공학박사 학위논문

Regulation of Hsf1 and Msn2 activity by CK2 kinase in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae 에서 CK2 인산화 효소에 의한 Hsf1 과 Msn2 의 활성 조절

2017 년 2 월

서울대학교 대학원

협동과정 바이오엔지니어링

조 보 람

Regulation of Hsf1 and Msn2 activity by CK2 kinase in *Saccharomyces cerevisiae*

by

Bo-Ram Cho

Advisor : Professor Ji-Sook Hahn, Ph.D.

Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in Seoul National University

February, 2017

Interdisciplinary Program for Bioengineering

Graduate School

Seoul National University

ABSTRACT

Regulation of Hsf1 and Msn2 activity by CK2 kinase in *Saccharomyces cerevisiae*

Bo-Ram Cho
Interdisciplinary Program for Bioengineering
The Graduate School
Seoul National University

Saccharomyces cerevisiae is one of the best-studied useful eukaryotic model organisms for basic biological research. In addition, *S. cerevisiae* is also very attractive microorganism as an industrial cell factory due to several advantages such as assured safety, well-established genetic and metabolic engineering methods, excellent fermentation ability, and rapid growth under anaerobic condition. Especially, it has great tolerance to a wide range of harsh environmental challenges not only a high concentration of substrate and product but also facing the fermentation processes and cell culture. In response to unfavorable environmental changes, cells rapidly adjust global gene expression programs with inducing many

stress-related genes such as heat shock proteins (HSPs) for maintaining cellular homeostasis and cell survival. Stress-induced transcriptional activation of HSP genes is mainly regulated by two stress transcription factors, Hsf1 and Msn2/4.

In this dissertation, regulation mechanisms of Hsf1 and Msn2 by CK2 in response to ethanol and several environmental stress were elucidated and applied to stress-tolerant yeast strain.

Firstly, we found that CK2-dependent phosphorylation on S608 is an ethanol stress-specific repression mechanism of Hsf1, which does not affect the basal or heat-induced activity of Hsf1. This repression is relieved by dephosphorylation by Ppt1 which directly interacts with Hsf1 via its tetratricopeptide repeat (TPR) domain. In response to ethanol stress, *PPT1* deletion and CK2 overexpression exert synergistic inhibitory effects on Hsf1 activation, whereas $Hsf1^{S608A}$ mutant shows enhanced activation. Therefore, regulation of the Hsf1 S608 phosphorylation status by reciprocal actions of CK2 and Ppt1 might play an important role to determine Hsf1 sensitivity towards ethanol stress.

Secondly, the reciprocal regulation mechanism of Hsf1 by CK2 and Ppt1 was applied to improve alcohols tolerance for industrial yeast strain. Improving yeast tolerance to alcohols is an important stage toward enabling high titer production. To enhance the ethanol stress-specific activation of Hsf1, CK2-dependent phosphorylation site S608 in Hsf1 was substituted with alanine. The $Hsf1^{S608A}$ strain was showed the improved tolerance to alcohols not just ethanol but also a variety of alcohols, such as methanol, 2,3-butandiol, 2-phenylethanol, 3-methyl-1-

butanol, isobutanol, and hexanol. Therefore, CK2-dependent repression mechanism of Hsf1 is applicable for improving yeast tolerance to various alcohols.

Thirdly, we demonstrate that CK2-dependent phosphorylation positively regulates Msn2/4, the general stress response transcriptional activators in *Saccharomyces cerevisiae*, in response to various types of environmental stress conditions. CK2 overexpression elicits hyperactivation of Msn2/4, whereas deletion of one of the CK2 catalytic subunits, especially *CKA2*, leads to reduced transcriptional activity of Msn2/4 in response to glucose starvation, H_2O_2, and lactic acid. The *CKA2* deletion mutant also shows increased stress sensitivity. CK2 phosphorylates Ser194 and Ser638 in Msn2 and replacement of these residues with alanine leads to reduced Msn2 activity upon stress and reduced tolerance to H_2O_2 and lactic acid. *CKA2* deletion mutant shows shorter nuclear retention time of Msn2 upon lactic acid stress, suggesting that CK2 might regulate nuclear localization of Msn2. However, $Msn2^{S194A,\ S638A}$ mutant shows normal nuclear import and export patterns upon stress, suggesting that CK2 might positively regulate the general stress response not only by direct phosphorylation of Msn2/4, but also by regulating cellular translocation machinery.

Keywords : CK2 kinase, Ppt1 phosphatase, Hsf1, Msn2, Msn4,
Phosphorylation, Stress transcription factor, Stress tolerance
Saccharomyces cerevisiae

***Student Number* :** 2007-21278

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
2,3-BD	2,3-butandiol
BiFC	bimolecular fluorescence complementation
cAMP	cyclic adenosine monophosphate
CK2	casein kinase 2
CTA	C-terminal activation domain
DAPI	4'-6-Diamidino-2-phenylindole
DBD	DNA binding domain
EDTA	ehylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ESR	environmental stress response
EtOH	ethanol
GPD	glycerol-3-phosphate dehydrogenase
GSK3B	Glycogen synthase kinase 3 beta
GSR	general stress response
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HSE	heat shock response element

HSF	Heat Shock transcription Factor
HSP	heat shock protein
HSR	heat shock response
LA	lactic aicd
LB	lysogeny broth
Leu	leucine
MAP	mitogen-activated protein
3MB	3-methyl-1-butanol
MeOH	Methano
NTA	N-terminal activation domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	PCR polymerase chain reaction
PIC	Protease inhibitor cocktail
PMSF	phenylmethylsulfonyl fluoride
pNPP	para-nitrophenol phosphate
PPT1	protein phosphatase T
OD	optical density
2-PE	2-phenylethanol
qRT-PCR	quantitative real time polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SC	synthetic complete

SDS	sodium dodecyl sulfate
SS	silver staining
STRE	stress response element
TBB	4,5,6,7-tetrabromobenzotriazole
TEF	translation elongation factor
TPR	tetratricopeptide repeat
TRD	trimerization domain
Ura	uracil
VC	C-terminal Venus fragment
VN	N-terminal Venus fragment
WB	western blotting
YPD	yeast extract-peptone-dextrose
ZnF	zinc finger

Chapter 1.

Research background and objective

Cells exposed environmental stresses rapidly changes their global gene expression to cope with detrimental conditions, such as heat shock, nutrient starvation, oxidative stress, osmotic stress, extreme pH, and toxic chemicals. Transcripts levels of house-keeping genes such as ribosomal proteins are quickly repressed in response to environmental stress, whereas stress-related genes, including the heat shock proteins (HSP), are drastically increased. In *Saccharomyces cerevisiae*, the stress-induced extensive expression levels of HSP genes are mainly governed by two stress-responsive transcription factors, Hsf1 and Msn2/4. The two stress-responsive transcription factors, Hsf1 and Msn2/4, are cooperatively regulating gene expression under multiple stress conditions. These transcription factors share some common regulatory mechanisms, primarily regulated by phosphorylation.

Although, it has been known that ethanol induces extensive activation of Hsf1-depentent genes, the regulation mechanism of ethanol-dependent Hsf1 activation has not yet been reported. Several kinases regulate the transcriptional activity of Hsf1 in positive and negative roles. However, phosphatase implicated in Hsf1 regulation has not identified.

Msn2 and Msn4 transcription factors are a major regulator of the general stress response (GSR) and their activation is essential for cell growth and viability. In spite of the fact that Msn2 are regulated by two nutrient-sensing pathways in response to nutrient depletion, however, how the transcriptional activity of Msn2 is regulated in response to multiple stress conditions is still unknown.

CK2 is a highly conserved Ser/Thr protein kinase involved in a large number

of cellular processes such as transcription, translation, signal transduction, cell cycle progression, and cell growth. Although it has been well known that CK2 plays a role for regulation of vital cellular functions, mechanisms implicated in stress response are largely unknown.

This dissertation focuses on the identification of the novel regulatory mechanism of two stress-responsive transcription factors by CK2 in response to several environmental challenges. In addition, application for stress tolerant yeast strain was attempted base on the regulatory mechanism of the stress response.

Chapter 2.

Literature review

2.1. Stress response in *Saccharomyces cerevisiae*

Living cells are constantly exposed to a variety of environmental stresses resulting in cellular confusions including reduced enzyme activity, cellular structures, and a collapse of membrane functions and chemical gradients (6). Thus, cells must be able to respond to stressful environmental conditions to maintain cellular homeostasis and cell survival. The stress response to stressful environmental signals is composed of highly complex networks of signal transduction leading to adaptations including extensive alterations of gene expression programs (7). Therefore, comprehensive understanding is required for investigating regulation mechanism of the stress response and its application. Global gene expression analysis in response to environmental stresses can provide comprehensive insights how cells adapt to environmental fluctuations through reprogramming of the gene expressions (8-10). In response to sudden environmental changes, cells quickly adjust gene expression programs with rebalancing between expression levels of growth-related genes and stress-related genes (9, 11). In *Saccharomyces cerevisiae*, various types of stress, such as heat shock, oxidative or reductive stress, osmotic shock, nutrient starvation, DNA damage and extreme pH, lead to a drastic change in gene expressions nearly 20% of whole genes, termed the environmental stress response (ESR) (8, 9). The ESR genes are classified into two groups of genes according to their opposite expression patterns, which are stress-repressed genes and stress-

induced genes (12). The stress-repressed ESR genes are involved in fundamental cellular processes, such as ribosome biosynthesis, RNA metabolism, protein synthesis, and associated cell growth. on the other hand, stress-induced ESR genes are involved in a wide range of cellular processes to rapidly adapt to abrupt environmental changes, including carbohydrate metabolism, metabolite transport, maintenance of the cellular redox potential, protein folding and degradation, oxidative stress defense, autophagy, cytoskeletal reorganization, DNA damage repair, intracellular signaling and others. These expression of ESR genes are governed by a variety of regulatory mechanisms including stress-responsive transcription factors, such as Msn2/4 and Hsf1 (6).

2.2. Stress-responsive transcription factors

2.2.1. Msn2 and Msn4

Expression of stress-induced ESR genes is primarily regulated by functionally redundant transcription factors Msn2 and Msn4, already known as a regulator of the general stress response (GSR) (6). Interestingly, *msn2/4*Δ cells are hypersensitive to stressful conditions, apparently indicating that transcriptional activity of Msn2/4 and induction of the Msn2/4-dependent GSR genes are important for yeast cell survival under various environmental challenges (13).

Msn2 and Msn4 are transcription factors integrated into bow-tie shaped

signaling pathway as a central player (14). The general stress transcription factors Msn2/4 activate expression of a vast number of their target genes in response to a variety of stresses, such as nutrient starvation, heat shock, osmotic shock, oxidative stress, alteration of pH, and noxious chemicals in *Saccharomyces cerevisiae* (8, 9, 13). Msn2/4-dependent genes have the stress response element (STRE) consisting CCCCT sequence (or reverse complementary sequence, AGGGG) in their promoters (8, 9, 13). Msn2 and Msn4 are shown 41% identity and similarity of size and amino acid composition (6, 13, 15). Msn2 contains four part of functional domain, such as transcriptional activation domain (TAD), nuclear export signal (NES), nuclear localization signal (NLS), and zinc finger DNA-binding domain (ZnF) (16-19). Overexpression of Msn2/4 leads to reduced growth rate (20), implying that Msn2/4 activity is strictly regulated in response to both normal and stressful conditions. Transcriptional activities of Msn2/4 are regulated at multiple levels, including nuclear translocation, DNA binding, and stability, influenced by phosphorylation status of Msn2/4 (13, 21-23). Several protein kinase and phosphatase, including the cAMP-protein kinase A (PKA), the target of rapamycin complex 1 (TORC1), Yak1, Rim15, PP1 phosphatase, and PP2A phosphatase, are involved in regulation of Msn2/4 activity and stress response.

2.2.2. Regulation of Msn2 and Msn4 transcriptional activity

Regulation mechanism of Msn2 activity in response to nutrient starvation is comparatively well-studied. Two nutrient-sensing pathways, the cAMP-PKA pathway and the TORC1 pathway, negatively regulate transcriptional activity of Msn2/4. In normal condition, PKA is activated by a high concentration of cAMP and effectively repress the nuclear localization of Msn2 in a direct phosphorylation-dependent manner (18, 21). Indeed, mutation of the PKA-dependent phosphorylation site, S620, S625, and S633 in NES domain of Msn2 lead to constitutive nuclear accumulation of Msn. Nutrient depletion such as glucose starvation leads to down regulation of PKA activity and result in the nuclear accumulation of Msn2 by the protein phosphatase 1 (PP1) and release from Bmh2, a yeast 14-3-3 protein (21, 23, 24). In addition, Yak1 kinase involves in regulation of Msn2 activation leading to an induction of STRE-mediated gene expression (1). Yak1 kinase activates Msn2 activity upon glucose starvation through a direct phosphorylation. Nuclear localization of Yak1 kinase is also blocked under high-glucose condition by PKA-dependent phosphorylation. PKA-dependent phosphorylation of Yak1 on S295 and two minor sites, S127 and S128, inhibits nuclear localization of Yak1 (25).

The TORC1 signaling pathway also participates in the regulation of Msn2 and Msn4. TORC1 negatively regulates the expression of Msn2/4-dependent genes by enhancing the association between Msn2/4 and Bmh2 (26). Interestingly, Bmh1, another yeast 14-3-3 protein, also binds to Yak1 and

inhibits its catalytic activity under high glucose condition (25). The protein phosphatase 2A (PP2A), negatively regulated by TORC1, is also required for nuclear accumulation of Msn2 in response to stresses (27). Taken together, under nutrient starvation such as glucose depletion, both PKA and TORC1 are inactivated and Msn2 is rapidly accumulated in the nucleus through dephosphorylation by the protein phosphatase 1 (PP1) and release from Bmh2. Furthermore, inactivation of PKA and TORC1 lead to activation and nuclear accumulation of both Yak1 and Rim15, resulting in induction of many Msn2/4-dependent gene expressions.

Although Transcriptional activation of Msn2 is primarily regulated by nuclear accumulation of Msn2, it is insufficient to stress-induced activation of Msn2/4. The deletion of Msn5 mediating the nuclear export of Msn2 results in constant nuclear accumulation of Msn2, even under normal condition, but could not affect the transcriptional activation of STRE-controlled genes (22), implying that another regulatory steps, such as phosphorylation, are required for Msn2 activation apart from its nuclear accumulation.

2.2.3. Hsf1

Expression of stress-induced ESR genes is also regulated by heat shock transcription factor (HSF), known as a regulator of the heat shock response (HSR) (6). The HSR can be considered a subset of the ESR since all HSR genes are accounted for within the ESR genes (28). Four heat shock factors, HSF1 to

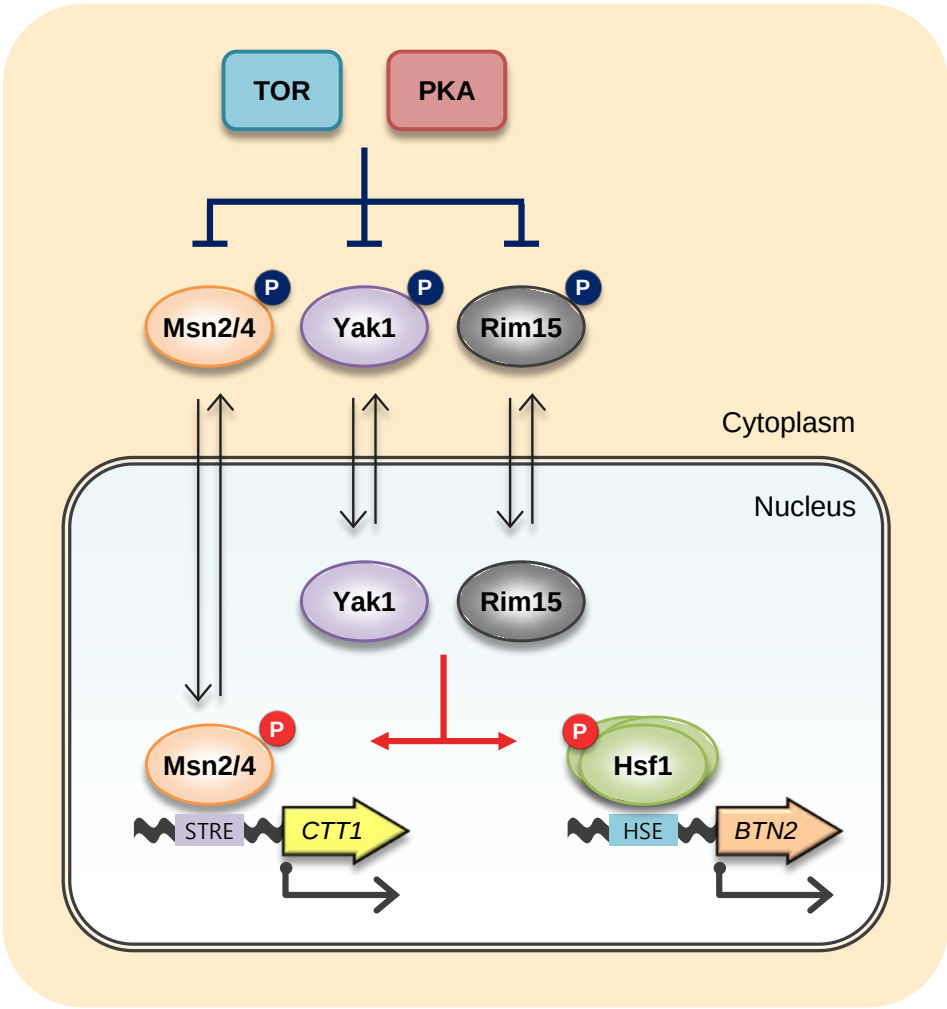


Figure 2.1 Regulation of Msn2/Msn4 and Hsf1 transcription activity

HSF4, in vertebrates and single HSF1 in yeast have been identified (28, 29). All HSF1 play a central role in the expression of heat shock proteins (HSPs) in response to various stress conditions perturbing protein homeostasis including heat shock, oxidative stress, heavy metals, and pharmacological reagents (29). The fundamental architectural elements of Hsf1 are conserved in the functional domains including winged helix-turn-helix DNA-binding domain (DBD), leucine zipper motif for trimerization of Hsf1, and a carboxyl-terminal transactivation domain (CTA). In addition, *Saccharomyces cerevisiae* Hsf1 has two additional domain, N-terminal activation domain (NTA) and yeast-specific heptapeptide region, termed CE2 (28).

2.2.4. Regulation of Hsf1 transcriptional activity

Hsf1 is activated through several regulatory processes including release from Hsp90 complex, homo-trimerization of Hsf1, nuclear localization, binding to the heat shock element (HSE) on promoter of their target genes and extensive posttranslational modifications such as phosphorylation, acetylation, and sumoylation in mammalian cells (30). However, *Saccharomyces cerevisiae* Hsf1 bind constitutively to HSE on target genes promoter as a trimeric complex and it is essential for cell viability (28). In addition, several functional studies with truncated yeast Hsf1 have revealed that sustained activity of CTA is repressed by both NTA, which has a transient transcriptional activity, and CE2 region (31, 32).

Phosphorylation is a conserved common regulatory mechanism of Hsf1 transcriptional activity, although mammalian HSF1 is known to be regulated by other post-translational modifications such as sumoylation and acetylation (30). In mammalian cells, several kinases have been identified which modulate Hsf1 in both positive and negative ways (33). Indeed, 12 serine residues implicated in modulation of Hsf1 activity in both activation and repression (34). The extracellular signal–regulated kinases 1/2 (ERK1/2) MAP kinases and Glycogen synthase kinase 3 beta (GSK3B) and constitutively phosphorylate the S303 and S307 on Hsf1, thereby inhibiting the transcriptional activity of Hsf1 during stress recovery (35, 36). In addition, transactivation activity of Hsf1 is also repressed through its S363 phosphorylation by protein kinases C alpha and zeta (PKCα/ζ) (36). On the other hand, Hsf1 is activated by phosphorylation at S230 and S326, leading to stimulation of Hsf1 DNA binding (37). Phosphorylation of Hsf1 at S419 and S320 by polo-like kinase 1 (PLK1) and protein kinase A (PKAcα),leads to Hsf1 activation through regulating HSF1 oligomerization, nuclear translocation and DNA binding (34). In *Saccharomyces cerevisiae*, Hsf1 is also regulated by several kinase in response to heat shock and glucose starvation (28). Snf1 kinase, a homolog of mammalian AMP-activated kinase, is required for induction of Hsf1 target gene in response to glucose starvation (38). However, heat shock-induced Hsf1 activation is Snf1-independent (39). Another nutrient-sensing pathway, the cAMP-PKA pathway is implicated in Hsf1 regulation. Deletion of PKA catalytic subunits lead to increased

phosphorylation status of Hsf1, implying that PKA negatively regulates Hsf1 activity (40). In addition, both Yak1 and Rim15, whose activity is negatively regulated by PKA, are also involved in the activation of Msn2 by direct phosphorylation (1, 41).

However, phosphatases that regulate Hsf1 are not well known. In a previous report, human PP5 negatively modulates DNA binding activity of Hsf1 under heat shock and chemical stresses including exposure to ethanol (42). Moreover, substitution of serine residues on CE2 region with alanine lead to an increased transcriptional activity of *K. lactis* Hsf1, highly conserved with *S. cerevisiae* Hsf1, giving a clue for yeast Hsf1 regulation by phosphatase (43). In *Saccharomyces cerevisiae*, Ppt1 protein phosphatase, a PP5 homologue, is known to dephosphrylate Hsp90, the repressor of mammalian Hsf1, and it positively regulates the chaperone activity of Hsp90 (44). Recently, Buchner and Daub have identified 33 proteins including Hsf1 as Ppt1 substrates with mass spectrometry analysis (45). Another interesting report demonstrated that in modern sake yeast, *PPT1* is spontaneously deleted, exhibiting constitutive hyperphosphorylation of Hsf1 and down-regulation of HSE-lacZ reporter expression during sake fermentation and under acute ethanol stress (46). These results suggest that Hsf1 activity is positively regulated by Ppt1 under ethanol stress in a dephosphorylation-dependent manner. On the other hand, even though two kinases Yak1 and Snf1 have been reported to which positively regulate Hsf1 activity in response to glucose starvation, Hsf1-repressor kinase

as an antagonist of Ppt1 has not yet been identified in *Saccharomyces cerevisiae* (1, 39).

Chapter 3.

Materials and methods

3.1. Strains and media

Yeast strains used in this study are listed in table 3.1. E. coli strains used in this study are also listed in table 3.2. All yeast strains were derived from the BY4741 genetic background. The *ppt1Δ* mutant was generated by PCR-based homologous recombination with plasmid pCg*URA3* (47). For the generation of cells expressing *HSF1*^{WT} or *HSF1*^{S608A}, a DNA fragment containing *HSF1*^{WT} or *HSF1*^{S608A} ORF with its own 379-bp terminator and *KlURA3* cassette was PCR amplified from pFA6a-HSF1^{WT}-KlURA3 or pFA6a-HSF1^{S608A}-KlURA3 plasmid, respectively, and then integrated into a chromosome by substituting the endogenous *HSF1* ORF and terminator by homologous recombination. To generate *msn2/4*Δ strain, *MSN2* was deleted in *msn4*Δ strain by PCR-based homologous recombination with pUG72 plasmid. Strains JHY135, JHY136, JHY137, and JHY138 were generated by utilizing the Cre/*loxP* recombination system. Briefly, a DNA fragment containing *MSN2*^{WT}, *MSN2*^{S194A}, *MSN2*^{S638A}, or *MSN2*$^{S194A,\ S638A}$ ORF with its own 176-bp terminator and *loxP-URA3-loxP* cassette was PCR amplified from JHP041, JHP042, JHP043, and JHP044, respectively. And then, each DNA fragment was integrated into the chromosome of *msn4*Δ strain by substituting the endogenous *MSN2* ORF and terminator by homologous recombination. Following the integration, *URA3* marker gene was removed by transformation of Cre recombinase-expression plasmid, pSH62. For the generation of strains JHY139, JHY140, JHY141, and JHY142, *GIS1* was deleted in JHY135, JHY136, JHY137,

and JHY138 strain, by PCR-based homologous recombination with pUG72 plasmid.

Yeast cells were grown in YPD medium (1% yeast extract, 2% bacto-peptone, and 2% dextrose) or synthetic complete (SC) medium containing 0.67% yeast nitrogen base without amino acids (Sigma-Aldrich), 2% glucose, and 0.2% amino acids dropout mixture suitable for plasmid selection. For the purification of CK2, selective SC medium containing 2% raffinose or 4% galactose was used.

Table 3.1 *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
WT	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
ppt1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ppt1Δ::::URA3*	This study
T7-HSF1	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::loxP-LEU2-loxP URA::TEF-T7-HSF1*	This study
T7-HSF1^{S478A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::loxP-LEU2-loxP URA::TEF-T7-HSF1^{S478A}*	This study
T7-HSF1^{S608A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::loxP-LEU2-loxP URA::TEF-T7-HSF1^{S608A}*	This study
HSF1^{WT}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::HSF1^{WT}-KlURA3*	This study
HSF1^{S608A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::HSF1^{S608A}-KlURA3*	This study
HSF1S608A	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsf1Δ::HSF1^{S608A}-loxP*	This study
PPT1-VN	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ppt1::PPT1-VN-KlURA3*	(5)
CKA1-VN	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka1::CKA1-VN-KlURA3*	(5)
CKA2-VN	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka2::CKA2-VN-KlURA3*	(5)
CKB1-VN	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ckb1::CKB1-VN-KlURA3*	(5)
CKB2-VN	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ckb2::CKB2-VN-KlURA3*	(5)
msn4Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6*	EUROSCARF
msn2/4Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6 msn2Δ::loxP-URA3-loxP*	This study

Table 3.1 *S. cerevisiae* strains used in this study (*Continued*)

Strain	Genotype	Reference
*cka1*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka1Δ::kanMX6*	EUROSCARF
*cka2*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cka2Δ::kanMX6*	EUROSCARF
MSN^{WT}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6* $msn2\Delta::MSN2^{WT}$*-loxP*	This study
MSN^{S194A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6* $msn2\Delta::MSN2^{S194A}$*-loxP*	This study
MSN^{S638A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6* $msn2\Delta::MSN2^{S638A}$*-loxP*	This study
$MSN^{S194A, S638A}$	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6* $msn2\Delta::MSN2^{S194A, S638A}$*-loxP*	This study
*gis1*Δ MSN^{WT}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6 gis1Δ::loxP-URA3-loxP* $msn2\Delta::MSN2^{WT}$*-loxP*	This study
*gis1*Δ MSN^{S194A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6 gis1Δ::loxP-URA3-loxP* $msn2\Delta::MSN2^{S194A}$*-loxP*	This study
*gis1*Δ MSN^{S638A}	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6 gis1Δ::loxP-URA3-loxP* $msn2\Delta::MSN2^{S638A}$*-loxP*	This study
*gis1*Δ $MSN^{S194A, S638A}$	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 msn4Δ::kanMX6 gis1Δ::loxP-URA3-loxP* $msn2\Delta::MSN2^{S194A, S638A}$*-loxP*	This study
RSY620	*MATa ade2 leu2-3,112 his3 trp1 ura3 pep4::TRP1*	(2)
L40	*MATa his3, D200 trp1-910 leu2-3,112 ade2 LYS::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4*	(3)

Table 3.2 *E. coli* strains used in this study

Strain	Genotype	Reference
DH5α	F- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ^- *th*i-*1 gyrA96 relA1*	
Rosetta-gami(DE3)pLysS	*Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) F´[*lac+ lacIq pro*] *gor522::Tn10 trxB* pLysSRARE2 (CamR, StrR, TetR)	Novagen
BL21(DE3)pLysS	F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S) pLysS[*T7p20 ori*$_{p15A}$](Cm^R)	Novagen

3.2. Plasmids

Plasmids used in this study are listed in table 3.3-4. Plasmids were generated by standard restriction digestion cloning or by site-directed mutagenesis. All insert DNA fragments were generated by PCR from genomic DNA of the BY4741 strain and were verified by sequencing.

Table 3.3 Plasmids used for yeast in this study

Plasmid	Description	Reference
JHP012	pRS416TEF-PPT1	This study
JHP013	pRS416TEF-PPT1ΔTPR	This study
JHP014	pRS426-GAL-GST-CKA2	This study
	pRS423GPD	(4)
	pRS425GPD	(4)
JHP015	pRS423GPD-CKA1	This study
JHP016	pRS425GPD-CKA2	This study
JHP017	pRS306TEF-T7-HSF1	This study
JHP018	pRS306TEF-T7-HSF1^{S478A}	This study
JHP019	pRS306TEF-T7-HSF1^{S608A}	This study
JHP027	pFA6a-HSF1^{WT}-HSF1 terminator-KlURA3	This study
JHP028	pFA6a-HSF1^{S608A}- HSF1 terminator – KlURA3	This study
	pUG72M-HSF1^{WT}-T_{HSF1}	This study
	pUG72M-HSF1^{S608A}-T_{HSF1}	This study
	pRS415ADH-VC	This study
	pRS415ADH-VC-HSF1	This study
JHP020	pVP16-HSF1	This study
	pVP16-MSN2	This study
	pVP16-MSN4	This study
JHP021	pBTM116-LexA-CKA1	This study
JHP022	pBTM116-LexA-CKA2	This study
JHP023	pBTM116-LexA-CKB1	This study
JHP024	pBTM116-LexA-CKB2	This study

Table 3.3 Plasmids used for yeast in this study (*Continued*)

Plasmid	Description	Reference
	pRS415ADH-EGFP	This study
JHP039	pRS415ADH-MSN2-EGFP	This study
	pRS415ADH-MSN2^{S194A}-EGFP	This study
	pRS415ADH-MSN2^{S638A}-EGFP	This study
JHP040	pRS415ADH-MSN2$^{S194A, S638A}$-EGFP	This study
pSH62	Plasmid containing Cre-recombinase gene	EUROSCARF
pUG72	Plasmid containing *loxP-LEU2-loxP* cassette	EUROSCARF
pUG72M	pUG72 plasmid containing multiple cloning site consisting of *Hind* III, *Pac*I, *Nhe*I, *BamH*I, *Sam*I, *EcoR*I, *Apa*I, *Mlu*I, *Asc*I, and *Pst*I	This study
JHP041	pUG72M-P$_{MSN2}$-MSN2^{WT}-T$_{MSN2}$	This study
JHP042	pUG72M-P$_{MSN2}$-MSN2^{S194A}-T$_{MSN2}$	This study
JHP043	pUG72M-P$_{MSN2}$-MSN2^{S638A}-T$_{MSN2}$	This study
JHP044	pUG72M-P$_{MSN2}$-MSN2$^{S194A, S638A}$-T$_{MSN2}$	This study

Table 3.4 Plasmids used for *E. coli* in this study

Plasmid	Description	Reference
JHP001	pET28b-His-PPT1	This study
JHP002	pET28b-His/T7-PPT1	This study
JHP025	pET28b-His/T7-TPR	This study
	pET15b-His-HSC82	This study
	pET28b- His/T7-CKA1	This study
	pET28b- His/T7-CKA2	This study
	pET28b- His/T7-CKB1	This study
	pET28b- His/T7-CKB2	This study
JHP003	pGEX4T-1-HSF1(1-833)	This study
JHP004	pGEX4T-1-HSF1(1-155)	This study
JHP005	pGEX4T-1-HSF1(1-260)	This study
JHP006	pGEX4T-1-HSF1(261-833)	This study
JHP007	pGEX4T-1-HSF1(261-570)	This study
JHP008	pGEX4T-1-HSF1(571-833)	This study
JHP009	pGEX4T-1-HSF1 ^{S608A}(571-833)	This study
JHP010	pGEX4T-1-HSF1(571-680)	This study
JHP011	pGEX4T-1-HSF1(681-833)	This study
JHP026	pGEX4T-1-HSF1 ^{S608A}(571-680)	This study
JHP029	pGEX-4T-1-MSN2(1-704)	This study
JHP030	pGEX-4T-1-MSN4(1-630)	This study
JHP031	pGEX-4T-1-MSN2(1-165)	This study
JHP032	pGEX-4T-1-MSN2(1-300)	This study
JHP033	pGEX-4T-1-MSN2(1-400)	(1)
JHP034	pGEX-4T-1-MSN2(401-704)	(1)

Table 3.4 Plasmids used for *E. coli* in this study (*Continued*)

Plasmid	Description	Reference
JHP035	pGEX-4T-1-MSN2(401-570)	This study
JHP036	pGEX-4T-1-MSN2(571-704)	This study
JHP037	pGEX-4T-1-MSN2^{S194A}(1-300)	This study
JHP038	pGEX-4T-1-MSN2^{S638A}(571-704)	This study

3.3. RNA preparation and analysis

Total RNA was extracted from yeast cells using the hot phenol method as previously described (48). The relative amount of specific mRNA was determined by semi quantitative reverse transcription-PCR (RT-PCR) or quantitative real time-PCR (qRT-PCR). Briefly, 1 μg of heat-denatured total RNA was subjected to reverse transcription in a 30 μl reaction mixture containing 200 unit of M-MLV reverse transcriptase (Thermo scientific), 0.2 μg oligo $(dT)_{15}$ (IDT) and 1 μl each of 10 mM dNTPs at 42°C for 60 min, and reverse transcription was terminated by heating at 75°C for 15 min.

For RT-PCR analysis, 1 μl of cDNA was amplified by endpoint PCR (Eppendorf) using 10 pmol each of specific primers with 15–25 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The PCR products of size 300-500 bp were separated by 1.2% agarose gel electrophoresis. The number of amplification cycles was adjusted to avoid reaching a plateau phase during PCR.

For qRT-PCR analysis, 1 μl of cDNA (diluted 1:20) was amplified by SYBR Green I master mix (Roche Applied Science) using 5 pmol each of specific primers with 45 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s on a Lightcycler 480 II System (Roche Applied Science). The crossing point (Cp) values were obtained using method of Second Derivative Maximum of the LightCycler 480 Software version 1.5 (Roche Applied Science) and mRNA levels were represented as normalized target/reference ratios.

Table 3.5 Primer sequences used for semiquantitative or quantitative RT-PCR

Primer	Sequence	PCR product size
Primers for semiquantitative RT-PCR		
BTN2 RT-F	ATGTTTTCCATATTCAATTC	500 bp
BTN2 RT-R	AATATGCTCAATTCATCACC	
HSP26 RT-F	ATGTCATTTAACAGTCCA	500 bp
HSP26 RT-R	TAGTCTGGCAAAGTGATG	
HSP30 RT-F	TCTGGGTTGTTTCGCTATTAG	500 bp
HSP30 RT-R	CTAAGCAGTATCTTCGACAGC	
HSP42 RT-F	TCACCTACAAGCGCCTTCCCC	478 bp
HSP42 RT-R	TCAATTTTCTACCGTAGGGTT	
18sRNA RT-F	AGCCGATGGAAGTTTGAGGC	300 bp
18sRNA RT-R	GCCCCCTTCTCTAAGCAGAT	
Primers for quantitative RT-PCR		
BTN2 qRT-F	AGCAATTCTGGTTCAGCAGAAAG	201 bp
BTN2 qRT-R	TTATATCTCCTCAATAATAGAGTTT	
HSP42 qRT-F	CAACAACGGTCTACTACAAATTAAGG	138 bp
HSP42 qRT-R	TCTACCGTAGGGTTGGGATTTTCTTC	
CTT1 qRT-F	CAGAAGAAATTATTCGTTCATAACG	202 bp
CTT1 qRT-R	TTAATTGGCACTTGCAATGGACC	
HSP12 qRT-F	GACAACAAGGGTGTCTTCCAAGGTG	154 bp
HSP12 qRT-R	GACCGGAAACATATTCGACGGCATC	
ACT1 qRT-F	CCCCAGAAGCTTTGTTCCATCCTTC	154 bp
ACT1 qRT-R	CCTGGGAACATGGTGGTACCACCG	

3.4. Proteins preparation

For *in vitro* kinase assay, CK2 was expressed and purified from yeast cells. Briefly, overnight cultured RSY620 yeast cells harboring the pRS426GAL-GST-CKA2 were grown from an A_{600} of 0.2 to 0.8 in SC-Ura medium containing 2% raffinose, washed twice with distilled water and transfer into SC-Ura medium containing 4% galactose for induction. After 18 h cultivation, cells were harvested, lysed by vortex with a glass bead in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM EDTA, 1 mM DTT, 10% glycerol, and 0.5% NP-40] containing 0.1% protease inhibitor cocktail (Calbiochem) and purified using a glutathione agarose resin (Novagen). Purified CK2 was stored at -75°C after dialysis against a dialysis buffer [50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, and 15% Glycerol].

For purification of CK2 subunits, *E. coli* Rosetta gami2 (DE3) pLysS strain was transformed with pET28b-His/T7-CKA1, pET28b-His/T7-CKA2, pET28b-His/T7-CKB1, or pET28b-His/T7-CKB2 plasmids. The His/T7 tag fusion CK2 subunits were purified using Ni-NTA resin (Qiagen). The purified proteins were dialyzed against a dialysis buffer [50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, and 15% Glycerol], and stored at -75°C.

In order to purify GST-fused Hsf1 proteins, *E. coli* Rosetta gami2 (DE3) pLysS strain was transformed with various HSF1 plasmids (see table 3.4). The recombinant GST-Hsf1 proteins were purified using glutathione agarose resin following the manufacturer's recommendations, and dialyzed against a dialysis

buffer [50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 15% Glycerol].

Recombinant Ppt1 proteins containing His or His/T7 tag were prepared as described previously (44). The expression plasmids were transformed in *E. coli* strain BL21 (DE3) pLysS and the proteins were purified using Ni-NTA resin (Qiagen). The purified proteins were dialyzed against a dialysis buffer [50 mM HEPES-KOH (pH 7.0), 150 mM KCl, 5 mM glycine, 3.5 mM DTT, 2 mM EDTA, and 15% Glycerol] and stored at -75°C.

In order to purify GST-fused Msn2 proteins, *E. coli* Rosetta gami2 (DE3) pLysS strain was transformed with various MSN2 plasmids (see table 3.4). The recombinant GST-Msn2 proteins were purified using glutathione agarose resin following the manufacturer's recommendations, and dialyzed against a dialysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 15% Glycerol].

3.5. Western blot analysis

Phosphorylation state of Hsf1 was detected as described previously (1). Briefly, Yeast cells were incubated in ice for 20 min with a final concentration of 5% trichloroacetic acid (Sigma-Aldrich). Precipitated pellets were washed three times with 100% acetone, dried, resuspended with boiling buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1% SDS], and the samples were broken by vigorous vortex mixing for 1 min in the presence of glass beads (Sigma-Aldrich). After boiling in SDS sample buffer, the samples were separated by 6% SDS-PAGE, and analyzed

by western blotting with an anti-Hsf1 antibody.

For loading control, GST-fused Hsf1 proteins, GST-fused Msn2 proteins, and GST-fused Msn4 protein were subjected to SDS-PAGE, and detected by anti-GST antibody (Santa Cruz). His/T7-CKA1, His/T7-CKA2, His/T7-CKB1, or His/T7-CKB2 proteins were detected by anti-T7 tag antibody (Novagen).

3.6. *In vitro* kinase and phosphatase assays

For *in vitro* kinase assay, 5 μg of GST-fused Hsf1 proteins, GST-fused Msn2 proteins, and GST-fused Msn4 protein purified from *E. coli* were labeled with [γ-^{32}P] ATP using 0.2 μg of GST-Cka2 purified from yeast cells in 20 μl of kinase reaction buffer [25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, and 50 μM cold ATP containing 2 μCi of [γ-^{32}P] ATP] at room temperature for 1 h. For dephosphorylation of Hsf1 by Ppt1, *in vitro* phosphatase assay was performed as previously described (44). Following the *in vitro* kinase assay, free [γ-^{32}P] ATP was hydrolyzed by treatment with 0.3 unit apyrase (Sigma-Aldrich) at room temperature for 45 min. The mixture was subjected to dephosphorylation by 20 μM of Ppt1 purified from *E. coli* in a phosphatase reaction buffer [200 mM Tris-HCl (pH 7.8), 150 mM KCl, 25 mM $MgCl_2$, 5 mM glycine, and 1 mM DTT] at room temperature for 1-4 h. Reactions were terminated by boiling in SDS sample buffer and the reaction products were separated by SDS-PAGE. The phosphorylated proteins were detected and quantified using a phosphor-image analyzer (BAS-2500,

Fujifilm).

3.7. GST pull-down assay

1 μM of GST or GST-Hsf1 proteins were incubated with glutathione agarose beads (Novagen) at 4°C for 2 h in GST PD buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 5 mM MgCl2, and 0.1% NP-40] containing 0.1% protease inhibitor cocktail (Calbiochem), and the beads were washed four times with GST PD buffer. Fallowing the washing step, 1 μM His/T7-tagged Ppt1 or His/T7-tagged TPR was added to the beads and incubated with gentle rocking at 4°C for 2 h. After washing the beads four times with GST PD buffer, samples were analyzed by western blotting with an anti-GST (Santa Cruz Biotechnology) and anti-T7 tag antibody (Novagen). Resulting images were captured using a G:box iChemi XL device (Syngene).

3.8. pNPP assay

The phosphatase activity of Ppt1 was determined by monitoring hydrolysis of *para*-nitrophenol phosphate (*p*NPP) as previously described (44). 0.1 μM of Ppt1 was mixed with increasing concentrations of Hsf1 or GST in a phosphatase reaction buffer [200 mM Tris-HCl (pH 7.8), 150 mM KCl, 25 mM $MgCl_2$, 5 mM glycine, and 1 mM DTT] containing 20 mM *p*NPP. Production of *p*NP was monitored every 3 min for 2 h at 410 nm in a microplate spectrophotometer

(Multiscan GO, Thermo scientific). The results were represented as a reaction velocity according to the concentrations of Hsf1 or GST.

3.9. Fluorescence microscopy

BY4741 and *cka2*Δ cells harboring pRS415ADH-MSN2-EGFP were grown to early exponential phase, and treated with a final concentration of 2% lactic acid. Subsequently, cells were harvested at different time points, fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, and washed with cold PBS. Cellular localization of Msn2-GFP was monitored by a confocal microscope (Leica SP8 X) and visualized using the Leica Application Suite X software (Leica Microsystems).

Chapter 4.

CK2-dependent inhibitory phosphorylation is relieved by Ppt1 phosphatase for the ethanol stress-specific activation of Hsf1 in *Saccharomyces cerevisiae*

4.1. Introduction

The evolutionally conserved heat shock transcription factors (HSFs) transactivate genes encoding heat shock proteins (HSPs) in response to various stress conditions perturbing protein homeostasis including heat shock, oxidative stress, heavy metals, and pharmacological reagents (28, 49). Vertebrates and plants harbor multiple HSF isoforms, HSF1 to HSF4, of which HSF1 is the master regulator for the expression of HSP genes (29). On the other hand, yeast and other invertebrates have a single HSF (28).

Unlike mammalian HSF1, which is activated through several regulatory steps including trimerization, nuclear localization, and DNA binding, *Saccharomyces cerevisiae* Hsf1 always exist in the nucleus as a trimer, being engaged in both constitutive and stress-inducible binding to the heat shock elements (HSEs) (28, 50). Multiple phosphorylations are common regulatory mechanisms shared by all HSFs, although mammalian HSF1 is known to be regulated by other post-translational modifications such as sumoylation and acetylation (30). In mammals, several kinases have been identified which activate or inhibit HSF1 activity in response to heat shock (30). However, it is still not well understood how the phosphorylation events integrate multiple stress signals into the differential regulation of HSF activity. Previously, we have shown that Snf1 kinase, a homologue of mammalian AMP-activated protein kinase (AMPK), and Yak1 kinase, a downstream effector of protein kinase A (PKA), activate the

transcriptional activity of Hsf1 upon glucose starvation, without affecting heat shock activation of Hsf1 (1, 39). The glucose starvation-specific effects of Snf1 and Yak1 correlate with the fact that both kinases are activated under glucose-limiting conditions (51). Additionally, Hsf1 is phosphorylated even under normal conditions, suggesting that phosphorylation might also regulate the basal activity of Hsf1. Although it has been suggested that phosphorylation of serine residues adjacent to a yeast-specific repressive region (CE2) might restrain HSF in an inactive state in *Kluyveromyces lactis* (43), no repressive kinase has been identified for yeast HSF.

Whereas, not much is known about protein phosphatases involved in HSF regulation. Protein phosphatase 5 (PP5), a member of PPP family of Ser/Thr phosphatase, has been suggested as a negative regulator of HSF1 (42). Rat PP5 overexpressed in *Xenopus* oocytes inhibited DNA binding activity of HSF1 and accelerated recovery after heat shock (42). PP5, one of the tetratricopeptide repeat (TPR)-containing Hsp90 cochaperones, interacts with the C-terminal MEEVD domain of Hsp90 (52). Hsp90 chaperone machinery also interacts with HSF1, inhibiting HSF1 activity via negative feedback regulation (53). Although physical interaction between PP5 and Hsp90-HSF1 complex was observed in *Xenopus* oocytes (42), it has not yet been elucidated whether HSF1 is a direct substrate of PP5. On the contrary, Ppt1, a yeast homologue of PP5, has been recently identified as a positive regulator of Hsf1 (46). In modern sake yeast where *PPT1* is spontaneously deleted, Hsf1 is constitutively hyperphosphorylated and shows

defects in activation during sake fermentation and upon acute ethanol stress, while exhibiting normal activation upon heat shock (46). However, the molecular details of the Ppt1-dependent regulation of Hsf1 are largely unknown.

In this chapter, we identified that CK2 (casein kinase 2) counteracts Ppt1 by phosphorylating Hsf1 on S608, thereby inhibiting Hsf1 activation upon ethanol stress, without affecting the basal and heat-induced activity of Hsf1. Such ethanol-specific regulatory mechanisms of Hsf1 might play an important role in balancing cell growth and stress response in *Saccharomyces* species, which produce high concentrations of ethanol during the fermentation.

4.2. Ppt1-dependent dephosphorylation and activation of Hsf1 during growth

To investigate the role of Ppt1 in Hsf1 regulation, we first examined the effects of Ppt1 on Hsf1 phosphorylation and activation during growth. As shown previously (1), Hsf1 showed growth phase-dependent increase in phosphorylation levels, which correlates with the increase in mRNA expression levels of its target genes such as *HSP26*, *HSP30*, and *BTN2* (Fig. 4.1). *PPT1* deletion mutant showed higher levels of Hsf1 phosphorylation than wild type throughout the growth phase, while maintaining the growth-dependent phosphorylation pattern (Fig. 4.1). Therefore, Ppt1 might dephosphorylate constitutive phosphorylation sites of Hsf1, which are phosphorylated from early to late exponential phase. Even with the higher levels of Hsf1 phosphorylation, *ppt1Δ* exhibited reduced growth-dependent induction of

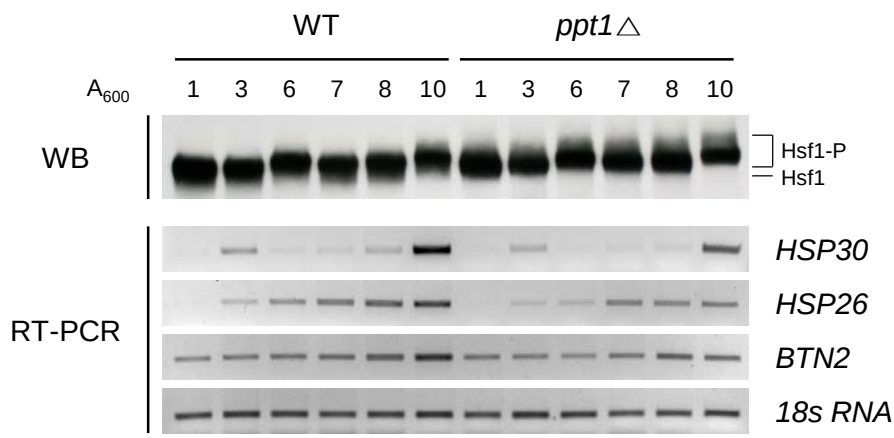


Figure 4.1 Ppt1 dephosphorylates and activates Hsf1 during growth.

BY4741 (WT) and *ppt1Δ* strains were cultured from early to late exponential phase in YPD medium. Hsf1 was detected by western blotting with antibody against Hsf1, and the expression levels of *HSP30*, *HSP26*, and *BTN2* mRNAs were detected by RT-PCR using *18S rRNA* as a control.

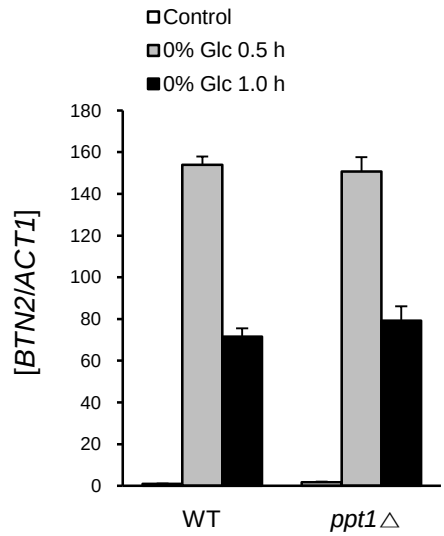
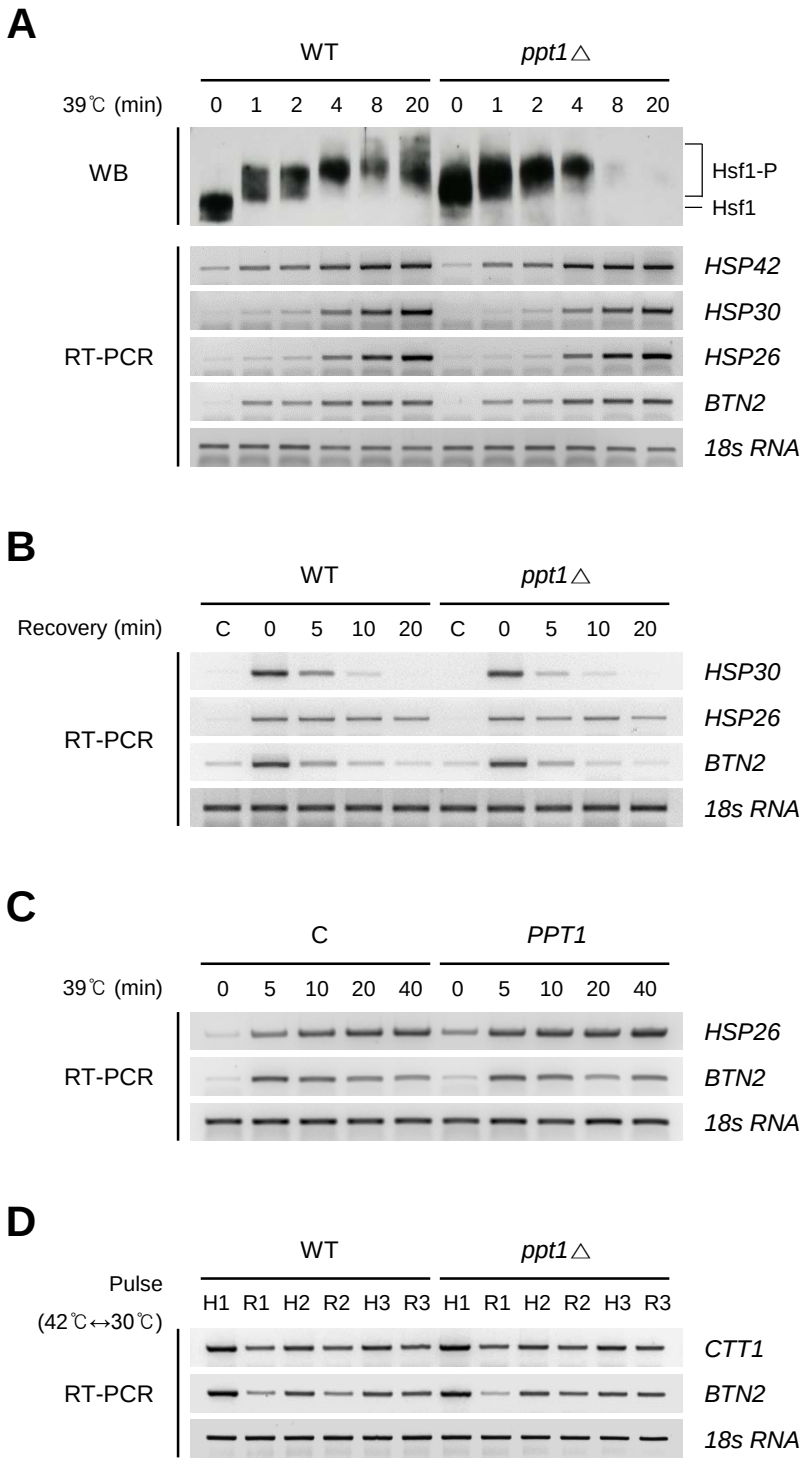


Figure 4.2 Ppt1 is not involved in glucose starvation-dependent activation of Hsf1.

WT and *ppt1Δ* cells were grown to early exponential phase in YPD medium, and then transferred to a medium lacking glucose for 0.5 and 1 h. *BTN2* mRNA levels were detected by qRT-PCR normalized with *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the control of wild-type (*n*=3).


A
WT
ppt1△
39℃ (min)
0 1 2 4 8 20 0 1 2 4 8 20
WB
Hsf1-P
Hsf1
RT-PCR
HSP42
HSP30
HSP26
BTN2
18s RNA
B
WT
ppt1△
Recovery (min)
C 0 5 10 20 C 0 5 10 20
RT-PCR
HSP30
HSP26
BTN2
18s RNA
C
C
PPT1
39℃ (min)
0 5 10 20 40 0 5 10 20 40
RT-PCR
HSP26
BTN2
18s RNA
D
WT
ppt1△
Pulse
(42℃↔30℃)
H1 R1 H2 R2 H3 R3 H1 R1 H2 R2 H3 R3
RT-PCR
CTT1
BTN2
18s RNA

E

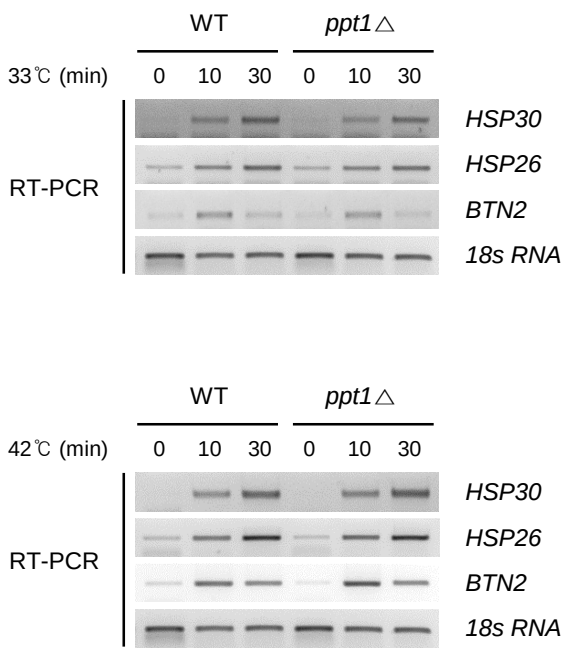

WT
ppt1△
33℃ (min)
0
10
30
0
10
30
RT-PCR
HSP30
HSP26
BTN2
18s RNA
WT
ppt1△
42℃ (min)
0
10
30
0
10
30
RT-PCR
HSP30
HSP26
BTN2
18s RNA

Figure 4.3 Ppt1 is not involved in heat shock-dependent activation of Hsf1.

WT and *ppt1Δ* cells were grown to early exponential phase in YPD medium at 30°C and heat shocked at 39°C for the indicated times. Hsf1 was detected by western blotting with antibody against Hsf1, and the expression levels of *HSP42*, *HSP30*, *HSP26*, *CTT1*, and *BTN2* mRNAs were detected by RT-PCR using *18S rRNA* as a control.

For heat shock recovery, WT and *ppt1Δ* cells were heat shocked at 39°C for 20 min and then recovered at 30°C for the indicated times. mRNA expression levels were detected by RT-PCR.

Over-expression of Ppt1 does not affect Hsf1 activation upon heat-shock. The *ppt1Δ* cells harboring the empty vector (C) or plasmid expressing full-length *PPT1* (*PPT1*). Over-night cultured cells were inoculated and cultured from O.D 0.1 to O.D 1 in YPD medium previous to heat-shock.

Pulse between heat shock at 42°C and recovery at 30°C was repeated three times for 20 minutes each.

Expression of Hsf1 target genes are not affected by heat shock at different temperatures, 33℃ and 42℃

Hsf1 target genes, supporting the notion that Ppt1 plays a positive role for Hsf1 activity. However, deletion of *PPT1* did not affect Hsf1 activation upon glucose starvation (Fig. 4.2), implying that Ppt1-dependent activation of Hsf1 at late growth phase might reflect the recently identified role of Ppt1 in Hsf1 activation upon ethanol stress (46).

We also checked the effect of *PPT1* deletion on heat shock activation of Hsf1. However, neither heat shock activation nor recovery of Hsf1 target genes was affected by the lack of *PPT1* (Fig. 4.3A-B). In addition, We further examined the effect of Ppt1 on heat shock activation of Hsf1 with several heat shock conditions such as overexpression of Ppt1 upon heat shock (Fig. 4.3C), the repeated pulses of heat shock and recovery (Fig. 4.3D), heat shock at different temperatures (Fig. 4.3E). Although heat shock-dependent activation of Hsf1 has been well known, Ppt1 did not affect Hsf1 activation upon various heat shock conditions. Taken together, these data suggest a stress-specific regulatory role of the Ppt1-dependent dephosphorylation of Hsf1.

4.3. Ppt1 physically interacts with Hsf1 *in vitro*

Next, we tested for the interaction between Ppt1 and Hsf1. The interaction between Ppt1 and Hsf1 was investigated by bimolecular fluorescent complementation (BiFC) assay to monitor transient interaction between phosphatase and substrate *in vivo* (54). Cells co-expressing the C-terminally VN-tagged Ppt1 (Ppt1-VN) and

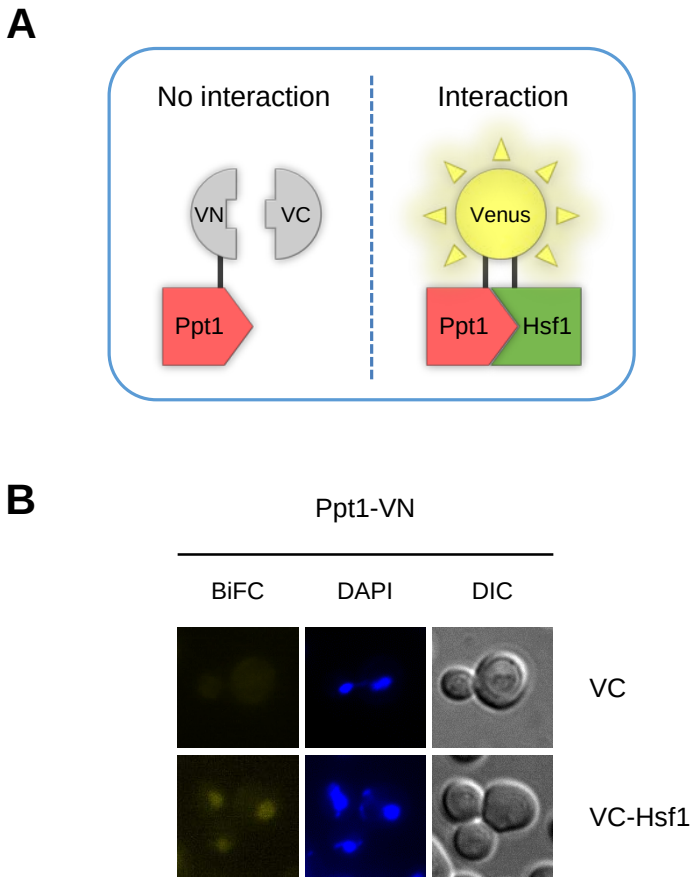


Figure 4.4 Ppt1 physically interact with Hsf1 *in vivo*.

A. Schematic representation of the principle of the bimolecular fluorescence complementation (BiFC) assay. VN, N-terminal Venus fragment; VC, C-terminal Venus fragment.

B. Interaction between Ppt1 and Hsf1 was monitored by BiFC assay. Cells co-expressing the C-terminally VN-tagged Ppt1 (Ppt1-VN) and the N-terminally VC-tagged Hsf1 (VC-Hsf1) was subjected to fluorescence microscopy. Cells co-expressing the C-terminally VN-tagged Ppt1 (Ppt1-VN) and the VC fragment (VC) was used as a negative control.

the N-terminally VC-tagged Hsf1 (VC-Hsf1) was subjected to fluorescence microscopy. As shown in Figure 4.4B, the BiFC signals were detected only when the Ppt1-VN were co-expressed with VC-Hsf1. These data demonstrate successful interaction between Ppt1 and Hsf1 *in vivo*.

Hsp90-dependent inhibition of Hsf1 activity (55) suggests a potential physical interaction between Hsf1 and Hsp90, which has been demonstrated for mammalian HSF1 and Hsp90 after cross-linking (53). Considering the fact that Ppt1 is one of the TPR-containing Hsp90 cochaperones, Hsp90 could possibly mediate the interaction between Hsf1 and Ppt1. However, *in vitro* GST pull-down assay showed a direct interaction between GST-Hsf1 and T7-Ppt1 purified from *Escherichia coli* even in the absence of Hsp90 (Fig. 4.5). We also detected a direct interaction between GST-Hsf1 and T7-TPR, suggesting that the interaction between Hsf1 and Ppt1 was mediated by the TPR domain of Ppt1 (Fig. 4.5). Therefore, Hsp90 seems not to be required for the interaction between Hsf1 and Ppt1. On the contrary, it has been shown that Hsp90-mediated targeting is necessary for the Ppt1-dependent phosphorylation of Cdc37, an Hsp90 cochaperone (56).

The TPR domain of Ppt1 is engaged in an autoinhibition through intramolecular interaction with the phosphatase domain, which can be relieved by TPR-binding proteins such as Hsp90 or by fatty acid ligands (52, 57). Therefore, we investigated whether Hsf1 binding to the TPR domain can affect the phosphatase activity of Ppt1. To investigate whether Hsf1 binding to TPR

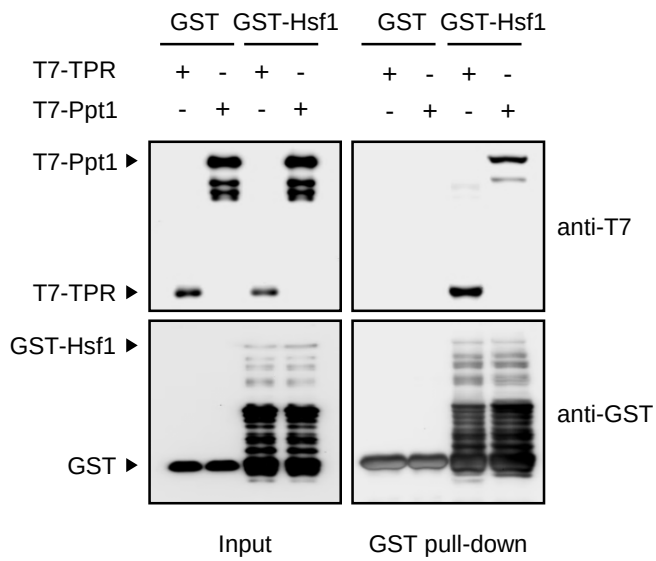
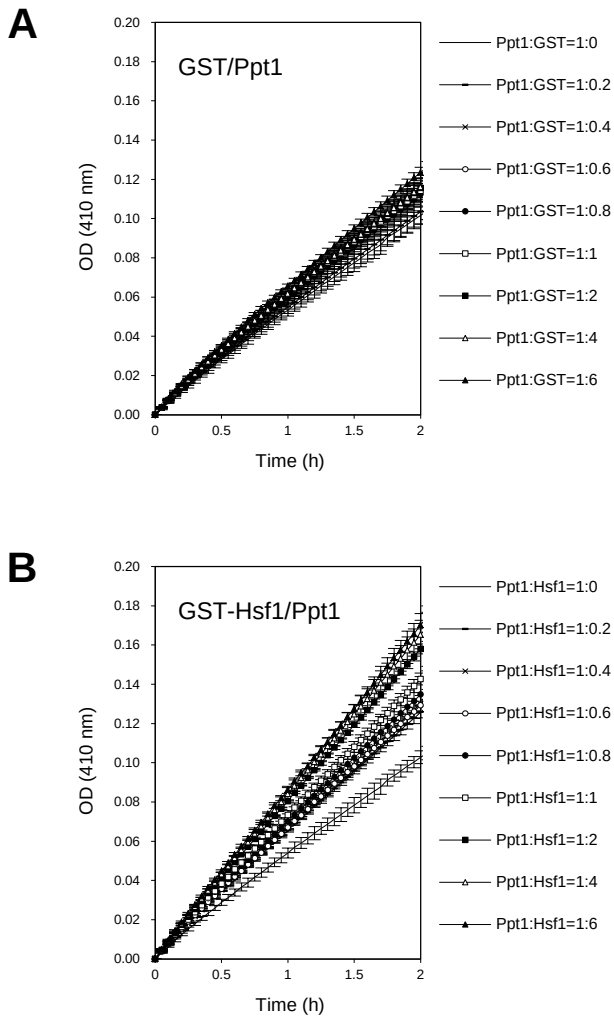


Figure 4.5 Ppt1 directly interact with Hsf1 through its TPR domain *in vitro*.

Interaction between Hsf1 and Ppt1 *in vitro*. GST or GST-Hsf1 and His/T7-Ppt1 or His/T7-TPR proteins were incubated with glutathione-agarose beads, and the precipitates were analyzed by western blotting.


A
GST/Ppt1
OD (410 nm)
Time (h)
Ppt1:GST=1:0
Ppt1:GST=1:0.2
Ppt1:GST=1:0.4
Ppt1:GST=1:0.6
Ppt1:GST=1:0.8
Ppt1:GST=1:1
Ppt1:GST=1:2
Ppt1:GST=1:4
Ppt1:GST=1:6
B
GST-Hsf1/Ppt1
OD (410 nm)
Time (h)
Ppt1:Hsf1=1:0
Ppt1:Hsf1=1:0.2
Ppt1:Hsf1=1:0.4
Ppt1:Hsf1=1:0.6
Ppt1:Hsf1=1:0.8
Ppt1:Hsf1=1:1
Ppt1:Hsf1=1:2
Ppt1:Hsf1=1:4
Ppt1:Hsf1=1:6

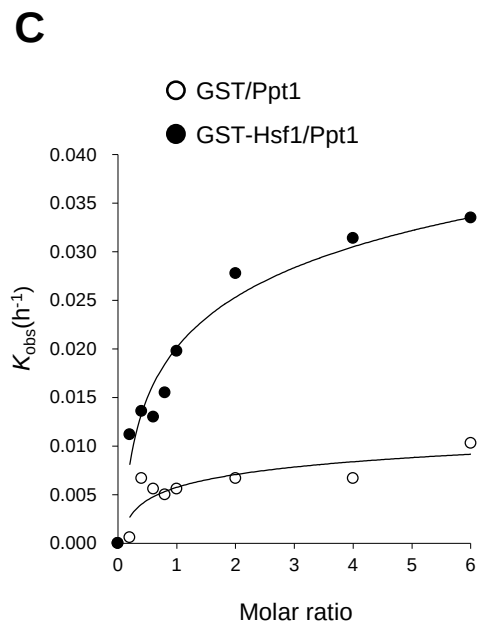


Figure 4.6 Hsf1 activates Ppt1 phosphatase activity

A.-B. The kinetics of pNP production over time by incubating Ppt1 with increasing concentrations of GST (A, upper panel) or GST-Hsf1 (B, under panel). His-Ppt1 was incubated with increasing concentrations of GST or GST-Hsf1 with the indicated molar ratios, and Ppt1 activity was measured by using 20 mM pNPP.

C. The data plotted are corrected for Ppt1 activity in the absence of Hsf1 and represent the average of three independent experiments.

domain can stimulate the Ppt1 activity, dose-dependent activation of Ppt1 was measured by monitoring hydrolysis of para-nitrophenol phosphate (*p*NPP) with increasing concentrations of GST or GST-Hsf1 (Fig. 4.6A-B). We measured the Ppt1 activity using low concentration of His-Ppt1 (0.1 μM) to avoid the saturation of Ppt1 activity without adding Hsf1. Production of *p*NP was monitored every 3 min for 2 h at 410 nm in a linear range. As shown in Fig. 4.6C, GST-Hsf1 exhibited a dose-dependent activation of Ppt1 *in vitro*, whereas the effect of GST control on Ppt1 activity was marginal.

We also confirmed the role of Ppt1 TPR domain in the dephosphorylation of Hsf1 *in vivo*. The hyperphosphorylation of Hsf1 in *ppt1Δ* was restored to the normal phosphorylation levels by expressing full-length Ppt1, but not Ppt1 mutant lacking the TPR domain, suggesting that the TPR domain is necessary for Ppt1 to target Hsf1 (Fig. 4.7).

Taken together, these data suggest that Hsf1 directly binds to the TPR domain of Ppt1, relieving its autoinhibition. It has been shown that Ppt1 also activates Hsp90 by dephosphorylating two serine residues (58). Since Ppt1 interacts with Hsf1 and Hsp90 through the same domain, Hsp90 might compete with Hsf1 for Ppt1 binding in an Hsp90-Hsf1 complex, exerting a negative effect on the Ppt1-dependent dephosphorylation of Hsf1.

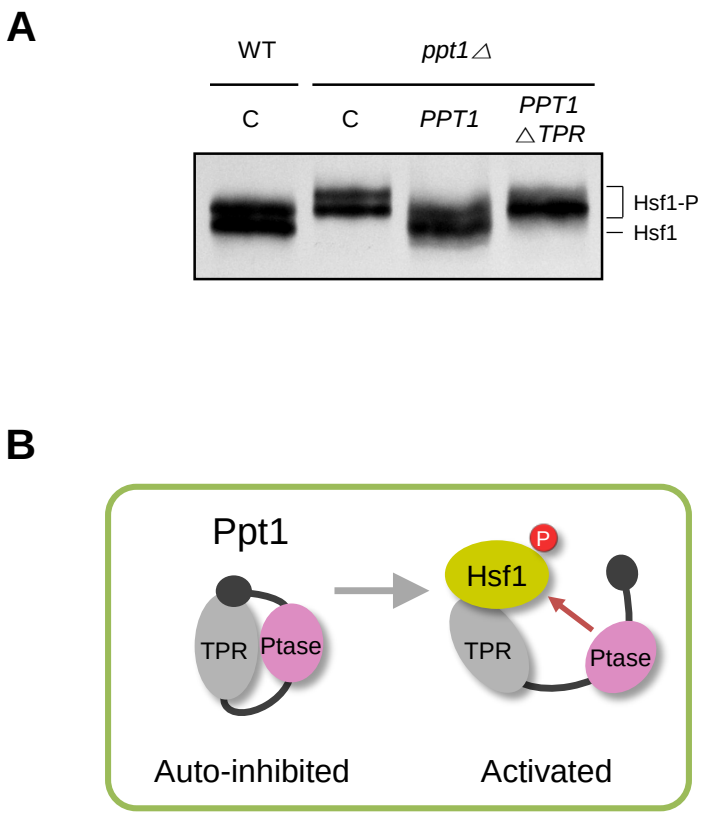


Figure 4.7 *in vivo* complementation

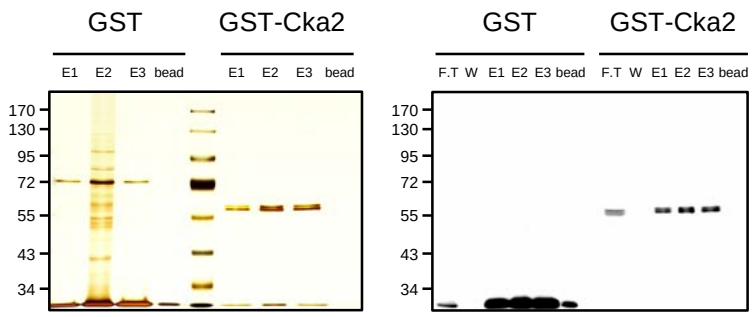
A. The *ppt1Δ* cells harboring the empty vector (C), or plasmid expressing full-length *PPT1* or *PPT1ΔTPR* were grown in a selective minimal medium. The phosphorylation status of Hsf1 was detected by western blotting with antibody against Hsf1.

B. Schematic representation of the role of Ppt1 TPR domain in the dephosphorylation of Hsf1.

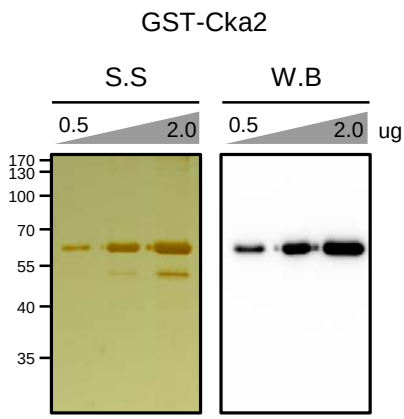
4.4. CK2 phosphorylates S608 in the C-terminal activation domain of Hsf1

Next, we searched for the kinase counteracting Ppt1 to repress Hsf1. So far, two kinases, Snf1 and Yak1, have been shown to activate Hsf1 in response to glucose starvation (1, 39), but no repressive kinase has been identified. Although Ppt1 might counteract several protein kinases, we focused on the fact that Ppt1 dephosphorylates CK2-dependend phosphorylation site in Cdc37 (56). Additionally, Hsp90 phosphorylated by CK2 was dephosphorylated by Ppt1 *in vitro* (44). Therefore, we investigated the possibility of CK2 as a counteracting kinase of Ppt1 for Hsf1 regulation. CK2 is a ubiquitous, highly conserved, and constitutively active Ser/Thr protein kinase involved in a wide range of cellular functions. CK2 is a tetrameric enzyme composed of two catalytic and two regulatory subunits, and yeast has two isoforms of catalytic subunits (Cka1 and Cka2) and regulatory subunits (Ckb1 and Ckb2) (59). CK2 holoenzyme was purified from yeast cells expressing GST-Cka2 (Fig. 4.8A-B). GST was also purified from yeast cells expressing GST to use as negative control (Fig. 4.8A). The purified CK2 activity successfully phosphorylated Hsp90 known for CK2 substrate (Fig. 4.8C). To investigate the possibility that CK2 might phosphorylate Hsf1, CK2 purified from yeast cells expressing GST-Cka2 was incubated with His tag-Hsp90 and GST-Hsf1 purified from *E. coli* in the presence of [γ-^{32}P]ATP. CK2 successfully phosphorylated Hsf1 as well as Hsp90 *in vitro* (Fig. 4.9A). In addition,

A


GST
GST-Cka2
GST
GST-Cka2
E1 E2 E3 bead
E1 E2 E3 bead
F.T W E1 E2 E3 bead
F.T W E1 E2 E3 bead
170
130
95
72
55
43
34

B


GST-Cka2
S.S
W.B
0.5
2.0
0.5
2.0
ug
170
130
100
70
55
40
35

C

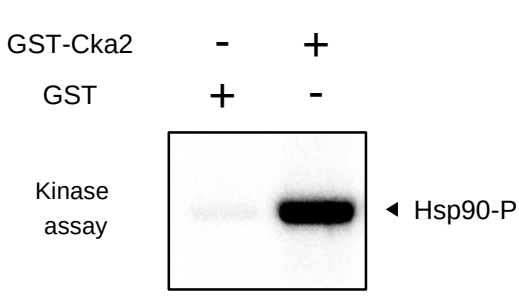

GST-Cka2
-
+
GST
+
-
Kinase
assay
Hsp90-P

Figure 4.8 CK2 holoenzyme was purified from yeast cells expressing GST-Cka2.

A. Confirmation of GST and GST-Cka2 purification process. GST or GST-Cka2 was purified from yeast cells using glutathione agarose resin. GST and GST-Cka2 were detected by using silver staining (left panel) and western blotting (right panel) .

B. Confirmation of the purified GST-Cka2. GST-Cka2 was detected by using silver staining (S.S) and western blotting (W.B).

C. CK2 activity test. The purified CK2 activity was tested with Hsp90 known for CK2 substrate. *in vitro* kinase assay was performed using His tag fusion Hsp90 purified from *E. coli* as a substrate. GST purified from yeast cells was used as negative control.

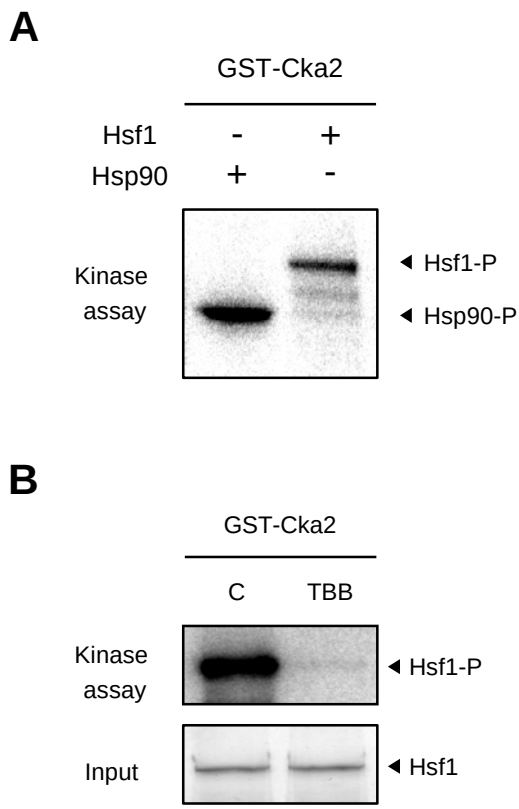
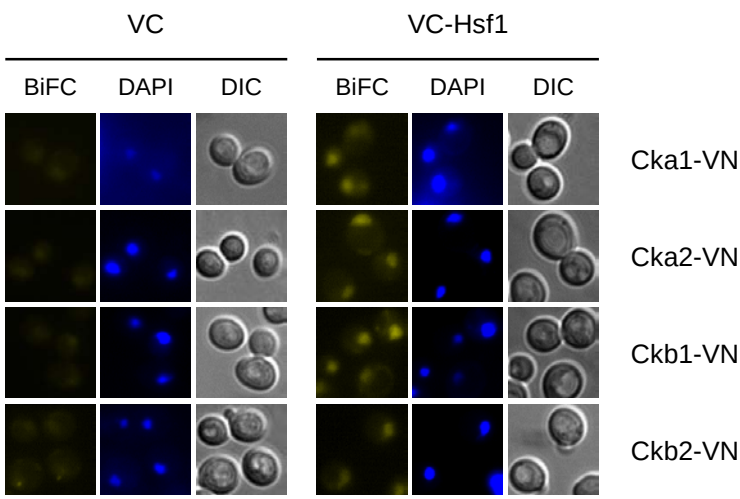


Figure 4.9 CK2 phosphorylates Hsf1.

A. Phosphorylation of Hsf1 by CK2 was investigated by *in vitro* kinase assay using GST-Hsf1 purified from *E. coli* as a substrate. The His tag fusion Hsp90 purified from *E. coli* was used as positive control.

B. GST-Cka2 was purified from *S. cerevisiae* and *in vitro* kinase assay was performed using GST-Hsf1 as a substrate in the presence of 10% DMSO (C) or 20 μM TBB. The input proteins were detected by Coomassie Blue staining.

A


VC
VC-Hsf1
BiFC
DAPI
DIC
BiFC
DAPI
DIC
Cka1-VN
Cka2-VN
Ckb1-VN
Ckb2-VN

B

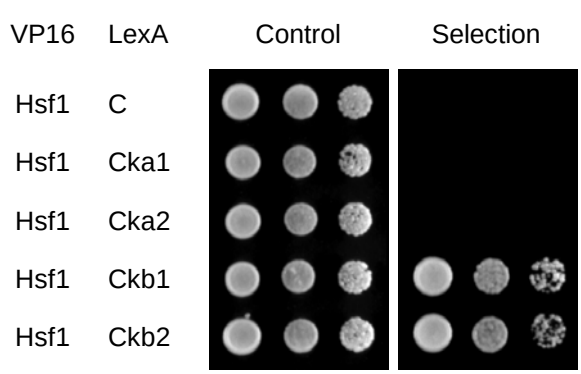

VP16
LexA
Control
Selection
Hsf1
C
Hsf1
Cka1
Hsf1
Cka2
Hsf1
Ckb1
Hsf1
Ckb2

Figure 4.10 CK2 interacts with Hsf1 *in vivo*.

A. CK2 subunits interact with Hsf1 in the nucleus. Interactions between Hsf1 and CK2 subunits were monitored by BiFC assay. Cells co-expressing the C-terminally VN-tagged CK2 subunits (Cka1-VN, Cka2-VN, Ckb1-VN, or Ckb2-VN) and the N-terminally VC-tagged Hsf1 (VC-Hsf1) was subjected to fluorescence microscopy. Cells co-expressing the C-terminally VN-tagged CK2 subunits and the VC fragment (VC) was used as a negative control.

B. *S. cerevisiae* strain L40 expressing VP16- Hsf1 and LexA-fused Cka1, Cka2, Ckb1, or Ckb2 were spotted on a control plate medium containing histidine and a selection plate medium containing 0.1 mM 3-aminotriazole (3-AT) but lacking histidine to detect protein-protein interactions.

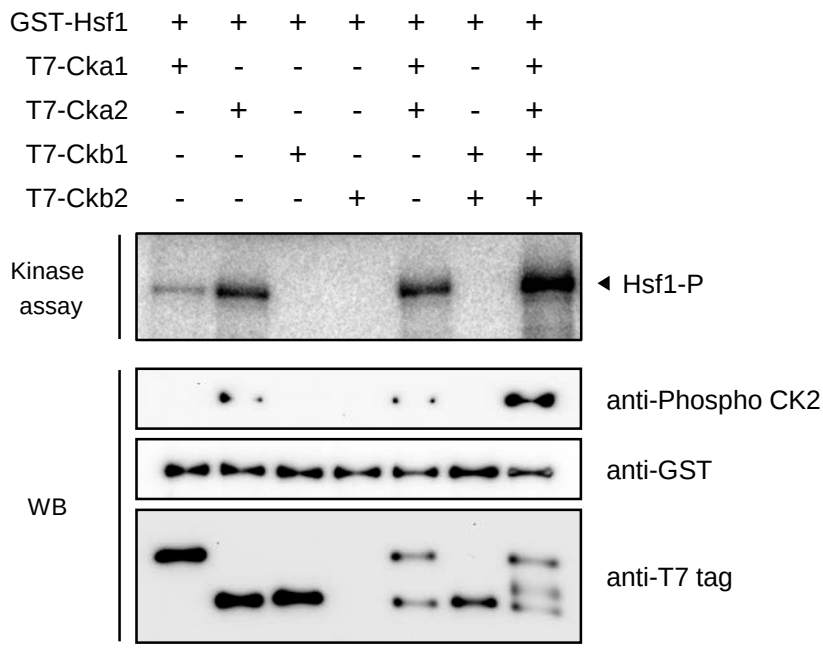


Figure 4.11 CK2 activity toward Hsf1 is enhanced by regulatory subunits.

GST-Hsf1 was phosphorylated by each 0.2 μM CK2 subunits in the presence of [γ-^{32}P] ATP for 1 h. The phosphorylation status was detected by a phosphorimager and western blotting with antibody against phospho CK2 substrate. The input proteins were detected by western blotting with antibody against GST and T7 tag.

the CK2-dependent phosphorylation of Hsf1 was abolished by the treatment of 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2 (Fig. 4.9B), confirming the specificity of the phosphorylation event.

We further examined whether CK2 is capable of interacting with Hsf1 *in vivo*. The interaction between CK2 subunits and Hsf1 was investigated by bimolecular fluorescent complementation (BiFC) assay to monitor transient interaction between kinase and substrate *in vivo* (54). Cells co-expressing the C-terminally VN-tagged CK2 subunits (Cka1-VN, Cka2-VN, Ckb1-VN, or Ckb2-VN) and the N-terminally VC-tagged Hsf1 (VC-Hsf1) was subjected to fluorescence microscopy. As shown in Figure 4.10A, the BiFC signals were detected only when the VN-tagged CK2 subunits, but not the VC fragment (VC) used as a negative control, were co-expressed with VC-Hsf1. These data demonstrate successful interaction between CK2 holoenzyme and Hsf1 *in vivo*. However, in yeast two-hybrid assay, the regulatory subunits, Ckb1 and Ckb2, but not the catalytic subunits, Cka1 and Cka2, showed interaction with Hsf1 (Fig. 4.10B). Therefore, the interaction between Hsf1 and CK2 holoenzyme might be mediated by the regulatory subunits. Unlike CK2 catalytic subunits, CK2 regulatory subunits are not essential for yeast cell viability. However, the regulatory subunits of CK2 have been shown to play an important role in CK2 regulation through modulating tetramer assembly, substrate selectivity, or catalytic activity and stability (59). Indeed, CK2 activity toward Hsf1 is enhanced by regulatory subunits (Fig. 4.11).

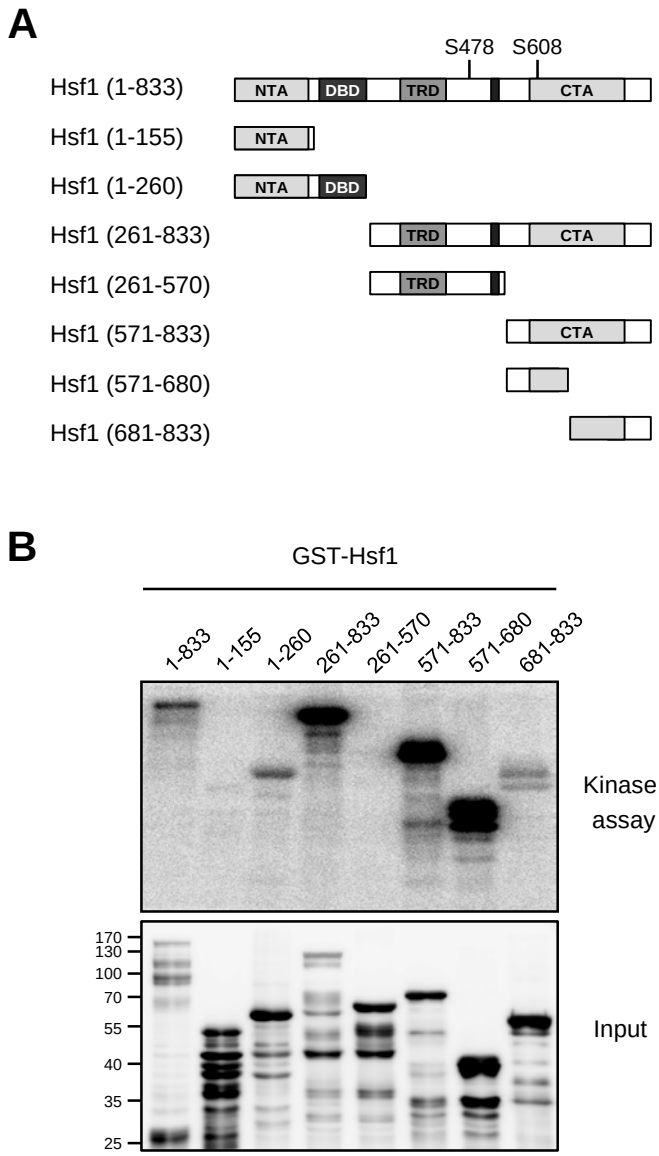

A
S478
S608
Hsf1 (1-833)
NTA
DBD
TRD
CTA
Hsf1 (1-155)
Hsf1 (1-260)
Hsf1 (261-833)
Hsf1 (261-570)
Hsf1 (571-833)
Hsf1 (571-680)
Hsf1 (681-833)
B
GST-Hsf1
1-833
1-155
1-260
261-833
261-570
571-833
571-680
681-833
Kinase assay
Input
170
130
100
70
55
40
35
25

Figure 4.12 Mapping of the CK2-dependent phosphorylation domain of Hsf1.

A. Schematic representation of full-length and truncated Hsf1 derivatives. NTA, N-terminal activation domain; DBD, DNA binding domain; TRD, trimerization domain; CTA, C-terminal activation domain.

B. GST-Hsf1 derivatives purified from *E. coli* were phosphorylated by GST-Cka2 *in vitro*. The input proteins were detected by western blotting with antibody against GST.

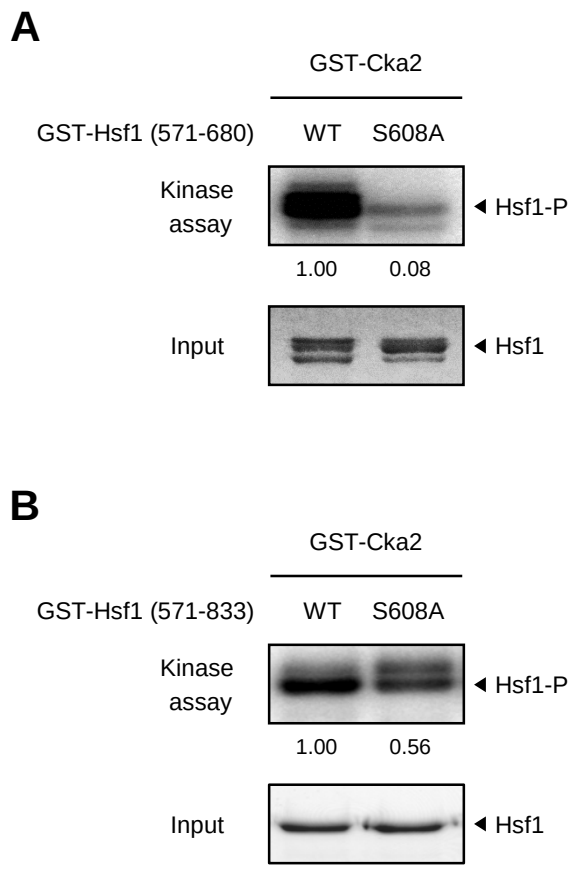


Figure 4.13 CK2 phosphorylates S608 in the C-terminal activation domain of Hsf1.

A.- B. CK2 phosphorylates S608 in Hsf1. GST-Hsf1 (571-833) wild type (WT) or S608A mutant (A, upper panel), and GST-Hsf1 (571-680) wild type (WT) or S608A mutant (B, under panel) were subjected to *in vitro* kinase assay with GST-Cka2. The phosphorylation levels were quantified with a phosphorimager and the relative intensities were indicated. The amounts of input proteins were detected by Coomassie Blue staining.

To map the CK2-dependent phosphorylation sites in Hsf1, we generated 7 truncation derivatives of Hsf1 (Fig. 4.12A) and tested for their phosphorylation by CK2 *in vitro* (Fig. 4.12B). Truncation mutants containing the C-terminal activation domain (CTA) were strongly phosphorylated by CK2, narrowing down the phosphorylation sites between 571 and 680 amino acid residues of Hsf1 (Fig. 4.12B). In this region, we could identify S608 (S^{608}DDD) as a putative CK2 phosphorylation site based on the CK2 consensus sequence (SXXE/D). $Hsf1^{S608A}$ (571-680) mutant substituting S608 with A showed about 92% reduction in the CK2-dependent phosphorylation level *in vitro*, indicating that S680 is the major CK2-dependent phosphorylation site in Hsf1 CTA (Fig. 4.13A). However, Hsf1 (1-260) and Hsf1 (681-833) also showed weak phosphorylation signals by CK2 (Fig. 4.12B), indicating the presence of additional CK2 phosphorylation sites in Hsf1. Accordingly, when we introduced S608A mutation to a longer fragment, Hsf1 (571-833), phosphorylation level was reduced only by 44%, suggesting the presence of additional CK2-dependent phosphorylation sites in the C-terminal half of the CTA (Fig. 4.13B). In a previous global phosphoproteome analysis, higher level of S608 phosphorylation was detected in *ppt1Δ* compared with wild type (45). Although the effect of *ppt1Δ* on S608 phosphorylation was detected only once out of two replicate experiments, this result raises the possibility that S608 might be the site for the reciprocal regulation between CK2 and Ppt1. The same phosphoproteome analysis also identified S478 in Hsf1 as a Ppt1-dependent dephosphorylation site. However, Hsf1 (261-570) containing the S478 residue was

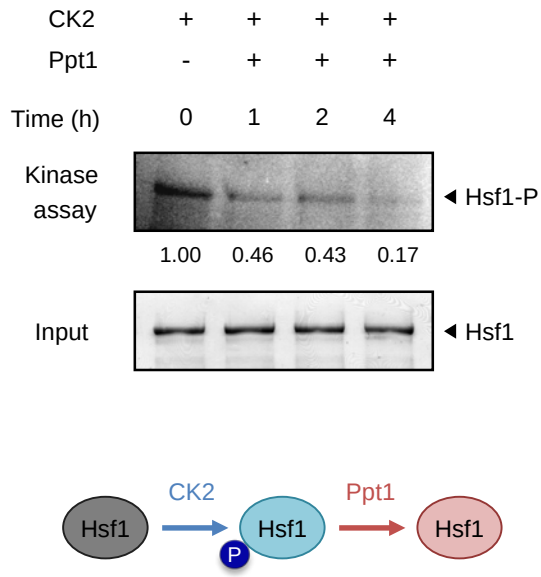


Figure 4.14 Ppt1 dephosphorylates Hsf1 phosphorylated by CK2.

GST-Hsf1 was phosphorylated by GST-Cka2 in the presence of [γ-^{32}P] ATP for 1 h, incubated with apyrase to hydrolyze the remaining [γ-^{32}P] ATP, and then incubated with His-Ppt1 for the indicated times. The phosphorylation levels were quantified with a phosphorimager and the relative intensities were indicated. The input proteins were detected by Coomassie Blue staining.

not phosphorylated by CK2 (Fig. 4.12B). Therefore, S478 might be phosphorylated by another yet unidentified kinase.

Since Hsf1 S608, the identified CK2-dependent phosphorylation site, is also a potential target site for Ppt1, we checked whether Ppt1 and CK2 share common target sites in Hsf1. To test this, Hsf1 was first phosphorylated by CK2 in the presence of [γ-^{32}P] ATP, and then incubated with Ppt1. As shown in Fig. 4.14, Ppt1 successfully reduced Hsf1 phosphorylation levels, indicating that Ppt1 might dephosphorylate the CK2-dependent phosphorylation site, although Ppt1 and CK2 might also have unshared target sites in Hsf1.

4.5. Hsf1 is reciprocally regulated by CK2 and Ppt1 upon ethanol stress

Based on the potential regulation of Hsf1 S608 by CK2 and Ppt1, we next examined the interplay between CK2 and Ppt1 for the regulation of Hsf1. Since Ppt1 is known to be involved in Hsf1 activation upon ethanol stress (46), we first examined the effects of Ppt1 on Hsf1 phosphorylation and activation in response to ethanol stress. In agreement with the previous report (46), *PPT1* deletion mutant showed higher levels of Hsf1 phosphorylation than wild type throughout 10% ethanol stress (Fig. 4.15).

Next, we investigated the effects of CK2 overexpression and *PPT1* deletion on the ethanol-dependent induction of Hsf1 target genes, *BTN2* and *HSP42*. Neither

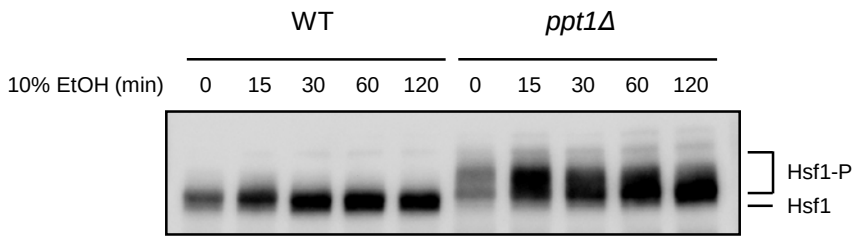


Figure 4.15 Ppt1-dependent dephosphorylation of Hsf1 upon ethanol stress.

WT and *ppt1Δ* strain was transformed with pRS413 TEF-T7 tag HSF1. Over-night cultured cells were inoculated and cultured from O.D 0.1 to O.D 0.8 in SC-HIS medium previous to ethanol stress. Hsf1 was detected by immunoblotting with antibody against T7 tag.

PPT1 deletion nor CK2 overexpression exhibited significant effect on basal expression levels of Hsf1 target genes (Fig. 4.16A). However, in agreement with the previous report (46), *ppt1Δ* showed reduced induction levels of Hsf1 target genes upon ethanol stress (Fig. 4.16A). In addition, overexpression of *CKA1* and *CKA2* also reduced ethanol-dependent induction of Hsf1 target genes, suggesting that CK2 plays an inhibitory role for Hsf1 activation in response to ethanol. Moreover, *ppt1Δ* strain overexpressing CK2 catalytic subunits showed a dramatic defect in the ethanol-dependent induction of Hsf1 target genes, supporting the synergistic inhibitory effects of *PPT1* deletion and CK2 overexpression on Hsf1 activation.

We also investigated the effects of CK2 and Ppt1 on heat shock activation of Hsf1. As shown previously (Fig. 4.3), *PPT1* deletion did not show any significant effect on heat shock activation of *BTN2* (Fig. 4.16B). Furthermore, heat shock activation of Hsf1 was not affected either by CK2 overexpression alone or in combination with *PPT1* deletion. Therefore, Hsf1 phosphorylated by CK2 might be readily activated by heat shock, but might not be competent for activation by ethanol.

Next, we examined whether the synergistic effects of *PPT1* deletion and CK2 overexpression on Hsf1 activation can affect cellular ethanol tolerance. Neither *PPT1* deletion nor overexpression of *CKA1* or *CKA2* alone affected ethanol sensitivity on plate medium containing up to 9% ethanol (Fig. 4.16C). However, overexpression of both *CKA1* and *CKA2* exerted clear growth defects in the

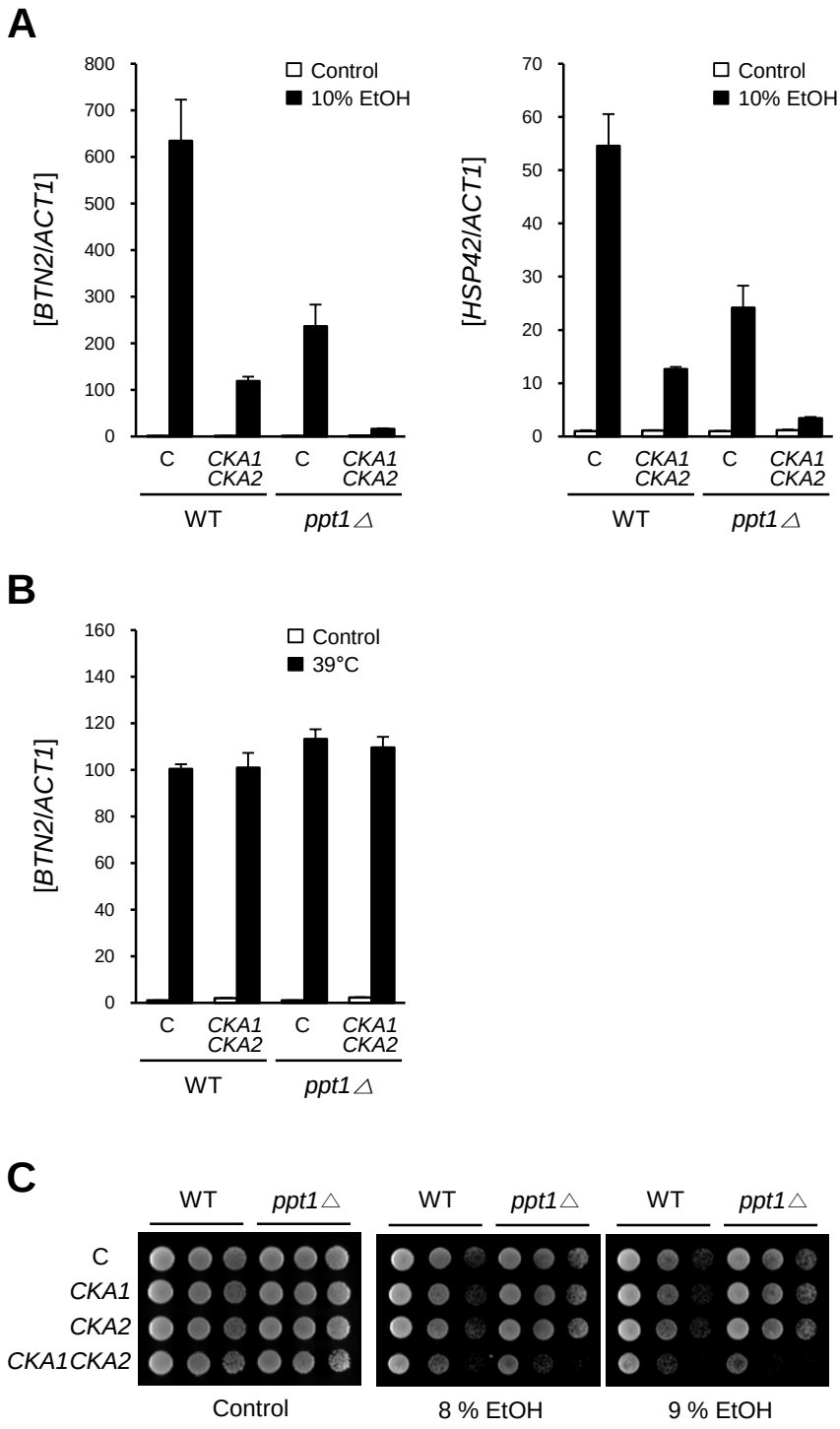

A
[BTN2/ACT1]
Control
10% EtOH
800
700
600
500
400
300
200
100
0
C
CKA1 CKA2
C
CKA1 CKA2
WT
ppt1△
[HSP42/ACT1]
70
60
50
40
30
20
10
0
B
[BTN2/ACT1]
Control
39°C
160
140
120
100
80
60
40
20
0
C
C
CKA1
CKA2
CKA1CKA2
WT
ppt1△
WT
ppt1△
WT
ppt1△
Control
8 % EtOH
9 % EtOH

Figure 4.16 Reciprocal actions of CK2 and Ppt1 regulate Hsf1 activation upon ethanol but not upon heat shock stress.

A. WT and *ppt1Δ* cells harboring the vector controls (C) or plasmids for overexpressing *CKA1* and *CKA2* were treated with 10% ethanol for 2 h, and mRNA expression levels of *BTN2* and *HSP42* were detected by qRT-PCR normalized with *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated control of wild type (n=3).

B. The same cells described in panel A were heat shocked at 39°C for 15 min and *BTN2* mRNA levels were detected by qRT-PCR.

C. WT and *ppt1Δ* cells harboring the empty vectors (C), or plasmid overexpressing *CKA1* and *CKA2* alone or in combination, were tested for growth by spotting serially diluted cultures onto minimal plate media with or without ethanol.

presence of ethanol (Fig. 4.16C), consistent with the inhibitory effect of their overexpression on ethanol-dependent Hsf1 activation (Fig. 4.16A). Overexpression of *CKA1* and *CKA2* in *ppt1Δ* further exacerbated the growth defects on ethanol plates, suggesting the synergistic effects of CK2 overexpression and *PPT1* deletion on reducing ethanol tolerance (Fig. 4.16C).

Taken together, in *ppt1Δ* cells overexpressing *CKA1* and *CKA2*, most Hsf1 molecules might be phosphorylated on S608 and impaired for ethanol-induced activation, resulting in ethanol sensitivity. However, the phosphorylation status of S608 seems not affect the Hsf1 basal activity or Hsf1 activation upon heat shock.

4.6. Hsf1^{S608A} mutant showed enhanced activation by ethanol, but not by heat shock

Next, we confirmed the role of S608 phosphorylation status in the regulation of Hsf1 activation. Cells expressing *HSF1*^{WT} or *HSF1*^{S608A} from its own promoter was generated by substituting the endogenous *HSF1* gene with either wild type *HSF1* or *HSF1*^{S608A} bearing its own terminator and *KlURA3* cassette. Cells expressing *HSF1*^{S608A} exhibited about 2.2-fold increase in *BTN2* induction levels upon ethanol stress than wild type control, supporting our hypothesis that dephosphorylation of S608 might sensitize Hsf1 for activation by ethanol (Fig. 4.17A). However, basal expression levels of *BTN2* was not significantly changed in Hsf1^{S608A} strain, implying that the phosphorylation status of S608 does not affect Hsf1 activity

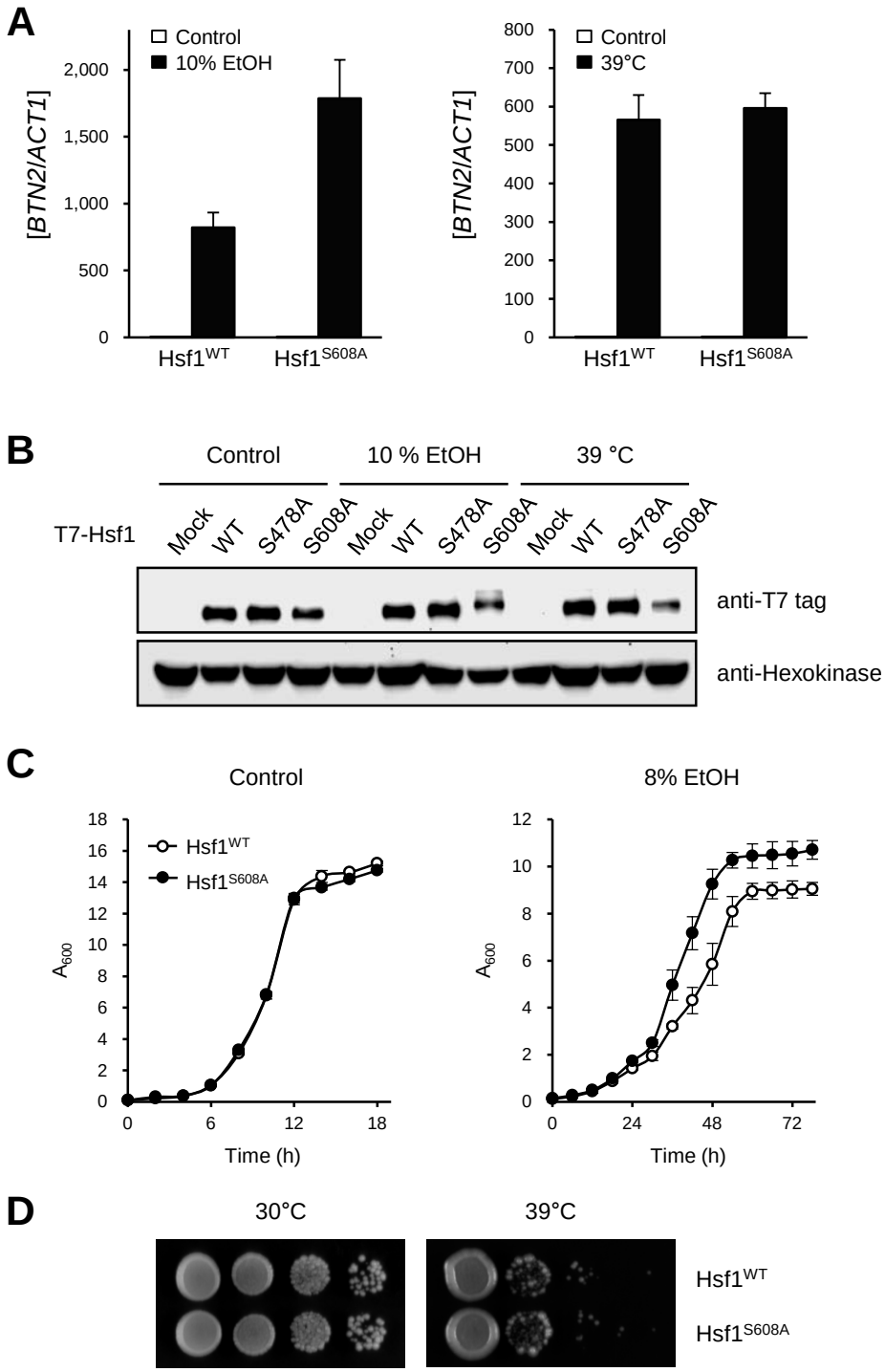

A
Control
10% EtOH
[BTN2/ACT1]
2,000
1,500
1,000
500
0
Hsf1WT
Hsf1S608A
Control
39°C
800
700
600
500
400
300
200
100
0
Hsf1WT
Hsf1S608A
B
Control
10 % EtOH
39 °C
T7-Hsf1
Mock
WT
S478A
S608A
anti-T7 tag
anti-Hexokinase
C
Control
8% EtOH
Hsf1WT
Hsf1S608A
A600
Time (h)
D
30°C
39°C
Hsf1WT
Hsf1S608A

Figure 4.17 Cells expressing *HSF1*^{S608A} shows enhanced Hsf1 activation and stress tolerance in response to ethanol.

A. Cells expressing *HSF1*^{WT} and *HSF1*^{S608A} were grown to early exponential phase, and then incubated in the presence of 10% ethanol for 3 h or heat shocked at 39°C for 15 min. The expression levels of *BTN2* were measured by qRT-PCR normalized to *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the control of wild type (*n*=3).

B. Hsf1 proteins in the cells expressing *T7-HSF1*^{WT}, *T7-HSF*^{S478A}, and *T7-HSF*^{S608A} were analyzed by western blotting with antibody against T7 tag. BY4741 cells (Mock) were used as a negative control. The hexokinase protein was detected as a loading control.

C. The growth of cells expressing *HSF1*^{WT} and *HSF1*^{S608A} were monitored in YPD medium in the absence or presence of 8% ethanol. The data were shown as the average ± SD of triplicates.

D. Serially diluted cells expressing *HSF1*^{WT} and *HSF1*^{S608A} were spotted onto YPD plate medium and incubated at 30°C and 39°C for 2 to 5 days.

under normal conditions. In accordance with the fact that CK2 and Ppt1 do not affect heat shock activation of Hsf1, heat-induced expression levels of *BTN2* was not affected by introducing *HSF1*^{S608A} mutation (Fig. 4.17A).

We also confirmed the role of S608 phosphorylation status in regulation of Hsf1 activation. We generated a mutant strain by integrating T7-tagged *HSF1*^{S608A} gene in the chromosome under the control of *TEF2* promoter and *CYC1* terminator, while deleting the essential wild type gene. We also generated control strains expressing wild type *T7-HSF1* or *T7-HSF1*^{S478A} under the *TEF2* promoter and analyzed for Hsf1 activation upon ethanol and heat shock stress. Consistent with its higher activity of *HSF1*^{S608A} mutation (Fig. 4.17A), T7-Hsf1^{S608A} showed higher levels of phosphorylation than T7-Hsf1 or T7-Hsf1^{S478A} upon ethanol stress, which might reflects yet uncharacterized positive phosphorylation events (Fig. 4.17B). These results clearly demonstrate the repressive role of S608 phosphorylation in the activation of Hsf1 upon ethanol stress.

We further investigated whether the phosphorylation status of S608 can affect cellular stress tolerance. Cells expressing *HSF1*^{S608A} showed higher tolerance to ethanol than cells expressing *HSF1*^{WT}, reflecting the stronger activation of Hsf1^{S608A} upon ethanol stress (Fig. 4.17C). However, consistent with the similar activities of the wild type and mutant Hsf1 proteins upon heat shock stress, cells expressing *HSF1*^{WT} and *HSF1*^{S608A} showed no significant difference in heat shock sensitivity (Fig. 4.17D). Although we cannot rule out the potential roles of other yet uncharacterized CK2 phosphorylation sites in ethanol-specific regulation of

Hsf1, *Hsf1*^{S608A} mutant faithfully mimic the ethanol stress-sensitive unphosphorylated status of Hsf1, suggesting that S608 might be the major site for the reciprocal regulations by CK2 and Ppt1. Taken together, these results clearly demonstrate that CK2-dependent S608 phosphorylation is an ethanol stress-specific repression mechanism of Hsf1, which can be relieved by Ppt1 phosphatase.

4.7. Conclusions

In this chapter, it has been shown that ethanol-induced Hsf1 activity reciprocally regulated by CK2 and Ppt1. The ethanol specific regulatory mechanism does not affect the basal or heat-induced activity of Hsf1. Ppt1 can bind to the Hsf1 via its TPR domain, resulting in dephosphorylation and activation of Hsf1 repressed by CK2. CK2-dependent phosphorylation was reciprocally dephosphorylated by Ppt1. The Ser608 of Hsf1 was mainly phosphorylated by CK2. Deletion of *PPT1* or overexpression of CK2 reduced the induction levels of Hsf1 target genes in response to ethanol stress. Notably, ppt1 deletion mutant cells overexpressing CK2 showed the lowest induction levels of Hsf1 target genes. The mutation of Hsf1 S608 with alanine leads to enhanced activation and tolerance in response to ethanol. Hsf1S608A mutant also shows increased tolerance to multiple alcohols. Taken together, CK2 and Ppt1 reciprocally regulate the Hsf1 activity through phosphorylation/dephosphorylation of its serine 608 residue. In addition, phosphorylation status of Ser608 site on Hsf1 might play a role in regulating the

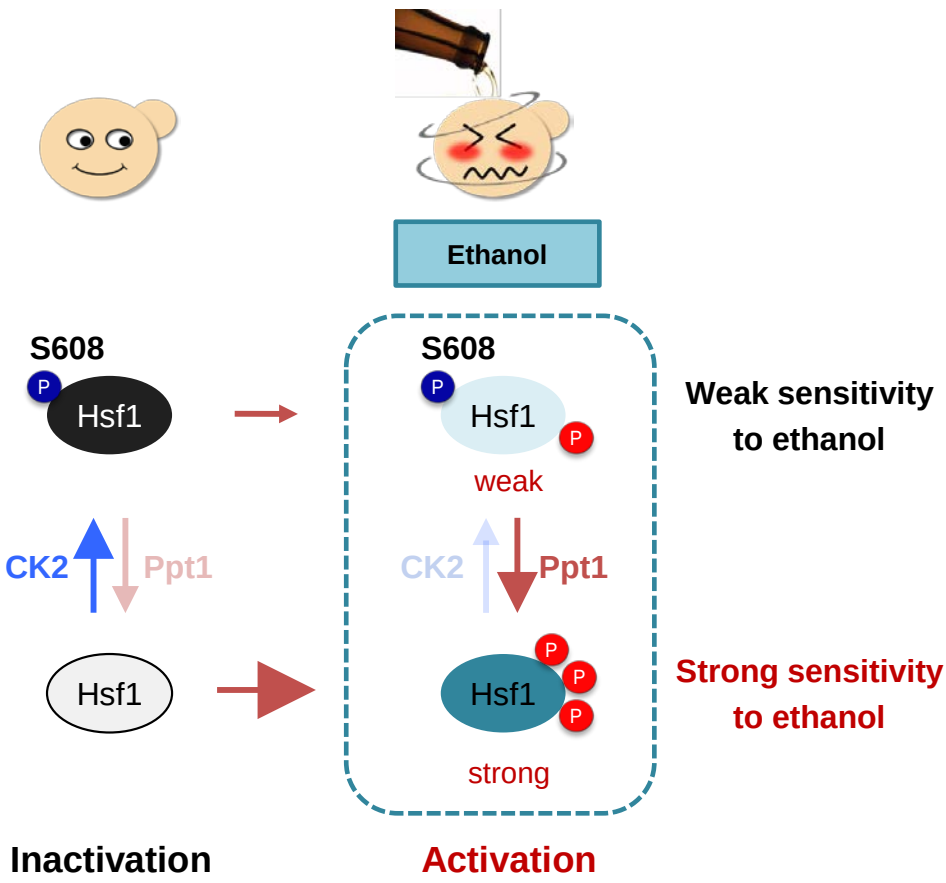


Figure 4.18 Graphical summary

Hsf1 sensitivity to ethanol is determined by S608 phosphorylation status through reciprocal actions of CK2 and Ppt1.

Hsf1 ethanol sensitivity of depending on cell growth and resistance to ethanol. Our findings will provide an understanding the regulation mechanism of Hsf1 and alcohols tolerant industrial strain.

Chapter 5.

CK2-dependent phosphorylation positively regulates stress-induced activation of Msn2 in *Saccharomyces cerevisiae*

5.1. Introduction

In response to changes in environmental conditions, cells rapidly adjust gene expression programs to maintain cellular homeostasis and cell survival (11). In *Saccharomyces cerevisiae*, various types of stress, such as heat shock, oxidative or reductive stress, osmotic shock, nutrient starvation, DNA damage, and extreme pH, lead to an extensive alternation of gene expression, termed the environmental stress response (ESR) (8, 9). The ESR genes are categorized into two groups based on their opposite expression patterns, which are stress-repressed genes and stress-induced genes (12). The stress-repressed ESR genes are mainly involved in protein synthesis, whereas stress-induced ESR genes are involved in a wide range of cellular processes to rapidly adapt to abrupt environmental changes.

Expression of stress-induced ESR genes is primarily regulated by functionally redundant transcription factors Msn2 and Msn4, known as general stress transcription factors (6). The *msn2*Δ*msn4*Δ cells are hypersensitive to stressful conditions, indicating that Msn2/4 activity and expression levels of their target genes are crucial for yeast cell survival under various environmental challenges (13). Msn2/4 activate expression of a large number of genes containing stress response element (STRE) in their promoters in response to variety of stresses, such as nutrient starvation, heat shock, osmotic shock, oxidative stress, alteration of pH, and noxious chemicals (8, 9, 13). Transcriptional activity of Msn2/4 is regulated at multiple levels, including nuclear translocation, DNA binding, and stability, which

are primarily influenced by the phosphorylation status of Msn2/4 (13, 21, 22).

Two nutrient-sensing pathways, the cAMP-protein kinase A (PKA) pathway and the target of rapamycin complex 1 (TORC1) pathway, are implicated in Msn2/4 regulation. Under non-stress conditions, Msn2 localizes in the cytoplasm and is inactivated by both PKA-dependent phosphorylation of the nuclear localization signal (NLS) and association with Bmh2, a yeast 14-3-3 protein, in a TORC1-dependent manner (18, 26). When cells face acute glucose starvation, both PKA and TORC1 are inactivated and Msn2 rapidly enters the nucleus through dephosphorylation by the protein phosphatase 1 (PP1) and release from Bmh2, leading to transcriptional activation of many STRE-controlled genes (24, 26). In addition, both Yak1 and Rim15, whose activity is restrained by PKA and TORC1, are also involved in the activation of Msn2 by direct phosphorylation (1, 41). Apart from nutrient-sensing pathways, the mitogen-activated protein kinase (MAPK) Hog1 and protein phosphatase 2A (PP2A) associated with Cdc55, a regulatory B subunit, regulate the Msn2/4-dependent transcriptional activation through modulating chromatin association and nuclear accumulation of Msn2/4 in response to osmotic stress (60-62).

Msn2/4 and Hsf1 are two primary stress-responsive transcription factors cooperatively regulating gene expression under multiple stress conditions. These transcription factors share some common regulatory mechanisms such as activation by direct phosphorylation by Yak1 and Rim15 (1, 41). In addition, we recently reported that Hsf1 activity is negatively regulated by CK2-dependent

phosphorylation for ethanol stress-specific activation (63), raising the possibility that Msn2/4 might also be regulated by CK2.

CK2 is a highly conserved and constitutively active Ser/Thr protein kinase. CK2 is a tetrameric enzyme, composed of two catalytic subunits (encoded by *CKA1* and *CKA2*) and two regulatory subunits (encoded by *CKB1* and *CKB2*) in *S. cerevisiae* (59). CK2 plays a role in a large number of fundamental cellular processes for cell growth and viability (59, 64, 65). In addition, several reports have recently demonstrated that CK2 is linked to stress-related responses including autophagy, osmotic stress, ethanol stress, and DNA damage (63, 66-69). Interestingly, protein stability and phosphorylation status of CK2 regulatory subunits, considered a modulator of substrate selectivity, were changed under stress condition (70, 71), implying that CK2 activity toward to substrate might also be regulated in response to stress conditions. Nevertheless, little is known about the function of CK2 engaged in stress-related responses, in contrast to numerous findings on the growth-related roles for CK2.

In this chapter, we demonstrated that CK2-dependent phosphorylation of Msn2 is required for Msn2 activation to cope with several stress conditions. These results demonstrate an important role for CK2 in Msn2-mediated stress responses.

5.2. Both Msn2 and Msn4 are phosphorylated by CK2 *in vitro*

Based on the fact that Hsf1 and Msn2/4 share some kinases for their regulation, we investigated whether Msn2 and Msn4 can be phosphorylated by CK2, which had been shown to phosphorylate Hsf1 (63). CK2 purified from yeast cells expressing GST-Cka2 was incubated with GST-Msn2 and GST-Msn4 purified from *E. coli* in the presence of [γ-^{32}P] ATP. CK2 successfully phosphorylated Msn2 and Msn4 as well as Hsf1 (Fig. 5.1A), and the CK2-dependent phosphorylation of Msn2 and Msn4 were abolished by the treatment of 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2 (Fig. 5.1B), confirming the specificity of the phosphorylation event.

We further examined whether CK2 is capable of interacting with Msn2 and Msn4 *in vivo*. In yeast two-hybrid assay, the regulatory subunits, Ckb1 and Ckb2, but not the catalytic subunits, Cka1 and Cka2, showed interaction with both Msn2 and Msn4 (Fig. 5.2). Thus, the interaction between Msn2 or Msn4, and CK2 holoenzyme might be mediated by the regulatory subunits. Unlike CK2 catalytic subunits, CK2 regulatory subunits are unessential for yeast cell viability. However, the regulatory subunits of CK2 are regulator considered to play a crucial role in CK2 regulation through modulating tetramer assembly, substrate selectivity, or catalytic activity and stability (59). Taken together, these result suggest that both Msn2 and Msn4 are regulated by CK2 through the phosphorylation and interaction.

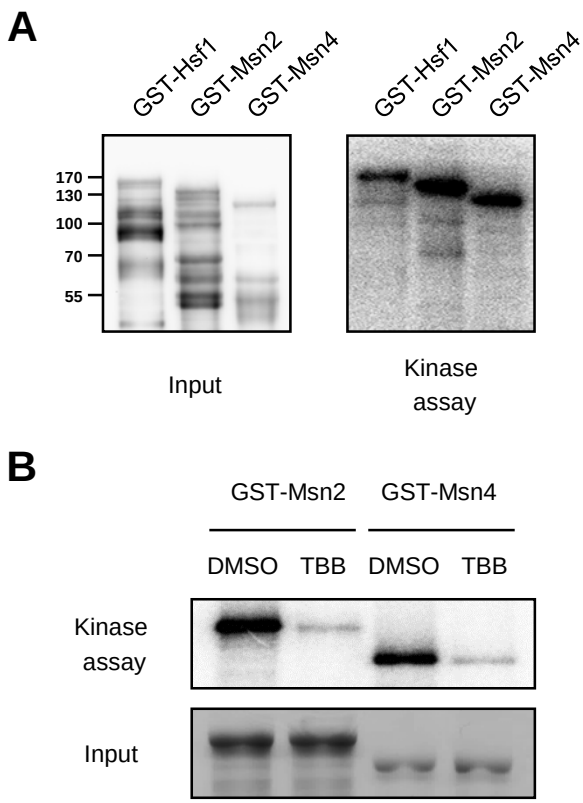


Figure 5.1 CK2 phosphorylates both Msn2 and Msn4 *in vitro*.

A. GST-Cka2 was purified from *S. cerevisiae* and *in vitro* kinase assay was performed using GST-Hsf1, GST-Msn2, and GST-Msn4 purified from *E. coli* as substrates. The input proteins were detected by western blotting with antibody against GST.

B. GST-Msn2 and GST-Msn4 were phosphorylated by GST-Cka2 in the presence of [γ-^{32}P] ATP, and 5% DMSO or 50 μM TBB. The input proteins were detected by Coomassie Blue staining.

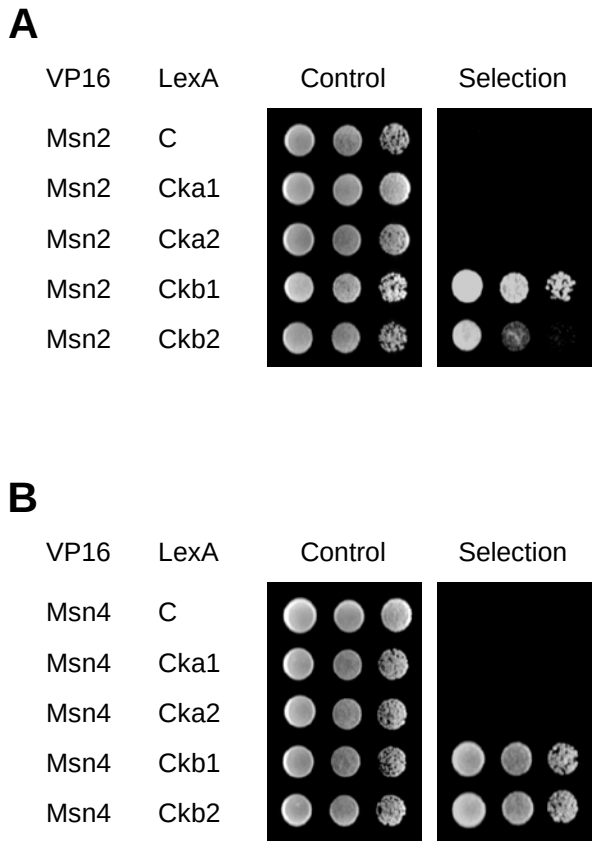


Figure 5.2 CK2 interacts with Hsf1 *in vivo.*

The intercation between CK2 subunits and Msn2, and CK2 subunits and Msn4 were ascertained by using yeast two hybrid assay. *S. cerevisiae* strain L40 expressing VP16-Msn2, or VP16-Msn4 and LexA-fused Cka1, Cka2, Ckb1, or Ckb2 were spotted on a control plate medium containing histidine and a selection plate medium containing 0.5 mM 3-aminotriazole (3-AT) but lacking histidine to detect protein-protein interactions.

5.3. Msn2 activity is regulated by CK2 catalytic subunits

To investigate the role of CK2 in Msn2/4 regulation, we examined the effects of CK2 overexpression on the transcriptional activation of Msn2/4 targets in response to stresses such as glucose starvation and treatment of H_2O_2 or lactic acid (Fig. 5.3A-C). Transcript levels of Msn2/4 target genes, *HSP12* and *CTT1*, were induced in wild type upon all three stress conditions tested, but these inductions were drastically reduced in *msn2*Δ*msn4*Δ, confirming the major role of Msn2/4 in the transcriptional induction of *HSP12* and *CTT1*. Overexpression of *CKA1* and *CKA2*, encoding the catalytic subunits of CK2, led to enhanced induction levels of Msn2/4 target genes in response to all three stresses. However, overexpression of *CKA1* and *CKA2* showed marginal effects in *msn2*Δ*msn4*Δ, suggesting that Msn2/4 activity might be positively regulated by CK2.

We next examined the effect of reducing CK2 activity on Msn2/4 activation. Since the CK2 activity is essential for yeast cell viability, we used the single gene deletion mutants lacking one of the CK2 catalytic subunits. Stress-dependent induction levels of *HSP12* and *CTT1* were reduced in both *cka1*Δ and *cka2*Δ except for *CTT1* induction by glucose starvation (Fig. 5.4A-C). Considering the fact that *CTT1* induction by glucose starvation is less dependent on Msn2/4 compared to the induction by H_2O_2 or lactic acid, other regulatory pathways involving other transcriptional activators might be responsible for the hyper glucose starvation-dependent induction of *CTT1* in *cka1*Δ and *cka2*Δ (Fig. 5.4A).

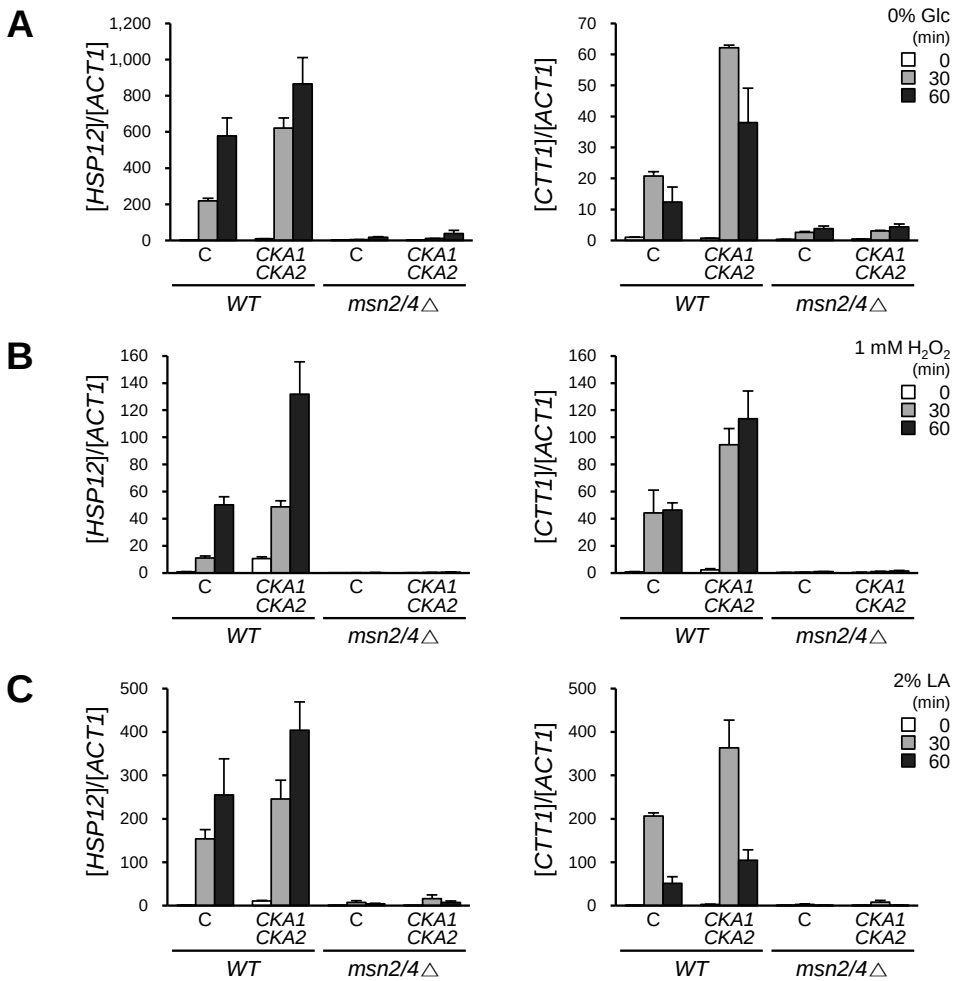

A
[HSP12]/[ACT1]
1,200
1,000
800
600
400
200
0
C
CKA1 CKA2
C
CKA1 CKA2
WT
msn2/4△
[CTT1]/[ACT1]
70
60
50
40
30
20
10
0
0% Glc
(min)
0
30
60
B
160
140
120
100
80
60
40
20
1 mM H2O2
C
Cka1 Cka2
WT
msn2/4△
C
500
400
300
200
100
2% LA

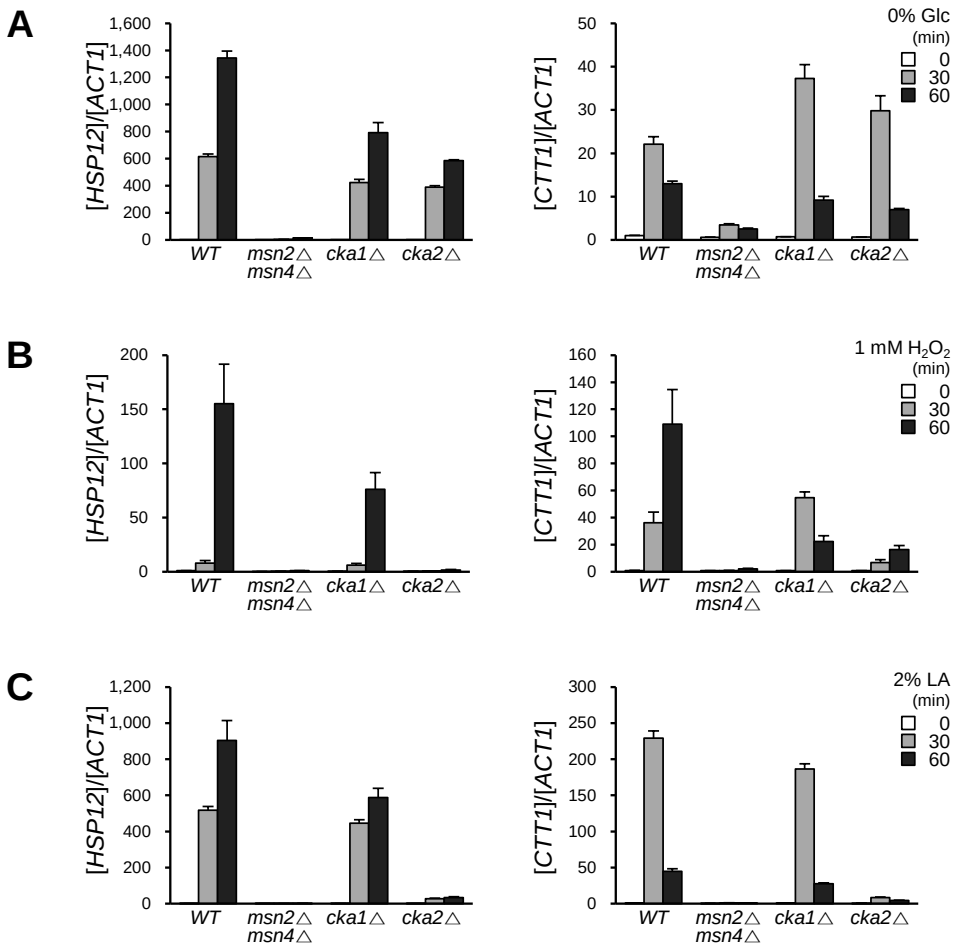
Figure 5.3 Overexpression of CK2 catalytic subunits enhances the transcriptional activation of Msn2/4 target genes upon various stress conditions.

A..-C. WT and *msn2/4*Δ cells harboring the vector controls (C) or plasmids for overexpressing *CKA1* and *CKA2* were grown to early exponential phase in SC-Leu-Ura medium containing 2% glucose, and then transferred to SC-Leu-Ura medium lacking glucose (A) or treated with a final concentration of 1 mM H_2O_2 (B) or 2% lactic acid (C) for the indicated times. *HSP12* and *CTT1* mRNA levels were detected by qRT-PCR normalized with *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated WT cells harboring the vector control (n=3).

Notably, expression levels of Msn2/4 target genes were severely reduced in *cka2*Δ upon the treatment of H_2O_2 or lactic acid (Fig. 5.4B and C). Taken together, these results indicate that CK2, especially Cka2 catalytic subunit, is required for stress-induced activation of Msn2/4. In the case of glucose starvation, the presence of other regulatory pathways might mask the effect of CK2 on the activation of Msn2/4.

To investigate the relative of CK2 activity toward Msn2, we examined the phosphorylation strength of Msn2 by Cka1 or Cka2 purified from *E. coli*. As shown Fig. 5.5, both Cka1 and Cka2 successfully phosphorylated Msn2 *in vitro*. In accordance with the transcript levels of Msn2/4 target genes (Fig. 5.4A-C), Msn2 was strongly phosphorylated by Cka2 more than Cka1, implying that Msn2 is regulated by CK2, primarily Cka2 catalytic subunit.

Since it has been known that transcriptional activity of Msn2/4 is essential for yeast cell survival in response to severe stress conditions (13), we tested whether the CK2-dependent activation of Msn2/4 can affect cellular stress tolerance. In accordance with the transcript levels of Msn2/4 target genes, both *cka1*Δ and *cka2*Δ exhibited reduced tolerance to H_2O_2 or lactic acid (Fig. 5.4D). Particularly, *cka2*Δ strain, showing severe defects in Msn2/4 activity (Fig. 5.4B and C), exhibited hypersensitivity to both H_2O_2 and lactic acid, implying that impaired CK2 activity leads to growth defect of yeast cells upon stress conditions. Taken together, these results demonstrate that CK2 positively regulates stress-induced Msn2/4 activation and stress tolerance in *S. cerevisiae*.


A
[HSP12]/[ACT1]
1,600
1,400
1,200
1,000
800
600
400
200
0
WT
msn2△ msn4△
cka1△
cka2△
[CTT1]/[ACT1]
50
40
30
20
10
0
0% Glc
(min)
0
30
60
B
[HSP12]/[ACT1]
200
150
100
50
0
[CTT1]/[ACT1]
160
140
120
100
80
60
40
20
0
1 mM H2O2
(min)
0
30
60
C
[HSP12]/[ACT1]
1,200
1,000
800
600
400
200
0
[CTT1]/[ACT1]
300
250
200
150
100
50
0
2% LA
(min)
0
30
60

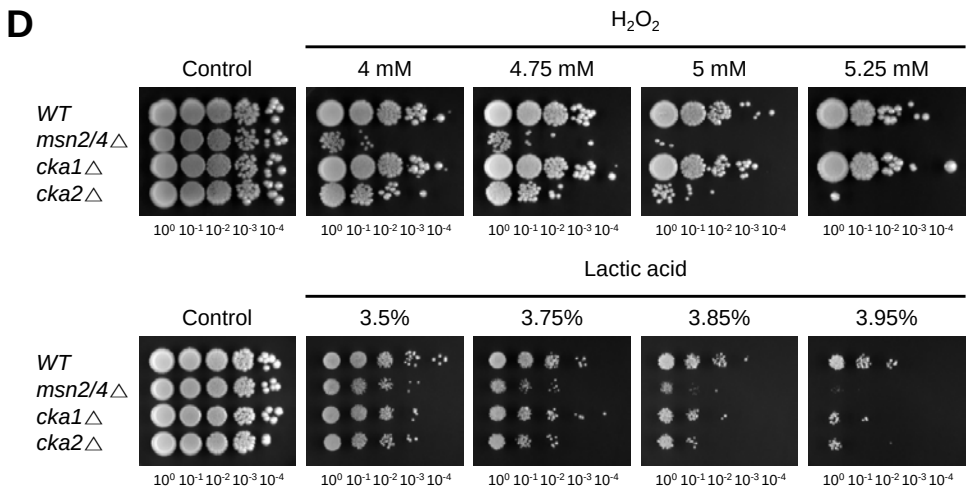


Figure 5.4 Deletion of CK2 catalytic subunits reduced transcriptional induction of Msn2/4 target genes and stress tolerance upon various stress conditions

A.-C. WT, *msn2*/*4*Δ, *cka1*Δ, and *cka2*Δ cells were cultured until early exponential phase in YPD medium containing 2% glucose, and then transferred to YP medium lacking glucose (A) or treated with a final concentration of 1 mM H_2O_2 (B) or 2% lactic acid (C) for the indicated times. Expression levels of *HSP12* and *CTT1* mRNA were detected by qRT-PCR normalized with *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated WT cells (*n*=3).

D. WT, *msn2*/*4*Δ, *cka1*Δ, and *cka2*Δ cells were tested for growth by spotting serially diluted cultures onto YPD agar plate with/without H_2O_2 or lactic acid.

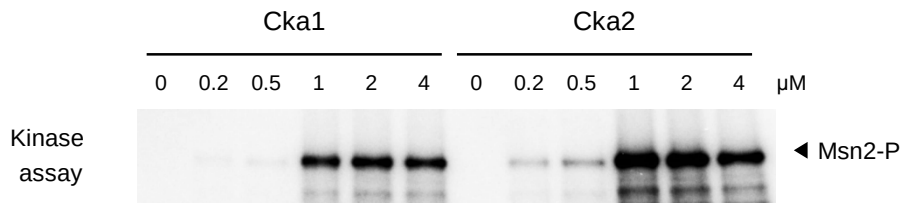
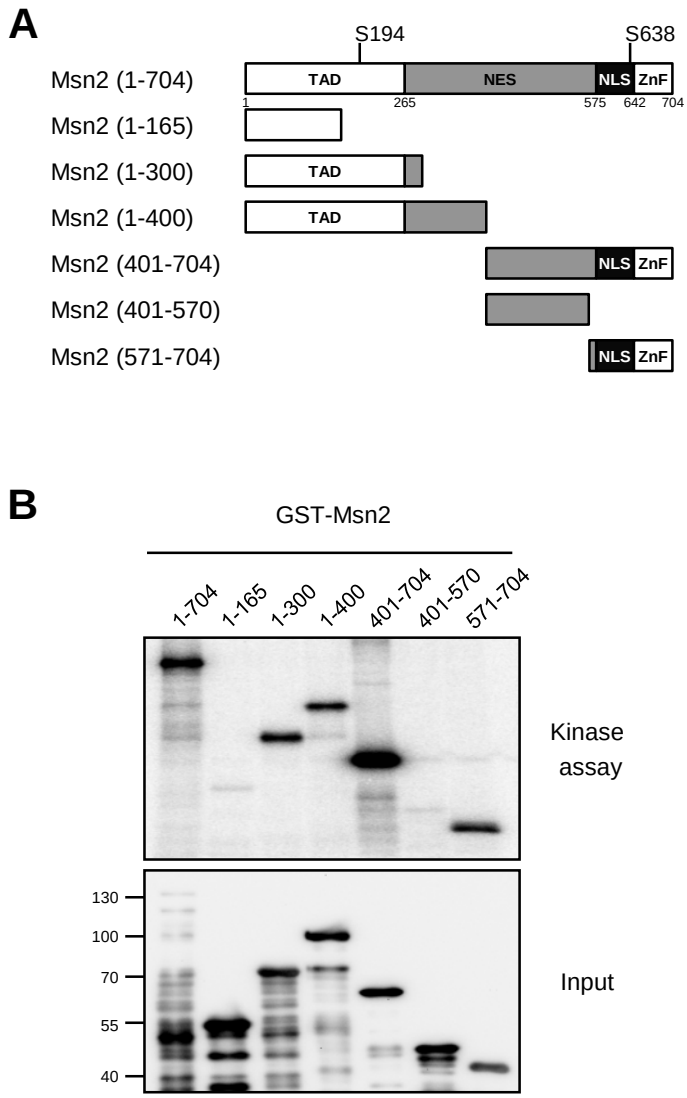


Figure 5.5 Msn2 was strongly phosphorylated by Cka2 more than Cka1 *in vitro*.

GST-Msn2 purified from *E. coli* was phosphorylated by increasing molar concentrations of GST-Cka1 or GST-Cka2 in the presence of [γ-^{32}P] ATP for 1 h.


A
S194
S638
Msn2 (1-704)
TAD
NES
NLS
ZnF
1
265
575
642
704
Msn2 (1-165)
Msn2 (1-300)
TAD
Msn2 (1-400)
TAD
Msn2 (401-704)
NLS
ZnF
Msn2 (401-570)
Msn2 (571-704)
NLS
ZnF
B
GST-Msn2
1-704
1-165
1-300
1-400
401-704
401-570
571-704
Kinase assay
130
100
70
55
40
Input

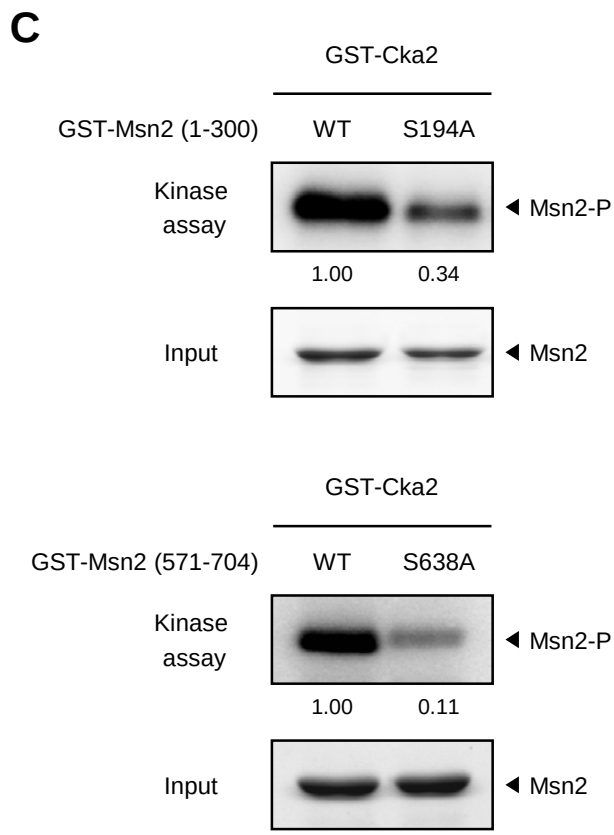


Figure 5.6 CK2 phosphorylates S194 and S638 in Msn2.

A. Schematic representation of full-length and truncated Msn2 derivatives. TAD, transcriptional activation domain; NES, nuclear export signal; NLS, nuclear localization signal; ZnF, zinc finger-binding domain.

B. GST-Msn2 derivatives purified from *E. coli* were phosphorylated by GST-Cka2 *in vitro*. The input proteins were detected by western blotting with antibody against GST.

C. CK2 phosphorylates both S194 and S638 in Msn2 *in vitro*.

5.4. CK2 phosphorylates S194 and S638 in Msn2

Next, we mapped the CK2-dependent phosphorylation sites in Msn2. We generated 6 truncation derivatives of Msn2 (Fig. 5.6A) and CK2-dependent phosphorylation of these mutants were tested *in vitro* (Fig. 5.6B). Truncation mutants containing N terminus and C terminus of Msn2 were strongly phosphorylated by CK2, narrowing down the phosphorylation sites between 1 and 300 amino acid residues and between 571 and 704 amino acid residues of Msn2. In these two regions, we predicted Ser194 and Ser638 residues as putative CK2 phosphorylation sites based on the CK2 consensus sequence (**S**XXE/D). CK2-dependent phosphorylation levels were significantly reduced by 66% in $Msn2^{S194A}$ (1-300) and by 89% in $Msn2^{S638A}$ (571-704) *in vitro* (Fig. 5.6C), demonstrating that both Ser194 and Ser638 are major CK2-dependent phosphorylation sites in Msn2.

5.5. CK2-dependent phosphorylation of S638 is required for Msn2 activation

To assess the role of Ser194 and Ser638 phosphorylation status in the regulation of Msn2 activity, we generated *msn4*Δ strains expressing $MSN2^{WT}$, $MSN2^{S194A}$, $MSN2^{S638A}$, or $MSN2^{S194A,\ S638A}$ from its own promoter and terminator (Fig. 5.7). $Msn2^{S638A}$ strain showed significantly reduced induction levels of *HSP12* and *CTT1* by H_2O_2 (Fig. 5.8B) and *CTT1* by lactic acid (Fig. 5.8C), while showing milder negative effect on *HSP12* induction by glucose starvation and lactic acid (Fig. 5.8A

and C). In agreement with the result showing no negative effect of *CKA1* or *CKA2* deletion on *CTT1* induction upon glucose starvation (Fig. 5.4A), glucose starvation-dependent *CTT1* induction was not affected by expressing $MSN2^{S638A}$ (Fig. 5.8A). On the other hand, $Msn2^{S194A}$ mutation only showed moderate reduction of H_2O_2-dependent induction of *CTT1* (Fig. 5.8B). In addition, $Msn2^{S638A}$ and $Msn2^{S194A, S638A}$ strains exhibited similar induction levels of Msn2 target genes, suggesting that S638 is the major CK2-dependent phosphorylation site positively regulating Msn2 activity in response to stress, with minor contribution of S194. Nevertheless, $Msn2^{S194A, S638A}$ strain showed a moderate effect on impairment of stress-induced Msn2 activation compared with the *cka2*Δ strain (Fig. 5.4A-C and Fig. 5.8A-C), raising the possibility that CK2 might regulate the expression of Msn2 target genes not only by phosphorylating Msn2, but also by regulating other players.

Gis1, a transcription factor responsible for regulating gene expression in response to glucose depletion at the diauxic shift, has functional overlaps with Msn2/4. Msn2/4 and Gis1 are activated upon shared stress conditions such as nutrient starvation, and cooperatively regulate transcription of many common target genes by binding to very similar consensus sequences, stress response element (STRE, AGGGG) and post diauxic shift (PDS, TT/AAGGGAT) element, respectively (21, 72, 73), suggesting the possibility that the effect of loss of function mutations of Msn2 might be masked by Gis1. Therefore, to exclude the potential effect of Gis1, strains expressing $MSN2^{WT}$, $MSN2^{S194A}$, $MSN2^{S638A}$, or

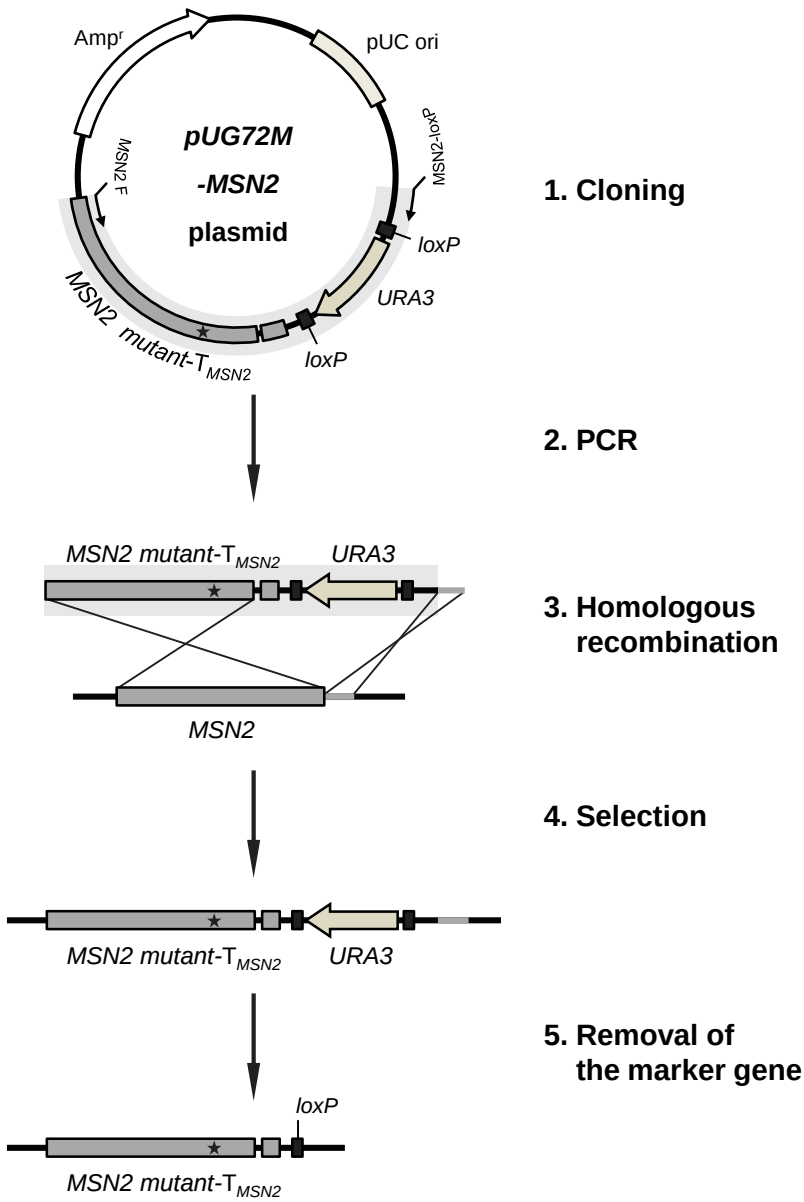


Figure 5.7 Schematic representation of plasmid construction and manipulation processes to generate yeast strain expressing Msn2 mutated at CK2-dependent phosphorylation site.

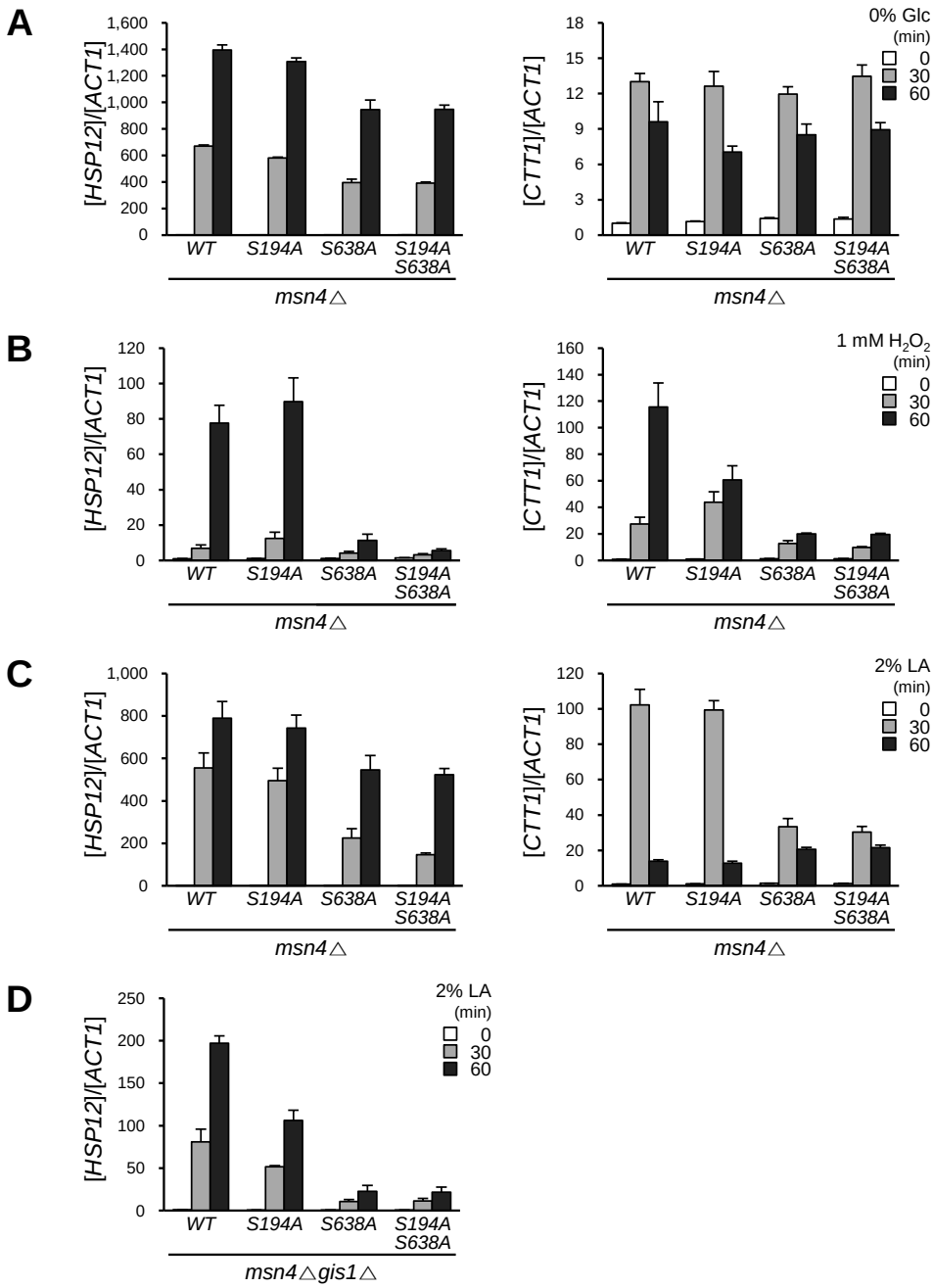

A
[HSP12]/[ACT1]
1,600
1,400
1,200
1,000
800
600
400
200
0
WT
S194A
S638A
S194A S638A
msn4△
[CTT1]/[ACT1]
18
15
12
9
6
3
0
0% Glc
(min)
0
30
60
B
1 mM H2O2
C
2% LA
D
msn4△gis1△

Figure 5.8 Mutations in the CK2-dependent phosphorylation sties affect transcriptional activity of Msn2 in response to various stress conditions.

A.-D. Cells expressing *MSN2*^{WT}, *MSN2*^{S194A}, *MSN2*^{S638A}, or *MSN2*$^{S194A, S638A}$ in *msn4Δ* (A-C) or in *msn4Δgis1Δ* were grown to early exponential phase in YPD medium containing 2% glucose, and then transferred to YP medium lacking glucose (A) or treated with a final concentration of 1 mM H_2O_2 (B) or 2% lactic acid (C, D) for the indicated times. The expression levels of *HSP12* and *CTT1* were measured by qRT-PCR normalized to *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated cells expressing *MSN2*^{WT} (*n*=3).

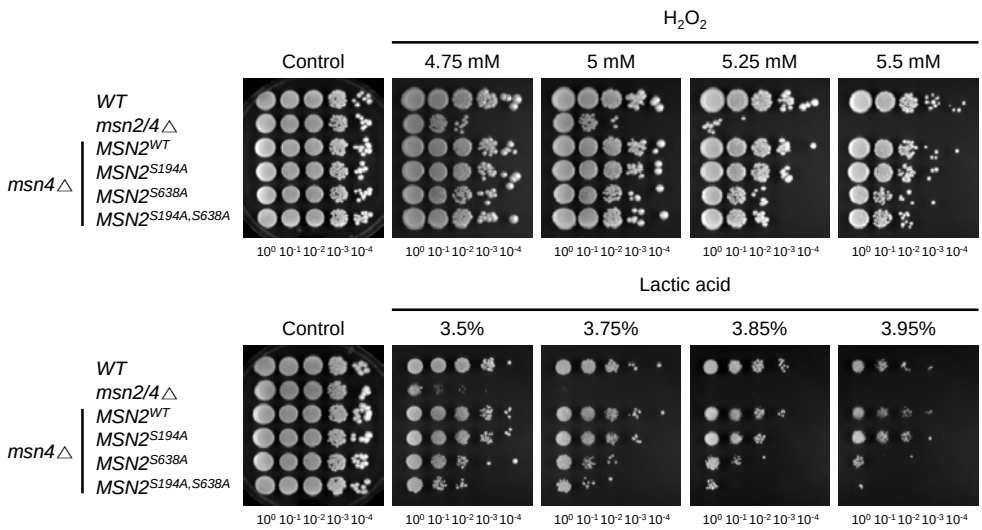


Figure 5.9 Mutation of CK2-dependent phosphorylation sites in Msn2 affects stress tolerance upon hydrogen peroxide and lactic acid.

WT, *msn2/4*Δ, $MSN2^{WT}$, $MSN2^{S194A}$, $MSN2^{S638A}$, and $MSN2^{S194A, S638A}$ cells were tested for growth by spotting serially diluted cultures onto YPD agar plate with/without H_2O_2 or lactic acid.

MSN2$^{S194A,\ S638A}$ were generated in *msn4*Δ*gis1*Δ background. *msn4*Δ*gis1*Δ cells expressing *MSN2*^{WT} exhibited about 4-fold reduction in *HSP12* induction levels at 60 min after lactic acid treatment compared with *msn4*Δ cells expressing *MSN2*^{WT} (Fig. 5.8C and D), supporting the assumption that Gis1 is partially accountable for the induction of *HSP12*. Furthermore, *msn4*Δ*gis1*Δ cells expressing *MSN2*^{S194A}, *MSN2*^{S638A}, and *MSN2*$^{S194A,\ S638A}$ showed a clear defect in the induction of *HSP12* (Fig. 5.8D), demonstrating that Gis1-dependent *HSP12* induction might in part mask the negative effects of the *MSN2* mutants on *HSP12* induction upon lactic acid treatment (Fig. 5.8C).

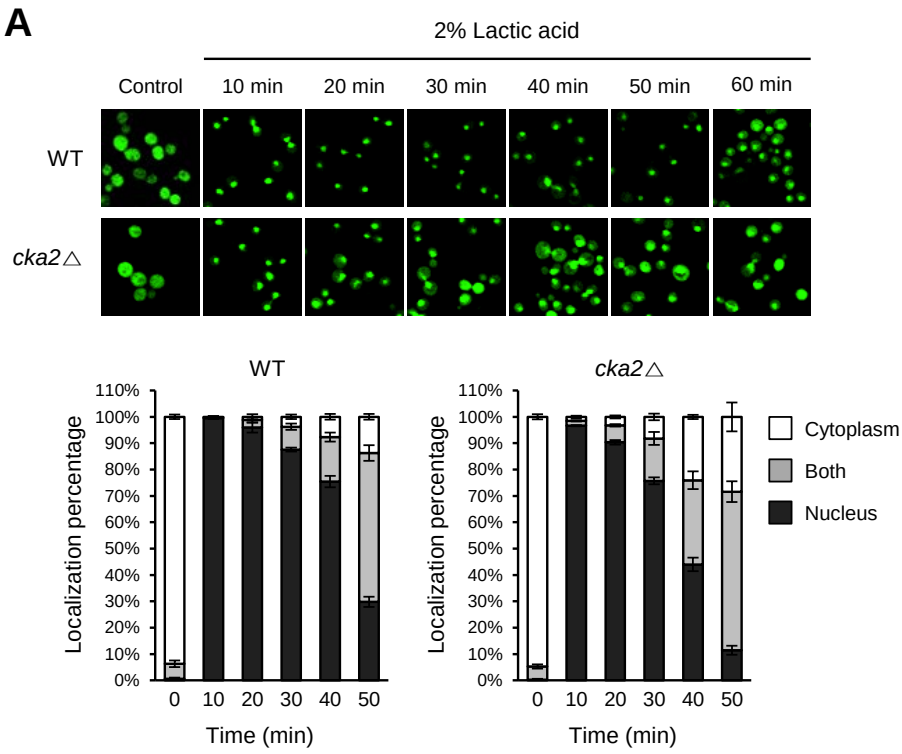
Next, we further investigated whether the phosphorylation status of S194 and S638 can affect cellular stress tolerance. In accordance with the transcript levels of Msn2/4 target genes, cells expressing *MSN2*^{S638A} and *MSN2*$^{S194A,\ S638A}$ showed higher sensitivity to both H_2O_2 and lactic acid, whereas *MSN2*^{S194A} mutant showed minor effect on stress tolerance (Fig. 5.9). Taken together, these results clearly demonstrate that CK2-dependent phosphorylation of S194 and S638 is required for stress-induced Msn2 activation and stress tolerance.

5.6. CK2 regulates nuclear accumulation of Msn2

To understand how the transcriptional activity of Msn2 is regulated by CK2, we examined the stress-induced nuclear accumulation of Msn2, which is one of the main mechanisms of Msn2 regulation. Under normal conditions, Msn2-GFP was

mainly localized to the cytoplasm and rapidly accumulated in the nucleus within 10 min after the treatment of lactic acid in both wild-type and *cka2*Δ cells (Fig. 5.10A). In wild-type cells, Msn2-GFP was primarily retained in the nucleus for up to 40 min, and then exported to the cytoplasm. In *cka2*Δ cells, Msn2-GFP accumulated in the nucleus showed faster redistribution to the cytoplasm than in wild-type cells. 40 min after lactic acid treatment, 75.42% of wild type, but only 44.01% of *cka2*Δ cells showed nuclear localization of Msn2-GFP (Fig. 5.10A). These results imply that CK2 might increase the duration of the stress-induced nuclear localization of Msn2.

We next tested whether the identified CK2-dependent phosphorylation sites in Msn2 are involved in the nuclear localization of Msn2. Although $Msn2^{S194A,\ S638A}$ clearly showed reduced transactivation activity compared with wild type Msn2 upon lactic acid treatment (Fig. 5.8), the kinetics of nuclear import and export in response to lactic acid treatment was almost indistinguishable between the wild-type Msn2 and $Msn2^{S194A,\ S638A}$ (Fig. 5.10B). Furthermore, the same as wild type Msn2, $Msn2^{S194A,\ S638A}$ showed shorter nuclear retention time in *cka2*Δ cells. Therefore, CK2-dependent phosphorylation of S194 and S638 in Msn2 might not regulate the subcellular localization of Msn2, and the retardation of Msn2 export observed in *cka2*Δ might be due to the CK2-dependent regulation of other target proteins yet to be identified.


A
2% Lactic acid
Control
10 min
20 min
30 min
40 min
50 min
60 min
WT
cka2△
WT
cka2△
Localization percentage
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
10
20
30
40
50
Time (min)
Cytoplasm
Both
Nucleus

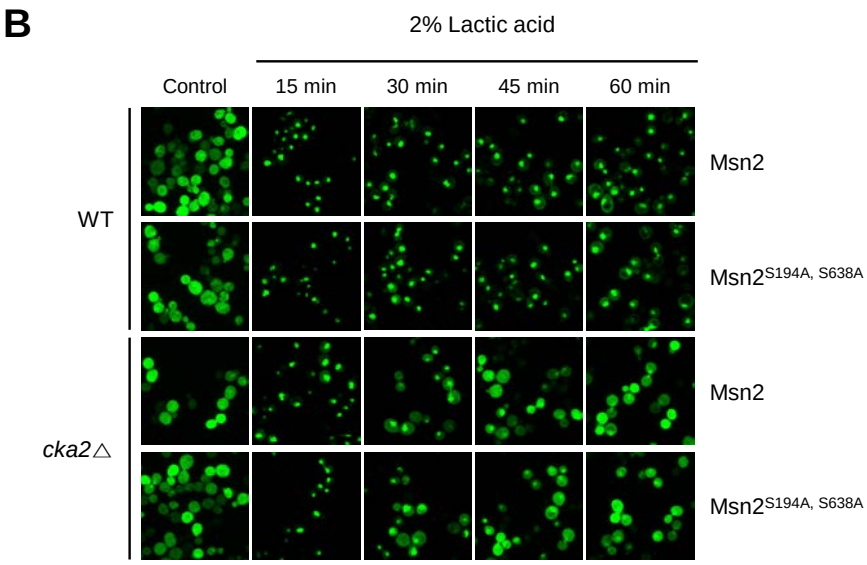

B
2% Lactic acid
Control
15 min
30 min
45 min
60 min
WT
cka2△
Msn2
Msn2S194A, S638A
Msn2
Msn2S194A, S638A

Figure 5.10 Mutation of CK2-dependent phosphorylation sites in Msn2 affects stress tolerance upon hydrogen peroxide and lactic acid.

A. WT and *cka2*Δ cells expressing *MSN2-GFP* were cultured until early exponential phase, and then treated with a final concentration of 2% lactic acid. Fluorescence signals were monitored at indicated time points. The number of individual cells displaying Msn2-GFP in the nuclear, cytoplasm, or both compartments was counted by manual inspection. Each value represents the average percentage ± SD of the number of individual cells (n>200).

B. WT and *cka2*Δ cells expressing *MSN2-GFP* or *MSN2-GFP*$^{S194A,\ S638A}$ were cultured until early exponential phase, and then treated with a final concentration of 2% lactic acid. Fluorescence signals were monitored at the indicated time points.

5.7. Conclusions

In this chapter, it has been shown that Msn2 activity is regulated by CK2-dependent phosphorylation in response to various types of environmental stress. Overexpression of CK2 catalytic subunits *CKA1* and *CKA2* enhances transcription of Msn2/4-dependent genes and cells lacking one or both CK2 genes show diminished Msn2/4-dependent transcription and stress tolerance in response to glucose starvation, H_2O_2, and lactic acid. In addition, Ser 194 and Ser 638 in Msn2 are phosphorylated by Cka2 *in vitro* and replacement of these residues with alanine results in reduction of stress-induced Msn2 activity and tolerance to H_2O_2, and lactic acid.

Chapter 6.

Overall discussion and recommendations

Ethanol-specific regulation of Hsf1 by CK2 and Ppt1

Ethanol has long been known as an inducer of heat shock response, but the underlying mechanisms by which ethanol activates HSF have not yet been investigated. Cellular adaptation to ethanol stress might be important especially for yeast which produces ethanol as a major fermentation product. Although *S. cerevisiae* produces high concentrations of ethanol, its cell growth and viability are inhibited by accumulation of ethanol during the fermentation, and understanding the ethanol tolerance mechanisms is one of the key issues in the brewing and biofuel industries (74, 75).

Ethanol can disorder cellular membranes and induce conformational changes of proteins by substituting the water surrounding biological molecules. Therefore, similar to the situations occurring in heat shocked cells, ethanol-induced accumulation of misfolded proteins could be sensed by Hsp70 and Hsp90 chaperones, which then dissociate from Hsf1, relieving its repression (28). However, the specific requirement of S608 dephosphorylation for ethanol-dependent activation of Hsf1 suggests that heat shock and ethanol might activate Hsf1 via different mechanisms. It has been shown that *Drosophila* HSF1 can be activated by direct sensing of heat and H_2O_2, but not ethanol (76). However, considering the conformation-specific activation of *S. cerevisiae* Hsf1 by ethanol, it is still possible that ethanol can directly interact with Hsf1, inducing its conformational changes leading to the activation. The activator function of CTA and a yeast-specific N-terminal activation domain (NTA) is repressed under

unstressed conditions by intramolecular interactions with the central regulatory domains including the DNA-binding domain (32, 77). Phosphorylation of S608 in the CTA might induce conformational changes of Hsf1 to a form resistant to be activated by ethanol, but not by heat shock, without affecting the basal activity of Hsf1 (Fig. 6.1). Further elucidation of the stress-sensing mechanisms might be necessary to understand how the S608 phosphorylation status regulates Hsf1 sensitivity towards different types of stresses. Phosphorylations by Snf1 and Yak1 are required for Hsf1 activation upon glucose starvation (1, 39), whereas phosphorylation by CK2 inhibits Hsf1 activation upon ethanol stress. Therefore, differential phosphorylation of Hsf1 might play an important role in stress-specific regulation of Hsf1 activity in both positive and negative ways.

Interestingly, the S608 residue in the CTA of Hsf1 is conserved only in the *Saccharomyces* sensu strict yeast species including *S. mikatae*, *S. bayanus*, *S. kudriavzevii*, and *S. paradoxus*, but not in other yeast species. These yeast species are specialized for high concentrations of ethanol production compared with the non-*Saccharomyces* yeast species (78). Therefore, regulation of the ethanol-dependent activation of Hsf1 by CK2 and Ppt1 might have provided some advantages for these high-ethanol producers during the evolution. Since induction of stress response can exert inhibitory effects on glycolysis and ethanol production, sophisticated balance between cell growth and stress response might be essential to ensure high levels of ethanol production while maintaining cell growth. The reciprocal actions of CK2 and Ppt1 might play a role in regulating ethanol

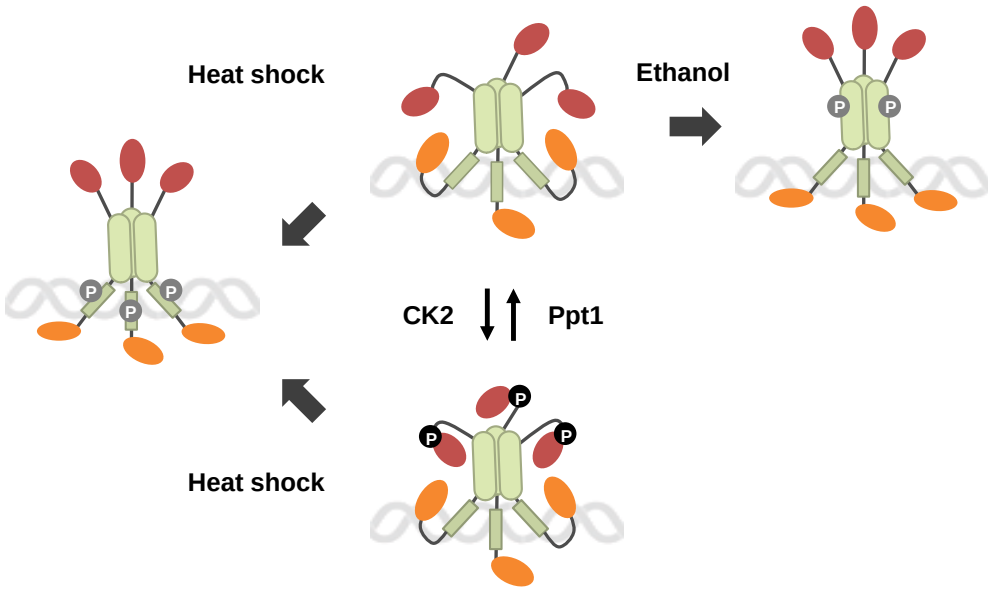


Figure 6.1 Model for the stress-specific regulation of Hsf1 by CK2 and Ppt1.

Phosphorylation of S608 in the CTA by CK2 (black circles) might restrain Hsf1 in a conformation resistant to the ethanol-induced activation, which can be relieved by Ppt1-dependent dephosphorylation. On the other hand, the phosphorylation status of S608 does not affect Hsf1 activation upon heat shock. Additional positive phosphorylation events (grey circles) might occur during the activation of Hsf1 by ethanol and heat shock.

sensitivity of Hsf1 depending on cell growth and environmental conditions. However, it remains unclear whether the activities of CK2 and Ppt1 towards Hsf1 are altered in response to ethanol, resulting in different S608 phosphorylation status depending on ethanol concentrations. Although catalytic subunit of CK2 has been known to be constitutively active, the catalytic activity can be enhanced or attenuated by regulatory subunit through modulating substrate selectivity (59). Since we identified that CK2 interacts with Hsf1 through the regulatory subunits Ckb1 and Ckb2, it would be interesting to investigate whether ethanol can affect the function or stability of the regulatory subunits of CK2. Further elucidation of the genetic and environmental factors regulating the activity balance between CK2 and Ppt1 would provide more clear view of the roles of Hsf1 regulation by this kinase-phosphatase pair.

Regulation of stress-induced Msn2 activation by CK2

Msn2 and Msn4, which are typical transcription factors implicated in bow-tie shaped signaling pathway as a central regulator, mediate transcriptional activation of numerous genes in response to multiple different environmental signals (14). Interestingly, overexpression of Msn2/4 leads to reduced growth rate (20), implying that Msn2/4 activity is strictly regulated in response to both normal and stressful conditions. In this study, we presented compelling evidence that CK2 positively regulates transcriptional activity of Msn2 in response to multiple environmental stress conditions including glucose starvation, H_2O_2, and lactic acid

stress.

We showed that CK2-dependent phosphorylation of S194 and S638, especially S638, in Msn2 are required for stress-induced activation of Msn2 and cell survival in response to severe stress conditions. CK2 is considered constitutively active kinase, and CK2-dependent phosphorylation activates Msn2 upon multiple stress conditions involving different signal transduction pathways. Therefore, unlike Yak1 and Rim15, which phosphorylate Msn2 upon stress conditions inactivating PKA or TORC1, CK2 might constitutively phosphorylate Msn2 in a stress-independent manner. CK2-dependent phosphorylation might make Msn2 competent to activate transcription under various stress conditions possibly by regulating interaction with transcriptional machinery and other regulatory proteins. However, it is not clear yet how the CK2-dependent phosphorylation of S194 and S638 activates transcriptional activity of Msn2. Although we showed that CK2 promotes stress-induced nuclear retention of Msn2 (Fig. 6.2), it seems to be independent of the two phosphorylation sites identified in this study. We cannot rule out the possibility that yet unidentified CK2-dependent phosphorylation sites in Msn2 might be involved in the regulation of Msn2 translocation between the nucleus and cytoplasm, but it is also plausible that CK2 might regulate nuclear retention of Msn2 by phosphorylating other components.

Msn2 is known to be secluded in the cytoplasm by physical interaction with Bmh2, a 14-3-3 protein (26). The 14-3-3 family proteins are highly conserved dimeric proteins participating in a wide range of cellular processes through binding

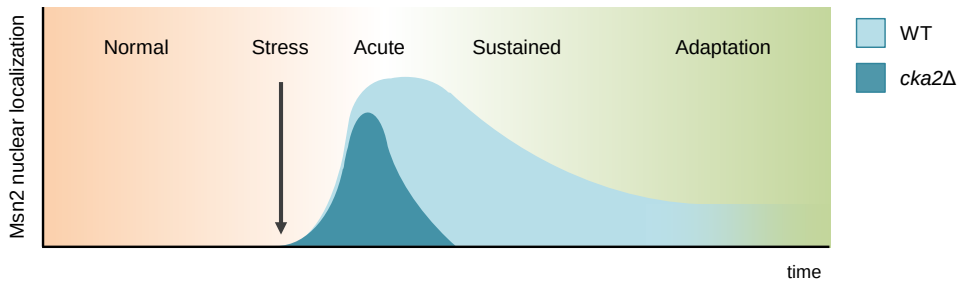


Figure 6.2 CK2 promotes stress-induced nuclear retention of Msn2.

Schematic representation of CK2-dependent stress-induced nuclear retention of Msn2

to hundreds of proteins containing the phosphorylated 14-3-3 binding motif (79). 14-3-3 proteins are also phosphorylated at multiple residues, inhibiting the interaction with their partner proteins (79, 80). Particularly, it has been purposed that phosphorylation of 14-3-3 protein by CK2 may disrupt interaction with their partner proteins in mammals (81), raising the possibility that CK2 might repress the Bmh2-mediated cytoplasmic sequestration of Msn2 through Bmh2 phosphorylation. In addition, nuclear export of Msn2 is mediated by Msn5, a yeast karyopherin (18). Disruption of *MSN5* leads to permanent nuclear accumulation of Msn2, even under normal condition, but could not elicit induction of its target genes (22), implying that another regulatory steps, such as phosphorylation, are require for Msn2 activation apart from its nuclear accumulation. Taken together, these results support the notion that CK2 synergistically regulates transcriptional activation of Msn2-dependent genes through phosphorylation of not only Msn2 but also other factors regulating accumulation of Msn2 in the nucleus. In fact, disruption of a catalytic subunit *CKA2*, even with a partial CK2 activity by intact *CKA1*, more successfully reduced transcriptional activity of Msn2/4, compared with the mutation of the CK2-dependent phosphorylation sites in Msn2 (Fig. 5.4 and 5.8). In addition, CK2 might affect transcription of Msn2 targets by regulating other players. For example, it has been shown that CK2 regulates transcriptional elongation and gene expression of a number of heat-shock induced genes through tyrosine phosphorylation of histone H2A (82).

Although CK2 phosphorylated Msn4 as well as Msn2 (Fig. 5.1), the two CK2-

dependent phosphorylation sites in Msn2 are not conserved in Msn4. Thus, Msn2 and Msn4 might be differently regulated by CK2-dependent phosphorylation. Msn2 and Msn4, which have 41% identity and similarity in size and amino acid composition, are often considered as functionally redundant transcription factors (6, 13, 15). However, Msn2 and Msn4 are dissimilar in regulating a subset of target genes, and show different expression and phosphorylation patterns in response to stressful conditions (9, 23). PKA-dependent phosphorylation sites are partially conserved between Msn2 and Msn4, and only Msn2, but not Msn4, has PKA-dependent oscillatory behavior, shuttling repeatedly between the nucleus and cytoplasm (83).

In summary, our study has revealed that CK2 positively regulates Msn2 activity in a phosphorylation-dependent manner in response to glucose starvation, H_2O_2, and lactic acid. These findings shed light on the crucial role for CK2 as a central regulator administering not only growth-related but also stress-related responses to ensure proper cellular adaptation and survival in response to environmental fluctuations. In addition, we newly demonstrated that lactic acid induces nuclear accumulation and activation of Msn2, and its CK2-dependent activation is essential for cell survival in response to lactic acid. These findings might provide information to develop lactic acid-tolerant yeast strain for industrial applications.

Bibliography

1. Lee P, Cho BR, Joo HS, & Hahn JS (2008) Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. *Molecular microbiology* 70(4):882-895.
2. Miller EA, *et al.* (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* 114(4):497-509.
3. Vojtek AB, Hollenberg SM, & Cooper JA (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74(1):205-214.
4. Mumberg D, Muller R, & Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156(1):119-122.
5. Sung MK, *et al.* (2013) Genome-wide bimolecular fluorescence complementation analysis of SUMO interactome in yeast. *Genome Res* 23(4):736-746.
6. Gasch AP (2003) The environmental stress response: a common yeast response to diverse environmental stresses. *Yeast Stress Responses*, eds Hohmann S & Mager WH (Springer Berlin Heidelberg, Berlin, Heidelberg), pp 11-70.
7. Hohmann S & Mager WH (2007) Yeast stress responses. (Springer Science & Business Media).
8. Causton HC, *et al.* (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* 12(2):323-337.
9. Gasch AP, *et al.* (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* 11(12):4241-4257.
10. Chen D, *et al.* (2003) Global transcriptional responses of fission yeast to environmental stress. *Mol Biol Cell* 14(1):214-229.

11. Lopez-Maury L, Marguerat S, & Bahler J (2008) Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. *Nat Rev Genet* 9(8):583-593.
12. Gasch AP & Werner-Washburne M (2002) The genomics of yeast responses to environmental stress and starvation. *Funct Integr Genomics* 2(4-5):181-192.
13. Martinez-Pastor MT, *et al.* (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J* 15(9):2227-2235.
14. Hansen AS & O'Shea EK (2016) Encoding four gene expression programs in the activation dynamics of a single transcription factor. *Curr Biol* 26(7):R269-271.
15. Estruch F & Carlson M (1993) Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol Cell Biol* 13(7):3872-3881.
16. Boy-Marcotte E, *et al.* (2006) The transcriptional activation region of Msn2p, in Saccharomyces cerevisiae, is regulated by stress but is insensitive to the cAMP signalling pathway. *Mol Genet Genomics* 275(3):277-287.
17. Garmendia-Torres C, Goldbeter A, & Jacquet M (2007) Nucleocytoplasmic oscillations of the yeast transcription factor Msn2: evidence for periodic PKA activation. *Curr Biol* 17(12):1044-1049.
18. Gorner W, *et al.* (2002) Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *EMBO J* 21(1-2):135-144.
19. Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H, & Jacquet M (1998) Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in Saccharomyces cerevisiae. *J Bacteriol* 180(5):1044-1052.

20. Smith A, Ward MP, & Garrett S (1998) Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. *EMBO J* 17(13):3556-3564.
21. Gorner W, *et al.* (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev* 12(4):586-597.
22. Durchschlag E, Reiter W, Ammerer G, & Schuller C (2004) Nuclear localization destabilizes the stress-regulated transcription factor Msn2. *J Biol Chem* 279(53):55425-55432.
23. Garreau H, *et al.* (2000) Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*. *Microbiology* 146 (Pt 9):2113-2120.
24. 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(23):4115-4123.
25. Lee P, Paik SM, Shin CS, Huh WK, & Hahn JS (2011) Regulation of yeast Yak1 kinase by PKA and autophosphorylation-dependent 14-3-3 binding. *Mol Microbiol* 79(3):633-646.
26. Beck T & Hall MN (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* 402(6762):689-692.
27. Santhanam A, Hartley A, Duvel K, Broach JR, & Garrett S (2004) PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. *Eukaryot Cell* 3(5):1261-1271.
28. Verghese J, Abrams J, Wang Y, & Morano KA (2012) Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiol Mol Biol Rev* 76(2):115-158.

29. Akerfelt M, Morimoto RI, & Sistonen L (2010) Heat shock factors: integrators of cell stress, development and lifespan. *Nature reviews. Molecular cell biology* 11(8):545-555.
30. Anckar J & Sistonen L (2011) Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual review of biochemistry* 80:1089-1115.
31. Sorger PK (1990) Yeast heat shock factor contains separable transient and sustained response transcriptional activators. *Cell* 62(4):793-805.
32. Jakobsen BK & Pelham HR (1991) A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO J* 10(2):369-375.
33. Zorzi E & Bonvini P (2011) Inducible hsp70 in the regulation of cancer cell survival: analysis of chaperone induction, expression and activity. *Cancers (Basel)* 3(4):3921-3956.
34. Guettouche T, Boellmann F, Lane WS, & Voellmy R (2005) Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem* 6:4.
35. Kline MP & Morimoto RI (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Molecular and cellular biology* 17(4):2107-2115.
36. Chu B, Zhong R, Soncin F, Stevenson MA, & Calderwood SK (1998) Transcriptional Activity of Heat Shock Factor 1 at 37 oC Is Repressed through Phosphorylation on Two Distinct Serine Residues by Glycogen Synthase Kinase 3α and Protein Kinases Cα and Cζ. *Journal of Biological Chemistry* 273(29):18640-18646.
37. Holmberg CI, *et al.* (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *EMBO J* 20(14):3800-3810.
38. Tamai KT, Liu X, Silar P, Sosinowski T, & Thiele DJ (1994) Heat shock transcription factor activates yeast metallothionein gene expression in

response to heat and glucose starvation via distinct signalling pathways. *Mol Cell Biol* 14(12):8155-8165.

39. Hahn JS & Thiele DJ (2004) Activation of the Saccharomyces cerevisiae heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. *J Biol Chem* 279(7):5169-5176.
40. Ferguson SB, *et al.* (2005) Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in Saccharomyces cerevisiae. *Genetics* 169(3):1203-1214.
41. Lee P, *et al.* (2013) Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*. *FEBS Lett* 587(22):3648-3655.
42. Conde R, Xavier J, McLoughlin C, Chinkers M, & Ovsenek N (2005) Protein phosphatase 5 is a negative modulator of heat shock factor 1. *The Journal of biological chemistry* 280(32):28989-28996.
43. Hoj A & Jakobsen BK (1994) A short element required for turning off heat shock transcription factor: evidence that phosphorylation enhances deactivation. *The EMBO journal* 13(11):2617-2624.
44. Wandinger SK, Suhre MH, Wegele H, & Buchner J (2006) The phosphatase Ppt1 is a dedicated regulator of the molecular chaperone Hsp90. *EMBO J* 25(2):367-376.
45. Schreiber TB, *et al.* (2012) Global analysis of phosphoproteome regulation by the Ser/Thr phosphatase Ppt1 in Saccharomyces cerevisiae. *J Proteome Res* 11(4):2397-2408.
46. Noguchi C, Watanabe D, Zhou Y, Akao T, & Shimoi H (2012) Association of constitutive hyperphosphorylation of Hsf1p with a defective ethanol stress response in Saccharomyces cerevisiae sake yeast strains. *Appl Environ Microbiol* 78(2):385-392.

47. Kitada K, Yamaguchi E, & Arisawa M (1995) Cloning of the Candida glabrata TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation. *Gene* 165(2):203-206.
48. Gasch AP (2002) Yeast genomic expression studies using DNA microarrays. *Methods in enzymology* 350:393-414.
49. Gidalevitz T, Prahlad V, & Morimoto RI (2011) The stress of protein misfolding: from single cells to multicellular organisms. *Cold Spring Harbor perspectives in biology* 3(6).
50. Hahn JS, Hu Z, Thiele DJ, & Iyer VR (2004) Genome-wide analysis of the biology of stress responses through heat shock transcription factor. *Molecular and cellular biology* 24(12):5249-5256.
51. Broach JR (2012) Nutritional control of growth and development in yeast. *Genetics* 192(1):73-105.
52. Yang J, *et al.* (2005) Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. *The EMBO journal* 24(1):1-10.
53. Zou J, Guo Y, Guettouche T, Smith DF, & Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94(4):471-480.
54. Pusch S, Dissmeyer N, & Schnittger A (2011) Bimolecular-fluorescence complementation assay to monitor kinase-substrate interactions in vivo. *Methods Mol Biol* 779:245-257.
55. Duina AA, Kalton HM, & Gaber RF (1998) Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. *The Journal of biological chemistry* 273(30):18974-18978.
56. Vaughan CK, *et al.* (2008) Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37. *Molecular cell* 31(6):886-895.
57. Chinkers M (2001) Protein phosphatase 5 in signal transduction. *Trends in endocrinology and metabolism: TEM* 12(1):28-32.

58. Soroka J, *et al.* (2012) Conformational switching of the molecular chaperone Hsp90 via regulated phosphorylation. *Molecular cell* 45(4):517-528.
59. Litchfield DW (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* 369(Pt 1):1-15.
60. Schuller C, Brewster JL, Alexander MR, Gustin MC, & Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. *EMBO J* 13(18):4382-4389.
61. Alepuz PM, de Nadal E, Zapater M, Ammerer G, & Posas F (2003) Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. *EMBO J* 22(10):2433-2442.
62. Reiter W, *et al.* (2013) Yeast protein phosphatase 2A-Cdc55 regulates the transcriptional response to hyperosmolarity stress by regulating Msn2 and Msn4 chromatin recruitment. *Mol Cell Biol* 33(5):1057-1072.
63. Cho BR, Lee P, & Hahn JS (2014) CK2-dependent inhibitory phosphorylation is relieved by Ppt1 phosphatase for the ethanol stress-specific activation of Hsf1 in *Saccharomyces cerevisiae*. *Mol Microbiol* 93(2):306-316.
64. Ahmed K, Gerber DA, & Cochet C (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* 12(5):226-230.
65. Kim MS & Hahn JS (2016) Role of CK2-dependent phosphorylation of Ifh1 and Crf1 in transcriptional regulation of ribosomal protein genes in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1859(8):1004-1013.
66. Watabe M & Nakaki T (2011) Protein kinase CK2 regulates the formation and clearance of aggresomes in response to stress. *J Cell Sci* 124(Pt 9):1519-1532.
67. Kanki T, *et al.* (2013) Casein kinase 2 is essential for mitophagy. *EMBO Rep* 14(9):788-794.

68. Burns LT & Wente SR (2014) Casein kinase II regulation of the Hot1 transcription factor promotes stochastic gene expression. *J Biol Chem* 289(25):17668-17679.
69. Montenarh M (2016) Protein kinase CK2 in DNA damage and repair. *Translational Cancer Research* 5(1):49-63.
70. Zhang C, Vilk G, Canton DA, & Litchfield DW (2002) Phosphorylation regulates the stability of the regulatory CK2beta subunit. *Oncogene* 21(23):3754-3764.
71. Sanchez-Casalongue ME, *et al.* (2015) Differential phosphorylation of a regulatory subunit of protein kinase CK2 by target of rapamycin complex 1 signaling and the Cdc-like kinase Kns1. *J Biol Chem* 290(11):7221-7233.
72. Roosen J, *et al.* (2005) PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. *Mol Microbiol* 55(3):862-880.
73. 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(11):2569-2579.
74. Ma M & Liu ZL (2010) Mechanisms of ethanol tolerance in Saccharomyces cerevisiae. *Applied microbiology and biotechnology* 87(3):829-845.
75. Zhao XQ & Bai FW (2009) Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. *Journal of biotechnology* 144(1):23-30.
76. Zhong M, Orosz A, & Wu C (1998) Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. *Molecular cell* 2(1):101-108.
77. Chen T & Parker CS (2002) Dynamic association of transcriptional activation domains and regulatory regions in Saccharomyces cerevisiae heat shock factor. *Proceedings of the National Academy of Sciences of the United States of America* 99(3):1200-1205.

78. Sicard D & Legras JL (2011) Bread, beer and wine: yeast domestication in the Saccharomyces sensu stricto complex. *Comptes rendus biologies* 334(3):229-236.
79. Aitken A (2011) Post-translational modification of 14-3-3 isoforms and regulation of cellular function. *Semin Cell Dev Biol* 22(7):673-680.
80. Paul AL, Denison FC, Schultz ER, Zupanska AK, & Ferl RJ (2012) 14-3-3 phosphoprotein interaction networks - does isoform diversity present functional interaction specification? *Front Plant Sci* 3:190.
81. Sachs NA & Vaillancourt RR (2004) Cyclin-dependent kinase 11p110 and casein kinase 2 (CK2) inhibit the interaction between tyrosine hydroxylase and 14-3-3. *J Neurochem* 88(1):51-62.
82. Basnet H, *et al.* (2014) Tyrosine phosphorylation of histone H2A by CK2 regulates transcriptional elongation. *Nature* 516(7530):267-271.
83. Jacquet M, Renault G, Lallet S, De Mey J, & Goldbeter A (2003) Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*. *J Cell Biol* 161(3):497-505.

Abstract in Korean

국 문 초 록

Saccharomyces cerevisiaes 는 연구가 활발히 진행된 진핵 모델 시스템으로서, 다른 미생물들과 비교하여 다양한 스트레스에 강한 저항성을 나타내어 연료 및 화학 물질 생산을 위한 미생물 세포 공장으로써의 높은 가능성을 지닙니다. 본 연구에서는 효모의 스트레스 전사 조절인자들 중 고온 충격과 다양한 스트레스에 의해 활성화되는 Hsf1 과 Msn2/4 가 CK2 인산화 효소에 의한 인산화를 통해 활성이 조절됨을 규명하였으며 이러한 조절 메커니즘을 이용하여 다양한 알코올 생산 균주로서 그 가능성을 확인하였습니다.

첫 번째로, *Saccharomyces cerevisiae* 의 주요 발효 산물인 에탄올은 heat shock response 를 일으키는 유도인자로 오래전부터 알려져 왔으나 에탄올이 스트레스 전사 조절인자 HSF 를 활성화하는 조절기작은 잘 알려져 있지 않았습니다. 따라서 본 연구는 CK2 에 의한 Hsf1 608 번 세린의 인산화는 Hsf1 의 활성을 에탄올 스트레스에 특이적으로 저해함을 증명하였습니다. 그러나, CK2 에 의한 Hsf1 인산화는 Hsf1 기저 활성과 고온충격에 의한 Hsf1 활성화에는 영향을 주지 않음을 확인하였습니다. 이러한 Hsf1 활성 저해는 Ppt1 의 tetratricopeptide repeat (TPR) 도메인과 Hsf1 의 직접적인 결합을 통한 Hsf1 탈인산화에 의해 완화되었습니다. PPT1 결손과 CK2 과발현은 에탄올 스트레스에 반응하여 Hsf1 활성화에 상승 저해 효과를 나타내었으며 반면에 Hsf1 S608A 돌연변이는 강화된 Hsf1 활성을 나타내었습니다. 따라서 CK2 와 Ppt1 의 상호작용에 의한 Hsf1 608 번 세린의 인산화 상태 조절은 에탄올

스트레스에 반응하는 Hsf1 활성 민감도를 결정하는 데 중요한 역할을 할 것입니다.

두 번째로, CK2 에 의한 인산화에 의해 조절되는 Hsf1 활성 조절 메커니즘을 이용하여 다양한 알코올에 저항성을 갖는 알코올 생산용 균주를 제작하였습니다. CK 에 의해 인산화되는 Hsf1 S608 를 alanine 으로 치환하여 Hsf1 전사 활성을 강화시킨 균주를 제작하였으며 Hsf1 S608A 균주가 높은 농도의 에탄올뿐만 아니라 2-Penylethanol 과 2,3-butandiol 과 같은 다양한 알코올에 저항성을 갖는 것을 확인하였으며 다양한 알코올 생산용 균주로서 가능성을 확인하였습니다.

세 번째로, 스트레스 전사 조절인자 Msn2/4 는 고온, 삼투압, pH, 산화적 스트레스 등 다양한 스트레스에 의해 활성화되어 스트레스 저항성에 관여하는 유전자의 발현을 유도하는 전사 조절인자로서 스트레스 상황에서 효모의 생존에 매우 중요합니다. 그러나 다양한 스트레스 상황에서 Msn2/4 전사 활성이 어떻게 조절되는지 잘 알려져 있지 않았습니다. 따라서, 본 연구에서 Msn2/4 를 CK2 인산화 효소가 포도당 고갈, 산화 스트레스 및 lactic acid 에 반응하여 인산화를 통해 전사 활성을 강화함을 규명하였습니다. CK2 인산화 효소의 과발현에 의한 Msn2/4 전사활성 증가와 CK2 인산화 효소 결손에 따른 Msn2/4 전사활성 감소를 qRT-PCR 을 통해 확인하였습니다. 효모에서 발현/정제한 CK2 에 의한 Msn2/4 의 직접적인 인산화와 Msn2 194 번 세린과 638 번 세린의 인산화 를 *in vitro* kinase assay 를 통해 확인하였습니다. 또한, CK2 에 의해 인산화되는 Msn2 잔기의 돌연변이를 통해 포도당 고갈, 산화 스트레스 및 lactic acid 에 대한 Msn2 전사활성과 스트레스 저항성이 감소함을 qRT-PCR 과 stress sensitivity assay 를 통해

확인하였습니다. 스트레스에 의해 유도되는 핵으로의 Msn2 응집 시간이 CK2 에 의해 강화됨을 확인하였으나, 이것은 Msn2 194 번 세린과 638 번 세린의 직접적인 인산화에 의한 효과가 아님을 확인하였습니다. 종합적으로 다양한 스트레스에 의해 유도되는 Msn2 의 전사활성에 CK2 에 의한 인산화가 중요한 조절작용임을 확인하였습니다. 또한, CK2 에 의한 Msn2 의 전사활성화가 스트레스 저항성에 중요함을 확인함으로써 Msn2/4 의 전사 활성을 강화하여 다양한 스트레스에 대해 복합적인 저항성을 갖는 산업용 효모 균주를 개발하는 연구의 가능성을 확인하였습니다.

주 요 어 : CK2 kinase, Ppt1 phosphatase, Hsf1, Msn2, Msn4, 인산화, 스트레스 전사인자, 스트레스 저항성, *Saccharomyces cerevisiae*

학 번 : 2007-21278