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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 (crz1Δ) 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, C2H2-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 glo1Δ 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 elf2α 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 CaCl₂ induces the expression of GLO1, which is strictly dependent upon Hog1, Msn2/Msn4, and two STREs in the GLO1 promoter. Notably, the Ca²⁺-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 Ca²⁺/calmodulin. Full induction of the expression of GLO1 following treatment with CaCl₂ 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 Ms2/Msn4 in the nuclei of cells treated with CaCl₂ by facilitating the degradation of Msn2 and Msn4. Consequently, a further increase in the Ca²⁺-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 CaCl₂.

**EXPERIMENTAL**

**Strain, plasmid, and medium**

Unless otherwise stated, yeast strains used in this study have the YPH250 background (MATα trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52). Construction of the hog1Δ, pbs2Δ, ssk1Δ, sho1Δ, ssk1Δsho1Δ, cnb1Δ, crz1Δ, msn2Δ, and msn2Δmsn4Δ mutants in YPH250 was described previously [13, 18]. An erg6Δ mutant has the BY4741 background (MATα 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 28ºC 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 coefficient of 3.37 mM⁻¹cm⁻¹ [14]. One unit of β-galactosidase activity was defined as the amount of enzyme that increases the A₄₂₀ by 1000 per h [13]. Protein concentrations were determined by the method of Bradford [28].

**Northern blotting**

Cells were cultured in YPD medium until A₆₁₀ = 1.0, and 300 mM CaCl₂ 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-³²P)dCTP was prepared as described previously [13].

**Detection of Hog1 phosphorylation**

Cells were cultured in YPD medium until A₆₁₀ = 1.0, and 300 mM CaCl₂ 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 CaCl₂ 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 MgCl₂, 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 SalI site of YIp5 (YIp5+GLO1-lacZ), and the cassette was then amplified by PCR with 5TO358F (5’-AGGGCATCGGTCTCGAGGTAAATTC-3’) and 5TO358R (5’-TAAGGGAATTCTCCCAGGTCTTCTCAAT-3’) using YIp5+GLO1-lacZ as a template. SalI and EcoRI sites were designed in 5TO358F and 5TO358R, respectively (underlined). The PCR fragment was digested with SalI and EcoRI, and cloned into the SalI-EcoRI site of YIp358R. The resultant plasmid (YIp358R+GLO1-lacZ) was digested with Ncol, 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’-AATAGGTTAAAGAGATGGGGGTGGGTTGG-3’; GSTRE1R, 5’-CCACCCCCACCCCCATCTCTTACCTATT-3’; GSTRE2, 5’-CTGAATTTAAAGATGTCTCTTTGATGG-3’; and GSTRE2R, 5’-CCATCGTAAAGCATCTTTATTCTACGAGGTG-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 SalI and EcoRI, and the resultant fragment was inserted into the SalI-EcoRI 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 \textit{GLO1-lacZ} gene carrying both mutated \textit{STRE1} and \textit{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 \textit{SalI} and \textit{EcoRI}, and the resultant fragment was inserted into the \textit{SalI}-\textit{EcoRI} site of YIp358R to yield YIp358R+PMS12. Introduction of the mutation was verified by DNA sequencing.

\textbf{Construction of GPX1-lacZ reporter gene}

To amplify the region between -365 and +8 of \textit{GPX1}, taking the translation initiation codon (ATG) as +1, PCR was done with GPX1-lacZ-F (5'-GGAGTCGACGGACTTGATAGAATCCACCTT-3') and GPX1-lacZ-R (5'-GGTGAAAAAGAATGAAATCCTTGCATCGTT-3'). \textit{SalI} and \textit{EcoRI} sites were designed in GPX1-lacZ-F and GPX1-lacZ-R, respectively (underlined). The PCR fragment was digested with \textit{SalI} and \textit{EcoRI}, and cloned into the \textit{SalI}-\textit{EcoRI} site of YIp358R. The resultant plasmid (YIp358R-GPX1-lacZ) was digested with \textit{NcoI}, and the linearized fragment was integrated into the \textit{ura3} locus of YPH250.

\textbf{Construction of HOG1-overexpression plasmid}

The \textit{HOG1} gene was amplified by PCR with HOG1S (5'-GGTGTTAGGAAGCATGCTTTATCTCCAAG-3') and HOG1R (5'-CCTTTTATGGAATTCCTTACATTTAAGGAGG-3') using the genomic DNA of YPH250 as a template. \textit{SphI} and \textit{BamHI} sites were designed in HOG1S and HOG1R, respectively (underlined). The PCR fragment was digested with \textit{SphI} and \textit{BamHI}, and cloned into the \textit{SphI}-\textit{BamHI} site of a 2 \mu-type plasmid pRS423 to yield pRS423+HOG1.

\textbf{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 \(\mu\)g/ml FK506. After incubation for the prescribed time at 28\(^\circ\)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.

\textbf{RESULTS}

\textbf{Expression of GLO1 is induced by Ca\textsuperscript{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 \textit{GLO1} encoding an enzyme crucial for the metabolism of MG is altered [2, 13]. Here we found that levels of \textit{GLO1} mRNA were increased following treatment with CaCl\(_2\) (Figure 1A). We constructed the \textit{GLO1-lacZ} reporter gene to verify the Ca\textsuperscript{2+}-induced expression of \textit{GLO1}, and found that the activity of
β-galactosidase driven by GLO1-lacZ was increased following treatment with CaCl₂ (see Figure 5B). The activity of glyoxalase I (Glo1) was also increased in cells treated with CaCl₂ (Figure 1B). Therefore, we concluded that CaCl₂ 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 hog1Δ mutant. As shown in Figure 1A, the level of GLO1 mRNA was not increased in hog1Δ cells following the treatment with CaCl₂. Induction of Glo1 by CaCl₂ occurred in neither the hog1Δ nor pbs2Δ 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, C2H2 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 Ca²⁺-induced expression of GLO1, we conducted Northern blotting in an msn2Δmsn4Δ mutant. As shown in Figure 1A, the levels of GLO1 mRNA in msn2Δmsn4Δ cells were not increased following the treatment with CaCl₂, indicating that Msn2 and Msn4 are necessary for the response of GLO1 to Ca²⁺.

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 Ca²⁺-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 ssk1Δsho1Δ double mutant.

Curious response of GLO1 to Ca²⁺ and FK506
Although we have shown that the Hog1 MAP kinase cascade is necessary for the Ca²⁺-induced expression of GLO1, we determined whether the Ca²⁺/calmodulin-dependent calcineurin system is also involved in this response, because the system is a well-known pathway for Ca²⁺ signaling in eukaryotic cells, including S. cerevisiae [40]. In the Ca²⁺/calcineurin system, extracellular Ca²⁺ 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 Ca²⁺-induced genes regulated by the Ca²⁺/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 Ca²⁺-induced expression of GLO1, we determined the effect of FK506. Surprisingly, and rather bizarrely, the Ca²⁺-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$\Delta$ and sho1$\Delta$ mutants, but not in the ssk1$\Delta$sho1$\Delta$ 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$\Delta$ and crz1$\Delta$ 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-inducible 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$\Delta$ and crz1$\Delta$ 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 CaCl2. As shown in Figure 3, the amount of GLO1 mRNA in wild-type cells gradually increased upon the addition of CaCl2, reaching a maximum after 30-40 min of incubation. The timing of the increase in the level of GLO1 mRNA in cells treated with CaCl2 plus FK506 was almost the same as that in cells treated with CaCl2 alone, although the amount of GLO1 mRNA was obviously higher than that in cells treated with CaCl2 alone. The pattern of the changes in the level of GLO1 mRNA in cnb1Δ cells treated with CaCl2 was substantially the same as that in wild-type cells treated with CaCl2 plus FK506. The expression profile of GLO1 in crz1Δ cells in response to Ca2+ was essentially similar to that in cnb1Δ cells (data not shown). Therefore, the calcineurin pathway seems to negatively influence the maximal level of GLO1 mRNA in response to Ca2+.

**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 Ca2+-induced expression of GLO1 was strictly dependent upon Hog1, we determined the level of Hog1 phosphorylation following treatment with CaCl2. As shown in Figure 4A, Hog1 was phosphorylated in cells treated with CaCl2, however, the simultaneous treatment of cells with CaCl2 and FK506 did not enhance the phosphorylation of Hog1. Similarly, the level of phosphorylated Hog1 in cnb1Δ as well as crz1Δ cells treated with CaCl2 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 CaCl2, and the intensity of the fluorescence derived from Hog1-GFP in the nuclei in cells treated with CaCl2 was the same as that observed in cells treated with CaCl2 and FK506 simultaneously.

We determined the nuclear localization of Hog1 following the treatment with CaCl2 and FK506. In wild-type cells, Hog1 was immediately accumulated in the nucleus upon the treatment with CaCl2, and redistributed to the cytoplasm after 30-45 min of incubation in the presence of CaCl2. 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 cnb1Δ and crz1Δ cells (Figure 4C). Therefore, even though Hog1 is necessary for GLO1 to respond to extracellular Ca2+, 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 Ca2+-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].
Ca\textsuperscript{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\Delta mutant. As Msn2 is the primary transcription factor for the STRE-dependent gene, the Ca\textsuperscript{2+}-induced expression of GLO1-lacZ was impaired in msn2\Delta cells when compared with that in wild type cells (Supplementary Figure 1). Even though the induction rate was quite small, the Ca\textsuperscript{2+}-induced expression of GLO1 occurred in msn2\Delta cells, which may be caused by Msn4; however, only limited increase in \(\beta\)-galactosidase activity was observed in the presence of FK506 (Supplementary Figure 1). Since the Ca\textsuperscript{2+}-induced expression of PMS1 was comparable to that of wild-type STRE construct in msn2\Delta cells, Msn4 may bind to STRE2 in response to Ca\textsuperscript{2+}. No induction was observed in msn2\Deltamsn4\Delta 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\textsuperscript{2+}-induced expression of GLO1 was markedly reduced in a hog1\Delta 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\textsubscript{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'-TTCAAGGGGATACAACGGAAGGGGCAAG-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\textsuperscript{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\Delta as well as crz1\Delta cells following treatment with CaCl\textsubscript{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\textsubscript{2} in an Mns2/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\textsuperscript{2+} [50]. On the other hand, the response in terms of the activity of catalase as well as STRE\textsubscript{CTT1}-lacZ to Ca\textsuperscript{2+} is dependent upon Hog1 (Figures 6A and 6B). Collectively, the STRE regulated by both Hog1 and Msn2/Msn4 seems able to mediate
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 Ca²⁺-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 Ca²⁺ was determined in cells overexpressing MSN2 or CRZ1. As shown in Figure 7A, the activity of Glo1 in MSN2-overexpressing cells following treatment with CaCl₂ 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 CaCl₂ in CRZ1-overexpressing cells was repressed, but the activity increased to a maximal level when CaCl₂ and FK506 were added simultaneously.

We have previously reported that Msn2 is accumulated in the nucleus following treatment with CaCl₂ (ref. [42], also see Figure 7B). Msn2 was accumulated in the nucleus immediately upon the treatment of cells with CaCl₂, 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 cnb1Δ cells was the same as that in wild-type cells (Figure 7B). The nucleocytoplasmic localization of Msn2 in response to Ca²⁺ and FK506 in crz1Δ cells was substantially the same as that in wild-type and cnb1Δ cells (data not shown). Meanwhile, Crz1 was also accumulated in the nucleus upon treatment with CaCl₂, 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 CaCl₂, 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 Ca²⁺

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 Ca²⁺ is present. To address this possibility, we determined the level of Msn2 protein following treatment with CaCl₂ with or without FK506. As shown in Figure 8A, the level of Msn2 protein in wild-type cells gradually decreased in the presence of CaCl₂ but the reduction was blocked when FK506 was present; whereas in crz1Δ cells, the reduction in the level of Msn2 protein following the treatment with CaCl₂ was repressed even in the absence of FK506 (Figure 8A). Substantially, the same results in terms of the stability of Msn2 were obtained in cnb1Δ 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\textsuperscript{2+}.

Since the bizarre response was observed in genes whose expression is dependent upon Hog1, we determined the stability of Msn2 in hog1Δ cells and Hog1-overexpressed cells. The Ca\textsuperscript{2+}-induced degradation of Msn2 was observed in hog1Δ cells, which was blocked by the addition of FK506 (Figure 8C). Meanwhile, in Hog1-overexpressed cells, the Ca\textsuperscript{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\textsubscript{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\textsubscript{2}. During the course of this study, intriguingly, we found that the Ca\textsuperscript{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\textsubscript{2}. Therefore, the bizarre response occurred only with a combination of Ca\textsuperscript{2+} and FK506, not with a combination of FK506 and other ions (Na\textsuperscript{+} and K\textsuperscript{+}) 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\textsuperscript{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\textsuperscript{2+} was virtually unaffected by FK506. In addition, Durchschlag et al. [52] have reported that Msn2 is constitutively concentrated in the nucleus in msn5\textDelta 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\textDelta mutant were normal [52], which is accounted for by a decrease in the total amount of Msn2 protein in msn5\textDelta 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\textDelta 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\textsubscript{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\textsubscript{2}. Additionally, if Crz1 is titrated by Hog1, the induction of the expression of Crz1-dependent gene by Ca\textsuperscript{2+} would be impaired in Hog1-overexpressed cells. So, we determined the response of GPX2 to Ca\textsuperscript{2+}, the expression of which is regulated by the calcineurin/Crz1 pathway [42]. However, GPX2 normally responded to Ca\textsuperscript{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\textsuperscript{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 CaCl₂ for 30 min. Among them, we looked for the gene whose expression was induced further when FK506 is present together with CaCl₂. Next, we calculated the ratio of the induction fold between the Ca²⁺-induced expression ([Ca²⁺]) and the Ca²⁺ plus FK506-induced expression [Ca²⁺+FK506]. We searched for the gene whose [Ca²⁺+FK506]/[Ca²⁺] 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 Ca²⁺**

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 Ca²⁺ 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 CaCl₂-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 Ca²⁺-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 erg6Δ mutant because MG132 is hardly taken up by wild type cells [55]. As shown in Figure 8E, Msn2 was degraded in erg6Δ cells following treatment with CaCl₂, which was blocked by FK506. We confirmed that the bizarre response occurred in the erg6Δ mutant also (data not shown). However, the addition of MG132 did not repress the degradation of Msn2 in the presence of CaCl₂.

Importantly, the CDMD was not seen in the gene whose response to Ca²⁺ is independent of Hog1, even though its Ca²⁺-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


27 Cyert, M. S. (2003) Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go
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
signaling pathway. FEBS Lett. 569, 301–306
50 Ohdate, T., Izawa, S., Kita, K. and Inoue, Y. Regulatory mechanism for expression of GPX1 in response to glucose starvation and Ca²⁺ in Saccharomyces cerevisiae: Involvement of Snf1 and Ras/cAMP pathway in Ca²⁺ signaling. Genes Cells (in press)
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 Ca²⁺ 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 CaCl₂ 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 CaCl₂ 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 nM 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\(\Delta\) 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\(\Delta\) or crz1\(\Delta\) 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\(\Delta\) 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\(\Delta\) mutation (defective in peroxisomal catalase) in the YPH250 background was cultured in a log phase of growth,
and treated with CaCl₂ 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 msn2Δmsn4Δ (msn2Δ::KanMX4 msn4Δ::TRP1) strain were cultured in YPD medium until a log phase of growth, and treated with CaCl₂ 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ 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 Ca²⁺ on degradation of Msn2** (A) Cells of wild type (WT) or crz1Δ 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 CaCl₂ or 300 mM CaCl₂ 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 hog1Δ 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 CaCl₂ or 300 mM CaCl₂ plus 1 µg/ml FK506. At each prescribed time, the level of Msn2 protein was determined. (E) erg6Δ::KanMX4 cells in the BY4741 background carrying pRS316+Msn2-GFP were treated with CaCl₂ as described in (A) for 90 min. Lane 1, control (YPD); lane 2, 300 mM CaCl₂; lane 3, 300 mM CaCl₂ plus 1 µg/ml FK506; lane 4, 300 mM CaCl₂ plus 50 µM MG132; and lane 5, 300 mM CaCl₂ plus 1% dimethyl sulfoxide (solvent to dissolve MG132).

**Figure 9 A tentative model for the CDMD** Extracellular Ca²⁺ activates the Hog1 MAP kinase cascade, and subsequently, phosphorylated Hog1 is translocated into the nucleus. Extracellular Ca²⁺ induces the nuclear accumulation of Msn2. Meanwhile, extracellular Ca²⁺ enters the cell, and activates calcineurin in a Ca²⁺/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 (cnb1Δ) to block the nuclear accumulation of Crz1, or if CRZ1 per se is destroyed (crz1Δ), the degradation of Msn2 is suppressed, which leads to full activation of Msn2. Details are described in the text.