Crz1 destabilizes Msn2/Msn4 in the nucleus in response to Ca^{2+} in *Saccharomyces cerevisiae*: FK506 has an additive effect on the Ca^{2+}-induced expression of *GLO1 via* Msn2, Hog1, and the stress response element

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Running Title: Crz1 affects stability of Msn2/Msn4 in yeast nucleus

Synopsis

Although methylglyoxal is derived from glycolysis, it has adverse effects on cellular function. Hence, the intrinsic role of methylglyoxal *in vivo* remains to be determined. Glyoxalase I is a pivotal enzyme in the metabolism of methylglyoxal in all types of organisms. To learn about the physiological roles of methylglyoxal, we have screened conditions that alter the expression of the gene encoding glyoxalase I (*GLO1*) in *Saccharomyces cerevisiae*. Here we show that the expression of *GLO1* is induced following treatment with $CaCl_2$ dependent on Hog1-MAP kinase and the Msn2/Msn4 transcription factors. Intriguingly, the Ca^{2+}-induced expression of *GLO1* was enhanced in the presence of FK506, a potent inhibitor of calcineurin. Consequently, the Ca^{2+}-induced expression of *GLO1* in a mutant defective in calcineurin or defective in the sole transcription factor functioning under the control of calcineurin (*crz1*Δ) was much greater than that in the wild-type strain even without FK506. This phenomenon was dependent upon a *cis*-element, the STRE (stress response element), in the promoter that is able to mediate Ca^{2+} signaling together with Hog1 and Msn2/Msn4. The level of Ca^{2+}-induced expression of *GLO1* reached a maximum in cells overexpressing *MSN2* even when FK506 was not present; whereas, that in cells overexpressing *CRZ1* was greatly reduced, but increased markedly when FK506 was present. We found that the levels of Msn2 and Msn4 proteins in $CaCl_2$–treated cells decreased gradually, and FK506 blocked the degradation of Msn2/Msn4. Here we propose that Crz1 destabilizes Msn2/Msn4 in the nucleus of cells in response to Ca^{2+} signaling.

Key words: Calcineurin, Calcium, Crz1, FK506, Methlglyoxal, Msn2

INTRODUCTION

Methylglyoxal (MG) is a typical 2-oxoaldehyde in all types of organism, because MG is synthesized during glycolysis, a ubiquitous energy-generating pathway [1, 2]. Though a natural metabolite, MG inhibits the growth of cells [1-3]. In some cases, MG induces apoptosis or necrosis, but the mode of cell death depends upon the cell line examined [4-6]. MG was once believed to be a major intermediate of glycolysis, but the hypothesis has been disproved; nevertheless, MG and its metabolic enzymes have received considerable attention, because MG is involved in diseases such as diabetes and its complications, cancers, Alzheimer disease, and autism [7-12]. Hence, there must be an ingenious system to avoid any metabolic disorder involving MG in cells, thereby reducing the risk of suffering such diseases; however, the molecular mechanism by which MG causes such diseases is not well understood.

To find a clue as to the physiological role of MG, we searched for conditions that alter intracellular levels of MG as well as the activities of MG-metabolizing enzymes. We have previously reported that the expression of *GLO1* is specifically induced by osmotic stress in a Hog1 MAP (mitogen-activated protein) kinase-dependent manner [13]. The *GLO1* gene encodes glyoxalase I, an enzyme crucial for the metabolism of MG [14]. The *GLO1* promoter contains a characteristic *cis*-acting element, the STRE (stress response element). Genes possessing the STRE usually respond to a wide variety of stress stimuli, such as oxidative stress, heat shock stress, and osmotic stress [15]. Msn2 and Msn4, C_2H_2-type zinc finger transcription factors, have been proven to translocate into the nucleus, and bind to the STRE under environmental stress. Even though the *GLO1* promoter possesses two STREs, intriguingly, *GLO1* did not respond to any stress other than osmotic stress [13]. *S. cerevisiae* produces glycerol as a compatible osmolyte under high osmotic stress [16]. We found that the uptake of glucose, and subsequently, the flux of glucose into glycolysis, were enhanced when cells were exposed to high osmotic stress to facilitate glycerol production [13]. Consequently, intracellular MG increased [13], because the major source of MG is glycolysis. Therefore, we have previously proposed that the physiological significance of the specific expression of *GLO1* under high osmotic stress is most likely to the efficient metabolism of MG, which increases during the response to osmotic stress [2, 13].

On the other hand, we have studied the effect of MG on cellular function. As a result, we revealed that MG functions as a signal initiator in yeasts [17-19]. For example, we found that an AP-1-like bZIP transcription factor Yap1 is constitutively activated in *glo1*Δ cells through the modification of cysteine residues, which are crucial for the determination of its nucleocytoplasmic localization, by MG, the level of which is elevated due to the disruption of Glo1 [17]. Yap1 plays crucial roles in the response to oxidative stress as well as drug stress in *S. cerevisiae* (for a review, see ref. [20], and references therein). Recently, we found that MG attenuates overall protein synthesis through phosphorylation of the translation initiation factor eIF2α in a target of rapamycin (TOR)-independent manner [21], and another AP-1-like transcription factor Gcn4 plays a role in the adaptive response to MG stress [22]. So, cellular levels of MG

must be controlled adequately to warrant normal cellular functions.

Here we show that $CaCl_2$ induces the expression of *GLO1*, which is strictly dependent upon Hog1, Msn2/Msn4, and two STREs in the *GLO1* promoter. Notably, the Ca^{2+}-induced expression of *GLO1* was further augmented by FK506, a potent inhibitor of calcineurin [23]. Calcineurin is a protein phosphatase whose activity is regulated by Ca^{2+}/calmodulin. Full induction of the expression of *GLO1* following treatment with $CaCl_2$ was observed in a mutant defective in either calcineurin or *CRZ1*, which encodes the sole transcription factor functioning under the control of calcineurin [24-27]. We provide evidence that Crz1 negatively affects the functions of Msn2/Msn4 in the nuclei of cells treated with $CaCl_2$ by facilitating the degradation of Msn2 and Msn4. Consequently, a further increase in the Ca^{2+}-induced expression of *GLO1* was seen when FK506 was present, or calcineurin or Crz1 was disrupted. Our data suggest that Crz1 destabilizes Msn2 and Msn4 proteins in the nucleus when cells are treated with $CaCl_2$.

EXPERIMENTAL

Strain, plasmid, and medium

Unless otherwise stated, yeast strains used in this study have the YPH250 background (*MAT***a** *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52*). Construction of the *hog1*Δ, *pbs2*Δ, *ssk1*Δ, *sho1*Δ, *ssk1*Δ*sho1*Δ, *cnb1*Δ, *crz1*Δ, *msn2*Δ, and *msn2*Δ*msn4*Δ mutants in YPH250 was described previously [13, 18]. A *rlg1*Δ mutant has the BY4741 background (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Cells were cultured in YPD medium (2% glucose, 1% yeast extract, and 2% peptone), or SD minimal medium (2% glucose and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases at 28°C with reciprocal shaking.

Enzyme assay

The preparation of cell extracts and measurement of the activities of glyoxalase I (Glo1) and *β*-galactosidase were described previously [13, 14]. One unit of the activity of Glo1 was defined as the amount of enzyme forming 1 μmol of *S*-D-lactoylglutathione per min using a millimolar extinction coefficience of 3.37 $mM^{-1}cm^{-1}$ [14]. One unit of *β*-galactosidase activity was defined as the amount of enzyme that increases the A_{420} by 1000 per h [13]. Protein concentrations were determined by the method of Bradford [28].

Northern blotting

Cells were cultured in YPD medium until A_{610} = 1.0, and 300 mM $CaCl_2$ and/or 1 μg/ml FK506 were added. After 30 min of incubation, total RNA was prepared according to the method of Schmitt [29]. The probe DNA labeled with (α-^{32}P)dCTP was prepared as described previously [13].

Detection of Hog1 phosphorylation

Cells were cultured in YPD medium until A_{610} = 1.0, and 300 mM $CaCl_2$ and/or 1 μg/ml

FK506 were added. After 60 min of incubation, cell lysates were prepared as described by Bell *et al.* [30]. Yeast cellular proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Anti-phospho-p38 monoclonal antibody (Sigma-Aldrich) was used as the primary antibody, and anti-rabbit IgG antibody conjugated with horseradish peroxidase (New England Biolabs) was used as the secondary antibody. To measure the level of Hog1 protein, anti-Hog1 antibody (Santa Cruz Biotechnology) was used as a primary antibody.

Detection of Msn2 and Msn4

To determine the levels of Msn2 and Msn4 proteins, cells carrying pRS316+Msn2-GFP or pGR247 (pAdh1-Msn4-GFP) [31] were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ at 28°C. Cells were collected at a prescribed time, and disrupted with glass beads in 100 mM Tris-HCl buffer (pH 7.0), 5 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, 50 mM NaCl, 1 mM dithiothreitol, and protease inhibitor cocktail (Nacalai tesque). Cell extracts (20 μg proteins) were subjected to SDS-PAGE. Anti-GFP antibody (Santa Cruz Biotechnology) was used to detect Msn2-GFP and Msn4-GFP. To detect Cdc28 as a loading control, the membrane was treated in 0.2 M glycine (pH 2) for 5 min, and reprobed with anti-Cdc2 antibody (Santa Cruz Biotechnology).

Construction of *GLO1-lacZ* reporter gene

The *GLO1-lacZ* cassette in pRSGlac415 [13] was cloned into the *Sal*I site of YIp5 (YIp5+GLO1-lacZ), and the cassette was then amplified by PCR with 5TO358F (5′-AGGGCATCGGTCGACGGATCCGGGTAATTC-3′) and 5TO358R (5′-TAAAACGACGGAATTCCCGGGTTTCTCAAT-3′) using YIp5+GLO1-lacZ as a template. *Sal*I and *Eco*RI sites were designed in 5TO358F and 5TO358R, respectively (underlined). The PCR fragment was digested with *Sal*I and *Eco*RI, and cloned into the *Sal*I-*Eco*RI site of YIp358R. The resultant plasmid (YIp358R+GLO1-lacZ) was digested with *Nco*I, and the linearized fragment was integrated into the *ura3* locus of YPH250.

To insert a point mutation into the STREs (5′-AGGGG-3′ → 5′AGATG-3′), an overlap extension PCR was conducted [32]. For the site-directed mutagenesis, four internal primers (GSTRE1, 5′-AATAGGTAAAGAGATGGGTGGGGGTGG-3′; GSTRE1R, 5′-CCACCCCCACCCATCTCTTTACCTATT-3′; GSTRE2, 5′-CTGAATAAACAAGATGCTTTACGATGG-3′; and GSTRE2R, 5′-CCATCGTAAAGCATCTTGTTTATTCAG-3′) were designed. To create the mutation in STRE1 (-432 to -428), the first PCR was performed with the following primer sets: 5TO358F plus GSTRE1R, and 5TO358R plus GSTRE1. The second PCR was done with 5TO358F and 5TO358R as the primers, and a mixture of the products of each first PCR as the template. The PCR product was digested with *Sal*I and *Eco*RI, and the resultant fragment was inserted into the *Sal*I-*Eco*RI site of YIp358R to yield YIp358R+PMS1. Similarly, to create the mutation in STRE2 (-229 to -225), the first PCR was performed with the following primers: 5TO358F plus GSTRE2R and 5TO358R plus GSTRE2. The second PCR was done with 5TO358F and 5TO358R, and a mixture of the products of each first PCR as the template. The PCR product was cloned into YIp358R as described

above (YIp358R+PMS2). To construct the *GLO1-lacZ* gene carrying both mutated STRE1 and STRE2 (PMS12), the first PCR was performed with 5TO358F plus GSTRE2R, and 5TO358R plus GSTRE2 using YIp358R+PMS1 as a template. The second PCR was done using 5TO358F and 5TO358R, and a mixture of the products of each first PCR as the template. The PCR product was digested with *Sal*I and *Eco*RI, and the resultant fragment was inserted into the *Sal*I-*Eco*RI site of YIp358R to yield YIp358R+PMS12. Introduction of the mutation was verified by DNA sequencing.

Construction of *GPX1-lacZ* reporter gene

To amplify the region between -365 and +8 of *GPX1*, taking the translation initiation codon (ATG) as +1, PCR was done with GPX1-lacZ-F (5′-GGAGTCGACGGACTTGATAGAATCCACCTT-3′) and GPX1-lacZ-R (5′-GGTGAAAAAGAATGAATTCCTTGCATCGTT-3′). *Sal*I and *Eco*RI sites were designed in GPX1-lacZ-F and GPX1-lacZ-R, respectively (underlined). The PCR fragment was digested with *Sal*I and *Eco*RI, and cloned into the *Sal*I-*Eco*RI site of YIp358R. The resultant plasmid (YIp358R-GPX1-lacZ) was digested with *Nco*I, and the linearized fragment was integrated into the *ura3* locus of YPH250.

Construction of *HOG1*-overexpression plasmid

The HOG1 gene was amplified by PCR with HOG1S (5′-GTTGTTAGGAAAGCATGCTTTATCTCCAAG-3′) and HOG1R (5′-CCTTTTATGGGATCCTAATTTCTTAAGGAG-3′) using the genomic DNA of YPH250 as a template. *Sph*I and *Bam*HI sites were designed in HOG1S and HOG1R, respectively (underlined). The PCR fragment was digested with *Sph*I and *Bam*HI, and cloned into the *Sph*I-*Bam*HI site of a 2 μ-type plasmid pRS423 to yield pRS423+*HOG1*.

Fluorescence microscopy

Cells expressing GFP-tagged proteins were cultured in YPD medium to A_{610} = 0.5, and 300 mM $CaCl_2$ was added in the presence or absence of 1 μg/ml FK506. After incubation for the prescribed time at 28°C, the intracellular localization of each GFP-tagged protein was observed using a fluorescence microscope (Olympus BX51, Tokyo, Japan). Plasmids carrying Hog1-GFP, Msn2-GFP, and Crz1-GFP were pRS4-Hog1-GFP [18], pAMG (pAdh1-Msn2-GFP) [33], and pASM463 [34], respectively.

RESULTS

Expression of *GLO1* is induced by Ca^{2+} in a Hog1 MAP kinase cascade-dependent manner

We have searched for conditions under which the cellular concentration of MG is changed, or the expression of *GLO1* encoding an enzyme crucial for the metabolism of MG is altered [2, 13]. Here we found that levels of *GLO1* mRNA were increased following treatment with $CaCl_2$ (Figure 1A). We constructed the *GLO1-lacZ* reporter gene to verify the Ca^{2+}-induced expression of *GLO1*, and found that the activity of

β-galactosidase driven by *GLO1-lacZ* was increased following treatment with $CaCl_2$ (see Figure 5B). The activity of glyoxalase I (Glo1) was also increased in cells treated with $CaCl_2$ (Figure 1B). Therefore, we concluded that $CaCl_2$ induces the expression of *GLO1*.

Hog1 is one of the MAP kinases in *S. cerevisiae*, and plays crucial roles in the response to osmotic stress (for a review, see ref. [35, 36]). So, first, we conducted a Northern blot analysis of *GLO1* in a *hog1*Δ mutant. As shown in Figure 1A, the level of *GLO1* mRNA was not increased in *hog1*Δ cells following the treatment with $CaCl_2$. Induction of Glo1 by $CaCl_2$ occurred in neither the *hog1*Δ nor *pbs2*Δ mutant, which is defective in a MAP kinase kinase responsible for the activation of Hog1 (Figure 1B).

Besides Hog1, we have reported that Msn2 and Msn4, C_2H_2 zinc finger transcription factors, are necessary for response of *GLO1* to osmotic stress [13]. To determine whether these transcription factors are also involved in the Ca^{2+}-induced expression of *GLO1*, we conducted Northern blotting in an *msn2*Δ*msn4*Δ mutant. As shown in Figure 1A, the levels of *GLO1* mRNA in *msn2*Δ*msn4*Δ cells were not increased following the treatment with $CaCl_2$, indicating that Msn2 and Msn4 are necessary for the response of *GLO1* to Ca^{2+}.

Next, we determined the roles of the osmosensors functioning upstream of the Hog1 MAP kinase cascade. In *S. cerevisiae*, two osmosensors (Sln1 and Sho1) have been thus far identified. The Sln1 branch consists of Ypd1 (phosphorelay protein) and Ssk1 (response regulator), constituting a two-component system [37]. Two redundant MAP kinase kinase kinases (Ssk2 and Ssk22) function downstream of the Sln1 branch. In the Sho1 branch, Sho1 physically interacts with Pbs2 through the SH3 domain [38, 39]. To determine whether these two osmosensors are involved in the Ca^{2+}-induced expression of *GLO1*, we measured the Glo1 activity in cells defective in these branches. Since *SLN1* is an essential gene, we disrupted *SSK1* and/or *SHO1* to inactivate the osmotic-stress signaling pathways to Hog1. As shown in Figure 1B, the induction of *GLO1* expression was observed in the mutant defective in either *SSK1* or *SHO1*, although it was repressed in the *ssk1*Δ*sho1*Δ double mutant.

Curious response of *GLO1* to Ca^{2+} and FK506

Although we have shown that the Hog1 MAP kinase cascade is necessary for the Ca^{2+}-induced expression of *GLO1*, we determined whether the Ca^{2+}/calmodulin-dependent calcineurin system is also involved in this response, because the system is a well-known pathway for Ca^{2+} signaling in eukaryotic cells, including *S. cerevisiae* [40]. In the Ca^{2+}/calcineurin system, extracellular Ca^{2+} enters the cell and binds calmodulin to activate a protein phosphatase calcineurin, thereby dephosphorylating Crz1, a transcription factor functioning downstream of calcineurin [24, 25]. Since FK506 is a potent inhibitor of calcineurin [23], the expression of the Ca^{2+}-induced genes regulated by the Ca^{2+}/calcineurin system (*e. g. FKS2* and *GPX2*) [41, 42] is markedly repressed in its presence. To address whether the calcineurin system is involved in the Ca^{2+}-induced expression of *GLO1*, we determined the effect of FK506. Surprisingly, and rather bizarrely, the Ca^{2+}-induced expression of *GLO1* was further increased when FK506 was present (Figure 1A). Similarly, the activities of *β*-galactosidase driven by *GLO1-lacZ* as well as Glo1 were further increased by the

simultaneous treatment of cells with $CaCl_2$ and FK506 (Figures 1A and 5B). We have shown that two osmosensors function as the upstream modules for the transduction of Ca^{2+} signal to Hog1, and subsequently, such an unexpected phenomenon in terms of the response of *GLO1* to Ca^{2+} and FK506 was observed in the *ssk1*Δ and *sho1*Δ mutants, but not in the *ssk1*Δ*sho1*Δ mutant (Figure 1B).

Calcineurin/Crz1-mediated pathway is involved in the bizarre response of *GLO1*

We confirmed this curious response of *GLO1* to Ca^{2+} and FK506 is caused by much lower concentrations (50 mM) of $CaCl_2$ (data not shown). However, since the maximal induction of *GLO1* is brought about by 200-300 mM $CaCl_2$, we analyzed this response in the presence of 300 mM $CaCl_2$.

To verify whether this curious phenomenon caused by FK506 in terms of the Ca^{2+}-induced expression of *GLO1* is brought about by a certain action of FK506 on the machinery involved in Ca^{2+} signaling, or is dependent upon the calcineurin system, we conducted a genetic analysis. We disrupted a regulatory subunit of calcineurin (*CNB1*), which abolishes the activity of this protein phosphatase [43], and Crz1, the sole transcription factor identified to date that functions under the control of calcineurin [24, 25]. As shown in Figure 2A, the expression of *GLO1* in both *cnb1*Δ and *crz1*Δ mutants reached a maximum level on treatment with $CaCl_2$ alone, and the treatment of these mutants with $CaCl_2$ and FK506 simultaneously did not enhance the expression of *GLO1* any further. Therefore, the effect of FK506 on the expression of *GLO1* with respect to the response to Ca^{2+} was confirmed to be exerted through the calcineurin/Crz1-mediated pathway.

This positive effect of FK506 was not observed for the NaCl-induced expression of *GLO1* (Figure 2B). This was also the case for the KCl- and sorbitol-induced expression of *GLO1* (data not shown). Meanwhile, basal levels of *GLO1* mRNA as well as Glo1 activity did not increase in the *cnb1*Δ and *crz1*Δ mutants (Figures 2A and 2B), therefore, the calcineurin/Crz1 pathway *per se* does not act as a negative regulator of the expression of *GLO1* under normal conditions. It is likely that the effect of FK506 on the Hog1-dependent induction of the expression of *GLO1* occurs only when Ca^{2+} is present, and FK506 does not enhance the response to osmotic stress in general. Here we temporally refer to this phenomenon as the "bizarre response", because Crz1 is a transcription factor that essentially has a positive effect on its target gene, nevertheless, Crz1 seems to act as a negative regulator for the Ca^{2+}-induced expression of *GLO1*.

One possible explanation for the bizarre response is that some newly synthesized protein(s), the synthesis of which is regulated by the calcineurin/Crz1 pathway, functions as a negative regulator of the expression of *GLO1* in response to Ca^{2+}. To address this possibility, we treated yeast cells with $CaCl_2$ and FK506 in the presence of cycloheximide. As shown in Figure 2C, the bizarre response was observed even under conditions where protein synthesis is blocked. Hence, the bizarre response is likely to be caused by the preexisting machinery in yeast cells.

Time course of the expression of *GLO1*

To gain further insights into the role of the calcineurin pathway in the Ca^{2+}-induced

expression of *GLO1*, we monitored the level of *GLO1* mRNA in cells treated with $CaCl_2$. As shown in Figure 3, the amount of *GLO1* mRNA in wild-type cells gradually increased upon the addition of $CaCl_2$, reaching a maximum after 30-40 min of incubation. The timing of the increase in the level of *GLO1* mRNA in cells treated with $CaCl_2$ plus FK506 was almost the same as that in cells treated with $CaCl_2$ alone, although the amount of *GLO1* mRNA was obviously higher than that in cells treated with $CaCl_2$ alone. The pattern of the changes in the level of *GLO1* mRNA in *cnb1*Δ cells treated with $CaCl_2$ was substantially the same as that in wild-type cells treated with $CaCl_2$ plus FK506. The expression profile of *GLO1* in *crz1*Δ cells in response to Ca^{2+} was essentially similar to that in *cnb1*Δ cells (data not shown). Therefore, the calcineurin pathway seems to negatively influence the maximal level of *GLO1* mRNA in response to Ca^{2+}.

Phosphorylation and nucleocytoplasmic localization of Hog1

Hog1 is phosphorylated by Pbs2, and then translocates into the nucleus in response to high osmotic stress [33, 44, 45]. Since the Ca^{2+}-induced expression of *GLO1* was strictly dependent upon Hog1, we determined the level of Hog1 phosphorylation following treatment with $CaCl_2$. As shown in Figure 4A, Hog1 was phosphorylated in cells treated with $CaCl_2$, however, the simultaneous treatment of cells with $CaCl_2$ and FK506 did not enhance the phosphorylation of Hog1. Similarly, the level of phosphorylated Hog1 in *cnb1*Δ as well as *crz1*Δ cells treated with $CaCl_2$ was the same as that in wild-type cells, and FK506 did not give rise to an additive effect in terms of Hog1's phosphorylation.

Next, we determined the nucleocytoplasmic localization of Hog1. As shown in Figure 4B, Hog1 was concentrated in the nucleus upon the treatment with $CaCl_2$, and the intensity of the fluorescence derived from Hog1-GFP in the nuclei in cells treated with $CaCl_2$ was the same as that observed in cells treated with $CaCl_2$ and FK506 simultaneously.

We determined the nuclear localization of Hog1 following the treatment with $CaCl_2$ and FK506. In wild-type cells, Hog1 was immediately accumulated in the nucleus upon the treatment with $CaCl_2$, and redistributed to the cytoplasm after 30 - 45 min of incubation in the presence of $CaCl_2$. No distinct difference was observed in the timing of the change in the nucleocytoplasmic localization of Hog1 even when FK506 was present (Figure 4C). This was also the case in *cnb1*Δ and *crz1*Δ cells (Figure 4C). Therefore, even though Hog1 is necessary for *GLO1* to respond to extracellular Ca^{2+}, neither the level of phosphorylation nor the nucleocytoplasmic localization of Hog1 is likely to be a cause of the bizarre response.

Roles of STREs in the *GLO1* promoter for the bizarre response

The *GLO1* promoter contains two STREs (STRE1, -432 to -428; and STRE2, -229 to -225) [13]. Since the Ca^{2+}-induced expression of *GLO1* was dependent upon the Msn2/Msn4 transcription factors, (Figure 1A), and it has been reported that Msn2 and Msn4 bind to the STRE [33], we determined whether STREs are involved in the bizarre response. A point mutation was introduced into the STRE (5′-AGGGG-3′ → 5′-AGATG-3′) (Figure 5A), a mutation by which the function of STRE was reported to be lost [46]. The

Ca^{2+}-induced expression of *GLO1-lacZ* was reduced approximately 45% by introduction of a mutation into either STRE1 or STRE2, and greatly repressed by the simultaneous introduction into both STREs (Figure 5B). Importantly, the bizarre response was repressed by the mutation in either STRE1 or STRE2, although STRE2 seems to shoulder a more important role in the response.

Next, we determined the response of *GLO1-lacZ* with various STRE constructs in an *msn2*Δ mutant. As Msn2 is the primary transcription factor for the STRE-dependent gene, the Ca^{2+}-induced expression of *GLO1-lacZ* was impaired in *msn2*Δ cells when compared with that in wild type cells (Supplementary Figure 1). Even though the induction rate was quite small, the Ca^{2+}-induced expression of *GLO1* occurred in *msn2*Δ cells, which may be caused by Msn4; however, only limited increase in β-galactosidase activity was observed in the presence of FK506 (Supplementary Figure 1). Since the Ca^{2+}-induced expression of PMS1 was comparable to that of wild-type STRE construct in *msn2*Δ cells, Msn4 may bind to STRE2 in response to Ca^{2+}. No induction was observed in *msn2*Δ*msn4*Δ cells (Figure 1A).

We have demonstrated that Hog1 plays a crucial role in the bizarre response, we determined the effect of Hog1-deficiency on the expression of *GLO1-lacZ* carrying various STRE constructs. As shown in Fig. 5C, since Hog1 is crucial, the level of Ca^{2+}-induced expression of *GLO1* was markedly reduced in a *hog1*Δ mutant (it should be noted that the scale of vertical axis of Fig 5C is one fiftieth of that of Fig. 5B), and the bizarre response was not clearly observed in the presence of FK506 together with $CaCl_2$ even in *GLO1-lacZ* with wild-type STRE construct. We determined the response of *GLO1-lacZ* in Hog1-overexpressed cells also. Intriguingly, as shown in Fig. 5D, the bizarre response was not observed.

STRE regulated by both Mns2/Msn4 and Hog1 exhibits the bizarre response

To verify whether the STRE is sufficient for the bizarre response, we used the *STRE-lacZ* reporter gene containing two STREs from the *CTT1* promoter (5'-TTCAAGGGGATCACCGGTAAGGGGCCAAG-3', STREs are underlined) followed by the TATA box of *CYC1* [47]. As shown in Figure 6A, the bizarre response was observed in this reporter gene also. The *CTT1* gene encodes a cytoplasmic catalase [48], so we measured the catalase activity. The Ca^{2+}-induced increase in catalase activity was further increased by addition of FK506 (Figure 6B). In addition, as observed in Glo1, catalase activity was markedly increased in *cnb1*Δ as well as *crz1*Δ cells following treatment with $CaCl_2$ alone, but did not increase any further when FK506 was present (Figure 6B).

Very recently, we found that the expression of *GPX1*, encoding a homologue of mammalian glutathione peroxidase in *S. cerevisiae* [49], is induced following treatment with $CaCl_2$ in an Msn2/Msn4-dependent manner (Figure 6C), and the *GPX1* promoter contains two functional STREs [50]; nevertheless, *GPX1* did not exhibit the bizarre response (Figure 6C). We have found that Hog1 is dispensable for *GPX1*'s response to Ca^{2+} [50]. On the other hand, the response in terms of the activity of catalase as well as $STRE_{CTT1}$-*lacZ* to Ca^{2+} is dependent upon Hog1 (Figures 6A and 6B). Collectively, the STRE regulated by both Hog1 and Msn2/Msn4 seems able to mediate

the bizarre response.

Effect of Crz1 on the function of Msn2

The data obtained so far imply two possibilities, *i. e.* Crz1 competes with Msn2 to bind to the STRE in the *GLO1* promoter to reduce the effect of Msn2 on the Ca^{2+}-induced expression of *GLO1*, or Crz1 inhibits the activity of Msn2 toward the STRE. However, Crz1 binds to a distinct DNA sequence, the calcineurin-dependent response element (CDRE) [24], the consensus sequence of which (5'-GAGGCTG-3') does not resemble the STRE. In addition, since the *GLO1* promoter does not contain a sequence exactly coinciding with that of the CDRE around the STREs, the former is not likely. To address the possibility of the latter, the response of *GLO1* to Ca^{2+} was determined in cells overexpressing *MSN2* or *CRZ1*. As shown in Figure 7A, the activity of Glo1 in *MSN2*-overexpressing cells following treatment with $CaCl_2$ was increased to a much greater extent than that in cells carrying the vector alone, and the addition of FK506 did not bring about a further increase in the activity. On the other hand, the induction of Glo1 activity by $CaCl_2$ in *CRZ1*-overexpressing cells was repressed, but the activity increased to a maximal level when $CaCl_2$ and FK506 were added simultaneously.

We have previously reported that Msn2 is accumulated in the nucleus following treatment with $CaCl_2$ (ref. [42], also see Figure 7B). Msn2 was accumulated in the nucleus immediately upon the treatment of cells with $CaCl_2$, resided in the nucleus for up to 30 min, and redistributed in the cytoplasm after 45 – 60 min of incubation. The behavior of Msn2 in terms of its nucleocytoplasmic localization, *i. e.* the timing of the beginning of the nuclear accumulation, and the retention period in the nucleus, was not affected by the presence of FK506 (Figure 7B). Similarly, the behavior of Msn2 in *cnb1*Δ cells was the same as that in wild-type cells (Figure 7B). The nucleocytoplasmic localization of Msn2 in response to Ca^{2+} and FK506 in *crz1*Δ cells was substantially the same as that in wild-type and *cnb1*Δ cells (data not shown). Meanwhile, Crz1 was also accumulated in the nucleus upon treatment with $CaCl_2$, which is blocked by FK506 (ref. [51], also see Figure 7C). The nuclear accumulation of Crz1 did not occur following treatment with NaCl (Figure 7C). Therefore, when Crz1 is concentrated in the nucleus in cells treated with $CaCl_2$, this transcription factor seems to function as a negative regulator of Msn2 in the nucleus.

Crz1 affects stability of Msn2 in the nucleus in response to Ca^{2+}

Durchschlag *et al.* [52] have reported that Msn2 is degraded in the nucleus. Hence, one feasible explanation for the bizarre response is that the nuclear Crz1 affects the rate of stability of Msn2 in the nucleus when Ca^{2+} is present. To address this possibility, we determined the level of Msn2 protein following treatment with $CaCl_2$ with or without FK506. As shown in Figure 8A, the level of Msn2 protein in wild-type cells gradually decreased in the presence of $CaCl_2$, but the reduction was blocked when FK506 was present; whereas in *crz1*Δ cells, the reduction in the level of Msn2 protein following the treatment with $CaCl_2$ was repressed even in the absence of FK506 (Figure 8A). Substantially, the same results in terms of the stability of Msn2 were obtained in *cnb1*Δ cells (data not shown). Additionally, the same results were obtained with respect to the

stability of Msn4 (Figure 8B). Taken together, Crz1 seems to destabilize Msn2 in response to Ca^{2+}.

Since the bizarre response was observed in genes whose expression is dependent upon Hog1, we determined the stability of Msn2 in *hog1*Δ cells and Hog1-overexpressed cells. The Ca^{2+}-induced degradation of Msn2 was observed in *hog1*Δ cells, which was blocked by the addition of FK506 (Figure 8C). Meanwhile, in Hog1-overexpressed cells, the Ca^{2+}-induced degradation of Msn2 was not blocked in the presence of FK506 (Figure 8D), and subsequently, further increase in the expression of *GLO1-lacZ* in the presence of $CaCl_2$ and FK506 (bizarre response) was not observed in Hog1-overexpressed cells (Figure 5D).

DISCUSSION

Negative effect of Crz1 on Msn2

To learn about the physiological role of MG in yeast cells, we searched for conditions that alter the expression of *GLO1*, and revealed that *GLO1* is expressed following treatment with $CaCl_2$. During the course of this study, intriguingly, we found that the Ca^{2+}-induced expression of *GLO1* was further enhanced if FK506 was present, which we temporally referred to as the bizarre response.

In the present study, we proposed that Crz1 negatively influences the function of Msn2 on the STRE in the *GLO1* promoter in cells treated with $CaCl_2$. Therefore, the bizarre response occurred only with a combination of Ca^{2+} and FK506, not with a combination of FK506 and other ions (Na^+ and K^+) or sorbitol at concentrations that provoke the nuclear accumulation of Msn2. This is presumably because such chemicals do not activate the calcineurin system, subsequently, Crz1 does not accumulate in the nucleus (Figure 7C); and therefore, Msn2 does not encounter Crz1 in the nucleus. However, even though the expression is regulated by Msn2 and the STRE, if it was not dependent upon Hog1, such a gene (*e. g. GPX1*) would not exhibit the bizarre response (Figure 5B). In other words, a gene whose expression is dependent upon these three factors will exhibit the bizarre response (*e. g. CTT1*). Since calcineurin is a protein phosphatase, a simple model explaining the role of Hog1 in the bizarre response is that calcineurin dephosphorylates Hog1, thereby preventing the Ca^{2+} signal from reaching the target gene. However, as shown in Figure 4A, disruption of *CNB1* or *CRZ1* did not affect the level of phosphorylation or the nuclear accumulation of Hog1. These results support our conclusion that Crz1 affects the function of Msn2/Msn4, but not Hog1.

We have collected evidence that Crz1 acts negatively on the expression of genes regulated by Hog1, Msn2/Msn4, and STRE. There are several explanations for this. For example, Crz1 directly binds to Msn2/Msn4 to reduce its function as a transcription factor. To address this possibility, we tried to detect direct interaction between Crz1 and Msn2 by conducting a GST pulldown assay as well as a co-immunoprecipitation assay, however, we were unable to obtain evidence of physical interaction as far as the experimental conditions we could determine (data not shown).

Another possibility is that Crz1 influences the machinery involved in the nuclear localization of Msn2. However, as shown in Figure 7B, the behavior of Msn2 in terms of its nucleocytoplasmic localization in response to Ca^{2+} was virtually unaffected by FK506. In addition, Durchschlag *et al.* [52] have reported that Msn2 is constitutively concentrated in the nucleus in *msn5*Δ cells because Msn5 is an exportin for Msn2 [34, 53]; nonetheless, the basal level of *CTT1* mRNA and the stress response of *CTT1's* expression in the *msn5*Δ mutant were normal [52], which is accounted for by a decrease in the total amount of Msn2 protein in *msn5*Δ cells (described below). The expression of *CTT1* under environmental stress is regulated by Hog1, Msn2/Msn4, and the STRE [48]; and consequently, the *CTT1* promoter shows the bizarre response (Figures 6A and 6B). Although Msn2 is a positive regulator for the expression of *GLO1*, the basal activity of Glo1 did not increase in the *msn5*Δ mutant (data not shown) as observed in the case of *CTT1* despite of the constitutive nuclear accumulation of Msn2. Therefore, the cause of the bizarre response is not likely to be the period of Msn2's nuclear retention.

Next, we examined the possibility that Crz1 titrates Hog1 away from Msn2, thereby reducing the activity as transcription factor in the presence of $CaCl_2$. Although the direct physical interaction between Crz1 and Hog1 has not been reported so far, if the nuclear Crz1 titrates Hog1 away from Msn2 thereby reducing Msn2 activity, further increase in the expression of *GLO1-lacZ* is expected to occur in the presence of FK506 that blocks the nuclear accumulation of Crz1. However, this was not the case (Figure 5D). Furthermore, in contrast to Msn2-overexpressed cells (Figure 7A), full activation of Msn2 in terms of the expression of *GLO1-lacZ* was not observed in Hog1-overexpressed cells (Figure 5D). We supposed that this might occur because the proportion of Msn2 that is not bound to STRE in the *GLO1* promoter is increased. It has been reported that Hog1 forms a complex with Msn2, although Hog1 *per se* is not a transcription factor. Therefore, overexpression of Hog1 may increase the proportion of Hog1-Msn2 complex that is not bound to the STRE thereby removing Msn2 away from the target sequence (STRE), which reduces the opportunity of Msn2 to function as transcription factor on the promoter. Subsequently, full activation was not observed following treatment with $CaCl_2$. Additionally, if Crz1 is titrated by Hog1, the induction of the expression of Crz1-dependent gene by Ca^{2+} would be impaired in Hog1-overexpressed cells. So, we determined the response of *GPX2* to Ca^{2+}, the expression of which is regulated by the calcineurin/Crz1 pathway [42]. However, *GPX2* normally responded to Ca^{2+} even in Hog1-overexpressed cells (Supplementary Figure 2A). Taken together, we thought that Hog1 was not titrated by Crz1 in the presence of Ca^{2+}.

Genome-wide search for the gene showing bizarre response

Although a typical sequence (5'-GAGGCTG -3'), to which Crz1 binds to, has not been found in the *GLO1* promoter, Ruiz *et al.* [54] have recently reported that 5'-GGGGCTG-3' sequence in the *HXT2* promoter is functional as CDRE. A similar sequence (5'-GGGGCTT-3'; -228 to -222) overlaps the STRE2 (5'-AGGGG-3'; -229 to -225) in the *GLO1* promoter. So, to address the possibility that the bizarre response can be explained by the coexistence or the proximity of STRE and Crz1 binding sites, we searched for the CDRE-like sequence (5'-GGGGCT(G/T)-3') in the promoter that

exhibits the bizarre response. We used the DNA microarray data set made by Yoshimoto *et al.* [26] to find such genes.

First, we looked for the gene whose expression was induced more than 2-fold when cells were treated with $CaCl_2$ for 30 min. Among them, we looked for the gene whose expression was induced further when FK506 is present together with $CaCl_2$. Next, we calculated the ratio of the induction fold between the Ca^{2+}-induced expression ([Ca^{2+}]) and the Ca^{2+} plus FK506-induced expression [Ca^{2+}+FK506]. We searched for the gene whose [Ca^{2+}+FK506]/[Ca^{2+}] value is >1.67, because the value of *GLO1* is 1.67 when calculated based on the database. Consequently, 22 genes, including *CTT1* (ranking, #4), were found. Finally, we searched for the STRE and CDRE-like sequence in the promoter region (~ -500) of such 22 genes. As a result, all of them have one or more STREs, although only 6 genes contained the typical CDRE or CDRE-like sequence (Supplementary Table 1). Therefore, the coexistence or the proximity of STRE and CDRE seems not necessary for the bizarre response. Regarding the function of such gene product, they are not likely to be involved in a certain common biological process. However, some of them are involved in the energy metabolism. We are now trying to find the physiological relevance of the bizarre response using the gained information as a clue.

Crz1 destabilizes Msn2/Msn4 in the nucleus in response to Ca^{2+}

A striking feature of the negative effect of Crz1 on Msn2 is that nuclear Crz1 affects the stability of Msn2 and Msn4 in the nucleus when Ca^{2+} is present (Figure 8A). We named this phenomenon "the Crz1-dependent Msn2/4 degradation (CDMD)". Taking into account the overall results of the present study, one explanation for the curious response found in this study is as follows (Figure 9): Crz1 accumulated in the nucleus upon $CaCl_2$-treatment reduces the stability of Msn2/Msn4 by a yet unknown mechanism, and the blockade of the simultaneous accumulation of Crz1 and Msn2/Msn4 in the nucleus by the addition of FK506 to inhibit calcineurin, or disruption of a gene encoding calcineurin or Crz1 *per se* inhibits the degradation of Msn2/Msn4, thereby allowing a further increase in the Ca^{2+}-induced expression of *GLO1*.

Msn2 under conditions of chronic stress or low PKA activity is supposed to be degraded by the 26 S proteasome in the nucleus [52]. To examine whether Crz1 enhances the activity of this protein degradation pathway, we treated yeast cells with a proteasome inhibitor MG132. In this experiment, we used an *erg6*Δ mutant because MG132 is hardly taken up by wild type cells [55]. As shown in Figure 8E, Msn2 was degraded in *erg6*Δ cells following treatment with $CaCl_2$, which was blocked by FK506. We confirmed that the bizarre response occurred in the *erg6*Δ mutant also (data not shown). However, the addition of MG132 did not repress the degradation of Msn2 in the presence of $CaCl_2$.

Importantly, the CDMD was not seen in the gene whose response to Ca^{2+} is independent of Hog1, even though its Ca^{2+}-induced expression is dependent upon both Msn2/Msn4 and the STRE (*e. g. GPX1*). So, the reduction in the level of Msn2 protein might decrease the integrity of a complex of Hog1-Msn2, which may limit the efficacy of the expression of its target gene possessing an STRE. As far as we could determine, the

behavior of Msn2 with respect to its nuclear localization in response to Ca^{2+} closely resembles that of Hog1 (Figures 4C and 7C). Hence, the Ca^{2+}-induced expression of *GLO1* may reach a limit due to the degradation of Msn2 in addition to the nuclear export of Msn2 and Hog1. The disruption of Crz1 or inhibition of the nuclear accumulation of Crz1 by inhibiting calcineurin with FK506 block the breakdown of Msn2, thereby stabilizing a Hog1-Msn2 complex to warrant a full response to Ca^{2+}. However, intriguingly, we found that FK506 did not block the degradation of Msn2 in the presence of Ca^{2+} in Hog1-overexpressed cells (Figure 5D). As a clue of the answer to this question, we found that Msn2 resided in the nucleus for longer period in Hog1-overexpressed cells in the presence of $CaCl_2$ + FK506; *i. e.* Crz1 was concentrated in the nucleus following treatment with Ca^{2+} and redistributed in the cytoplasm after 30 – 45 min in cells carrying the control vector; however, Crz1 resided in the nucleus after 60 min of treatment with $CaCl_2$ (Supplementary Figure 2B). Durchschlag *et al.* [52] have reported that longer residence of Msn2 in the nucleus causes the degradation of Msn2 in a 26 S proteasome-dependent manner. Thus, in this case, we supposed that Msn2 might have been degraded in Hog1-overexpressed cells due to the longer retention in the nucleus.

Our findings suggest that an indirect interaction between transcription factors by affecting their stability may be a regulatory point of the response to stress besides the nucleocytoplasmic dynamics of transcription factors, changes to the affinity of transcription factors for their target sequence through posttranslational modifications as well as chromatin remodeling, or interaction with RNA polymerase. For example, Williams and Cyert [56] have reported that an oxidative stress-responsive transcription factor Skn7 regulates the turnover of Crz1, yet the protease involved has not been identified. A network system regulating the stability of transcription factors might exist in yeast cells to warrant an appropriate and distinct stress response to a wide variety of environmental stimuli that partially overlap, *i. e.* a high concentration of $CaCl_2$ provokes both an osmotic stress response and a Ca^{2+} signal response simultaneously. One possible physiological interpretation of the CDMD is that it minimizes unnecessary Hog1-Msn2/Msn4-dependent osmotic responses induced by Ca^{2+} by destabilizing Msn2/Msn4 through Crz1.

AUTHOR CONTRIBUTION

Yoshifumi Takatsume and Takumi Ohdate equally contributed to this study. Yoshifumi Takatsume undertook most of the experimentation shown in Figures 1-5ABC, 6, and 7. Takumi Ohdate undertook most of the experimentation shown in Figures 5D, 8, and Supplementary Figures and Table. Importantly, this author found the Crz1-dependent degradation of Msn2/Msn4 in the presence of $CaCl_2$. Kazuhiro Maeta, Wataru Nomura, and Shingo Izawa gave valuable discussion about this study. Yoshiharu Inoue initially found the fundamental phenomenon of this study, and wrote the paper.

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REFERENCES

1 Inoue, Y. and Kimura, A. (1995) Methylglyoxal and regulation of its metabolism in microorganisms. Adv. Microbial. Physiol. **37,** 177–227

2 Inoue, Y. and Kimura, A. (1999) Glycolytic-methylglyoxal pathway. Molecular evolution and stress response of glyoxalase I in *Saccharomyces cerevisiae*. Proc. Japan Acad. **75B,** 127–132

3 Kalapos, M. P. (1999) Methylglyoxal in living organisms. Chemistry, biochemistry, toxicology and biological implications. Toxicol. Lett. **110,** 145–175

4 Du, J., Suzuki, H., Nagase, F., Akhand, A. A., Yokoyama, T., Miyata, T., Kurokawa, K. and Nakashima, I. (2000) Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase. J. Cell. Biochem. **77,** 333–344

5 Du, J., Suzuki, H., Nagase, F., Akhand, A. A., Ma, X. Y., Yokoyama, T., Miyata, T. and Nakashima, I. (2001) Superoxide-mediated early oxidation and activation of ASK1 are important for initiating methylglyoxal-induced apoptosis process. Free Rad. Biol. Med. **31,** 469–478

6 Maeta, K., Mori, K., Takatsume, Y., Izawa, S. and Inoue, Y. (2005) Diagnosis of cell death induced by methylglyoxal, a metabolite derived from glycolysis, in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **243,** 87–92

7 Ranganathan, S., Walsh, E. S., Godwin, A. K. and Tew, K. D. (1993) Cloning and characterization of human colon glyoxalase-I. J. Biol. Chem. **268,** 5661–5667

8 Thornalley, P. J., Hooper, N. I., Jennings, P. E., Florkowski, C. M., Jones, A. F., Lunec, J. and Barnett, A. H. (1989) The human red blood cell glyoxalase system in diabetes mellitus. Diabetes Res. Clin. Prac. **7,** 115–120

9 Ramasamy, R., Yan, S. F. and Schmidt, A. M. (2006) Methylglyoxal comes of AGE. Cell **124,** 258-260

10 Ahmed, N., Ahmed, U., Thornalley, P. J., Hager, K., Fleischer, G. and Munch, G. (2005) Protein glycation, oxidation and nitration adduct residues and free adducts of cerebrospinal fluid in Alzheimer's disease and link to cognitive impairment. J. Neurochem. **92,** 255–263

11 Junaid, M. A., Kowal, D., Barua, M., Pullarkat, P. S., Sklower Brooks, S. and Pullarkat, R. K. (2004) Proteomic studies identified a single nucleotide polymorphism in glyoxalase I as autism susceptibility factor. Am. J. Med. Genet. **131,** 11–17

12 Chen, F., Wollmer, M. A., Hoerndli, F., Münch, G., Kuhla, B., Rogaev, E. I., Tsolaki, M., Papassotiropoulos, A. and Götz, J. (2004) Role for glyoxalase I in Alzheimer's disease. Proc. Natl. Acad. Sci. USA **101,** 7687-7692

13 Inoue, Y., Tsujimoto, Y. and Kimura, A. (1998) Expression of the glyoxalase I gene of *Saccharomyces cerevisiae* is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. J. Biol. Chem. **273,** 2977–2983
14 Inoue, Y. and Kimura, A. (1996) Identification of the structural gene for glyoxalase I from *Saccharomyces cerevisiae*. J. Biol. Chem. **271,** 25958–25965
15 Ruis, H. and Schüller, C. (1995) Stress signaling in yeast. Bioessays **17,** 959-965
16 Albertyn, J., Hohmann, S., Thevelein, J. M. and Prior, B. A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. **14,** 4135– 4144
17 Maeta, K., Izawa, S., Okazaki, S., Kuge, S. and Inoue, Y. (2004) Activity of the Yap1 transcription factor in *Saccharomyces cerevisiae* is modulated by methylglyoxal, a metabolite derived from glycolysis. Mol. Cell. Biol. **24,** 8753–8764
18 Maeta, K., Izawa, S. and Inoue, Y. (2005) Methylglyoxal, a metabolite derived from glycolysis, functions as a signal initiator of the high osmolarity glycerol-mitogen-activated protein kinase cascade and calcineurin/Crz1-mediated pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **280,** 253-260
19 Takatsume, Y., Izawa, S. and Inoue, Y. (2006) Methylglyoxal as a signal initiator for activation of the stress-activated protein kinase cascade in the fission yeast *Schizosaccharomyces pombe*. J. Biol. Chem. **281,** 9086-9092
20 Gulshan, K. and Moye-Rowley, W. S. (2007) Multidrug resistance in fungi. Eukaryot. Cell **6,** 1933-1942
21 Nomura, W., Maeta, K., Kita, K., Izawa, S. and Inoue, Y. Methylglyoxal activates Gcn2 to phosphorylate eIF2α independently of the TOR pathway in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. (in press)
22 Nomura, W., Maeta, K., Kita, K., Izawa, S. and Inoue, Y. (2008) Role of Gcn4 for adaptation to methylglyoxal in *Saccharomyces cerevisiae*: methylglyoxal attenuates protein synthesis through phosphorylation of eIF2α. Biochem. Biophys. Res. Commun. **376,** 738–742
23 Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66,** 807-815
24 Stathopoulos, A. M. and Cyert, M. S. (1997) Calcineurin acts through the *CRZ1/TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11,** 3432-3444
25 Matheos, D. P., Kingsbury, T. J., Ahsan, U. S. and Cunningham, K. W. (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. Genes Dev. **11**, 3445-3458
26 Yoshimoto, H., Saltsman, K., Gasch, A. P., Li, H. X., Ogawa, N., Botstein, D., Brown, P. O. and Cyert, M. S. (2002) Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **277,** 31079-31088
27 Cyert, M. S. (2003) Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go

crazy in response to stress. Biochem. Biophys. Res. Commun. **311,** 1143-1150

28 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254

29 Schmitt, M. E., Brown, T. A. and Trumpower, B. L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18,** 3091–3092

30 Bell, M., Capone, R., Pashtan, I., Levitzki, A. and Engelberg, D. (2001) Isolation of hyperactive mutants of the MAPK p38/Hog1 that are independent of MAPK kinase activation. J. Biol. Chem. **276,** 25351-25358

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

32 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77,** 51-59

33 Görner, W., Durchschlag, E., Martínez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schüller, C. (1998) Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. **12,** 586-597

34 Boustany, L. M. and Cyert, M. S. (2002) Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. Genes Dev. **16,** 608-619

35 Gustin, M. C., Albertyn, J., Alexander, M. and Davenport, K. (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **62,** 1264-1300

36 O'Rourke, S. M. Herskowitz, I. and O'Shea. E. K. (2002). Yeast go the whole HOG for the hyperosmotic response. Trends Genetics **18,** 405-412

37 Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. and Saito, H. (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell **86,** 865–875

38 Maeda, T., Wurgler-Murphy, S. M. and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369,** 242–245

39 Maeda, T., Takekawa, M. and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **269,** 554–558

40 Cohen, P. (1989) The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58,** 453–508

41 Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S. and Levin, D. E. (1998) Temperature-induced expression of yeast *FKS2* is under the dual control of protein kinase C and calcineurin. Mol. Cell. Biol. **18,** 1013–1022

42 Tsuzi, D., Maeta, K., Takatsume, Y., Izawa, S. and Inoue, Y. (2004) Distinct regulatory mechanism of yeast *GPX2* encoding phospholipid hydroperoxide glutathione peroxidase by oxidative stress and a calcineurin/Crz1-mediated Ca^{2+}

signaling pathway. FEBS Lett. **569,** 301–306

43 Cyert, M. S. and Thorner, J. (1992) Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. **12,** 3460-3469

44 Ferrigno, P., Posas, F., Koepp, D., Saito, H. and Silver. P. A. (1998) Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin β homologs NMD5 and XPO1. EMBO J. **17,** 5606–5614

45 Rep, M., Krantz, M., Thevelein, J. M. and Hohmann, S. (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. **275,** 8290–8300

46 Zähringer, H., Thevelein, J. M. and Nwaka, S. (2000) Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol. Microbiol. **35,** 397-406

47 Stanhill, A., Schick, N. and Engelberg, D. (1999) The yeast Ras/cyclic AMP pathway induces invasive growth by suppressing the cellular stress response. Mol. Cell. Biol. **19,** 7529-7538

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

49 Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S. and Kimura, A. (1999) Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. J. Biol. Chem. **274,** 27002–27009

50 Ohdate, T., Izawa, S., Kita, K. and Inoue, Y. Regulatory mechanism for expression of *GPX1* in response to glucose starvation and Ca^{2+} in *Saccharomyces cerevisiae*: Involvement of Snf1 and Ras/cAMP pathway in Ca^{2+} signaling. Genes Cells (in press)

51 Stathopoulos-Gerontides, A., Guo, J. J. and Cyert, M. S. (1999) Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev. **13,** 798–803

52 Durchschlag, E., Reiter, W., Ammerer, G. and Schüller, C. (2004) Nuclear localization destabilizes the stress-regulated transcription factor Msn2. J. Biol. Chem. **279,** 55425-55432

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

54 Ruiz, A., Serrano, R. and Ariño J. (2008) Direct regulation of genes involved in glucose utilization by the calcium/calcineurin pathway. J. Biol. Chem. **283,** 13923-13933

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

56 Williams, K. E. and Cyert, M. S. (2001) The eukaryotic response regulator Skn7p

regulates calcineurin signaling through stabilization of Crz1p. EMBO J. **20,** 3473-3483

Figure legends

Figure 1 Effects of Ca^{2+} and FK506 on the expression of *GLO1* (A) Cells were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506.After 30 min of incubation, total RNA was prepared, and a Northern blot analysis was conducted. (B) Cells were treated with $CaCl_2$ and/or FK506 as described in (A). After 60 min of incubation, cell extracts were prepared, and glyoxalase I (Glo1)

activity was measured.

Figure 2 Calcineurin/Crz1 pathway is involved in the Ca^{2+}-induced expression of *GLO1* (A) Cells were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 30 min of incubation, total RNA was prepared, and a Northern blot analysis was conducted. (B) Cells were treated with $CaCl_2$ and/or FK506 as described in (A). NaCl concentration was 500 mM. After 60 min of incubation, cell extracts were prepared, and glyoxalase I (Glo1) activity was measured. (C) Cells were treated with $CaCl_2$ and/or FK506 as described in (A), except that cells had been pretreated with 10 μg/ml cycloheximide (CHX) for 10 min before treatment with $CaCl_2$.

Figure 3 Time course of the expression of *GLO1* in response to Ca^{2+} Cells were cultured in YPD medium until a log phase of growth, and 300 mM $CaCl_2$, or 300 mM $CaCl_2$ plus 1 μg/ml FK506 was added (time 0 min). Total RNA was prepared periodically, and Northern blotting was conducted.

Figure 4 Effects of Ca^{2+} and FK506 on the phosphorylation and nucleocytoplasmic localization of Hog1 (A) Cells were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506. After 10 min of incubation, phosphorylation of Hog1 as well as the level of Hog1 protein was determined. (B) *hog1*Δ cells carrying a plasmid for Hog1-GFP (pRS4-Hog1-GFP) [18] were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506 for 10 min, after which the nucleocytoplasmic localization of Hog1-GFP was determined with fluorescence microscopy. (C) Cells of wild type (WT), *cnb1*Δ or *crz1*Δ mutant carrying a plasmid for Hog1-GFP were cultured in YPD medium until a log phase of growth, 300 mM $CaCl_2$, or 300 mM $CaCl_2$ plus 1 μg/ml FK506 was added, and the nucleocytoplasmic localization of Hog1-GFP was determined periodically.

Figure 5 Role of STRE in the bizarre response (A) Schematic diagram of STRE construct. (B, C) Cells of wild type (WT) or a *hog1*Δ mutant carrying each *GLO1-lacZ* reporter gene were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. (D) Wild type cells carrying pRS423 or pRS423+*HOG1* were cultured in SD medium until a log phase of growth. Cells were collected by centrifugation, and suspended in fresh YPD medium containing 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined.

Figure 6 STRE is involved in the bizarre response (A) Cells carrying a plasmid for $STRE_{CTT1}$-*lacZ* were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 60 min of incubation, β-galactosidase activity was determined. (B) Each strain carrying a *cta1*Δ mutation (defective in peroxisomal catalase) in the YPH250 background was cultured in a log phase of growth,

and treated with $CaCl_2$ with or without FK506 as described in (A). After 60 min of incubation, cell extracts were prepared, and catalase activity was measured. (C) Cells (YPH250 background) carrying *GPX1-lacZ* in the *ura3* locus in wild type (WT) and an *msn2*Δ*msn4*Δ (*msn2*Δ::*KanMX4 msn4*Δ::*TRP1*) strain were cultured in YPD medium until a log phase of growth, and treated with $CaCl_2$ and/or FK506 as described in (A), after which *β*-galactosidase activity was determined.

Figure 7 Negative effect of Crz1 on Msn2 (A) Cells carrying the *MSN2*-overexpression plasmid (pAMG, *MSN2-OP*) [33] or its control vector (YCplac111), and *CRZ1*-overexpression plasmid (pAMS453, *CRZ1-OP*) [24] or its control vector (YEp351) were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 60 min of incubation, Glo1 activity was determined. (B) Cells carrying pAdh1-Msn2-GFP were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ in the presence or absence of 1 μg/ml FK506, after which the nucleocytoplasmic localization of Msn2-GFP was determined periodically. (C) Cells carrying a plasmid for GFP-Crz1 (pAMS463) [34] were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$ and/or 1 μg/ml FK506. After 10 min of incubation, the nucleocytoplasmic localization of GFP-Crz1 was determined. The distribution of GFP-tagged Crz1 in cells treated with 500 mM NaCl for 10 min was determined also.

Figure 8 Effect of Ca^{2+} on degradation of Msn2 (A) Cells of wild type (WT) or *crz1*Δ carrying a *CEN*-type plasmid for Msn2-GFP (pRS316+Msn2-GFP) were cultured in YPD medium until a log phase of growth, and treated with 300 mM $CaCl_2$, or 300 mM $CaCl_2$ plus 1 μg/ml FK506. At each prescribed time, the level of Msn2 protein was determined using anti-GFP antibody. The level of Cdc28 protein is shown as a loading control. (B) Cells carrying a plasmid for Msn4-GFP (pGR247) [31] were treated as described in (A). (C) Cells of wild type (WT) or *hog1*Δ carrying pRS316+Msn2-GFP were treated as described in (A). (D) Wild type cells carrying pRS316+Msn2-GFP and pRS423, or pRS316+Msn2-GFP and pRS423+*HOG1* were cultured in SD medium until a log phase of growth. Cells were collected by centrifugation, and suspended in a fresh YPD medium containing 300 mM $CaCl_2$, or 300 mM $CaCl_2$ plus 1 μg/ml FK506. At each prescribed time, the level of Msn2 protein was determined. (E) *erg6*Δ::*KanMX4* cells in the BY4741 background carrying pRS316+Msn2-GFP were treated with $CaCl_2$ as described in (A) for 90 min. Lane 1, control (YPD); lane 2, 300 mM $CaCl_2$; lane 3, 300 mM $CaCl_2$ plus 1 μg/ml FK506; lane 4, 300 mM $CaCl_2$ plus 50 μM MG132; and lane 5, 300 mM $CaCl_2$ plus 1% dimethyl sulfoxide (solvent to dissolve MG132).

Figure 9 A tentative model for the CDMD Extracellular Ca^{2+} activates the Hog1 MAP kinase cascade, and subsequently, phosphorylated Hog1 is translocated into the nucleus. Extracellular Ca^{2+} induces the nuclear accumulation of Msn2. Meanwhile, extracellular Ca^{2+} enters the cell, and activates calcineurin in a Ca^{2+}/calmodulin-dependent manner. Calcineurin dephosphorylates Crz1, thereby allowing its nuclear accumulation. Crz1 in the nucleus, in turn, enhances the

degradation of Msn2, which leads to the insufficient induction of a gene whose expression is dependent upon both STRE and Msn2-Hog1. Hence, if calcineurin is inhibited by FK506 or inactivated by disruption of its regulatory subunit (*cnb1*Δ) to block the nuclear accumulation of Crz1, or if *CRZ1 per se* is destroyed (*crz1*Δ), the degradation of Msn2 is suppressed, which leads to full activation of Msn2. Details are described in the text.