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Signalling mucin Msb2 Regulates adaptation to thermal stress in *Candida albicans*

Darpan Saraswat,[1] Rohitashw Kumar,[1] Tanaya Pande,[2] Mira Edgerton[1] and Paul J. Cullen[2]*

Departments of [1]Oral Biology and [2]Biological Sciences, University at Buffalo, Buffalo, NY, 14260-1300, USA.

Summary

Temperature is a potent inducer of fungal dimorphism. Multiple signalling pathways control the response to growth at high temperature, but the sensors that regulate these pathways are poorly defined. We show here that the signalling mucin Msb2 is a global regulator of temperature stress in the fungal pathogen *Candida albicans*. Msb2 was required for survival and hyphae formation at 42°C. The cytoplasmic signalling domain of Msb2 regulated temperature-dependent activation of the CEK mitogen activated proteins kinase (MAPK) pathway. The extracellular glycosylated domain of Msb2 (100-900 amino acid residues) had a new and unexpected role in regulating the protein kinase C (PKC) pathway. Msb2 also regulated temperature-dependent induction of genes encoding regulators and targets of the unfolded protein response (UPR), which is a protein quality control (QC) pathway in the endoplasmic reticulum that controls protein folding/degradation in response to high temperature and other stresses. The heat shock protein and cell wall component Ssa1 was also required for hyphae formation and survival at 42°C and regulated the CEK and PKC pathways.

Introduction

An important environmental stimulus encountered by most organisms is temperature. The adaptation to thermal stress is a common feature of animals, plants and microorganisms. The response to temperature stress involves the regulation of protein folding by molecular chaperones, and the turnover of mis-folded proteins in the cytosol and other compartments. Cells respond to temperature stress in different ways, including the reorganization of the cytoskeleton, alteration of membrane fluidity, and the regulated arrest of cell-cycle progression (Zeuthen, 1971; Lindquist, 1980; Yost and Lindquist, 1986; Richter *et al*., 2010). Temperature sensing and thermal adaptation mechanisms have been well-established in bacteria (Klinkert and Narberhaus, 2009) and fungal microorganisms (Nemecek *et al*., 2006). Temperature sensing has been intensively studied in pathogens, which are thought to detect high temperature as an indicator of entry into a warm-blooded host. Accordingly, much effort has been made to identify the signalling pathways and proteins that regulate the adaptation to high temperature in microbial pathogens.

Fungal species undergo distinctive morphogenetic and developmental behaviours in response to environmental challenges (Madhani and Fink, 1998; Lengeler *et al*., 2000; Heitman, 2005; d'Enfert, 2009; Leach and Brown, 2012). *Candida albicans* is an opportunistic fungal species present as a normal flora in the majority of the human population. Under pre-disposing conditions in the host, this species can be pathogenic and cause mucosal and systemic candidiasis (Scully *et al*., 1994; Moran *et al*., 2011; Leach and Brown, 2012; Polvi *et al*., 2015). One of the major virulence factors involved in *C. albicans* pathogenesis is its conversion from the yeast to the hyphal form (Sudbery *et al*., 2004). Other virulence traits include genome plasticity (Selmecki *et al*., 2010; Goodenough and Heitman, 2014), cell-surface variegation (Nather and Munro, 2008; Latge, 2010), and biofilm formation (Kumamoto and Vinces, 2005; Nobile and Mitchell, 2006). Multiple environmental signals induce morphogenetic responses in *C. albicans*, such as altered nutrient levels, temperature, pH, CO_2, pheromones and the release of quorum-sensing compounds (Biswas *et al*., 2007). These diverse stimuli are sensed by a variety of different sensors, which regulate signal transduction pathways that control morphogenetic and biochemical changes to promote host invasion and colonization (Rispail *et al*., 2009). Among the major signalling pathways that regulate dimorphism in *C. albicans*

Accepted 6 January, 2016. *For correspondence. E-mail pjcullen@buffalo.edu; Tel. (716)-645-4923; Fax (716)-645-2975.

are Mitogen Activated Protein Kinase (MAPK) pathways, the RAS pathway, the calcium-calcineurin pathway and amino acid sensing and signalling pathways (Biswas *et al*., 2007; Zhao *et al*., 2007; Rispail *et al*., 2009).

The response to temperature stress and filamentation in *C. albicans* has been well characterized (Shapiro *et al*., 2009; Brown *et al*., 2010; Shapiro and Cowen, 2010; Shapiro and Cowen, 2012a,b; Shapiro *et al*., 2012a; Leach and Cowen, 2013; Leach and Cowen, 2014a,b; O'Meara and Cowen, 2014). One aspect of thermoregulation is mediated by an evolutionarily conserved heat shock response (HSR). The major role of the response is to promote protein folding by the action of heat shock proteins (HSPs) such as the major molecular chaperone Hsp90 (Morimoto, 1998). Other pathways sense and respond to changes in protein folding, such as the unfolded protein response (UPR) that operates in the endoplasmic reticulum (Patil and Walter, 2001; Wimalasena *et al*., 2008; Gardner *et al*., 2013). Short-term responses include stabilization of the essential transcription factor Cta8/Hsf1, which is a 'client protein' for Hsp90 (Leach *et al*., 2012a). Prolonged thermal adaptation requires coordination between Hsp90 and MAPK pathways (Liu and Chang, 2008; Leach *et al*., 2012a,). In this case, Hsp90 stabilizes other client proteins that include Cek1 (Leach *et al*., 2012b), the MAP kinase that regulates the CEK pathway (Leberer *et al*., 1996; Csank *et al*., 1998). Hsp90 also stabilizes the MAP kinase Hog1 (Diezmann *et al*., 2012), which regulates the high osmolarity glycerol response (HOG) pathway (Alonso-Monge *et al*., 1999; Saito, 2010). In addition, Hsp90 stabilizes the MAP kinase Mkc1 (LaFayette *et al*., 2010; Leach *et al*., 2012a), which regulates the protein kinase C (PKC) pathway (Navarro-Garcia *et al*., 1998). The RAS pathway is also a global nutrient-sensing pathway that regulates the response to high temperature (Shapiro *et al*., 2009; Huang *et al*., 2010; Langford *et al*., 2013; Rao *et al*., 2013).

Although much work has been done to characterize the signalling pathways that regulate thermoregulation in *C. albicans* and other species, the 'sensors' that detect changes in temperature have not been well defined. One candidate protein is the signalling mucin Msb2. Signalling mucins are transmembrane (TM) glycoproteins that regulate signalling pathways in eukaryotes (Kufe, 2009; Tian and Ten Hagen, 2009; Bafna *et al*., 2010; Cullen, 2011; Birchenough et al., 2015). In fungal species, Msb2 is a regulator of environmental stress, cell wall biogenesis and the CEK pathway (Cullen *et al*., 2004; Roman *et al*., 2009; Puri *et al*., 2012). Because the CEK pathway is activated by growth in high temperatures, Msb2 may be a candidate regulator of thermal stress. We show here that Msb2 plays an important role in thermo-adaptation in *C. albicans*. Msb2 was required for hyphae formation and growth at 42°C. Msb2 regulated the response to high temperatures by regulating the CEK pathway. In addition, Msb2 regulated the activity of the PKC pathway and the expression of genes that encode proteins that regulate the UPR. Ssa1, a Hsp70 chaperone and abundant cell wall protein (Sun *et al*., 2010), was also required for thermal adaptation and MAP kinase (CEK and PKC) pathway regulation. These results extend the understanding of the signalling pathways that regulate thermo-tolerance in *C. albicans* to include signalling mucins and cell-wall-associated HSP-type sensors. The identification of such regulatory proteins is critical to understand thermo-tolerance regulation in fungi and possibly other systems.

Results

The signalling mucin Msb2 is required for hyphae formation and survival at 42°C

Msb2 is a signalling glycoprotein that regulates the CEK MAP kinase pathway (Cullen *et al*., 2004; Roman *et al*., 2009; Puri *et al*., 2012). To better understand the role of Msb2 in regulating signalling and behavioural responses in *C. albicans*, wild-type cells and the *msb2*Δ mutant were examined by microscopy under different growth conditions. Nutrient limitation and growth at elevated temperatures are potent inducers of fungal dimorphism in *C. albicans* (Puri *et al*., 2012). The formation of hyphae was therefore examined in response to growth in different carbon sources and different temperatures. Growth of *C. albicans* is optimal at 37°C, but the organism can tolerate temperature stresses exceeding 55°C. We found that the *msb2*Δ mutant was defective for hyphae formation at 42°C. Wild-type cells showed a reduction in the formation of hyphae at 42°C, but the *msb2*Δ mutant was completely defective (Fig. 1A, arrows point to examples of hyphae at 42°C; see Supporting Information Fig. S1 for quantitation). These results show that Msb2 plays a role in hyphae formation at 42°C.

To better define the basis for the defect in hyphae production at 42°C, the *msb2*Δ mutant was examined in detail. The *msb2*Δ mutant showed a growth defect at 30°C and 37°C and had a striking growth defect at 42°C (Fig. 1B). The growth (OD_{600}) of wild-type cells and the *msb2*Δ mutant was assessed by growth curves in liquid media. Wild-type cells grew optimally at 37°C and showed a reduced growth rate at 30°C and 42°C (Fig. 1C). By comparison, the *msb2*Δ was severely defective for growth at 42°C (Fig. 1C). It may be that cells compromised for viability at 42°C are unable to produce hyphae. This is probably in part true. However, measuring colony forming units (CFU) at the time point tested showed that the *msb2*Δ mutant was viable at 42°C under conditions when it was defective for hyphae

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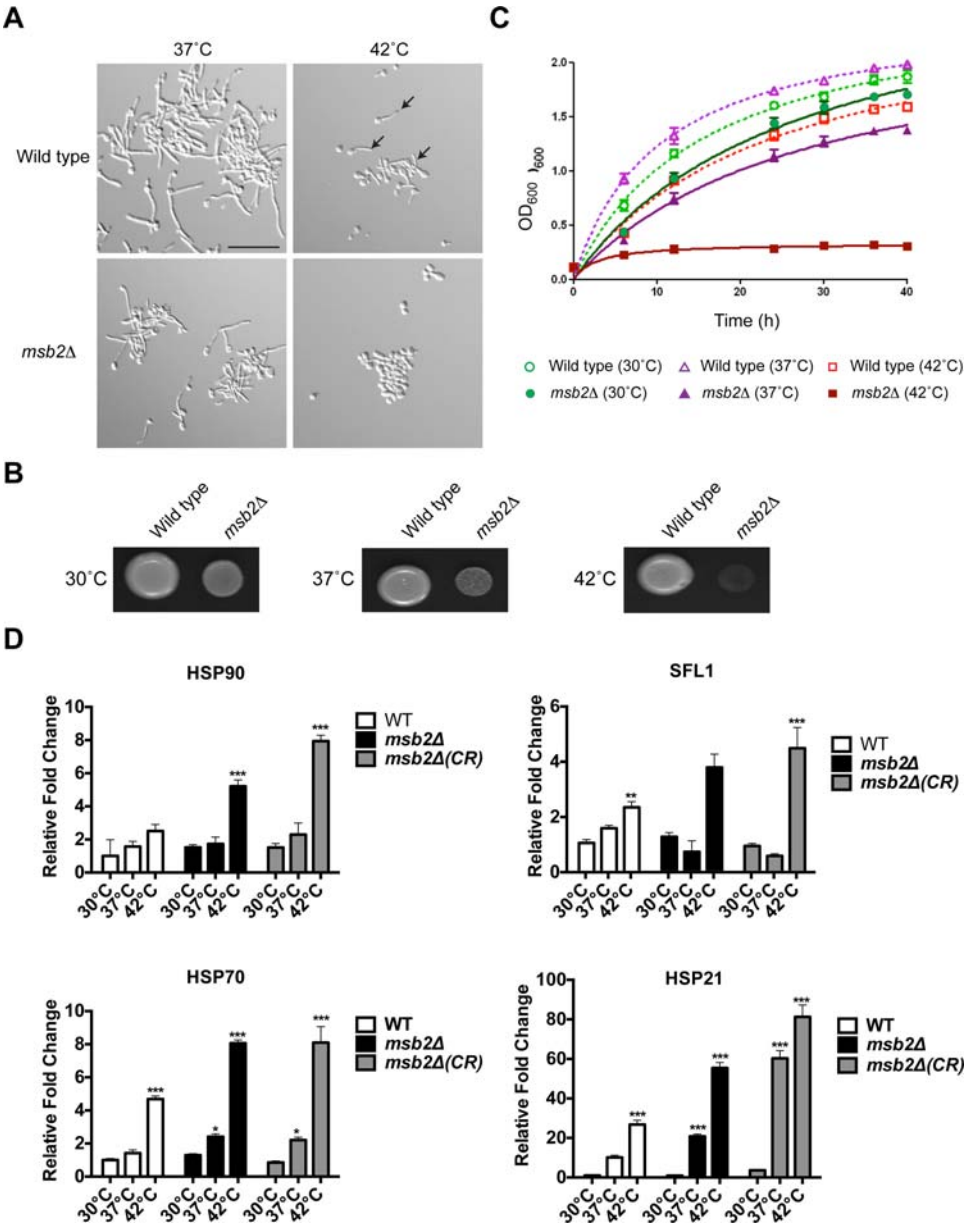

Fig. 1. Role of Msb2 in hyphae formation and survival at 42°C.
A. Wild type and the *msb2*Δ mutant were grown in YNB + 2% Glu for 3 h at 37°C and 42°C and observed by microscopy at 20X magnification. Arrows, examples of hyphae at 42°C. Bar, 10 microns.
B. Growth of wild-type cells and the *msb2*Δ mutant in YPD media at 30°C, 37°C, 42°C. Equal concentrations of cells were spotted onto plates for 48 h.
C. Growth curves of wild-type cells and the *msb2*Δ mutant in YPD media at 30°C, 37°C, 42°C. Results are the average of two independent experiments. Error bars represent the standard deviation between experiments.
D. Quantification of HSP gene expression was measured by quantitative RT-PCR of RNA extracted from cells grown in 30°C, 37°C, 42°C for 3 h. Transcript levels were normalized to *GAPDH2* gene expression. Levels are expressed relative to wild type, which was set to 1. Data are the mean and SD of three independent biological replicates. Error bars refer to the standard deviation between trials. The *** indicates P value < 0.001, ** indicates $P < 0.01$ and * indicates $P < 0.5$. CR, congo red.

formation (Supporting Information Fig. S2A). Furthermore, at an intermediate temperature (39°C), the *msb2*Δ mutant was defective for hyphae formation but viable (Supporting Information Fig. S2B). Therefore, in addition to its growth defect at 42°C, the *msb2*Δ mutant is defective for hyphae formation at 42°C.

In response to growth at high temperatures, cells induce a Heat Shock Response (HSR). One feature of this response is the transcriptional induction of genes encoding heat shock proteins (HSPs) that include Hsp90 (Leach *et al.*, 2012a), Hsp70 (Leach *et al.*, 2012a), Hsp21 (Mayer *et al.*, 2012) and the transcription factor Sfl1 (Znaidi *et al.*, 2013). Quantitative PCR (qPCR) analysis was used to measure the expression of *HSP90*, *HSP21*, *HSP70* and *SFL1* in wild-type cells and the *msb2*Δ mutant at 30°C, 37°C and 42°C. The expression of heat shock genes was elevated by more than twofold in wild-type cells grown at 42°C in YPD medium for 3 h (Fig. 1D, white bars). Thus, growth of *C. albicans* at 42°C induces thermal stress. The expression of heat shock genes was also elevated in the *msb2*Δ mutant, by more than twofold above that seen in wild-type cells (Fig. 1D, black bars). These results indicate that the *msb2*Δ mutant experiences an elevated HSR compared to wild-type cells. We also examined HSR targets in response to Congo Red (CR) stress at three temperatures (Fig. 1D,

CR). For at least some targets, an additive effect of temperature and cell wall stress was observed. Elevated HSR in the *msb2*Δ mutant did not rescue the growth defect, which suggests that Msb2 regulates a specific pathway. Below we show that Msb2 regulates the CEK, PKC, and UPR. Altogether, the findings identify a new role for Msb2 in regulating hyphae formation and survival at 42°C.

The extracellular domain of Msb2 is required for survival and hyphae formation at 42°C

Like other signalling mucins (Singh and Hollingsworth, 2006; Tian and Ten Hagen, 2009; Kufe, 2013), Msb2 is a type I single-pass transmembrane (TM) protein that contains a highly glycosylated extracellular domain, a cleavage domain (CD) and a cytosolic signalling domain (CYT; Fig. 2A). The domain structure of Msb2 has been well characterized (Szafranski-Schneider *et al.*, 2012; Swidergall *et al.*, 2015). The extracellular domain of Msb2 is highly modified by O-linked glycans in a repeat region that is rich in proline, threonine and serine residues (PTS region, 100-900 aa residues). A highly glycosylated PTS region is shared with all mucin members (Desseyn *et al.*, 2008; Tarp and Clausen, 2008). The PTS region of Msb2 also contains a Low Complexity Region [LCR (Coletta *et al.*, 2010)] in its N-terminus (aa 100–450). Versions of Msb2 were constructed that lacked each of the major functional domains, including the PTS region (*ED1*Δ*, ED2*Δ and *ED1/2*Δ), the CD (*ED3/4*Δ and *ED4*Δ) and the TM and CYT domains (*TM-CYT*Δ, Fig. 2A). The deletion derivatives were tested for growth at 30°C, 37°C and 42°C. Versions of Msb2 lacking the PTS region (*ED1*Δ*, ED2*Δ and particularly *ED1/2*Δ) were required for growth at high temperatures (Fig. 2B). The CD (*ED3/4*Δ and *ED4*Δ) and TM-CYT domains (*TM-CYT*Δ) were not required (Fig. 2B). A similar phenotype was observed by examining the formation of hyphae at 42°C. The *ED1*Δ*, ED2*Δ*, ED1/2*Δ and *ED3/4*Δ mutants were defective for hyphae production at 42°C, but the *TM-CYT*Δ mutant was not (Fig. 2C, Supporting Information Fig. S1). The ED2 domain also contributed to hyphae formation at 37°C (Fig. 2C, Supporting Information Fig. S1). CFU analysis showed that the cells were viable (Supporting Information Fig. S2C). These results identify the extracellular domain of Msb2 (primarily the PTS region) as being required for survival and hyphae formation at 42°C.

Msb2 regulates temperature-dependent activation of the CEK pathway

Msb2 is an established regulator of the CEK MAP kinase pathway (Roman *et al.*, 2009; Puri *et al.*, 2012). The CEK pathway requires both limiting nutrients [e.g. growth in the poor carbon source *N*-Acetyl-Glucosamine (GlcNAc)] and high temperature (37°C) for activation (Puri *et al.*, 2012). Deletion derivatives of Msb2 were tested for the regulation of the CEK pathway by measuring phosphorylation of the MAP kinase Cek1 (P∼Cek1). Consistent with a previous report (Puri *et al.*, 2012), growth of wild-type cells in glucose (Glu) did not induce P∼Cek1 at 30°C or 37°C (Fig. 3A, wild type). However, growth of cells in GlcNac induced P∼Cek1 at 37°C (Fig. 3A, wild type). Growth of cells at 42°C also induced the CEK pathway but to a lesser extent than growth at 37°C (see below). Two mutants were defective for this induction, the *ED1/2*Δ and the *TM-CYT*Δ domain (Fig. 3A). Thus, Msb2 regulates temperature-dependent CEK pathway activation through the ED1/2 and TM-CYT domains. Moreover, the TM-CYT domain is dispensable for survival (Fig. 2B) and hyphae formation at 42°C (Fig. 2C, Supporting Information Fig. S1) but is required for CEK pathway activation at 37°C (Fig. 3A) and at 42°C (see below).

The activation of the CEK pathway involves Sap-dependent Msb2 processing and shedding of the extracellular domain (Puri *et al.*, 2012; Vadaie et al., 2008). Growth at 37°C resulted in elevated shedding of HA-Msb2 in wild-type cells (Fig. 3B, wild type), which correlates with the activation of the CEK pathway. The deletion derivatives were all detected by slot blot, indicating that they are all expressed (Fig. 3B). *ED1*Δ*, ED2*Δ and *ED1/2*Δ were present at lower levels, which may indicate that these domains contribute to the stability of the protein. The reduced levels of these versions of Msb2 may account for the phenotypes described above. Slot blot analysis showed that *ED3/4*Δ and ED4Δ were shed at higher levels than wild-type Msb2 in glucose, which may indicate this region is inhibitory to shedding in some contexts. It is possible that different Saps differentially impact Msb2 shedding in a condition-dependent manner. In related studies (Szafranski-Schneider *et al.*, 2012; Swidergall *et al.*, 2015), deletion derivatives of Msb2 showed functional differences to those reported here. This may result, in part, from differences in *MSB2* expression, because *MSB2* was expressed from an exogenous (strong) promoter in (Szafranski-Schneider *et al.*, 2012; Swidergall *et al.*, 2015) or from functional differences arising from the precise deletions constructed.

The MAP kinase Cek1 was itself required for hyphae formation at 42°C (Fig. 3C, Supporting Information Figs. S1 and S2A). The *cek1*Δ mutant [and other CEK pathway mutants, like *sho1*Δ (Roman *et al.*, 2009)] also showed a growth defect at 42°C, but not to the same extent as the *msb2*Δ mutant, because the growth defect was detected at early (24 h) but not late (48 h) time points (Supporting Information Fig. S3). These results

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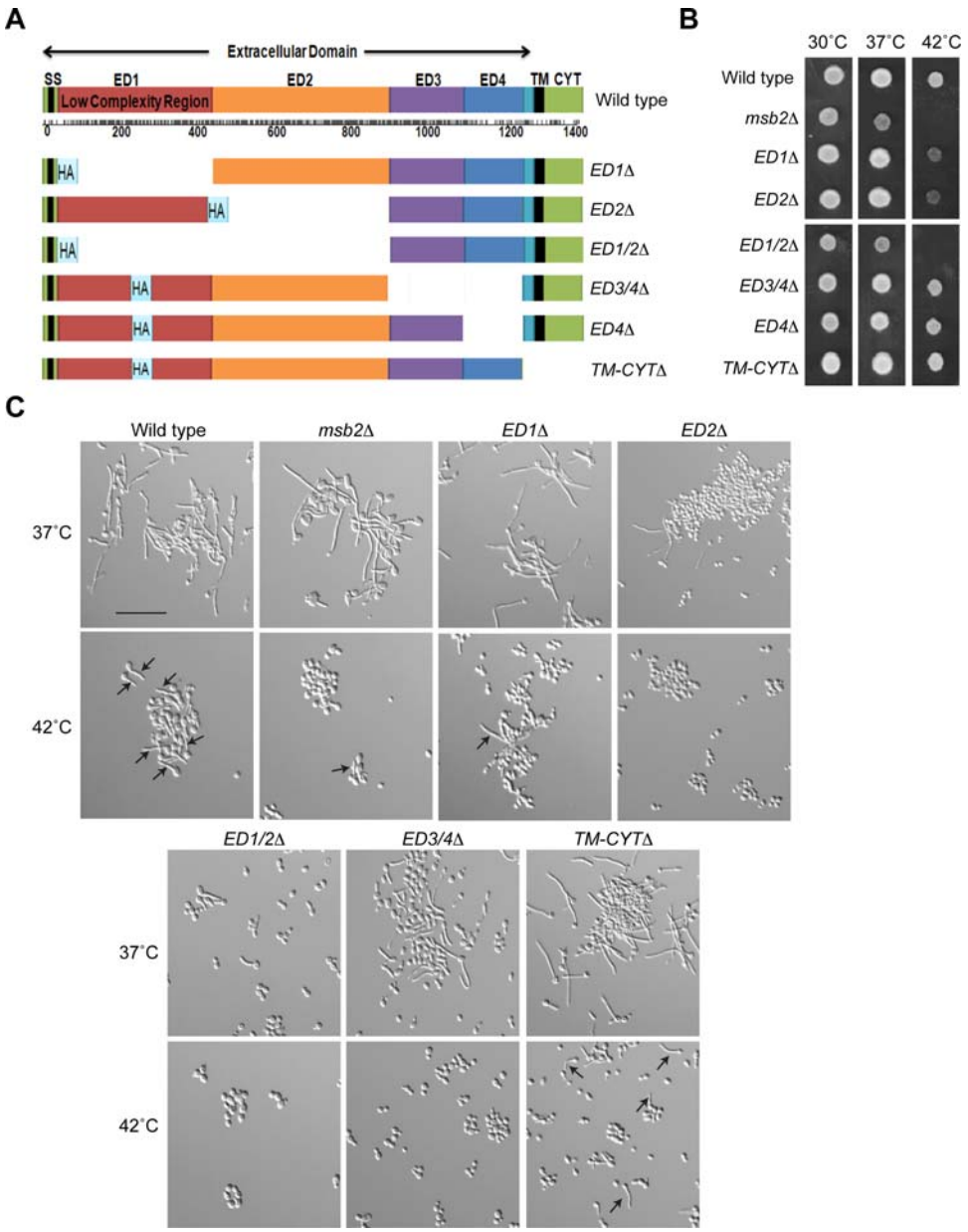

Fig. 2. Role of different domains of Msb2 in regulating survival and hyphae production at high temperatures.
A. Full length Msb2 and deletion derivatives used in the study. The signal sequence (ss), low-complexity region, extracellular domain, transmembrane domain (TM) and cytosolic (CYT) domains are indicated. The deletions are shown as indicated in the figure, with corresponding replacement of the domains of the Msb2 protein by the hemagglutinin (HA) epitope.
B. Strains containing Msb2 deletions were spotted on YPD media at 30°C, 37°C and 42°C for 48 h.
C. Strains containing Msb2 deletions were grown in YNB + 2% Glu for 3 h at 37°C and 42°C and observed by microscopy at 20X magnification. Arrows, examples of hyphae at 42˚C. Bar, 10 microns.

agree with other studies where Msb2 deletions were constructed and analyzed (Szafranski-Schneider *et al*., 2012; Swidergall *et al*., 2015). Altogether, the results show that one aspect of Msb2's temperature response is mediated through its canonical role in regulating the CEK pathway.

Msb2 also regulates the PKC pathway

Several observations indicate that Msb2 regulates survival at 42°C beyond its role in regulating the CEK pathway. First, the extracellular domain (but not the TM-CYT domain) is required for survival at 42°C (Fig. 2B). Second, the *msb2*Δ mutant has a more severe growth defect than other CEK pathway mutants (Supporting Information Fig. S3). To identify how Msb2 might regulate thermo-tolerance outside of its role in regulating the CEK pathway, a panel of mutants was examined that were defective for pathways that impact stress signalling and dimorphism including the HOG (*sln1*Δ, *sho1*Δ, *ssk1*Δ, *ssk2*Δ and *hog1*Δ), RAS (*ras1*Δ, *cyr1*Δ, *tpk1*Δ, *tpk2*Δ and *efg1*Δ), PKC (*wsc1*Δ, *wsc2*Δ, *wsc4*Δ, *bck1*Δ, *mkk2*Δ and *mkc1*Δ) and UPR (*ire1*Δ and *hac1*Δ) pathways, as well as several proteins that function in the cell wall (*mp65*Δ, *muc1*Δ, *hyp1*Δ, *hwp2*Δ, *ssa2*Δ, *ssa1*Δ and *sgt1*Δ). Several mutants showed growth defects after 24 h

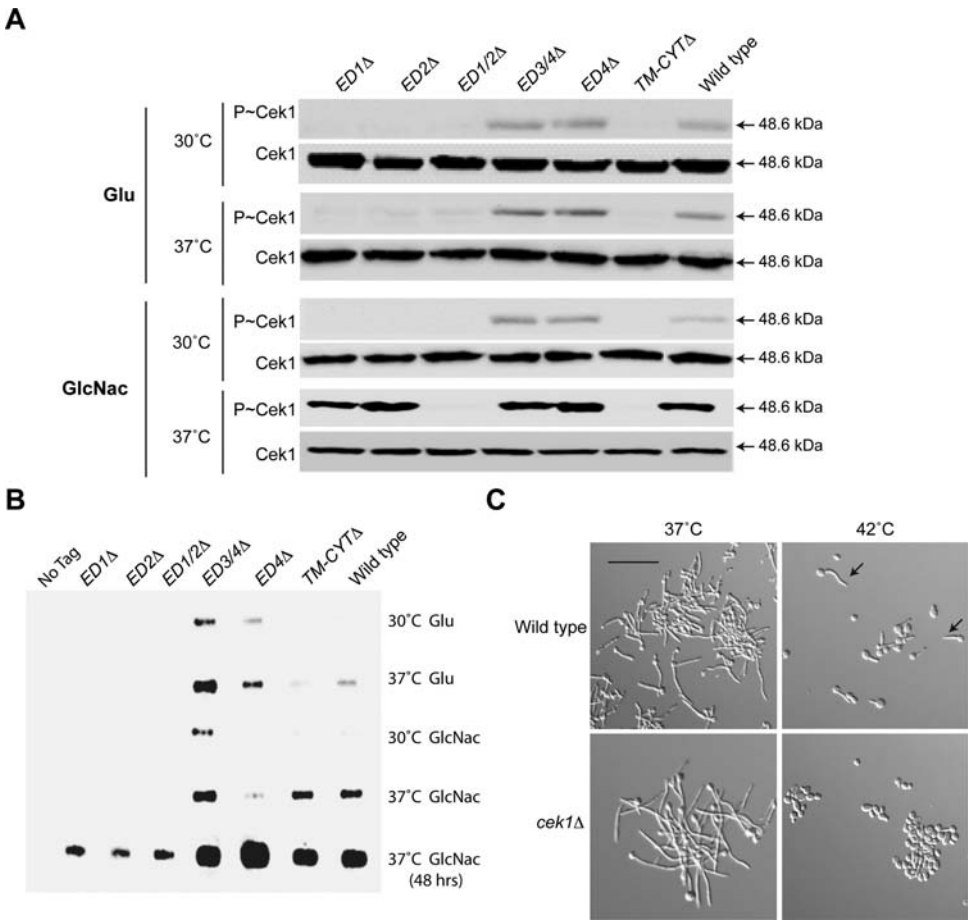

Fig. 3. Msb2 regulates the response to high temperature through the CEK pathway. A. Immunoblot of P∼Cek1 levels in Msb2 deletions. Strains were incubated in pre-warmed YNB media with 1.25% GlcNac at 30˚C or 37°C for 3 h. Total cellular protein (20 μg) from cell lysates was examined by immunoblot analysis with α-phospho p42/44 MAPK antibodies or α-Cek1 antibodies as a control. B. To determine Msb2 shedding, control (CAI4) or cells expressing HA-Msb2 and derivatives were grown in YNB + Glu or YNB + GlcNac media (pre-warmed to 30°C or 37°C) for 3 h. Cells were removed by centrifugation, and proteins in the supernatant were precipitated by TCA. Normalized protein (20 μg) was examined by slot blot using anti-HA antibodies. C. Cells were grown in YNB + 2% Glu for 3 h at 37°C and 42°C and observed by microscopy at 20X magnification. Arrows, examples of hyphae at 42˚C. Bar, 10 microns.

incubation at 42°C, including *mkc1*Δ, *ire1*Δ, and *ssa1*Δ (Fig. S3), which were examined in detail.

The first mutant that showed a growth defect at 42°C was the *mkc1*Δ mutant. The growth defect of the *mkc1*Δ mutant resembled that of the *cek1*Δ mutant, which had slow growth at 24 h but grew normally at 48 h (Supporting Information Fig. S3). Mkc1 is the MAP kinase that regulates the PKC or cell wall integrity pathway and is an established regulator of the response to high-temperature stress (Levin, 2005; LaFayette *et al*., 2010; Diezmann *et al*., 2012). Thus, we tested whether Msb2 regulates the PKC pathway. The PKC pathway protects cells against a variety of stresses to the cell wall (Diezmann *et al*., 2012). The *msb2*Δ mutant was sensitive to cell wall perturbing agents such as Congo Red (CR) and Calcofluor White (CFW) to the same degree as the *mkc1*Δ mutant (Fig. 4A). The cell wall sensitivity was mediated by the PTS region of the protein, because the *ED1*Δ and *ED1/2*Δ mutants showed sensitivity whereas other mutants did not (Fig. 4B). *ED2*Δ was not sensitive which distinguished its role in cell-wall integrity from its role in regulating survival at 42°C (Fig. 2B).

We next tested whether Msb2 was required to activate the PKC pathway by temperature or cell wall stresses by examining phosphorylation of Mkc1 (P∼Mkc1). The *msb2*Δ mutant was defective for phosphorylation of P∼Mkc1 (and P∼Cek1; P∼Mkc1 and P∼Cek1 are recognized by the same p42/p44 P∼ERK antibodies) at all temperatures tested and in response to CR and CFW (Fig. 4C). Optimal phosphorylation of Mkc1 was seen at 37°C (for YPD and CFW) or 30°C (for CR). These results demonstrate a role for Msb2 in regulating the PKC pathway in *C. albicans*. Furthermore, the results show that the extracellular domain of Msb2 (but not the CYT domain) regulates the PKC pathway. The *mkc1*Δ mutant was defective for hyphae production at 42°C (Fig. 4D, Supporting Information Figs. S1 and S2A). Therefore, two MAP kinase pathways controlled by Msb2 (CEK and PKC) are involved in regulating the response to high temperature stress. Because the *cek1*Δ and *mkc1*Δ mutants show intermediate temperature sensitivity compared to the *msb2*Δ mutant (Supporting Information Fig. S3), Msb2 may regulate survival at 42°C by a mechanism that involves combinatorial action of the CEK and PKC pathways. Based on

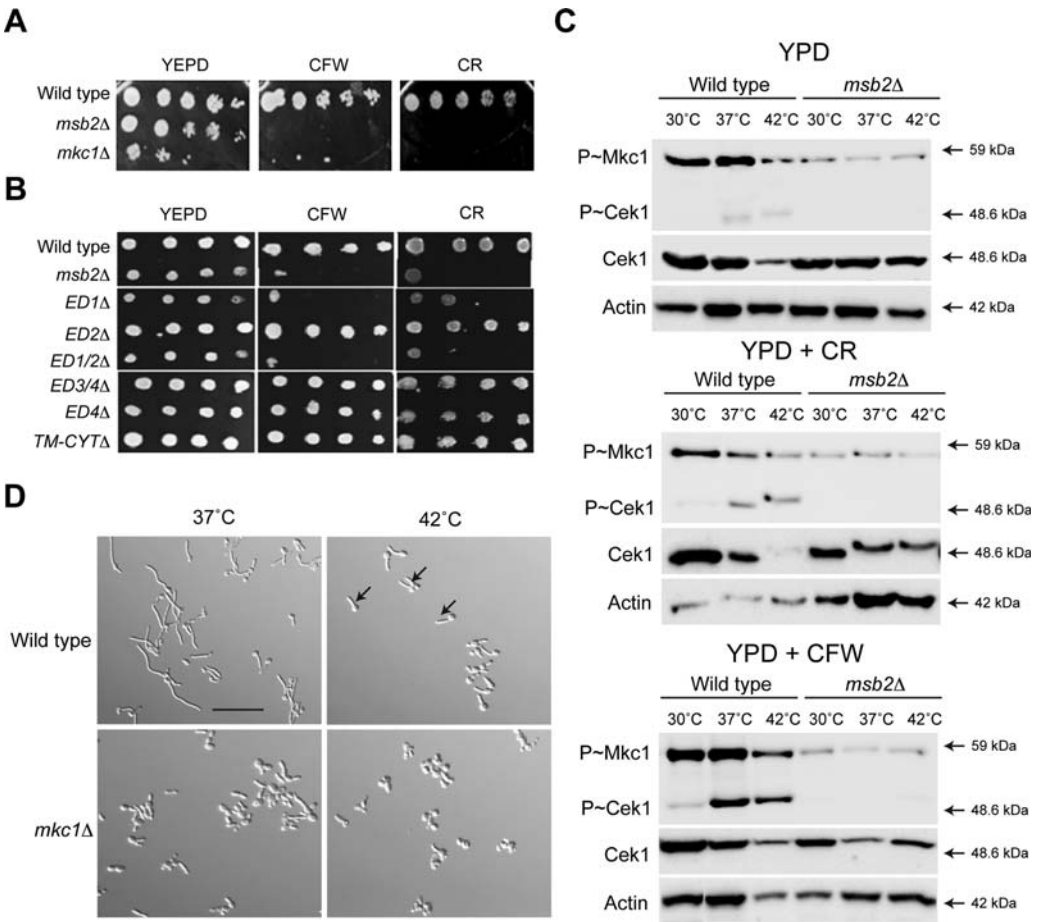

Fig. 4. Msb2 regulates the PKC pathway.
A. Growth of strains in YPD, YPD + CFW (100 μg/ml) and YPD + CR (100 μg/ml). Serial dilutions were spotted, and plates were incubated for 48 h at 37°C.
B. Growth of Msb2 deletion mutants was examined as in panel 4A.
C. Activity of the CEK and PKC pathways in response to different stimuli. Mid-log phase cells in YPD were incubated in YPD media alone, YPD + CFW (100 μg/ml) and YPD + CR (100 μg/ml) for 3 h at the indicated temperatures. Total protein (20 μg) was examined by immunoblot analysis with α-phospho p42/44 MAPK rabbit monoclonal antibodies, α-Cek1 and α-Act1 as a control for total protein levels.
D. Cells were grown to mid-log phase in YNB + 2% Glucose for 3 h at 37°C and 42°C and observed by microscopy at 20X magnification. Arrows, examples of hyphae at 42°C. Bar, 10 microns.

phospho-MAPK analysis, the MKC pathway is not activated at 42°C, and although the CEK pathway is activated at 37°C, it is not further induced at 42°C. It is possible that the two pathways (CEK and MKC), which are redundant for growth at 42°C, buffer each other's activities at 42°C. Alternatively, growth at 42°C may also involve a third pathway.

Msb2 is required to survive UPR stress and for induction of genes encoding UPR regulators and targets

Among the pathways that the PKC pathway regulates is the UPR (Patil and Walter, 2001; Wimalasena *et al*., 2008; Scrimale *et al*., 2009; Gardner *et al*., 2013). The UPR is a QC pathway that regulates the folding and degradation of proteins in the endoplasmic reticulum in response to high temperature and other stresses. The UPR is regulated by the sensor kinase Ire1, which functions in the endoplasmic reticulum (Cox *et al*., 1993; Mori *et al*., 2000). The *ire1*Δ mutant had a growth defect that resembled the *cek1*Δ and *mkc1*Δ mutants (Supporting Information Fig. S3), which indicates that Ire1 is required for growth at 42°C. In *S. cerevisiae*, Msb2 and the Kss1 MAP kinase pathway (Cek1 equivalent pathway) regulate the UPR (Adhikari *et al*., 2015). We therefore examined the possible connection between Msb2 and the UPR in *C. albicans*. The *msb2*Δ mutant was sensitive to chemical inducers of the UPR including the reducing agent dithiothreitol (DTT) and tunicamycin (Fig. 5A). In response to these compounds, the *msb2*Δ mutant had the same phenotype as the *mkc1*Δ mutant (Fig. 5A).

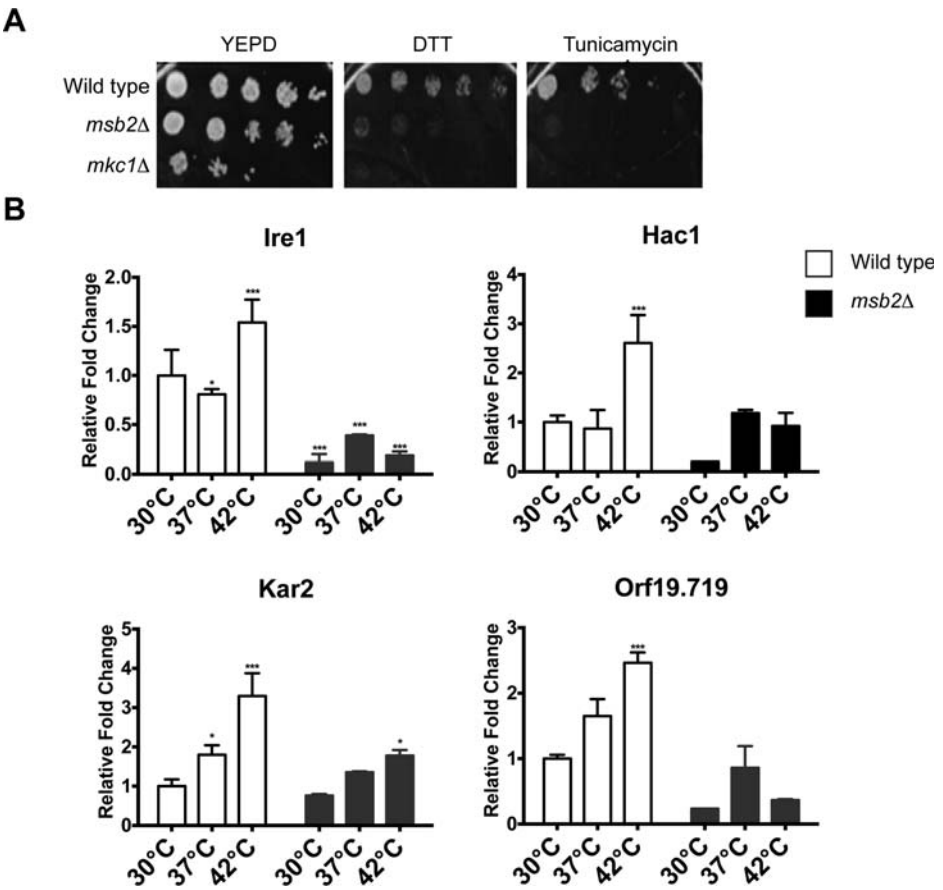

Fig. 5. Msb2 regulates the response to ER stress and expression of regulators of the UPR and other QC pathways in the endoplasmic reticulum.
A. Growth of the indicated strains on YPD, YPD + DTT (10mM) and YPD + Tunicamycin (2 μg/ml). Equal concentrations of cells were spotted in serial dilutions onto the indicated medium. Plates were incubated for 48 h at 37°C.
B. Expression of genes encoding UPR regulators by quantitative RT-PCR analysis. Cells were grown for 3 h at 30°C, 37°C and 42°C. Transcript levels were normalized to the *ACT1* gene. Levels are expressed relative to wild type, which was set to 1. Data are the mean and SD of three independent biological replicates. Error bars refer to the standard deviation between trials. The *** indicates P value <0.001, ** indicates $P<0.01$ and * indicates $P<0.5$.

Ire1 is the sensor kinase for the UPR pathway, and Hac1 is the major transcription factor for the UPR (Cox and Walter, 1996). Among the transcriptional targets of the UPR is the heat shock protein Kar2 (de Keyzer *et al*., 2009; Hale *et al*., 2010). Proteins that become mis-folded in the endoplasmic reticulum can be degraded in the cytoplasm by ERAD, which requires the ubiquitin ligase Hrd1 [(Scrimale *et al*., 2009; Kanehara *et al*., 2010; Miller *et al*., 2010) Orf19.719 in *C. albicans*]. We examined whether Msb2 played a role in regulating expression of genes encoding UPR pathway regulators, targets, or ERAD regulators. In wild-type cells, the expression of *IRE1, HAC1, HRD1* and *ORF19.719* were found to be induced by growth at elevated temperatures by a factor of >1.5-fold (Fig. 5B, white). The expression of these genes was reduced in the *msb2*Δ mutant by a factor of at least twofold at 42°C (Fig. 5B, black). These results connect Msb2 to the regulation of QC pathways that operate in the endoplasmic reticulum in *C. albicans*. It is possible that the connection is mediated by Msb2 regulation of the PKC pathway. In addition, the CEK pathway may be involved, which is supported by the intriguing observation that the temperature-dependent depletion of Cek1 is seemingly Msb2 dependent (Fig. 4C).

Ssa1 is required for survival at 42°C and regulates the CEK and PKC pathways

A third protein that was required for normal growth at 42°C was the heat-shock regulator and abundant cell-wall protein Ssa1 (Sun *et al*., 2010). Among ~40 mutants tested, the growth defect of the *ssa1*Δ and *msb2*Δ mutants were the most severe (Supporting Information Fig. S3). The *ssa1*Δ mutant was defective for hyphae formation at 42°C (Fig. 6A, Supporting Information Figs. S1 and S2A). The *ssa1*Δ mutant was also required for activation of the CEK and PKC pathways (Fig. 6B). P~MAPK levels showed some differences depending on growth conditions. Thus, Ssa1 may be part of the sensing/signalling mechanism that allows cells to respond to high-temperature stress.

Growth at 37°C induces, among other responses, the formation of hyphae. Inhibiting the function of Hsp90 mimics the heat-shock response at 30°C (Dai *et al*., 2012; Diezmann *et al*., 2012; Leach *et al*., 2012b). We

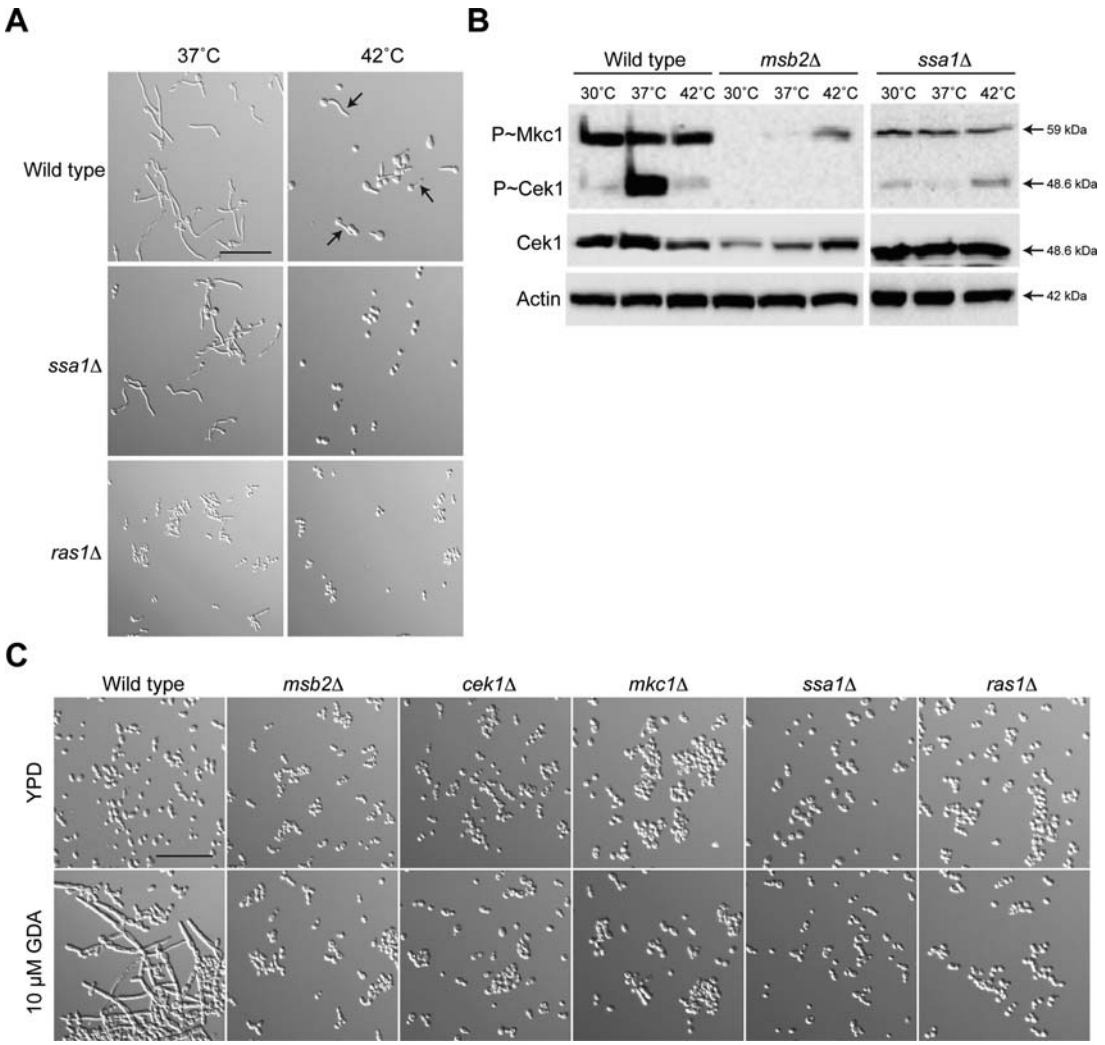

Fig. 6. Role of Ssa1 in hyphae formation and regulation of the CEK and PKC pathways.
A. Cells were grown in YNB + 2% Glu for 3 h at 37°C or 42°C. Cells were examined by microscopy at 20X magnification. Arrows refer to examples of hyphae at 42°C. Bar, 10 microns.
B. Cells were grown to mid-log phase and incubated in YNB + GlcNac media pre-warmed to 37°C for 3 h at the indicated temperatures. Total cellular protein (20 μg) from cell lysates was examined by immunoblot analysis with α-phospho p42/44 MAPK, α-Cek1 and α-Act1 antibodies (as a control for protein loading).
C. Wild type cells and the indicated mutants were incubated in YPD or YPD with 10 μM GDA at 30°C for 6 h. Cells were examined by microscopy at 20X magnification. Bar, 10 microns.

used geldanamycin (GDA), a inhibitor of Hsp90 (Wehrli and Staehelin, 1971), to induce hyphae formation at 30°C. When added to wild-type cells, GDA induced hyphae at 30°C (Fig. 6C). The *msb2*Δ, *cek1*Δ, *mkc1*Δ, and *ssa1*Δ mutants did not (Fig. 6C). The RAS pathway also regulates the response to high temperature (Shapiro *et al*., 2009; Huang *et al*., 2010; Langford *et al*., 2013; Rao *et al*., 2013). The *ras1*Δ mutant was required for hyphae production at 42°C (Fig. 6A) and GDA-dependent hyphae formation at 30°C (Fig. 6C). This experiment provides validation of the above findings. Therefore, the morphogenetic response to high temperature stress involves the action of the CEK, PKC, UPR and RAS pathways, in part through the action of the signalling pathway regulators Msb2 and Ssa1.

Discussion

Temperature is a common inducer of fungal dimorphism. Although many signalling pathways that respond to temperature stress have been identified, the sensors have not been well characterized. We show here that the signalling mucin-like glycoprotein Msb2 is a regulator of

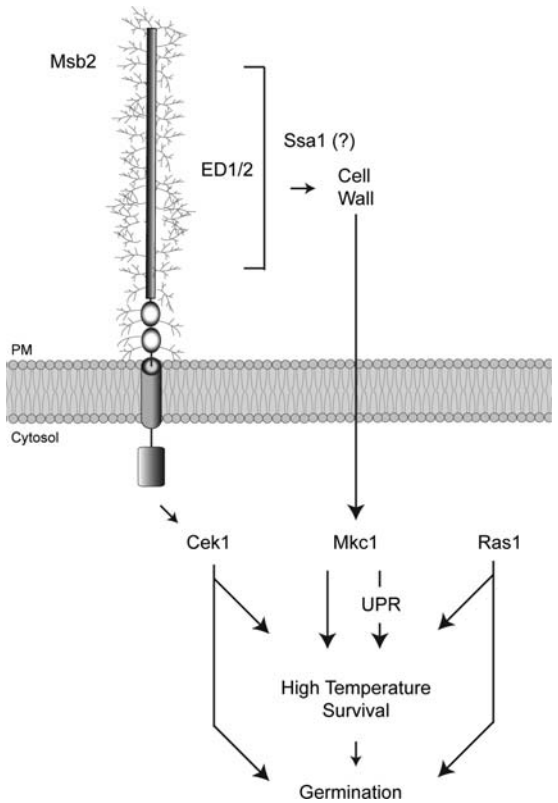

Fig. 7. The role of Msb2 and Ssa1 in regulating pathways that control the high temperature response. The signalling mucin Msb2 plays two roles in regulating the response to high temperature. Through its extracellular and cytosolic domains, Msb2 regulates the activity of the CEK pathway (Cek1). Through its extracellular (but not cytosolic domain), Msb2 regulates the PCK pathway (Mkc1). Msb2 also regulates the UPR, potentially through the PKC pathway. Together with the RAS pathway (Ras1), the CEK (Cek1) and PKC (Mkc1) pathways regulate survival and hyphae formation at high temperatures. Ssa1 regulates the CEK and PKC pathways, potentially at the level of the cell wall, through a mechanism that has yet to be elucidated, as indicated by the question mark.

thermal stress in the fungal pathogen *C. albicans*. Msb2 was required for a variety of responses that occur at higher temperatures, including optimal growth (at 37°C), survival (at 42°C), and hyphae formation (at 42°C). The identification of a regulatory protein that functions at the plasma membrane and is required for the response to thermal stress is an important step in understanding how changes in temperature are detected by fungal cells.

Msb2 controls the response to high temperature by regulating at least three different signalling pathways (Fig. 7). Msb2 is an established regulator of the CEK pathway (Puri *et al.*, 2012; Szafranski-Schneider *et al.*, 2012; Perez-Nadales and Di Pietro, 2014; Swidergall *et al.*, 2015) and was required for activation of the CEK pathway during growth at high temperatures. The extracellular and CYT domains of Msb2 were required for this response. It is reasonable to speculate that Msb2 regulates the CEK pathway by binding to and regulating cytosolic components of that pathway (Fig. 7). Msb2 also regulates thermo-tolerance through the PKC pathway. The PKC pathway is an established regulator of the response to high temperatures (Levin, 2005; LaFayette *et al.*, 2010; Diezmann *et al.*, 2012), but Msb2 has not been previously shown to regulate the PKC pathway. Interestingly, the extracellular domain of Msb2 – but not the cytosolic domain – was required to regulate the PKC pathway (Fig. 7). Although it is not clear how the extracellular domain connects to the PKC pathway, one possibility is that the highly glycosylated extracellular domain of Msb2 impacts the integrity or stability of proteins or carbohydrates in the cell wall. A second possibility is that Msb2 regulates one or more of the sensors that regulate the PKC pathway. The third pathway that Msb2 regulates is the UPR. The role of Msb2 in regulating the UPR may be mediated by its regulation of the PKC pathway (Fig. 7). The *cek1*Δ, *mkc1*Δ, and *ire1*Δ mutants show temperature sensitivity at 42°C but not to the same degree as the *msb2*Δ mutant. Thus, Msb2 may function as a 'master regulator' that coordinates the combinatorial response of these pathways to growth at high temperatures. In humans, signalling mucins similarly coordinate the action of many different pathways (Regimbald et al., 1996; Kam *et al.*, 1998; Rahn et al., 2004; Wei *et al.*, 2005; Singh and Hollingsworth, 2006; Wei *et al.*, 2006).

We also show that Ssa1 plays a critical role in thermoregulation in *C. albicans*. Ssa1 is putative heat shock protein that is present on the cell surface and in the cytosol (Sun *et al.*, 2010). Given that Ssa1 is a cell wall protein, Ssa1 may interact with Msb2 or the cell wall to detect and regulate adaptation to thermal stress. Alternatively, Ssa1 may regulate MAPK signalling as a heat shock protein in the cytosol. These possibilities are not mutually exclusive.

Given that multiple pathways regulate the response to thermal stress, it may not be surprising that different phenotypes emerge by examining different mutants. For example, Msb2 controls dimorphism by regulating the CEK and PKC pathways; however, the *msb2*Δ mutant forms hyphae at 37°C. Furthermore, cells lacking Msb2 retain the ability to mount one type of thermotolerance response (as evinced by the induction of cytosolic HSP encoding genes), but not another (based on the diminished capacity of UPR targets and regulators). Thus, Msb2 may play a particular role in the cell's overall heat shock response. In the future, it will be important to understand how Msb2 function may connect to Hsp90, the key regulator of temperature-dependent morphogenesis (Shapiro *et al.*, 2009; Shapiro and Cowen, 2010; Senn *et al.*, 2012; Shapiro *et al.*, 2012b). Hsp90 controls

temperature sensing through many pathways, including the RAS pathway (Shapiro *et al*., 2009), and it may be interesting to know whether Msb2 function impacts the RAS pathway. Like Ssa1, Hsp90 also localizes on the cell surface as well as in the cytoplasm (Urban *et al*., 2003; Pitarch *et al*., 2004), so it will be instructive to define from where different HSPs regulate the response.

Intriguingly, several connections between signalling mucins and the heat shock response have been made in other systems. In humans, HSP90 targets MUC1 to the mitochondria (Ren *et al*., 2006). A related HSP, HSP70, regulates MUC1 secretion (Fang *et al*., 2013). It will be interesting to learn more about how heat shock proteins regulate signalling mucin function and vise versa.

In summary, we have identified a new role for Msb2 (and Ssa1) in regulating survival and dimorphism at high temperatures in *C. albicans*. Msb2 regulates multiple pathways to regulate the response. Given that temperature is critical for dimorphism and virulence in *C. albicans* and other fungal species, the identification of new regulators may provide insight into the molecular basis of fungal pathogenesis.

Experimental procedures

Strains, media and growth conditions

C. albicans strains used in this study are listed in Table 1. CAI4 strain was the wild type control for all experiments. Strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) or YNB (2% glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% agar). For growth sensitivity assays, cells were grown to saturation for 16 h, and cells were diluted to OD_{600} 0.1 for spotting serial dilutions onto media. For immunoblot analysis, cells were grown in YPD, YNB and YNB with 1.25% *N*-Acetyl-D Glucosamine (GlcNac) for 3 h.

Msb2 deletion derivative strains were constructed by a PCR-based approach (Wilson *et al*., 2000) using the URA-Blaster technique (Fonzi and Irwin, 1993). PCR primers were designed to amplify the 3HA-URA3–3HA cassette in plasmid pCaMPY-3XHA (Liu *et al*., 2007), tailed with an additional 80 nucleotides of sequence flanking the open reading frame (ORF) of the region to be disrupted. PCR products were verified by gel electrophoresis. The purified PCR product (10 μg) was transformed into the MSB2/msb2Δ strain with Frozen-EZ Yeast Transformation II Kit (Zymo Research, CA, USA). Uracil-deficient agar (YNB-URA) media was used for selection of *URA* positive colonies. PCR-based analysis of transformants was performed with primer pairs internal to the wild-type locus and the *URA* cassette. Homozygous transformants were reverted to the URA negative phenotype by selection on 5-fluororotic acid (5-FOA). Domain deletion knockouts were verified by immunoblot analysis. Hence, all deletions represent the sole copy of *MSB2* expressed at the *MSB2* locus from its endogenous promoter.

Protein and immunoblot Analysis

Anti-phospho p42/44 MAPK ERK1/2 Thr202/Tyr204 rabbit monoclonal antibody was used to detect P∼Cek1 and P∼Mkc1 (Signalling Technology). Anti-HA antibodies were used to detect HA-Msb2 (Abcam, ab75640). To detect actin, anti-Act1 antibody was used (Santa Cruz Biotechnology, sc47778). Goat anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories, Inc.) was used as the secondary antibody.

For protein extraction, cell lysis was performed as described (Puri *et al*., 2012). Cells were grown to mid-log phase and harvested by centrifugation. Cell pellets were resuspended in 300 μl of 10% TCA Buffer (10 mM Tris-HCl pH 8.0, 10% trichloroacetic acid, 25 mM NH_4OAc, 1 mM sodium EDTA). Cells were lysed using acid washed glass beads by vortexing for 40 s × 10 cycles using Fast PrepH-24 Instrument (MP Biomedicals LLC). Cell debris and glass beads were separated by centrifugation. Cell lysates were then centrifuged at 4°C for 10 min at 13,000 rpm. 150 μl of resuspension buffer (0.1 M Tris-HCl pH 11.0, 3% SDS) was added to the pellets. For immunoblotting, normalized protein content (20 μg) was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% Milk or Bovine Serum albumin (BSA) in TBS containing 0.1% Tween-20 at (TBST) at room temperature for 1 h. Blots were incubated in primary antibody for 16 h at 4°C. Membranes were washed twice with TBST and probed with a secondary antibody for 1 h at 25°C. Secondary antibodies were detected using SuperSignal West Pico detection kit (Thermo Scientific).

For quantifying Msb2 shedding in liquid culture media, overnight cultures were diluted to OD_{600} 0.3 and allowed to grow for 3 h under different conditions. Culture supernatants were collected, filter sterilized and precipitated with acetone. Precipitated proteins were quantified using BCA protein quantification kit (Thermo Scientific) and normalized protein content (20 μg) was examined by slot blot with anti-HA antibody.

Sensitivity to drugs and temperature

Aliquots of overnight cultures were washed two times with phosphate buffer saline. Drop tests were performed by spotting 5 μl of serial 10-fold dilutions of overnight culture of an optical density OD_{600} of 0.1 on YPD plates, YPD plates were supplemented with CR (100 μg/ml), CFW (100 μg/ml), DTT (30 mM) and tunicamycin (2 μg/ml) at the indicated concentrations. For temperature sensitivity, plates were incubated at 30°C, 37°C, 42°C for 48 h and photographed. Growth curves for each strains were recorded by measuring their OD_{600} at 6 h interval for up to 40 h using a spectrophotometer (Lambda 25, UV/VS Spectrophotometer, Perkin Elmer).

Germination assay

Overnight YPD cultures were diluted in YNB+2%Glucose, YNB+1.25%GlcNAc, Spider and YPD+10% Serum media

Table 1. *C. albicans* strains used in the study.

Strain	Genotype	Reference
CAI4	Δ*ura3::imm434/*Δ*ura3::imm434*	(Fonzi and Irwin, 1993)
Msb2-HA/*msb2*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA*	(Puri *et al*., 2012)
Msb2/*msb2*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2*	(Puri *et al*., 2012)
*msb2*Δ	Δ*ura3::imm434/*Δ*ura3::imm434* Δ/Δ*msb2::URA*	(Puri *et al*., 2012)
*ED1*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 100-450*Δ	This study
*ED2*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 450-900*Δ	This study
*ED1/2*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 100-900*Δ	This study
*ED3/4*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 900-1250*Δ	This study
*ED4*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 1050-1250*Δ	This study
*TM-CYT*Δ	Δ*ura3::imm434/*Δ*ura3::imm434 Msb2/msb2*Δ*::FRT/Msb2-HA 1250-1409*Δ	This study
*cek1*Δ	*ura3/ura3 cek1*Δ*::hisG/cek1*Δ*::hisG*	(Csank *et al*., 1998)
*mkc1*Δ	*CAI-4*, mkc1Δ::hisG/mkc1Δ::hisG mkc1*::pCK70* (URA3*)*	(Kumamoto, 2005)
*ire1*Δ	*Aaron Mitchell kinase collection*	(Blankenship *et al*., 2010)
*hac1*Δ	*ura3::*λ*imm434/ura3::*λ*imm434,his1::hisG/his1::hisG,arg4::hisG/arg4::hisG, hac1::loxP/hac1::loxP Clp30 (URA3 HIS1 ARG4)*	(Wimalasena *et al*., 2008)
*ssa1*Δ	Δ*ura3::imm434* Δ*ssa1::FRT/*Δ*ura3::imm434*Δ*ssa1::FRT*	(Sun *et al*., 2010)
*ssa2*Δ	Δ*ura3::imm434* Δ*ssa2::FRT/*Δ*ura3::imm434*Δ*ssa2::FRT*	(Sun *et al*., 2010)
*muc1*Δ	*his1*Δ*/his1*Δ *leu2*Δ*/leu2*Δ *arg4*Δ*/arg4*Δ*::C.dubliniensis ARG4 URA3/ura3*Δ*:: imm434,IRO1/iro1*Δ*::imm434orf19.4183*Δ*::C.dubliniensisHIS1/orf19.4183*Δ*::: C.maltosaLEU2*	(Nobile *et al*., 2003)
*sgt1*Δ	*his1*Δ*/his1*Δ *leu2*Δ*/leu2*Δ *arg4*Δ*/arg4*Δ*::C.dubliniensisARG4 URA3/ura3*Δ*::imm434 IRO1/iro1*Δ*::imm434orf19.4183*Δ*::C.dubliniensisHIS1/orf19.4089*Δ*:::C.maltosaLEU2*	(Nobile *et al*., 2003)
*cpp1*Δ	*ura3/ura3 cpp1*Δ*::hisG/cpp1*Δ*::hisG*	(Csank *et al*., 1998)
*cph1*Δ	*cph1* Δ*::hisG/cph1* Δ*::hisG-URA3-hisG, ura3* Δ*/ura3* Δ	(Fonzi and Irwin, 1993)
*sap1/2/3*Δ	*sap1*Δ*::hisG/sap1*Δ*::hisG sap2*Δ*::hisG/sap2*Δ*::hisG sap3*Δ*::hisG sap3*Δ*::hisG*	(Kretschmar *et al*., 2002)
*sap4/5/6*Δ	*sap6*Δ*::hisG/sap6*Δ*::hisG sap4*Δ*::hisG/sap4*Δ*::hisG sap5*Δ*::hisG/sap5*Δ*::hisG*	(Sanglard *et al*., 1997)
*sap6*Δ	*sap6::hisG/sap6::hisG-URA3-hisG*	(Kumar *et al*., 2015)
*sap8*Δ	Δ*sap8::hisG/*Δ*sap8::hisG-URA3-hisG*	(Puri *et al*., 2012)
*sap9/10*Δ	Δ*sap8::hisG/*Δ*sap8::hisG-URA3-hisG/SAP8*	(Albrecht *et al*., 2006)
*ssk1*Δ	Δura3::imm434/Δura3::imm434 Δssk1::hisG/Δssk1::hisG-URA3-hisG	(Chauhan *et al*., 2003)
*ssk2*Δ	*ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP*	(Cheetham *et al*., 2007)
*hog1*Δ	*ura3:: λimm434/ura3:: λimm434, his1:: hisG/his1:: hisG, hog1:: LoxP-ura3-LoxP, hog1:: LoxP-HIS1-LoxP* Clp20 (*URA3, HIS1*)	(Smith *et al*., 2004)
*ras1*Δ	*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 ras1::hisG/ras1::hisG::RAS1*^{G13V}*-URA3*	(Piispanen *et al*., 2011)
*cyr1*Δ	*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 cyr1*Δ*::hisG::cyr1*Δ*::hisG*	(Lindsay *et al*., 2012)
*tpk1*Δ	*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 his1::hisG/his1::hisGarg4::hisG/arg4::hisG tpk1::ARG4/tpk1::URA3*	(Piispanen *et al*., 2011)
*tpk2*Δ	*ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 his1::hisG/his1::hisGarg4::hisG/arg4::hisG tpk2::ARG4/tpk2::URA3*	(Piispanen *et al*., 2011)
*efg1*Δ	*ura3::*λ*imm434/ura3::*λ*imm434 efg1::hisG/efg1::hisG-URA3-hisG*	(Lo *et al*., 1997)
*sho1*Δ	Δ*ura3::imm434/*Δ*ura3::imm434,* Δ*/*Δ*sho1::URA*	Dr. Edgerton lab strain
*sln1*Δ	Δ*ura3::imm434* Δ*sln1::FRT/*Δ*ura3::imm434*Δ*sln1::FRT*	Dr. Edgerton lab strain
*wsc1*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*wsc2*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*wsc4*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*bck1*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*mkk2*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*hwp1*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*hwp2*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*mp65*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*cdc28*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*ste11*Δ	Aaron Mitchell kinase collection	(Blankenship *et al*., 2010)
*hst7*Δ	Aaron Mitchell kinase collection	Mitchell collection
*cst20*Δ	Aaron Mitchell kinase collection	Mitchell collection
*cek2*Δ	Aaron Mitchell kinase collection	Mitchell collection

and incubated for 3 h with shaking at 37°C, 42°C and visualized at 20X magnification using differential interference contrast (DIC) microscopy using a Zeiss Axio Fluorescence microscope. For Hsp90 compromise mediated germination, diluted cultures were grown for 6 h in YPD and YPD media supplemented with 10 μM GDA at 30°C. The percentage of hyphae or yeast form cells was determined by the number of yeast/hyphal cells divided by total number of cells by microscopy (500 cells were counted/strain). The germinated cells were screened for survival at various temperatures by determining Colony Forming Units (CFU). Cells grown at the indicated temperatures

and plated on YPD agar media and compared to control cells to determine percent survival.

Quantitative real time PCR (qRT-PCR)

Primers for qPCR used in the study are listed in Table 2. To monitor expression levels of heat shock genes and unfolded protein response genes at different temperatures, overnight cultures of wild-type cells and the *msb2*Δ mutant were inoculated in YPD media to 5ml final volume and incubated for 3 h with shaking at 30°C, 37°C and 42°C. Cultures were harvested and cell pellets were resuspended in 1 ml of TRIzol® Reagent (Life Technologies) and vortexed (4 cycle, 6m/s) with acid washed glass beads using a Fast Prep instrument (MP Biomedicals). Chloroform (200 μl) was added in the lysed cells and mixed vigorously for 15 s and kept at room temperature for 2 min. Cell lysates were centrifuged at 11,500 × *g* for 10 min at 4°C. RNA containing upper aqueous layer was mixed with 0.5 volume of 100% ethanol to precipitate total RNA. Total precipitated RNA was purified using an RNeasy kit from Qiagen according to manufacturer's instructions. Following isolation, RNA purity and concentrations were determined using gel electrophoresis and Nanodrop 1000 (Thermo Scientific). Total cDNA was synthesized for each sample using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instruction with equal amounts of RNA (1 μg in 20 μl reaction).

To quantify the transcript levels of heat shock genes and UPR regulators, PCR primers were designed to amplify 100 to 150 bps of the target gene. Synthesized cDNA (1 μl) was used to amplify transcripts of selected genes. Amplification and detection were carried out in 96-well plates on an iCycler iQ real-time detection system (Bio-Rad). All samples contained 10 μl iQ SYBR Green supermix (2× concentration), 1 μl forward primer, 1 μl reverse primer, 1 μl template (cDNA) and 17 μl nuclease-free water. Fluorescent data were collected and analyzed with iCycler iQ software. Threshold value (ΔC_T) was obtained by difference between C_T values of the target gene and the control genes (*ACT1* and *GAPDH2*, which gave the same results and were used interchangeably). Results represent the mean of at least three independent biological replicates. Statistical analysis was determined by Student's *t*-test using Expert qPCR Analysis software.

Table 2. Primers for qPCR used in the study.

SFL1 F	
ACAACAGCAACAGCAACAGC	
SFL1R	
GTGGAATTGGTCCGCTAAAA	
HSP21F	
TGCAGAAATTGGTGAGCAAG	
HSP21 R	
TTGCAGCTGCTTTGGAAATA	
HSP 90 F	
AGTTGAAACCGATGGAGCTG	
HSP 90 R	
ATGGTTCGTCCAAGGTGAAA	
HSP 70 F	
AACCTACTGCTGCTGCCATT	
HSP 70 R	
AAGTACCACCACCCAAATCG	
IRE1 F	
TGCCCCATCCTTTGAAAGTG	
IRE1 R	
TCTGAAACTCATAGCCACCCA	
HAC1 F	
GAGGATGAACACCAAGAAGAAGG	
HAC1 R	
AGATGGTGGTGTAGACGTCA	
KAR2 F	
CAATGAACCTACTGCTGCCG	
KAR2 R	
AGCCAAGACTTCGAAAACACC	
Orf19.719 F	
ACACTGTTAAGAGGATGGCAAG	
Orf19.719 R	
TCCACTTGCCCAGACTCATT	
ACTIN1-F	
TCGGTGACGAAGCTCAATCCAAGA	
ACTIN1-R	
CAATGGATGGACCACTTTCGTCGT	
TDH3/GAPDH-F	
AAGAGTTGCTTTGGGCAGAA	
TDH3/GAPDH-R	
GTCGTCACCAGAAGCAGTGA	

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