Cbk1 kinase and Bck2 control MAP kinase activation and inactivation during heat shock

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ABSTRACT Saccharomyces cerevisiae Cbk1 kinase is a LATS/NDR tumor suppressor orthologue and component of the Regulation of Ace2 and Morphogenesis signaling network. Cbk1 was previously implicated in regulating polarized morphogenesis, gene expression, and cell integrity. Here we establish that Cbk1 is critical for heat shock and cell wall stress signaling via Bck2, a protein associated with the Pkc1-Mpk1 cell integrity pathway. We demonstrate that cbk1 and bck2 loss-of-function mutations prevent Mpk1 kinase activation and Mpk1-dependent gene expression but do not disrupt Mpk1 Thr-190/Tyr-192 phosphorylation. Bck2 overexpression partially restores Mpk1-dependent Rlm1 transcription factor activity in cbk1 mutants, suggesting that Bck2 functions downstream of Cbk1. We demonstrate that Bck2 precisely colocalizes with the mitogen-activated protein kinase (MAPK) phosphatase Sdp1. During heat shock, Bck2 and Sdp1 transiently redistribute from nuclei and the cytosol to mitochondria and other cytoplasmic puncta before returning to their pre-stressed localization patterns. Significantly, Cbk1 inhibition delays the return of Bck2 and Sdp1 to their pre-stressed localization patterns and delays Mpk1 Thr-190/Tyr-192 dephosphorylation upon heat shock adaptation. We conclude that Cbk1 and Bck2 are required for Mpk1 activation during heat shock and cell wall stress and for Mpk1 dephosphorylation during heat shock adaptation. These data provide the first evidence that Cbk1 kinase regulates MAPK-dependent stress signaling and provide mechanistic insight into Sdp1 phosphatase regulation.

INTRODUCTION

Stress response and cell growth must be precisely coordinated to ensure cell survival during adverse environmental conditions (Brauer et al., 2008). Typically, stress signaling promotes adaptive mechanisms by influencing the pattern of gene expression (Mager and De Kruijff, 1995). Adverse environmental conditions frequently lead to cell cycle delays, reflecting a link between stress and growth signaling (Pearce and Humphrey, 2001; Clotet and Posas, 2007). Similarly, aberrant cell growth can reduce the effectiveness of stress response pathways, leading to decreased cellular resistance to environmental stress.

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Abbreviations used: CR, Congo red; CW, calcofluor white; CWI, Cell Wall Integrity; Hyg B, hygromycin B; MAPK, mitogen-activated protein kinase; RAM, Regulation of Ace2 and Morphogenesis.

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Two Saccharomyces cerevisiae signaling pathways, the Cell Wall Integrity (CWI) and the Regulation of Ace2 and Morphogenesis (RAM) signaling networks, provide insight into the mechanisms that coordinate cell growth control and stress signaling (Nelson et al., 2003; Levin, 2005; Chen and Thorner, 2007; Fuchs and Mylonakis, 2009). The S. cerevisiae CWI pathway is a protein kinase C–dependent and mitogen-activated protein kinase (MAPK) stress-signaling pathway that is activated in response to heat shock and cell wall damage (Martin et al., 2000; Harrison et al., 2004; Levin, 2005; Fuchs and Mylonakis, 2009). Heat shock and cell wall stress cause protein kinase C (Pkc1) to activate a downstream MAP kinase cascade comprising three protein kinases, MAPKKK (Bck1), MAPKK (Mkk1/2), and MAPK (Mpk1/Slt2). The terminal CWI kinase Mpk1 induces a variety of cellular responses by influencing gene expression and is subject to negative regulation by MAPK kinase phosphatases (Mattison et al., 1999; Martin et al., 2000, 2005; Collister et al., 2002; Hahn and Thiele, 2002; Flandez et al., 2004; Harrison et al., 2004; Chen and Thorner, 2007). A key substrate for Mpk1 during heat shock and cell wall stress is Rlm1, a MADS-box transcription factor that promotes the expression of cell wall maintenance proteins (Watanabe et al., 1995; Dodou and Treisman, 1997; Heinisch et al., 1999; Jung et al., 2002; Garcia et al., 2004; Fuchs and Mylonakis,
Cbk1- and Bck2-regulated mRNAs, suggesting that Cbk1 also influences cell integrity via Ssd1-independent mechanisms (Kurischko et al., 2008). Although the relationship between the RAM and CWI signaling networks has not been established, it seemed plausible that these two signaling networks are functionally linked. Here we provide molecular and genetic evidence that Cbk1 kinase regulates Mpk1 activation and Mpk1-dependent transcription during heat shock and cell wall stress via the CWI pathway-associated Bck2. We establish that Cbk1 and Bck2 are required for Mpk1 inactivation during heat shock adaptation via the MAP kinase phosphatase Sdp1. Our experiments provide the first evidence that Cbk1 and MAP kinase signaling networks are functionally linked. Given the conservation of RAM and CWI signaling proteins among eukaryotes, this work may anticipate conserved mechanisms for LATS/NDR tumor suppressor kinases in regulating cell growth and stress signaling via MAPK pathways.

RESULTS

BCK2 is a dosage suppressor of conditional cbk1-8 mutant cells

Conditional cbk1-8 mutant cells display severe defects in cell integrity when shifted to restrictive temperature (Kurischko et al., 2008, 2011a). To gain insight into the role of Cbk1 in maintenance of cell integrity, we screened a yeast DNA library for dosage suppressors of the conditional lethality of cbk1-8 mutant cells (see Materials and Methods). We previously identified and described several cbk1 dosage suppressors that encode cell wall biosynthesis proteins whose expression is influenced by the Cbk1 substrate and mRNA-binding protein Ssd1 (Kurischko et al., 2008, 2011a). These findings are consistent with the model that Cbk1 modulates the synthesis of a subset of cell wall biosynthesis proteins via Ssd1 regulation (Jansen et al., 2009; Kurischko et al., 2011a).

We also identified cbk1 dosage suppressors whose mRNAs are not known to interact with Ssd1 (Kurischko et al., 2008). One such dosage suppressor plasmid (pGPL64-BCK2-RPH1) contained a chromosomal fragment encoding BCK2 and RPH1 (Figure 1A). Bck2 is a bypass suppressor of the protein kinase C- and MAPK-dependent CWI pathway and has been implicated in regulating gene expression (Lee et al., 1993; Di Como et al., 1995; Ferrezuelo et al., 2009). Rph1 is a histone demethylase that is involved in regulating gene expression and mediating DNA damage response and has not been previously implicated in regulating cell integrity or polarized growth (Jang et al., 1999; Kim et al., 2002; Liang et al., 2003; Ohyama et al., 2010; Kurischko et al., 2011b). Conditional cbk1 mutants exhibit severe cell morphology defects and die by cellular lysis (Kurischko et al., 2008).

Recent data indicate that Cbk1 kinase influences cell integrity by modulating the function and localization of the mRNA-binding protein Ssd1 (Jansen et al., 2009; Kurischko et al., 2011a, 2011b). Ssd1 associates with a subset of mRNAs, many of which encode cell wall biosynthesis proteins (Hogan et al., 2008; Jansen et al., 2009; Ohyama et al., 2010; Kurischko et al., 2011a). In the absence of Cbk1 phosphorylation, Ssd1 and its associated mRNAs constitutively localize to mRNA-processing bodies (P-bodies) and stress granules, which are known to repress translation during cellular stress (Kurischko et al., 2011a). These data suggest that conditional cbk1 mutants die by lysis because the cell wall proteins encoded by Ssd1-associated mRNAs are translationally repressed. In support, several dosage suppressors of conditional cbk1 mutants encode Ssd1-associated mRNAs of cell wall biosynthesis proteins, including the mannoproteins Sim1, Srl1, and Ccw12 (Kurischko et al., 2005, 2011a; Hogan et al., 2008). These data suggest that Cbk1 regulates cell integrity during polarized growth and stress response via Ssd1 by modulating the expression of a subset of cell wall proteins (Jansen et al., 2009; Kurischko et al., 2011a).

It is intriguing that not all cbk1 dosage suppressors encode Ssd1-associated mRNAs, suggesting that Cbk1 also influences cell integrity via Ssd1-independent mechanisms (Kurischko et al., 2008). Although the relationship between the RAM and CWI signaling networks has not been established, it seemed plausible that these two signaling networks are functionally linked. Here we provide molecular and genetic evidence that Cbk1 kinase regulates Mpk1 activation and Mpk1-dependent transcription during heat shock and cell wall stress via the CWI pathway-associated Bck2. We establish that Cbk1 and Bck2 are required for Mpk1 inactivation during heat shock adaptation via the MAP kinase phosphatase Sdp1. Our experiments provide the first evidence that Cbk1 and MAP kinase signaling networks are functionally linked. Given the conservation of RAM and CWI signaling proteins among eukaryotes, this work may anticipate conserved mechanisms for LATS/NDR tumor suppressor kinases in regulating cell growth and stress signaling via MAPK pathways.
CWI pathway mutants, including sensitivity to CW, CR, caffeine, and hygromycin B, were assayed for integrity and CWI signaling have not been reported. We therefore focused the remainder of this study on defining the roles of Bck1 and Bck2 with respect to CWI signaling.

The phenotypes of cbk1-8 mutants are also hypersensitive to CW, rapamycin, and hygromycin B, as were cbk1-8 and mpk1Δ cells, but not to CR and caffeine. In contrast, rph1Δ cells were not hypersensitive to any of the drugs tested. These data suggest that Bck2, but not Rph1, is important for CWI pathway function. To investigate the role of Cbk1 and Bck2 in CWI signaling, we analyzed Rlm1 reporter activation in cbk1-8 and bck2Δ mutants (Figure 2A). Rlm1 is a MADS-family transcription factor that is phosphorylated and activated by Mpk1 during heat shock, cell wall stress, and caffeine treatment (Jung et al., 2002). Once activated, Rlm1 induces the expression of a subset of cell wall biosynthesis genes that help maintain cell wall integrity and ensure stress survival. We introduced a previously described reporter plasmid that bears both a lexA-RLM1 fusion and a lacZ reporter gene with lexA-binding sites and measured lacZ activity in cells after stress induction (Kirchrath et al., 2000). As a control, we conducted parallel experiments with wild-type, mpk1Δ, and knr4Δ cells, the latter of which lacks a Bck2-associated protein and a putative scaffold protein required for Mpk1-dependent Rlm1 activation (Lee et al., 1993; Martin-Yken et al., 2002, 2003). In wild-type cells, heat shock and caffeine treatment induce the Mpk1-dependent phosphorylation of Rlm1 and hence the activation of the Rlm1 reporter (Watanabe et al., 1995; Dodou and Treisman, 1997; Jung et al., 2002). Peak reporter activity occurs in wild-type cells at 60 min upon heat shock and at 120 min upon caffeine treatment (Figure 2A). In contrast, Rlm1 reporter activation was completely inhibited in cbk1 mutant cells, indicating that Cbk1 kinase is essential for Rlm1 activation during CWI stress signaling. Rlm1 reporter activity was also diminished by ~6- to 7-fold in bck2Δ and ~10-fold in knr4Δ mutants relative to peak reporter activities in stressed wild-type cells (Figure 2A). In contrast, RPH1 deletion only modestly diminished peak Rlm1 activity (<10% reduction; Supplemental Figure 2A). These data indicate that Cbk1, Bck2, and Knr4 are critical for Mpk1-dependent Rlm1 activation during heat shock and cell wall stress.

Low Rlm1 reporter activities could reflect impaired Mpk1 kinase activity or impaired Rlm1-dependent gene expression. Thus, as an independent method to determine whether Cbk1 and Bck2 influence Mpk1 kinase activity during stress signaling, we immunoprecipitated Mpk1-GFP from heat-shocked cells and conducted in vitro kinase assays using myelin basic protein (MBP) as substrate. As expected, immunoprecipitated Mpk1 from wild-type cells had significant kinase activity toward MBP (Figure 2B). In contrast, the Mpk1 activity from cbk1-8, bck2Δ, and knr4Δ cells was as low as that of Mpk1 from bck1Δ cells, which lack the activating MAPKKK (Figure 2B). These data establish that Cbk1, Bck2, and Knr4 are essential for Mpk1 kinase activation during heat shock.
Our data suggest that Bck2-mediated dosage suppression of cbk1 mutants occurs by restoring Mpk1 kinase activity and CWI-dependent gene expression. To test this hypothesis, we introduced BCK2 suppressor plasmids into conditional cbk1-8 mutants and quantified Rlm1 reporter activity after heat shock induction and caffeine treatment. We observed that high-copy BCK2 plasmids restored Rlm1 reporter activity in heat-shocked and caffeine-treated cells to ∼30% (heat shock) and ∼20% (caffeine) of the levels of cells expressing wild-type Cbk1 (Figure 2C). It is intriguing that Rph1 overexpression also partially restored the diminished Rlm1 reporter activity in heat-shocked and caffeine-treated cbk1-8 cells, suggesting that Rph1 overexpression elevates Rlm1-dependent transcription (Supplemental Figure S2B). The high-copy BCK2-RPH1 plasmid restores Rlm1 reporter activity in cbk1 mutants to the same degree as the BCK2 and RPH1 plasmids (Supplemental Figure S2B). These data support the hypothesis that Bck2 and Rph1 overexpression suppresses the conditional lethality of cbk1 mutants by elevating Rlm1-dependent gene expression. Our data are consistent with the model that Cbk1 functions via Bck2 to mediate Mpk1 activation during stress signaling. Because Rph1 is a histone H3 demethylase and functions as a transcriptional repressor (Kim et al., 2002), we hypothesize that Rph1 overexpression induces epigenetic changes that indirectly enhance Rlm1-mediated gene expression.

**Cbk1, Bck2, and Knr4 are not required for CWI pathway activation**

It is well established that heat shock, cell wall stress, and caffeine treatment lead to CWI pathway and Mpk1 activation (Kamada et al., 1995; Harrison et al., 2004; Imazu and Sakurai, 2005; Levin, 2005). MAPKK-dependent Mpk1 Thr-190/Tyr-192 phosphorylation is a common marker for CWI pathway activation and is readily detected via immunoblots probed with a phosphorylation-specific p44/42 MAPK antibody (Martin et al., 2000). To determine whether Cbk1 and Bck2 are required for Mpk1 Thr-190/Tyr-192 phosphorylation, we monitored Mpk1 phosphorylation in heat-shocked and caffeine-treated cbk1-8 and bck2Δ cells. Representative immunoblots are presented (Figure 3). Because heat shock leads to increased Mpk1 expression (Jung and Levin, 1999; Mattison et al., 1999; Hahn and Thiele, 2002), we plotted the ratios of Thr-190/Tyr-192-phosphorylated Mpk1 to total Mpk1 (pMpk1/tMpk1), as determined from two independent experiments (Figure 3 and Supplemental Table S1). Within 15 min of heat shock and 45–60 min of caffeine treatment, the levels of phosphorylated Mpk1 increased significantly (approximately fourfold) in cbk1-8, bck2Δ, and wild-type cells (Figure 3, A and B). The same is true for knr4Δ cells (Figure 3, A and B; Martin-Yken et al., 2002). These data indicate that Cbk1, Bck2, and Knr4 are not essential for CWI activation and Mpk1 phosphorylation. Thus Cbk1, Bck2 and Knr4 must function after Mkk1/Mkk2-dependent phosphorylation with respect to Mpk1 kinase activation, similar to the chaperone Hsp90 and the cochaperone Cdc37 (Hawle et al., 2007; Truman et al., 2007).
Bck2 localizes to the nucleus, cytosol, and bud neck during logarithmic growth

Our data suggest that Cbk1 and Bck2 are important for Mpk1 activation, perhaps by promoting Mpk1 protein interactions. If this model is correct, Bck2 should at least transiently localize to the nucleus or to sites of polarized growth (bud neck and bud cortex) where Mpk1 and Cbk1 localize (Kamada et al., 1995; Hahn and Thiele, 2002; Jung et al., 2002; van Drogen and Peter, 2002; Weiss et al., 2002). To investigate Bck2 localization, we generated strains expressing Bck2–green fluorescent protein (GFP) under the control of its own promoter. The cells expressing Bck2-GFP displayed no obvious phenotypes, suggesting that Bck2-GFP is fully functional. We discovered that during logarithmic growth, Bck2-GFP localizes to the cytosol and is enriched in the nucleoplasm throughout the cell cycle (Figure 4A). We confirmed the nuclear localization by coexpressing a red fluorescent protein (RFP)–tagged nuclear marker. In addition, Bck2-GFP localized to a prominent spot at the nuclear periphery in all cells (n = 100; Figure 4A, arrowhead). We also occasionally observed modest enrichment of Bck2 at the bud neck in a small fraction of cells (<1% of cells; Figure 4B). It is likely that the number of cells with Bck2 at the bud neck is an underestimate because the Bck2-GFP fluorescence signal is difficult to detect over the diffuse cytosolic Bck2-GFP signal. Bck2 overexpression via the constitutive GPD promoter enhanced detection of Bck2 at the bud neck in small- and large-budded cells (Figure 4C; 38%; n = 60). These data support the proposed functional interactions among Bck2, Mpk1, and Rlm1 in the nucleus and cytosol.

Bck2 localizes to cytoplasmic puncta upon heat shock

To determine how cellular stress influences Bck2 localization, we monitored Bck2-GFP in caffeine-, CW-, salt-, and heat-stressed cells. Caffeine, CW treatment, and hypertonic stress did not trigger any obvious changes in Bck2 nuclear or cytosolic localizations (Figure 5A and data not shown). In contrast, within 15 min of heat shock, Bck2 radically redistributed from the nucleoplasm and cytosol to 5–11 prominent cytoplasmic puncta (Figure 5, A and B). Corresponding time-lapse microscopy indicates that the puncta derive from redistributed cytoplasmic and nuclear Bck2 (Figure 5B and Supplemental Movie S1). Heat shock did not appear to eliminate or disrupt the single Bck2 nuclear spot (Figure 5B, arrowheads, and Supplemental Movie S1). Significantly,
The punctate pattern of Bck2 localization in heat-shocked cells was localized similarly during heat shock or cell wall stress (Kamada et al., 2002; and data not shown). The localization of Bck2 to cytoplasmic puncta in heat-shocked cells (Figure 5C) logically expressed Bck2, moderately overexpressed Bck2 did not localize to cytoplasmic puncta during heat shock (Figure 5C); however, curiously, and in contrast to physiologically expressed Bck2-GFP faintly localizes to bud necks (arrowhead) in ∼1% of medium- and large-budded cells. (C) Moderate Bck2 overexpression enhances detection of Bck2 at bud necks (arrowhead). For Bck2 overexpression, a plasmid with Bck2-GFP under the control of the constitutive GDP promoter (pGPD-BCK2-GFP) was introduced into bck2Δ cells (FLY3276). All images represent single optical sections.

Bck2 overexpression disrupts its recruitment to cytoplasmic puncta

Because Bck2 is spatially regulated and is a robust cbk1 dosage suppressor, we wondered whether Bck2 translocation to cytoplasmic puncta correlates with dosage suppression. We therefore investigated Bck2 localization when expressed from a low-copy plasmid under the control of the constitutive GDP promoter. pGDP-Bck2-GFP suppressed the conditional lethality of cbk1-8 mutants (Supplemental Figure S1C); however, curiously, and in contrast to physiologically expressed Bck2, moderately overexpressed Bck2 did not localize to cytoplasmic puncta during heat shock (Figure 5C). Instead, most Bck2 remained in the nucleus during heat shock. These data indicate that Bck2 overexpression disrupts its recruitment to cytoplasmic puncta and suggest that Bck2 does not need to translocate to cytoplasmic puncta in order to suppress the conditional lethality of cbk1 mutants.

Mitochondria

Mitochondria are known to play an important role in mediating heat shock response (Lanneau et al., 2008), we introduced mitochondrial-binding dyes and mitochondria-targeted RFP into GFP-tagged cells to test whether Bck2 and Sdp1 colocalize with mitochondria during heat shock. It is striking that ∼53% of the Bck2 cytoplasmic puncta (n = 50 cells) colocalized with mitochondria within 15–30 min of heat shock (Figure 7A). Three-dimensional models of the microscopy data support the conclusion that many of the Bck2 puncta colocalize with mitochondria (Figure 7B and Supplemental Movie S2). We obtained similar results with Sdp1-GFP (Figure 7C).

To corroborate the mitochondrial localization of Bck2 and Sdp1, we fractionated unstressed and heat-shocked cells and probed mitochondria fractions for Bck2 and Sdp1 by immunoblot. As a control for specificity, we also probed immunoblots with antibodies to the mitochondrial protein Tim23 and the endoplasmic reticulum (ER) protein Dpm1. We were unable to obtain conclusive fractionation data for Bck2 due to unresolved protein stability issues (data not shown); however, Sdp1 was greatly enriched in the mitochondrial-enriched fraction P2 from heat-shocked cells (Figure 7D). Notably, Sdp1 was not enriched in fraction P2 from unstressed cells. Because Bck2 and Sdp1 colocalize, these experiments suggest that a significant fraction of both proteins associate with mitochondria during heat shock. It is intriguing that Sdp1 was also present in fraction P3 in both unstressed and heat-shocked cells, which is enriched for microsomes and ER. It is not clear whether some of the heat shock–induced Bck2 or Sdp1 puncta associate with microsomes.

Cbk1 promotes the return of Bck2 and Sdp1 to their unstressed localization patterns during heat shock adaptation

The genetic relationship between Cbk1 and Bck2 with respect to Mpk1 activation suggests that Cbk1 regulates Bck2 function. To determine whether Cbk1 influences Bck2 or Sdp1 localization, we monitored Bck2 and Sdp1 in cells carrying the analogue-sensitive cbk1-as allele, which encodes mutant Cbk1-as, which is specifically inhibited by the drug 1NA-PP1 (Weiss et al., 2002), thereby allowing Cbk1 inhibition in the absence of heat shock.

We first exploited the effect of Cbk1 inhibition on Bck2 localization in unstressed cbk1-as cells. At 22°C in the absence of 1NA-PP1, the overall pattern of Bck2 nuclear localization was similar to that of wild-type cells. Curiously, Bck2 also localized to approximately one or two cytoplasmic Bck2 puncta in 25% of the cbk1-as cells (Figure 8A). On Cbk1 inhibition (1NA-PP1 addition) at 22°C, the percentage of cells with one or two cytoplasmic puncta increased to ∼42% (n = 90), and the number of cells with three or more cytoplasmic puncta per cell increased from 0 to 7% (Figure 8A). Parallel experiments with cells expressing an RFP-tagged mitochondria marker indicate that ∼49% (n = 63) of the Bck2 cytoplasmic puncta colocalize with mitochondria (Figure 8B, arrowheads). These data suggest that...
Cbk1 activity is modestly diminished in cbk1-as cells (in the absence of 1NA-PP1) and that robust Cbk1 kinase inhibition enhances the formation of Bck2 cytoplasmic puncta in unstressed cells.

We also tested whether Cbk1 inhibition influences Bck2 and Sdp1 localization during heat shock and heat shock adaptation (Figure 9). We inhibited Cbk1 at the same time as heat shock and observed that Bck2 and Sdp1 rapidly (within 15 min) relocated from the nucleus to cytoplasmic puncta in nearly all cells, similar to heat-shocked wild-type cells (Figure 9, A and B). In contrast, Cbk1 inhibition significantly delayed the release of Bck2 and Sdp1 from cytoplasmic puncta during heat shock adaptation. By 90 min of continual heat stress, when ∼20% of wild-type cells contained three or more Bck2 and Sdp1 cytoplasmic puncta, ∼58% of 1NA-PP1-treated cbk1-as cells retained three or more Bck2 and Sdp1 cytoplasmic puncta (Figure 9B). Moreover, the percentage of cbk1-as cells with three or more Bck2 and Sdp1 cytoplasmic puncta remained high for at least 2 h during heat stress. Parallel experiments with cells expressing the RFP-tagged mitochondrial marker established that ∼59% of the Bck2 puncta colocalize with mitochondria in heat-shocked cbk1-as cells throughout the experiment (Supplemental Figure S3). These data indicate that Cbk1 is important for mediating the recovery of Bck2-Sdp1 from a heat shock–induced localization pattern to the unstressed localization pattern and are consistent with the model that Cbk1 regulates heat shock recovery by controlling Bck2 and Sdp1 release from cytoplasmic puncta.

Cbk1, Bck2, and Knr4 are required for Mpk1 dephosphorylation during heat shock

Sdp1 was shown to play a major role in Mpk1 Thr-190/Tyr-192 dephosphorylation during heat shock adaptation (Collister et al., 2002; Hahn and Thiele, 2002). In light of the Bck2-Sdp1 colocalization data and the role of Cbk1 in regulating Bck2-Sdp1 release from cytoplasmic puncta, we postulated that Cbk1, Bck2, and Sdp1 cooperatively function to inactivate Mpk1 during heat shock adaptation. To test this hypothesis, we compared the dynamics of Mpk1 Thr-190/Tyr-192 phosphorylation in heat-stressed wild-type and mutant cells over time.

All images were captured via wide-field fluorescence microscopy. All images in A and C represent single optical sections, and the images in B are merged from $3 \times 0.2 \mu m$ optical sections.
Mpk1 phosphorylation levels in comparison to similarly treated wild-type cells (Figure 3A). Likewise, the pMpk1/tMpk1 ratios of heat-shocked bck2Δ and knr4Δ cells peaked within 15–30 min, dropped slightly between 30 and 45 min, and remained elevated for >120 min. These data indicate that, like Sdp1, also Cbk1, Bck2, and Knr4 are required for efficient Mpk1 dephosphorylation during heat shock adaptation. Moreover, these data support the model that Bck2 and Sdp1 recruitment to cytoplasmic puncta during heat shock prolongs Mpk1 activity by effectively reducing nuclear and cytosolic MAPK phosphatase activity. Consistent with this model, most Bck2 and Sdp1 disappear from the nucleus shortly after heat shock induction (Hahn and Thiele, 2002) and reappear in the nucleus after prolonged heat stress, concurrent with Mpk1 dephosphorylation (Figure 6).

**Cbk1, Bck2, and Knr4 influence Mpk1 phosphorylation in caffeine-treated cells in an Sdp1-independent manner**

Because Sdp1 localization is not affected by caffeine treatment (data not shown), we hypothesized that Sdp1 would not affect the timing of Mpk1 dephosphorylation during prolonged caffeine exposure. To test this hypothesis, we compared the timing of Mpk1 Thr-190/Tyr-192 dephosphorylation in caffeine-treated sdp1Δ cells to that of wild-type cells. In caffeine-treated wild-type and sdp1Δ cells, we observed that pMpk1/tMpk1 ratios peaked at ~60 min and then steadily dropped until reaching basal levels at ~120–180 min (Figure 3B). Thus sdp1Δ had no major effect on the timing or extent of Mpk1 dephosphorylation in caffeine-treated cells, consistent with the model that Sdp1 is specific for heat shock adaptation (Figure 3B).

We conducted parallel experiments to determine whether Cbk1, Bck2, or Knr4 influenced adaptation to caffeine stress. Of note, the pMpk1/tMpk1 ratios consistently peaked higher (greater than two-fold) in caffeine-treated cbk1-8, bck2Δ, and knr4Δ cells than in corresponding wild-type cells. Moreover, Mpk1 phosphorylation remained higher at every subsequent time point for the duration of the experiment (180 min). In cbk1-8 cells, Mpk1 Thr-190/Tyr-192 phosphorylation levels peaked at ~60 min, similar to wild-type cells, before steadily decreasing to ~50% of peak levels by 120 min (Figure 3B). In bck2Δ and knr4Δ cells, the pMpk1/tMpk1 ratios peaked 30 and 90 min later than in similarly treated wild-type cells. Collectively, these data indicate that Cbk1, Bck2, and Knr4 influence the proper timing and extent of Mpk1 dephosphorylation (adaptation) during prolonged caffeine exposure; however, they likely regulate Mpk1 dephosphorylation during caffeine treatment independent of the MAPK phosphatase Sdp1.

**DISCUSSION**

Our data support the working model that Cbk1 controls two Bck2 functions with regard to stress signaling (Figure 10). The first Cbk1-dependent Bck2 function is to promote Mpk1 activation and Mpk1-dependent gene expression during heat shock and cell wall stress, in collaboration with Knr4 and Hsp90. It is significant that Cbk1, Bck2, and Knr4 are not essential for Mpk1 Thr-190/Tyr-192 phosphorylation during heat shock, caffeine treatment, and cell wall stress; however each protein is essential for Mpk1 kinase activity and Rlm1 activation. These data indicate that Cbk1 and Bck2 function after MAPKK-dependent Mpk1 phosphorylation with regard to CWI pathway signaling, similar to the chaperone Hsp90 and cochaperone Cdc37 (Hawle et al., 2007; Truman et al., 2007). Given the similar phenotypes of cbk1-8, bck2Δ, knr4Δ, and hsp90 cells with respect to Mpk1 activation, we propose that Bck2 and Knr4 cooperate with or function in parallel to Hsp90 for Mpk1-dependent Rlm1

**FIGURE 6:** Bck2 colocalizes with the MAP kinase phosphatase Sdp1. Cells expressing Bck2-GFP and Sdp1-RFP were monitored by wide-field fluorescence microscopy prior to (T = 0) and during heat shock. Bck2 and Sdp1 colocalize prior to and throughout heat shock. Greater than 98% of the Sdp1 puncta colocalize with Bck2 (n > 800 puncta in ~550 cells). The strain and plasmid used in this experiment are FLY3503 and FLE1283, respectively.

Table 1: Mpk1 phosphorylation levels in comparison to similarly treated wild-type cells (Figure 3A). Likewise, the pMpk1/tMpk1 ratios of heat-shocked bck2Δ and knr4Δ cells peaked within 15–30 min, dropped slightly between 30 and 45 min, and remained elevated for >120 min. These data indicate that, like Sdp1, also Cbk1, Bck2, and Knr4 are required for efficient Mpk1 dephosphorylation during heat shock adaptation. Moreover, these data support the model that Bck2 and Sdp1 recruitment to cytoplasmic puncta during heat shock prolongs Mpk1 activity by effectively reducing nuclear and cytosolic MAPK phosphatase activity. Consistent with this model, most Bck2 and Sdp1 disappear from the nucleus shortly after heat shock induction (Hahn and Thiele, 2002) and reappear in the nucleus after prolonged heat stress, concurrent with Mpk1 dephosphorylation (Figure 6).

Cbk1, Bck2, and Knr4 influence Mpk1 phosphorylation in caffeine-treated cells in an Sdp1-independent manner

Because Sdp1 localization is not affected by caffeine treatment (data not shown), we hypothesized that Sdp1 would not affect the timing of Mpk1 dephosphorylation during prolonged caffeine exposure. To test this hypothesis, we compared the timing of Mpk1 Thr-190/Tyr-192 dephosphorylation in caffeine-treated sdp1Δ cells to that of wild-type cells. In caffeine-treated wild-type and sdp1Δ cells, we observed that pMpk1/tMpk1 ratios peaked at ~60 min and then steadily dropped until reaching basal levels at ~120–180 min (Figure 3B). Thus sdp1Δ had no major effect on the timing or extent of Mpk1 dephosphorylation in caffeine-treated cells, consistent with the model that Sdp1 is specific for heat shock adaptation (Figure 3B).

We conducted parallel experiments to determine whether Cbk1, Bck2, or Knr4 influenced adaptation to caffeine stress. Of note, the pMpk1/tMpk1 ratios consistently peaked higher (greater than two-fold) in caffeine-treated cbk1-8, bck2Δ, and knr4Δ cells than in corresponding wild-type cells. Moreover, Mpk1 phosphorylation remained higher at every subsequent time point for the duration of the experiment (180 min). In cbk1-8 cells, Mpk1 Thr-190/Tyr-192 phosphorylation levels peaked at ~60 min, similar to wild-type cells, before steadily decreasing to ~50% of peak levels by 120 min (Figure 3B). In bck2Δ and knr4Δ cells, the pMpk1/tMpk1 ratios peaked 30 and 90 min later than in similarly treated wild-type cells. Collectively, these data indicate that Cbk1, Bck2, and Knr4 influence the proper timing and extent of Mpk1 dephosphorylation (adaptation) during prolonged caffeine exposure; however, they likely regulate Mpk1 dephosphorylation during caffeine treatment independent of the MAPK phosphatase Sdp1.

**DISCUSSION**

Our data support the working model that Cbk1 controls two Bck2 functions with regard to stress signaling (Figure 10). The first Cbk1-dependent Bck2 function is to promote Mpk1 activation and Mpk1-dependent gene expression during heat shock and cell wall stress, in collaboration with Knr4 and Hsp90. It is significant that Cbk1, Bck2, and Knr4 are not essential for Mpk1 Thr-190/Tyr-192 phosphorylation during heat shock, caffeine treatment, and cell wall stress; however each protein is essential for Mpk1 kinase activity and Rlm1 activation. These data indicate that Cbk1 and Bck2 function after MAPKK-dependent Mpk1 phosphorylation with regard to CWI pathway signaling, similar to the chaperone Hsp90 and cochaperone Cdc37 (Hawle et al., 2007; Truman et al., 2007). Given the similar phenotypes of cbk1-8, bck2Δ, knr4Δ, and hsp90 cells with respect to Mpk1 activation, we propose that Bck2 and Knr4 cooperate with or function in parallel to Hsp90 for Mpk1-dependent Rlm1
Bck2 and Sdp1 associate with mitochondria during heat shock. (A) Some heat shock–induced Bck2 puncta colocalize with mitochondria. A plasmid expressing an RFP-tagged mitochondrial marker (Mito-RFP; pHCRED) was introduced into Bck2-GFP cells. All images represent single optical sections captured by spinning disk confocal microscopy 15 min after shifting cells to 39°C (heat shock). (B) The cell is presented as a three-dimensional (3D) model via Velocity software (PerkinElmer). Left, a merge/projection of 21 × 0.2 μm Z-sections. Middle and right, different angles of a 3D model of the same cell. See Supplemental Movie S2 for this model in rotation. (C) Sdp1 localizes to mitochondria in heat-shocked cells. Cells expressing Sdp1-GFP and Mito-RFP (FLY3570, plasmids pRS425-SDP1-GFP, pHCRED) were monitored after 15 min of heat stress, as described in A. (D) Immunoblots of organelle fractions of unstimulated (22°C) and heat-shocked (39°C) Sdp1-GFP cells (see Materials and Methods). Western blots are probed with antibodies to GFP, Tim23 (a mitochondrial marker), and Dpm1 (ER/microsome marker). Mitochondria are enriched in P2, microsomes and ER are enriched in P3, and S3 is enriched for cytosolic proteins. Note that Sdp1 is enriched in fraction P2 (mitochondria) of heat-shocked cells and not in P2 of unstimulated cells. Some Sdp1 is also present in fraction P3 (ER/microsome), regardless of heat shock.

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The role of Bck2 in heat shock signaling

Our data clearly establish that Bck2 is subject to heat shock–specific regulation, as previously shown for Sdp1 (Hahn and Thiele, 2002). Most notably, heat shock causes a rapid change in Bck2 and Sdp1 phosphatase localization that is not brought about by other CWI pathway–activating stresses (caffeine or cell wall–disrupting drugs). Sdp1-mediated Mpk1 dephosphorylation also appears to be specific for heat shock adaptation (Hahn and Thiele, 2002; Figure 3). Likewise, Bck2 has a more pronounced effect on Mpk1 dephosphorylation during caffeine stress adaptation. We cannot rule out the possibility that Sdp1 contributes to Mpk1 dephosphorylation during caffeine treatment or other stresses; however, our data indicate that other MAPK phosphatases must play a more significant role in Mpk1 Thr-190/Tyr-192 dephosphorylation during cell wall or caffeine stress adaptation. We speculate that Cbk1 regulates Bck2-Sdp1 release from puncta via indirect mechanisms because it does not localize to cytoplasmic puncta during heat shock.

Prior to heat shock, a significant fraction of Bck2 and Sdp1 localizes to the nucleus. This is particularly noteworthy with respect to Mpk1 regulation because Mpk1-mediated Rlm1 phosphorylation is thought to take place in the nucleus since Rlm1 is only detectable in the nucleus (Jung et al., 2002; data not shown). Thus we propose that Bck2 and Sdp1 sequestration in cytoplasmic puncta prevents premature Mpk1 dephosphorylation/inactivation during heat stress.

activation during heat shock and cell wall stress. Furthermore, because Bck2 overexpression suppresses the stress sensitivity of mpklΔ mutants (Lee et al., 1993), it is likely that in the absence of Mpk1, Bck2 stimulates other MAP kinases to activate Rlm1 and other Mpk1-dependent transcription factors during heat shock and cell wall stress. It is intriguing that Bck2 contains consensus sequences for Cbk1 phosphorylation, which is consistent with the model that Cbk1 promotes Mpk1 activation via Bck2 phosphorylation. Alternatively, Cbk1 and Bck2 may promote Mpk1 activation by inhibiting an Mpk1 inhibitor. Nevertheless, because Mpk1 Thr-190/Tyr-192 phosphorylation occurs on schedule in heat-shocked and caffeine-treated cbk1Δ, bck2Δ, and knr4Δ cells, it is unlikely that Cbk1 and Bck2 promote Mpk1 activation by inactivating MAPK phosphatases, such as Sdp1.

A second function for Cbk1 and Bck2 is to mediate Mpk1 dephosphorylation during heat stress adaptation (Figure 10). In support, Cbk1 and Bck2 are essential for efficient Mpk1 dephosphorylation during heat stress adaptation, and Cbk1 influences Bck2 and Sdp1 release from heat stress–induced cytoplasmic/mitochondrial puncta. During heat shock (but not during caffeine treatment), both Bck2 and Sdp1 are rapidly targeted to cytoplasmic puncta, many of which colocalize with mitochondria, where they are sequestered from Mpk1. On heat shock adaptation and CWI signaling attenuation (as detected by Mpk1 dephosphorylation), Bck2 and Sdp1 disappear from the mitochondria and other cytoplasmic puncta and concurrently reappear in the cytosol and nucleus. We propose that the Cbk1-dependent release of Bck2 and Sdp1 from puncta promotes Mpk1 dephosphorylation/inactivation during heat shock adaptation. We speculate that Cbk1 regulates Bck2-Sdp1 release from puncta via indirect mechanisms because it does not localize to cytoplasmic puncta during heat shock.

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There is precedent for stress-dependent differences in CWI signaling. Heat shock and cell wall stress stimulate the same plasma membrane receptors that activate the CWI signaling, whereas caffeine activates the CWI pathway independent of upstream CWI pathway components (Inagaki et al., 1999; Ketela et al., 1999; Martin et al., 2000; Harrison et al., 2004). Heat shock also stimulates parallel physiological and transcriptional responses via Hsf1 transcription factor, which induces the expression and activity of a variety of proteins and chaperones (Imazu and Sakurai, 2005; Truman et al., 2007), including Hsp90, which was shown to mediate Mpk1 activation and the mitochondrial targeting of a variety of proteins (Young et al., 2003).

FIGURE 8: Cbk1 inhibition enhances the appearance of Bck2 puncta. (A) cbk1-as cells expressing Bck2-GFP were monitored before and after Cbk1 inhibition. The percentages of cells with one or two cytoplasmic and three or more cytoplasmic Bck2 puncta are plotted (n = 90). Bck2 localizes to one or two cytoplasmic puncta in ~24% of cbk1-as cells at 22°C. By 15 min of Cbk1 inhibition (1NA-PP1 addition), the number of cells with one or two Bck2 puncta nearly doubles. Cbk1 inhibition also increases the percentage of cells with three or more Bck2 puncta per cell. Representative cells are shown below the graph. (B) Many (~49%, n = 63) of the Bck2 puncta colocalize with Mito-RFP. All images were captured via wide-field fluorescence microscopy and represent single optical sections.

Bck2 and Sdp1 mitochondrial functions
Our data support the model that Bck2 and Sdp1 are specifically sequestered at mitochondria and other cytoplasmic puncta during heat shock to prevent premature Mpk1 dephosphorylation/inactivation. Bck2 and Sdp1 mitochondrial localization may also reflect specific mitochondrial functions during heat shock. Indeed, many proteins that transiently associate with mitochondria are known to mediate heat shock, oxidative stress response, and apoptosis (Fairn et al., 2007; Lanneau et al., 2008). Both Hsf1 and the chaperone Hsp90 were shown to associate with mitochondrial proteins (Young et al., 2003; Reinders et al., 2006). Thus it is tempting to speculate that Hsp90 or other chaperones contribute to Bck2 and Sdp1 mitochondrial localization and Mpk1 signaling. Alternatively, Bck2 or Sdp1 may be subject to functionally important posttranslational modifications or protein interactions at mitochondria that in turn are important for Mpk1 inhibition during heat shock adaptation.

Regardless of the specific function of mitochondrial Bck2 and Sdp1, it is unlikely that Bck2-Sdp1 mitochondrial recruitment is essential for cbk1 dosage suppression because moderate Bck2 overexpression disrupts mitochondrial targeting. Rather, we expect that Bck2 overexpression suppresses the lethality of cbk1 mutants by enhancing Bck2 protein interactions in the nucleus or cytosol that promote Mpk1 signaling. Although it is not known how Bck2 overexpression disrupts mitochondrial recruitment, we speculate that Bck2 overexpression interferes with the function or expression of important Bck2 regulators or mitochondrial-targeting factors.

Other Bck2 functions
Our data regarding Bck2-dependent Mpk1 activation also have important implications regarding cell cycle and growth control. We
FIGURE 9: Cbk1 inhibition prolongs Bck2 and Sdp1 puncta localization during heat shock. (A) Bck2-GFP localization was monitored at various intervals after heat shock in wild-type cells (left) and in cbk1-as cells upon Cbk1 inhibition in cbk1-as cells (right). For cbk1-as cells, Cbk1 inhibition (1NA-PP1 addition) and heat shock were done simultaneously. NLS-RFP shows the nuclear localization. Bck2 localized to cytoplasmic puncta in all cells (n = 35) within 15 min of heat shock. All images were captured via wide-field fluorescence microscopy and represent two merged optical sections. See Supplemental Figure S3 for a temporal analysis of Bck2 localization at mitochondria. (B) Parallel experiments were done in cells coexpressing Sdp1-RFP, and the number of cells with three or more Bck2 and Sdp1 cytoplasmic puncta was plotted over time. The data were tabulated from two independent experiments (n = 29–55 cells per time point). Cbk1 kinase inhibition significantly delayed the disappearance of the heat shock–induced Bck2 and Sdp1 puncta. See Supplemental Figure S4 for additional controls. The strains used for these experiments are FLY3503 and FLY3559.
in other organisms is to regulate MAPK signaling via similar regulatory circuits.

**MATERIALS AND METHODS**

**Yeast growth conditions and strain construction**

Standard yeast genetics and culture methods were used as described (Guthrie and Fink, 1991; Kurischko et al., 2005). For heat shock, mid-log-phase cells were transferred from 25 to 39°C. For caffeine-mediated CWI pathway activation, cells were transferred to media containing 15 mM caffeine and maintained at 25°C. The strains used in this study are listed in Table 1. The strain expressing C-terminally GFP-tagged Bck2 was constructed by integration of a PCR-based GFP cassette using the oligos FLO655 and FLO656 (Table 2), as described (Longtine et al., 1998).

**cbk1-8 dosage suppressor screen**

We performed a genome-wide screen for dosage suppressors of the cbk1-8 mutant strain (FLY2884), using an ordered array of 1588 high-copy plasmids of overlapping yeast genomic DNA that was obtained from Open Biosystems/Thermo Fisher (Waltham, MA) and described in Jones et al. (2008). Plasmid YGPM13c23, which contained both BCK2 and RPH1, suppressed the conditional lethality of cbk1-8 cells at 34 and 37°C.

**Plasmid construction**

The oligonucleotides and plasmids used in this study are listed in Tables 2 and 3. pGP564-BCK2 (FLE1255) was generated by inserting PCR-amplified BCK2 into the SacI and XhoI sites of pGP564. BCK2 (from base pair −550 to +220) was amplified from YGPM13c23 with oligos FLO696 and FLO697. pGP564-RPH1 (FLE1254) was constructed by digesting YGPM13c23 (containing both BCK2 and RPH1) with XhoI and subcloning the 12.0-kb fragment containing RPH1 into pGP564. pENTRY-BCK2 and pENTRY-SDP1 were generated by PCR amplifying BCK2 and SDP1 open reading frames with oligos FLO686, FLO687, FLO704, and FLO705 (Table 2) and subcloning the PCR products into the Gateway vector pDONR221 (Invitrogen, Carlsbad, CA). BCK2 and SDP1 were transferred from their pENTRY vectors into pAG416-GDP-ccdB-GFP to yield pGDP-BCK2-GFP and pGDP-SDP1-GFP (C-terminally tagged), as described (Alberti et al., 2007), pAG416-GDP-ccdB-GFP was provided by Aaron Gitler (University of Pennsylvania, Philadelphia, PA). All constructs were confirmed by sequencing.

**Mpk1 immunoblot analysis**

For Mpk1 immunoblots, yeast cells were grown to mid-logarithmic phase (OD$_{600}$ = 0.6) in synthetic complete media at 25°C. Proteins from 1.8 ml of the yeast culture were precipitated with 10% trichloroacetic acid, as described (Baerends et al., 2000). Protein precipitates were washed twice with ice-cold acetone and dissolved in equal volume of 0.1 N NaOH 1% SDS, and SDS protein sample buffer and processed for immunoblots. To detect phosphorylated Mpk1, the immunoblots were probed with 1/2500 rabbit anti–phospho-p44/42 MAPK (Thr-202/Tyr-204) antibody.
**TABLE 1: Yeast strains.**

(4370S; Cell Signaling Technology, Beverly, MA) as described (Martin et al., 2000), followed by secondary alkaline phosphatase (AP)-conjugated anti-rabbit immunoglobulin G (IgG; S373B; Promega, Madison, WI). The immunoblots were processed for enhanced chemifluorescence (ECF), as described by the manufacturer’s protocol (GE Healthcare, Piscataway, NJ), and analyzed with a STORM phosphorimager (GE Healthcare).

**In vitro Mpk1 kinase assays**

For Mpk1-GFP immunoprecipitation, cells containing pRS425-Mpk1-GFP (kindly provided by Dennis J Thiele, Duke University, Durham, NC) were grown to mid-log phase (OD$_{600}$ = 0.8–1.0) and heat shocked for 30 min at 39°C. The cells were harvested and lysed as described (Kamada et al., 1995; Kurischko et al., 2011a). The cell extracts were normalized for protein concentration, and 100 μg was immunoprecipitated with anti-GFP antibody (1181446001; Roche, Indianapolis, IN). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004). In vitro kinase assays were carried out with immunoprecipitated Mpk1-GFP and exogenous MBP as substrate, as described (Kamada et al., 2004).
et al., 1995). MBP phosphorylation was detected by a STORM phorimager and quantified with ImageQuant software.

Subcellular fractionation and isolation of mitochondria
Mitochondrial fractionation was carried out as described (Sepuri et al., 2007). Briefly, yeast cells were grown in selective synthetic media to mid-log phase (OD_{600} = 1.2) at 22°C and heat shocked for 30 min at 39°C. Unstressed and heat-shocked cells were harvested and converted to protoplasts by treating them with zymolyase (MP Biomedicals, Solon, OH). Differential centrifugations of protoplasts were carried out as described previously. The mitochondrial-enriched fraction was collected as pellet P2 from 8000 × g centrifugation for 10 min. P2 was resuspended in 0.5 ml SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 1 mM phenylmethylsulfonyl fluoride). The 8000 × g supernatant (S1) was further subjected to 35,000 × g for 2 h to enrich for ER/microsome (P3) and cytosolic proteins (S3). After fractionation, protein concentrations were measured, and 50 μg of each fraction was loaded onto 10% polyacrylamide gels and immunoblotted. Immunoblots of cytosol, mitochondrial, and microsomal fractions were probed with antibodies for the mitochondrial marker Tim23 (Santa Cruz Biotechnology), the ER protein Dpm1 (A6429; Invitrogen), and anti-GFP antibody (Roche) for Sdp1-GFP protein. Anti-Dpm1 and anti-Tim23 antibodies were kindly provided by Narayan Avadhani.

Rlm1 reporter assays
Rlm1-lacZ reporter plasmid, which bears both a lexA-RLM1 fusion and a lacZ reporter gene with upstream lexA-binding sites, was obtained from Jürgen Heinisch (Universität Osnabrück, Osnabrück, Germany). The Rlm1-lacZ reporter plasmid was introduced into yeast cells, and β-galactosidase activity was measured at the designated time points following heat shock and caffeine treatment, as previously described (Kirchrath et al., 2000; Martin-Yken et al., 2003). Rlm1-dependent lacZ expression was quantified by measuring the nanomoles per minute per milligram of o-nitrophenyl phosphate produced from o-nitrophenyl β-D-galactoside substrate, as previously described (Kirchrath et al., 2000; Martin-Yken et al., 2003). Rlm1 activity was represented as the mean value of three independent experiments.

Fluorescence microscopy
All microscopy was carried out as described (Kurischko et al., 2011a). Wide-field fluorescence and time-lapse microscopy was carried out with a Leica (Wetzlar, Germany) DMR5 fluorescence microscope equipped with a 100× PL Apo 1.46 numerical aperture (NA) oil objective and an Imager EM 16-bit cooled Hamamatsu EMCCD camera (Hamamatsu, Japan), as previously described (Nelson et al., 2003; Kurischko et al., 2008). For time-lapse microscopy, cells were heat shocked by raising the temperature on an objective heater (Biopics, Butler, PA) from room temperature to 37°C. Spinning disc confocal microscopy was conducted with a Leica Inverted DMI4000 microscope equipped with a 100× HCX PL Apo 1.46 NA oil objective, a Yokogawa (Sugarland, TX) CSU-10 spinning disc confocal system, and an Imager EM 16-bit cooled Hamamatsu EMCCD camera. Laser excitation was provided by a 488-nm laser (Spectra Physics, Newport Corporation, Irvine, CA) and a 561-nm laser (Cooldt Jive, Solna, Sweden) controlled through LMMS (Spectral Applied Research, Richmond Hill, Canada). The emissions were collected at 503–552 nm for GFP and 583–650 nm for RFP. Z-stacks were taken for a total thickness of 1.8–3.4 μm at a step size of 0.2 μm. Image capture was controlled by MetaMorph software (MDS Analytical Technologies, Sunnyvale, CA), and image analysis and three-dimensional modeling were conducted with Velocity software (PerkinElmer, Waltham, MA).

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