Running title: Regulation of *GPX1* expression

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

Takumi Ohdate, Shingo Izawa[§], Keiko Kita and Yoshiharu Inoue*

Laboratory of Molecular Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

*Correspondence: y_inoue@kais.koyoto-u.ac.jp.

[§]Present address: Laboratory of Microbial Engineering, Division of Applied Biology, Kyoto Institute of Technology.

Saccharomyces cerevisiae has three homologues of the glutathione peroxidase gene, *GPX1*, *GPX2*, and *GPX3*. We have previously reported that the expression of *GPX3* was constitutive, but that of *GPX2* was induced by oxidative stress and $CaCl_2$, and uncovered the regulatory mechanisms involved. Here we show that the expression of *GPX1* is induced by glucose starvation and treatment with $CaCl_2$. The induction of *GPX1* expression in response to glucose starvation and Ca^{2+} was dependent on the transcription factors Msn2 and Msn4 and *cis*-acting elements (stress response element, STRE) in the *GPX1* promoter. The Ras/cAMP pathway is also involved in the expression of *GPX1*. We found that Snf1, a Ser/Thr protein kinase, is involved in the glucose starvation- and Ca^{2+}-induced expression of *GPX1*. The activation of Snf1 is accompanied by phosphorylation of Thr^{210}. We found that the Ca^{2+}-treatment as well as glucose starvation causes the phosphorylation of Thr^{210} of Snf1 in a Tos3, Sak1, and Elm1 protein kinase-dependent manner. Since the timing of the initiation of the Ca^{2+}-induced expression of *GPX1* was retarded in an *snf1*Δ mutant, the activation of Snf1 seems pivotal to the early-stage-response of *GPX1* to Ca^{2+}.

Introduction

Cells alter their pattern of gene expression in response to environmental stress, such as oxidative stress, heat shock stress and osmotic stress. The change in the availability of nutrients, such as amino acids and glucose, is also crucial to the remodeling of gene expression. The budding yeast *Saccharomyces cerevisiae* has provided an excellent model with which to analyze the regulatory mechanism of gene expression as well as cell signaling systems. Cellular responses to glucose have also been studied extensively using yeasts as models. In the presence of glucose, two Ras proteins, Ras1 and Ras2, activate adenylate cyclase. The activated adenylate cyclase catalyzes the synthesis of cAMP, which in turn activates the protein kinase A (PKA) pathway (Gancedo 1998; Carlson 1999; Rolland *et al.* 2002). PKA is involved in many important cellular events, such as gene expression, cell cycle control, and stress response (Santangelo 2006). Regarding the response to stress, PKA regulates the nucleocytoplasmic localization of the transcription factors Msn2 and Msn4 (Görner *et al.* 1998). Msn2 and Msn4, C_2H_2 zinc-finger proteins, are known to be important in responses to various types of environmental stress (Estruch 2000). Msn2 and Msn4 bind to a characteristic DNA sequence referred to as the stress response element (STRE, 5'-AGGGG-3' or 5'-CCCCT-3') in the promoter region of various stress-responsive genes, and induce their expression. PKA phosphorylates Msn2, thereby sequestering it to the cytoplasm (Görner *et al.* 2002). Therefore, the low level of PKA activity is associated with an increasing proportion of nuclear Msn2, leading to transcriptional activation.

Glucose repression is a well-known phenomenon in not only yeasts but also many organisms. Snf1, a Ser/Thr protein kinase and a tentative counterpart of a mammalian AMP-activated protein kinase (AMPK), is a key regulator for the derepression of the glucose-repressed genes in yeast (Gancedo 1998; Carlson 1999).

Thr^{172} of mammalian AMPK is phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) during the activation process (Hawley *et al.* 2005; Hurley *et al.* 2005; Woods *et al.* 2005). In the activation of Snf1 in cells starved of glucose, Thr^{210} of Snf1 is phosphorylated by three redundant upstream protein kinases, Tos3, Elm1, and Sak1, homologues of mammalian LKB1 and CaMKKα (Hong *et al.* 2005). Snf1 thus activated phosphorylates its target such as Mig1 (Treitel *et al.* 1998).

The availability of nitrogen also affects many cellular events. The target of rapamycin (TOR) pathway plays crucial roles in nitrogen signaling in yeasts and mammals (De Virgilio & Loewith 2006). The pathway regulates transcription, translation, ribosome biogenesis, nutrient transport, autophagy, and the organization of the actin cytoskeleton (Schmelzle & Hall 2000). Regarding the correlation between stress responses and the TOR pathway, it has been proven that TOR kinase negatively regulates the nuclear localization of Msn2 and Msn4 (Beck & Hall 1999).

When starved of nutrient, yeast cells undergo a remodeling of metabolism and gene expression to survive extended periods: accumulating storage carbohydrates such as glycogen and glycerol, increasing the expression of stress-responsive genes, and thickening the cell wall (Herman 2002; Longo *et al.* 1996). Oxidative stress is one of the most notable features in the stationary phase, in which the availability of nutrients would be limited. Organisms are armed with several antioxidants to scavenge reactive oxygen species, such as superoxide dismutase, catalase, and peroxidases. The expression of yeast *CTT1* (cytosolic catalase), *TSA2* (cytosolic thiol peroxidase), and *PRX1* (mitochondrial thiol peroxidase) is induced by glucose starvation in an Msn2 and Msn4-dependent manner (Hong *et al.* 2002; Martínez-Pastor *et al.* 1996; Monteiro & Netto 2004). We have previously cloned the *GPX1*, *GPX2*, and *GPX3* genes in *S. cerevisiae* encoding a homologue of mammalian glutathione peroxidase (GPx), a major antioxidant in higher eukaryotes (Inoue *et al.* 1999).

In contrast to Gpx2 and Gpx3, information about Gpx1 has been largely lacking. To gain a clue as to the physiological function of Gpx1, here we searched for the conditions that induce the expression of *GPX1*, and investigated the regulatory mechanism involved. We found that the expression of *GPX1* was induced by glucose starvation, nitrogen starvation, and treatment with $CaCl_2$ in an Msn2 and Msn4-dependent manner. The Ras/cAMP pathway, TOR pathway and Snf1 kinase were involved in the upstream modules for the glucose starvation-induced expression of *GPX1*. Notably, we found that Snf1 is involved in the Ca^{2+} signaling pathway. Hence, in the present study, we aimed to clarify the regulatory mechanism of the expression of *GPX1*, and also tried to show the role of Snf1 in Ca^{2+} signaling through the elucidation of the expression of *GPX1*.

Results

Expression of *GPX1* is induced by glucose starvation

We have previously reported that expression of *GPX1* is induced when logarithmically growing cells in YPD medium are transferred to glucose-starved medium (Inoue *et al.* 1999). To quantify the expression of *GPX1*, a *GPX1-lacZ* reporter gene was constructed. As shown in Fig. 1A, mRNA levels of *GPX1* increased upon glucose starvation as we reported previously (Inoue *et al.* 1999). *β*-Galactosidase activity derived from *GPX1-lacZ* was also increased in glucose-starved cells. We constructed a HA-tagged *GPX1* gene (*GPX1-3HA*) also to monitor the Gpx1 protein level. As shown in Fig. 1A, Gpx1 protein levels also increased with glucose starvation. Collectively, the monitoring systems (*GPX1-lacZ* and *GPX1-3HA*) constructed here are valid to evaluate the expression of *GPX1*.

Expression of *GPX1* is induced by Ca^{2+}

By using *GPX1-lacZ*, we screened the conditions that induce the expression of *GPX1*, and found that the expression was induced following treatment with $CaCl_2$. The maximum induction of *GPX1-lacZ* expression in terms of the $CaCl_2$ concentration was attained at 400 mM (data not shown). As shown in Fig. 1A, mRNA levels of *GPX1* as well as protein levels of Gpx1 also increased following treatment with $CaCl_2$.

High concentrations of $CaCl_2$ cause an osmotic stress response in yeast. However, the expression of *GPX1* was not substantially induced by osmotic stress caused by 1 M sorbitol (data not shown). Hog1, a mitogen-activated protein (MAP) kinase responsible for the osmotic stress response in *S. cerevisiae* (Brewster *et al.* 1993; Errede & Levin 1993), was phosphorylated and accumulated in the nucleus following treatment with 300 mM $CaCl_2$ (Takatsume, Y., Ohdate, T., Maeta, K., Nomura, W., Izawa, S., and Inoue, Y., submitted). To determine whether Hog1 is involved in the Ca^{2+}-induced expression of *GPX1*, *HOG1* was disrupted. As a result, expression of *GPX1* was induced in *hog1*Δ cells following treatment with $CaCl_2$ when evaluated using *GPX1-lacZ* (Fig. 1B), indicating that the Hog1-MAP kinase pathway is not involved in the Ca^{2+}-induced expression of *GPX1*. Hence, the induction of the expression of *GPX1* in response to Ca^{2+} seems not a response to osmotic stress.

The calcineurin/Crz1 pathway is a major device of Ca^{2+} signaling in yeast (Cohen 1989; Cyert 2003; Sugiura *et al.* 2001; Yoshimoto *et al.* 2002). A gene whose expression is regulated by the calcineurin/Crz1-mediated pathway has a distinct DNA sequence called CDRE (calcineurin-dependent response element). Yoshimoto *et al.* (2002) have reported a genome-wide analysis of gene expression that is regulated in a calcineurin/Crz1-dependent manner in *S. cerevisiae*. The computational analysis revealed that the calcineurin/Crz1-dependent genes contain a common sequence

(5'-GAGGCTG-3') in their promoter region (Yoshimoto *et al.* 2002). We have previously reported that the expression of *GPX2*, another gene of GPx homologue in *S. cerevisiae*, was induced following treatment with $CaCl_2$ in a calcineurin/Crz1-dependent manner (Tsuzi *et al.* 2004b). Thus, we looked for this motif in the *GPX1* promoter, although no such a sequence was found. Indeed, the induction of *GPX1* expression following treatment with Ca^{2+} did occur even in a mutant lacking Crz1 (Fig. 1B).

Msn2 and Msn4 act as key transcription factors of *GPX1*

The *GPX1* promoter has two putative STREs (consensus sequence: 5'-CCCCT-3' or 5'-AGGGG-3') located between -303 and -298 (STRE1; 5'-CCCCT-3'), and between -248 and -243 (STRE2; 5'-CCCCT-3') upstream of the translation initiation codon of *GPX1* (Fig. 2A). Msn2 and Msn4 are known to be responsible for general stress responses, and bind to STRE to induce the expression of a wide variety of stress-responsive genes. Although the *GPX2* promoter has the consensus sequence of STRE, neither Msn2 nor Msn4 regulates the expression of *GPX2* under oxidative stress and $CaCl_2$-treatment (Tsuzi *et al.* 2004a, 2004b). To determine whether Msn2 and Msn4 are involved in the expression of *GPX1* induced by glucose starvation and Ca^{2+}, we disrupted *MSN2* and *MSN4*. The induction of *GPX1* expression under glucose starvation and Ca^{2+}-treatment was vastly repressed in *msn2*Δ*msn4*Δ cells (Fig. 2B). Therefore, Msn2 and Msn4 are key transcription factors in the expression of *GPX1*.

Roles of two putative STREs in *GPX1* promoter as *cis*-element

To determine whether the two STREs in the *GPX1* promoter function as *cis*-elements in response to glucose starvation and Ca^{2+} signaling, the consensus sequence of STRE1 and/or STRE2 was changed to 5'-CATCT-3' (Fig. 2A), the mutation which was proven to disable STRE's function (Zahringer *et al.* 2000). Each STRE-mutated *GPX1* promoter

was fused with the *lacZ* gene, and the resulting reporter genes (STRE1mut-, STRE2mut-, and STRE1/2mut-*lacZ*) were integrated into the genome of *S. cerevisiae*. All STRE mutations resulted in a marked decrease in the *GPX1* promoter activity in response to glucose starvation and $CaCl_2$ (Fig. 2C).

To verify further whether the STREs found in the *GPX1* promoter function as the *cis*-acting elements in terms of glucose-starvation as well as Ca^{2+}-induced responses, the corresponding region (-345 to -182) was introduced upstream of the $CYC1_{TATA}$-*lacZ* reporter gene in pTBA30 (Galius-Duerner *et al.* 1997). As shown in Fig. 2D, the *β*-galactosidase activity derived from p*GPX1-CYC1-lacZ* was increased by glucose starvation and Ca^{2+} as observed for *GPX1-lacZ*, which has the original promoter of *GPX1*. When p*GPX1-CYC1-lacZ* was introduced into the *msn2*Δ*msn4*Δ mutant, no increase in *β*-galactosidase activity was observed in response to glucose starvation or $CaCl_2$ treatment (Fig. 2D). Taken together, Msn2 and Msn4 are responsible for the glucose starvation and Ca^{2+}-induced signaling response of *GPX1*, and STREs in the *GPX1* promoter function as the *cis*-acting elements for such events.

Role of Ras/cAMP pathway in expression of *GPX1*

The Ras/cAMP pathway negatively regulates the nucleocytoplasmic localization of Msn2 and Msn4 via PKA (Görner *et al.* 1998). We determined the expression of *GPX1* in *ras2*Δ cells in response to glucose starvation and $CaCl_2$. As we expected, the deletion of *RAS2* enhanced the basal level of Gpx1 protein under non-stressed conditions. The glucose starvation and $CaCl_2$ treatment increased Gpx1 protein levels further even in *ras2*Δ cells (Figs. 3A and 3B). So, first, we explored the machinery involved in the Ca^{2+}-induced expression of *GPX1* in *ras2*Δ cells. Since we have demonstrated that Msn2 and Msn4 play crucial roles in the Ca^{2+}-induced expression of *GPX1*, and the PKA pathway negatively regulates Msn2 and Msn4 (Görner *et al.* 1998), we determined whether or not

the increase in the basal level of *GPX1* expression in *ras2*Δ cells is suppressed by the disruption of *MSN2* and *MSN4*. As shown in Fig. 3B, basal level the *GPX1-lacZ* expression in *msn2*Δ*msn4*Δ*ras2*Δ cells was reverted to that in wild type cells. Next, to verify the involvement of the Ras/cAMP pathway in the expression of *GPX1* in response to Ca^{2+}, the phosphorylation state of Msn2 was determined. It has been reported that Ser^{582} and Ser^{620} of Msn2 are phosphorylated by PKA, thereby sequestering this transcription factor in the cytoplasm when enough glucose is supplied in the medium (Görner *et al.* 2002). We confirmed that Msn2 is concentrated in cells starved of glucose (Fig. 3C). We have previously reported that Msn2 is concentrated in the nucleus in cells treated with $CaCl_2$, which was also confirmed in this study (Fig. 3C). In *ras2*Δ cells, Msn2 was partially distributed in the nucleus even though enough glucose is present, although upon glucose starvation or $CaCl_2$-treatment, nuclear Msn2 was clearly observed (Fig. 3C). It has been reported that these two phospho-serines are dephosphorylated upon glucose starvation, which was confirmed in this study (Fig. 3D). As shown in Fig. 3D, both Ser^{582} and Ser^{620} of Msn2 in wild-type cells were slightly dephosphorylated following treatment with $CaCl_2$ also. These results suggest that Ca^{2+} gives rise to a negative effect on the PKA pathway.

Since Gpx1 protein levels increased further in *ras2*Δ cells starved of glucose, in which cells Msn2 and Msn4 may be activated (Fig. 3A) even in the presence of glucose, in the next paragraph we explored another machinery involved in the glucose starvation- and Ca^{2+}-induced expression of *GPX1*.

Effect of Snf1 kinase on expression of Gpx1

Snf1, a Ser/Thr protein kinase, is one of the key regulators for the derepression of glucose-repressed genes in yeast. To determine whether Snf1 is involved in the expression of *GPX1*, we examined the Gpx1 level in an *snf1*Δ mutant. In wild type cells,

Gpx1 level increased after 30 min upon glucose starvation, and the level was kept almost constant until 180 min (Fig. 4A). In *snf1*Δ cells, Gpx1 level was also increased after 30 min of glucose starvation, and the level was almost constant within the period of experiment; however, overall level of Gpx1 in *snf1*Δ cells was lower compared with that of wild type cells (Fig. 4A). By contrast, in wild type cells Gpx1 level began to increase after 30 min of $CaCl_2$ treatment, peaked at 60 min, and then declined. Whereas in *snf1*Δ cells, intriguingly, the beginning of the Ca^{2+}-induced expression of *GPX1* was retarded for 30 min compared with that in wild type cells (Fig. 4A). The Gpx1 level reached a maximum after 90 min of $CaCl_2$ treatment, and it decreased slowly compared with that of wild type. These results suggest the following two possibilities: (i) Snf1 is involved in the early-stage-response (~30 min) of *GPX1* to Ca^{2+}, and (ii) Snf1 is involved in turnover of Gpx1 protein in the presence of Ca^{2+}.

Snf1 is involved in Ca^{2+} signaling pathway to *GPX1*

Next, we determined the effect of Ca^{2+} on phosphorylation of Snf1. In the activation of Snf1 in cells starved of glucose, Thr^{210} is phosphorylated (McCartney & Schmidt 2001, also see Fig. 4B). So, we determined whether Thr^{210} of Snf1 is phosphorylated upon treatment with $CaCl_2$. As shown in Fig. 4B, Thr^{210} was phosphorylated following treatment with $CaCl_2$.

Three upstream protein kinases, Tos3, Elm1 and Sak1, have been identified to phosphorylate Snf1 (Hong *et al.* 2003; Nath *et al.* 2003; Sutherland *et al.* 2003). In a *tos3*Δ*elm1*Δ*sak1*Δ mutant, Snf1 was not phosphorylated at all by glucose starvation (Hong *et al.* 2003), which was confirmed in this study (Fig. 4B). We determined whether Snf1 is activated by treatment with $CaCl_2$ in the *tos3*Δ*elm1*Δ*sak1*Δ mutant. As shown in Fig. 4B, Snf1 phophorylation did not occur in this mutant. Nonetheless, the phosphorylation level of Snf1 in cells treated with $CaCl_2$ was slightly lower compared

with that in cells starved of glucose. Calcineruin is a protein phosphatase that is activated by Ca^{2+}/calmodulin (Cohen 1989; Cyert 2003), following treatment with $CaCl_2$. To address the possibility that calcineruin dephosphorylates Snf1 in the presence of Ca^{2+}, we determined the phosphorylation level of Snf1 in a mutant defective in calcineurin (*cnb1*Δ). As shown in Fig. 4C, no distinct differences in the phosphorylation states of Snf1 in cells starved of glucose or treated with $CaCl_2$ were observed between wild type and *cnb1*Δ mutant. So, calcineurin is not likely to be involved in the control of phosphorylation state of Snf1 in the presence of Ca^{2+}.

Next, we determined the nucleocytoplasmic localization of Snf1. Snf1 distributes in the cytoplasm when glucose is supplied sufficiently, but upon glucose starvation, it is concentrated in the nucleus (Vincent *et al.* 2001). We confirmed the nuclear accumulation of Snf1 in cells starved of glucose (Fig. 4D). Indirect immunofluorescence microscopy revealed that Snf1 is concentrated in the nuclei in wild-type cells treated with $CaCl_2$; however, in the *tos3*Δ*elm1*Δ*sak1*Δ mutant, Snf1 resided in the cytoplasm (Fig. 4D). Together with the phosphorylation of Snf1, three upstream protein kinases (Tos3, Elm1 and Sak1) are activated following treatment with $CaCl_2$, thereby phosphorylating Snf1, which allows Snf1 to accumulate in the nucleus.

Chronic response of *GPX1* to glucose-limited conditions

Under the experimental conditions thus far employed, yeast cells at a log phase of growth were suddenly transferred to a glucose-starved (0.1% glucose) medium. Next, we determined whether the expression of *GPX1* was also induced when the glucose level in the medium was decreased gradually in accordance with the growth of yeast cells. So, the amount of Gpx1 protein in the cells cultured in YPD medium was monitored periodically. The glucose concentration in the medium was also measured. The level of Gpx1 reached a plateau after 37 h from the beginning of the culture, and concomitantly, the glucose in

the medium was depleted after 33-37 h (Fig. 5A). The diauxic shift is defined as the period in which yeast cells consume glucose in the medium completely, and change from fermentation to respiration as a means to obtain energy. The Gpx1 level was high during the diauxic shift (37-49 h), and it was kept constant in the post-diauxic shift (61-73 h).

Contribution of Gis1 to the post-diauxic response of *GPX1*

The chronic response of Gpx1 was monitored using the *msn2*Δ*msn4*Δ mutant also (Fig. 5B). Basically, the level of Gpx1 in *msn2*Δ*msn4*Δ cells was low compared with that in wild-type cells, although immunoreative bands corresponding to Gpx1-3HA were observed even in *msn2*Δ*msn4*Δ cells at the diauxic shift. Therefore, other transcription factor(s) may be involved in the expression of *GPX1* in the stationary phase. Pedruzzi *et al.* (2000) have reported that the transcription factor Gis1, responsible for genes induced post-diauxic shift, can partially mediate the induction of some genes possessing STRE. Hence, we constructed an *msn2*Δ*msn4*Δ*gis1*Δ mutant, and analyzed the expression of *GPX1-3HA* by Western blotting. The level of Gpx1 protein at the diauxic shift in a *gis1*Δ mutant was comparable with that in the wild type (data not shown), although no Gpx1 protein was observed in the *msn2*Δ*msn4*Δ*gis1*Δ mutant (Fig. 5B). Since the induction of Gpx1 in response to glucose starvation and Ca^{2+}-treatment occurred in *gis1*Δ cells at the log phase of growth (data not shown), Gis1 seems to contribute little in the response of *GPX1* to stress, but does contribute partially to the post-diauxic response of this gene.

Response of *GPX1* to nitrogen starvation

We have demonstrated that the expression of *GPX1* is induced by glucose-starvation as well as entry into the stationary phase, where not only glucose but also nitrogen sources may be limited. So, we next determined whether the expression of *GPX1* is induced when nitrogen is limited. The Gpx1 protein level was monitored at 6, 12 and 24-h after transfer

to a nitrogen-starved medium, because protein synthesis under such conditions may proceed slowly due to the limited amount of nitrogen sources such as amino acids. As a result, the amount of Gpx1 in cells shifted to the nitrogen-starved medium was increased (Fig. 6A). The level of Gpx1 was also increased when cells were cultured in a nitrogen-sufficient medium, which is due to the decrease in glucose in the medium. Glucose in the nitrogen-starved medium was consumed slowly compared with the medium supplemented with a sufficient amount of nitrogen; nonetheless, the Gpx1 level increased to a much greater extent in cells cultured in the nitrogen-starved medium than in cells in the nitrogen-sufficient medium. Therefore, we concluded that the expression of *GPX1* is induced by nitrogen starvation also.

The TOR pathway regulates the expression of *GPX1*

To gain an insight into the regulatory mechanism behind the nitrogen starvation-induced expression of *GPX1*, we examined whether the TOR pathway, which is mainly controlled by nitrogen, is involved. We have demonstrated that Msn2 and Msn4 play crucial roles in the induction of *GPX1* expression. It has been reported that these transcription factors are negatively regulated by the TOR pathway by being sequestered in the cytoplasm (Beck & Hall 1999). *S. cerevisiae* has two TOR kinases, Tor1 and Tor2 (Heitman *et al.* 1991). The two TOR kinases in TORC1 (TOR complex I) have a redundant function in, for example, regulating transcription, translation, ribosome biogenesis and autophagy; whereas, TORC2 (TOR complex II), where only Tor2 is involved, has a unique function in regulating the organization of the actin cytoskeleton (Schmelzle & Hall 2000). To determine whether the TOR pathway is involved in expression of the *GPX1* gene in response to starvation, cells were treated with rapamycin, a potent inhibitor of TORC1 (Heitman *et al.* 1991). As shown in Fig. 6B, the mRNA levels of *GPX1* were greatly increased following treatment with rapamycin. Therefore, the TOR pathway negatively

regulates the expression of *GPX1*.

Role of Gpx1 under glucose starved conditions

To explore the physiological relevance to induction of *GPX1*, we determined the capability of wild type and *gpx1*Δ cells, which had been adapted to glucose-limited conditions, to grow in the presence of H_2O_2 in glucose-starved medium. As shown in Fig. 7A, the growth of a *gpx1*Δ mutant in the glucose-starved medium containing H_2O_2 was impaired compared with that of wild type. Similarly, cell growth of the *msn2*Δ*msn4*Δ mutant was also impaired under the same conditions. We examined the growth of yeast cells in the presence of both Ca^{2+} and H_2O_2, although no additive effect of Ca^{2+} on the H_2O_2-induced growth defect of *gpx1*Δ and *msn2*Δ*msn4*Δ mutants was observed (data not shown).

On the other hand, we have shown that the Gpx1 level increased in the post-diauxic shift (Fig. 5A). Glycerol medium is sometimes used to mimic the conditions of post-diauxic shift (Roosen *et al.* 2005), so we monitored the growth of yeast cells in glycerol medium. As shown in Fig. 7B, no substantial difference was observed in the generation time of wild type, *gpx1*Δ, and *gpx1*Δ*gpx2*Δ*gpx3*Δ cells in the log-phase of growth in the glycerol medium, although maximum growth of the *gpx1*Δ mutant and *gpx1*Δ*gpx2*Δ*gpx3*Δ mutant was lower by approximately 20% and 30%, respectively, compared with that of wild type.

Discussion

Regulatory mechanism for expression of *GPX1* in response to glucose starvation and Ca^{2+}

In the present study, we found that the expression of *GPX1* in *S. cerevisiae* is induced by not only glucose starvation, which we have reported previously (Inoue *et al.* 1999), but also by $CaCl_2$ treatment, and investigated the regulatory mechanisms involved. The C_2H_2 zinc-finger transcription factors Msn2 and Msn4, and two *cis*-acting elements (STREs) in the *GPX1* promoter played crucial roles. Snf1 kinase was also involved in response to both glucose starvation and Ca^{2+} of *GPX1*.

The Mig1–Ssn6–Tup1 global complex is the major repressor of glucose-repressed genes, which binds to the upstream repression site (URS) of the respective genes, when glucose is present (Treitel *et al.* 1998). When glucose is limited, Snf1 is activated and phosphorylates Mig1, thereby dissociating the repressor complex form URS, and consequently, glucose repression is cancelled to induce the expression of glucose-repressive genes (Gancedo 1998; Calrson 1999). First, we suspected that Mig1 is involved in the glucose-starvation-induced expression of *GPX1*, because Mig1 binds to the G cluster preceded by the AT-rich sequence (Lundin *et al.* 1994), and the *GPX1* promoter contains such a sequence (5'-TTAAAGCCGGGG-3') 182-bp upstream of the translational initiation codon. We then disrupted *MIG1* and its paralogue *MIG2*, however, the basal expression level of *GPX1* did not increase in such mutants (data not shown). This was also the case in *ssn6*Δ and *tup1*Δ cells (data not shown). In addition, the glucose starvation-induced expression of *GPX1* occurred in these mutants (data not shown); and therefore, the Mig1–Ssn6–Tup1 global repressor seems not involved in the regulatory mechanism of the expression of *GPX1* in response to glucose starvation. Nonetheless, since the glucose starvation-induced expression of *GPX1* did not occur sufficiently in *snf1*Δ cells (Fig. 4A), it is conceivable that Snf1 plays some important role in induction of the expression of *GPX1* under glucose-limited conditions, rather than in derepression of glucose repression of *GPX1*.

Both Ser^{582} and Ser^{620} of Msn2 are dephosphorylated in response to glucose

starvation (De Wever *et al.* 2005; Görner *et al.* 1998), which was confirmed in this study (Fig. 3D). These two serine residues are believed to be phosphorylated by PKA, and their phosphorylation state is correlated with the nucleocytoplasmic localization of Msn2 (Görner *et al.* 1998). Here we showed that these serine residues are dephosphorylated in response to Ca^{2+}. Therefore, Ca^{2+} may partially regulate PKA activity negatively.

Msn2 is concentrated in the nucleus by a wide variety of environmental stresses such as heat shock, osmotic stress, and oxidative stress, and such stress signals converge on the nuclear export signal (NES) at the N terminus of Msn2, which causes the phosphorylation of several serine residues in the NES to block the nuclear export of Msn2 (Görner *et al.* 2002). Hyperphosphorylation of Msn2 is observed when cells are challenged by heat shock (Garreau *et al.* 2000). On the other hand, Ser^{582} and Ser^{620} lie within the nuclear localization signal (NLS) of Msn2, and the phosphorylation of these serine residues by PKA inhibits the nuclear import of Msn2 (Görner *et al.* 2002). We have previously reported that Msn2 is concentrated in the nucleus following treatment with $CaCl_2$ (Tsuzi *et al.* 2004b), but Msn2 was not hyperphosphorylated upon treatment with $CaCl_2$, instead Ser^{582} and Ser^{620} were dephosphorylated (Fig. 3D). In *ras2*Δ cells, the steady state activity of PKA is expected to be lowered due to the insufficient supply of cAMP, which would lead to dephosphorylation of Ser^{598} and Ser^{620} of Msn2, and consequently Msn2 is activated. Indeed, Msn2 was partially distributed in the nucleus in *ras2*Δ cells even though enough glucose is supplied (Fig. 3C). Therefore, the observation that the basal level of Gpx1 protein was increased in the *ras2*Δ mutant is rational (Figs. 3A and 3B). Nonetheless, we found that Msn2 was concentrated in the nucleus in *ras2*Δ cells following treatment with $CaCl_2$, and consequently, the levels of Gpx1 were further increased (Figs. 3A-3C). Therefore, Ca^{2+} signal is sent to Msn2 besides deactivation of the PKA pathway, thereby activating the transcription of *GPX1* (Fig. 8).

A striking feature of the regulatory mechanism of *GPX1* is that Snf1 was

involved not only in the glucose starvation-induced but also in the Ca^{2+}-induced expression of *GPX1*. Intriguingly, here we first demonstrated that Thr^{210} in Snf1 is phosphorylated following treatment with $CaCl_2$ in a Tos3, Elm1, and Sak1 protein kinase-dependent manner. These three protein kinases are homologues of mammalian CaMKKα, a Ca^{2+}/calmodulin-dependent kinase kinase, but it has never been examined whether Ca^{2+} activates them in yeast. Since Tos3, Elm1, and Sak1 are homologues of CaMKKα, it is conceivable that Ca^{2+} activates them in yeast. Recently, Hong and Carlson (2007) have reported that Snf1 is phosphorylated in cells challenged by Na^+ or alkaline stress. Denis and Cyert (2002) have demonstrated that Ca^{2+} in vacuole is released into the cytoplasm upon exposure of yeast cells to Na^+. Meanwhile, alkaline stress induces the uptake of extracellular Ca^{2+} to bind calmodulin, thereby activating calcineurin (Viladevall et al. 2004). Additionally, mammalian CaMKKα is able to phosphorylate Thr^{210} of Snf1 in a *tos3*Δ*elm1*Δ*sak1*Δ mutant following treatment with NaCl or alkaline stress (Hong & Carlson 2007). Together these earlier reports, our data obtained in the present study suggest that an increase in the level of Ca^{2+} in cells is a trigger of the activation of Snf1 via environmental stress.

Even though Snf1 is activated following treatment with $CaCl_2$, the Gpx1 level increased in *snf1*Δ cells in the presence of Ca^{2+} (Fig. 4A). However, the timing of the initiation of the Ca^{2+}-induced expression was delayed for 30 min in the *snf1*Δ mutant (Fig. 4A). Therefore, Ca^{2+} signal seems to be sent to *GPX1* through three different routes; i. e. (i) Ca^{2+} gives rise to a negative effect on to the Ras/cAMP/PKA pathway, which leads to dephosphorylation of Ser^{598} and Ser^{620} of Msn2, thereby accumulating this transcription factor in the nucleus to activate the expression of *GPX1*, (ii) Ca^{2+} activates Msn2 by yet unknown mechanism independent of the Ras/cAMP/PKA pathway, and (iii) Ca^{2+} activates Snf1 through three upstream protein kinases (Tos3, Elm1, and Sak1) to cause the early-stage-response of *GPX1* (Fig. 8).

Physiological relevance to induction of *GPX1* under glucose starved conditions

GPX1, *GPX2*, and *GPX3* were cloned as structural homologues of mammalian glutathione peroxidase (Inoue *et al.* 1999), although Gpx2 and Gpx3 have been identified as atypical 2-Cys peroxiredoxins that prefer to use thioredoxin as a reducing agent in vitro (Delaunay *et al.* 2002; Tanaka *et al.* 2005). The biochemical characterization of Gpx1 remains to be conducted, however, the enzyme is presumably an atypical 2-Cys peroxiredoxin as judged by the similarity of amino acid sequence with Gpx2 and Gpx3 (Tanaka *et al.* 2005). Of glutathione peroxidase homologues, as *GPX3* is constitutively expressed, a *gpx3*Δ mutant is sensitive to peroxides (Inoue *et al.* 1999). Meanwhile, a *gpx1*Δ mutant at log phase does not show any obvious phenotypes in terms of oxidative stress, which may be due to that the level of Gpx1 protein in a log phase of growth is basically quite low (Inoue *et al.* 1999), and therefore, the loss of Gpx1 may not give rise to a serious effect. We found that expression of *GPX1* is induced under glucose-starved conditions and in the diauxic shift, so, first, we compared the susceptibility to H_2O_2 between wild type and *gpx1*Δ cells under glucose-starved conditions. When cells pre-cultured in SD medium (2% glucose) were transferred to the glucose-starved medium containing 2 mM H_2O_2, cells were not able to grow. By contrast, when cells pre-cultured in the glucose-starved medium (0.1% glucose) were transferred to the fresh glucose-starved medium containing 2 mM H_2O_2, cells were able to grow, which is presumably due to the induction of antioxidant enzyme such as Gpx1 during adaptation period. As expected, the growth of a *gpx1*Δ mutant in the glucose-starved medium containing H_2O_2 was impaired (Fig. 7A). Since the expression of *GPX1* under glucose-starved conditions depends upon Msn2 and Msn4 as we have demonstrated in this study, cell growth of the *msn2*Δ*msn4*Δ mutant was also impaired.

On the other hand, we found that the Gpx1 level was kept high in the

post-diauxic shift (Fig. 5A), therefore, Gpx1 may have a role in the post-diauxic shift also. The mode of energy production of yeast changes from fermentation by glycolysis to respiration in mitochondria during diauxic shift (Werner-Washburne *et al.* 1993). It has been well known that functional mitochondria are necessary for assimilation of glycerol, and glycerol medium is sometimes used to mimic the conditions of post-diauxic shift. We then monitored the growth of yeast cells in the medium in which the amount of glucose is restricted. As a result, the growth of the *gpx1*Δ mutant in the glycerol medium was lower by approximately 20% compared with that of wild type (Fig. 7B). Recently, Lee *et al.* have reported that Gpx3 protein physically interacts with dihydroxyacetone kinase 1 (Dak1), which is involved in glycerol metabolism (Lee *et al.* 2008); however, the role of Gpx3 in glycerol metabolism has not yet been elucidated. Since Gpx1 shows higher similarity with Gpx3 in amino acid sequence, Gpx1 may share the redundant function with Gpx3 in terms of the energy metabolism, especially in the assimilation of glycerol. As shown in Fig. 7B, the maximum growth of a *gpx1*Δ*gpx2*Δ*gpx3*Δ mutant was further reduced in glycerol medium compared with that of the *gpx1*Δ single mutant. Yeast glutathione peroxidase homologues may have some roles in the energy metabolism of non-fermentable carbon sources besides the function as an antioxidant enzyme.

The differences in the regulatory mechanism of the expression of three glutathione peroxidase homologues may reflect the distinct roles under different physiological conditions. Since *GPX3* is constitutively expressed and the amount of Gpx3 protein is much higher compared with other two Gpxs, the deficiency in *GPX3* increases the susceptibility to peroxides (Inoue *et al.* 1999). In addition, Gpx3 plays an important role in activation of Yap1, an oxidative-stress-responsive transcription factor (Delaunay *et al.* 2002; Okazaki *et al.* 2007). So, Gpx3 may be standing by for a sudden increase in the oxidative stress under normal growth conditions. On the other hand, the expression of *GPX2* is induced under conditions of oxidative stress (Inoue *et al.* 1999;

Tsuzi *et al.* 2004a), and in the presence of $CaCl_2$ (Tsuzi *et al.* 2004b). It is known that Ca^{2+} increases the flux of electron in the electron transport chain in mitochondria, thereby accelerating the generation of reactive oxygen species (Nicotera *et al.* 1988; Pozniakovsky *et al.* 2005). We have previously reported that viability of a *gpx2*Δ mutant decreased when cells were treated with $CaCl_2$ and *tert*-butyl hydroperoxide simultaneously (Tanaka *et al.* 2005). Regarding the expression of *GPX1*, we have demonstrated in this study that the expression of *GPX1* is induced when cells are starved of glucose or nitrogen. It has been reported that a limited supply of glucose increases oxygen respiration in yeast cells (Lin *et al.* 2002). Similarly, the reduction of TOR activity also enhances the oxygen consumption (Bonawitz *et al.* 2007). The increase in oxygen respiration accelerates the generation of reactive oxygen species in yeast cells (Sugiyama *et al.* 2000). In addition, oxidative stress occurs in cells of stationary phase of growth, in which medium nutrients are exhausted (Jakubowski *et al.* 2000; Herker *et al.* 2004; Favre *et al.* 2008). Hence, the complicated regulatory mechanism of the expression of *GPX1* (Ras/cAMP/PKA pathway, Snf1 pathway, and TOR pathway) may be necessary for yeast cells to adapt to the nutrient starvation-induced oxidative stress.

Experimental procedures

Strains and media

The *S. cerevisiae* strains used in this study are summarized in Table 1 in Supplementary material. Cells were cultured in YPD (2% glucose, 1% yeast extract, and 2% peptone), synthetic dextrose (SD) medium (2% glucose and 0.67% yeast nitrogen base without amino acid), or glycerol medium (3% glycerol, 0.05% glucose, and 0.67% yeast nitrogen base without amino acids) with appropriate amino acids and bases at 28ºC. For glucose

starvation, cells were initially cultured in YPD or SD medium to a log phase of growth, collected by centrifugation, and suspended in a glucose-starved medium (0.1% glucose, 1% yeast extract and 2% peptone). For $CaCl_2$ treatment, 400 mM $CaCl_2$ was added to the medium. For nitrogen starvation, cells were initially cultured in SD medium to a log phase of growth, collected by centrifugation, and suspended in a nitrogen-starved medium (2% glucose, and 0.17% yeast nitrogen base without amino acids and ammonium sulfate) with appropriate amino acids and bases at 28ºC. As a control, cells were suspended in a nitrogen-sufficient medium (nitrogen-starved medium plus 0.5% ammonium sulfate) with appropriate amino acids and bases.

Gene disruption

The *RAS2* gene was amplified by PCR using primers RAS2-F and RAS2-R. The products were digested with *Bam*HI and *Hin*dIII, and cloned into the *Bam*HI-*Hin*dIII site of pUC19. The resultant plasmid (pUC19-*RAS2*) was digested with *Pst*I and *Bal*I, and the fragment containing a part of and the open reading frame (ORF) of *RAS2* was replaced with *TRP1*. This plasmid (pUC19-*ras2*Δ::*TRP1*) was digested with *Bam*HI and *Hin*dIII, and the *Bam*HI/*Hin*dIII fragment containing a *ras2*Δ::*TPR1* cassette was introduced into *S. cerevisiae* to disrupt *RAS2*.

The *GIS1* gene was amplified by PCR using GIS1-F and GIS1-R. *Sac*I and *Sph*I sites were designed in GIS1-F and GIS1-R, respectively (underlined). The PCR products were digested with *Sac*I and *Sph*I, and cloned into the *Sac*I-*Sph*I site of pUC19. The resultant plasmid (pUC19-*GIS1*) was digested with *Nsi*I, and the fragment containing a part of ORF of *GIS1* was replaced by *LEU2*. This plasmid (pUC19-*gis1*Δ::*LEU2*) was digested with *Sac*I and *Sph*I, and the *Sac*I/*Sph*I fragment containing a *gis1*Δ::*LEU2* cassette was introduced into *S. cerevisiae* to dispurt *GIS1*.

To construct a *msn4*Δ::*TRP1* mutant, pUCmsn4 (Inoue *et al.* 1998) was

digested with *Eco*RV, and the *Eco*RV fragment was replaced by *TRP1*. The resulting plasmid (pUC-*msn4*Δ::*TRP1*) was digested with *Nru*I and *Acc*I, and the *Nru*I/*Acc*I fragment containing a *msn4*Δ::*TRP1* cassette was introduced into *S. cerevisiae*.

To construct *gpx1*Δ::*HIS3*, *gpx3*Δ::*LEU2*, *msn2*Δ::*HIS3*, *snf1*Δ::*HIS3*, *hog1*Δ::*LEU2*, *cnb1*Δ::*HIS3*, *crz1*Δ::*LEU2*, and *mig1*Δ::*HIS3* mutants, each mutated allele was amplified by PCR using the following primer sets: GPX1-F and GPX1-R, GPX3-F and GPX3-R, MSN2ΔHIS3F and MSN2ΔHIS3R, SNF1 -F and SNF1-R, HOG1-F and HOG1-R, CNB1FhincII and CNB1RhincII, CRZ1-S and CRZ1-R, or MIG1-F and MIG1-R, and the genomic DNA of the respective mutants constructed in our previous studies (Inoue *et al.* 1999; Tsujimoto *et al.* 2000; Tsuzi *et al.* 2004a, 2004b; Maeta *et al.* 2005). To construct *gpx2*Δ::*kanMX4*, *msn2*Δ::*kanMX4*, *msn4*Δ::*kanMX4*, *mig2*Δ::*kanMX4*, *ssn6*Δ::*kanMX4* and *tup1*Δ::*kanMX4* mutants in the YPH250 background, genomic DNA of each mutant in the BY4741 background (Invitorogen) was used as a template for PCR with the following primer sets: GPX2-F and GPX2-R, MSN2ΔHIS3F and MSN2ΔHIS3R, MSN4-F and MSN4-R, MIG2-F and MIG2-R, SSN6-F and SSN6-R, and TUP1-F and TUP1-R. Each PCR fragment, which contains the disrupted allele of each gene replaced by *kanMX4*, was introduced into YPH250. Disruption of each gene was confirmed by PCR.

All PCR primers used in this study are summarized in Table 2 in Supplementary material.

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 and GPX1-lacZ-R. *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.

To construct the *GPX1-lacZ* gene carrying the mutated STRE (STRE1mut and STRE2mut), an overlap extension PCR was conducted (Ho *et al.* 1989). GPX1-lacZ-F and GPX1-lacZ-R were used as the external primers. For the site-directed mutagenesis, four internal primers (STRE1mut-F, STRE1mut-R, STRE2mut-F and STRE2mut-R) were designed to change the consensus sequence of STRE (5'-CCCCT-3') to 5'-CATCT-3' (Zahringer *et al.* 2000), where the mutated sequence is underlined. To create the mutation in STRE1 (-303 to -298), the first PCR was performed with the following primer sets: GPX1-lacZ-F plus STRE1mut-R, and GPX1-lacZ-R plus STRE1mut-F. 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-STRE1mut-GPX1-lacZ. To create the mutation in STRE2 (-248 to -243), the first PCR was performed with the following primers: GPX1-lacZ-F plus STRE2mut-R and GPX1-lacZ-R plus STRE2mut-F. The second PCR was done with GPX1-lacZ-F and GPX1-lacZ-R as the primers, and the mixture of the PCR products of each first PCR as the template. The PCR product was cloned into YIp358R as described above (YIp358R-STRE2mut-GPX1-lacZ). To construct the *GPX1-lacZ* gene carrying both STRE1mut and STRE2mut (STRE1/2mut), the first PCR was performed with the following primer sets: GPX1-lacZ-F plus STRE2mut-R, and GPX1-lacZ-R plus STRE2mut-F using YIp358R-STRE1mut-GPX1-lacZ as a template. The second PCR was done using GPX1-lacZ-F and GPX1-lacZ-R, and the mixture of the PCR 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-STRE1/2mut-GPX1-lacZ. Introduction of the mutation was verified by DNA sequencing.

Construction of *GPX1-lacZ* promoter fusion plasmid with pTBA30

The promoter region of *GPX1* was amplified with the primers, GPX-pTBA-2F and GPX1-pTBA-2R (-345 to -182). The PCR product was digested with *Xho*I, and inserted into the *Xho*I site of pTBA30, which contains the TATA box of *CYC1* followed by *lacZ* (Galius-Duerner *et al.* 1997). The resultant plasmid containing the region from -345 to -182 was named p*GPX1-CYC1-lacZ*. The insertion of the fragment in the correct orientation was verified by DNA sequencing.

Construction of *GPX1-3HA*

To add the HA-tag at the C terminus of Gpx1, PCR primers were designed (GPX1-HA-F and GPX1-HA-R) to amplify the region between the 5'-flanking region of *GPX1* and the triplet before the translation termination codon of *GPX1*. The *Xho*I and *Bgl*II sites were designed in GPX1-HA-F and GPX1-HA-R, respectively (underlined). The PCR fragment was digested with *Xho*I and *Bgl*II, and the resultant fragment was inserted into the *Xho*I-*Bgl*II site of the plasmid pSLF172 (Forsburg & Sherman 1997). The resultant plasmid (pSLF172-*GPX1*) was digested with *Hind*III and *Sal*I, and the DNA fragment containing *GPX1-3HA* was cloned into the *Hin*dIII-*Sal*I site of YIp5. The resultant plasmid (YIp5-*GPX1-3HA*) was linearlized with *Nco*I, and integrated into the *URA3* locus of *S. cerevisiae*.

β-Galactosidase assay

Cells carrying a genome-integrated *GPX1-lacZ* reporter gene were cultured in YPD medium until an appropriate phase of growth, and disrupted with glass beads in 10 mM potassium phosphate buffer (pH 7.0). To culture the cells carrying the plasmid (pTBA30)-borne *GPX1-lacZ*, cells were grown in SD medium with appropriate amino

acids and bases until the mid-log phase. β-Galactosidase activity was measured as described previously (Inoue *et al.* 1998). One unit of β-galactosidase activity was defined as the amount of enzyme that increases the A_{420} by 1000 per h at 30ºC. The protein concentration was determined by the method of Bradford (1976).

Western blotting

Cells carrying *GPX1-3HA* were cultured in YPD medium, and disrupted with glass beads in 10 mM Tris-HCl buffer (pH 7.0) containing 1mM phenylmethylesulfonyl fluoride. Cell homogenate was centrifuged at 14000 rpm for 10 min at 4ºC, and the supernatant (cell extract) was subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Immobilon; Millipore). Anti-HA monoclonal antibody (6E2, Cell Signaling) was used to detect Gpx1-3HA. 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). Anti-mouse IgG or anti-rabbit IgG conjugated with alkaline phosphatase (Cell Signaling) or horseradish peroxidase was used as a secondary antibody. Immunoreacting bands were visualized with a BCIP-NBT kit (Nacalai tesque).

Northern blotting

Total RNA was prepared by the method of Schmitt *et al.* (1990), and 20 µg of RNA was loaded onto a denaturing agarose gel. To prepare ^{32}P-labeled DNA probes, *GPX1* was amplified using primers GPX1-GFP-F2 and GPX1-GFP-R, and the PCR fragment was labeled with [α-^{32}P]dCTP (Amersham) using a kit (Takara).

Phosphorylation of Msn2

To determine the phosphorylation state of Msn2 at Ser^{582} and Ser^{620}, cells carrying

Msn2-GFP (Görner *et al.* 1998) were cultured in SD medium until a log phase of growth, and starved of glucose or treated with 400 mM $CaCl_2$ at 28 ºC. Cells were collected at a prescribed time, and disrupted with glass beads in 100 mM Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, 50 mM NaCl, 1 mM dithiothreitol, and protease inhibitor cocktail (Nakalai tesque). Cell extracts (20 μg proteins) were subjected to SDS-PAGE. Anti-GFP antibody was used to detect Msn2-GFP. Anti-phospho-Ser^{582} of Msn2 antibody, kindly provided by Dr. C. Brocard (De Wever *et al.* 2005), was used to determine the phosphorylation state of Ser^{582}. Anti-phospho-CREB (phospho-Ser^{133}) antibody (Cell Signaling) was used to determine the phosphorylation state of Ser^{620} (Görner *et al.* 2002).

Phoshorylation of Snf1

Cells were cultured in YPD medium until a log phase of growth, and then starved of glucose or treated with 400 mM $CaCl_2$ at 28ºC for a prescribed time. To determine the phophorylation state of Thr^{210} of Snf1, cellular proteins were prepared essentially as described by Bell *et al.* (2001). After preparation of the samples for SDS-PAGE, the protein concentration was determined with a RC DC Protein Assay kit (BIORAD). Anti-phospho-Thr^{172} of mammalian AMPK polyclonal antibody (Cell Signaling) was used to determine the phosphorylation state of Thr^{210} of Snf1 (Momcilovic *et al.* 2006). To detect Snf1 protein, anti-poly-His monoclonal antibody (HIS-1, Sigma) was used (Momcilovic *et al.* 2006).

Measurement of glucose in the medium

Cells were cultured in 500-ml Sakaguchi flasks containing 300 ml of YPD medium, and 1 ml of culture was withdrawn periodically. After the removal of cells by centrifugation, the glucose concentration in the supernatant was measured using a kit (Glucose test Wako

C, Wako).

Fluorescence microscopy

The localization of Msn2 was determined using a GFP-tagged Msn2 as we described previously (Tsuzi *et al.* 2004b). The localization of Snf1 was examined by indirect immunofluorescence microscopy using anti-poly-His monoclonal antibody.

Acknowledgements

We thank Drs. C. Brocard, M. Carlson, and C. Schüller for generously providing an antibody, strain, and plasmid.

References

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

Bell, M., Capone, R., Pashtan, I., Levitzki, A. & 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.

Bonawitz, N.D., Chatenay-Lapointe, M., Pan, Y., Shadel, G.S. (2007) Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell Metab.* **5,** 265-277.

Bradford, M.N. (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.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. & Gustin, M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259,** 1760-1763.

Carlson, M. (1999) Glucose repression in yeast. *Curr. Opin. Microbiol.* **2,** 202-207.

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

Cyert, M.S. (2003) Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. *Biochem. Biophys. Res. Commun.* **311,** 1143-1150.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J. & Toledano, M.B. (2002) A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* **111,** 471–481.

De Virgilio, C. & Loewith, R. (2006) The TOR signaling network from yeast to man. *Int. J. Biochem. Cell Biol.* **38,** 1476-1481.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G. & Brocard, C. (2005) A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation. *EMBO J.* **24,** 4115-4123.

Denis, V. & Cyert, M.S. (2002) Internal Ca^{2+} release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J. Cell Biol.* **156,** 29-34.

Errede, B. & Levin, D.E. (1993) A conserved kinase cascade for MAP kinase activation in yeast. *Curr. Opin. Cell Biol.* **5,** 254-260.

Estruch, F. (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol. Rev.* **24,** 469-486.

Favre, C., Aguilar, P.S., Carrillo, & M.C. (2008) Oxidative stress and chronological aging in glycogen-phosphorylase-deleted yeast. *Free Radic. Biol. Med.* **45,** 1446-1456.

Forsburg, S.S. & Sherman, D.A. (1997) General purpose tagging vectors for fission yeast. *Gene* **191,** 191-195.

Galius-Duerner, V., Chintamaneni, C., Wilson, R., Brill, S.J. & Vershon, A.K. (1997)

Analysis of a meiosis-specific URS1 site: sequence requirements and involvement of replication protein A. *Mol. Cell. Biol.* **17,** 3536-3546.

Gancedo, J.M. (1998) Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* **62,** 334-361.

Garreau, H., Hasan, R.N., Renault, G., Estruch, F., Boy-Marcotte, E. & Jacquet, M. (2000) Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*. Micobiology **146,** 2113-2120.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis H. & 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.

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

Hawley, S.A., Pan, D.A., Mustard, K.J., Ross, L., Bain, J., Edelman, A.M., Frengueli, G. & Hardie. D.G. (2005) Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* **2,** 9–19.

Heitman, J., Movva, N.R. & Hall, M.N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253,** 905-909.

Herker, E., Jungwirth, H., Lehmann, K.A., Maldener, C., Fröhlich, K.U., Wissing, S., Büttner, S., Fehr, M., Sigrist, S., & Madeo, F. (2004) Chronological aging leads to apoptosis in yeast. *J. Cell Biol.* **164,** 501-507.

Herman, P.K. (2002) Stationary phase in yeast. *Curr. Opin. Microbiol.* **5,** 602-607.

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

Hong, S.K., Cha, M.K., Choi, Y.S., Kim, W.C. & Kim, I.H. (2002) Msn2p/Msn4p act as a key transcriptional activator of yeast cytoplasmic thiol peroxidase II. *J. Biol. Chem.* **277,** 12109-12117.

Hong, S.P., Leiper, F.C., Woods, A., Carling, D. & Carlson, M. (2003) Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc. Natl. Acad. Sci. USA* **100,** 8839–8843.

Hong, S.P., Momcilovic, M. & Carlson, M. (2005) Function of Mammalian LKB1 and Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase α as Snf1-activating Kinases in Yeast. *J. Biol. Chem.* **280,** 21804-21809.

Hong, S.P. & Carlson, M. (2007) Regulation of Snf1 Protein Kinase in Response to Environmental Stress. *J. Biol. Chem.* **282,** 16838-16845.

Hurley, R.L., Anderson, K.A., Franzone, J.M., Kemp, B.E., Means, A.R. & Witters, L.A. (2005). The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J. Biol. Chem.* **280,** 29060–29066.

Inoue, Y., Tsujimoto, Y. & 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.

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

Jakubowski, W., Biliński, T., & Bartosz G. (2000) Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae. Free Radic. Biol. Med.* **28,** 659-664.

Lee, P.Y., Bae, K.H., Kho, C.W., Kang, S., Lee, do H., Cho, S., Kang, S., Lee, S.C., Park, B.C. & Park, S.G. (2008) Interactome analysis of yeast glutathione peroxidase 3. *J.*

Microbiol. Biotechnol. **18,** 1364-1367.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R., & Guarente, L. (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418,** 344-348.

Longo, V.D., Gralla, E.B. & Valentine, J.S. (1996) Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species *in vivo*. *J. Biol. Chem.* **271,** 12275–12280.

Lundin, M., Nehlin, J.O. & Ronne, H. (1994) Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein *MIG1*. *Mol. Cell. Biol.* **14,** 1979-1985.

Maeta, K., Izawa, S. & 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.

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

McCartney, R.R. & Schmidt, M.C. (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J. Biol. Chem.* **276,** 36460-36466.

Momcilovic, M., Hong, S.P. & Carlson, M. (2006) Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase *in vitro*. *J. Biol. Chem.* **281,** 25336-25343.

Monteiro, G. & Netto, L.E. (2004) Glucose repression of *PRX1* expression is mediated by Tor1p and Ras2p through inhibition of Msn2/4p in *Saccharomyces cerevisiae*. *FEBS*

Lett. **241,** 221-228.

Nath, N., McCartney, R.R. & Schimidt, M.C. (2003) Yeast Pak1 kinase associates with and activates Snf1. *Mol. Cell. Biol.* **23,** 3909–3917.

Nicotera, P., McConkey, D., Svensson, S.A., Bellomo, G. & Orrenius, S. (1988) Correlation between cytosolic Ca^{2+} concentration and cytotoxicity in hepatocytes exposed to oxidative stress. *Toxicology* **52,** 55-63.

Okazaki, S., Tachibana, T., Naganuma, A., Mano, N., & Kuge, S. (2007) Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H_2O_2 stress signal. *Mol. Cell* **27,** 675-688.

Pedruzzi, I., Burckert, N., Egger, P. & De Virgilio, C. (2000) *Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. *EMBO J.* **19,** 2569-2579.

Pozniakovsky, A.I., Knorre, D.A., Markova, O.V., Hyman, A.A., Skulachev, V.P. & Severin, F.F. (2005) Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. *J. Cell Biol.* **168,** 257-269.

Rolland, F., Winderickx, J. & Thevelein, J.M. (2002) Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Res.* **2,** 183-201.

Roosen, J., Engelen, K., Marchal, K., Mathys, J., Griffioen, G., Cameroni, E., Thevelein, J.M., De Virgilio, C., De Moor, B. & Winderickx, J. (2005) PKA and Sch9 control a molecular switch important for the proper daptation to nutrient availability. *Mol. Microbiol.* **55,** 862-880.

Santangelo, G.M. (2006) Glucose signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70,** 253-282.

Schmelzle, T. & Hall, M.N. (2000) TOR, a central controller of cell growth. *Cell* **103,** 253-262.

Schmitt, M.E., Brown, T.A. & Trumpower, B.L. (1990) A rapid and simple method for

preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic. Acids. Res.* **18,** 3091-3092.

Sugiyama, K., Izawa, S. & Inoue, Y. (2000) The Yap1p-dependent induction of glutathione biosynthesis in heat shock response in *Saccharomyces cerevisiae. J. Biol. Chem.* **275,** 15535-15540.

Sugiura, R., Sio, S.O., Shuntoh, H. & Kuno, T. (2001) Molecular genetic analysis of the calcineurin signaling pathways. *Cell Mol. Life Sci.* **58,** 278-288.

Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J., Schmidt, M.C. & Hardie, D.G. (2003) Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. *Curr. Biol.* **13,** 1299–1305.

Tanaka, T., Izawa, S. & Inoue Y. (2005) *GPX2*, encoding a phospholipid hydroperoxide glutathione peroxidase homologue, codes for an atypical 2-Cys peroxiredoxin in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280,** 42078-42087.

Treitel, M.A., Kuchin, S. & Carlson, M. (1998) Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18,** 6273-6280.

Tsujimoto, Y., Izawa, S. & Inoue, Y. (2000) Cooperative regulation of *DOG2*, encoding 2-deoxyglucose-6-phosphate phosphatase, by Snf1 kinase and the high-osmolarity glycerol-mitogen-activated protein kinase cascade in stress responses of *Saccharomyces cerevisiae*. *J. Bacteriol.* **182,** 5121-5126.

Tsuzi, D., Maeta, K., Takatsume, Y., Izawa, S. & Inoue, Y. (2004a) Regulation of the yeast phospholipid hydroperoxide glutathione peroxidase *GPX2* by oxidative stress is mediated by Yap1 and Skn7. *FEBS Lett.* **565,** 148-154.

Tsuzi, D., Maeta, K., Takatsume, Y., Izawa, S. & Inoue, Y. (2004b) 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.

Werner-Washburne, M.E.B., Johnston, G.C. & Singer, R.A. (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol Rev.* **57,** 383-401.

Viladevall, L., Serrano, R., Ruiz, A., Domenech, G., Giraldo, J., Barceló, A. & Ariño, J. (2004) Characterization of the Calcium-mediated Response to Alkaline Stress in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279,** 43614-43624.

Vincent, O,, Townley, R., Kuchin, S. & Carlson M. (2001) Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev.* **15,** 1104-1114.

Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., Carlson, M. & Carling, D. (2005) Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* **2,** 21–33.

Yoshimoto, H., Saltsman, K., Gasch, A.P., Li, H.X., Ogawa, N., Botstein, D., Brown. P.O. & 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.

Zahringer, H., Thevelein, J.M. & 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.

Figure legends

Figure 1 Expression of *GPX1* induced by glucose starvation and $CaCl_2$. (A) Cells carrying a *GPX1-lacZ* reporter gene were cultured in YPD medium until A_{610}=0.8, and then treated with 400 mM $CaCl_2$ or transferred to fresh medium containing 0.1% glucose (Low Glc) for 90 min, and β-galactosidase activity was measured. In control (Cont.) experiments, cells were transferred to fresh YPD medium. Values are the average of three independent experiments ± standard deviation. For Western blotting, cells carrying *GPX1-3HA* were cultured in YPD medium until A_{610}=0.8, and treated with $CaCl_2$ or

starved of glucose for 90 min. Gpx1 protein levels were then determined using anti-HA monoclonal antibody. Cdc28 is a loading control of Western blotting. For Northern blotting, cells were cultured in YPD medium until A_{610}=0.8, and treated with $CaCl_2$ or starved of glucose for 30 min. Total RNA was then prepared. (B) Each mutant carrying the *GPX1-lacZ* reporter gene was cultured in YPD medium until A_{610}=0.8, and treated with 400 mM $CaCl_2$ for 90 min, then β-galactosidase activity was measured. Values are the average for three independent experiments ± standard deviation.

Figure 2 Effect of disruption of *MSN2* and *MSN4* on *GPX1* expression. (A) Configuration of the *GPX1* promoter. (B) Cells of the wild-type (WT) or *msn2*Δ*msn4*Δ cultured in YPD medium until A_{610}=0.8 were treated as described in the legend of FIG. 1, and Northern blotting were done. (C) Wild-type cells carrying each mutant STRE-driven *GPX1-lacZ* were cultured in YPD medium until A_{610}=0.8, and treated as described in the legend of FIG. 1. The values of β-galactosidase activity are the average for three independent experiments ± standard deviation. Slot 1, control; slot 2, low glucose; and slot 3, $CaCl_2$. (D) Cells of the wild type or an *msn2*Δ::*HIS3 msn4*Δ::*TRP1* mutant carrying the *GPX1* promoter fusion plasmid (p*GPX1-CYC1-lacZ*) were cultured in SD medium until A_{610}=0.8. After being collected by centrifugation, cells were suspended in 0.1% glucose+YP medium (Low Glc) or YPD medium containing 400 mM $CaCl_2$. Cells were culture for another 90 min, and β-galactosidase activity was measured. Values are the average for three independent experiments ± standard deviation.

Figure 3 (A) Induction of *GPX1* expression in mutants defective in glucose signaling. Cells of the wild type (WT) and *ras2*Δ mutant carrying *GPX1-3HA* were grown in YPD medium until A_{610}=0.8, and treated with 400 mM $CaCl_2$ or starved of glucose (Low Glc) for 30 min. Gpx1 protein levels were then determined. In control (Cont.) experiments,

cells were transferred to fresh YPD medium. (B) Ca^{2+} signal converges on Msn2/Mns4. Cells (wild type, *ras2*Δ, *msn2*Δ*msn4*Δ, and *msn2*Δ*msn4*Δ*ras2*Δ) carrying a *GPX1-lacZ* reporter gene were cultured in YPD medium until A_{610}=0.8, and then treated with 400 mM $CaCl_2$ for 90 min, and β-galactosidase activity was measured. In control experiments, cells were transferred to fresh YPD medium. Values are the average of three independent experiments ± standard deviation. (C) Localization of Msn2. Cells of wild type or *ras2*Δ mutant carrying GFP-tagged Msn2 were cultured in SD medium until A_{610}=0.3, treated as described in (A) for 10 min, and then localization of Msn2-GFP was monitored. (D) Phophorylation of Ser^{582} and Ser^{620} of Msn2. Cells of the wild type carrying the Msn2-GFP plasmid were cultured in SD medium until A_{610}=0.8, and treated as described in (A) for 30 or 60 min. The phosphorylation state of each serine residue in Msn2 was determined using respective specific antibodies. The Msn2-GFP protein level was verified using anti-GFP antibody.

Figure 4 Role of Snf1 for expression of *GPX1* in response to glucose starvation and Ca^{2+}. (A) Cells of the wild type (WT) and *snf1*Δ mutant carrying *GPX1-3HA* were grown in YPD medium until A_{610}=0.8, and treated with 400 mM $CaCl_2$ or starved of glucose (Low Glc) for the prescribed time as indicated in the figure, and Gpx1 protein levels were determined by Western blotting. (B) Cells of the wild type or a *tos3*Δ*sak1*Δ*elm1*Δ mutant were cultured in YPD medium until A_{610}=0.5, and treated as described in (A). Cells were collected at the prescribed time, and the phosphorylation of Thr^{210} of Snf1 was monitored. (C) Cells of wild type or a *cnb1*Δ were cultured in YPD medium until A_{610}=0.5, treated as described in (A) for 30 min, and the phosphorylation of Thr^{210} of Snf1 was monitored. (D) Localization of Snf1 was monitored by indirect immunofluorescence microscopy using anti-poly-His monoclonal antibody. Since no signal was detected in *snf1*Δ cells, this monoclonal antibody is valid to detect Snf1. DAPI (4',6-diamino-2-phenylindole) was

used to stain the nucleus.

Figure 5 Change of Gpx1 level during growth. (A) Cells carrying *GPX1-3HA* were cultured in YPD medium, and cell growth was monitored periodically (*squares*). At each point, glucose concentration in the medium (*closed circles*) were also determined. Gpx1 protein level was determined by Western blotting at the time indicated in the figure. (B) Cells of *msn2*Δ*msn4*Δ and *msn2*Δ*msn4*Δ*gis1*Δ were cultured in YPD medium, and the Gpx1 protein level was determined by Western blotting at the time indicated in the figure.

Figure 6 Effect of nitrogen starvation and TOR pathway on expression of *GPX1*. (A) Cells carrying *GPX1-3HA* were cultured in YPD medium until A_{610}=0.8, transferred to nitrogen-starved medium or nitrogen-sufficient medium, and further cultured for the period as indicated in the figure to monitor the levels of Gpx1 protein. The glucose concentration in the medium is shown below each panel. Cdc28 is a loading control of Western blotting. (B) Wild-type cells were cultured in YPD medium until A_{610}=0.8 and treated with 200 ng/ml rapamycin for the period indicated in the figure, and total RNA was prepared.

Figure 7 Physiological relevance to induction of *GPX1* under glucose starved conditions. (A) Cells of wild type (WT), *gpx1*Δ, and *msn2*Δ*msn4*Δ were cultured in low glucose (0.1%) medium for 16 h, and an aliquot was transferred to the fresh low glucose medium containing 2 mM H_2O_2. The data indicate A_{610} of culture for 28 h. (B) Cells of wild type (WT), *gpx1*Δ, and *gpx1*Δ*gpx2*Δ*gpx3*Δ (*gpx1/2/3*Δ) were cultured in glycerol medium, and A_{610} was monitored periodically.

Figure 8 A schematic diagram of the regulation of *GPX1* expression. (*Left panel*) Ca^{2+}

induces the phosphorylation of Snf1 at Thr^{210}, leading to the expression of *GPX1* in an early-stage response. However, the Ca^{2+}-induced expression of *GPX1* was observed in *snf1*Δ cells, because Msn2 is activated following treatment with $CaCl_2$. Dephosphorylation of Ser^{582} and Ser^{620} of Msn2 in response to Ca^{2+} might be achieved through an inhibition of the Ras/cAMP/PKA pathway. In addition, Ca^{2+} signal seems sent to Msn2/Msn4 independent of the Ras/cAMP/PKA pathway. (*Right panel*) Glucose starvation causes the activation of Snf1 via phosphorylation of Thr^{210} by three upstream protein kinases. Meanwhile, glucose starvation deactivates the Ras/cAMP/PKA pathway, which leads to dephosphorylation of Ser^{582} and Ser^{620} of Msn2, and subsequently nuclear Msn2 activates transcription of *GPX1*. The details for the model are given in the text.