS can be phosphorylated by the two sphingoid base kinases Lcb4p and Lcb5p to yield DHS-1P and PHS-1P (Nagiec et al., 1998). Lcb4p is responsible for about 97% of total phosphorylation activity. The kinase activity seems to have some stereospecificity because the nonbiological threo-DHS isomers were less well phosphorylated (Lanterman and Saba, 1998; Nagiec et al., 1998; Skrzypek et al., 1999). In contrast, purified Lcb4p phosphorylated all 4 stereo isomers with the same efficiency in an *in vitro* reaction (I. Andrey and H. Riezman, personal

communication). Although none of these kinases contain a membrane localization signal about two thirds of the Lcb4p and about one third of the Lcb5p kinase activity was found in the membrane fraction. Lcb4p was localized to the ER whereas Lcb5p was found to cofractionate with Golgi membranes (Funato et al., 2003)

Once phosphorylated, sphingoid bases are generated they can be dephosphorylated or cleaved. Dephosphorylation is achieved by Lcb3p or Ysr3p in yeast (Qie et al., 1997). These phosphatases are specific for sphingoid base phosphates since they can not dephosphorylate ceramides or phospholipids (Mao et al., 1997). *LCB3* and *YSR3* encode for integral membrane proteins with several transmembrane domains. Neither of these genes is essential and both of the encoded proteins were localized to the ER. Lcb3p and Ysr3p are highly identical but differ in several physiological aspects. Whereas *LCB3* transcripts are very abundant, mRNA of *YSR3* is barely detectable (Mao and Obeid, 2000; Mao et al., 1999).

Cleavage of sphingoid base phosphates is performed by a lyase encoded by *DPL1*. Dpl1p is specific for D-erythro-sphingoid bases and prefers C16-DHS-1P (Zhang et al., 2001) and cleavage produces ethanolamine phosphate and fatty aldehyde (Saba et al., 1997). Dpl1p localizes to the ER (Grote et al., 2000), has one putative transmembrane domain and is membrane integrated despite of having a translocation sequence (K.D. Meier and H.Riezman, unpublished). A deletion of *DPL1* is viable but is hypersensitive to sphingoid bases and accumulates sphingoid base phosphates (Kim et al., 2000; Saba et al., 1997; Skrzypek et al., 1999; Zhang et al., 2001; and K.D. Meier and H. Riezman, unpublished).

Exogenous sphingoid bases and sphingoid base phosphates can also enter into the sphingolipid biosynthesis pathway, but their incorporation requires a specific mechanism. Deletion of *LCB3* or both *LCB3* and *YSR3* was shown to almost completely block the incorporation of exogenous sphingoid bases into ceramide (Mao et al., 1999). In addition Lcb4p but not Lcb5p is required for incorporation of exogenous sphingoid bases into ceramide (Funato et al., 2003). This suggests a phosphorylation, dephosphorylation cycle for the uptake of exogenous sphingoid bases and incorporation into ceramide (Funato et al., 2003; Mao et al., 1997).

Upon condensation with a very long chain fatty acyl (VLCFA) -CoA DHS and PHS can be used to produce ceramides. The VLCFA-CoA is usually of either C20:0, C24:0 or C26:0 type whereas wild type *Saccharomyces cerevisiae* uses almost exclusively the C26:0 type. This indicates a requirement for fatty acid elongation in ceramide production. Palmitoyl-CoA is elongated by the products of *ELO2*, *ELO3*, *TSC13* and *YBR159w* genes in a set of reactions which take place in the ER, yielding a VLCFA-CoA (Funato et al., 2002; Leonard et al., 2004). The simultaneous deletion of *ELO2* and *ELO3*, as well as the deletion of *TSC13* is lethal in yeast (Kohlwein et al., 2001; Oh et al., 1997; Rossler et al., 2003; Toke and Martin, 1996).

A C26:0 or sometimes a C24:0 VLCFA-CoA is then fused to DHS or PHS to produce dihydroceramide or phytoceramide. Based on the precursor, two types of ceramides can be produced that can be diverted into five different forms, depending on subsequent modifications (Figure 1-3).Ceramide synthesis is catalyzed by the ceramide synthase. The ceramide synthase consists of three subunits, the highly homologous Lac1p and Lag1p as well as the recently identified Lip1p (Guillas et al., 2001; Schorling et al., 2001; Vallee and Riezman, 2005). Lac1p and Lag1p are both several transmembrane spanning proteins localized to the ER (Guillas et al., 2001; Schorling et al., 2001). Lip1p also localizes to the ER and spans the membrane once, with a short N-terminal cytoplasmatic tail. So far it has been unclear if ceramide synthesis occurs at the cytosolic surface or in the lumen of the ER. Deletion of the cyptoplasmic N-term of Lip1p did not abrogate ceramide synthesis, suggesting that ceramide synthesis does not take place on the cytoplasmic site of the ER (Vallee and Riezman, 2005).

In mammals DHS or sphinganine is usually consumed with the use of a fatty acyl-CoA for the synthesis of dihydroceramide which is rapidly converted into ceramide. Ceramide is the central metabolite of mammalian sphingolipid biosynthesis (Hannun and Luberto, 2000). For instance sphingosine-1 phosphate (S1P) in mammals is produced by the cleavage of ceramide by ceramidases (see below) which yields sphingosine and a fatty acid. Sphingosine is then subsequently phosphorylated by the two known mammalian sphingosine kinases SphK1 and SphK2 (Spiegel and Milstien, 2003). In addition, ceramides can be phosphorylated to yield ceramide-1 phosphate, glycosylated to yield the glycosylceramides or modified by addition of phosphocholine to form the sphingomyelins (Futerman and Hannun, 2004; Reynolds et al., 2004).

Like in mammals, ceramides can also be cleaved into their building blocks in yeast. This reaction is performed by the two ceramidases Ydc1p and Ypc1p, both integral ER membrane proteins (Mao et al., 2000a; Mao et al., 2000b). Upon a total loss of ceramide synthase activity, Ydc1p and Ypc1p can revert their activity and produce small amounts of ceramides again (Mao et al., 2000a; Mao et al., 2000b; Schorling et al., 2001).

Up to the formation of ceramide, all steps are located in or within the ER. After its synthesis, ceramide then needs to travel from the ER to the Golgi apparatus, the site of IPC synthesis (see below). This transport was reported to occur in a vesicular or in a non-vesicular pathway in yeast whereas the non-vesicular pathway was shown to be ATP independent and likely to occur via direct ER-Golgi membrane contacts (Funato and Riezman, 2001). In mammals a recent study describes the discovery of a protein, CERT, that binds to ceramides in the ER and allows their transport in a non-vesicular way to the Golgi apparatus (Hanada et al., 2003; Riezman and van

Meer, 2004). It will be of great interest to learn more about the regulation of each of these individual transport steps with respect to the diverse roles of sphingolipids and metabolites in different compartments of the cell (Hannun and Luberto, 2004).

In the Golgi apparatus, ceramide gets further converted to inositol phosphorylceramide (IPC). IPC belongs to the class of the complex sphingolipids in yeast, each containing an inositol phosphate coupled to the C1 OH group of ceramide. This reaction is performed by the IPC synthase Aur1p, which is a membrane bound enzyme (Becker and Lester, 1980) and localizes to the Golgi apparatus with the active side in the lumen of the Golgi (Levine et al., 2000).

Several proteins were shown to modify the two types of IPC into a diverse set of subtypes (Figure 1-3). Scs7p specifically hydroxylates VLCFA chains. SCS7 is not essential and contains an ER retrieval sequence (Dunn et al., 1998). Ccc2p, that is localized to the Golgi apparatus, was further shown to be responsible for hydroxylation of the fatty acid chain. This indicates that this hydroxylation event occurs most likely only on complex sphingolipids (Beeler et al., 1997; Yuan et al., 1997; and Figure 1-3).

The proteins encoded by the *CSG1*, *CSH1* and *CSG2* genes are then involved in the conversion of IPC by mannosylation to mannosyl inositol-P-ceramide (MIPC). Deletion of either of those genes does not prevent vegetative growth. A recent study proposes two distinct IPC-mannosyltransferase complexes in yeast. Where Csg1p interacts with Csg2p to produce MIPC type A, B, C and B' , Csh1p interacts with Csg2p to mainly produce MIPC type C (Uemura et al., 2003). Based on sequence homology to other proteins Csg1p and Csh1p are likely to have catalytic function and mannosyltransferase activity was localized to the Golgi apparatus. The role of Csg2p is far less understood. Overexpressed Csg2p localizes to the ER (Beeler et al., 1994; Takita et al., 1995). In addition, Csg2p contains a $Ca^{2+}$ binding EF domain and deletion of *CSG2* raised calcium sensitivity (Tanida et al., 1996). This supported the idea that calcium sensitivity raises when IPC type C is accumulated (Uemura et al., 2003). The cause for calcium sensitivity is not known but the *csg2* mutant has been very successful for the discovery of many genes in the sphingolipid pathway. Yeast mutants that accumulated IPC and were sensitive to calcium could be screened for mutations that decreased accumulation of IPC and calcium sensitivity. Many of these mutants were found to be in the sphingolipid biosynthetic pathway (Beeler et al., 1997; Zhao et al., 1994). Another gene that is required for mannosylation of IPC, although not taking place on the reaction itself, is *VRG4*. *VRG4* encodes a GDP-mannose transporter that delivers GDP-mannose from the cytosol to the lumen of the Golgi (Dean et al., 1997).

By transfer of another inositol phosphate group to MIPC, $M(IP)_2C$ is formed. This reaction is catalyzed by Ipt1p. The active site of Ipt1p is suspected to be on the luminal side of the Golgi. Deletion of *IPT1* leads to a complete depletion of $M(IP)_2C$ and accumulation of MIPC, which has

no effect on vegetative growth (Dickson et al., 1997b). $M(IP)_2C$ is the most abundant sphingolipid in yeast cells. Since the total mass of sphingolipids, estimated to make 8% of total plasma membrane mass (Daum et al., 1998), does not change in *ipt1*Δ cells it appears that yeast cells are able to sense and adjust the relative levels of sphingolipids.

The three types of complex sphingolipids, IPC, MIPC and M(IP)2C can also be degraded. The degradation of the complex sphingolipids into ceramide is catalyzed by Isc1p (Sawai et al., 2000). Isc1p contains two putative transmembrane domains but its localization is yet unclear. Deletion of *ISC1* reduced sphingolipase activity greatly, suggesting that Isc1p is the only sphingolipase in yeast.

Under normal conditions, the complex sphingolipids travel to the plasma membrane and to the vacuole. This occurs via vesicular transport since all complex sphingolipids were found in secretory vesicles and in mutants affecting late secretory steps no inositol containing sphingolipids reached the plasma membrane (Hechtberger and Daum, 1995; Hechtberger et al., 1994). At the plasma membrane two other proteins important for the metabolism of sphingolipids were found, Rsb1p and Yor1p. Both were shown to pump sphingoid bases out of yeast cells and therefore to prevent their accumulation (Katzmann et al., 1999; Kihara and Igarashi, 2002). Overexpression of Rsb1p resulted in increased inward movement (also called flip) of glycerophospholipids and was therefore implicated in the establishment of plasma membrane asymmetry (Kihara and Igarashi, 2004).

## SPHINGOLIPID FUNCTION

Many of the cellular responses during heat stress depend on the upregulation of sphingolipid synthesis (reviewed in Jenkins, 2003).

The initial observation was made in mutants unable to produce sphingolipids. These mutant were shown to be hypersensitive to heat (Dickson et al., 1990; Patton et al., 1992; Zanolari et al., 2000; Chung et al., 2000) and supplementation of these mutants with exogenous sphingoid bases could restore the growth defect at high temperatures (Jenkins et al., 1997).

One of these mutants was shown to carry a temperature sensitive mutation in the *LCB1* gene, called *lcb1-100*. This mutant was initially discovered in a screen for temperature sensitive yeast cells deficient for endocytosis (Munn and Riezman, 1994; and below). *LCB1* encodes for the serine palmitoyltransferase (SPT), the first committed step of sphingolipid biosynthesis. *lcb1-100* cells have a reduced SPT activity. This gave evidence that de novo biosynthesis of sphingolipids was specifically needed to gain thermotolerance and to grow at 37°C (Chung et al., 2000; Zanolari et al., 2000).

As the sphingolipid biosynthesis pathway counts many steps with different intermediates (Figure 1-2), the response to heat shock of different knock out strains was analyzed. Some of the viable deletions were sensitive to heat stress but several were resistant to severe heat shock. A strain deleted for both sphingoid base phosphate phosphatases *LCB3* and *YSR3*, leading to an increase of phosphorylated sphingoid bases, was shown to have increased thermotolerance (Mandala et al., 1998; Mao et al., 1999). These results were in agreement with those obtained with a strain deleted in the sphingoid base phosphate lyase *DPL1* (Skrzypek et al., 1999). On the other hand, a strain deleted for the dihydroceramidase *YDC1* was shown to be hypersensitive to extreme heat stress at 50°C (Mao et al., 2000b). This lead to the hypothesis that sphingolipid metabolites could be mediators of thermotolerance.

Indeed, heat stress was shown to induce a transient increase of a vast majority of most of these metabolites. When grown at 24°C, yeast cells mainly contain sphingoid bases with 18 carbons and a small amount of 16 carbons (Ferguson-Yankey et al., 2002). Heat stress leads to a small but reproducible increase in C18 and a huge increase in C20 sphingoid bases. The total amount of increase varies from 6 to a 100 fold within different reports, probably due to different methods used to analyze the sphingoid bases (Dickson et al., 1997a; Jenkins et al., 1997). The level of C16 DHS also increases during heat stress (unpublished observation from Dickson and Lester, 2002). The increased levels of sphingoid bases upon heat stress were shown to peak after 10 to 15 minutes and to decrease to near basal levels after one hour (Dickson et al., 1997a; Jenkins et al.,

1997). However not only the sphingoid bases but also their phosphorylated counterparts were shown to increase about fivefold upon heat stress (Skrzypek et al., 1999). Ceramide levels in response to heat stress were also measured and shown to increase about five- to tenfold (Jenkins et al., 1997; Wells et al., 1998). The increased levels peaked after 1 hour and were maintained for 2 hours after heat stress (Wells et al., 1998). Fumonisin B1, a specific but weak inhibitor of the ceramide synthase, inhibited the increase in ceramide levels during heat stress, but only partially. Autralifungin, a very potent inhibitor of the ceramide synthase almost totally blocked this increase, showing, that de novo synthesis is the source of the increase in ceramides during heat stress (Jenkins et al., 1997; Wells et al., 1998). The transient changes in these metabolites (DHS, PHS, ceramides) are consistent with the idea that one ore more of this compounds could act as signaling molecules.

As mentioned above, DHS-1P and PHS-1P were implicated as mediators of heat stress based upon studies using strains deleted for *DPL1*, *LCB3* and *YSR3* (Lanterman and Saba, 1998; Mandala et al., 1998; Mao et al., 1999; Skrzypek et al., 1999). But not only the phosphorylated also the unphosphorylated forms of DHS and PHS increase during heat stress in this cells. This leads to the possibility that DHS and PHS alone or in combination with their phosphorylated forms are mediators of the heat stress resistance (Ferguson-Yankey et al., 2002).

It remains to be determined how heat stress regulates the increase in sphingolipid production. Since the increase in sphingoid bases during heat stress occurs extremely fast it is most likely that the first committed step, the serine palmitoyltransferase, has to be tightly regulated (either directly or indirectly) upon heat stress. Most studies of the regulation of this enzyme carried out in mammalian cells indicate that it's activity is controlled by the availability of its substrates, serine and palmitoyl-CoA (Merrill et al., 1988; Messmer et al., 1989).

Nevertheless, how these sphingolipid metabolites regulate the cellular responses to heat stress is still an open issue. An initial study showed that treatment of wild type cells with exogenous sphingoid bases lead to accumulation of trehalose. Addition of a solution of 50 μmolar DHS induced a *TPS2-lacZ* reporter construct even at low temperatures. The *TPS2* gene contains STRE's in its promoter which led to the hypothesis that sphingoid bases activate transcription of STRE containing genes, thereby directing thermotolerance and stress resistance (Dickson et al., 1997a). Intriguingly, addition of the nonendogenous L-threo-dihydrosphingosine and C2-ceramide also activated the induction of those genes (Dickson et al., 1997a). To get further insight into this point, mutant *lcb1-100* cells were used to display the specific transcriptional changes during heat stress. This study showed that HSE- and STRE- dependent transcription does not depend greatly on the production of sphingoid bases (Cowart et al., 2003). In this context it is important to note, that deletion of both *LCB3* and *DPL1* is lethal in many strain backgrounds. Lethality is thought to result

from a large accumulation of sphingoid base phosphates (Zanolari et al., 2000; Zhang et al., 2001). In addition, an unpublished observation (Jenkins, 2003) reported that treatment of cells with these amounts of sphingolipids can be lethal to yeast cells in liquid media. Most likely, addition of high amounts of sphingoid bases and analogs elicited a general stress response, leading to an activation of these genes.

Also the transient arrest of the cell cycle at $G_0/G_1$ phase that occurs after heat shock was shown to be dependent upon the synthesis of sphingolipids. An *lcb1-100* strain shows no transient arrest in cell cycle during heat stress and addition of exogenous sphingoid bases could induce such an arrest in the absence of heat stress in wild type cells. Strains deleted for the sphingoid base kinases *LCB4* and *LCB5*, the hydroxylase *SUR2*, the phosphatases *LCB3* and *YSR3,* displayed this transient arrest, showing that de novo synthesis of sphingoid bases is required for this response. Interestingly, deletion of *LCB4* and *LCB5* rendered cells unable to recover from the heat stress induced cell cycle arrest (Jenkins and Hannun, 2001). This could indicate that accumulation of sphingoid bases acts as a signal for this transient cell cycle arrest and that phosphorylation of the sphingoid bases is required to downregulate this signal.

The *lcb1-100* allele was initially characterized in a screen for temperature sensitive mutants defective for endocytosis (Munn and Riezman, 1994). At restrictive temperature *lcb1-100* cells were shown to be defective in the internalization step of the plasma membrane protein Ste2p and also in the vacuolar accumulation of the fluorescent dye Lucifer Yellow. Additionally, *lcb1-100* cells displayed defects in actin cytoskeleton organization. Subsequent studies showed that the lack of sphingoid base production at restrictive temperature was indeed responsible for these phenotypes (Zanolari et al., 2000). Overexpression of the yeast kinases *YCK2* and *PKC1* restored the defects observed in actin cytoskeleton and endocytosis in *lcb1-100* cells. Loss of the protein phosphatases *PPH21* and *PPH22* or of their regulatory subunit *CDC55* did also suppress the endocytic phenotype, proposing a complex signaling network downstream of sphingoid base involved in endocytosis (Friant et al., 2000). Interestingly, the plasma membrane uracil permease Fur4p could still be internalized in *lcb1-100* cells at 37°C (Dupre and Haguenauer-Tsapis, 2003). The discrepancy between this two results could be due to different experimental setups.

In addition, overexpression of one of the two kinases Pkh1p or Pkh2p, that are homologous to mammalian 3-phosphoinositide-dependent kinase-1 (PDK1), can suppress the sphingoid base synthesis requirement for endocytosis. Most importantly, this study showed that the Pkh1/2p kinases could be activated *in vitro* by nanomolar concentrations of sphingoid bases (Friant et al., 2001). In mammals, PDK1 phosphorylates and activates the serum and glucocorticoid-dependent kinase SGK (Kobayashi and Cohen, 1999) and in yeast two kinases were found to resemble SGK

and being phosphorylated by Pkh1/p, Ypk1 and Ypk2p. *YPK1* and *PKH1* overexpression were also found to suppress the growth defect mediated by myriocin, a specific inhibitor of the serine-palmitoyltransferase. Treatment of cells with myriocin inhibited phosphorylation of Ypk1p and inversely, addition of PHS induced phosphorylation of Ypk1p (Sun et al., 2000). And the protein kinases Ypk1/2p were also found to be required for endocytosis (deHart et al., 2002). This led to the proposal of a conserved sphingoid base activated signaling cascade that is required for endocytosis, actin organization and cell growth in yeast.

Moreover, sphingoid base synthesis was shown to be required for the degradation of proteins during heat stress. In contrast to the previously mentioned study by Dupré *et al.* (Dupre and Haguenauer-Tsapis, 2003), Chung *et al*. showed that in *lcb1-100* cells, unable to produce PHS during heat stress, degradation of Fur4p was disabled and could be reverted by the addition of PHS. PHS, and none of a variety of similar lipids, induced the degradation of Fur4p, even in the absence of heat. Deletion of the *NPI1* ubiquitin ligase and the *DOA4* deubiquitinase abrogated PHS induced Fur4p endocytosis (Chung et al., 2000) as expected from previous studies (Galan and Haguenauer-Tsapis, 1997). In the same study it was shown that PHS can stimulate the proteolysis of a Deg1-β-galactosidase fusion protein which is a substrate for the proteasome. PHS stimulated degradation of proteins requiring multi-ubiquitin chain formation through the stress responsive lysine 63 residue of ubiquitin (Chung et al., 2000). It remains to be determined if addition of PHS stimulated the degradation specifically or just induced a stress response leading to degradation of those proteins.

Sphingoid bases and ceramides have also been shown to play a role in secretion. Addition of PHS could restore protein secretion from the Golgi to the plasma membrane in Snc1/2p (v-SNARE) Sso1/2p (t-SNARE) mutants (Marash and Gerst, 2001). Overexpression of *DPL1* was additionally found to suppress a Snc1-M42A mutant which is defective in recycling of compounds from the plasma membrane (Grote et al., 2000). How overexpression of *DPL1* could restore recycling or PHS could restore secretion remains unclear. Moreover, ceramide synthesis was assigned to be required for transport of GPI-anchored proteins from the ER to the Golgi (Barz and Walter, 1999; Sutterlin et al., 1997) and for the stable attachment of GPI-anchored proteins to membranes (Watanabe et al., 2002).

Sphingolipids and cholesterol have the tendency to associate in membranes to form domains that are called rafts, detergent insoluble glycolipid-enriched complexes (DIG's) or detergent resistant membranes (DRM's) (Harder et al., 1998; London and Brown, 2000; Simons and Ikonen, 1997). Association of proteins with detergent resistant membranes was found in yeast (Bagnat et al., 2000; Dupre and Haguenauer-Tsapis, 2003) and mammalian cells and was attributed to play key roles in

signal transduction, membrane trafficking, cytoskeletal organization and pathogen entry (Munro, 2003). However, recent data indicate that Triton-X 100 itself induces the formation of ordered domains *in vitro* (Heerklotz, 2002). Therefore DRM's do not obligatory resemble functional, biological rafts. It can not be excluded that proteins, associated with DRM's *in vitro*, are associated with lipid rafts *in vivo* before the addition of the detergent. In addition, yeast proteins that were found together in biochemically purified DRM's could not be colocalized in living cells (Malinska et al., 2003).

Similarly, in mammalian cells, sphingolipid synthesis and the metabolites were shown to play crucial roles (reviewed in Hannun and Luberto, 2000; Jenkins, 2003; Spiegel and Milstien, 2003).

Heat shock caused a twofold increase of ceramide in NIH 3T3 and in HL-60 cells (Chang et al., 1995; Kondo et al., 2000a; Kondo et al., 2000b). In contrast to yeast cells, increase in ceramide levels did not result from an increase in sphingolipid synthesis but rather from an increase in hydrolysis of sphingomyelins (Kondo et al., 2000a). In contrast to the above mentioned cell types, de novo sphingolipid synthesis was upregulated during heat stress in Molt-4 cells. Detailed studies of this increase showed that ceramides, mostly of the C:16 type, were induced twofold (Jenkins et al., 2002). In mammalian cells, the increase in ceramide levels was shown to have several functions. In HL-60 cells, ceramide activates caspase-3 which causes an increase in *c-jun* mRNA, ultimately resulting in apoptosis (Kondo et al., 2000a). In Molt-4 cells, production of ceramide induced dephosphorylation of SR proteins which are implicated in mRNA splicing (Jenkins et al., 2002). A very recent study showed that SR proteins also play a crucial role in splicing events during heat stress (Shin et al., 2004).

In contrast to ceramide, Sphingosine-1-phosphate (S1P) has mainly anti-apoptotic effects. S1P is a ligand for a family of five G-protein coupled receptors, the Edg receptors (Hla et al., 2001). Binding of S1P to these receptors leads to activation of a variety of downstream signaling targets like PLC, PI3K, Rac, JNK and ERK. By this way, S1P can control diverse responses such as angiogenesis, vascular maturation, heart development and immunity (reviewed in Spiegel and Milstien, 2003).

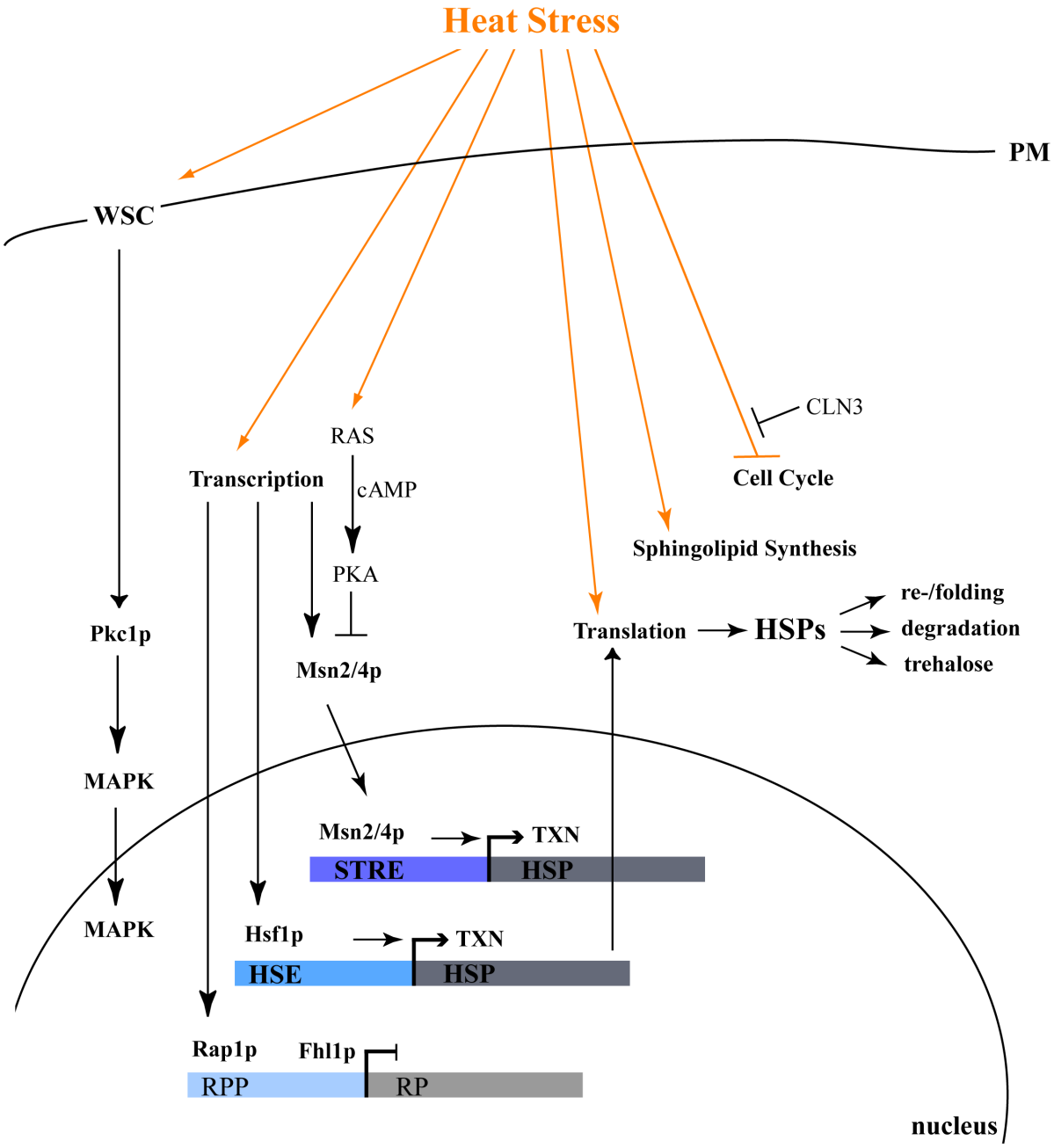



**Figure 1-1.** Simplified and schematic overview of the cellular events occurring throughout heat shock in *S. cerevisiae* cells. Heat stress activates several downstream targets, leading to transcriptional changes. Besides transcription, translation, sphingolipid synthesis and the cell cycle are affected. MAPK, mitogen activated protein kinase. HSPs, heat shock proteins. PM, plasma membrane.

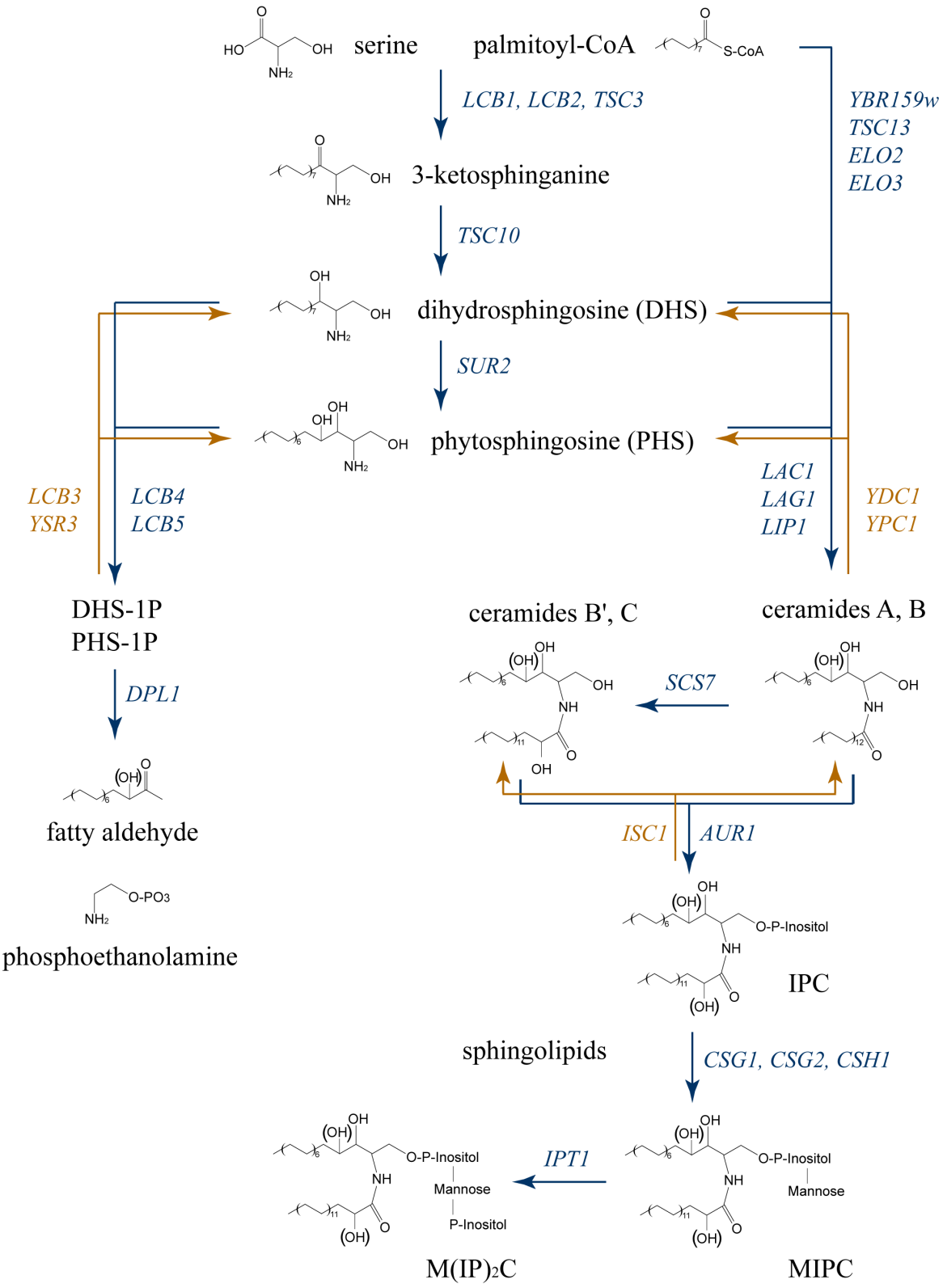



**Figure 1-2**. Schematic overview of the sphingolipid biosynthetic and degradation pathways. From the level of ceramides different isoforms can exist, which is simplified here and illustrated in greater detail in Figure 1-3. IPC inositol phosphorylceramide, MIPC mannose inositol phosphorylceramide, $M(IP)_2C$ mannose di-inositol phosphorylceramide.

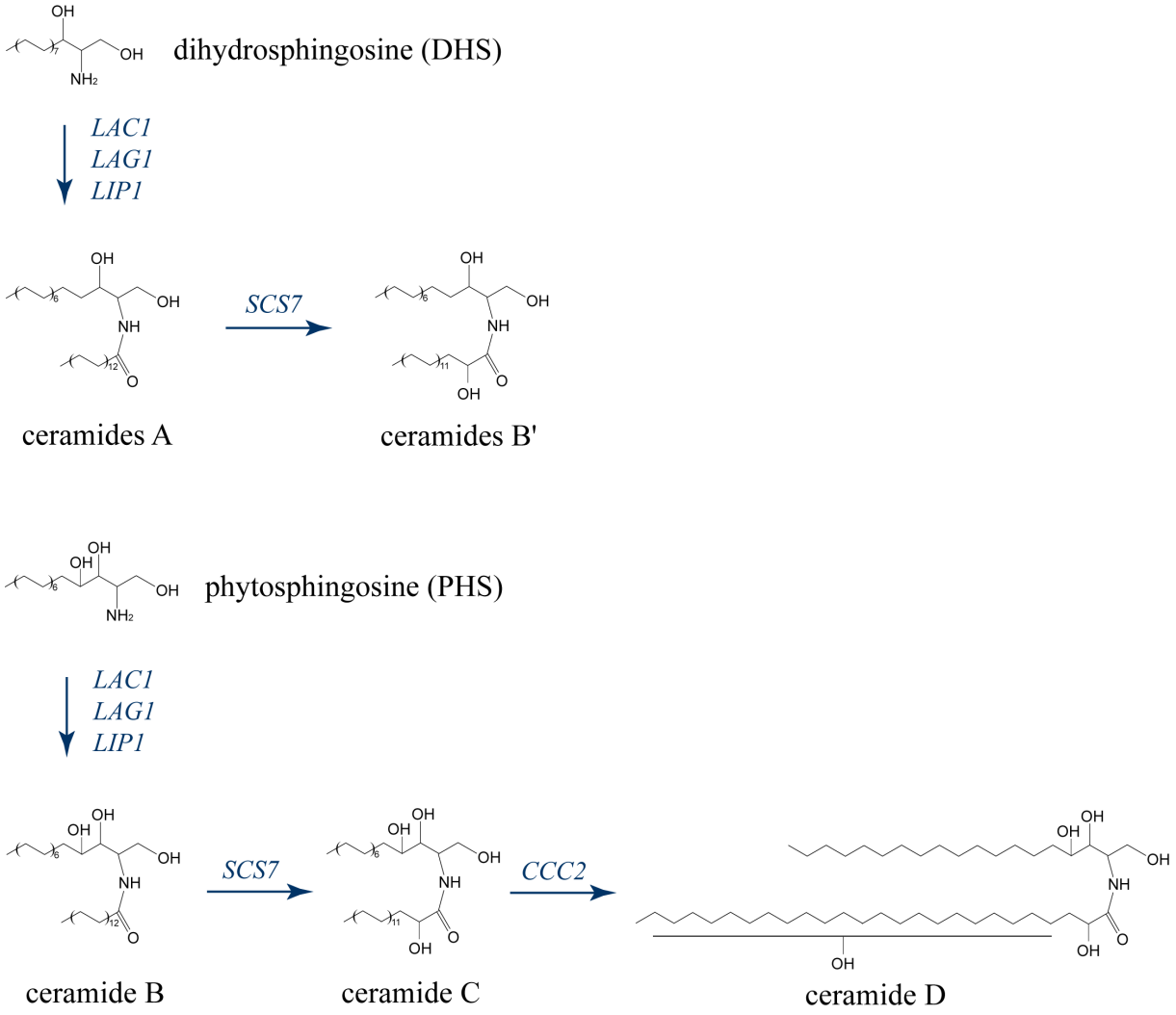



**Figure 1-3.** Illustration of the synthesis of the five different ceramide species. The long chain base moiety of ceramides A and B' is DHS, those of ceramides B, C, and D is PHS. The C-2 of ceramides B', C, and D is hydroxylated by Scs7p. Ceramide D contains another hydroxyl group on the very long chain fatty acid, although its precise position has not been determined. Because of the different hydroxylation states of ceramide (ceramides A, B', B, C, and D) five species of each IPC, MIPC, and $M(IP)_2C$ can be made.

## AIM OF THIS THESIS

Sphingolipid biosynthesis in animal cells and in the yeast *S. cerevisiae* is similar during the first steps and ongoing sphingolipid synthesis is required for a variety of cellular functions including response to heat stress.

During heat stress, *de novo* sphingolipid synthesis is upregulated and mutant cells that accumulate sphingoid bases show an increase in thermotolerance. Conversely, mutants defective in the biosynthesis of sphingolipids show a decrease in survival during and after heat stress. Roles for sphingolipid synthesis in heat stress were also found in the regulation of the transient cell cycle arrest, control of putative signaling pathways that govern cell integrity and the actin cytoskeleton, vesicle trafficking and protein breakdown in the plasma membrane. Biosynthesis of sphingolipids was therefore thought to be important for governing the specific responses to heat shock.

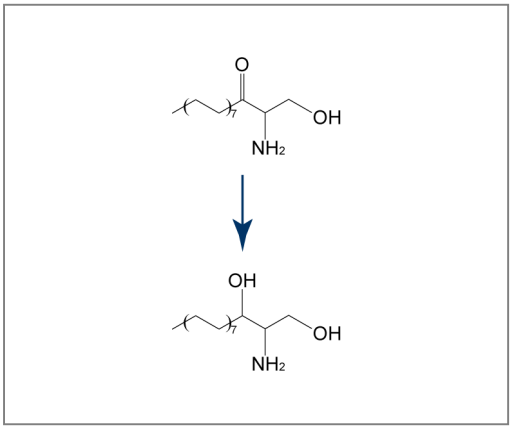
In this study we want to find and describe additional mechanisms of the function of sphingolipid synthesis during heat stress. To determine the role of sphingolipid synthesis in heat stress we make use of the yeast *lcb1-100* mutant which is impaired in serine palmitoyltransferase, the first enzyme in sphingolipid biosynthesis. In addition, this mutant shows a rapid and strong decrease in cell viability upon heat when compared to wild type cells, further indicating the requirement for sphingolipid synthesis during the response to heat stress.

We would also like to understand which sphingolipid metabolite is responsible for the response to heat shock. Looking at various mutants in the sphingolipid pathway is the primary choice to differentiate between the need of sphingoid bases, ceramides or complex sphingolipids for this response.

We also want to elucidate which signaling pathways mediate the sphingolipid synthesis dependent response to heat. It was previously shown that sphingoid bases can activate the yeast homologues of the 3-phosphoinositide-dependent protein kinase PDK1, *PKH1* and *PKH2*. The PKH kinases act upstream of *YPK1* and *YPK2*, the yeast homologues of the serum and glucocorticoid induced kinase SGK, which were also shown to affect cell viability.

In this study we want to achieve a more detailed view of the role of sphingolipid biosynthesis during rapid increases in temperature. This will help to understand the fundamental cellular processes that allow a cell to cope with heat stress.

# CHAPTER II



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

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Running title: Heat shock protein-independent heat resistance

## ABSTRACT

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 at high temperature. Normal heat shock resistance can be restored, without restoration of ceramide synthesis or induction of heat shock proteins, by overexpression of ubiquitin. This function of ubiquitin requires the proteasome. These data imply that the essential function of heat shock protein 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.

## 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 (Glover and Lindquist, 1998; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993). 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 heat shock protein 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 G1 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 (Jenkins et al., 1997; Patton et al., 1992; Ruis and Schuller, 1995). 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 the 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.

## 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 (Fig. 2-1 A), 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, *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 (Fig. 2-1 B). The *lcb1-100* mutant cells showed a clear defect in survival at high temperature when compared to wild-type 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 (Fig. 2-1 B). 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 (Amoros and Estruch, 2001; Boy-Marcotte et al., 1999; Simon et al., 1999). 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 (Fig. 2-1 A and 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 (Fig. 2-1 A). 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). This probably reflects the need for adequate ubiquitin levels to enable the ubiquitination 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 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 (Irie et al., 1993; Lee et al., 1993; Lee and Levin, 1992), 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 (Fig. 2-1 A, 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 base. 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 *lcb1-100* mutant strain. To investigate this possibility, sphingolipid synthesis was measured in cells overexpressing *UBI4*. Wild-type 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 (Fig. 2-2). At 24°C, both wild-type and mutant cells overexpressing *UBI4* or not, synthesize sphingolipids (inositolphosphoceramide (IPC), mannosylated inositolphosphoceramide (MIPC) and mannosylated di-inositolphosphoceramide (M(IP)$_2$C)), although the *lcb1-100* strains showed less sphingolipid synthesis than the wild-type cells. After mild heat-shock (37°C), wild-type 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* (Fig. 2-2). 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, wild-type 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. Wild-type 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 wild-type and *lcb1-100* mutant strains with or without a *UBI4* plasmid (Fig. 2-3 A). The wild-type cells (WT) 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 (Fig. 2-3 A). 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 wild-type and *lcb1-100* mutant strains (Fig. 2-3 B). Both wild-type (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 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 heat shock proteins 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 heat shock protein synthesis upon heat stress, the general heat shock response was analyzed in the *lcb1-100* mutant cells with our without the *UBI4* plasmid and compared to wild-type cells. Heat shock protein synthesis was induced by a temperature shift from 24°C 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 heat shock proteins is transient, with a maximum expression at 15-20 min (Martinez-Pastor et al., 1996; Smith and Yaffe, 1991). Proteins were separated by SDS-PAGE and visualized using a phosphorimager in wild-type, *lcb1-100*, and *lcb1-100* overexpressing *UBI4* strains (Fig. 2-4 A). 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 wild-type strain (WT) showed the expected production of heat shock proteins, whereas the *lcb1-100* strain overexpressing *UBI4* or not, showed a strong reduction in all heat shock protein synthesis (Fig. 2-4 A). Hsp104p and Hsp82p were barely detected in the *lcb1* mutant strain overexpressing *UBI4* or not, whereas these two proteins were expressed in the wild-type cells. Hsp90p and Hsp70p proteins synthesis was greatly reduced in the *lcb1* mutant strain when compared to wild-type cells. Mutant *lcb1+UBI4* cells, which retain viability under these conditions similarly to wild type cells (Fig. 2-1 B), were also defective in heat shock protein 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 nonreducing 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 wild-type cells and in *lcb1-100* mutant with or without a *UBI4* plasmid (Fig. 2-4 B). 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 wild type 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 Francois, 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 heat shock proteins 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 heat shock proteins 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 to wild-type 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 reincorporation 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 fragments (Fig. 2-5 A). At 37°C, protein degradation in wild-type cells exceeded that in the *lcb1-100* mutant by about two to three 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 ubiquitinated proteins after a shift to 37°C in wild-type 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 ubiquitinated proteins was determined by Western blotting with anti-ubiquitin antibody (Fig. 2-5 B). The *lcb1-100* mutant displayed an increase in protein ubiquitination at 24°C compared to wild-type 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 wild type cells at 24°C (Fig. 2-3 B). 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, wild-type cells and the *lcb1-100* mutant overexpressing *UBI4* displayed an increase in ubiquitinated proteins, but in the *lcb1-100* mutant cells where protein degradation was low (Fig. 2-5 A), the content of ubiquitinated proteins decreased (Fig. 2-5 B). 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 (Fig. 2-3 B) and that protein ubiquitination 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 to wild-type 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 nonionic detergent, of wild-type 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 (Fig. 2-5 C). The amount of aggregated proteins increased in wild type 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 done in the *lcb1-100* cells transformed by the *UBI4* plasmid (Fig. 2-5 C). The *lcb1-100* cells overexpressing *UBI4* showed only a small accumulation of aggregated proteins similar to wild-type 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 ubiquitinated 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 ubiquitinated proteins and shows reduced growth at 37°C (Fig. 2-6 A) (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 (Fig. 2-6 A). 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 like 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 wild-type 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 (Fig. 2-6 B). 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 (Fig. 2-6 C). 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.

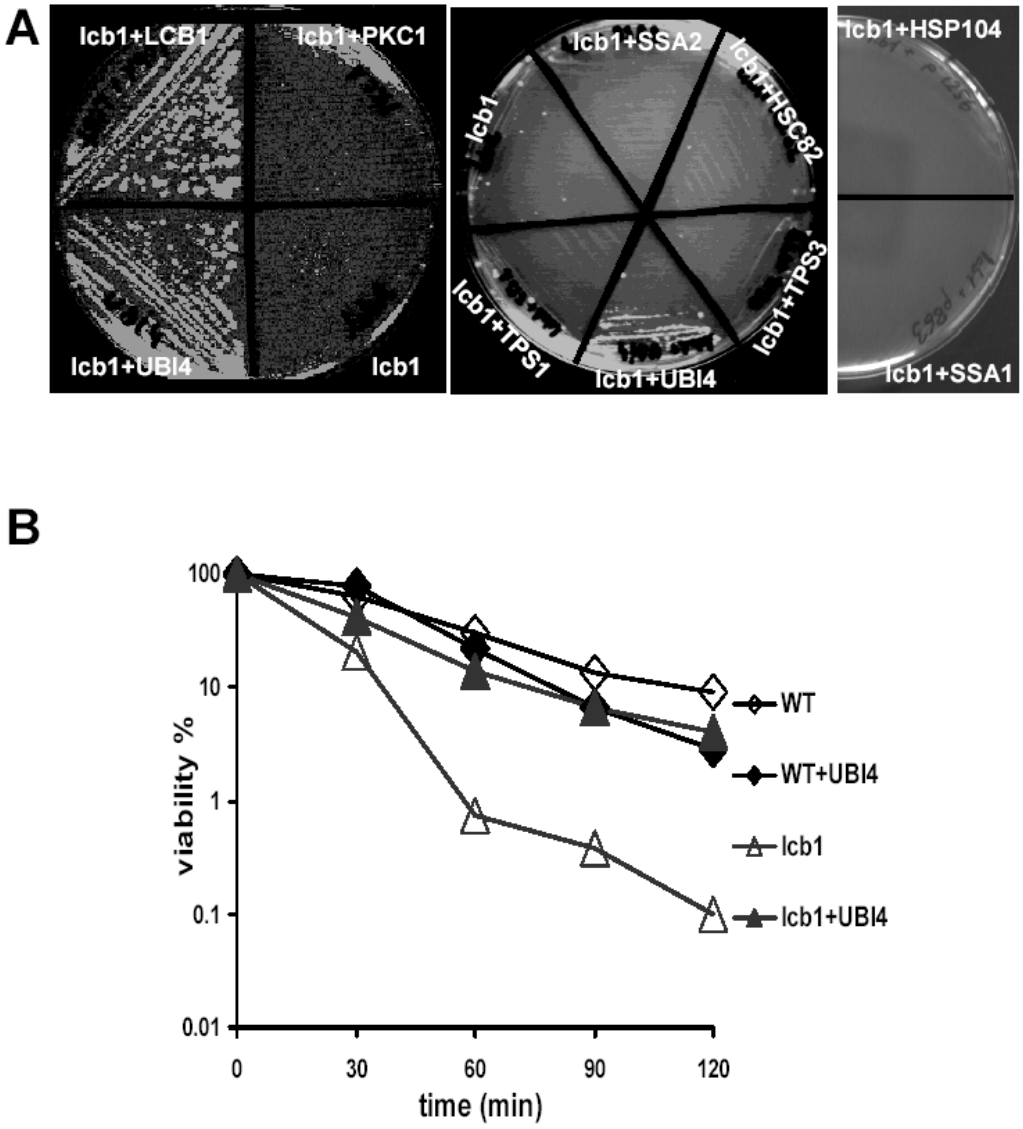


**Figure 2-1. A**. Increased ubiquitin expression suppresses the *lcb1-100* temperature-sensitive phenotype. RH3809 (*lcb1-100*) cells carrying plasmids that overexpress either *LCB1*, *UBI4*, *PKC1*, *TPS1*, *TPS3*, *HSC82* or *SSA2* were streaked onto YPUAD plates and grown at the non-permissive temperature 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 wild-type (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.

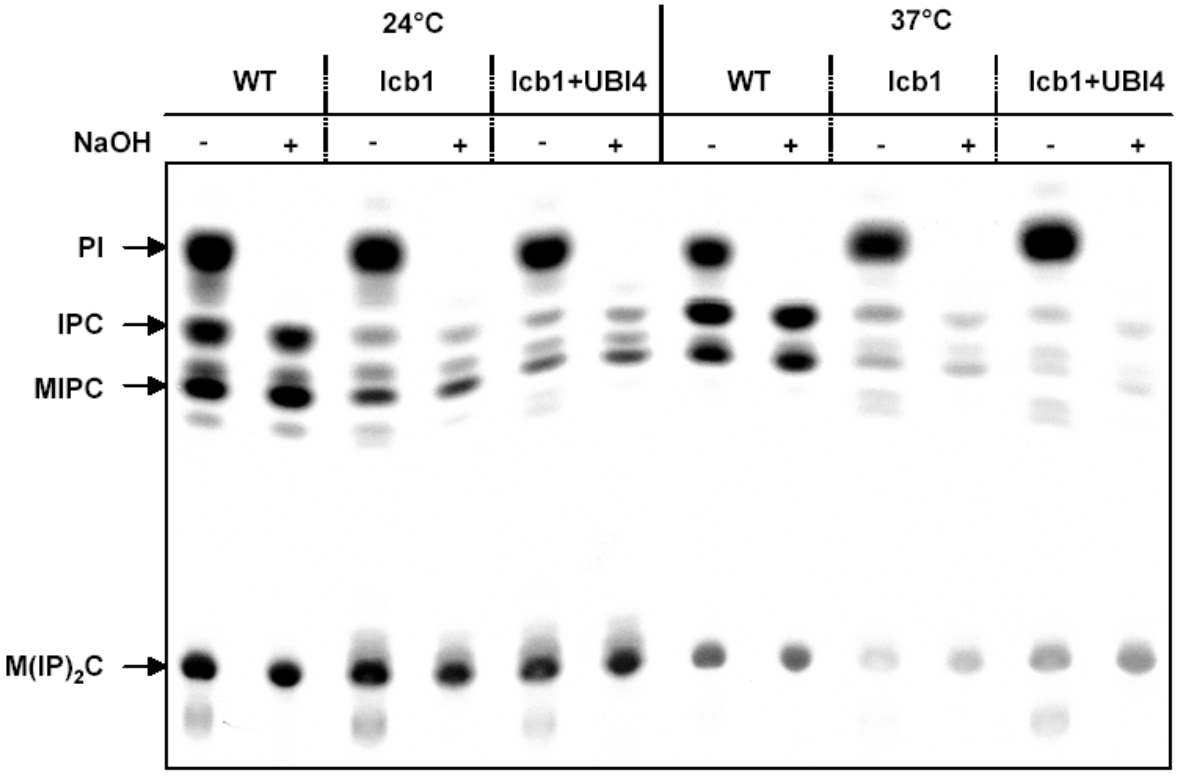


**Figure 2-2.** Increased ubiquitin expression does not restore sphingolipid synthesis in *lcb1-100* cells. Wild-type (WT), *lcb1-100* mutant (lcb1) or *lcb1-100* mutant cells overexpressing *UBI4* (+UBI4) were grown in SDYE at 24°C, preshifted to 24°C or 37°C and labeled with [$^3$H]*myo*inositol. Incorporation 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).

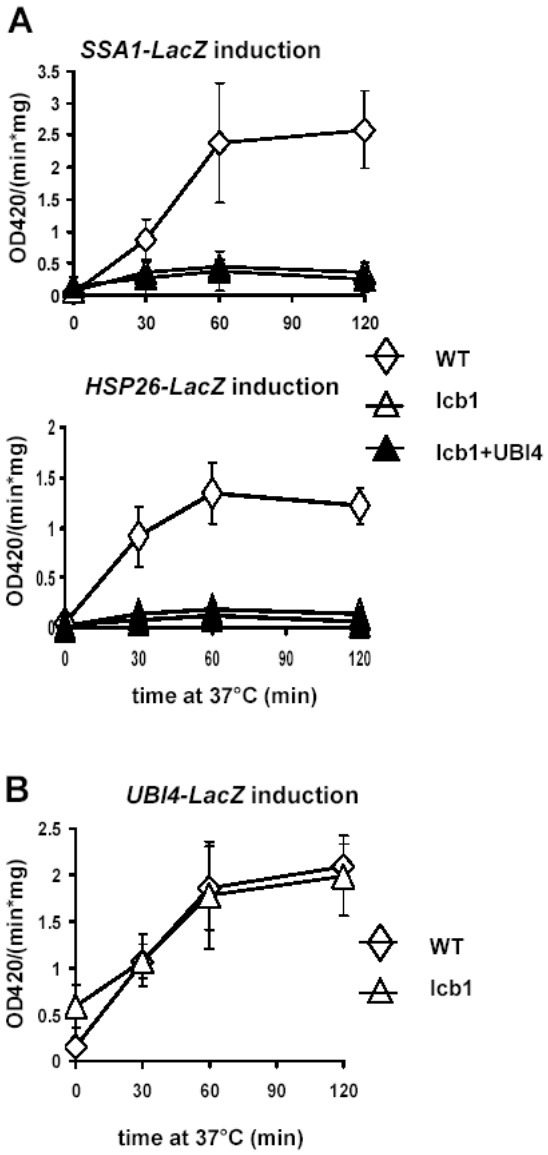


**Figure 2-3. A.** *lcb1-100* mutant cells are defective in Hsp induction. Wild-type (WT), *lcb1-100* mutant (lcb1) or *lcb1-100* mutant cells overexpressing *UBI4* (+UBI4) were transformed with plasmids carrying *SSA1-LacZ* or *HSP26-LacZ* reporter constructs. After growth at 24°C, transformants were 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. Wild-type (WT) or *lcb1-100* mutant (lcb1) cells were transformed with a plasmid carrying the *UBI4-LacZ* reporter gene and treated as described for panel A.

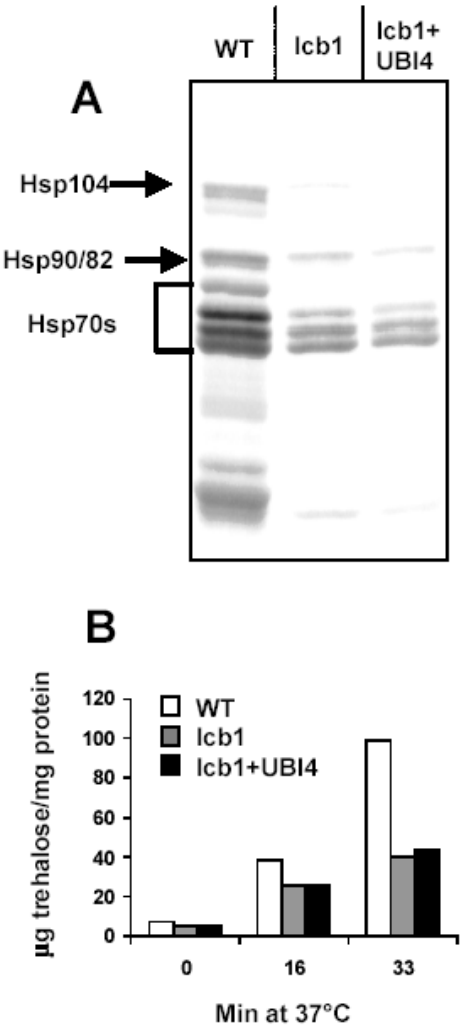


**Figure 2-4.** Heat shock protein synthesis and sustained trehalose accumulation are defective in the *lcb1-100* cells. **A.** Cells actively dividing at 24°C were transferred to 44°C. The production of heat shock proteins was assessed after [$^{35}$S]-Met/Cys labeling, followed by extraction, separation by SDS-PAGE and phosphorimager analysis of labeled proteins. Heat shock protein bands are indicated. **B.** Wild-type (WT) and *lcb1-100* (lcb1) cells bearing a plasmid without insert or *lcb1-100* cells overexpressing *UBI4* (lcb1+UBI4) were shifted from 24°C 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.

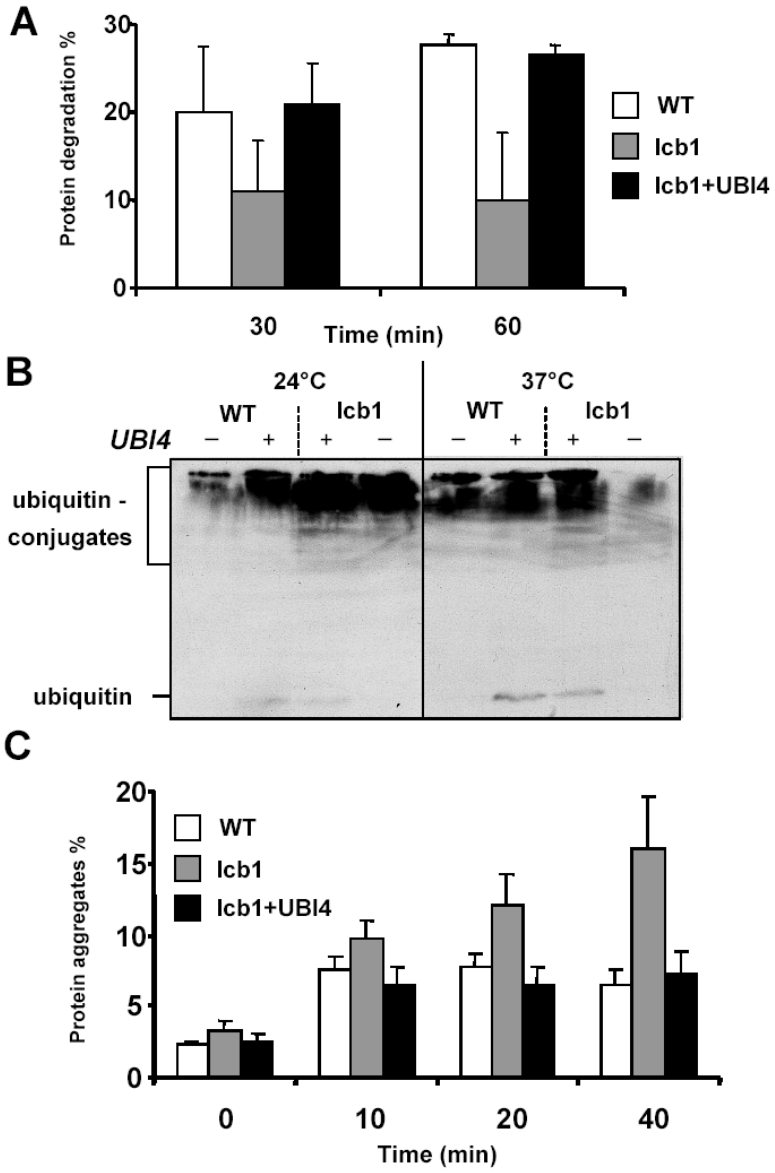


**Figure 2-5.** Increased protein degradation and removal of protein aggregates in the *lcb1-100* strain overexpressing *UBI4*. **A.** Wild-type (WT), *lcb1-100* (lcb1) and *lcb1-100* cells overexpressing *UBI4* (lcb1+UBI4) were pulse-labelled at 37°C with [$^{35}$S]-Met/Cys and the rate of protein degradation was measured during the chase period. The data shown are mean values and standard errors obtained from four independent experiments are shown. **B.** Wild-type (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 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 wild-type (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 15000xg for 15 min in glycerol and non-ionic detergent at physiological salt concentrations. Total and aggregated labeled proteins were quantified by liquid scintillation counting.

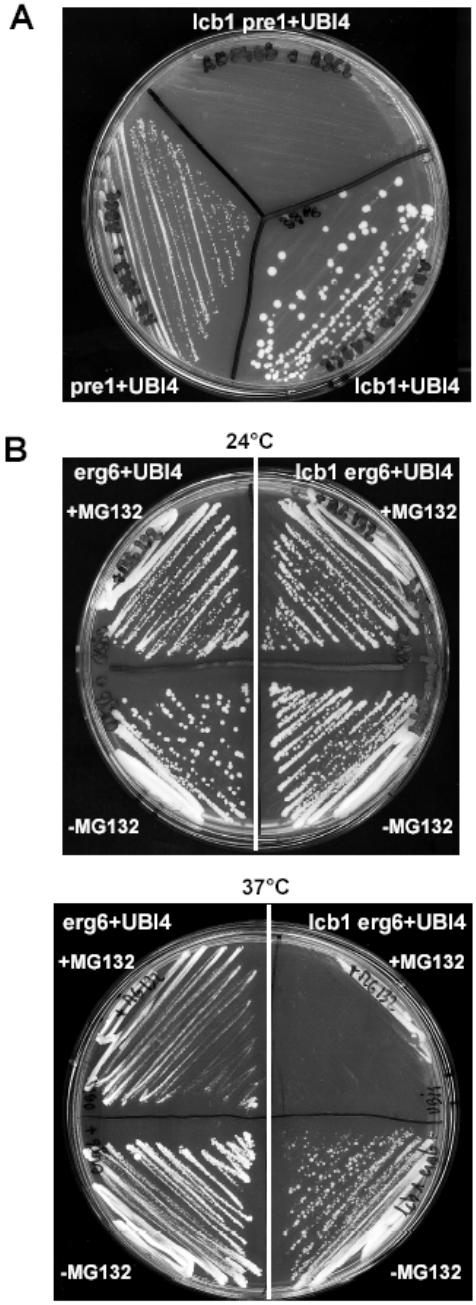


**Figure 2-6.** The proteasome is required for *UBI4* suppression of *lcb1-100*. **A.** The *pre1-1* strain is mutated in one subunit of the proteasome complex. Single mutant strains *lcb1-100* and *pre1-1*, and the double mutant strain *lcb1-100 pre1-1* were transformed with a high copy number *UBI4* 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.

## MATERIALS AND METHODS

### Plasmids and Yeast Strains

Previously described plasmids used in this study were pSH24 (*PKC1*), YEp195-PKH1, YEp195-PKH2 (Friant et al., 2001; Friant et al., 2000), expressing different ubiquitin genes, 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 (Lindquist and Kim, 1996; Nathan and Lindquist, 1995)), 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 (*Mat**a** 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 *ΔCTD::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 CuSO4 (0.1 mM final concentration) at 37°C.

**Viability Assay**

Mid log-phase cultures of wild-type (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 thin layer chromatography 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 (Guarente, 1983; Miller, 1972). For each sample, time, $OD_{420}$ and protein concentration, using a Bradford assay kit (BioRad), 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 (Easy Tag 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 were 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 hours and that total proteins were extracted using the NaOH/2-mercaptoethanol and TCA procedure (Horvath and Riezman, 1994), dissolved in 0.1N 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 600nm 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 (Easy Tag EXPRESS-[$^{35}$S] mix from NEN) for 3 minutes as described. Chase was initiated by adding a 1/100 volume of a mixture of 0.3% methionine/cysteine in 0.3M $(NH_4)_2SO_4$ and cycloheximide (0.5mg/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 x $10^6$ cells/ml in synthetic (SD) media containing 0.5% yeast extract and 40 mg/l of the appropriate amino acids. 1.2 x $10^8$ cells were harvested and washed in 10 ml SD without yeast extract. 3 x $10^7$ 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 (Easy Tag EXPRESS-[$^{35}$S] mix from NEN) for 10 minutes at 24°C. 5 µl 100x chase mix (0.3% methionine and cysteine, 0.3 M $(NH_4)_2SO_4$) was 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 (1mM EDTA, 150mM KCl, 1mM EGTA, 50mM 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 minutes 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 5 individual experiments.

## ACKNOWLEDGMENTS

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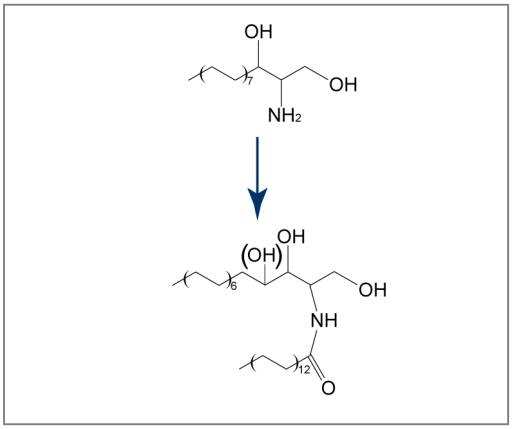
# CHAPTER III



# Sphingoid Base is Required for Translation during Heat Stress in *Saccharomyces cerevisiae*

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Running title: Sphingolipid regulation of translation initiation

## ABSTRACT

### Background

Ongoing sphingolipid synthesis is required for a variety of cellular functions including response to heat shock. The yeast *lcb1-100* mutant is conditionally impaired in the first step of sphingolipid biosynthesis and shows a strong decrease in cell viability upon heat-shock. The decrease in viability is caused by insufficient synthesis of heat-shock proteins.

### Results

Transcription and nuclear export of heat shock protein mRNAs is not affected in *lcb1-100* cells. However, these cells exhibited a strong decrease in protein synthesis and polysome analyses demonstrated a defect in translation initiation under heat-stress conditions. The relevant lipid is sphingoid base, not ceramide or sphingoid base phosphates. Deletion of the eIF4E binding protein Eap1p in *lcb-100* cells partially restored translation of heat shock proteins and increased viability of *eap1*Δ*lcb1-100* during heat stress. Ubiquitin overexpression allowed recovery of translation after heat stress, but not at the initial stages where heat shock proteins were made. The translation defect at later times during a heat stress in *lcb1-100* correlated with depletion of the translation initiation factor eIF4G. Translation initiation during heat stress depended at least partially on the function of the sphingoid base activated PKH1/2 protein kinases.

### Conclusion

We have uncovered a novel lipid-mediated regulation of translation initiation that is operative when cells are exposed to heat stress conditions. Sphingoid bases signal to the cap-dependent translation initiation apparatus to allow heat shock protein synthesis. This is required for the recovery from heat shock.

## INTRODUCTION

Eukaryotic cells have developed several mechanisms to respond to rapid increases in temperature. Upon heat stress, cells reduce the rate of synthesis of proteins that were expressed before the heat shock and change their transcription profile dramatically to produce mainly heat shock proteins (Gasch et al., 2000; Murray et al., 2004). In the budding yeast *S. cerevisiae* changes in transcription upon heat stress are fairly well understood, involving control by two transcription factors, the heat shock factor Hsf1p and Msn2p/4p. Hsf1p binds to heat shock elements (HSE's) found in the promoter region of many heat shock protein genes (Amin et al., 1988; Wu, 1995). Genes that do not contain HSEs, but whose transcription is induced by heat and other stress signals, including osmotic shock, DNA damage and oxidative stress, contain stress response elements (STRE's) in their promoter. Upon these stresses, Msn2/4p shuttles from the cytosol to the nucleus and activates transcription through binding the STRE (Gorner et al., 1998; Schmitt and McEntee, 1996). After transcription, the corresponding mRNAs are exported from the nucleus (Stutz and Rosbash, 1998). Proteins encoded by heat stress responsive genes are responsible for the synthesis of the thermoprotectant trehalose (Singer and Lindquist, 1998), for the folding of proteins and for the degradation of unfolded and aggregated proteins (Imai et al., 2003; Riezman, 2004).

In addition to the induction of heat shock proteins, yeast cells arrest transiently in the G1 phase of the cell cycle during heat stress (Johnston and Singer, 1980). A very early event in the heat shock response in yeast is the induction of *de novo* synthesis of free sphingoid bases, followed by ceramides and sphingolipids (Dickson et al., 1997; Jenkins et al., 1997; Wells et al., 1998). The first steps in the biosynthesis of sphingolipids in animal cells and in yeast are similar, but differ in production of complex sphingolipids. In yeast, two sphingoid bases, dihydrosphingosine (DHS) and phytosphingosine (PHS) can be converted upon addition of a $C_{26}$-CoA into ceramides. These ceramides are precursors for the three major classes of complex sphingolipids (Dickson and Lester, 2002; Funato et al., 2002).

Interestingly, many of the cellular responses during heat stress depend on the upregulation of sphingolipid synthesis (reviewed in Jenkins, 2003) and yeast mutants unable to produce sphingolipids are hypersensitive to heat (Chung et al., 2000; Patton et al., 1992; Zanolari et al., 2000). One of these mutants carries a temperature sensitive mutation in the *LCB1* gene, called *lcb1-100*. The *LCB1* gene encodes a subunit of the serine palmitoyl-transferase which catalyzes the first step in sphingolipid synthesis (Buede et al., 1991). *lcb1-100* mutants are therefore unable to produce sphingoid bases, ceramides and sphingolipids during heat stress. Addition of high concentrations of sphingoid bases to the growth media induces the synthesis of heat shock proteins

at low temperatures (Dickson et al., 1997) and an *lcb1-100* mutant was shown to be deficient in the synthesis of heat shock proteins (Friant et al., 2003). Mutant *lcb1-100* cells also displayed specific transcriptional changes during heat stress (Cowart et al., 2003). Interestingly, this study showed that HSE- and STRE- dependent transcription does not depend greatly on the production of sphingoid bases.

Apart from sphingolipid synthesis, translation initiation is one of the key points for the regulation of gene expression and adaptation to various stresses (Dever, 2002). In eukaryotes, the small 40S ribosomal subunit interacts with the ternary complex composed of eIF2-GTP and the charged Met-tRNAi$^{Met}$ to form the 43S preinitiation complex which then binds to the mRNA at the 5′ end, scans for the initiator codon and associates with the 60S ribosomal subunit to initiate translation (Kapp and Lorsch, 2004). Translation initiation can be regulated by various mechanisms including phosphorylation of the translation initiation factor eIF2α on serine 51 by the Gcn2p kinase, which down-regulates the overall translation initiation rate (Hinnebusch, 2000). The Gcn2p kinase is activated by several stimuli including low nutrients (Wilson and Roach, 2002) and membrane stress (Deloche et al., 2004).

The integrity of the 5'cap binding complex is also a target for general control of translation initiation for most cellular mRNAs (Gingras et al., 1999). One important regulator of cap dependent translation initiation is eIF4G, which binds eIF4E and associates with the cap structure and the poly(A)-binding protein leading to circularization of the mRNA (Sachs and Varani, 2000). The eIF4G protein is degraded upon nutrient deprivation and in yeast cells deficient for *YPK1/2* function (Berset et al., 1998; Gelperin et al., 2002; Powers and Walter, 1999). In addition, the eIF4E binding proteins (4E-BPs) act as specific inhibitors. Binding of the 4E-BPs to eIF4E abolishes the interaction of eIF4E with eIF4G, thus blocking ribosome recruitment to the mRNA. Two functional homologues of mammalian 4E-BPs, Caf20p and Eap1p, have been described in *S.cerevisiae* but how these proteins regulate translation initiation is still poorly understood (Altmann et al., 1997; Cosentino et al., 2000; de la Cruz et al., 1997; Deloche et al., 2004).

The major question of this study was how is heat shock protein expression controlled by sphingolipid biosynthesis. Wild type cells challenged with a heat stress initially decrease general translation initiation but increase synthesis of heat shock proteins. Subsequently, general translation is recovered and even surpasses pre-heat shock rates. We demonstrate that the defect in expression of heat shock proteins lies at the level of translation initiation. The initial translation initiation defect does not depend on the phosphorylation status of eIF2α, however deletion of the 4E binding protein Eap1p partially restored translation of heat shock proteins and cell viability. The recovery process seems to depend upon the function of the heat shock proteins made during the initial phase.

The function of heat shock proteins in the subsequent recovery process can be partially replaced by overexpression of ubiquitin. Lack of recovery of translation initiation correlates with depletion of the initiation factor eIF4G. Finally, sphingoid base signaling to translation initiation depends at least partially on the sphingoid base activated kinases, Pkh1/2p.

# RESULTS

### Synthesis of heat shock proteins is reduced in *lcb1-100* cells

We have shown previously that the synthesis of heat shock proteins is severely reduced in cells deficient for the production of sphingolipids using promoters from the heat shock genes *SSA1* and *HSP26* that were fused to the *E.coli* β-galactosidase. Also, heat shock protein labeling in a pulse-chase assay showed reduced induction of heat shock proteins in *lcb1-100* cells (Friant et al., 2003). Since the promoters of both reporter constructs were shown to be dependent on Hsf1p (Amoros and Estruch, 2001; Stone and Craig, 1990), we repeated these assays using a stress inducible promoter which contains seven artificially introduced STRE sequences and is solely dependent on Msn2/4p. Again β-galactosidase activity was greatly reduced in *lcb1-100* cells compared to wild type (Fig. 3-1 A) during heat stress. In addition, protein levels of this construct could barely be detected by western blot against β-galactosidase (Fig. 3-1 B). The decrease in activity or protein level was not due to an increase in β-galactosidase turnover since this protein remained stable over hours in both wild type and mutant cells as determined by pulse chase analysis (data not shown). This shows again that induction of stress- and heat-inducible proteins is reduced in *lcb1-100* cells.

To elucidate how sphingolipid synthesis governs the production of heat shock proteins we analyzed the nuclear import of the STRE activating transcription factor Msn2p. Msn2p translocates to the nucleus upon a wide variety of stresses including heat stress in a PKA/cAMP dependent manner (Gorner et al., 1998; Schmitt and McEntee, 1996). Translocation upon heat stress of Msn2p to the nucleus was normal in *lcb1-100* cells compared to wild type (Fig. 3-1 D). Therefore, cells defective for the synthesis of sphingolipids during heat stress are not impaired for the translocation of the stress responsive transcription factor Msn2p to the nucleus.

We then assessed the transcription of the *7xSTRE-lacZ* mRNA in *lcb1-100* cells by reverse transcriptase PCR. This assay clearly demonstrated that the transcription of the *7xSTRE-lacZ* mRNA in *lcb-100* cells was upregulated normally during heat stress (Fig. 3-1 C). This result is in agreement with a recent study, showing that almost none of the heat-induced heat shock mRNAs are affected in *lcb1-100* cells after 15 or 30 minutes of heat stress compared to wild type (Cowart et al., 2003).

Recent reports have shown the involvement of pre-mRNA splicing as a post-transcriptional regulatory mechanism during heat shock. In MOLT-4 cells, increased sphingolipid synthesis was shown to cause SR protein dephosphorylation (Jenkins et al., 2002) and SR proteins play a crucial role in splicing during heat stress (Shin et al., 2004). To analyze sphingolipid synthesis dependent splicing during heat stress in yeast cells, we performed a primer extension of *SNR17a* exon2. Wild

type as well as *lcb1-100* cells were able to properly splice and join exons 1 and 2, resulting in an 81 base product, during up to one hour of heat stress (Fig. 3-2). Based on these results we exclude a role for sphingolipid synthesis dependent splicing during heat stress in *S. cerevisiae*.

**Export of total and *HSP104* mRNA is functional in *lcb1-100* cells**

A reduced rate in heat shock protein synthesis was previously observed in cells defective for nuclear export of mRNA (Miller et al., 1979; Stutz and Rosbash, 1998). Consequently we analyzed both export of total and *HSP104* mRNA from the nucleus during heat stress in wild type, *lcb1-100* and *mex67-5* cells. For total mRNA we used a poly-dT probe that was labeled at its 3' end using digoxigenin (DIG-) modified dUTP. *HSP104* mRNA was detected using oligos that hybridize specifically to this gene and were internally modified with Cy3 as described (Jensen et al., 2001). In wild type and in *lcb1-100* cells, total and *HSP104* mRNA could be detected in the cytosol after 30 minutes of heat stress at 37°C as seen by a diffuse cytosolic staining with no accumulation in the nucleus (Fig. 3-3). Control *mex67-5* cells, accumulated total and heat induced mRNA within the nucleus as previously reported (Hurt et al., 2000; Segref et al., 1997). *HSP104* mRNA staining in wild type and *lcb1-100* cells before heat stress was not detectable (data not shown). We conclude that export of total and heat induced mRNA is not affected in *lcb1-100* mutant cells.

**The rate of protein synthesis and translation initiation is reduced during heat stress in cells deficient for sphingolipid synthesis**

Having ruled out the involvement of sphingolipid synthesis in a transcriptional event in heat shock protein synthesis we concluded that protein synthesis itself must be defective in *lcb1-100* cells. Translation can be regulated to cope with a diverse set of cellular responses to stress (Deloche et al., 2004; Miller et al., 1979; Uesono and Toh, 2002). First, we determined the rate of protein synthesis in wild type and *lcb1-100* cells by comparing the amounts of [$^{35}$S]methionine incorporation into protein during heat stress. Yeast cells were shifted from 24°C to 37°C to induce heat stress and radiolabeled for 5 minutes as indicated (Fig. 3-4 A). Incorporation was stopped by adding azide and fluoride and the amount of [$^{35}$S] incorporated into protein was determined by TCA precipitation and scintillation counting.

In contrast to wild type cells the total uptake of [$^{35}$S]methionine decreased in the *lcb1-100* mutant cells during heat stress (data not shown). Since the decrease in the TCA-precipitated counts in an aliquot of cells reflects both a decrease in uptake and incorporation into protein, the ratio of

TCA-precipitated versus total cell-associated [$^{35}$S]methionine for each aliquot was used to determine the rate of protein synthesis. The initial ratio of TCA-precipitable to total [$^{35}$S]methionine at 24°C was set to 100% (Fig. 3-4 A). Wild type cells showed a transient decrease in protein synthesis after heat stress at 37°C but recovered and increased their rate of synthesis to about 150% of the initial rate and maintained this for at least one hour at 37°C. Mutant *lcb1-100* cells showed the same decrease in protein synthesis upon heat stress, but there was no recovery. The rate of protein synthesis remained stable at about 50% of the initial synthesis rate (Fig. 3-4 A). Therefore the mechanisms involved in initial down regulation of protein synthesis are operative in *lcb1-100* cells, but these cells are unable to resume normal protein synthesis afterwards.

To assess how sphingolipid synthesis affects translation during heat stress, we analyzed the sedimentation profiles of polysomes on linear 5 to 50% sucrose gradients. After the indicated times of heat stress, cycloheximide was added to the cultures to arrest translation elongation and to preserve the polysomes during preparation. Compared to wild type cells, *lcb1-100* already showed an increase in 80S monosomes, relative to 40S and 60S ribosomal subunits, at permissive temperature (Fig. 3-4 B). After 15 minutes of heat stress at 37°C, the sedimentation analyses showed that both cell types attenuated translation initiation as monitored by the increase in monosomes and the slight decrease in polysomes. The analyses of later time points revealed that this attenuation was transient for wild type cells. In contrast, monosomes continued to increase in *lcb1-100* cells. After 60 minutes at 37°C, wild type cells returned to an almost normal distribution, but *lcb1-100* cells showed a substantial defect in translation initiation as monitored by the large monosome peak with only very few actively translating polysomes (Fig. 3-4 B).

The samples from wild type and *lcb1-100* cells after 60 minutes of heat stress were then loaded onto 5 to 50% linear sucrose gradients containing 0.7M NaCl. High salt concentration leads to the dissociation of randomly formed monosomes that are non-translating and not tightly bound to mRNA. In contrast to wild type cells, the accumulated monosomes in *lcb1-100* cells were non-translating because almost the entire peak consisted of randomly formed 80S particles that could be dissociated into their 40S and 60S subunits (Fig. 3-4 C). Therefore, *lcb1-100* mutant cells show the hallmarks of a translation initiation defect; a decrease in the poylsome to monosome ratio and a majority of non-translating monosomes.

To determine whether the synthesis of sphingolipids is required for translation initiation in general or only during heat stress, wild type cells grown at 24 or 37°C were treated with the antifungal compound myriocin (ISP-1) that inhibits serine palmitoyltransferase (Miyake et al., 1995). At 24°C the rate of protein synthesis remained stable at 100% for wild type cells in the presence or absence of 10 μg/myriocin whereas the same cells showed a decrease in the upregulation of protein synthesis during heat stress in the presence of myriocin (data not shown). Polysome distribution on sucrose gradients was normal for wild type cells at 24°C after up to two

hours of treatment with myriocin (Fig. 3-4 D). In contrast, wild type cells treated with myriocin at 37°C showed an increase in monosomes and a decrease in polysomes over time (Fig. 3-4 D). This demonstrates that sphingolipid synthesis is required for efficient translation initiation only during heat stress. The effects of myriocin are weaker than those seen with the *lcb1-100* mutant, but this is consistent with previous data (Horvath et al., 1994; Sutterlin et al., 1997) and probably results from an incomplete inhibition of serine palmitoyltransferase by the compound.

**The synthesis of sphingoid base is required for translation initiation during heat shock**

Metabolites of the sphingolipid biosynthesis pathway, including sphingoid base, ceramide and sphingoid base 1-phosphate have been shown to be important second messengers in eukaryotic cells, regulating diverse biological processes such as cell growth, differentiation, apoptosis, stress responses, endocytosis, calcium homeostasis, and cell migration (reviewed in Dickson and Lester, 2002; Hannun and Obeid, 2002; Spiegel and Milstien, 2003). Our attention first focused on the phosphorylation of the sphingoid bases since sphingoid base phosphates were shown to be important regulators of heat-induced cell cycle arrest (Chung et al., 2001; Jenkins and Hannun, 2001) and accumulation of phosphorylated sphingoid bases resulted in cell growth inhibition (Kim et al., 2000; Zhang et al., 2001). Yeast cells have two long chain sphingoid base kinases, *LCB4* and *LCB5,* which phosphorylate DHS and PHS (Nagiec et al., 1998). Concomitant with the increase in sphingoid bases during heat stress, yeast cells show an increase in the amount of sphingoid base phosphates with a peak 15 min after heat stress (Ferguson-Yankey et al., 2002; Skrzypek et al., 1999). Therefore we tested if *LCB4* and *LCB5* are required for the regulation of translation during heat stress.

Deletion of the sphingoid base kinases did not lead to a reduction in the production of heat shock proteins as measured by the induction of β-galactosidase from *SSA1* and *HSP26* heat shock gene promoters (Fig. 3-5 A). Moreover, the rate of protein synthesis during heat stress was similar in wild type and *lcb4,5* double mutant cells. Interestingly, *lcb4,5* cells did not show the typical, transient down-regulation in the rate of protein synthesis at 30 min of heat stress (Fig. 3-5 B). The distribution of polysomes on sucrose gradients was analyzed from wild type and *lcb4,5* cells maintained at 24°C or after 15, 30 or 60 minutes of heat stress at 37°C, respectively. Polysomes remained unchanged in *lcb4,5* cells as shown after 60 minutes of heat stress (Fig. 3-5 C). During early time points of heat stress wild type and *lcb4,5* cells showed the same distribution of polysomes (data not shown).

Sphingolipids and their precursor ceramide fulfill important functions in eukaryotic cells. Ceramide was shown to induce differentiation, cell cycle arrest in G0/G1 phase, senescence and

apoptosis whereas sphingolipids have been implicated in cell-cell or ligand-receptor interactions, differentiation and apoptosis in mammalian cells (Hannun and Obeid, 2002). Although ceramides and sphingolipids are upregulated during heat stress in yeast, so far no direct involvement of these metabolites could be shown during this response. Therefore we analyzed the role of ceramide and complex sphingolipid synthesis in translation during heat stress.

Two homologous, redundant genes, *LAG1* and *LAC1*, have been shown to be required for ceramide synthesis (Guillas et al., 2001; Schorling et al., 2001) and to be essential subunits of ceramide synthase (Vallée and Riezman, 2005) . Upon deletion of these genes, yeast cells produce vastly reduced quantities of ceramides. Deletion of both genes leads to severe defects in cell viability and to decreases in heat stress resistance (Barz and Walter, 1999). To rule out the formal possibility that these phenotypes are of secondary nature and not directly connected to the loss of the *de novo* synthesis of ceramides, we introduced a temperature-sensitive allele of *LAG1* on a plasmid in a strain deleted for both *LAG1* and *LAC1* (Schorling et al., 2001). The *lac1lag1*$^{ts}$ strain showed no defects in the induction of reporter constructs for heat shock proteins (Fig. 3-5 A). The rate of protein synthesis was also not affected in *lac1lag1*$^{ts}$ cells compared to wild type cells (Fig. 3-5 B). In agreement with these results, the distribution of polysomes on sucrose gradients was similar in *lac1lag1*$^{ts}$ at 24°C and after heat stress at 37°C (Fig. 3-5 C) when compared to wild type cells. To provide further evidence that ceramides are not required for regulation of protein synthesis after heat shock, we tested whether addition of the 4 different stereoisomers of DHS could complement the defects of *lcb1-100* cells. Only two of these stereoisomers can be incorporated into ceramide (Watanabe et al., 2002). All four stereoisomers could restore normal regulation of protein synthesis providing further proof that ceramide is not the sphingolipid required for regulation of translation (Fig. 3-5 D).

**Phosphorylation of eIF2α is similar in wild type and *lcb1-100* cells**

Since a block in sphingolipid synthesis could possibly induce a membrane stress, leading to a decrease of translation initiation by activating the Gcn2p kinase, we next monitored the phosphorylation status of eIF2α (encoded by *SUI2* in yeast) on serine 51 during heat stress in wild type and *lcb1-100* cells. Western blots against total and phosphorylated eIF2α were performed. Wild type and *lcb1-100* cells showed an increase in phosphorylation of eIF2α shortly after shift to 37°C. During prolonged times of heat stress decreased amounts of phosphorylated eIF2α were observed in *lcb1-100* cells compared to wild type cells (Fig. 3-6). The lack of difference at 15 and

30 min allow us to exclude a role of the Gcn2p-eIF2α pathway for regulation of heat shock protein translation in our mutant cells, but it could play some role in the recovery process.

**Deletion of the yeast eIF4E-binding protein, Eap1p, restores translation initiation and synthesis of heat shock proteins**

A second control step in translation initiation is achieved by the control of the availability of eIF4E proteins and deletion of *EAP1* was shown to partially restore translation initiation of membrane stressed cells (Deloche et al., 2004). Therefore, we analyzed the rate of protein synthesis in *eap1*Δ, and *eap1*Δ *lcb1-100* cells. Deletion of *EAP1* alone had no effect on the rate of protein synthesis during heat stress (Fig. 3-7 A). In contrast, deletion of *EAP1* in *lcb1-100* cells was able to restore the incorporation of [$^{35}$S]methionine into protein to almost wild type levels. Interestingly, similarly to *lcb4,5* cells, *eap1*Δ*lcb1-100* cells showed no transient down-regulation in the synthesis rate shortly after heat stress (Fig. 3-7 B). Deletion of *CAF20* in wild type, *lcb1-100*, *eap1*Δ or *eap1*Δ*lcb1-100* cells did not lead to any phenotypes different from presented above (data not shown).

Polysome analyses of *eap1Δ*, *lcb1-100* and *eap1Δlcb1-100* cells demonstrated that *eap1Δ* cells behave as wild type cells before and after 60 minutes of heat stress at 37°C (Fig 3-7 B). As before, mutant *lcb1-100* cells showed an accumulation of monosomes (80S peak) at permissive temperature and strong accumulation of non-translating monosomes after 60 minutes of heat stress. In contrast, *eap1*Δ*lcb1-100* cells showed a less severe loss of translating ribosomes (Fig. 5B). The increase in the protein synthesis rate at early time points during heat stress did not lead to any change in the distribution of polysomes or in the size of the 80S peak in *eap1Δlcb1-100* cells, as was observed for the *lcb4,5* mutant (data not shown).

Next we determined if deletion of *EAP1* also restored synthesis of heat shock proteins to *lcb1-100* cells. Induction of *SSA1* and *HSP26* heat shock gene promoters fused to β-galactosidase was increased in *eap1*Δ cells compared to wild type cells especially for the *HSP26* fusion (Fig. 3-7 C). The induction of the same β-galactosidase gene promoters was also improved in *lcb1-100* cells deleted for *EAP1* although the profile of induction was somewhat different.

Loss of production of heat shock proteins was shown to be a major cause of decreased viability during heat stress in cells deficient for sphingolipid synthesis (Friant et al., 2003). Therefore *lcb1-100* cells deleted for *eap1*Δ were predicted to have an improved viability due to the restoration of heat shock protein synthesis. Deletion of *EAP1* in wild type cells is lethal at high temperatures (Cosentino et al., 2000) and deletion of *EAP1* in *lcb1-100* cells did not restore growth

on plates (data not shown). Therefore, we assayed resistance to heat shock at an elevated temperature. Log-phase cultures of wild type, *lcb1-100*, *eap1*Δ and *eap1*Δ*lcb1-100* cells were heat shocked at 44°C and the percentage of cells able to form colonies was determined as a function of time (Fig. 3-7 D). The *lcb1-100* mutant showed a clear defect in survival at high temperature. In contrast, *lcb1-100* cells deleted for *EAP1* regained resistance to heat stress over the 2 hour period (Fig. 3-7 D).

Taken together these results show that deletion of the translation repressing 4E-BP Eap1p, increased translation of heat shock proteins in *lcb1-100* cells, indicating that synthesis of sphingoid bases during heat stress regulates translation initiation at a cap dependent step. In addition, these results show that recovery of heat shock protein synthesis allows *lcb1-100* cells to resist heat shock in the absence of sphingolipid synthesis. Therefore, the essential function of sphingolipids in resistance to heat shock is their function in heat shock protein expression.

**Translation initiation is regulated in part via the conserved *PKH/YPK* signaling cascade**

Sphingoid bases were previously shown to serve as signaling molecules. Addition of sphingoid base stimulated phosphorylation of the yeast serum- and glucocorticoid-inducible kinase (SKG) homologues Ypk1p and Ypk2p by the upstream yeast *PDK1* homologues Pkh1p and Pkh2p (Casamayor et al., 1999) in an *in vitro* assay (Friant et al., 2001). Also overexpression of *YPK1* conferred resistance to the serine palmitoyltransferase inhibitor myriocin, (Sun et al., 2000). Furthermore, it was shown that cells thermosensitive for *YPK* signaling, are defective for translation initiation and the translation initiation factor eIF4G is depleted with time at non-permissive temperature (Gelperin et al., 2002). If the proposed conserved signaling cascade is operative here, eIF4G should be depleted in *lcb1-100* at similar rates during heat stress as was observed for a $ypk^{ts}$ strain. To test this, western blots against eIF4G were performed.

eIF4G was stable in wild type cells up to 4 hours during heat stress at 37°C. In contrast, *lcb1-100* cells showed loss of eIF4G beginning at 60 minutes of heat stress and after 4 hours very small levels of eIF4G could be detected. Has1p, a member of the DEAD-box family of RNA helicases that is involved in 40S ribosomal subunit biogenesis (Emery et al., 2004), remained stable in both wild type and *lcb1-100* cells at all times tested (Fig 3-8 A). This demonstrates clearly that the stability of eIF4G in *lcb-100* cells is comparable to $ypk^{ts}$ cells and suggests a similar mechanism of regulation.

If translation initiation during heat stress is regulated via this signaling cascade, a strain defective for Pkh kinase signaling should also be defective for translation initiation during heat stress. We tested a $pkh^{ts}$ strain for the rate of protein synthesis and for polysome distribution on

sucrose gradients after 60 minutes of heat stress. The rate of protein synthesis during heat stress was clearly reduced in *pkh*$^{ts}$ cells compared to wild type cells, although not quite to the same extent as in *lcb-100* cells (compare Fig. 3-8 B and 3-8 A). Polysome distribution was also changed in *pkh*$^{ts}$ cells after 60 minutes of heat stress at 37°C. Thermosensitive *pkh*$^{ts}$ cells showed accumulation of 80S particles and a reduction in polysomes, indicating reduced translation rates (Fig. 3-8 C).

Since the arrest in translation initiation in *pkh*$^{ts}$ cells was rather slow compared to *lcb1-100* cells we wanted to know if induction of *SSA1* and *HSP26* heat shock gene promoters fused to β-galactosidase was affected in *pkh*$^{ts}$ and *ypk*$^{ts}$ cells. Whereas *pkh*$^{ts}$ cells showed a nearly 50% reduction in the induction of *SSA1-lacZ* and *HSP26-lacZ*, *ypk*$^{ts}$ cells displayed a induction profile similar to wild type cells (Fig. 3-8 D). These results suggest that translation is partially regulated by the sphingoid base dependent PKH-YPK signaling cascade during heat stress and that the Pkh kinases may have other targets than the Ypk kinases in this pathway.

**Ubiquitin overexpression can partially suppress the translation defect in *lcb1-100* cells**

Overexpression of the polyubiquitin gene *UBI4* restored growth of *lcb1-100* cells at elevated temperatures without restoring heat shock protein expression (Friant et al., 2003 and Fig. 3-9 A). *UBI4* overexpression led to higher levels of ubiquitin and enhanced degradation of un- or misfolded proteins in a proteasome-dependent pathway, preventing aggregation of those proteins (Friant et al., 2003). Overexpression of ubiquitin partially suppressed the decrease in the translation rate as determined by measuring the incorporation of [$^{35}$S]methionine into proteins (Fig. 3-9 B). Mutant *lcb1-100* cells overexpressing *UBI4* showed the same distribution of polysomes on sucrose gradients as wild type cells at permissive temperature. After 60 minutes of heat stress, *lcb1-100* cells overexpressing ubiquitin showed less accumulated monosomes and more polysomes compared to the polysomes of *lcb1-100* cells shown in Fig. 3-4 B. This was in agreement with the translation rates measured (Fig. 3-9 C). Therefore, the overexpression of ubiquitin in the *lcb1-100* mutant largely abrogates the need of sphingoid bases and heat shock proteins for the recovery of protein synthesis after a heat stress.

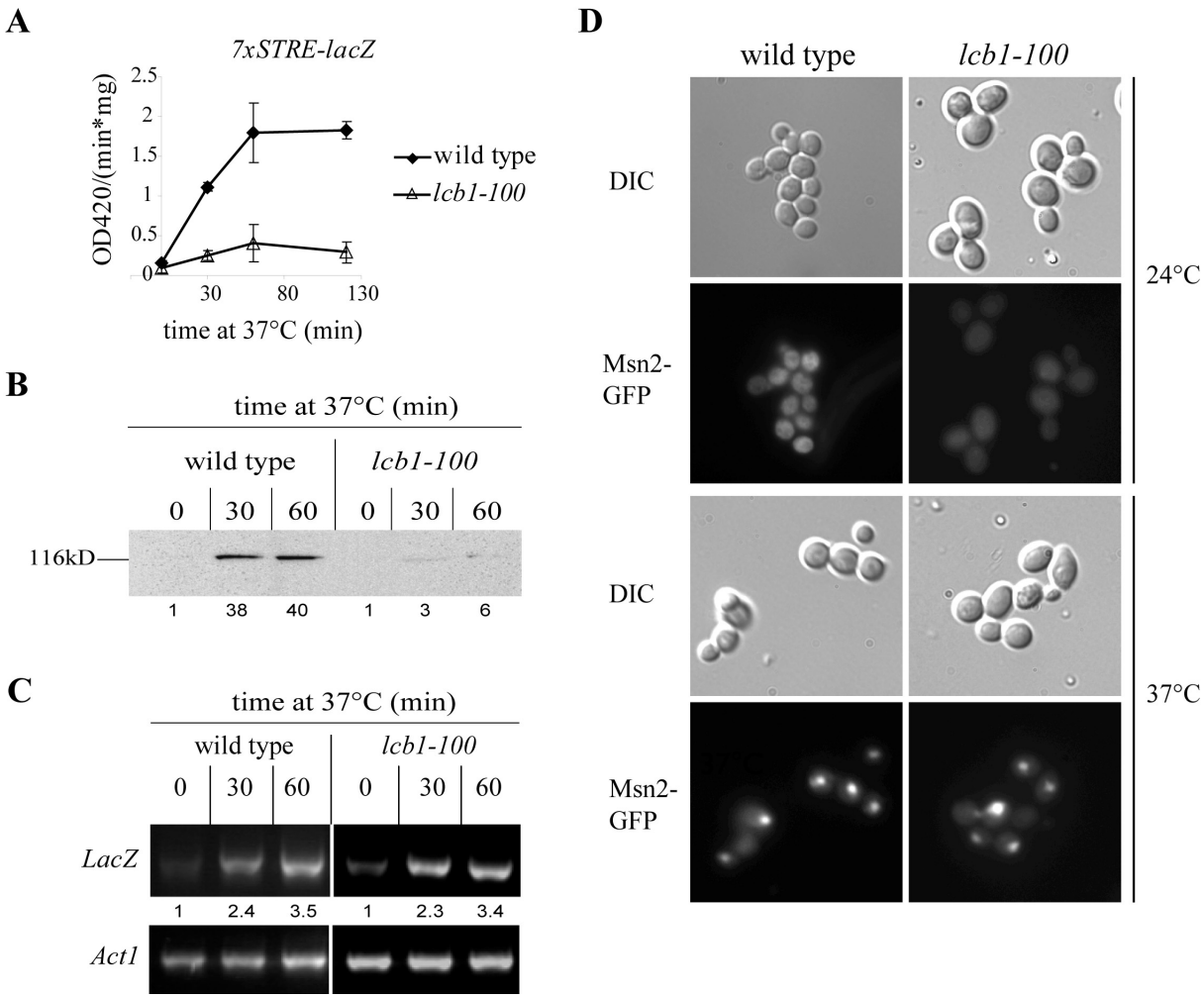


**Figure 3-1.** Synthesis of heat shock proteins in *lcb1-100* cells is not reduced at a transcriptional level. **A.** Wild type and *lcb1-100* cells transformed with *7xSTRE-lacZ* were heat stressed for up to two hours at 37°C. Whole cell lysates were assayed for β-galactosidase activity at indicated time points as described. **B**. Western blot showing induction of *7xSTRE-lacZ* in wild type and *lcb1-100* cells at 37°C. Yeast cells were treated as in A and protein extracts were analyzed using an antibody against β-galactosidase. Relative intensity of bands compared to the 0 time point is given. **C**. Reverse transcription analysis of *7xSTRE-lacZ* mRNA. At indicated times during heat stress at 37°C wild type and *lcb1-100* cells were collected, their RNA extracted and amplified using specific primers against *ACT1* and *LacZ* mRNA. Relative intensity of bands compared to time point 0 is shown. **D.** Translocation of Msn2-GFP to the nucleus is not inhibited in *lcb1-100* cells during heat stress. Wild type and *lcb1-100* cells were transformed with a plasmid carrying MSN2 fused to GFP and analyzed as described at permissive temperature and after 10 minutes heat stress at 37°C.

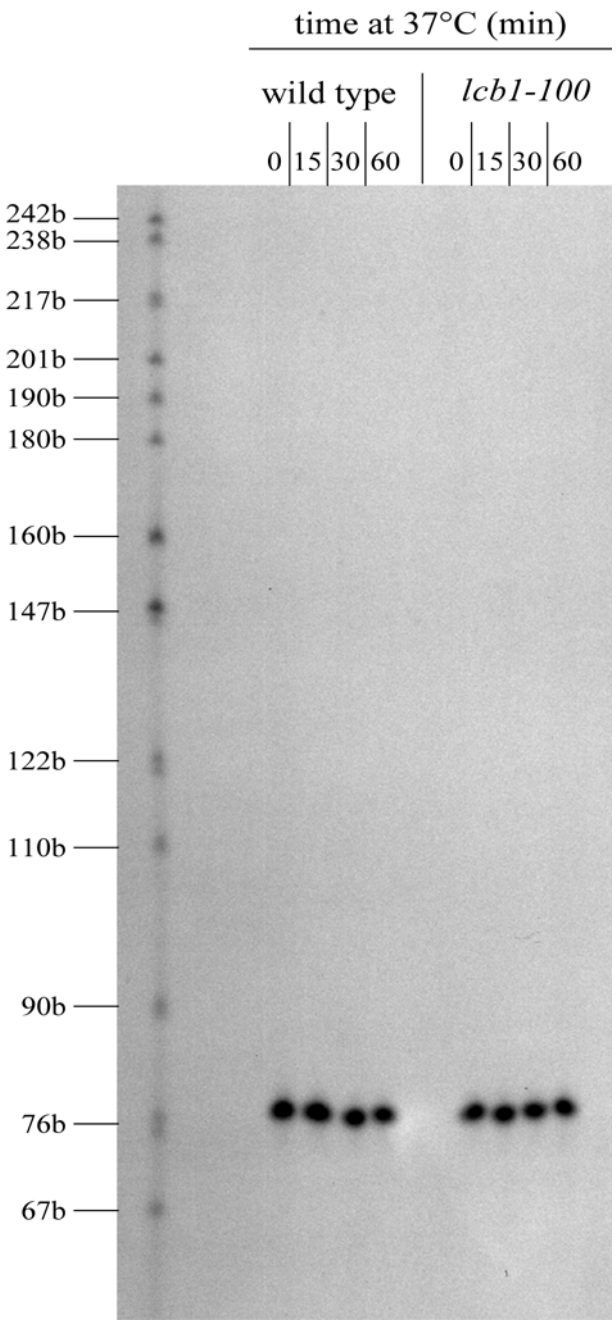


**Figure 3-2**. Splicing of U3 (SNR17a) snRNA is not affected in *lcb1-100* cells. Total RNA was extracted from cells at given time points after heat stress at 37°C as indicated. Primer extension was performed as described and autoradiographed. b, number of bases.

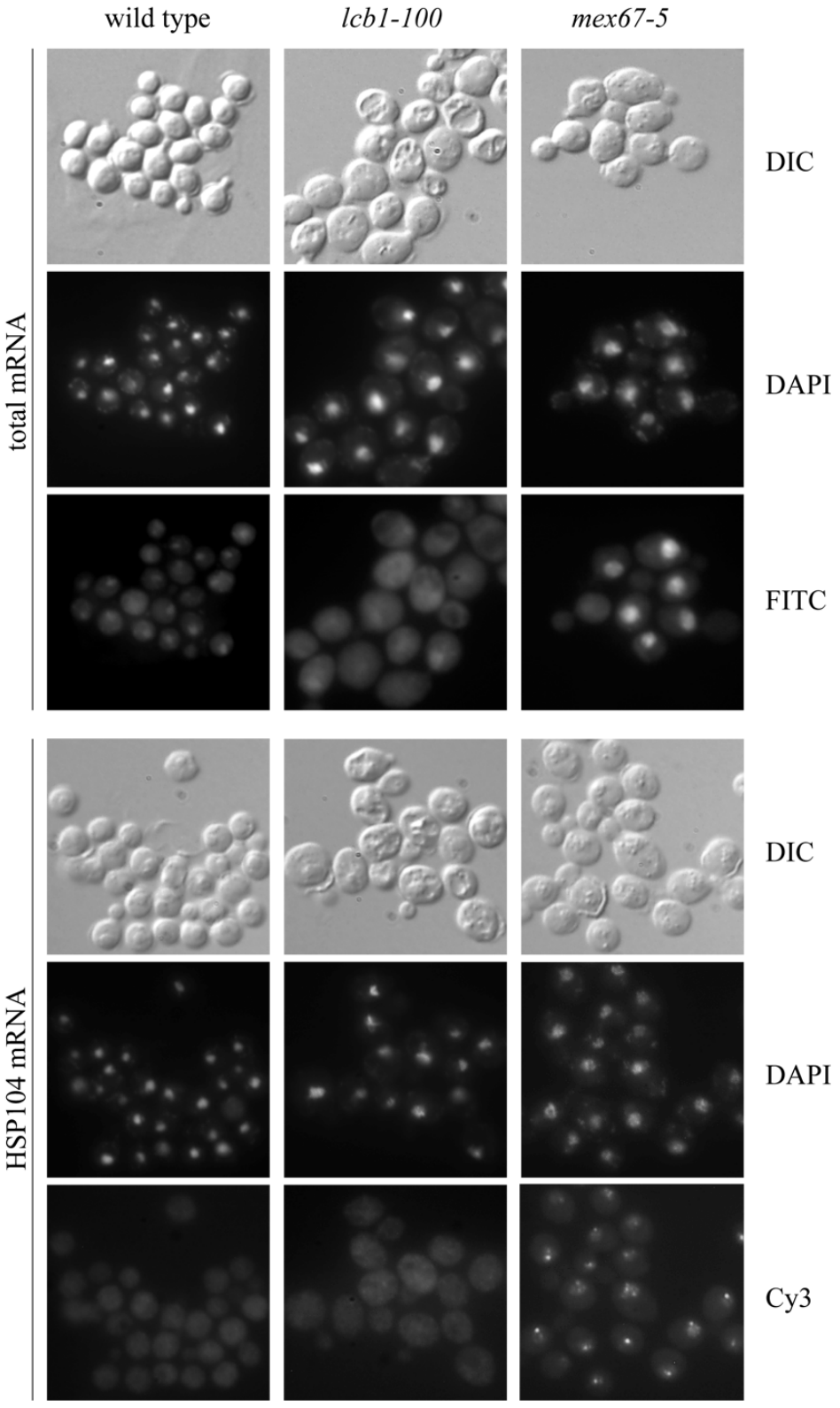


**Figure 3-3.** In situ hybridization of total and *HSP104* mRNA after 30 minutes at 37°C in wild type, *lcb1-100* and *mex67-5* cells showing normal export of mRNA in *lcb1-100* and wild type cells. Total mRNA was detected using DIG modified poly dT oligos with anti DIG antibodies coupled to fluorescein and visualized using a FITC filter set. *HSP104* mRNA was detected using specific Cy3 labeled oligos and a Cy3 filter set as described.

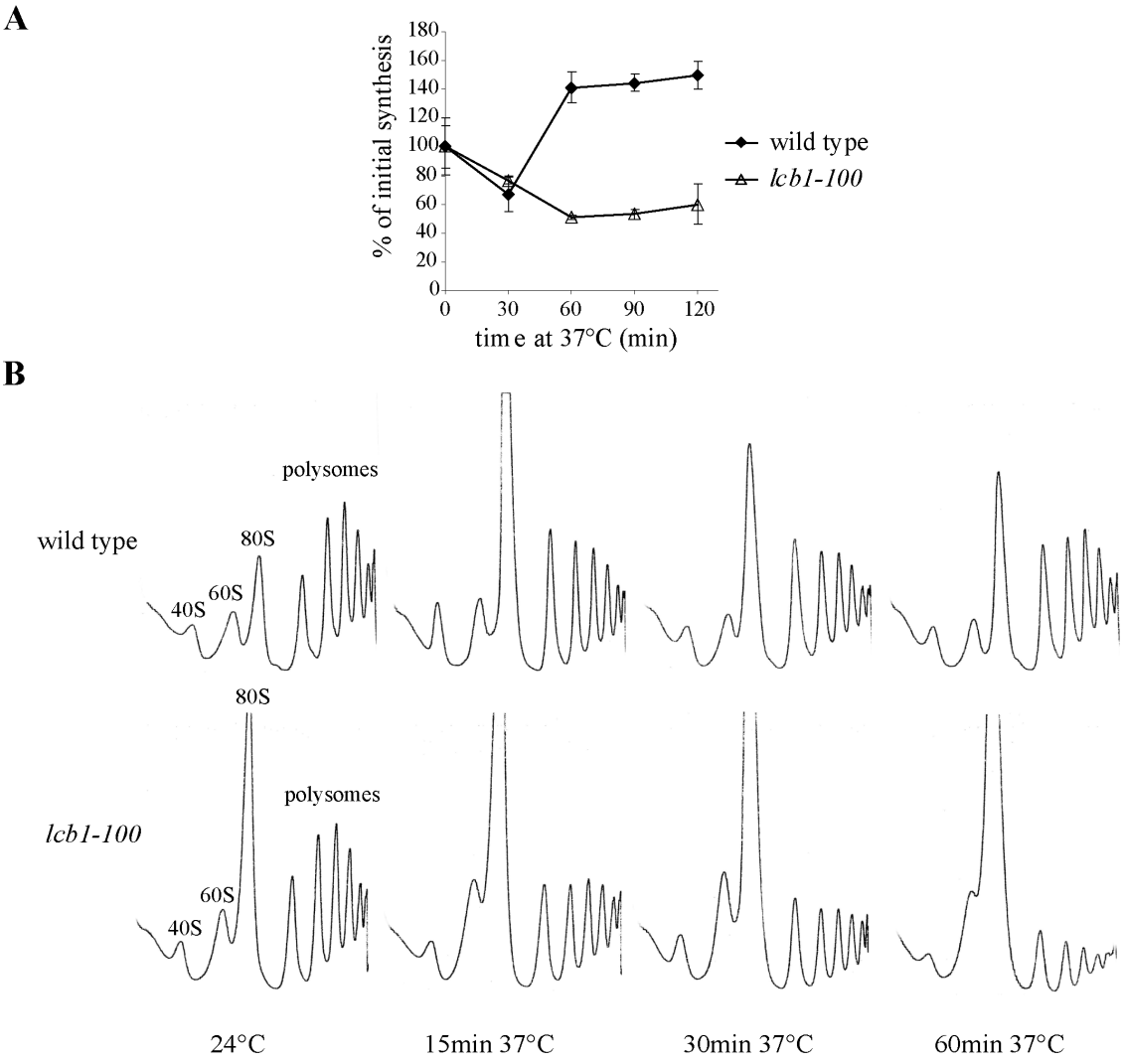


**Figure 3-4.** Translation is reduced in cells deficient for sphingolipid synthesis during heat stress. **A.** Wild type and *lcb1-100* cells were grown to early log phase at 24°C in synthetic media and shifted for the indicated time to 37°C. Each sample was pulsed using [$^{35}$S]methionine for the last 5 minutes and the amount of incorporated [$^{35}$S]methionine compared to cell associated [$^{35}$S]methionine was analyzed. **B.** Polysome analysis of wild type and *lcb1-100* cells during heat stress. Wild type and *lcb1-100* cells treated at 24°C or for the indicated time at 37°C were collected and lysed as described. 8 $OD_{260}$ were loaded on linear 5 to 50% sucrose gradients and spun at 39 krpm for 2h 45min.

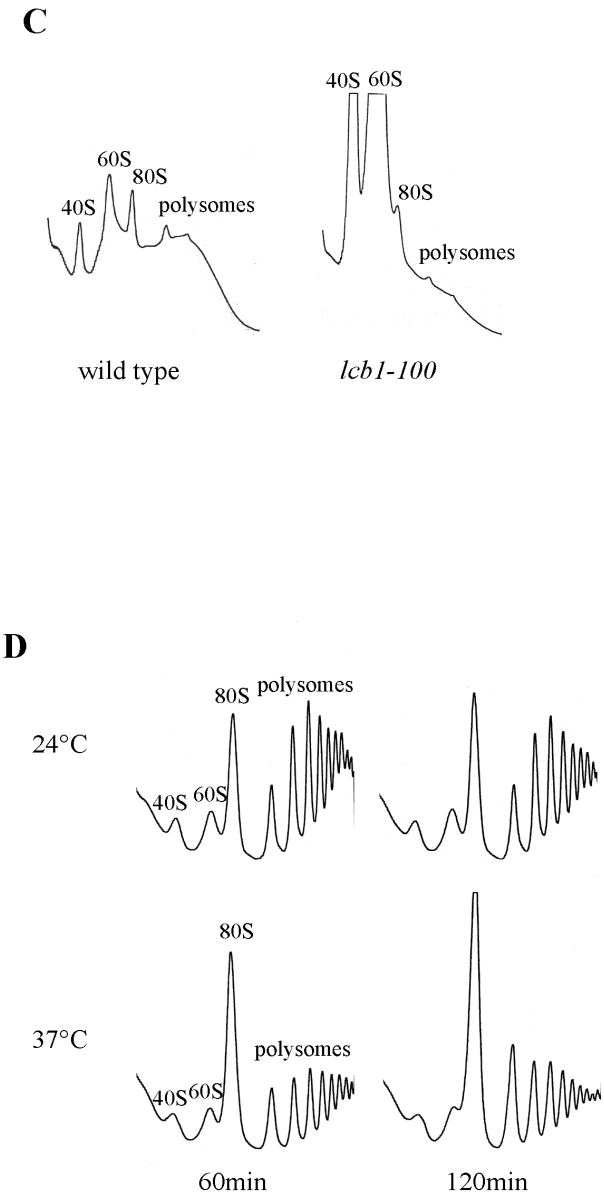


**Figure 3-4 cont**. **C**. Distribution of polysomes on high salt gradients for wild type and *lcb1-100* cells after 60 minutes heat stress at 37°C. Extracts were loaded on 5 to 50% linear sucrose gradients containing 0.7 M NaCl were spun at 35 k for 3h 20min and analyzed as before. **D**. Polysome analysis of wild type cells, treated for either 60 or 120 minutes at 24 or 37°C, with myriocin at a final concentration of 10μg/ml, respectively. Analysis was performed as in B except that centrifugation was for 3h 20min at 35 krpm. 40S, small ribosomal subunit; 60S large ribosomal subunit; 80S monosome.

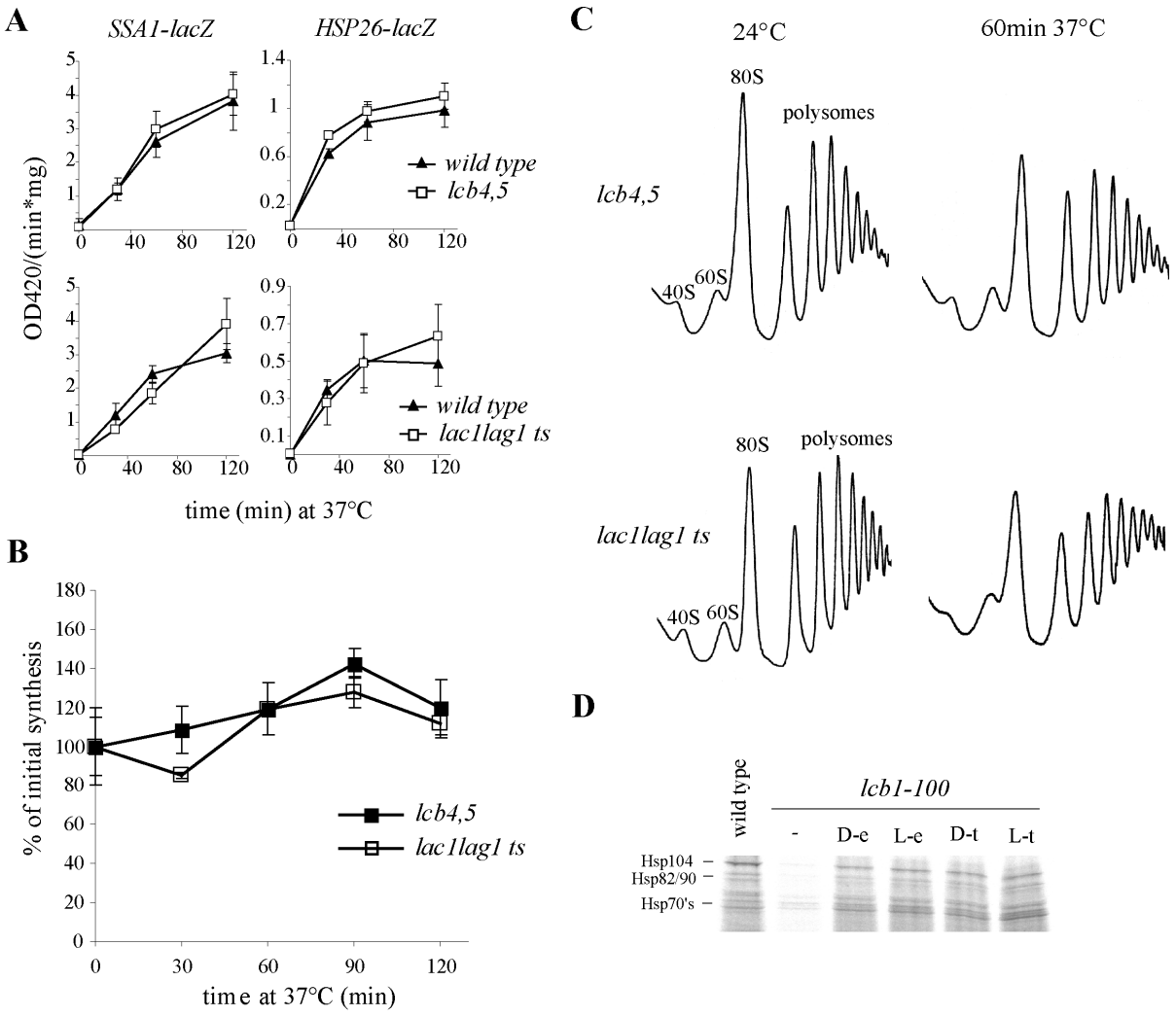


**Figure 3-5.** The synthesis of sphingoid base phosphates and ceramides is not required for proper translation initiation during heat shock. **A.** Wild type, *lcb4,5* and *lac1lag1*$^{ts}$ cells transformed with a plasmid containing either *SSA1-lacZ* or *HSP26-lacZ* were heat stressed for up to two hours at 37°C. β-galactosidase was extracted and assayed at indicated time points as described. **B.** Translation rate of *lcb4,5* and *lac1lag1*$^{ts}$ cells during heat stress at 37°C over two hours was analyzed as described above. **C**. Polysome analysis of *lcb4,5* and *lac1lag1*$^{ts}$ cells after 60 minutes of heat stress at 37°C in synthetic media was analyzed as described. 40S, small ribosomal subunit; 60S large ribosomal subunit; 80S monosome. **D**. Addition of all four stereo-isomers of DHS to *lcb1-100* cells induced heat shock protein synthesis. Cells actively dividing at 24°C were treated with isomer or carrier, transferred to 44°C and the production of heat shock proteins was assessed after [$^{35}$S]methionine labeling, followed by extraction and separation by SDS-PAGE of labeled proteins. Heat shock protein bands are indicated. e stands for erythro-DHS and t for threo-DHS.

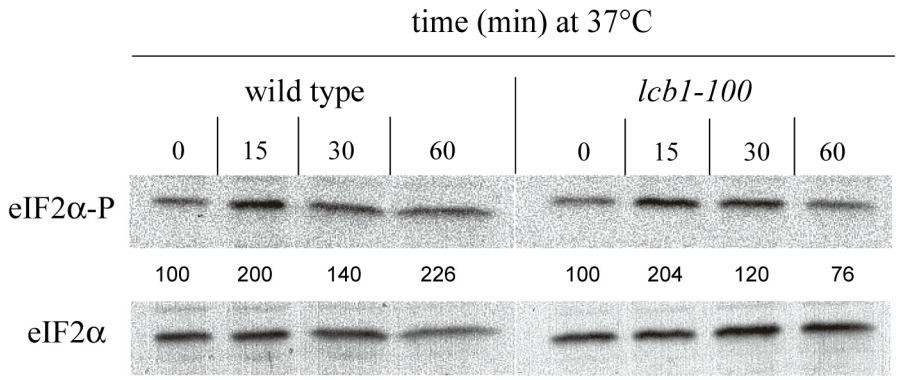


**Figure 3-6.** Phosphorylation of eIF2α is similar in wild type and *lcb1-100* cells. Western blots against eIF2α phosphorylated at Ser51 and total eIF2α before and after the indicated times during heat stress. Wild-type cells were grown to log phase in synthetic media and whole cell extracts were prepared. Phosphorylation of Ser51 was quantified and compared with the total amount of eIF2α protein, given in numbers below.

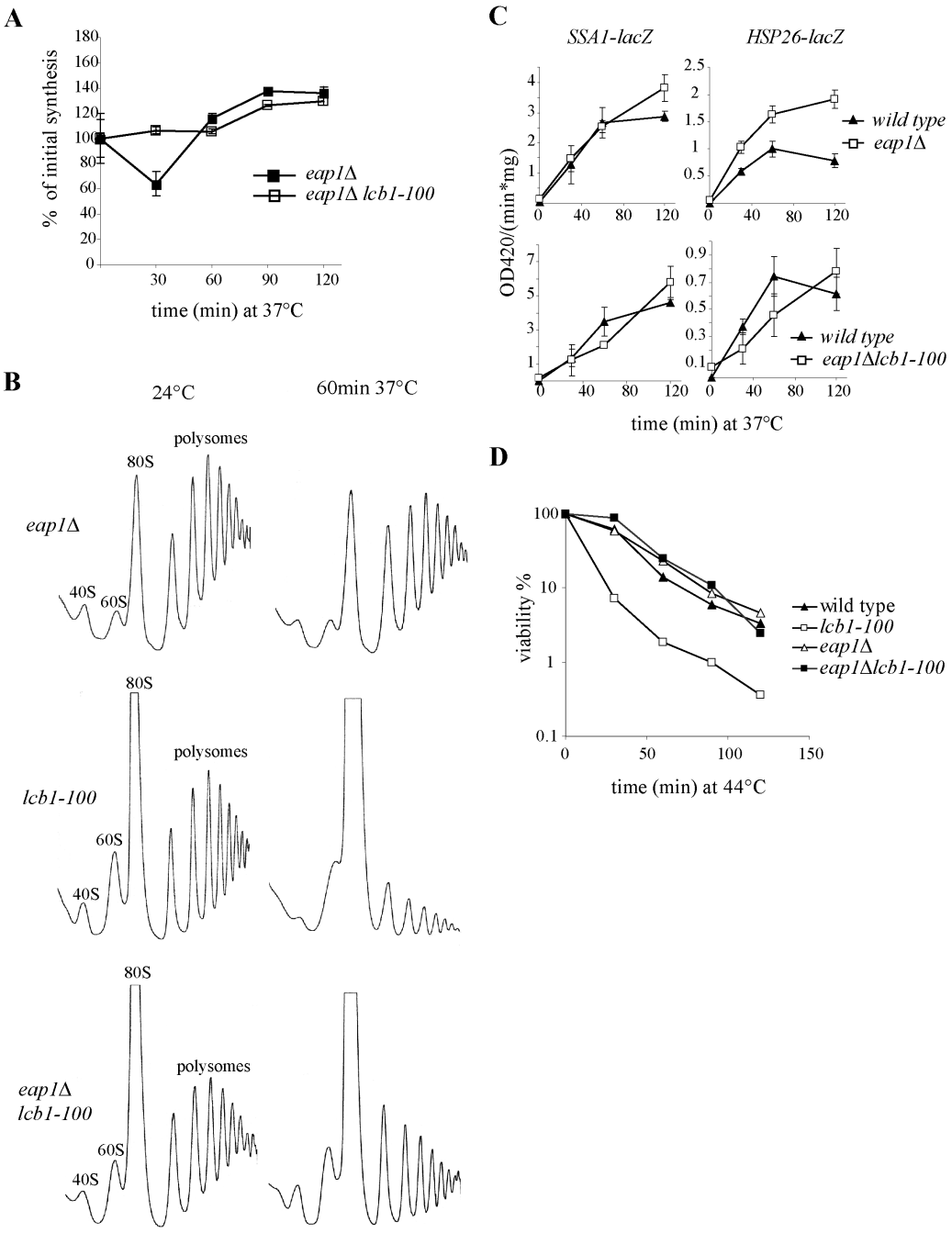


**Figure 3-7**. Deletion of *EAP1* partially restores translation initiation. **A**. Translation rate analysis of *eap1Δ* and *eap1Δlcb1-100* cells during heat stress at 37°C over two hours was performed as described above. **B**. Polysome analysis of *eap1Δ* and *eap1Δ lcb1-100* cells in synthetic media after 60 minutes of heat stress at 37°C, analyzed as described in Figure 3B. **C**. Wild type, *eap1*Δ and *eap1*Δ*lcb1-100* cells transformed with a plasmid containing either *SSA1-lacZ* or *HSP26-lacZ* were heat stressed for up to two hours at 37°C. β-galactosidase was extracted and assayed at indicated time points as described. **D**. Deletion of *EAP1* restores heat resistance to *lcb1-100* cells. Mid log-phase cultures were grown at 24°C and an aliquot was shifted to 44°C. Samples were taken in duplicate at the times indicated, diluted into ice-cold YPD, and immediately plated onto YPD 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 before the heat shock.

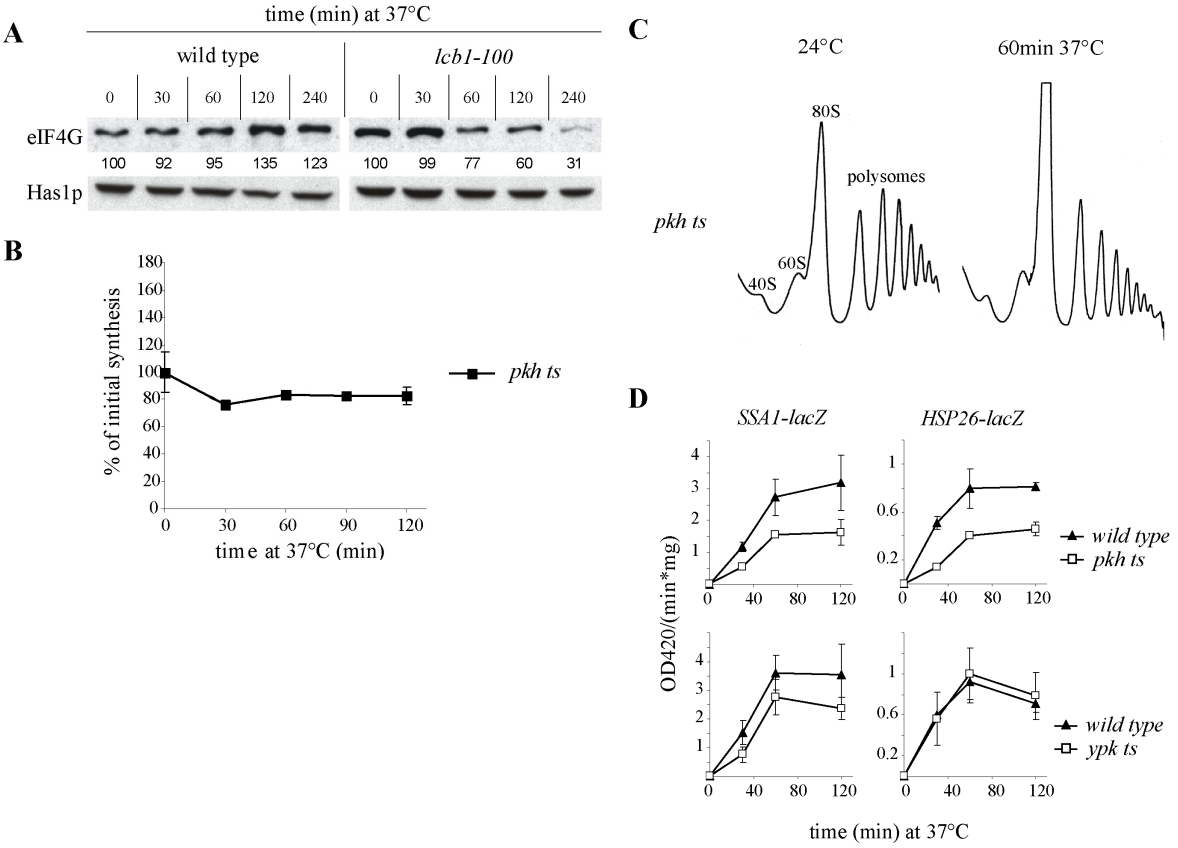


**Figure 3-8**. Translation during heat stress depends on the conserved PKH1/2 signaling cascade and leads to stability of eIF4G. **A**. Stability of eIF4G in wild type and *lcb1-100* cells during heat stress at 37°C. Cells were grown to log phase, shifted for the indicated time and total protein extracts were resolved on SDS-PAGE gels and analyzed by western blotting using antibodies against eIF4G and Has1p as control, respectively. **B**. Translation rate of $pkh^{ts}$ cells in response to heat stress at 37°C for two hours, analyzed as described. **C**. Polysome analysis of $pkh^{ts}$ cells in synthetic media after 60 minutes of heat stress at 37°C, analyzed as described in Figure 3B. **D**. Wild type, $pkh^{ts}$ and $ypk^{ts}$ cells transformed with a plasmid containing either *SSA1-lacZ* or *HSP26-lacZ* were heat stressed for up to two hours at 37°C. β-galactosidase was extracted and assayed as described.

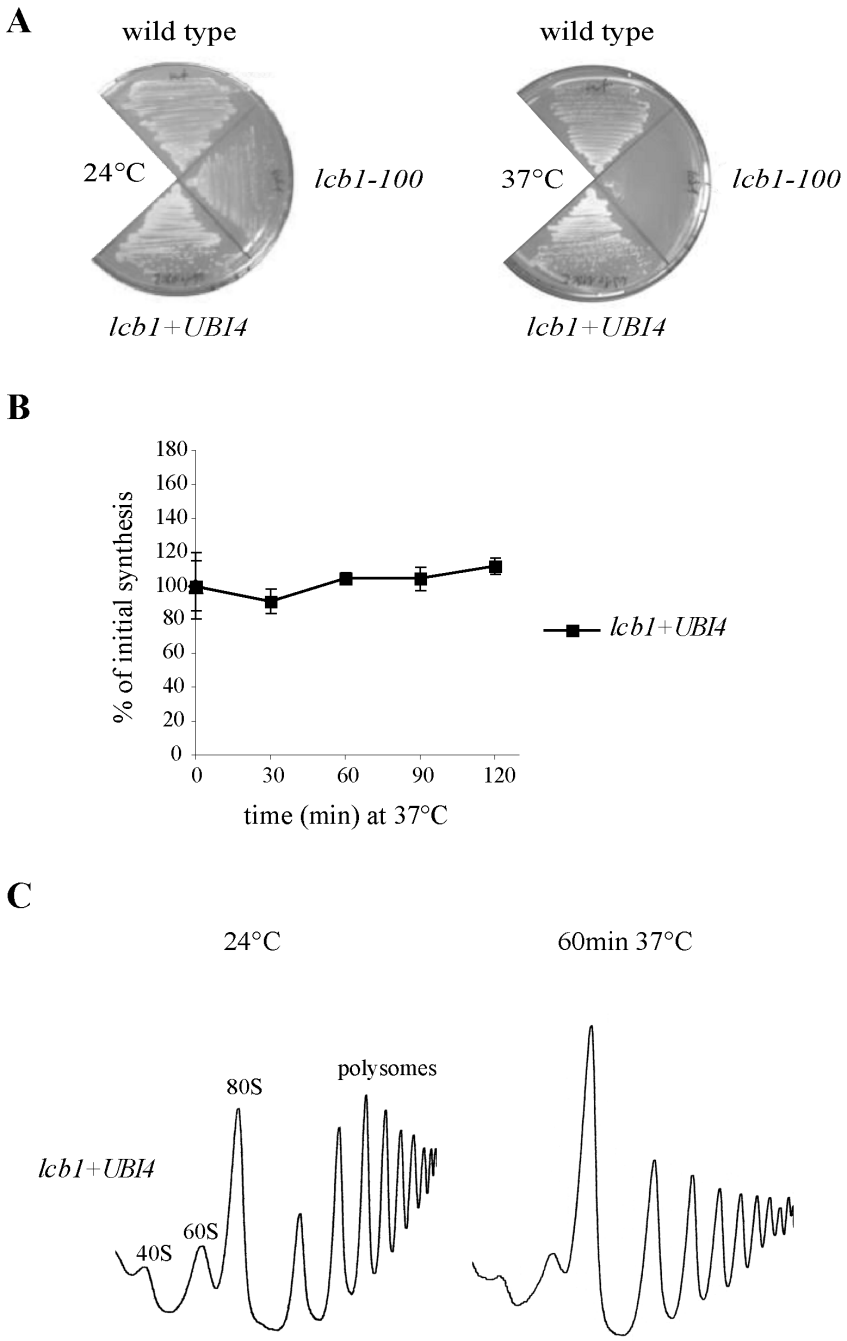


**Figure 3-9**. Overexpression of *UBI4* partially restores translation in *lcb1-100* cells. **A**. Wild type, *lcb1-100* and *lcb1-100* cells carrying plasmids that overexpress *UBI4* were streaked onto YPD plates and grown at 24°C and at the non-permissive temperature of 37°C **B**. Translation rate of *lcb1-100* transformed with a multicopy plasmid overexpressing *UBI4* cells in response to heat stress at 37°C, performed as described above. **C**. Polysome analysis of *lcb1-100* cells overexpressing *UBI4* in synthetic media after 60 minutes of heat stress at 37°C, analyzed as described in Figure 3B.

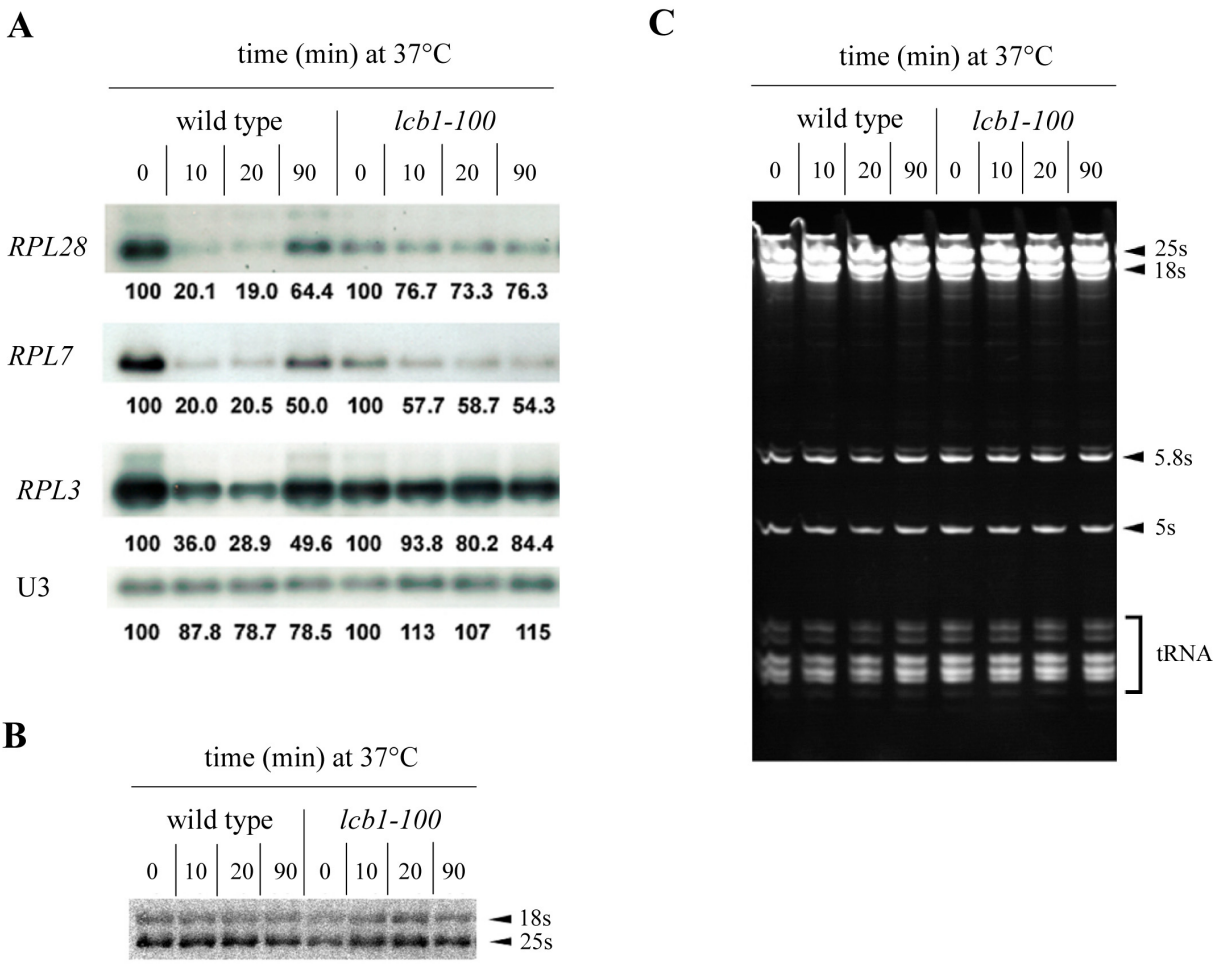


**Figure 3-10**. **A.** Transcription of ribosomal protein mRNA is less down regulated during heat stress in *lcb1-100* cells. Wild-type and *lcb1-100* cells were grown in synthetic media at 24°C. Log phase cultures were shifted to 37°C and cells were harvested before and at various times after the shift to 37°C. Total RNA was prepared and subjected to electrophoresis. Gels were transferred to nylon membranes and hybridized with the indicated probes to detect the steady state levels of the different RNA's. **B**. Transcription of ribosomal rRNA is not affected by the *lcb1-100 mutation*. Analysis was performed as described for A. **C**. Transcription of rRNA and total tRNA is not affected in *lcb1-100* cells during heat stress. RNA was extracted as described above and loaded on 8% polyacrylamide gels containing 8M Urea and stained using ethidium bromide.

# DISCUSSION

## Sphingoid base synthesis regulates translation initiation during heat stress

The major finding of this study is that a lipid mediator, in this case sphingoid base, regulates protein translation during a heat stress. Our results suggest that this regulation concerns two phases; the initial translation of heat shock protein mRNAs and the subsequent increase in translation rate. The latter phase probably depends upon proper execution of the first phase and the function of heat shock proteins. Sphingolipid synthesis has been suggested to play many roles in heat stress, but thus far little molecular insight into the pathways depending on sphingolipid synthesis has been forthcoming. Dickson and co-workers showed in a previous study that addition of high concentrations (μM range) of DHS could induce reporter genes driven by stress response elements (Dickson et al., 1997). These results could have implicated sphingoid bases in the induction of the heat stress response, in particular in mRNA induction, or could have meant that high amounts of sphingoid bases induce a stress themselves, in particular since high amounts of sphingoid bases are toxic to yeast cells (Mao et al., 1999). The role of sphingolipid biosynthesis in heat shock protein expression was then confirmed using the *lcb1-100* mutant (Friant et al., 2003). Interestingly, transcription of most heat shock protein genes was not dependent on sphingolipid biosynthesis (Cowart et al., 2003), which already suggested that the level of control was not transcriptional.

The results presented here demonstrate that synthesis of sphingoid base phosphates, ceramides and sphingolipids are not required for translation and synthesis of heat shock proteins during a heat shock. This result is both interesting and intriguing because all metabolites of the sphingolipid biosynthesis pathway were shown to be upregulated during heat stress. The precise roles for sphingoid base phosphates, ceramides and sphingolipid synthesis in the heat stress response in yeast still need to be determined. Alternatively, they could serve as sinks to remove sphingoid bases once they have performed their signaling function.

Heat stress leads to a rapid but temporary repression of ribosomal protein (RP) gene transcription (Eisen et al., 1998). In contrast to this, RP mRNA levels failed to be down regulated during heat stress in *lcb1-100* cells (Cowart et al., 2003) and Fig. 3-10 A). This is unlikely to affect translation since RP gene mRNA was not reduced to an amount below that of wild type cells during heat stress and quantification of ribosomal 40 and 60S subunits using low $Mg^{2+}$ extracts gave a ratio of 1.6 for wild type and *lcb1-100* cells at 24°C and after 60 minutes of heat stress at 37°C, respectively (data not shown). In addition, the levels of tRNA and rRNA were maintained normally for various time points after heat stress in *lcb1-100* cells (Fig. 3-10 B and Fig.

3-10 C), indicating that the translation initiation defect in the *lcb1-100* mutant is not due to an inhibition of tRNA and rRNA synthesis.

Surprisingly**,** the defect in sphingolipid synthesis did not elicit a membrane stress that is typically governed by strong phosphorylation of eIF2α (Deloche et al., 2004). Nevertheless, deletion of the 4E-BP *EAP1* partially restored heat shock protein and general translation in *lcb1-100* mutants in a fashion similar to cells showing a transient attenuation in translation initiation resulting from membrane stress. This result, together with the fact that eIF4G is depleted in *lcb1-100* cells, indicates that sphingoid bases regulate translation initiation through a cap-dependent step. Growth at high temperatures could not be restored by an *EAP1* deletion in *lcb1-100*, but this was to be expected because deletion of *EAP1* alone causes defects in genetic stability at 37°C (Chial et al., 2000). We do not know if this or the hyperactivation of the stress response is the primary cause for lethality in *eap1*Δ cells. The precise role of Eap1p needs to be determined.

Our data suggest that the defect in heat shock protein translation is likely to be regulated, at least in part, via the conserved sphingoid base dependent PKH/YPK signaling pathway. A mutant defective for YPK kinases showed a strong decrease in translation initiation during prolonged heat stress and depletion of eIF4G (Gelperin et al., 2002) was observed at similar rates in *lcb1-100* cells. Mutant cells defective for PKH1/2 or YPK1/2 signaling however showed moderate defects in the rate of heat shock protein induction for the former and almost no defect for the latter. This suggests that the role of this kinase cascade may be different in the two phases of response to heat shock. The first phase regulates immediate translation of heat shock mRNAs, requires sphingoid bases and is partially dependent upon Phk kinases, but independent of Ypk kinases. The second phase is the increase in the rate of translation in general. The second phase is likely to depend upon the function of heat shock proteins in the first phase because overexpression of ubiquitin partially restored the translation defect in *lcb1-100* cells. Ubiquitin has been shown to functionally substitute for heat shock proteins under these conditions and its role must be in the recovery phase because its overexpression does not restore heat shock protein induction in *lcb1-100* cells (Friant et al., 2003). One way to rationalize these results is that sphingoid bases are required for heat shock protein translation and that in the normal course of events, heat shock proteins play a crucial role in the recovery process. However, in the absence of heat shock proteins, protein aggregation or misfolded proteins could have an additional negative effect on translation. This latter effect could be circumvented by the overexpression of ubiquitin. It has been shown that the yeast Hsp70 homologue Ssa is required for efficient translation by promoting the interaction of Pab1p with eIF4G. Depletion of Ssa led to reduced translation and reduction of eIF4G even in the absence of heat stress (Horton et al., 2001). Therefore, accumulation of unfolded proteins and/or aggregates due to an inefficient heat shock protein induction could lead to a titrating out of Ssa proteins and a block in translation initiation. Overexpression of ubiquitin could remove the unfolded/aggregated

proteins thus making the limiting amount of Ssa available for its function in translation. Sphingolipid synthesis may also play a role in the recovery process because eIF4G is depleted in *lcb1-100* cells similarly to the reduction found in *ypk*$^{ts}$ mutants and the Ypk kinases are part of a sphingoid base activated protein kinase cascade in yeast (Casamayor et al., 1999; Friant et al., 2001).

**Sphingolipids as a general signal for heat stress**

The results presented here and previously demonstrate that the synthesis of sphingoid bases act as a general signal for the cellular responses to heat stress. In order to survive a heat stress, cells need to change their transcriptional program and the newly transcribed messages need to be processed and translated efficiently in order to change the developmental program of the cell (Preiss et al., 2003). During this time, the cell cycle is arrested in order to give the cell enough time to make these changes and to rearrange its actin cytoskeleton (Delley and Hall, 1999). In cells lacking sphingolipid synthesis, ribosomal proteins were found to be less down regulated and translation initiation failed to take place efficiently. In addition, cells deficient for sphingolipid synthesis fail to arrest in the cell cycle and cannot reorganize their actin cytoskeleton (Friant et al., 2000). The trigger for these responses is probably the increase in *de novo* sphingolipid biosynthesis, which is extremely fast and most likely controlled at the first committed step, serine palmitoyltransferase. Most studies of the regulation of this enzyme indicate that its activity is controlled by the availability of its substrates, serine and palmitoyl-CoA (Merrill et al., 1988; Messmer et al., 1989). One of the first events that ensues during a heat shock is a reduction in the rate of protein synthesis, which logically would lead to an increase in available serine, and could provide an explanation for the reason why sphingoid bases have evolved to regulate the heat shock response. Serine palmitoyltransferase activity also increases upon heat shock in mammalian cells (Jenkins, 2003).

Sphingolipid synthesis therefore acts as a sensor for heat stress, coupling an essential metabolic process to a diverse set of cellular responses like transcription, translation, cell cycle progression, actin organization and endocytosis. To further understand the responses to stress in connection to sphingolipid synthesis is of particular interest for many fields in biology and medicine. Recently it was shown that dihydromotuporamine C (dhMotC), a compound in preclinical development that inhibits angiogenesis and metastasis targets the sphingolipid pathway (Baetz et al., 2004) and therapeutic radiation stimulates the synthesis of ceramide in tumors (Santana et al., 1996). Understanding the molecular functions of the sphingolipid synthesis intermediates would greatly facilitate our understanding of disease states and therapeutic methods.

## MATERIALS AND METHODS

### Plasmids and Yeast Strains

The yeast strains used in this study were **wild type** (RH3435, Mat**a** *his4 leu2 lys2 ura3 bar1*), ***lcb1-100*** (RH3809, Mat**a** *his4 lcb1-100 leu2 ura3 bar1*), ***lcb4,5*** (RH4952, Mat**a** *his3 leu2 lcb4::HIS3 lcb5::LEU2 pep4 bar1*), ***pkh$^{ts}$*** (RH5410, Mat**a** *ade1 his2 leu2 ura3 trp1 pkh1$^{ts}$ pkh2::LEU2*), ***lac1lag1$^{ts}$*** (RH4859, Matα *ade2 his3 leu2 trp1 ura3 can1 lag1::HIS3 lac1::ADE2* transformed with plag1-1$^{TS}$::TRP1), ***eap1Δ*** (RH6178, Mat**a** *his3 his4 leu2 trp1 ura3 lys2 eap1::TRP1*, this study), ***eap1Δlcb1-100*** (RH6174, Mat**a** *his3 ura3 eap1Δ lcb1-100,* this study), ***ypk$^{ts}$*** (YPT-40, Matα *ypk1-1ts:HIS3 ypk2::TRP1 ade2 his3 leu2 lys2 trp1 ura3,* kindly provided by J. Thorner*),* ***mex67-5*** *(*Mat**a** ade2 his3 leu2 trp1 ura3 mex67:HIS3 (pUN100-LEU2-mex67-5),* kindly provided by F. Stutz*)*

Previously described plasmids used in this study were: pZJHSE2-137 containing an HSE, HSE2 from *SSA1* promoter fused to *LacZ*, the pUKC414 vector containing the *HSP26* promoter fused to *LacZ* (all described in (Friant et al., 2003)), plag1-1$^{TS}$ (Schorling et al., 2001), pAdh1-Msn2-GFP (Gorner et al., 1998) and pGM18/17 carrying a *7xSTRE-LacZ* fusion for genomic integration (Marchler et al., 1993).

### Polysome Analysis

All sucrose gradient analyses were performed exactly according to methods described in (Foiani et al., 1991) except that sucrose gradients were 5 to 50% (w/v) and sedimentation was performed for either 2h45 min at 39 krpm or for 3h20min at 35 krpm. Ribosomal subunit quantification was done in low $Mg^{2+}$ gradients as described (Foiani et al., 1991). All gradients were analyzed using an ISCO UV-6 gradient collector and continuously monitored at $A_{254}$.

### Western Blotting

Whole-cell extracts were prepared from 2 $OD_{600}$ of cells grown in synthetic media at 24°C and shifted to 37°C for the indicated time as published (Kushnirov, 2000). For the analysis of eIF4G stability, equal amounts of protein from the different extracts were resolved by SDS-PAGE and subjected to Western blotting a polyclonal antibody against eIF4G (kindly provided by M. Altmann). The blots were stripped and re-probed with polyclonal antibodies against Has1p (Emery

et al., 2004). Detection of β-galactosidase fusion constructs was performed using a monoclonal anti β-galactosidase antibody (Sigma).

**Fluorescent *in situ* Hybridization**

Fluorescent *in situ* hybridization and analysis of total mRNA export was performed exactly as described in (Cole et al., 2002). Analysis of *HSP104* mRNA export was done using 10 ng of a mixture of oligos thj203 and thj205 modified with Cy3 following the protocol as described (Jensen et al., 2001). Samples were analyzed with a Zeiss Axioplan microscope using the appropriate filter sets.

**Reverse Transcription-PCR**

Total mRNA was extracted using the RNeasy Kit (Qiagen). 5 μg of RNA was then treated with the DNA-free™ kit (Ambion) to remove all traces of remaining DNA. Integrity of the isolated mRNA was assayed by agarose gel electrophoresis. 2 μg of mRNA were then analyzed using primes against *lacZ* mRNA (forward: 5'- CCCCGTTTACAGGGCGGCTTC, reverse: 5'- CCCCGTTTACAGGGCGGCTTC) and *ACT1* (forward: 5'- CGGTTCTGGTATGTGTAA AGC, reverse: 5'- GGTGAACGATAGATGGACCAC) in a one step reverse transcription reaction (Access RT-PCR System, Promega). The reaction products were then analyzed on 1.5% agarose gels and visualized.

**Translation Rate Assay**

Translation rate measurements were adapted from (Deloche et al., 2004; Uesono and Toh, 2002). In brief: $2x10^8$ cells at early log phase were collected and resuspended in 3ml of synthetic dextrose media supplemented with appropriate nutrients. 0.5 ml aliquots were shifted to 37°C and labeled using [$^{35}$S]methionine mix (Easy Tag EXPRESS-[$^{35}$S] mix from NEN) for five minutes up to the indicated time. Uptake and incorporation of [$^{35}$S]methionine was stopped by bringing the solution to 10 mM each $NaN_3$ and NaF. Cells were put on ice and split in half. One half was mixed with an equal amount of 20% ice cold TCA and incubated for one hour one ice. The other half was resuspended in 10 more volumes of ice cold double distilled water, filtered using GF/C filters and washed with additional ice cold double distilled water. TCA precipitates were filtered using GF/C

filters. The filtrate was washed using ice cold 10% TCA and twice using ice cold ethanol. All filters were dried and counted in a liquid scintillation counter using a Packard Scintillation Counter (Packard Instrument Company, USA)

**Liquid β-Galactosidase Assay**

A liquid β-galactosidase assay was performed as described previously with slight modifications (Miller, 1972). For each sample, time, $OD_{420}$ and protein concentration, using a Bradford assay kit (BioRad), was determined. The values reported are the average of at least three independent measurements.

**Msn2 GFP**

Cells expressing MSN2-green fluorescent protein (GFP) were grown to logarithmic phase at 24°C, shifted to 37°C for 10 min and fixed for 2 h in phosphate-buffered saline-formaldehyde (3.7% final concentration) and analyzed essentially as described (Schmelzle et al., 2004).

**Viability Assay**

Mid log-phase cultures 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 the percentage of viable cells relative to the initial colony-forming units before heat shock. The viability experiments were repeated twice, yielding similar results.

***UBI4* Suppression of the *lcb1-100* Mutation**

The RH3809 (*lcb1-100*) strain carrying a temperature-sensitive allele of the *LCB1* gene was transformed with Yeplac181-UBI. *UBI4* rescued growth at 37°C only in high copy number as previously reported (Friant et al., 2003).

**Heat Shock Protein Labeling**

Strains were grown exponentially in SD medium at 24°C, shifted for 10 min at 44°C to induce heat-shock protein synthesis and pulse labeled with [$^{35}$S]methionine (Easy Tag 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). Dihydrosphingosine (DHS) isomers (Matreya Inc.) at 10 μM final concentration were added before shift.e

**IF2α Phosphorylation**

Whole-cell extracts were prepared from 2 $OD_{600}$ of cells grown in synthetic media at 24°C and shifted to 37°C for the indicated time as published (Kushnirov, 2000). For the analysis of eIF2α phosphorylation equal amounts of protein from the different extracts were resolved by SDS-PAGE and subjected to Western blotting using monospecific antibodies for phosphorylated serine 51 in eIF2α (Research Genetics/RG0001). The blots were stripped and reprobed with polyclonal antibodies against total eIF2α (Cherkasova and Hinnebusch, 2003).

**Primer Extension**

Primer extension analysis was done as described (Beltrame and Tollervey, 1992) using anti U3 (5'-CCAAGTTGGATTCAGTGGCTC) and reverse transcriptase from Stratagene (La Jolla, USA).

**Northern Blotting**

Mid log phase cell cultures grown in synthetic media were shifted to 37°C and were harvested at various times after the shift. Total RNA was prepared and 10 μg from each sample was subjected to 1.5% agarose gel electrophoresis and transferred to Nytran membranes as described (Eng and Warner, 1991) or alternatively on 8% polyacrylamide gels containing 8M Urea and stained using ethidium bromide.. The membranes were hybridized with different $^{32}$P-labeled DNA probes specific for mRNA such as RPL28, RPL7, RPL3 (Miyoshi et al., 2002; Tsuno et al., 2000). $^{32}$P-labeled oligonucleotide probes for snoRNA U3 were described (Shirai et al., 2004).

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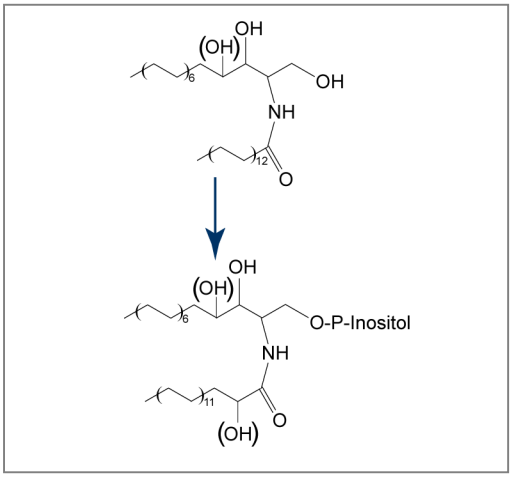
# CHAPTER IV



# Sphingolipid synthesis and heat stress:

# To maintain and change the balance

## ABSTRACT

Eukaryotic cells have the constant demand to respond to their changing environment. In order to do so, cells would have an advantage in coupling their response to a metabolic pathway that is altered during such a change. One of the most drastic changes in the environment is a rapid increase in temperature, called heat stress. Research over the past years, mainly in the budding yeast *Saccharomyces cerevisiae*, made clear that sphingolipid synthesis is such a pathway, that is regulated during heat stress and tightly coupled with a variety of functions that are important for a cell to survive a heat stress. During heat stress, *de novo* sphingolipid synthesis is upregulated which increases thermotolerance and mutants defective in the biosynthesis of sphingolipids show loss of viability when confronted with heat stress. Roles for sphingolipid synthesis in heat stress were found in the regulation of the transient cell cycle arrest, protein breakdown and in the control of signaling pathways that govern endocytosis and cell integrity. New data now connect sphingolipid synthesis with additional essential processes such as transcription and translation.

# DISCUSSION

## Sphingolipid synthesis and to keep the balance

The knowledge about the role of sphingolipid synthesis during heat stress expanded continuously during the past years. This knowledge arose mainly from work in the budding yeast *S. cerevisiae* in which essentially all of the important enzymes and their products in the sphingolipid synthesis pathway have been described. The finding that yeast and mammalian cells upregulate the synthesis of sphingolipids during heat stress led to the proposal that this special class of lipids could govern specific functions during heat stress (reviewed in Dickson and Lester, 2002; Jenkins, 2003). But sphingolipids do not simply need to be upregulated to make cells able to cope with heat, their amounts need to be regulated tightly. In unstressed cells, sphingolipid synthesis is in balance between synthesis and degradation. During heat stress, this balance is affected yielding primarily to a massive induction of sphingoid bases (Dickson et al., 1997; Ferguson-Yankey et al., 2002; Jenkins et al., 1997). The induction of sphingoid bases during heat stress is thought to be regulated at the first committed step, the serine palmitoyltransferase (SPT). In yeast and mammals, the serine palmitoyltransferase is encoded by to essential genes, *LCB1* and *LCB2*. In yeast, additionally Tcs3p interacts with the SPT and is required for optimal activity of the SPT at higher temperatures (Gable et al., 2000; Monaghan et al., 2002). How exactly the SPT gets activated by a raise in temperature remains unclear. Most studies however indicate that its activity is controlled by the availability of serine and palmitoyl-CoA (Merrill et al., 1988; Messmer et al., 1989) L.A. Cowart and Y.A. Hannun, personal communication).

The induction of the sphingoid bases (dihydrosphingosine (DHS) and phytosphingosine (PHS)) is the next crucial step. A lack in induction of the sphingoid bases during heat stress results in cell death, most drastically observed in the *lcb1-100* mutant which is defective in SPT activity upon heat stress (Friant et al., 2003; Jenkins and Hannun, 2001; Zanolari et al., 2000). The loss of viability in *lcb1-100* correlated with a lack of induction of the major heat shock proteins but could be suppressed by massive production of ubiquitin (Friant et al., 2003). This suggests that one of the major essential functions of sphingoid base synthesis is to control the expression of proteins involved in removal or refolding of denatured or aggregated proteins. Conversely, deletion of enzymes that lead to accumulation of DHS and PHS and their phosphates yielded strains that were hyper resistant to heat (Mandala et al., 1998; Mao et al., 1999; Skrzypek et al., 1999). An explanation for this phenomenon could be that sphingoid bases which accumulated before heat shock could be more rapidly made available for the translation of heat shock proteins, thereby

bypassing the limitation of serine at the first committed step (Meier et al., 2005). A massive increase in these metabolites however can also lead to a cell cycle arrest or death (Jenkins, 2003; Jenkins et al., 1997; Kim et al., 2000; Schorling et al., 2001; Zhang et al., 2001), indicating a requirement for readjusting the balance after heat stress. This readjustment, the down regulation of the sphingoid bases, is likely to be performed by Lcb4p and Lcb5p, which phosphorylate the sphingoid bases and make them accessible to the lyase Dpl1p (Saba et al., 1997). Another way for removal of the sphingoid bases as a signal would be their incorporation into ceramide. This is unlikely given that ceramides and sphingolipids themselves were assigned to have functions in signaling (see below). Furthermore, accumulation of ceramides and loss of ceramide synthesis lead to strong growth defects or cell death in yeast cells. Cells defective for ceramide synthesis additionally accumulate large levels of sphingoid bases (Guillas et al., 2001; Schorling et al., 2001; Vallee and Riezman, 2005). So far it is unclear if the lack of ceramides or the accumulation of sphingoid bases causes this phenotype. The same holds true for IPC synthesis. Cell treated with Aureobasidin A, an inhibitor of Aur1p dependent IPC synthesis, are also unable to grow. This is rather due to the accumulation of ceramide, or maybe sphingoid bases, than to the lack of IPC synthesis (Schorling et al., 2001).

However, to control the balance between these metabolites is crucial (Figure 4-1). This not at last because they turned out to be potent signaling molecules, regulating cellular processes like transcription, the actin cytoskeleton and cell integrity, cell cycle progression, exocytosis, endocytosis and translation (Dickson and Lester, 2002; Jenkins, 2003; Meier et al., 2005).

**Signaling via sphingoid bases**

In yeast, two genes, *PKH1* and *PKH2*, were found to be homologous to mammalian PDK1, the 3-phosphoinositide-dependent protein-kinase-1. PDK1 was shown to phosphorylate several other protein kinases including the serum and glucocorticoid-dependent kinase SGK (Kobayashi and Cohen, 1999), PKB (Chan et al., 1999) and PRK2 (Flynn et al., 2000). In agreement, the yeast PKH protein kinases were shown to phosphorylate three AGC family kinases: Ypk1/2p, the homologues of mammalian SGK (Casamayor et al., 1999), Pkc1p, the orthologue of mammalian PRK2 (Inagaki et al., 1999) and Sch9p, the orthologue of mammalian PKB (Fabrizio et al., 2001). The fact that yeast cells are unable to produce PI-3,4, 5-triphosphate and PI-3,4-bisphosphate and the lack of a PH domain that would bind to this lipids in *PKH1* and *PKH2* genes raised the question how this kinases get activated.

Initially it was observed that sphingosine can stimulate autophosphorylation of PDK1 (King et al., 2000) and later that sphingoid base and overexpression of the yeast PKH kinases can suppress the endocytic phenotype of *lcb1-100* cells (Friant et al., 2001; Friant et al., 2000; Zanolari et al., 2000). At about the same time it was described that overexpression of *YPK1* conferred resistance to the SPT inhibitor myriocin (Sun et al., 2000) and that sphingoid base could activate the Pkh1/2p dependent phosphorylation of Pkc1p *in vitro* (Friant et al., 2001). Also the YPK kinases were shown to be required for endocytosis (deHart et al., 2002). Further analysis demonstrated the involvement of the Pkhs, Ypks and the Pkc1p kinase in cytoskeleton organization and cell integrity (Friant et al., 2000; Schmelzle et al., 2002; Zanolari et al., 2000). This altogether lead to the establishment of a sphingoid base dependent signaling cascade (Figure 4-2). Unfortunately, the targets of the YPKs and the interplay between those kinases remain unknown (Schmelzle et al., 2002). Also the involvement of these pathways in translation initiation has not been entirely uncovered. Translation initiation was defective in *ypk*$^{ts}$ cells and lead to depletion of eIF4G after prolonged times at 37°C (Gelperin et al., 2002). Also *lcb1-100* showed defects in translation initiation and depletion of eIF4G after 4 hours at the restrictive temperature. Interestingly, heat shock protein induction was severely affected in *lcb1-100* cells (Friant et al., 2003; Meier et al., 2005) but not in *ypkt*$^{ts}$ cells (Meier et al., 2005). This, together with the fact that a *pkh*$^{ts}$ strain is only partially defective for translation initiation and heat shock protein induction indicates the existence of additional signaling modules downstream of sphingoid base.

Only recently, two new proteins have been identified as targets of the PKH kinases. Pil1p and Lsp1p. Both proteins were shown to negatively regulate the activity of the PKH kinases, affecting Ypk1/p and Pck1p kinases (Zhang et al., 2004). The exact role of these proteins however still needs to be determined. Since Pil1p and Lsp1p are highly abundant in yeast cells (R.C. Dickson, personal communication) they might serve as phosphoacceptors of Pkh1/2p and maybe other kinases and titrate away the activity from other downstream target.

Another interesting target of the PKH kinases is Sch9p. Sch9p was shown to be downstream of PKH signaling (Roelants et al., 2004) and to be phosphorylated by the PKH kinases *in vitro* (D. Mukhopadyay and H. Riezman, unpublished results). Deletion of Sch9p increased the chronologic life span of yeast cells and survival of extreme heat stress (Fabrizio et al., 2001; Roelants et al., 2004). How and if sphingoid bases regulate the activity of Sch9p remains to be determined.

Other cellular functions were also shown to be dependent upon sphingoid base synthesis although the connection to any signaling pathway is so far missing. One if these functions is the transient arrest in cell cycle, which naturally occurs in wild type cells during heat stress (Johnston and Singer, 1980; Shin et al., 1987) but not in *lcb1-100* cells. The relevant lipid for this was shown

to be sphingoid base (Jenkins et al., 1997). The importance to keep and turn the balance in sphingolipid synthesis is nicely illustrated in a series of experiments showing that *de novo* synthesis of sphingoid base is required to signal the transient cell cycle arrest. But deletion of *LCB4* and *LCB5* rendered cells unable to recover from heat stress induced cell cycle arrest (Jenkins and Hannun, 2001), indicating that phosphorylation of the sphingoid base is required to down regulate the sphingoid base mediated cell cycle arrest.

Another cellular function assigned to the synthesis of sphingoid base is transcription. *lcb1-100* cells showed specific defects in the induction or reduction of several genes during heat stress compared to wild type cells (Cowart et al., 2003). For instance heat stress leads to a rapid but temporary repression of ribosomal protein gene transcription (Eisen et al., 1998). In contrast to this, ribosomal protein mRNA levels failed to be down regulated during heat stress in *lcb1-100* cells (Cowart et al., 2003; Meier et al., 2005). It remains to be elucidated how sphingoid base synthesis regulates transcription during heat stress.

**Signaling via ceramides and complex sphingolipids**

Not only sphingoid bases, also ceramide and complex sphingolipids were involved in signaling events in yeast and mammalian cells. Ceramide mediates different cellular events, including apoptosis and stress response in mammalian cells (Hannun and Luberto, 2000; Obeid et al., 1993). In yeast, ceramides were implicated in aging because deletion (D'Mello N et al., 1994) or overxepression of *LAG1* (Jiang et al., 2004) affects longevity. In contrast, deletion or overxpression of *LAC1* had no effect (Jiang et al., 2004) on life span. So far it is not clear how *LAG1* affects longevity and other concrete roles for ceramides during heat stress and in signaling in yeast are still missing (Meier et al., 2005).

A complex sphingolipid that is likely to be involved in signaling is IPC. The IPC synthase members *CSG1* and *CSG2* were initially identified in a screen for $Ca^{2+}$ sensitive mutants (Beeler et al., 1994). $Ca^{2+}$ sensitivity of *csg2*Δ cells could be suppressed by several mutations that lead to a reduction in the level of IPC-C (Zhao et al., 1994). A further screen uncovered mutants in the TOR signaling pathway to suppress the $Ca^{2+}$ sensitivity in *csg2* mutants (Beeler et al., 1998), namely *tor2*, *mss4* and *avo3* (Loewith et al., 2002). If or how mutations in TOR signaling reduce the amount of IPC-C and the exact mechanism of how the accumulation of IPC-C renders cells sensitive to $Ca^{2+}$ remains to be revealed.

## Outlook

In wild type cells, flux through the sphingolipid synthesis pathway is in balance. A change in one step of this pathway can lead to a change in the amount of many individual metabolites. Since these metabolites have individual signaling functions it is obvious that a small change in this balance can elicit a variety of signals, with similar and opposing effects on the cell physiology. Of special importance will be to measure the overall lipid composition within a cell or a tissue at any state. This so called "Lipidomics" (Hannun and Obeid, 2002) will help to achieve a more detailed view of the role of sphingolipid metabolites and to understand many fundamental cellular processes.

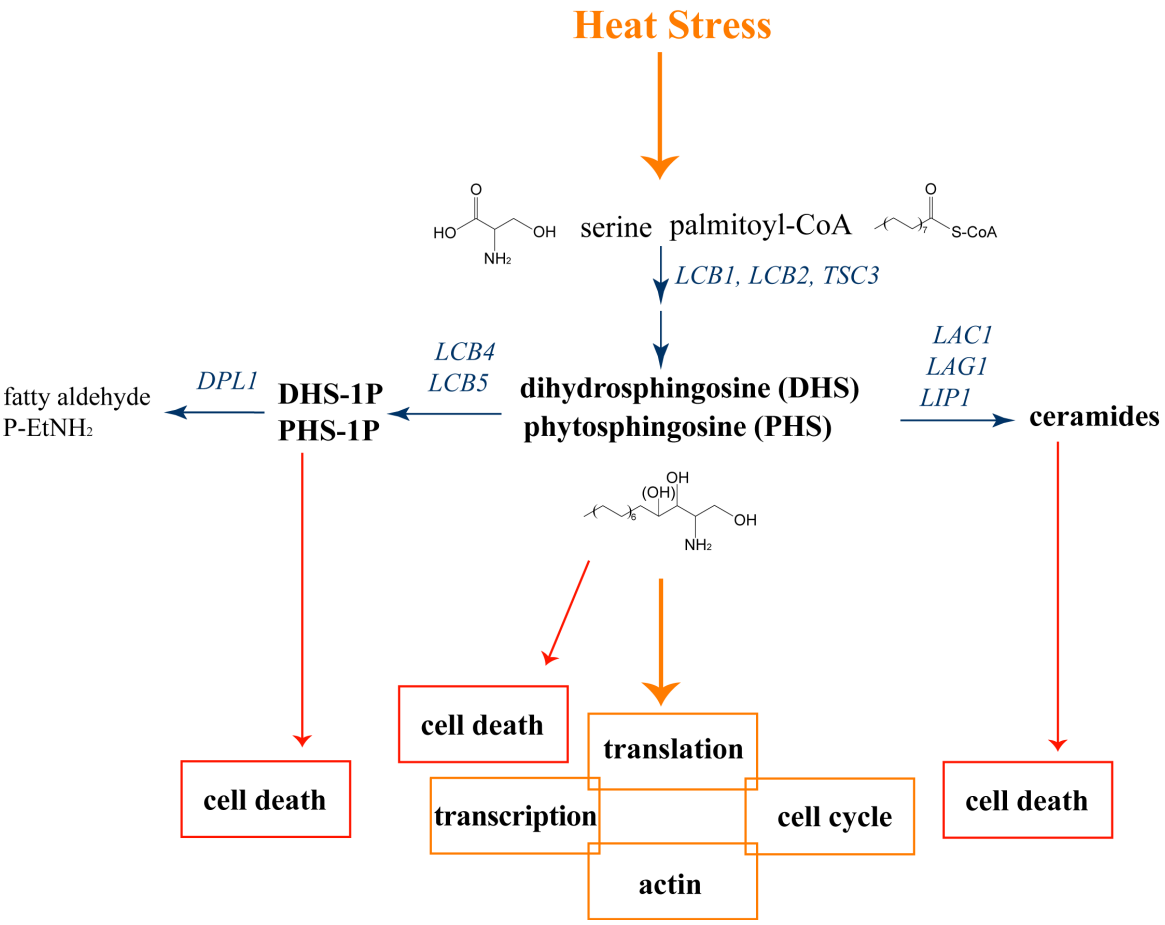



**Figure 4-1.** Schematic overview of the cellular readouts governed by sphingolipid synthesis during heat stress. Sphingolipid synthesis is activated by heat stress, leading to an increase in DHS, PHS, their phosphates and well ceramide. Balancing the amount of those molecules is crucial for appropriate response to heat stress. P-EtNH$_2$ , Phosphatidyl-ethanolamine.

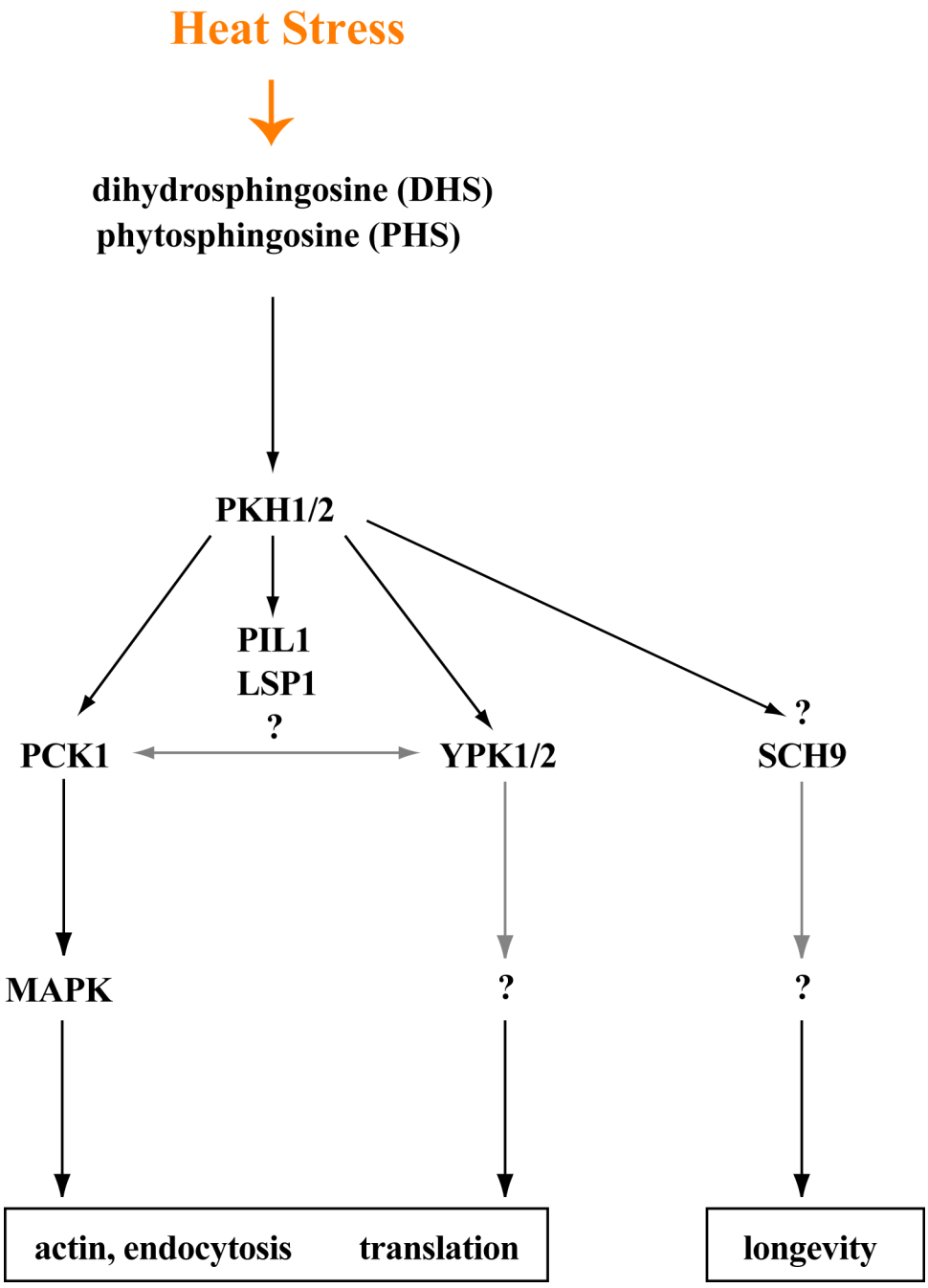



**Figure 4-2**. Schematic overview of the sphingoid base activated signalling cascade. Heat stress leads to an increase in sphingoid base synthesis. The two sphingoid bases dihydrosphingosine (DHS) and phytosphingosine (PHS) activate the PKH1/2 kinase, leading to the phosphorylation of several downstream targets.

# BIBLIOGRAPHY

Altmann, M., Schmitz, N., Berset, C. and Trachsel, H. (1997) A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *Embo J*, **16**, 1114-1121.

Amin, J., Ananthan, J. and Voellmy, R. (1988) Key features of heat shock regulatory elements. *Mol Cell Biol*, **8**, 3761-3769.

Amoros, M. and Estruch, F. (2001) Hsf1p and Msn2/4p cooperate in the expression of Saccharomyces cerevisiae genes HSP26 and HSP104 in a gene- and stress type-dependent manner. *Mol Microbiol*, **39**, 1523-1532.

Arnason, T. and Ellison, M.J. (1994) Stress resistance in Saccharomyces cerevisiae is strongly correlated with assembly of a novel type of multiubiquitin chain. *Mol Cell Biol*, **14**, 7876-7883.

Attfield, P.V. (1987) Trehalose accumulates in Saccharomyces cerevisiae during exposure to agents that induce heat shock response. *FEBS Lett*, **225**, 259-263.

Baetz, K., McHardy, L., Gable, K., Tarling, T., Reberioux, D., Bryan, J., Andersen, R.J., Dunn, T., Hieter, P. and Roberge, M. (2004) Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. *Proc Natl Acad Sci U S A*, **101**, 4525-4530.

Bagnat, M., Keranen, S., Shevchenko, A. and Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc Natl Acad Sci U S A*, **97**, 3254-3259.

Barz, W.P. and Walter, P. (1999) Two endoplasmic reticulum (ER) membrane proteins that facilitate ER-to-Golgi transport of glycosylphosphatidylinositol-anchored proteins. *Mol Biol Cell*, **10**, 1043-1059.

Beck, T. and Hall, M.N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature*, **402**, 689-692.

Becker, G.W. and Lester, R.L. (1980) Biosynthesis of phosphoinositol-containing sphingolipids from phosphatidylinositol by a membrane preparation from Saccharomyces cerevisiae. *J Bacteriol*, **142**, 747-754.

Beeler, T., Bacikova, D., Gable, K., Hopkins, L., Johnson, C., Slife, H. and Dunn, T. (1998) The Saccharomyces cerevisiae TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Delta mutant. *J Biol Chem*, **273**, 30688-30694.

Beeler, T., Gable, K., Zhao, C. and Dunn, T. (1994) A novel protein, CSG2p, is required for Ca2+ regulation in Saccharomyces cerevisiae. *J Biol Chem*, **269**, 7279-7284.

Beeler, T.J., Fu, D., Rivera, J., Monaghan, E., Gable, K. and Dunn, T.M. (1997) SUR1 (CSG1/BCL21), a gene necessary for growth of Saccharomyces cerevisiae in the presence of high Ca2+ concentrations at 37 degrees C, is required for mannosylation of inositolphosphorylceramide. *Mol Gen Genet*, **255**, 570-579.

Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A. and Thevelein, J.M. (1998) Composition and functional analysis of the Saccharomyces cerevisiae trehalose synthase complex. *J Biol Chem*, **273**, 33311-33319.

Beltrame, M. and Tollervey, D. (1992) Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. *Embo J*, **11**, 1531-1542.

Berset, C., Trachsel, H. and Altmann, M. (1998) The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **95**, 4264-4269.

Boy-Marcotte, E., Lagniel, G., Perrot, M., Bussereau, F., Boudsocq, A., Jacquet, M. and Labarre, J. (1999) The heat shock response in yeast: differential regulations and contributions of the Msn2p/Msn4p and Hsf1p regulons. *Mol Microbiol*, **33**, 274-283.

Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C.M. and Stefani, M. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*, **416**, 507-511.

Buede, R., Rinker-Schaffer, C., Pinto, W.J., Lester, R.L. and Dickson, R.C. (1991) Cloning and characterization of LCB1, a Saccharomyces gene required for biosynthesis of the long-chain base component of sphingolipids. *J Bacteriol*, **173**, 4325-4332.

Burdon, R.H. (1987) Temperature and animal cell protein synthesis. *Symp Soc Exp Biol*, **41**, 113-133.

Casamayor, A., Torrance, P.D., Kobayashi, T., Thorner, J. and Alessi, D.R. (1999) Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol*, **9**, 186-197.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A. (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell*, **12**, 323-337.

Chan, T.O., Rittenhouse, S.E. and Tsichlis, P.N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem*, **68**, 965-1014.

Chang, Y., Abe, A. and Shayman, J.A. (1995) Ceramide formation during heat shock: a potential mediator of alpha B-crystallin transcription. *Proc Natl Acad Sci U S A*, **92**, 12275-12279.

Cherkasova, V.A. and Hinnebusch, A.G. (2003) Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev*, **17**, 859-872.

Chial, H.J., Stemm-Wolf, A.J., McBratney, S. and Winey, M. (2000) Yeast Eap1p, an eIF4E-associated protein, has a separate function involving genetic stability. *Curr Biol*, **10**, 1519-1522.

Chung, N., Jenkins, G., Hannun, Y.A., Heitman, J. and Obeid, L.M. (2000) Sphingolipids signal heat stress-induced ubiquitin-dependent proteolysis. *J Biol Chem*, **275**, 17229-17232.

Chung, N., Mao, C., Heitman, J., Hannun, Y.A. and Obeid, L.M. (2001) Phytosphingosine as a specific inhibitor of growth and nutrient import in Saccharomyces cerevisiae. *J Biol Chem*, **276**, 35614-35621.

Ciechanover, A. (1994) The ubiquitin-proteasome proteolytic pathway. *Cell*, **79**, 13-21.

Ciechanover, A., Orian, A. and Schwartz, A.L. (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays*, **22**, 442-451.

Cliften, P., Wang, Y., Mochizuki, D., Miyakawa, T., Wangspa, R., Hughes, J. and Takemoto, J.Y. (1996) SYR2, a gene necessary for syringomycin growth inhibition of Saccharomyces cerevisiae. *Microbiology*, **142 ( Pt 3)**, 477-484.

Cole, C.N., Heath, C.V., Hodge, C.A., Hammell, C.M. and Amberg, D.C. (2002) Analysis of RNA export. *Methods Enzymol*, **351**, 568-587.

Cosentino, G.P., Schmelzle, T., Haghighat, A., Helliwell, S.B., Hall, M.N. and Sonenberg, N. (2000) Eap1p, a novel eukaryotic translation initiation factor 4E-associated protein in Saccharomyces cerevisiae. *Mol Cell Biol*, **20**, 4604-4613.

Cowart, L.A., Okamoto, Y., Pinto, F.R., Gandy, J.L., Almeida, J.S. and Hannun, Y.A. (2003) Roles for sphingolipid biosynthesis in mediation of specific programs of the heat stress response determined through gene expression profiling. *J Biol Chem*, **278**, 30328-30338.

Crespo, J.L. and Hall, M.N. (2002) Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*, **66**, 579-591, table of contents.

Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1992) Anhydrobiosis. *Annu Rev Physiol*, **54**, 579-599.

Daum, G., Lees N.D. , Bard M. and Dickson R. (1998) Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. *Yeast*. **14**:1471-1510.

de la Cruz, J., Iost, I., Kressler, D. and Linder, P. (1997) The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **94**, 5201-5206.

De Virgilio, C., Burckert, N., Bell, W., Jeno, P., Boller, T. and Wiemken, A. (1993) Disruption of TPS2, the gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in Saccharomyces cerevisiae, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. *Eur J Biochem*, **212**, 315-323.

Dean, N., Zhang, Y.B. and Poster, J.B. (1997) The VRG4 gene is required for GDP-mannose transport into the lumen of the Golgi in the yeast, Saccharomyces cerevisiae. *J Biol Chem*, **272**, 31908-31914.

deHart, A.K., Schnell, J.D., Allen, D.A. and Hicke, L. (2002) The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. *J Cell Biol*, **156**, 241-248.

Delley, P.A. and Hall, M.N. (1999) Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J Cell Biol*, **147**, 163-174.

Deloche, O., de la Cruz, J., Kressler, D., Doere, M. and Linder, P. (2004) A membrane transport defect leads to a rapid attenuation of translation initiation in Saccharomyces cerevisiae. *Mol Cell*, **13**, 357-366.

Dever, T.E. (2002) Gene-specific regulation by general translation factors. *Cell*, **108**, 545-556.

Dickson, R.C. and Lester, R.L. (2002) Sphingolipid functions in Saccharomyces cerevisiae. *Biochim Biophys Acta*, **1583**, 13-25.

Dickson, R.C., Nagiec, E.E., Skrzypek, M., Tillman, P., Wells, G.B. and Lester, R.L. (1997a) Sphingolipids are potential heat stress signals in Saccharomyces. *J Biol Chem*, **272**, 30196-30200.

Dickson, R.C., Nagiec, E.E., Wells, G.B., Nagiec, M.M. and Lester, R.L. (1997b) Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in Saccharomyces cerevisiae, requires the IPT1 (YDR072c) gene. *J Biol Chem*, **272**, 29620-29625.

Dickson, R.C., Wells, G.B., Schmidt, A. and Lester, R.L. (1990) Isolation of mutant Saccharomyces cerevisiae strains that survive without sphingolipids. *Mol Cell Biol*, **10**, 2176-2181.

D'Mello N, P., Childress, A.M., Franklin, D.S., Kale, S.P., Pinswasdi, C. and Jazwinski, S.M. (1994) Cloning and characterization of LAG1, a longevity-assurance gene in yeast. *J Biol Chem*, **269**, 15451-15459.

Duncan, R.F. and Hershey, J.W. (1989) Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. *J Cell Biol*, **109**, 1467-1481.

Dunn, T.M., Haak, D., Monaghan, E. and Beeler, T.J. (1998) Synthesis of monohydroxylated inositolphosphorylceramide (IPC-C) in Saccharomyces cerevisiae requires Scs7p, a protein with both a cytochrome b5-like domain and a hydroxylase/desaturase domain. *Yeast*, **14**, 311-321.

Dupre, S. and Haguenauer-Tsapis, R. (2003) Raft partitioning of the yeast uracil permease during trafficking along the endocytic pathway. *Traffic*, **4**, 83-96.

Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*, **95**, 14863-14868.

Emery, B., de la Cruz, J., Rocak, S., Deloche, O. and Linder, P. (2004) Has1p, a member of the DEAD-box family, is required for 40S ribosomal subunit biogenesis in Saccharomyces cerevisiae. *Mol Microbiol*, **52**, 141-158.

Emter, R., Heese-Peck, A. and Kralli, A. (2002) ERG6 and PDR5 regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms. *FEBS Lett*, **521**, 57-61.

Eng, F.J. and Warner, J.R. (1991) Structural basis for the regulation of splicing of a yeast messenger RNA. *Cell*, **65**, 797-804.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M. and Longo, V.D. (2001) Regulation of longevity and stress resistance by Sch9 in yeast. *Science*, **292**, 288-290.

Ferguson-Yankey, R., Skrzypek, M., Lester, R. and Dickson, R. (2002) Mutant analysis reveals complex regulation of sphingolipid long chain phosphates and long chain bases during heat stress in yeast. *yeast*, **19**, 573-586.

Finley, D., Ozkaynak, E. and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell*, **48**, 1035-1046.

Flynn, P., Mellor, H., Casamassima, A. and Parker, P.J. (2000) Rho GTPase control of protein kinase C-related protein kinase activation by 3-phosphoinositide-dependent protein kinase. *J Biol Chem*, **275**, 11064-11070.

Foiani, M., Cigan, A.M., Paddon, C.J., Harashima, S. and Hinnebusch, A.G. (1991) GCD2, a translational repressor of the GCN4 gene, has a general function in the initiation of protein synthesis in Saccharomyces cerevisiae. *Mol Cell Biol*, **11**, 3203-3216.

Fraser, J., Luu, H.A., Neculcea, J., Thomas, D.Y. and Storms, R.K. (1991) Ubiquitin gene expression: response to environmental changes. *Curr Genet*, **20**, 17-23.

Friant, S., Lombardi, R., Schmelzle, T., Hall, M.N. and Riezman, H. (2001) Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *Embo J*, **20**, 6783-6792.

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.

Friant, S., Zanolari, B. and Riezman, H. (2000) Increased protein kinase or decreased PP2A activity bypasses sphingoid base requirement in endocytosis. *Embo J*, **19**, 2834-2844.

Funato, K., Lombardi, R., Vallee, B. and Riezman, H. (2003) Lcb4p is a key regulator of ceramide synthesis from exogenous long chain sphingoid base in Saccharomyces cerevisiae. *J Biol Chem*, **278**, 7325-7334.

Funato, K. and Riezman, H. (2001) Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast. *J Cell Biol*, **155**, 949-959.

Funato, K., Vallee, B. and Riezman, H. (2002) Biosynthesis and trafficking of sphingolipids in the yeast Saccharomyces cerevisiae. *Biochemistry*, **41**, 15105-15114.

Futerman, A.H. and Hannun, Y.A. (2004) The complex life of simple sphingolipids. *EMBO Rep*, **5**, 777-782.

Gable, K., Slife, H., Bacikova, D., Monaghan, E. and Dunn, T.M. (2000) Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. *J Biol Chem*, **275**, 7597-7603.

Galan, J.M. and Haguenauer-Tsapis, R. (1997) Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J*, **16**, 5847-5854.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell*, **11**, 4241-4257.

Gelperin, D., Horton, L., DeChant, A., Hensold, J. and Lemmon, S.K. (2002) Loss of ypk1 function causes rapamycin sensitivity, inhibition of translation initiation and synthetic lethality in 14-3-3-deficient yeast. *Genetics*, **161**, 1453-1464.

Gingras, A.C., Raught, B. and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem*, **68**, 913-963.

Glover, J.R. and Lindquist, S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell*, **94**, 73-82.

Gorenstein, C. and Warner, J.R. (1976) Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. *Proc Natl Acad Sci U S A*, **73**, 1547-1551.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schuller, C. (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev*, **12**, 586-597.

Gorner, W., Durchschlag, E., Wolf, J., Brown, E.L., Ammerer, G., Ruis, H. and Schuller, C. (2002) Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *Embo J*, **21**, 135-144.

Grilley, M.M., Stock, S.D., Dickson, R.C., Lester, R.L. and Takemoto, J.Y. (1998) Syringomycin action gene SYR2 is essential for sphingolipid 4-hydroxylation in Saccharomyces cerevisiae. *J Biol Chem*, **273**, 11062-11068.

Grilley, M.M. and Takemoto, J.Y. (2000) Assay of the Saccharomyces cerevisiae dihydrosphingosine C-4 hydroxylase. *Methods Enzymol*, **311**, 9-14.

Grote, E., Vlacich, G., Pypaert, M. and Novick, P.J. (2000) A snc1 endocytosis mutant: phenotypic analysis and suppression by overproduction of dihydrosphingosine phosphate lyase. *Mol Biol Cell*, **11**, 4051-4065.

Guarente, L. (1983) Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol*, **101**, 181-191.

Guillas, I., Kirchman, P.A., Chuard, R., Pfefferli, M., Jiang, J.C., Jazwinski, S.M. and Conzelmann, A. (2001) C26-CoA-dependent ceramide synthesis of Saccharomyces cerevisiae is operated by Lag1p and Lac1p. *Embo J*, **20**, 2655-2665.

Haak, D., Gable, K., Beeler, T. and Dunn, T. (1997) Hydroxylation of Saccharomyces cerevisiae ceramides requires Sur2p and Scs7p. *J Biol Chem*, **272**, 29704-29710.

Hahn, J.S., Hu, Z., Thiele, D.J. and Iyer, V.R. (2004) Genome-wide analysis of the biology of stress responses through heat shock transcription factor. *Mol Cell Biol*, **24**, 5249-5256.

Han, G., Gable, K., Yan, L., Natarajan, M., Krishnamurthy, J., Gupta, S.D., Borovitskaya, A., Harmon, J.M. and Dunn, T.M. (2004) The topology of the lcb1p subunit of yeast serine palmitoyltransferase. *J Biol Chem*, **279**, 53707-53716.

Hanada, K. (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta*, **1632**, 16-30.

Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M. and Nishijima, M. (2003) Molecular machinery for non-vesicular trafficking of ceramide. *Nature*, **426**, 803-809.

Hannun, Y.A. and Luberto, C. (2000) Ceramide in the eukaryotic stress response. *Trends Cell Biol*, **10**, 73-80.

Hannun, Y.A. and Luberto, C. (2004) Lipid metabolism: ceramide transfer protein adds a new dimension. *Curr Biol*, **14**, R163-165.

Hannun, Y.A. and Obeid, L.M. (2002) The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem*, **277**, 25847-25850.

Harder, T., Scheiffele, P., Verkade, P. and Simons, K. (1998) Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J Cell Biol*, **141**, 929-942.

Hechtberger, P. and Daum, G. (1995) Intracellular transport of inositol-containing sphingolipids in the yeast, Saccharomyces cerevisiae. *FEBS Lett*, **367**, 201-204.

Hechtberger, P., Zinser, E., Saf, R., Hummel, K., Paltauf, F. and Daum, G. (1994) Characterization, quantification and subcellular localization of inositol-containing sphingolipids of the yeast, Saccharomyces cerevisiae. *Eur J Biochem*, **225**, 641-649.

Heerklotz, H. (2002) Triton promotes domain formation in lipid raft mixtures. *Biophys J*, **83**, 2693-2701.

Heinemeyer, W., Kleinschmidt, J.A., Saidowsky, J., Escher, C. and Wolf, D.H. (1991) Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. *Embo J*, **10**, 555-562.

Hendrick, J.P. and Hartl, F.U. (1993) Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem*, **62**, 349-384.

Hinnebusch, A.G. (2000) Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. In Sonenberg, N., Hershey, J.W.B. and Mathews (ed.), *Translational Control of Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 185-244.

Hjorth-Sorensen, B., Hoffmann, E.R., Lissin, N.M., Sewell, A.K. and Jakobsen, B.K. (2001) Activation of heat shock transcription factor in yeast is not influenced by the levels of expression of heat shock proteins. *Mol Microbiol*, **39**, 914-923.

Hla, T., Lee, M.J., Ancellin, N., Paik, J.H. and Kluk, M.J. (2001) Lysophospholipids--receptor revelations. *Science*, **294**, 1875-1878.

Hochstrasser, M. (1996) Ubiquitin-dependent protein degradation. *Annu Rev Genet*, **30**, 405-439.

Hochstrasser, M., Ellison, M.J., Chau, V. and Varshavsky, A. (1991) The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. *Proc Natl Acad Sci U S A*, **88**, 4606-4610.

Hohfeld, J., Cyr, D.M. and Patterson, C. (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep*, **2**, 885-890.

Hoj, A. and Jakobsen, B.K. (1994) A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. *Embo J*, **13**, 2617-2624.

Horton, L.E., James, P., Craig, E.A. and Hensold, J.O. (2001) The yeast hsp70 homologue Ssa is required for translation and interacts with Sis1 and Pab1 on translating ribosomes. *J Biol Chem*, **276**, 14426-14433.

Horvath, A. and Riezman, H. (1994) Rapid protein extraction from Saccharomyces cerevisiae. *Yeast*, **10**, 1305-1310.

Horvath, A., Sutterlin, C., Manning-Krieg, U., Movva, N.R. and Riezman, H. (1994) Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. *Embo J*, **13**, 3687-3695.

Hottiger, T., De Virgilio, C., Bell, W., Boller, T. and Wiemken, A. (1992) The 70-kilodalton heat-shock proteins of the SSA subfamily negatively modulate heat-shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast, Saccharomyces cerevisiae. *Eur J Biochem*, **210**, 125-132.

Hurt, E., Strasser, K., Segref, A., Bailer, S., Schlaich, N., Presutti, C., Tollervey, D. and Jansen, R. (2000) Mex67p mediates nuclear export of a variety of RNA polymerase II transcripts. *J Biol Chem*, **275**, 8361-8368.

Imai, J., Yashiroda, H., Maruya, M., Yahara, I. and Tanaka, K. (2003) Proteasomes and molecular chaperones: cellular machinery responsible for folding and destruction of unfolded proteins. *Cell Cycle*, **2**, 585-590.

Inagaki, M., Schmelzle, T., Yamaguchi, K., Irie, K., Hall, M.N. and Matsumoto, K. (1999) PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. *Mol Cell Biol*, **19**, 8344-8352.

Irie, K., Takase, M., Lee, K.S., Levin, D.E., Araki, H., Matsumoto, K. and Oshima, Y. (1993) MKK1 and MKK2, which encode Saccharomyces cerevisiae mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. *Mol Cell Biol*, **13**, 3076-3083.

Jacquet, M., Renault, G., Lallet, S., De Mey, J. and Goldbeter, A. (2003) Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in Saccharomyces cerevisiae. *J Cell Biol*, **161**, 497-505.

Jakobsen, B.K. and Pelham, H.R. (1988) Constitutive binding of yeast heat shock factor to DNA in vivo. *Mol Cell Biol*, **8**, 5040-5042.

Jenkins, G.M. (2003) The emerging role for sphingolipids in the eukaryotic heat shock response. *Cell Mol Life Sci*, **60**, 701-710.

Jenkins, G.M., Cowart, L.A., Signorelli, P., Pettus, B.J., Chalfant, C.E. and Hannun, Y.A. (2002) Acute activation of de novo sphingolipid biosynthesis upon heat shock causes an accumulation of ceramide and subsequent dephosphorylation of SR proteins. *J Biol Chem*, **277**, 42572-42578.

Jenkins, G.M. and Hannun, Y.A. (2001) Role for de novo sphingoid base biosynthesis in the heat-induced transient cell cycle arrest of Saccharomyces cerevisiae. *J Biol Chem*, **276**, 8574-8581.

Jenkins, G.M., Richards, A., Wahl, T., Mao, C., Obeid, L. and Hannun, Y. (1997) Involvement of yeast sphingolipids in the heat stress response of Saccharomyces cerevisiae. *J Biol Chem*, **272**, 32566-32572.

Jensen, T.H., Patricio, K., McCarthy, T. and Rosbash, M. (2001) A block to mRNA nuclear export in S. cerevisiae leads to hyperadenylation of transcripts that accumulate at the site of transcription. *Mol Cell*, **7**, 887-898.

Jiang, J.C., Kirchman, P.A., Allen, M. and Jazwinski, S.M. (2004) Suppressor analysis points to the subtle role of the LAG1 ceramide synthase gene in determining yeast longevity. *Exp Gerontol*, **39**, 999-1009.

Johnston, G.C. and Singer, R.A. (1980) Ribosomal precursor RNA metabolism and cell division in the yeast Saccharomyces cerevisiae. *Mol Gen Genet*, **178**, 357-360.

Jungmann, J., Reins, H.A., Schobert, C. and Jentsch, S. (1993) Resistance to cadmium mediated by ubiquitin-dependent proteolysis. *Nature*, **361**, 369-371.

Kamada, Y., Jung, U.S., Piotrowski, J. and Levin, D.E. (1995) The protein kinase C-activated MAP kinase pathway of Saccharomyces cerevisiae mediates a novel aspect of the heat shock response. *Genes Dev*, **9**, 1559-1571.

Kapp, L.D. and Lorsch, J.R. (2004) The molecular mechanics of eukaryotic translation. *Annu Rev Biochem*, **73**, 657-704.

Katzmann, D.J., Epping, E.A. and Moye-Rowley, W.S. (1999) Mutational disruption of plasma membrane trafficking of Saccharomyces cerevisiae Yor1p, a homologue of mammalian multidrug resistance protein. *Mol Cell Biol*, **19**, 2998-3009.

Kihara, A. and Igarashi, Y. (2002) Identification and characterization of a Saccharomyces cerevisiae gene, RSB1, involved in sphingoid long-chain base release. *J Biol Chem*, **277**, 30048-30054.

Kihara, A. and Igarashi, Y. (2004) Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry. *Mol Biol Cell*, **15**, 4949-4959.

Kim, C.H. and Warner, J.R. (1983) Mild temperature shock alters the transcription of a discrete class of Saccharomyces cerevisiae genes. *Mol Cell Biol*, **3**, 457-465.

Kim, S., Fyrst, H. and Saba, J. (2000) Accumulation of phosphorylated sphingoid long chain bases results in cell growth inhibition in Saccharomyces cerevisiae. *Genetics*, **156**, 1519-1529.

King, C.C., Zenke, F.T., Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A. and Bokoch, G.M. (2000) Sphingosine is a novel activator of 3-phosphoinositide-dependent kinase 1. *J Biol Chem*, **275**, 18108-18113.

Kobayashi, N. and McEntee, K. (1990) Evidence for a heat shock transcription factor-independent mechanism for heat shock induction of transcription in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **87**, 6550-6554.

Kobayashi, N. and McEntee, K. (1993) Identification of cis and trans components of a novel heat shock stress regulatory pathway in Saccharomyces cerevisiae. *Mol Cell Biol*, **13**, 248-256.

Kobayashi, T. and Cohen, P. (1999) Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J*, **339 ( Pt 2)**, 319-328.

Kohlwein, S.D., Eder, S., Oh, C.S., Martin, C.E., Gable, K., Bacikova, D. and Dunn, T. (2001) Tsc13p is required for fatty acid elongation and localizes to a novel structure at the nuclear-vacuolar interface in Saccharomyces cerevisiae. *Mol Cell Biol*, **21**, 109-125.

Kondo, T., Matsuda, T., Kitano, T., Takahashi, A., Tashima, M., Ishikura, H., Umehara, H., Domae, N., Uchiyama, T. and Okazaki, T. (2000a) Role of c-jun expression increased by heat shock- and ceramide-activated caspase-3 in HL-60 cell apoptosis. Possible involvement of ceramide in heat shock-induced apoptosis. *J Biol Chem*, **275**, 7668-7676.

Kondo, T., Matsuda, T., Tashima, M., Umehara, H., Domae, N., Yokoyama, K., Uchiyama, T. and Okazaki, T. (2000b) Suppression of heat shock protein-70 by ceramide in heat shock-induced HL-60 cell apoptosis. *J Biol Chem*, **275**, 8872-8879.

Kushnirov, V.V. (2000) Rapid and reliable protein extraction from yeast. *Yeast*, **16**, 857-860.

Lanterman, M.M. and Saba, J.D. (1998) Characterization of sphingosine kinase (SK) activity in Saccharomyces cerevisiae and isolation of SK-deficient mutants. *Biochem J*, **332 ( Pt 2)**, 525-531.

Lashkari, D.A., DeRisi, J.L., McCusker, J.H., Namath, A.F., Gentile, C., Hwang, S.Y., Brown, P.O. and Davis, R.W. (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proc Natl Acad Sci U S A*, **94**, 13057-13062.

Lee, D.H. and Goldberg, A.L. (1998) Proteasome inhibitors cause induction of heat shock proteins and trehalose, which together confer thermotolerance in Saccharomyces cerevisiae. *Mol Cell Biol*, **18**, 30-38.

Lee, D.H., Sherman, M.Y. and Goldberg, A.L. (1996) Involvement of the molecular chaperone Ydj1 in the ubiquitin-dependent degradation of short-lived and abnormal proteins in Saccharomyces cerevisiae. *Mol Cell Biol*, **16**, 4773-4781.

Lee, K.S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K. and Levin, D.E. (1993) A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. *Mol Cell Biol*, **13**, 3067-3075.

Lee, K.S. and Levin, D.E. (1992) Dominant mutations in a gene encoding a putative protein kinase (BCK1) bypass the requirement for a Saccharomyces cerevisiae protein kinase C homolog. *Mol Cell Biol*, **12**, 172-182.

Lee, T.I., Rinaldi, N.J., Robert, F., Odom, D.T., Bar-Joseph, Z., Gerber, G.K., Hannett, N.M., Harbison, C.T., Thompson, C.M., Simon, I., Zeitlinger, J., Jennings, E.G., Murray, H.L., Gordon, D.B., Ren, B., Wyrick, J.J., Tagne, J.B., Volkert, T.L., Fraenkel, E., Gifford, D.K. and Young, R.A. (2002) Transcriptional regulatory networks in Saccharomyces cerevisiae. *Science*, **298**, 799-804.

Leonard, A.E., Pereira, S.L., Sprecher, H. and Huang, Y.S. (2004) Elongation of long-chain fatty acids. *Prog Lipid Res*, **43**, 36-54.

Levine, T.P., Wiggins, C.A. and Munro, S. (2000) Inositol phosphorylceramide synthase is located in the Golgi apparatus of Saccharomyces cerevisiae. *Mol Biol Cell*, **11**, 2267-2281.

Li, B., Nierras, C.R. and Warner, J.R. (1999) Transcriptional elements involved in the repression of ribosomal protein synthesis. *Mol Cell Biol*, **19**, 5393-5404.

Lindquist, S. (1981) Regulation of protein synthesis during heat shock. *Nature*, **293**, 311-314.

Lindquist, S. and Kim, G. (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc Natl Acad Sci U S A*, **93**, 5301-5306.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M.N. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*, **10**, 457-468.

London, E. and Brown, D.A. (2000) Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim Biophys Acta*, **1508**, 182-195.

Malinska, K., Malinsky, J., Opekarova, M. and Tanner, W. (2003) Visualization of protein compartmentation within the plasma membrane of living yeast cells. *Mol Biol Cell*, **14**, 4427-4436.

Mandala, S.M., Thornton, R., Tu, Z., Kurtz, M.B., Nickels, J., Broach, J., Menzeleev, R. and Spiegel, S. (1998) Sphingoid base 1-phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. *Proc Natl Acad Sci U S A*, **95**, 150-155.

Mao, C. and Obeid, L.M. (2000) Yeast sphingosine-1-phosphate phosphatases: assay, expression, deletion, purification, and cellular localization by GFP tagging. *Methods Enzymol*, **311**, 223-232.

Mao, C., Saba, J.D. and Obeid, L.M. (1999) The dihydrosphingosine-1-phosphate phosphatases of Saccharomyces cerevisiae are important regulators of cell proliferation and heat stress responses. *Biochem J*, **342 Pt 3**, 667-675.

Mao, C., Wadleigh, M., Jenkins, G.M., Hannun, Y.A. and Obeid, L.M. (1997) Identification and characterization of Saccharomyces cerevisiae dihydrosphingosine-1-phosphate phosphatase. *J Biol Chem*, **272**, 28690-28694.

Mao, C., Xu, R., Bielawska, A. and Obeid, L.M. (2000a) Cloning of an alkaline ceramidase from Saccharomyces cerevisiae. An enzyme with reverse (CoA-independent) ceramide synthase activity. *J Biol Chem*, **275**, 6876-6884.

Mao, C., Xu, R., Bielawska, A., Szulc, Z.M. and Obeid, L.M. (2000b) Cloning and characterization of a Saccharomyces cerevisiae alkaline ceramidase with specificity for dihydroceramide. *J Biol Chem*, **275**, 31369-31378.

Marash, M. and Gerst, J.E. (2001) t-SNARE dephosphorylation promotes SNARE assembly and exocytosis in yeast. *Embo J*, **20**, 411-421.

Marchler, G., Schuller, C., Adam, G. and Ruis, H. (1993) A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *Embo J*, **12**, 1997-2003.

Martin, D.E., Soulard, A. and Hall, M.N. (2004) TOR Regulates Ribosomal Protein Gene Expression via PKA and the Forkhead Transcription Factor FHL1. *Cell*, **119**, 969-979.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1996) The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *Embo J*, **15**, 2227-2235.

McCarthy, J.E. (1998) Posttranscriptional control of gene expression in yeast. *Microbiol Mol Biol Rev*, **62**, 1492-1553.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M. and Cyr, D.M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol*, **3**, 100-105.

Meier, K.D., Deloche, O., Kajiwara, K., Funato, K. and Riezman, H. (2005) Sphingoid base is required for translation during heat stress in Saccharomyces cerevisiae. *submitted*.

Merrill, A.H., Jr., Wang, E. and Mullins, R.E. (1988) Kinetics of long-chain (sphingoid) base biosynthesis in intact LM cells: effects of varying the extracellular concentrations of serine and fatty acid precursors of this pathway. *Biochemistry*, **27**, 340-345.

Messmer, T.O., Wang, E., Stevens, V.L. and Merrill, A.H., Jr. (1989) Sphingolipid biosynthesis by rat liver cells: effects of serine, fatty acids and lipoproteins. *J Nutr*, **119**, 534-538.

Miller, J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor, N.Y.

Miller, M.J., Xuong, N.H. and Geiduschek, E.P. (1979) A response of protein synthesis to temperature shift in the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **76**, 5222-5225.

Miyake, Y., Kozutsumi, Y., Nakamura, S., Fujita, T. and Kawasaki, T. (1995) Serine palmitoyltransferase is the primary target of a sphingosine-like immunosuppressant, ISP-1/myriocin. *Biochem Biophys Res Commun*, **211**, 396-403.

Miyoshi, K., Tsujii, R., Yoshida, H., Maki, Y., Wada, A., Matsui, Y., Toh, E.A. and Mizuta, K. (2002) Normal assembly of 60 S ribosomal subunits is required for the signaling in response to a secretory defect in Saccharomyces cerevisiae. *J Biol Chem*, **277**, 18334-18339.

Moehle, C.M. and Hinnebusch, A.G. (1991) Association of RAP1 binding sites with stringent control of ribosomal protein gene transcription in Saccharomyces cerevisiae. *Mol Cell Biol*, **11**, 2723-2735.

Monaghan, E., Gable, K. and Dunn, T. (2002) Mutations in the Lcb2p subunit of serine palmitoyltransferase eliminate the requirement for the TSC3 gene in Saccharomyces cerevisiae. *Yeast*, **19**, 659-670.

Moskvina, E., Schuller, C., Maurer, C.T., Mager, W.H. and Ruis, H. (1998) A search in the genome of Saccharomyces cerevisiae for genes regulated via stress response elements. *Yeast*, **14**, 1041-1050.

Munn, A.L. and Riezman, H. (1994) Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new END genes. *J Cell Biol*, **127**, 373-386.

Munro, S. (2003) Lipid rafts: elusive or illusive? *Cell*, **115**, 377-388.

Murray, J.I., Whitfield, M.L., Trinklein, N.D., Myers, R.M., Brown, P.O. and Botstein, D. (2004) Diverse and specific gene expression responses to stresses in cultured human cells. *Mol Biol Cell*, **15**, 2361-2374.

Nadeau, K., Das, A. and Walsh, C.T. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J Biol Chem*, **268**, 1479-1487.

Nagiec, M.M., Skrzypek, M., Nagiec, E.E., Lester, R.L. and Dickson, R.C. (1998) The LCB4 (YOR171c) and LCB5 (YLR260w) genes of Saccharomyces encode sphingoid long chain base kinases. *J Biol Chem*, **273**, 19437-19442.

Nathan, D.F. and Lindquist, S. (1995) Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. *Mol Cell Biol*, **15**, 3917-3925.

Nelson, K.K. and Lemmon, S.K. (1993) Suppressors of clathrin deficiency: overexpression of ubiquitin rescues lethal strains of clathrin-deficient Saccharomyces cerevisiae. *Mol Cell Biol*, **13**, 521-532.

Neves, M.J. and Francois, J. (1992) On the mechanism by which a heat shock induces trehalose accumulation in Saccharomyces cerevisiae. *Biochem J*, **288 ( Pt 3)**, 859-864.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995) A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. *Embo J*, **14**, 5931-5938.

Obeid, L.M., Linardic, C.M., Karolak, L.A. and Hannun, Y.A. (1993) Programmed cell death induced by ceramide. *Science*, **259**, 1769-1771.

Oh, C.S., Toke, D.A., Mandala, S. and Martin, C.E. (1997) ELO2 and ELO3, homologues of the Saccharomyces cerevisiae ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. *J Biol Chem*, **272**, 17376-17384.

Parsell, D.A., Kowal, A.S., Singer, M.A. and Lindquist, S. (1994) Protein disaggregation mediated by heat-shock protein Hsp104. *Nature*, **372**, 475-478.

Parsell, D.A. and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet*, **27**, 437-496.

Parsell, D.A. and Lindquist, S. (1994) Heat shock proteins and stress tolerance. In Morimoto, R.I., Tissières, A. and Georgopoulus, C. (eds.), *The Biology of Heat Shock Proteins and Molecular Chaperones*. Cold Spring Harbor Laboratory Press, New York, pp. 457-494.

Patton, J.L. and Lester, R.L. (1991) The phosphoinositol sphingolipids of Saccharomyces cerevisiae are highly localized in the plasma membrane. *J Bacteriol*, **173**, 3101-3108.

Patton, J.L., Srinivasan, B., Dickson, R.C. and Lester, R.L. (1992) Phenotypes of sphingolipid-dependent strains of Saccharomyces cerevisiae. *J Bacteriol*, **174**, 7180-7184.

Pickart, C.M. (1997) Targeting of substrates to the 26S proteasome. *Faseb J*, **11**, 1055-1066.

Pickart, C.M. (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem*, **70**, 503-533.

Pinto, W.J., Srinivasan, B., Shepherd, S., Schmidt, A., Dickson, R.C. and Lester, R.L. (1992a) Sphingolipid long-chain-base auxotrophs of Saccharomyces cerevisiae: genetics, physiology, and a method for their selection. *J Bacteriol*, **174**, 2565-2574.

Pinto, W.J., Wells, G.W. and Lester, R.L. (1992b) Characterization of enzymatic synthesis of sphingolipid long-chain bases in Saccharomyces cerevisiae: mutant strains exhibiting long-chain-base auxotrophy are deficient in serine palmitoyltransferase activity. *J Bacteriol*, **174**, 2575-2581.

Pirkkala, L., Nykanen, P. and Sistonen, L. (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *Faseb J*, **15**, 1118-1131.

Powers, T. and Walter, P. (1999) Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. *Mol Biol Cell*, **10**, 987-1000.

Preiss, T., Baron-Benhamou, J., Ansorge, W. and Hentze, M.W. (2003) Homodirectional changes in transcriptome composition and mRNA translation induced by rapamycin and heat shock. *Nat Struct Biol*.

Prendergast, J.A., Ptak, C., Arnason, T.G. and Ellison, M.J. (1995) Increased ubiquitin expression suppresses the cell cycle defect associated with the yeast ubiquitin conjugating enzyme, CDC34 (UBC3). Evidence for a noncovalent interaction between CDC34 and ubiquitin. *J Biol Chem*, **270**, 9347-9352.

Qie, L., Nagiec, M.M., Baltisberger, J.A., Lester, R.L. and Dickson, R.C. (1997) Identification of a Saccharomyces gene, LCB3, necessary for incorporation of exogenous long chain bases into sphingolipids. *J Biol Chem*, **272**, 16110-16117.

Reynolds, C.P., Maurer, B.J. and Kolesnick, R.N. (2004) Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett*, **206**, 169-180.

Riezman, H. (2004) Why do cells require heat shock proteins to survive heat stress? *Cell Cycle*, **3**, 61-63.

Riezman, H. and van Meer, G. (2004) Lipid pickup and delivery. *Nat Cell Biol*, **6**, 15-16.

Roelants, F.M., Torrance, P.D. and Thorner, J. (2004) Differential roles of PDK1- and PDK2-phosphorylation sites in the yeast AGC kinases Ypk1, Pkc1 and Sch9. *Microbiology*, **150**, 3289-3304.

Rossler, H., Rieck, C., Delong, T., Hoja, U. and Schweizer, E. (2003) Functional differentiation and selective inactivation of multiple Saccharomyces cerevisiae genes involved in very-long-chain fatty acid synthesis. *Mol Genet Genomics*, **269**, 290-298.

Rowley, A., Johnston, G.C., Butler, B., Werner-Washburne, M. and Singer, R.A. (1993) Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast Saccharomyces cerevisiae. *Mol Cell Biol*, **13**, 1034-1041.

Ruis, H. and Schuller, C. (1995) Stress signaling in yeast. *Bioessays*, **17**, 959-965.

Saavedra, C., Tung, K.S., Amberg, D.C., Hopper, A.K. and Cole, C.N. (1996) Regulation of mRNA export in response to stress in Saccharomyces cerevisiae. *Genes Dev*, **10**, 1608-1620.

Saavedra, C.A., Hammell, C.M., Heath, C.V. and Cole, C.N. (1997) Yeast heat shock mRNAs are exported through a distinct pathway defined by Rip1p. *Genes Dev*, **11**, 2845-2856.

Saba, J.D., Nara, F., Bielawska, A., Garrett, S. and Hannun, Y.A. (1997) The BST1 gene of Saccharomyces cerevisiae is the sphingosine-1-phosphate lyase. *J Biol Chem*, **272**, 26087-26090.

Sachs, A.B. and Varani, G. (2000) Eukaryotic translation initiation: there are (at least) two sides to every story. *Nat Struct Biol*, **7**, 356-361.

Santana, P., Pena, L.A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E.H., Fuks, Z. and Kolesnick, R. (1996) Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell*, **86**, 189-199.

Santhanam, A., Hartley, A., Duvel, K., Broach, J.R. and Garrett, S. (2004) PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. *Eukaryot Cell*, **3**, 1261-1271.

Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domae, N. and Hannun, Y.A. (2000) Identification of ISC1 (YER019w) as inositol phosphosphingolipid phospholipase C in Saccharomyces cerevisiae. *J Biol Chem*, **275**, 39793-39798.

Schawalder, S.B., Kabani, M., Howald, I., Choudhury, U., Werner, M. and Shore, D. (2004) Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Ifh1. *Nature*, **432**, 1058-1061.

Schmelzle, T., Beck, T., Martin, D.E. and Hall, M.N. (2004) Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol Cell Biol*, **24**, 338-351.

Schmelzle, T., Helliwell, S.B. and Hall, M.N. (2002) Yeast Protein Kinases and the RHO1 Exchange Factor TUS1 Are Novel Components of the Cell Integrity Pathway in Yeast. *Mol Cell Biol*, **22**, 1329-1339.

Schmitt, A.P. and McEntee, K. (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **93**, 5777-5782.

Schorling, S., Vallee, B., Barz, W.P., Riezman, H. and Oesterhelt, D. (2001) Lag1p and Lac1p Are Essential for the Acyl-CoA-dependent Ceramide Synthase Reaction in Saccharomyces cerevisae. *Mol Biol Cell*, **12**, 3417-3427.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R. and Hurt, E. (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. *Embo J*, **16**, 3256-3271.

Seufert, W. and Jentsch, S. (1990) Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *Embo J*, **9**, 543-550.

Shih, S.C., Sloper-Mould, K.E. and Hicke, L. (2000) Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *Embo J*, **19**, 187-198.

Shin, C., Feng, Y. and Manley, J.L. (2004) Dephosphorylated SRp38 acts as a splicing repressor in response to heat shock. *Nature*, **427**, 553-558.

Shin, D.Y., Matsumoto, K., Iida, H., Uno, I. and Ishikawa, T. (1987) Heat shock response of Saccharomyces cerevisiae mutants altered in cyclic AMP-dependent protein phosphorylation. *Mol Cell Biol*, **7**, 244-250.

Shirai, C., Takai, T., Nariai, M., Horigome, C. and Mizuta, K. (2004) Ebp2p, the yeast homolog of Epstein-Barr virus nuclear antigen 1-binding protein 2, interacts with factors of both the 60 S and the 40 s ribosomal subunit assembly. *J Biol Chem*, **279**, 25353-25358.

Shore, D. (1994) RAP1: a protean regulator in yeast. *Trends Genet*, **10**, 408-412.

Simon, J.R., Treger, J.M. and McEntee, K. (1999) Multiple independent regulatory pathways control UBI4 expression after heat shock in Saccharomyces cerevisiae. *Mol Microbiol*, **31**, 823-832.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature*, **387**, 569-572.

Singer, M.A. and Lindquist, S. (1998a) Multiple effects of trehalose on protein folding in vitro and in vivo. *Mol Cell*, **1**, 639-648.

Singer, M.A. and Lindquist, S. (1998b) Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. *Trends Biotechnol*, **16**, 460-468.

Skrzypek, M.S., Nagiec, M.M., Lester, R.L. and Dickson, R.C. (1999) Analysis of phosphorylated sphingolipid long-chain bases reveals potential roles in heat stress and growth control in Saccharomyces. *J Bacteriol*, **181**, 1134-1140.

Slater, M.R. and Craig, E.A. (1987) Transcriptional regulation of an hsp70 heat shock gene in the yeast Saccharomyces cerevisiae. *Mol Cell Biol*, **7**, 1906-1916.

Smith, B.J. and Yaffe, M.P. (1991) Uncoupling thermotolerance from the induction of heat shock proteins. *Proc Natl Acad Sci U S A*, **88**, 11091-11094.

Sorger, P.K. (1990) Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell*, **62**, 793-805.

Sorger, P.K. and Pelham, H.R. (1987) Purification and characterization of a heat-shock element binding protein from yeast. *Embo J*, **6**, 3035-3041.

Spiegel, S. and Milstien, S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*, **4**, 397-407.

Stone, D.E. and Craig, E.A. (1990) Self-regulation of 70-kilodalton heat shock proteins in Saccharomyces cerevisiae. *Mol Cell Biol*, **10**, 1622-1632.

Stutz, F. and Rosbash, M. (1998) Nuclear RNA export. *Genes Dev*, **12**, 3303-3319.

Sun, Y., Taniguchi, R., Tanoue, D., Yamaji, T., Takematsu, H., Mori, K., Fujita, T., Kawasaki, T. and Kozutsumi, Y. (2000) Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol*, **20**, 4411-4419.

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.

Takita, Y., Ohya, Y. and Anraku, Y. (1995) The CLS2 gene encodes a protein with multiple membrane-spanning domains that is important Ca2+ tolerance in yeast. *Mol Gen Genet*, **246**, 269-281.

Tanaka, K., Matsumoto, K. and Toh-e, A. (1988) Dual regulation of the expression of the polyubiquitin gene by cyclic AMP and heat shock in yeast. *Embo J*, **7**, 495-502.

Tanida, I., Takita, Y., Hasegawa, A., Ohya, Y. and Anraku, Y. (1996) Yeast Cls2p/Csg2p localized on the endoplasmic reticulum membrane regulates a non-exchangeable intracellular Ca2+ pool cooperatively with calcineurin. *FEBS Lett*, **379**, 38-42.

Toke, D.A. and Martin, C.E. (1996) Isolation and characterization of a gene affecting fatty acid elongation in Saccharomyces cerevisiae. *J Biol Chem*, **271**, 18413-18422.

Tongaonkar, P., Chen, L., Lambertson, D., Ko, B. and Madura, K. (2000) Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. *Mol Cell Biol*, **20**, 4691-4698.

Treger, J.M., Schmitt, A.P., Simon, J.R. and McEntee, K. (1998) Transcriptional factor mutations reveal regulatory complexities of heat shock and newly identified stress genes in Saccharomyces cerevisiae. *J Biol Chem*, **273**, 26875-26879.

Tsuno, A., Miyoshi, K., Tsujii, R., Miyakawa, T. and Mizuta, K. (2000) RRS1, a conserved essential gene, encodes a novel regulatory protein required for ribosome biogenesis in Saccharomyces cerevisiae. *Mol Cell Biol*, **20**, 2066-2074.

Uemura, S., Kihara, A., Inokuchi, J. and Igarashi, Y. (2003) Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. *J Biol Chem*, **278**, 45049-45055.

Uesono, Y. and Toh, E.A. (2002) Transient inhibition of translation initiation by osmotic stress. *J Biol Chem*, **16**, 16.

Vainberg, I.E., Dower, K. and Rosbash, M. (2000) Nuclear export of heat shock and non-heat-shock mRNA occurs via similar pathways. *Mol Cell Biol*, **20**, 3996-4005.

Vallee, B. and Riezman, H. (2005) Lip1p: a novel subunit of acyl-CoA ceramide synthase. *Embo J*, *in press*

van Nocker, S., Sadis, S., Rubin, D.M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D. and Vierstra, R.D. (1996) The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. *Mol Cell Biol*, **16**, 6020-6028.

Verna, J., Lodder, A., Lee, K., Vagts, A. and Ballester, R. (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **94**, 13804-13809.

Vuorio, O.E., Kalkkinen, N. and Londesborough, J. (1993) Cloning of two related genes encoding the 56-kDa and 123-kDa subunits of trehalose synthase from the yeast Saccharomyces cerevisiae. *Eur J Biochem*, **216**, 849-861.

Wade, J.T., Hall, D.B. and Struhl, K. (2004) The transcription factor Ifh1 is a key regulator of yeast ribosomal protein genes. *Nature*, **432**, 1054-1058.

Warner, J.R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci*, **24**, 437-440.

Warner, J.R. and Udem, S.A. (1972) Temperature sensitive mutations affecting ribosome synthesis in Saccharomyces cerevisiae. *J Mol Biol*, **65**, 243-257.

Watanabe, R., Funato, K., Venkataraman, K., Futerman, A.H. and Riezman, H. (2002) Sphingolipids are required for the stable membrane association of glycosylphosphatidylinositol-anchored proteins in yeast. *J Biol Chem*, **277**, 49538-49544.

Wells, G.B., Dickson, R.C. and Lester, R.L. (1998) Heat-induced elevation of ceramide in Saccharomyces cerevisiae via de novo synthesis. *J Biol Chem*, **273**, 7235-7243.

Wilson, W.A. and Roach, P.J. (2002) Nutrient-regulated protein kinases in budding yeast. *Cell*, **111**, 155-158.

Wu, C. (1995) Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol*, **11**, 441-469.

Yasuda, S., Nishijima, M. and Hanada, K. (2003) Localization, topology, and function of the LCB1 subunit of serine palmitoyltransferase in mammalian cells. *J Biol Chem*, **278**, 4176-4183.

Yuan, D.S., Dancis, A. and Klausner, R.D. (1997) Restriction of copper export in Saccharomyces cerevisiae to a late Golgi or post-Golgi compartment in the secretory pathway. *J Biol Chem*, **272**, 25787-25793.

Zanolari, B., Friant, S., Funato, K., Sutterlin, C., Stevenson, B.J. and Riezman, H. (2000) Sphingoid base synthesis requirement for endocytosis in Saccharomyces cerevisiae. *Embo J*, **19**, 2824-2833.

Zhang, X., Lester, R.L. and Dickson, R.C. (2004) Pil1p and Lsp1p negatively regulate the PDK1-like kinase Pkh1p and downstream signaling pathways Pkc1p and Ypk1p. *J Biol Chem*.

Zhang, X., Skrzypek, M.S., Lester, R.L. and Dickson, R.C. (2001) Elevation of endogenous sphingolipid long-chain base phosphates kills Saccharomyces cerevisiae cells. *Curr Genet*, **40**, 221-233.

Zhao, C., Beeler, T. and Dunn, T. (1994) Suppressors of the Ca(2+)-sensitive yeast mutant (csg2) identify genes involved in sphingolipid biosynthesis. Cloning and characterization of SCS1, a gene required for serine palmitoyltransferase activity. *J Biol Chem*, **269**, 21480-21488.

Zou, J., Guo, Y., Guettouche, T., Smith, D.F. and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell*, **94**, 471-480.

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## Professional Experience

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| 1999 - 2001 | Diploma Thesis with Professor Howard Riezman<br>Div. of Biochemistry, Biozentrum, University of Basel<br>Thesis Title: Identification and Characterization of Yeast Mutants Defective in Fluid-Phase Endocytosis<br>Grade: *magna cum laude* |
| 2001 - present | Ph.D. Thesis with Professor Howard Riezman<br>Div. of Biochemistry, Biozentrum, University of Basel; and<br>Dept. of Biochemistry, Sciences II, University of Geneva<br>Thesis Title: Mechanisms of Sphingolipid Functions during Heat Stress in *Saccharomyces cerevisiae* |

## Teaching Experience

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| 1999 - 2004 | Assistant Biochemistry Block-Course for Biology II Students |
| 2000 - 2002 | "Introduction into the Basics of Biology" for 1. semester biology students |
| 2002 - 2004 | "Applied Ethics in Biological Research" for 4. semester biology students |

## Bibliography

1. Wiederkehr *, A., **K.D. Meier***, and H. Riezman. 2001. Identification and characterization of *Saccharomyces cerevisiae* mutants defective in fluid-phase endocytosis. *Yeast*. 18:759-73.
2. Friant*, S., **K.D. Meier***, and H. Riezman. 2003. Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction. *Embo J*. 22:3783-3791.
3. **Meier, K.D**., O. Deloche, K. Kaijiwara, K. Funato, and H. Riezman. 2004. Sphingoid base is required for translation during heat stress in *Saccharomyces cerevisiae*, *submitted*

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## Conference Participations

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| 2000 | Swiss Yeast Meeting, November, ISREC Lausanne, Switzerland |
| 2001 | Swiss Yeast Meeting, September, Biozentrum Basel, Switzerland |
| 2003 | HSFP Meeting, September 6-7, Sciences II, Geneva, Switzerland |
| 2004 | 1st Sapporo Summer Conference, Sapporo Sphingolipid Symposium, July 21-23, Sapporo, Japan |
| | Gordon Research Conference "Glycolipid and Sphingolipid Biology" July 25-30, Hyogo, Japan, invited speaker from abstract |

## Extra Curricular Activities

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| 1996 - 2000 | Class-Speaker, 1.-4. year students Biology II |
| 1999 - 2002 | President of the association of students at the Biozentrum "Verein Studierender am Biozentrum (VSB)" |
| 2001 - 2002 | Delegate of the interfaculty organization of assistants from the University of Basel "Dachverband der Assistierenden" |
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Cooperation in various search and appointment committees for ROCHE, the University of Basel and the Biozentrum. Quality audits and hearings in corporation with the Swiss Science and Technology Council (SWTR), the Center of Accreditation and Quality Assurance of the Swiss Universities (OAQ). Event organization for the student association (VSB) and for the Biozentrum ranging from 50 to 700 participants

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## ERKLÄRUNG

Hiermit erkläre ich, dass ich die Dissertation

**"Mechanisms of Sphingolipid Functions during Heat Stress in *Saccharomyces cerevisiae*"**

nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Karsten D. Meier

Don't cry, work!