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# Regulation of weak acid stress responses and ribosomal protein gene expression in *Saccharomyces cerevisiae*

***Saccharomyces cerevisiae*에서 약산 스트레스 반응과 리보좀 단백질 유전자의 발현 조절**

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# Regulation of weak acid stress responses and ribosomal protein gene expression in *Saccharomyces cerevisiae*

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## ABSTRACT

# Regulation of weak acid stress responses and ribosomal protein gene expression in *Saccharomyces cerevisiae*

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*Saccharomyces cerevisiae* is widely used in molecular and cell biology since it is one of the simplest eukaryotic single cell. Due to its easiness in manipulation and fast growth, *S. cerevisiae* has served as a model system for fundamental cellular processes for all eukaryotes. In nutrient rich conditions, yeast cells rapidly grow and proliferate. To maintain robust growth, yeast cells generate numerous ribosomes to synthesize proteins in need. In response to stresses, cells rapidly

adjust global gene expressions to adapt to stresses. For rapid growth and stress responses, cells activate transcription factors to regulate gene expressions. In this dissertation, regulatory mechanisms of transcription factors Haa1/War1 for weak organic acid stresses and Ifh1/Crf1 for the expression of ribosomal protein genes were studied.

Firstly, *S. cerevisiae* is known to activate transcription factors such as Haa1 and War1 for cellular adaption against weak acids. Haa1 plays important roles against less lipophilic acids such as acetic acid and lactic acid, whereas War1 exert protection against more lipophilic acids such as propionic acid, sorbic acid, and benzoic acid. However, it has been unknown how these transcription factor are activated in response to weak acid stresses. Using field-effect transistor (FET) type biosensor based on carbon nanofibers, it has been revealed that Haa1 and War1 directly bind to weak acid anions with varying affinities. In agreement with previous studies, Haa1 binds acetate most strongly followed by lactate, whereas War1 binds benzoate most strongly followed by sorbate. Upon activation of Haa1 by direct binding with acetate, Haa1 binds to DNA of target gene promoters through the N-terminal Zn-binding domain of Haa1. Acetate is shown to bind N-terminal 150 amino acid region of Haa1, however, further C-terminal region of Haa1 is required for acetic acid induced transcriptional

activation of its target genes. Therefore, it is proposed that conformation changes caused by direct binding of acetate may activate Haa1, being capable of DNA binding and transcriptional activation.

Secondly, transcription factors Ifh1 and Crf1 are involved in the regulation of ribosomal protein (RP) genes through interaction with a forkhead-associated (FHA) domain containing transcription regulator Fhl1. The FHA domain of Fhl1 interacts with FHB domains of RP gene co-activator Ifh1 or co-repressor Crf1. Ifh1$^{T681}$ and Crf1$^{T348}$, which are resides in the FHB domain, is phosphorylated by CK2 kinase. These phosphorylations play very critical roles for interaction with Fhl1. Cells expressing Ifh1$^{T681A}$ mutant showed reduced phosphorylation by CK2 followed by substantially decreased interaction with Fhl1. Decreased interaction resulted in defects in association of Ifh1 at the RP gene promoters and finally reduced RP gene transcriptional activation, thereby resulting in slow growth rates. On the contrary, cells expressing Crf1$^{T348A}$ failed to repress RP gene transcription upon inhibition of target of rapamycin complex 1 (TORC1) by rapamycin treatment. Taken together, these results propose that CK2-dependent phosphorylation of transcription factors, Ifh1 and Crf1, regulates recruitment transcription factors at the RP gene promoters, which

leads to transcription of RP genes.

**Keywords:** Haa1, War1, CNF-FET, weak organic acid,
Fhl1, Ifh1, Crf1, ribosomal protein,
Transcription factor, *Saccharomyces cerevisiae*

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# CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-AT | 3-amino-1,2,4-triazole |
| ABC | ATP binding cassette |
| ACRE | acetic acid-responsive element |
| BCS | bathocuproinedisulfonic acid |
| cAMP | cyclic adenosine monophosphate |
| CK2 | casein kinase 2 |
| CNF-FET | carbon nanofiber field effective transistor |
| CTA | C-terminal activation domain |
| DBD | DNA binding domain |
| EDTA | ehylenediaminetetraacetic acid |
| EGFP | enhanced green fluorescent protein |
| ESR | environmental stress response |
| FHA | forkhead-associated |
| FRET | florescence energy element |
| 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 |
| I-C | current-voltage |
| LA | lactic acid |
| LB | lysogenic broth |
| Leu | leucine |
| LSU | large subunit |
| MDR | multidrug resistance |
| PBS | phosphate-buffered saline |
| PDRE | pleiotropic drug response element |
| PCR | polymerase chain reaction |
| PIC | protease inhibitor cocktail |
| PMSF | phenylmethylsulfonyl fluoride |
| OD | optical density |
| PKA | protein kinase A |
| RP | ribosomal protein |
| RPL | large ribosomal protein |
| RPS | small ribosomal protein |

| | |
|---|---|
| qRT-PCR | quantitative real time polymerase chain reaction |
| RT-PCR | reverse transcription polymerase chain reaction |
| SC | synthetic complete |
| SDS | sodium dodecyl sulfate |
| SSU | small subunit |
| STRE | stress response element |
| TBB | 4, 5, 6, 7-tetrabromobenzotriazole |
| TEF | translation elongation factor |
| TPEN | N, N, N', N'-tetrakis (2-pyridinylmethyl)-1, 2-ethanediamine |
| TORC | target of rapamycin complex |
| Ura | uracil |
| WB | western blotting |
| YPD | yeast extract-peptone-dextrose |
| ZBD | zinc binding domain |
| ZnF | zinc finger |

# Chapter 1.

# Research background and objective

This dissertation focuses on the elucidation of the underlying regulatory mechanisms how *S. cerevisiae* regulates transcription factors in response to nutrient availability and stresses. Cells are exposed to constant challenges due to rapidly changing environments. Cells need to alter their global gene expressions to survive through such insults including nutrient starvation, oxidative stresses, weak acid stresses and pH changes. In *S. cerevisiae,* general stress responses are mainly regulated by transcription factors such as Msn2/4 and Hsf1. These transcription factors are major regulators of the general stress response (GSR). Activation of Msn2/4 is essential for survival and mainly activated by phosphorylation. However, transcription factors directly involved in weak organic acid responses including Haa1 and War1, are recently identified, but regulatory mechanism for their activation remain elusive. Haa1 and War1 are known to be phosphorylated upon weak acid stresses, but mutations on phosphorylation sites failed to show any relevance to their activating signaling pathways.

On the contrary, cells need to generate ribosomal proteins for cell growth and proliferation. Transcription factors such as Ifh1 and Sfp1 are involved in the transcriptional activation of ribosomal protein

genes. Expression of ribosomal proteins are mainly regulated by phosphorylation of many transcription factor and regulators by master kinases including TORC1 and PKA. Inhibition of TORC1 by starvation or rapamycin dramatically reduce ribosomal protein gene expression and hyper-activation of PKA can rescue repression of ribosomal protein gene expression. Furthermore, CK2, which is a highly conserved protein kinase involved in a large number of cellular processes, is proposed to play important roles for ribosomal protein gene expression by phosphorylating many transcription factors, chromatin remodeler such as Gcn5 and Sir2, and all three RNA polymerases.

This dissertation identifies activating regulatory mechanisms of transcription factors for both weak acid responses and ribosomal protein gene expression.

# Chapter 2.

# Literature review

## 2.1. Stress responses in *S. cerevisiae*

During growth of microbial cells, cells are constantly under the toxic environmental insults. It is important how efficient cells respond to potentially lethal stresses. Of many possible stresses cells may encounter, resistance to carboxylic weak acids including lactic, acetic, propionic, sorbic, and benzoic acids is particularly important. Carboxylic weak acids containing food and beverage preservatives are regularly used to prevent microbial cell growth (1-3). In contrast, the growth inhibition caused by weak acids becomes one of the major growth-limiting factors of efficient microbial fermentation. It is well known that weak acids produced during fermentation are the major factors for slow growth and cell arrests (4). For example, lignocellulosic hydrolysates contain acetic acid and other weak acids that can prevent efficient utilization of cellular biomass (5-7). Furthermore, if production of weak acids such as lactic acid is desired, it is improbable that high-levels of lactic acid production can be achieved due to toxicity of lactic acids (8). Nevertheless, *S. cerevisiae* remains to be one of the very important and promising model organisms to produce invaluable chemicals (9), since *S. cerevisiae* is generally considered more

tolerant and adaptable to toxic effects of chemicals produced than other model organisms (10). Therefore, understanding of molecular mechanisms how *S. cerevisiae* develop resistance and adaptation to weak acids is important.

### 2.1.1. An overview of adaptive response to weak acids

Hydrophobicity and p$K_a$ of carboxylic acids are key factors to determine how potent weak acids would be. When pH is below the p$K_a$ value of weak acid, undissociated form of acids predominates. Undissociated lipophilic acids such as sorbic acid and benzoic acid easily penetrated across the cell membrane by simple diffusion and become dissociated inside the cell (11). Furthermore, acetic acid is known to be transported into the cell by facilitated diffusion that is mediated by the aquaglyceroporin Fps1 (12,13). When high concentration of acetic acid is treated, acetic acid is able to enter cells though Fps1. It this case, resistance is partly established by activating Hog1, mitogen-activated protein kinase. Hog1 is known to response to osmoregulation which affects gene expression and cell cycle progression. Acetic acid penetrated into the cell activates the Sln1

branch of HOG pathway, thus activating upstream kinase of Hog1, Pbs2. Activated Hog1 phosphorylates at multiple sites on Fps1 in response to high concentration of acetic acid resulting in inactivation of Fps1 followed by reduced imports of acetic acid. Once inside the cell, dissociated protons and corresponding anions are not able to easily dissipate through the lipid plasma membranes due to their electric charges and accumulated protons and anions cause severe growth inhibition (7,14). In addition, dissociated acid anions inside the cell is known to disrupt the integrity and stability of cellular membranes (7,15). As a result, the loss of plasma membrane escalates permeability of cells leading to further growth inhibition, protein aggregation, and oxidative stress.

Severe acidification of cells caused by dissociation of weak acids and increased entry caused by disruption of cell membrane may leads to significantly decreased synthesis of RNAs and DNAs (16). To maintain permissive internal pH, yeast cells require Pma1, H+-ATPase, that pumps protons out of the cell at the expense of ATPs. Activity of Pma1 is known to be elevated following weak organic acid stresses (17,18). In addition, vacuolar membrane V-ATPase, which is responsible for acidification of vacuoles, intake excess protons into

vacuoles at the expense of ATPs, thus leading to abated acidification of cytoplasm of cells. (19-22).

Accumulated acid anions rely on several specific transporters to be pumped out of the cell. The most well studied such transporter is ATP-binding cassette, Pdr12. Pdr12 is the main transporter that facilitates efflux of propionate, sorbate and benzoate anions (23). Polyamine transporters of the major facilitator superfamily such as *TPO2* and *TPO3* are also responsible for the export of excess acids anions. These proteins are also membrane proteins that are originally known to transfer polyamines including spermine and spermidine (24).

To ensure that extruded anions do not re-enter the cells, it is essential for cells to reorganize cell wall structures. The glycosylphosphatidylinositol cell wall protein Spi1 is known to exert protective rolls against lipophilic acids via remodeling of cell wall structure (25).

It is known that the TORC1 pathway is activated in response to acetic acid (26). It is believed that activation of TORC1 kinase counteracts the limitation of nutrients caused by weak acid stressed conditions. Furthermore, cells increase the expression of genes

involved in biosynthesis of amino acid under weak acid stressed (27).

*S. cerevisiae* is not able to catabolize acetate, propionate, or benzoate. But it can degrade sorbic acid to hydrocarbon 1,3-pentadiene, although it is not a major resistance mechanisms for sorbate in yeast. On the other hand, the major spoilage yeast, *Z. bailii* can catabolized large amounts of acetate, sorbate, and benzoate by utilizing a broad-specificity mitochondrial monooxygenase with benzoate-4-hydrozylase, ZbYme2. *S. cerevisiae* can catabolize sorbate and benzoate when ZbYme2 is expressed.

### 2.1.2. Transcription factors involved in resistance to weak acids

#### Msn2/Msn4

Msn2 and Msn4 are general transcription factors that response to various stresses. Together, they regulate more than 200 genes in response to heat shock, osmotic shock, oxidative stress, low pH, and sorbic acid. They bind to STRE element that is located in their target gene promoter sequence (28). Msn2 is known to be constitutively expressed, whereas expression of Msn4 is Msn2/4 dependent and

induced by stresses. They have zinc-finger binding domain that can bind to the STRE elements. Msn2/4 is phosphorylated by PKA kinase, cAMP-dependent protein kinase (29-31). This phosphorylation inhibits activity of Msn2/4. Dephosphorylation by phosphatase, Ppt1, activates them in response to stresses (32). Msn2/4 interacts with 14-3-3 protein, Bmh2, in the cytoplasm and hyper-phosphorylated by stress responsive kinase including Yak1 and translocates into the nucleus (33-35). Msn2 is known to interact with Msn5 that is a karyopherin, which is involved, in nuclear import and export of many proteins. Nucleocytoplasmic shuttling is regulated by PKA (36).

Involvement of Msn2/4 in response to organic acids has been confirmed. Msn2/4 activates expression of genes involved in molecular chaperones, carbohydrate metabolism, and oxidative stress responses. Interestingly, many genes regulated by Msn2/4 in response to weak organic acids do not have STRE elements in their promoter sequences (25,37).

**Haa1**

Haa1 is a transcription factor that activates most of genes expressed in response to acetic acid, either directly or indirectly. Haa1 was

originally classified as a fungal Cu-regulated transcription factor due to highly conserved protein sequences with the well-known Cu-regulated transcription factor Ace1 (38). However, it was revealed that Haa1 is not regulated by the presence or absence of copper ions. Instead, Haa1 protects cells from weak organic acids such as lactic, acetic, and propionic acids by activating the expression of acid responsive genes. Among genes that are highly responsive to acetic acids includes polyamine antiporters of the major facilitator superfamily, *TPO2* and *TPO3*, a protein kinase involved in activation of $H^+$-ATPase Pma1, Hrk1 (17,24,39). Haa1 is mainly cytoplasmic in the absence of weak organic acid stress, but it rapidly translocates into the nucleus upon acetic acid or lactic acid treatment. Haa1 is known to bind to promoter sequences of target genes containing an acetic acid-responsive element (ACRE) (17,38). Haa1 is known to be heavily phosphorylated in both activation and inactivation. Hrr25, a Casein kinase I, was also shown to prevent the activation of Haa1 in response to acetic acid stress (40). However, whether the phosphorylation of Haa1 upon acid stresses is directly related to the activation of Haa1 and expression of Haa1 target genes still remains elusive. Furthermore, Haa1 is known to constantly shuttling from the nucleus

to the cytoplasm even in the absence of stresses by exportin known as Msn5. However, it is utterly unidentified how Haa1 is activated in response to weak acids.

**War1**

War1 is classified as a Zn(II)2Cys6 family member of transcription factor that contains a zinc-finger. War1 is known to form a homodimer at the promoter of target genes such as *ATO2* and *PDR12*, and is constitutively localized in the nucleus even in the absence of stresses. But the DNA binding affinity of War1 increases with weak-acid presence, more specifically by lipophilic acids such as sorbic acid. War1 is hyperphosphorylated upon propionic acid and sorbic acid, but mutations on the phosphorylation sites did not disclose any critical roles of phosphorylation in War1 activation (41). Lack of evidence that actually activated War1 led to the idea that War1 could be activated by direct binding with acid anions. This idea was supported by conformational changes of War1 detected by FRET analysis, florescence energy transfer in response to propionic acid (42). However, direct binding of weak acids to War1 has not yet been demonstrated.

### Other transcription factors

Other transcription factors such as Pdr1/3 or Rim101 are involved in weak organic acid responses. Pdr1/3 are transcription factors that are involved in pleiotropic drug responses, regulating about 200 genes. They are known to form homo- and heterodimers at pleiotropic drug response elements (PDREs) in the promoters of target genes. Pdr1/3 constitutively occupy target gene promoters. Their target genes include the ABC transporters, *PDR5*, *PDR10*, and *PDR15*, hexose transporter genes, and sphingolipid biosynthesis genes. Loss of both Pdr1 and Pdr3 shows severe drug hypersensitivity. Pdr1/3 are known to be involved in acetic acid, propionic acid, and sorbic acids and direct binding with pleiotropic drugs activates Pdr1 (43).

Rim101 is a zinc-finger containing transcriptional repressor that is involved in alkaline responsive repression. In addition, Rim101 is involved in transcriptional responses in response to propionic acid by regulating remodeling cell wall structures (44).

## 2.2. The expression of ribosomal protein genes in *S. cerevisiae*

Ribosomes are organelles that translate the genetic information stored in mRNA and synthesize proteins in all known organisms. These complexes consist of two ribonucleoprotein subunits, the small 40S subunit (SSU) with 18S rRNA and 33 ribosomal proteins and the large 60S subunits (LSU) with 5S, 5.8S, 25S rRNAs and 46 ribosomal proteins for yeast (45-47). The SSU brings mRNA and aminoacylated transfer RNAs (tRNAs) together, whereas the LSU performs peptidyltransferase reaction (48-50). Ribosomal RNAs are transcribed from the nucleolus, which are found on the chromosome XII. Ribosomal DNAs in about 150 tandem repeats are transcribed by RNA polymerase I and III. RNA polymerase I transcribed rRNAs represents about 60% of the total cellular RNA transcripts. All rRNAs are originally transcribed as pre-rRNA and heavily cleaved by endonucleolytic and exonucleolytic nucleases (48,51,52). pre-rRNAs and ribosomal proteins are assembled with the help of numerous assembly factors to form premature ribosomes followed by further trimming by RNases to form mature ribosomes. Recent studies revealed that rRNAs are

placed at the core of ribosomes and ribosomal proteins are embedded. Furthermore, ribosomal proteins are known to make direct interaction with rRNAs to help with the removal of unnecessary RNA sequences and the correct assembly of ribosomes (53).

Ribosome assembly is a very complex processes. To generate a single ribosome, more than 76 small nucleolar RNAs and 200 assembly factor are necessary in a time limiting manner. For robust cell growth and proliferation, cells must generate 2,000 ribosomes per minute to meet the protein synthesis needs for growing cells. These processes are greatly energy consuming, so are tightly regulated in response to nutrient availabilities and physiological stresses. *S. cerevisiae* has 138 ribosomal protein genes (RPGs) that consist of 20 single genes and 59 paralogous gene pairs. (54). About 8.5% of RNA polymerase II activity is devoted to the transcription of RP genes in a actively growing cell (55). Therefore, understanding of molecular mechanisms how *S. cerevisiae* regulates the expression of ribosomal proteins are important.

### 2.2.1. An overview of ribosomal protein gene expression

Nutrient-sensing master kinases such as TORC1 (target of rapamycin complex1) or PKA (protein kinase A) are known to play central roles in the transcriptional activation of RP genes under nutrient-rich conditions (56,57). TORC1, which was originally identified by rapamycin-resistant *TOR1* and *TOR2* mutation studies, is a central regulator of cell growth and proliferation. *TOR1* and *TOR2* are well known for the regulation in response to nutrient availability and cellular stresses. They are involved in diverse cellular processes such as protein synthesis, autophagy, transcription activation, cell cycle, and ribosome biogenesis. TORC1 can be inhibited by drug rapamycin, thus giving the name TOR. Rapamycin make direct contact with one of many subunits of TORC1, Frp1, resulting in inhibition of TORC1 activity. Tor1 and Tor2 share great sequence similarity and contain number of domains named HEAT (Huntingtin, Elongation factor 3, regulatory subunit A of PP2A, TOR1), FAT (FRAP, ATM, TTRAP), and kinase domain. HEAT domain is known to regulate protein interactions, whereas FAT domain mediate protein binding. Kinase domain is responsible for TORC1 the phosphatidylinositol-kinase-related kinase

family due to conserved lipid kinase motif. Under nutrient-rich conditions, TORC1 represses number of transcription factors, which are involved in nitrogen catabolite-repression, retrograde response, and stress-response, whereas TORC1 activates factors, involved in ribosome biosynthesis. TORC1 regulates these transcription factors or other regulators by phosphorylating them.

PKA is the cAMP-dependent protein kinase. PKA is well-conserved from yeast to human and regulates processes such as cell growth, nutrient sensing, energy metabolism and cell cycle. PKA consists of two catalytic subunits encoded by Tpk1~3 and two regulatory subunits of Bcy1. In the absence of cAMP, Bcy1-bound PKA remains inactive. However, cAMP binds to Bcy1, thus releasing Bcy1 from catalytic subunits followed by activation of PKA (56). Rapamycin causes down-regulation of the ribosomal protein gene expression. But this down-regulation can be suppressed by hyperactivation of PKA caused by high concentration of cAMPs. Furthermore, subcellular localization of PKA is controlled by TORC1. As a consequence, RP gene transcription is rapidly downregulated in response to physiological cues such as nutrient starvation and a wide range of stress conditions (57).

### 2.2.2. Transcription factors involved in ribosomal protein gene expression

Transcription regulators such as Rap1 and Hmo1 and transcription factors including Fhl1, Ifh1, Crf1, and Sfp1 have been proved to regulate RP gene transcription in *S. cerevisiae*. However, detailed mechanisms governing RP gene transcription remain elusive (58-62). It has been shown that essential regulator Rap1 (Repressor Activator Protein) is involved in many cellular processes especially for ribosomal protein transcription. Rap1 binds to DNA in a sequence specific manner and remodels the local chromatin structure (63). Hmo1, chromatin associated high mobility group family member, is also involved in remodeling the local chromatin structure where it occupies. Hmo1 is recently shown to binds to about half of the RP gene promoters and expedite the recruitment of other transcription factors such as Fhl1, Ifh1 and Crf1 (64-66). Fhl1, a forkhead (FH)-like DNA binding protein, serves as a platform by constitutively being bound at RP gene promoters to recruit its co-activator Ifh1 or co-repressor Crf1 in response to nutrient availability and stresses (57). In addition, Sfp1,

which positively regulates ribosomal protein gene transcription and ribosome biogenesis in response to nutrients and stresses, has been shown to be phosphorylated by TORC1 (59,67). It has been recently proved that redundant spatial organization of Fhl1 Ifh1, and Sfp1 at about 100 bps downstream of the Rpa1 binding sites (68). TORC1 is known to orchestrate these transcription factor according to nutrient conditions. Inhibition of TORC1 by either rapamycin or nutrient-limiting conditions, Ifh1, Sfp1, or Hmo1 are known to be dissociated from the RP gene promoters, whereas Rap1 and Fhl1 are constitutively bound (65,69-71).

Fhl1 interacts with Ifh1 in nutrient rich conditions and with Crf1 in nutrient limiting conditions. A forkhead-associated (FHA) domain of Fhl1 that is known to bind to forkhead-binding (FHB) domains of Ifh1 and Crf1 sharing about 59% identity over a core region (57). Upon inhibition of TORC1 Crf1 translocates into the nucleus from the cytoplasm to compete with Ifh1 for binding to Fhl1 (57). However, Crf1 is known to be not essential for the repression of RP gene transcription in some strains upon rapamycin treatment (72). Furthermore, the FHA domain of Fhl1 is well-known as a phosphothreonine-binding domain that recognizes a pTxxD/E site (73-75). This suggests that FHB

domains of Ifh1 and Crf1 must be phosphorylated to interact with FHA domain of Fhl1. Furthermore, Ifh1 is known to form a complex known as CURI complex with subunits of protein kinase CK2, rRNA processing proteins Utp22 and Rrp7. Ifh1 is known to be phosphorylated by CK2 *in vitro* (65). CK2 is a protein kinase involved in cell growth and proliferation. Two catalytic subunits, Cka1 and Cka2, form a heterodimer with two regulatory subunits, Ckb1 and Ckb2. The fact that CK2 phosphorylated Ifh1 may imply that CK2 phosphorylation on FHB domain of Ifh1 and Crf1 promotes the interaction with FHA domain of Fhl1, thereby activating transcriptional activation of ribosomal protein genes.

# Chapter 3.

# Materials and methods

## 3.1. Strains and media

Yeast and *E. coli.* strains used in this study are listed in table 3.1. All strains used were derived from the BY4741 generic background. Gene deletion or chromosomal tagging of 13Myc at the C-terminus of Haa1 or Ifh1 and tagging of 3HA at the C-terminus of Fhl1 were performed by using PCR-mediated homologous recombination from pFA6a-13Myc-his3MX6 and pFA6a-3HA-kanMX6, respectively (76). CKB2 was deleted in BY4741 *ckb1Δ* strain (EUROSCARF) by PCR-mediated homologous recombination based on Cre/loxP recombination system, followed by the removal of the marker gene by transformation with the Cre recombinase-expression vector, pSH62, resulting in strain *ckb1Δckb2Δ* (77). *CRF1* deletion strain was generated by PCR-mediated homologous recombination based on Cre/loxP recombination system in TB50 strain (57). To generate strains expressing either only Ifh1 T681 or Ifh1 T681A, FHL1 was C-terminally tagged with 3HA in the chromosome, and p416-$P_{IFH1}$-IFH1-13Myc-$T_{ADH1}$ or p416-$P_{IFH1}$-IFH1$^{T681A}$-13Myc-$T_{ADH1}$ was introduced, respectively, followed by deletion of endogenous IFH1. Yeast cells were grown in YPD medium (1% yeast extract, 2% bacto peptone, and

2% glucose) or synthetic complete (SC) medium containing 2% glucose, 0.67% yeast nitrogen base without amino acid, and 0.2% amino acids dropout mixture appropriate for plasmid selection.

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

| **Strain** | **Genotype** | **Reference** |
|---|---|---|
| WT (BY4741) | *MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | EUROSCARF |
| *haa1Δ* | BY4741 *haa1Δ:: kanMX6* | This study |
| *HAA1-13Myc* | BY4741 *HAA1-13Myc::his3MX6* | This study |
| *msn5Δ* | BY4741 *msn5Δ:: kanMX6* | This study |
| *msn5Δ HAA1-13Myc* | BY4741 *msn5Δ:: kanMX6 HAA1-13Myc::his3MX6* | This study |
| *war1Δ* | BY4741 *war1Δ:: kanMX6* | This study |
| *haa1Δ war1Δ* | BY4741 *haa1Δ:: kanMX6 war1Δ::loxP* | This study |
| *FHL1-3HA IFH1-13Myc* | BY4741 *FHL1-3HA::kanMX6 IFH1-13Myc::his3MX6* | This study |
| *ckb1Δ ckb2Δ* | BY4741 *ckb1Δ:: kanMX6 ckb2Δ::loxP* | This study |
| *ckb1Δ ckb2Δ FHL1-3HA IFH1-13Myc* | BY4741 *ckb1Δ::loxP ckb2Δ::loxP FHL1-3HA::kanMX6 IFH1-13Myc::his3MX6* | This study |
| *crf1Δ* | TB50 *crf1Δ::kanMX6* | This study |
| *FHL1-3HA IFH1-13Myc* | BY4741 *ifh1Δ::loxP FHL1-3HA*::*kanMX6* p416-$P_{IFH1}$-*IFH1-13Myc*-$T_{ADH1}$ | This study |
| *FHL1-3HA IFH1*$^{T681A}$*-13Myc* | BY4741 *ifh1Δ::loxP FHL1-3HA*::*kanMX6* p416-$P_{IFH1}$-*IFH1*$^{T681A}$*-13Myc*-$T_{ADH1}$ | This study |
| L40 | *MATa his3, D200 trp1-910 leu2-3,112 ade2 LYS::(lexAop)4-HIS3 URA3:: (lexAop)8-lacZ GAL4* | (78) |

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* $\lambda^-$ *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 |

## 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

| Plasmids | Description | Reference |
|---|---|---|
| p426GAL-GST | *GST* cloned between SpeI and HindIII of p426 GAL | (79) |
| p426GAL-GST-CKA2 | *CKA2* cloned between HindIII and XhoI of p42 6GAL-GST | (80) |
| | CEN/ARS plasmid, $P_{TEF1}$, $T_{CYC1}$, *HIS3* marker | |
| p413TEF | | (81) |
| p416TEF | CEN/ARS plasmid, $P_{TEF1}$, $T_{CYC1}$, *URA3* marker | (81) |
| pUG73 | Plasmid containing *loxP-LEU2-loxP* deletion ca ssette | EUROSCARF |
| pSH62 | Plasmid containing a gene for Cre-recombinas e, *HIS3* marker | EUROSCARF |
| p416TEF-GST | *GST* cloned between SpeI and BamHI of p41 6TEF | This study |
| p416TEF-GST-IFH1 (400-800) | *IFH1* (400-800) cloned between SmaI and Xh oI of p416TEF-GST | This study |
| p416TEF-GST-IFH1 (400-800)$^{S680A}$ | *IFH1* (400-800)$^{S680A}$ cloned between SmaI and XhoI of p416TEF-GST | This study |
| p416TEF-GST-IFH1 (400-800)$^{T681A}$ | *IFH1* (400-800)$^{T681A}$ cloned between SmaI and XhoI of p416TEF-GST | This study |
| p416TEF-GST-CRF1 (280-467) | *CRF1* (280-467) cloned between SmaI and Xh oI of p416TEF-GST | This study |
| p416TEF-GST-CRF1 (280-467)$^{S347A}$ | *CRF1* (280-467)$^{S347A}$ cloned between SmaI an d XhoI of p416TEF-GST | This study |
| p416TEF-GST-CRF1 (280-467)$^{T348A}$ | *CRF1* (280-467)$^{T348A}$ cloned between SmaI an d XhoI of p416TEF-GST | This study |
| p413TEF-T7-FHL1 (2 70-570) | T7 tag cloned between SpeI and BamHI and *FHL1* (270-570) cloned between SmaI and Xh oI of p416TEF | This study |

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

| Plasmids | Description | Reference |
|---|---|---|
| p413-CRF1 | *CRF1* with own promoter (600 bps) and te rminator (250 bps) cloned between SmaI a nd XhoI of pRS413 | This study |
| p413-CRF$^{T348A}$ | *CRF1$^{T348A}$* with own promoter (600 bps) an d terminator (250 bps) cloned between Sm aI and XhoI of pRS413 | This study |
| p416-P$_{IFH1}$-IFH1-13M yc-T$_{ADH1}$ | *IFH1* with own promoter (600 bps) and 13 Myc tag with *ADH1* terminator cloned betw een SpeI and XhoI of pRS416 | This study |
| p416-P$_{IFH1}$-IFH1$^{T681A}$-13Myc-T$_{ADH1}$ | *IFH1$^{T681A}$* with own promoter (600 bps) and 13Myc tag with *ADH1* terminator cloned b etween SpeI and XhoI of pRS416 | This study |
| pVP16-IFH1 (644-80 0) | *IFH1* (644-800) cloned between SmaI and XhoI of pVP16 | This study |
| pBTM116-LexA-CKA1 | *CKA1* ORF cloned between EcoRI and S alI of pBTM116 | (82) |
| pBTM116-LexA-CKA2 | *CKA2* ORF cloned between EcoRI and S alI of pBTM116 | (82) |
| pBTM116-LexA-CKB1 | *CKB1* ORF cloned between EcoRI and S alI of pBTM116 | (82) |
| pBTM116-LexA-CKB2 | *CKB2* ORF cloned between EcoRI and S alI of pBTM116 | (82) |
| p416TEF | CEN/ARS plasmid, P*TEF1*, T*CYC1*, URA3 marker | (1) |
| pUG27 | Plasmid containing loxP-HIS5-loxP deletion cassette | EUROSCARF |
| pSH47 | Plasmid containing a gene for Cre-recombinase, URA3 marker | EUROSCARF |
| p416-PHAA1-HAA1-13Myc-TADH1 | *HAA1* ORF with own promoter (600 bps) and 13 Myc tag with ADH1 terminator (250 bps) cloned between Not1 and Xho1 of pRS416 | This study |

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

| Plasmids | Description | Reference |
|---|---|---|
| p416-PHAA1-HAA1ΔZBD -13Myc- | Deletion of *HAA1*(6-40) from p416-PHAA1-HAA1-13Myc-TADH1 | This study |
| p416-PHAA1-HAA11-130-13Myc- | Deletion of *HAA1*(131-694) from p416-PHAA1-HAA1-13Myc- | This study |
| p416-PHAA1-HAA11-230-13Myc- | Deletion of *HAA1*(231-694) fromp416-PHAA1-HAA1-13Myc- | This study |
| p416-PHAA1-HAA11-483-13Myc- | Deletion of *HAA1*(484-694) from p416-PHAA1-HAA1-13Myc- | This study |
| p416-PHAA11-HAA1C11S-13Myc | *HAA1*$^{C11S}$ mutation from p416-PHAA1-HAA1-13Myc- | This study |
| p416-PHAA1-HAA1C63S-13Myc- | *HAA1*$^{C163S}$ mutation from p416-PHAA11-HAA1-13Myc- | This study |
| p416 ADH-VP16 AD-T7 | Activation domain from VP16 with T7 tag cloned between EcoRI and Xho1 of p416 ADH | This study |
| p416ADH-HAA11-40-VP16 AD-T7 | *HAA1*(1-40) cloned between BamH1 and EcoR1 of p416 ADH-VP16 AD-T7 | This study |
| p416ADH-HAA11-130-VP16 AD-T7 | *HAA1*(1-130) cloned between BamH1 and EcoR1 of p416 ADH-VP16 AD-T7 | This study |
| p416ADH-HAA11-150-VP16 AD-T7 | *HAA1*(1-150) cloned between BamH1 and EcoR1 of p416 ADH-VP16 AD-T7 | This study |
| p416ADH-HAA11-180-VP16 AD-T7 | *HAA1*(1-180) cloned between BamH1 and EcoR1 of p416 ADH-VP16 AD-T7 | This study |
| p416ADH-HAA11-230-VP16 AD-T7 | *HAA1*(1-230) cloned between BamH1 and EcoR1 of p416 ADH-VP16 AD-T7 | This study |
| p415ADH-HAA1-eGFP | *HAA1* ORF cloned between Spe1 and Xho1 of pRS415 ADH | This study |

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

| Plasmids | Description | Reference |
|---|---|---|
| pGEX4T-1-HAA1 | *HAA1* cloned between Sma1 and Xho1 | This study |
| pGEX4T-1-HAA1 (1-150) | *HAA1*(1-150) cloned between Sma1 and Xho1 | This study |
| pGEX4T-1-IFH1 (400-800) | *IFH1*(400-800) cloned between Sma1 and Xho1 | This study |
| pGEX4T-1-IFH1 (400-800)$^{S680A}$ | S680A mutation from pGEX4T-1-IFH1 (400-800) | This study |
| pGEX4T-1-IFH1 (400-800)$^{T681A}$ | T681A mutation from pGEX4T-1-IFH1 (400-800) | This study |
| pGEX4T-1-IFH1 (400-800)$^{S680AT681A}$ | S680AT681A mutation from pGEX4T-1-IFH1 (400-800) | This study |
| pGEX4T-1-IFH1 (644-710) | *IFH1*(644-710) cloned between Sma1 and Xho1 | This study |
| pGEX4T-1-IFH1 (644-710)$^{S680A}$ | S680A mutation from pGEX4T-1-IFH1 (644-710) | This study |
| pGEX4T-1-IFH1 (644-710)$^{T681A}$ | T681A mutation from pGEX4T-1-IFH1 (644-710) | This study |
| pGEX4T-1-IFH1 (644-710)$^{S680AT681A}$ | S680AT681A mutation from pGEX4T-1-IFH1 (644-710) | This study |
| pGEX4T-1-CRF1 (280-370) | *CRF1*(279-370) cloned between Sma1 and Xho1 | This study |
| pGEX4T-1-CRF1 (280-370)$^{S347A}$ | S347A mutation from pGEX4T-1-CRF1 (280-370) | This study |
| pGEX4T-1-CRF1 (280-370)$^{T348A}$ | T348A mutation from pGEX4T-1-CRF1 (280-370) | This study |
| pGEX4T-1-CRF1 (280-370)$^{S347AT348A}$ | S347AT348A mutation from pGEX4T-1-CRF1 (280-370) | This study |
| pET28b-His-FHL1 (270-570) | *FHL1*(270-570) cloned between Ecor1 and Bamh1 | This study |

## 3.3. Qauntitative reverse transcription PCR (qRT-PCR)

Yeast cells were grown in either YPD or selective SC medium up to $OD_{600}$ of 0.6-0.8 and total RNA was extracted by the hot phenol method. The relative transcription was determined by qRT-PCR as described previously (83).

## 3.4. Fluorescence microscopy analysis

Cells transformed with plasmid expressing eGFP-tagged Haa1 were grown in SC-Leu medium to the exponential phase and subjected to 30 mM acetic acid stress for 30 min. Cells were placed in a FluoView FV1000 confocal laser scanning unit with the IX81 inverted microscope (Olympus) and images were captured with a confocal PMT detector. IMARIS software (Bitplane) was used to process confocal images.

## 3.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed using cells expressing Haa1-13Myc as described

previously (83). Cells were inoculated in YPD or selective medium (SC-Ura) to $OD_{600}$ of 0.2, grown to $OD_{600}$ of 0.8 to 1.0 and treated with a final concentration of 1% formaldehyde for 25 min at room temperature followed by 5 min of quenching with glycine. Cells were collected and washed with cold 150 mM NaCl TBS [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] buffer. Cross-linked yeast cells were re-suspended with in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitors]. Sonication was performed in ice-cold water for 10 times for 20 sec with 100 sec intervals at 24% power output. After 30 min of centrifugation at 4 ºC, the supernatant was pre-cleared with 20 µl protein A/G beads (Santa Cruz Biotechnology) for 2 h followed by overnight immunoprecipitation with 1 µg of anti-Myc antibody (Santa Cruz Biotechnology) and 20 µl protein A/G beads for 1 h to capture antibodies. Reversal of DNA-protein cross-linking was performed followed by DNA purification with the QIAGEN DNA purification kit. To calculate the fold enrichment of Haa1-13Myc, the bound DNA was quantified by $2^{-\Delta\Delta CT}$ method (84) and analyzed by qPCR using Roche LightCycle 480 II. Primer sequences used for qPCR are listed in supplementary Table S2. The promoter occupancy of Haa1 was

normalized by the promoter occupancy of untagged negative controls and indicated as fold enrichment.

## 3.6. Weak acid tolerance test

After dilution of cells with water to $OD_{600}$ of 1, 0.1, 0.01, and 0.001, 5 μl of the cells was spotted on a control YPD plate and YPD plates with various concentrations of 60 acetic acid, lactic acid, propionic acid, sorbic acid, or benzoic acid and incubated at 30 ºC for 2-3 days.

## 3.7. Fabrication of carboxyl-functionalized carbon nanofibers (CNFs) and CNF-FET biosensor electrodes

To fabricate carbon nanofibers, electrospun polyacrylonitrile (PAN) fibers were used as starting materials. First, PAN solutions were prepared by dissolving 1.0 g of PAN (MW = 150,000) in 10 mL of DMF at 60 °C and stirred until achieving homogeneity. The resulting solution was spun into PAN nanofiber using an electrospinning apparatus (Nano NC, Korea). The diameter of electrospinning needle was 23

gauge and the voltage was at 15 kV with a flow rate of 10 μL/min. The distance from the needle tip to the grounded collector was fixed at 15 cm. The electrospun PAN nanofibers were collected and calcined at 270 °C for 2 h in air and carbonized at 800 °C for 1 h in flowing nitrogen. Carboxyl-functionalized carbon nanofiber (CNF) was prepared via oxidation process with strong acid solution. Carbonized PAN nanofibers (250 mg) were soaked into 20 ml of a 3:1 volume mixture of 1 M sulfuric acid ($H_2SO_4$) and 1 M nitric acid ($HNO_3$) for 12 h at room temperature with vigorous stirring. The resulting CNF acid mixture was centrifuged with DI water for several times until the pH of washing becomes neutral. Finally, collected CNFs were dried in vacuum oven for overnight.

To fabricate CNF-FET electrodes, gold interdigitated array (IDA) electrodes on glass substrate were prepared by thermal evaporation of Cr/Au (20 nm/200 nm) followed by lift-off process. To build the sensing electrodes, IDA electrodes were treated with 5% (w/v) aqueous (3-aminopropyl) triethoxysilane solution for 6 hr to introduce amine groups on the surface. A mixture of 0.1% (w/v) aqueous CNFs solution (5 μL) and 1% (w/v) aqueous 4-(4,6-dimethoxy-1,3,5-triazin-2-ly)-4-methylmorpholinium chloride (DMTMM, 5 μL) were used to

treat the aminosilane modified electrode surface for over 6 h at room temperature. The resulting electrodes were carefully rinsed with distilled water to remove excess DMTMM and unbound CNFs. GST, GST-Haa1, GST-Haa1 (1-150), GST-War1 proteins were purified from *E. coli* using glutathione agarose resin and dialysed with dialysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 15% glycerol]. Immobilization of proteins was carried out using a mixture of buffer with 2 μg/L of protein (10 μL) and 1% (w/v) aqueous DMTMM solution (10 μL) for overnight at 4 °C.

## 3.8. Measurement of real-time responses using CNF-FET electrodes

All weak acids (acetic, lactic, sorbic, and benzoic acids) were purchased from Sigma-Aldrich. Acid solutions were prepared as a 0.1 M stock solution in phosphate buffer saline (PBS, pH 7.4) and titrated to a pH of about 7 using NaOH solution. Subsequently, the stock solutions were serially diluted 1/10 times to prepare analyte samples having a final concentration of 1 fM to 10 μM. Electrical performance of the sensor electrodes was measured by a semiconductor analyzer

(Keithley 2612A) and a probe station (MS TECH, model 4000). To monitor the response of CNF-FETs in solution environment, a glass chamber (200 µL volume) was utilized. The chamber was filled with 100 µL of PBS (pH 7.4) electrolyte. The gate electrode was immersed in the PBS (pH 7.4) electrolyte and used to bias the sensor to the desired operating point. During the measurement, source-drain bias voltage ($V_{DS}$) was maintained as -0.01 V with a gate bias of 0.7 V. The solution (3 µL) containing the analyte was consecutively added onto electrolyte chamber and $I_{SD}$ was monitored. The measured $I_{SD}$ was normalized as $\Delta I/I_0 = (I - I_0)/I_0$, where $I$ is the measured real-time current and $I_0$ is the initial current.

## 3.9. Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described previously (85). *S. cerevisiae* L40-Ura cells with plasmids expressing VP16- and LexA DBD-fusion proteins were grown in liquid selective medium (SC-Leu-Trp) and harvested. After dilution of cells with water to $OD_{600}$ of 1, 0.1, and 0.01, 5 µl of the cells was spotted on a control plate (SC-Leu-Trp) and a selective plate (SC-Leu-Trp-His containing 1 mM 3-AT), and

incubated at 30 °C for 2–3 days.

## 3.10. *in vitro* phosphorylation

GST-tagged proteins were purified as described previously (79,82). For *in vitro* kinase assay, 5 μg of GST, 10 μg of GST-Ifh1, or 10 μg of GST-Crf1 protein purified from *E. coli* was incubated for 60 min with 0.2 μg GST-Cka2 purified from *S. cerevisiae* in a kinase buffer containing 1 μCi [γ-$^{32}$P] ATP. Samples were run on 10% SDS-PAGE gels, dried, and detected by autoradiography.

## 3.11. Western blotting

Western blotting was performed as described previously (79,82) by using anti-T7 antibody (Novagen), anti-GST antibody (Santa Cruz Biotechnology), anti-LexA antibody (Santa Cruz Biotechnology), anti-Myc antibody (Santa Cruz Biotechnology), and anti-phospho-CK2 substrate (Cell Signaling). To detect CK2-dependent phosphorylation levels of Ifh1 *in vivo*, JHY801 and JHY803 having genome-integrated *IFH1*-13Myc were grown at the exponential phase, and 500 μg of cell

lysis was immunoprecipitated in IP150 (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% NP-40 containing 0.1% protease inhibitor cocktail (Calbiochem) and 1 mM phenylmethylsulfonyl fluoride (Calbiochem) with anti-Myc antibody for 4 h, and phosphorylated Ifh1 levels were detected by western blotting with antibody against phospho-CK2 substrate.

## 3.12. GST pull-down assay

For *in vitro* GST pull-down assay, purified GST-Ifh1 (400-800) was phosphorylated by 0.2 μg GST-Cka2 and 50 μM ATP at 30 ºC for 30 min. Next, 5 μg of GST or 10 μg of GST-Ifh1 (400-800) was pre-bound to glutathione agarose beads (Novagen) in binding buffer (pH 7.5 HEPES/KOH, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.02% SDS) at 4 ºC for 2 h and washed three times. Then, 1 μg of T7-Fhl1 (270-570) was added, incubated for 30 min, washed three times, and the bound proteins were analyzed by western blotting with anti-GST and anti-T7 antibodies. For *in vivo* GST pull-down assay, cells expressing T7-Fhl1 (270-570) together with GST-Ifh1 (400-800) or GST-Crf1 (279-467) were grown up to $OD_{600}$ of

0.8 in SC-Leu-Ura medium. Cells were harvested, lysed in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP40, and protease inhibitors] and incubated with glutathione agarose beads at 4 ºC for 2 h. The bound proteins were analyzed by western blotting with anti-GST and anti-T7 antibodies.

# Chapter 4.

# Activation of Haa1 and War1 transcription factors by differential binding of weak acid anions in *S. cerevisiae*

## 4.1. Introduction

Monocarboxylic weak acids are commonly used to prevent unwanted food spoilages (1-3). On the other hand, weak acids also cause the severe growth inhibition, which is limiting steps for microbial fermentation. For this reason, *S. cerevisiae* is extensively exploited as an important model system to comprehend how cells adopt to and resist against weak acids, even though *S. cerevisiae* is not the best organisms for microbial fermentation (9). Therefore, resistance mechanisms against weak acids is important.

Acetic acid can penetrate into the cell by both passive and facilitated diffusion. At low pH, acetic acids are mainly present as the undissociated form. At this state, undissociated acetic acids readily enter cells by passive diffusion followed by dissociation into protein and acetate in the cytoplasm. Furthermore, acetic acid can be transported into the cell by aquaglyceroporin protein known as Fps1 (12,13). Increased concentration of protons and acetates are main reason for severe growth inhibition (86). To maintain internal pH and plasma membrane potential within physiological levels, it is

necessary for cells that defensive mechanisms against the weak acid stress are engaged. Plasma membrane $H^+$-ATPase and vacuolar membrane V-ATPase export excess protons out of the cell. Further, acid-anions exporting proteins such as multidrug resistance (MDR) transporter are highly expressed and remodeling of cell wall and plasma membrane takes place to prevent further diffusion of weak acids (19-22).

The cytotoxic effects of weak acids and genes induced by weak acids are variable depending on their chemical structures. Therefore, cells might have regulatory mechanisms sensing structurally different weak acids and induce proper responses. *S. cerevisiae* Haa1 and War1 are weak acid-specific transcriptional activators known to be activated in response to more hydrophilic and more lipophilic weak acids, respectively. However, it is not well understood how these transcription factors sense different weak acid stresses.

In *S. cerevisiae*¸ transcription factors such as Haa1 and War1 are known to play important roles against weak acids by activating transcription of anion-exporting membrane proteins (3). Haa1 was

first identified as a fungal Cu-regulated transcription factor (38).

However, Haa1 failed to show copper mediated regulation. Unexpectedly, Haa1 is known to play defensive roles against hydrophilic acids such as acetic acid, lactic acid, and propionic acid by activating the expression of genes such as *TPO2, TPO3* and *TDA6,* which are major facilitator superfamily of transporters invoked in the export of acid anions (2,17,87,88). Haa1 translocated from the cytoplasm to the nucleus and highly phosphorylated in response to acetic acid and lactic acid (17,40). However, it is completely unknown how Haa1 is activated in response to weak acids.

In addition to Haa1, War1 is a Zn(II)2Cys6 family member of transcription factor that is activated by propionic acid, sorbic acid, and benzoic acid. War1 is known to activate the expression of genes such as *PDR12* encoding an ABC transporter even in the absence of stress (25,89-92), but its DNA binding affinity increases upon weak acid stress (42). War1 is also highly phosphorylated upon sorbic acid (41) and is known to change its conformation in response to propionic acid confirmed by florescence energy

transfer (FRET) analysis (42). However, direct binding of weak acids to War1 has not yet been demonstrated.

In this chapter, using field-effect transistor (FET) type sensors based on carbon nanofibers (CNFs) (93,94), we propose dissociated acid anions can directly bind to Haa1 and War1 with varying binding affinities. In agreement with previous studies, Haa1 binds to more hydrophilic acid anions such as acetate and lactate, whereas War1 binds to more lipophilic acid anions such as sorbate and benzoate. These results reveals that Haa1 and War1 are differentially activated by various weak acid stresses.

## 4.2. Haa1 requires zinc ions for its proper functions

*S. cerevisiae* Haa1 is believed to be descended from ancestral dual functioning *Zygosaccharomyces bailii* Haa1. *Zb*Haa1 is known to regulate expression of genes involved in both hydrophilic acid inducible genes and high concentration of copper stresses. *Zb*Haa1 is functionally separated to *Sc*Haa1, for an acid responsive

transcription factor, and ScAce1, a copper inducible transcription factor (95). As a result, Haa1 and Ace1 shares a great extent of protein similarity. Many transcription factors are known to contain zinc binding regions for its DNA binding affinity (96).

The DNA binding affinity of Haa1 to its target gene promoters anticipated to be via the N-terminal region of Haa1 (Figure 4.1). It is predicted that Ace1 has a 40-residue Zn-binding domain followed by a 70-residue Cu regulatory module. Each has three and eight cysteine amino acid residues, respectively. It is well known that DNA binding of Ace1 is promoted by binding of not only Cu ions but also Zn ions (97-99), and all 11 Cys residues are critical for either DNA binding or Cu-induced gene expression (100).

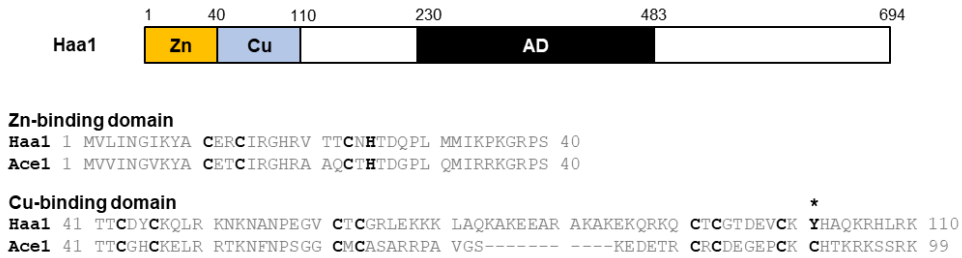



**Figure 4.1 Schematic diagram of Haa1 and its protein similarity with Ace1.**

Organization of predicted Zn-and Cu-binding domains and activation domain in Haa1. Amino acid sequence alignment of Haa1 and Ace1 is shown, where the highly conserved N-terminal Zn-binding domains and moderately conserved Cu-binding domains of Haa1 and Ace1 are compared.

All the cysteine residues are conserved in Haa1 except for the last cysteine residue that is substituted for tyrosine instead of cysteine. Nevertheless, cells grown in copper lacking medium did not show any differences in the expression of Haa1 target genes (38), leading to the idea that the copper binding module for Haa1 might be dispensable, thus placing the potential importance of zinc binding domain in DNA binding of Haa1.

To emphasize the importance of zinc binding domain in Haa1 activity, we examined whether lack of zinc ions affect Haa1 target gene transcription in response to acetic acid stress. Exponentially growing cells were treated with a cell-impermeable metal chelator, or a cell-permeable Zn-specific chelator, EDTA (101), or TPEN (102,103), respectively. Then the expression of Haa1 target genes tested, *TPO2* and *TDA6.* It revealed that transcription was significantly reduced upon acetic acid stress when compared to the untreated control (Figure 4.2).

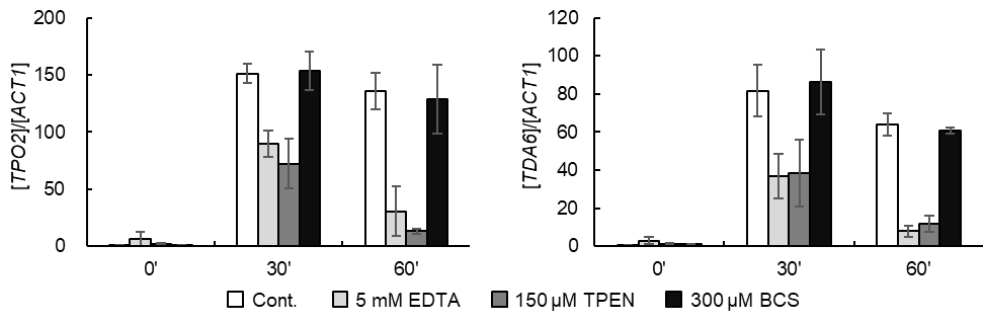
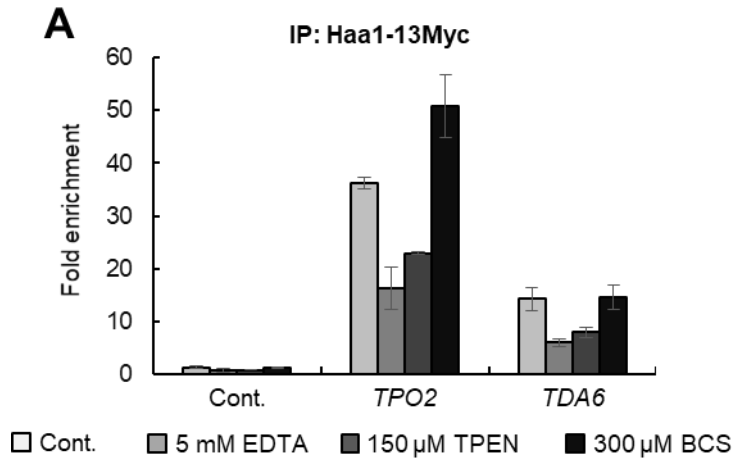



**Figure 4.2 The expression of Haa1 target genes upon acetic acid stress in the absence of zinc ions.**

*S. cerevisiae* BY4741 cells were grown in YPD medium to the exponential phase and pre-treated with chelating agents, 5 mM EDTA, 150 μM TPEN, or 300 μM BCS for 2 h, followed by 30 mM acetic acid treatment for the indicated times. mRNA levels of Haa1 target gene *TPO2* and *TDA6* were measured by qRT-PCR and normalized to the mRNA levels of *ACT1.* Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated WT cells (n = 3).

On the contrary, the copper depletion form the medium by BCS, a cell-impermeable copper specific chelator, did not show any reduction in Haa1 target gene transcription in response to acetic acid (38,104). These results are in agreement with the previous study that copper does not play important role for the activity of Haa1 (Figure 4.2). To investigate the DNA binding affinity of Haa1 upon Zn chelation, cells were treated with the same concentration of metal chelators followed by acetic acid stresses. ChIP assays showed that the chelation of Zn by either EDTA or TPEN but not by BCS shows severely reduced DNA binding of Haa1 onto its target gene promoters (Figure 4.3 A). Reduced DNA binding affinity of Haa1 is not due to reduced protein expression levels of Haa1. Cells were treated with the same concentration of chelators and acetic acid and changes in protein levels of Haa1 were confirmed to be negligible (Figure 4.3 B).


A
IP: Haa1-13Myc
Fold enrichment
60
50
40
30
20
10
0
Cont.
TPO2
TDA6
Cont.
5 mM EDTA
150 μM TPEN
300 μM BCS

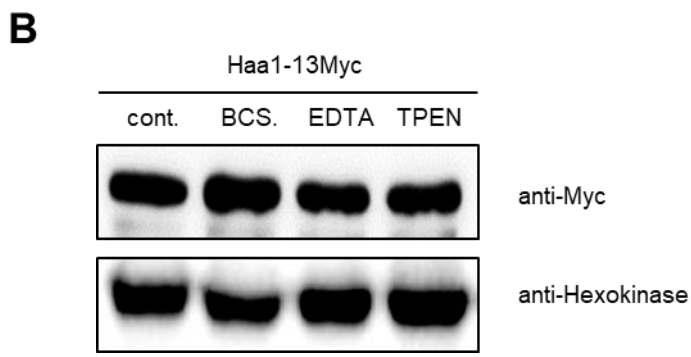

B
Haa1-13Myc
cont.
BCS.
EDTA
TPEN
anti-Myc
anti-Hexokinase

**Figure 4.3 DNA binding of Haa1 upon acetic acid stress in the absence of zinc ions.**

(A) BY4741 cells expressing Haa1-13Myc were treated with the chelating agents for 2 h, followed by addition of 30 mM acetic acid for 30 min. Binding of Haa1-13Myc to the *TPO2* or *TDA6* promoter was detected by ChIP with anti-Myc antibody, and indicated as fold enrichment relative to the untagged control. Non-transcribed region of Chr. V was used as a control promoter (105). Each value represents the average ± SD from three independent experiments.

(B) Protein levels of Haa1-13Myc with the identical incubation time and concentration of chelators followed by addition of 30 mM acetic acid for 30 min.

Since it can be inferred that zinc ions play very important for DNA binding affinity of Haa1 and the expression of Haa1 target genes in response to acetic acid stress, we further examined the significance of the N-terminal 40-residue denoted as Zn-binding domain (ZBD) in Haa1. As expected, *HAA1* deletion strain expressing the wild-type Haa1-13Myc under the control of its own promoter (600 bps) showed induction of its target genes following acetic acid stresses. However, induction of Haa1 target genes by cells expressing Haa1$^{\Delta ZBD}$-13Myc that lacks the N-terminal 6-40 residue deletion being unable to interact with zinc ions failed to show any target gene expression (Figure 4.4 A). The decrease in transcription activity of Haa1$^{\Delta ZBD}$ is not due to reduced protein levels (Figure 4.4 B) as protein levels were comparable. These results confirm that the predicted N-terminal Zn-binding binding domain is essential for Haa1 activity.

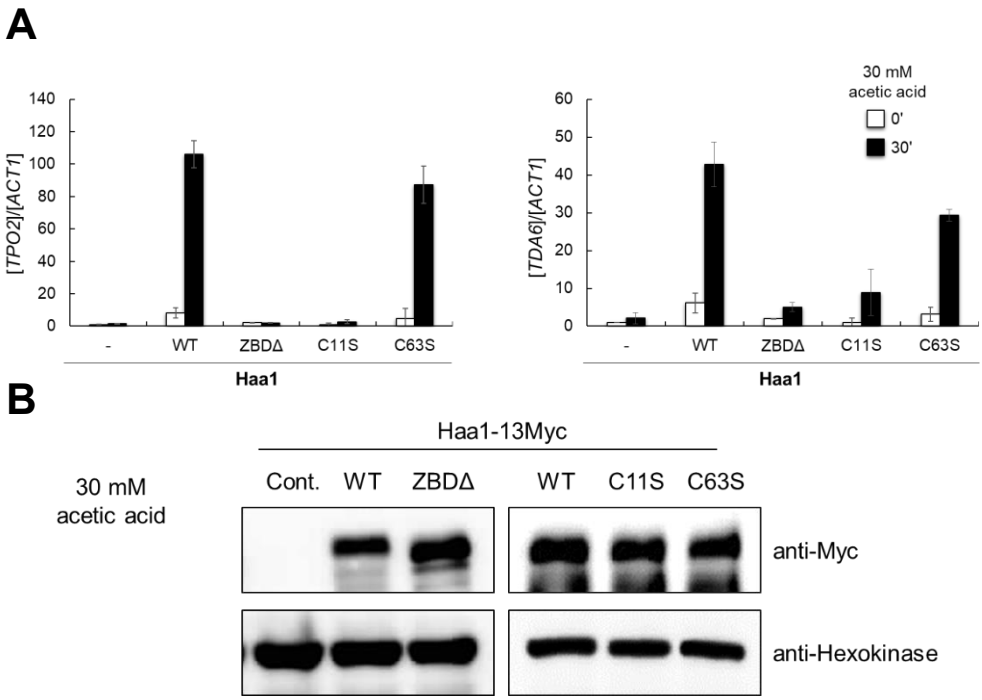



**Figure 4.4 The expression of Haa1 target genes upon acetic acid stress in Haa1$^{WT, C11S, or C63S}$.**

(A) *HAA1* deletion strain harboring plasmid expressing either wild-type Haa1-13Myc or mutant Haa1-13Myc (ΔZBD (Δ6-40), C11S, or C63S) under the control of own promoter was grown in SC-Ura medium to the exponential phase and treated with 30 mM acetic acid for 30 min. mRNA levels of *TPO2* and *TDA6* were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*.

(B) Protein levels of wild-type Haa1-13Myc or mutant Haa1-13Myc (ΔZBD (Δ6-40), C11S, or C63S) in response to 30 mM acetic acid.

## 4.3. N-terminal Zn-binding domain is required for DNA binding of Haa1

To further investigate the roles of Zn binding in Haa1 activity, Haa1$^{C11S}$ that cannot form the zinc-cysteine complex was generated. As expected, Haa1$^{C11S}$ resulted in a complete loss of target gene expression (Figure 4.4 A). Furthermore, DNA binding affinity of Haa1$^{C11S}$ was severely abated upon acetic acid stress (Figure 4.5). These results indicate that Zn binding to Cys residues including Cys11 is critical for DNA binding activity of Haa1. The decrease in transcription activity and DNA occupancy is not due to reduced protein levels (Figure 4.4B). In contrast, mutated Haa1, which is conserved in the Cu-binding sites in Ace1 and cannot form a complex with copper ion, Haa1$^{C63S}$, did not significantly alter the expression of Haa1 target gene (Figure 4.4A) nor DNA binding activity of Haa1 (Figure 4.5), further supporting the Cu-independent regulation of Haa1. Taken together, these results suggest that binding of Haa1 with Zn plays important roles for the activity and the DNA binding affinity of Haa1.

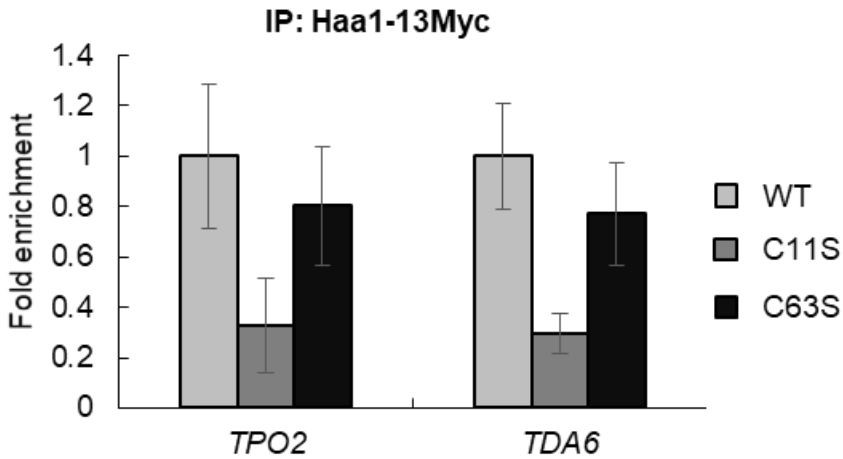



**Figure 4.5 DNA binding affinity of Haa1$^{WT, C11S, or C63S}$.**

*HAA1* deletion strain expressing Haa1-13Myc wild type or C11S or C63S mutant was treated with 30 mM acetic acid for 30 min, and DNA binding affinity of Haa1 to the *TPO2* and *TDA6* promoter was detected by ChIP with Myc antibody. Fold enrichment of each protein was normalized to that of wild-type Haa1-13Myc.

## 4.4. Nuclear localization of Haa1 is not sufficient for Haa1 target gene transcription

It is known that Haa1 translocates from the cytoplasm to the nucleus in response to acetic acid or lactic acid stresses (17,40). Haa1 interacts with the nuclear exportin Msn5, which is responsible for the export of many transcription factors out of the nucleus (106). As a consequence, Haa1 is present in the nucleus even in the absence of acetic acid stresses in *MSN5* deletion strain (17). However, it is still unclear whether nuclear localization of Haa1 is the major regulatory mechanisms for the transcription of Haa1 target genes. To investigate what triggers the expression of genes, localization of Haa1 with or without acetic acid stresses was tested. In agreement with the previous study, Haa1 is mainly cytoplasmic in the absence of acetic acid but, it translocates into the nucleus in the wild-type cells (Figure 4.6). Nonetheless, the nuclear localization of Haa1 induced by *MSN5* deletion was not sufficient to increase the basal expression levels of Haa1 target genes (Figure 4.7). These results suggest that additional activation mechanisms are required.

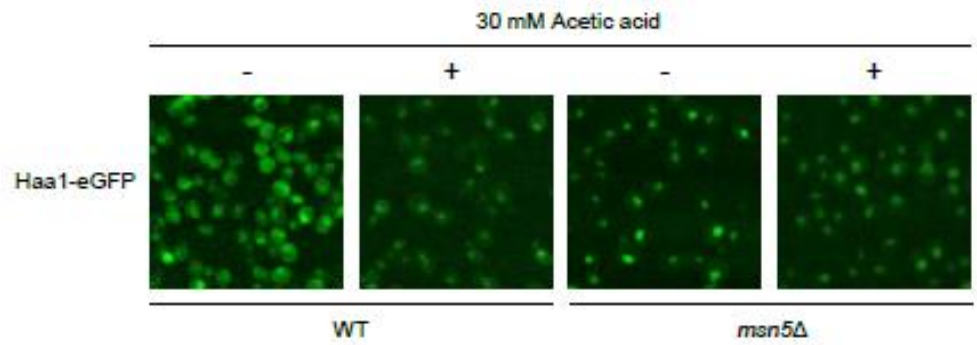



**Figure 4.6 Nuclear localization of Haa1 in response to acetic acid.**

Wild type or *msn5*Δ cells expressing Haa1-eGFP were grown in SC-Leu medium to the exponential phase and 30 mM acetic acid was treated for 30 min. Localization of Haa1-eGFP was analyzed by fluorescence microscope.

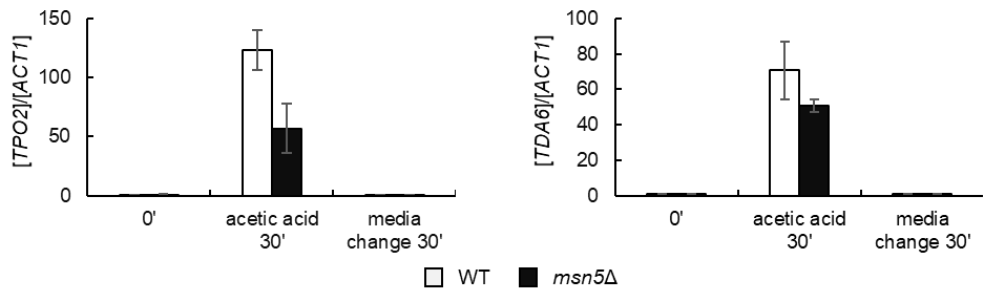



**Figure 4.7 Comparison of Haa1 target gene transcription in wild-type or *msn5*Δ.**

Wild type and *msn5*Δ cells were grown in YPD medium to the exponential phases and treated with 30 mM acetic acid for 30min. Cells were then washed and resuspended in fresh YPD medium for another 30 min. *TPO2* mRNA levels were detected by qRT-PCR. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated WT cells (n = 3).

In addition, an acetic acid induced transcriptional induction of Haa1 target genes showed moderately reduced responses in the absence of *MSN5* (Figure 4.7). This reduction in transcription might be due to reduced protein levels of Haa1 in *msn5*Δ (Figure 4.8). Furthermore, transcriptional induction caused by an acetic acid was completely eliminated when cells were resuspended in fresh medium. These results confirm that simple nuclear localization triggered by *MSN5* deletion is not sufficient for Haa1 activation (Figure 4.7). DNA binding affinity measured by ChIP assay revealed that Haa1 binds with DNA in an acetic acid stress dependent manner (Figure 4.9). Moreover, for *TPO2,* no significant increase in basal or stress-induced DNA binding of Haa1 was observed in *msn5Δ* compared to wild type (Figure 4.9). These results suggest that nuclear translocation of Haa1 is not enough for its activation, and Haa1 activity is regulated at the level of DNA binding upon weak acid stress.

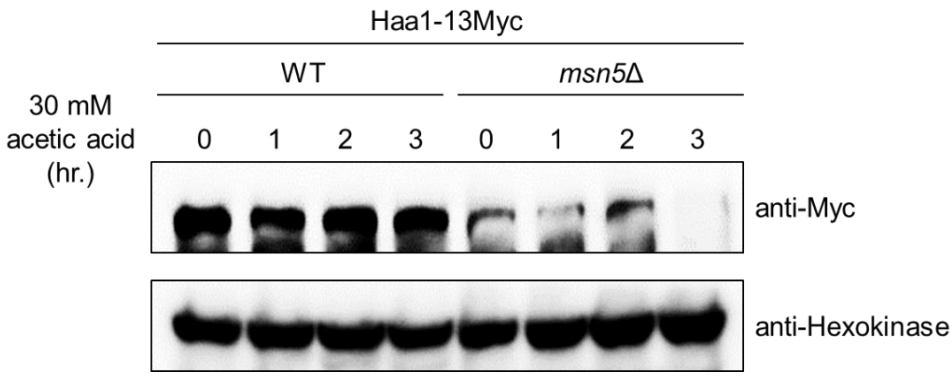



**Figure 4.8 Protein levels of Haa1 in wild-type and *msn5*Δ.**

Wild-type and *msn5*Δ having Haa1 tagged with13Myc in the chromosome were grown to the exponential phase in YPD medium and treated with 30 mM acetic acid for the indicated times. Cells were lysed in IP150 buffer and 100 μg protein was used to detect protein levels by western blotting.

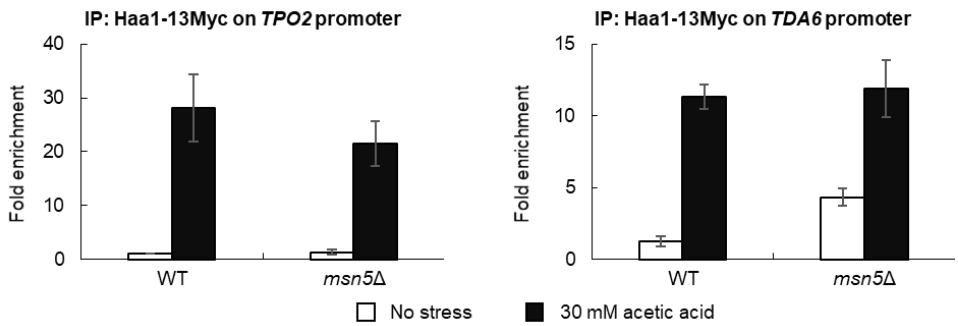



**Figure 4.9 DNA binding of Haa1 is induced by acetic acid stress.**

Wild-type and *msn5*Δ having Haa1 tagged with 13Myc in the chromosome were grown to the exponential phase in YPD medium and treated with 30 mM acetic acid for 30 min. Binding of Haa1-13Myc to the *TPO2* promoter was detected by ChIP. Each value represents the average ± SD of the relative fold enrichment, normalized to the untreated WT cells (n = 3).

## 4.5. Mapping the activation domain of Haa1

To discover the activation domain of Haa1, various lengths of Haa1 that lacks the C-terminal region were constructed. *HAA1* deletion strains expressing different lengths of Haa1 were tested in response to acetic acid stresses. $Haa1^{1-130}$ and $Haa1^{1-230}$ were not able to induce any transcription of Haa1 target genes. However, $Haa1^{1-483}$ showed both significantly increased basal levels of transcription in the absence of stresses and acetic acid-induced transcriptional activation although the induction fold was lower than that mediated by $Haa1^{1-694}$, a full-length Haa1 (Figure 4.10). Considering a previous finding that $Haa1^{1-483}$ showed comparable growth rates with wild type Haa1 (24), the activation domain spans a residues from 230 to 483, although the further C-terminal region of Haa1 is required to gain maximum transcriptional activation in response to acetic acid stresses.

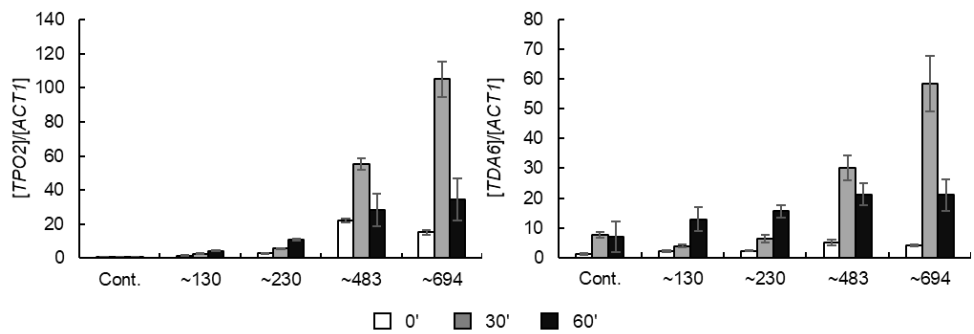



**Figure 4.10 Identification of activation domain of Haa1.**

*haa1Δ* cells harboring plasmid expressing various C-terminal truncation mutants of Haa1-13Myc under the control of own promoter were grown to the exponential phase and treated with 30 mM acetic acid for 30 min. *TPO2* mRNA levels were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated control containing empty vector (n = 3).

## 4.6. Mapping the acid responsible region of Haa1

We then searched the region that is responsible for sensing the presence of acetic acid. To map the possible regions on Haa1 that can interact with acetic acid, various lengths of Haa1 containing the own DNA binding domain was C-terminally fused to a heterologous VP16 activation domain. Although the N-terminal 40-residue Zn-binding domain was essential for Haa1 activity (Figure 4.4 A), DNA binding domain alone was not able to induce *TPO2* expression, suggesting that further down the C-terminal region of Haa1 might be necessary for either stable DNA binding or transcriptional activation of Haa1 target genes. Haa1$^{1-130}$-VP16 and Haa1$^{1-150}$-VP16 were able to show the increased basal transcription levels of *TPO2*, but did not show acetic acid-inducible transcription. On the other hand, Haa1$^{1-180}$-VP16 and Haa1$^{1-230}$-VP16 showed acetic acid-dependent induction of *TPO2,* suggesting that regions between 150 and 180 of Haa1 might hold amino acid residues responsible for sensing the acetic acid stress or for acetic acid stress-dependent regulation of Haa1 activity (Figure 4.11).

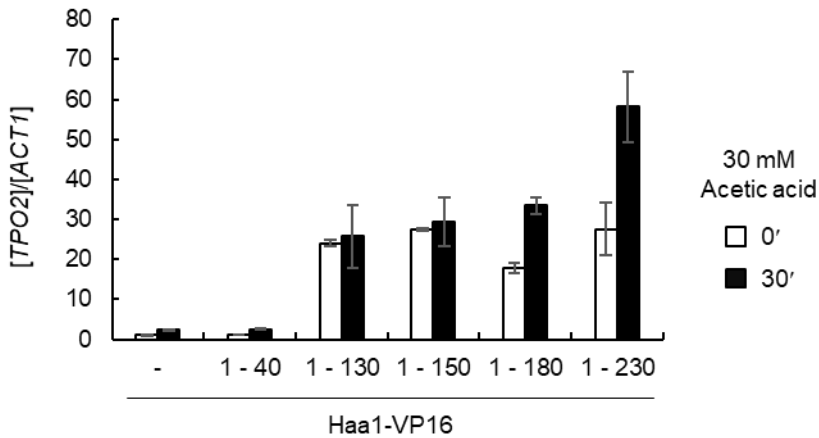



**Figure 4.11 Identification of acetic acid-responsible domain of Haa1.**

*haa1Δ* cells harboring plasmid expressing various C-terminal truncation mutants of Haa1-VP16 under the control of the *ADH1* promoter were grown to the exponential phase and treated with 30 mM acetic acid for 30 min. *TPO2* mRNA levels were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated control containing empty vector (n = 3).

## 4.7. Activation of Haa1 and War1 by different weak acids

To apprehend the activation mechanisms of Haa1, we studied the possible types of weak organic acids that can activate Haa1 and War1. It has long been known that Haa1 is responsible for transcriptional activation in response to hydrophilic acids, whereas War1 is for more lipophilic acids. Thus, relatively less lipophilic acids such as acetic acid and lactic acid activate Haa1. On the contrary, more lipophilic acids such as sorbic acid and benzoic acid activate War1, thereby exerting protective roles against the corresponding weak organic acid types (2,17,39,88,89). However, prior studies were separately focused on either only Haa1 or War1 under different experimental conditions such as acid concentration or pH of medium, making it problematic to comprehensively understand the relative contributions of each transcription factor in response to different types of weak acids. Therefore, we investigated the transcriptional activation caused by either Haa1 or War1 in response to increasing order of lipophilicity of, lactic acid, acetic acid, propionic acid, sorbic acid, and benzoic acid. To inspect activation of each transcription factor under various stress conditions, expression of a Haa1-specific target gene *TPO2, a* War1-

specific target gene *ATO2,* and a shared target gene *PDR12*, were observed in wild-type, *haa1*Δ*, war1*Δ*,* and *haa1*Δ*war1*Δ. Since acids that are tested have different lipophilicity, the lowest possible concentration of acids were used to induce comparable target gene transcription. Transcription of *TPO2*, Haa1 target gene, showed practically complete abolishment of expression in *haa1*Δ, but not in *war1*Δ, confirming Haa1-specific activation of this target gene (Figure 4.12). Interestingly, all the weak acids tested showed transcription of *TPO2*, revealing the activation of Haa1 even by lipophilic weak acids. However, the transcriptional activation of *TPO2* by benzoic acid was much lower when compared to other acids tested. On the contrary, expression of *ATO2* was observed in all acid tested except acetic acid (Figure 4.12). Unlike the preceding findings, these results demonstrate that Haa1 and War1 can be commonly activated by a broad range of weak acids, but have different preferences as previously suggested. Expression of *PDR12* showed the combined effects of both transcription factors, Haa1 and War1. Transcription of *PDR12* was induced by acetic acid in a Haa1-dependent manner, whereas mainly induced by War1 in other weak acids with having minor roles played by Haa1 (Figure 4.12).

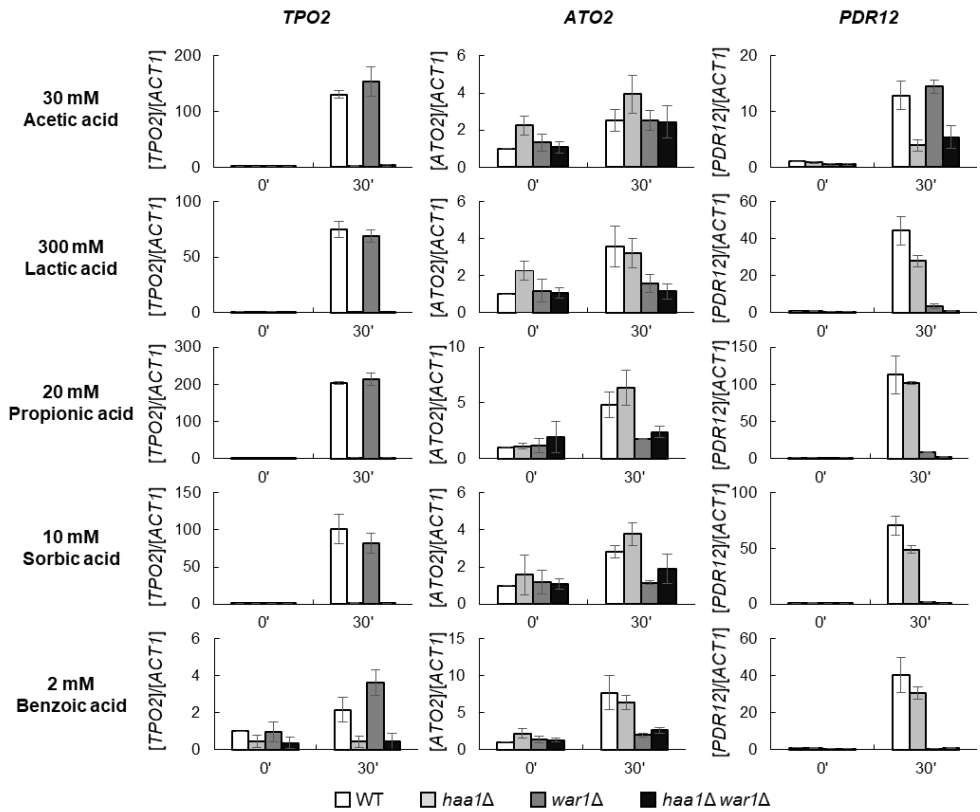



**Figure 4.12 Activation of Haa1 and War1 by various weak acids.**

Wild type, *haa1Δ*, *war1Δ*, and *haa1Δwar1Δ* cells were grown in YPD medium to the exponential phase and treated with 30 mM acetic acid, 300 mM lactic acid, 20 mM propionic acid, 10 mM sorbic acid, or 2 mM benzoic acid for 30 min, and mRNA levels of Haa1 or War1 target genes, *TPO2, ATO2,* and *PDR12*, were measured by qRT- PCR. Each value represents the average ± SD of the relative fold change in expression, normalized to the untreated wild type cells.

We next examined the role for Haa1 and War1 in tolerance to the weak acids tested by spot assays. Confirming the previously suggested role for Haa1 in tolerance to hydrophilic acid (41,89), *haa1*Δ showed significantly reduced tolerance to acetic acid and lactic acid, but not to sorbic acid and benzoic acid (Figure 4.13). Therefore, although Haa1 can be activated by more lipophilic acids such as sorbic acid and benzoic acid (Figure 4.12), Haa1-specific target genes might play a minor role in tolerance to more lipophilic weak acids. On the contrary, the role for War1 in weak acid stress tolerance was mainly correlated with the transcriptional activation patterns of War1. All weak acids tested except for acetic acid required War1 for tolerance. In the same manner, acetic acid was not able to activate War1 (Figure 4.12 and 4.13). Although Haa1 is known to be involved in tolerance to propionic acid (2), *war1*Δ exhibited greater sensitivity to propionic acid than *haa1*Δ did. Much higher concentration of propionic acid was necessary to detect the protective role of Haa1 against propionic acid (Figure 4.14).

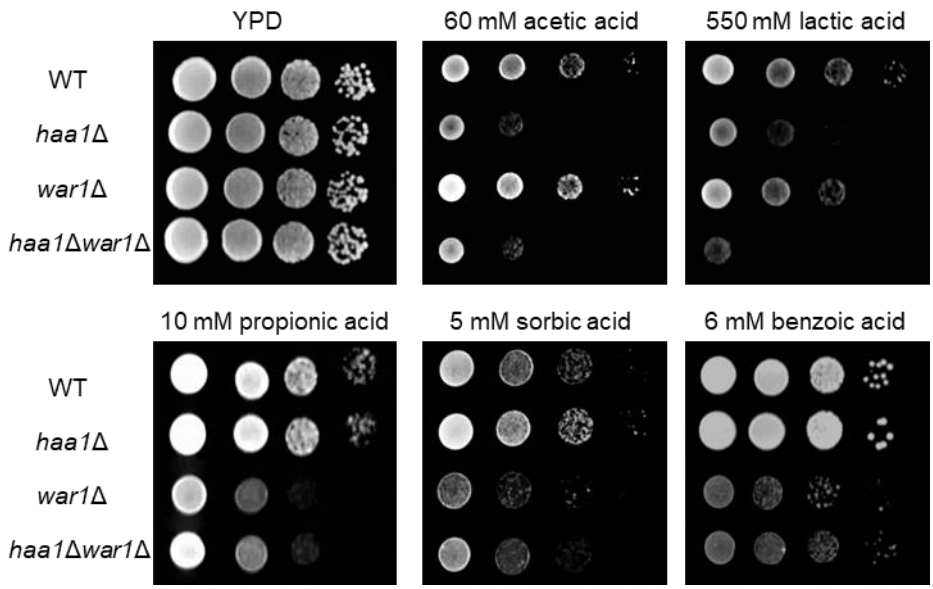



**Figure 4.13 Haa1 and War1 dependent tolerance to various weak acids.**

Wild type, *haa1Δ*, *war1Δ*, and *haa1Δwar1Δ* cells were grown in YPD medium and then $OD_{600}$ of 1 cells were serially diluted and spotted onto YPD solid medium containing the indicated weak acids.

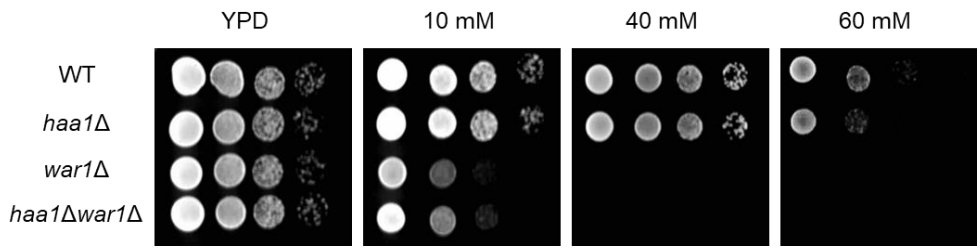



**Figure 4.14 .Growth inhibition by various concentrations of propionic acid.**

Wild type, *haa1*Δ, *war1*Δ, and *haa1*Δ*war1*Δ cells were grown in YPD medium and then $OD_{600}$ of 1 cells were serially diluted and spotted onto YPD solid medium with or without 10, 40, or 60 mM propionic acid.

For lactic acid stress, Haa1 seems to play a major role in stress tolerance, but War1 also showed minor contributions to the stress tolerance, resulting in an elevated sensitivity to lactic acid when both genes are deleted (Figure 4.13). Consequently, these results principally reach agreement with the previously suggested roles for Haa1 and War1 in weak acid stress responses, but offer more comprehensive interpretation regarding the types of weak acids involved in their activation, and their roles in defense against each weak acid.

## 4.8. Direct binding of acetate to Haa1 detected by CNF-FET sensor electrode

The fact that a wide range of weak acids can activate Haa1 and War1 may suggests a possibility of direct binding of weak acids with these transcription factors for their activation. To confirm protein-weak acid interactions, we used field-effect transistor (FET) type sensor based on carbon nanofibers (CNF-FET), which allows label-free, real-time monitoring of interaction with high sensitivity (107,108). The fabrication

process of CNF-FET biosensor electrodes are described (Figure 4.15). The electrodes include a gold microelectrode consisting of several pairs of source (S) and drain (D) electrodes, and transducer materials (CNF). To deposit transducer materials onto an electrode substrate and make a good electrical contact between them, the electrode surface was modified with aminosilane (109). CNFs are oxidized with strong acids to create carboxylic groups on the surface. The condensation reaction between amine groups on the electrode substrate and carboxylic acids of CNFs resulted in covalent immobilization of the CNFs onto the substrate. This covalent bonding strategy was also applied to attach Haa1 protein molecules on the CNF transducer. To characterize electrical properties of the CNF-FET electrodes, liquid-ion gated FET configuration was placed in PBS (pH 7.4), which offered the electrolytes that can provide solution gate control (Figure 4.16 A).

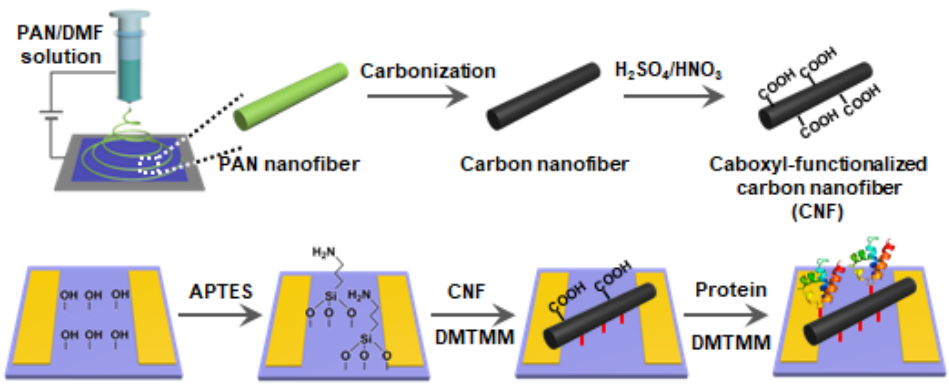



**Figure 4.15. Schematic diagram of the fabrication process of CNF-FET biosensor electrodes.** The electrode was processed with aminosilane followed oxidation with strong acid. The oxidation resulted in creation of carboxylic groups on the surface of the electrode. Recombinant proteins were attached to the electrode.

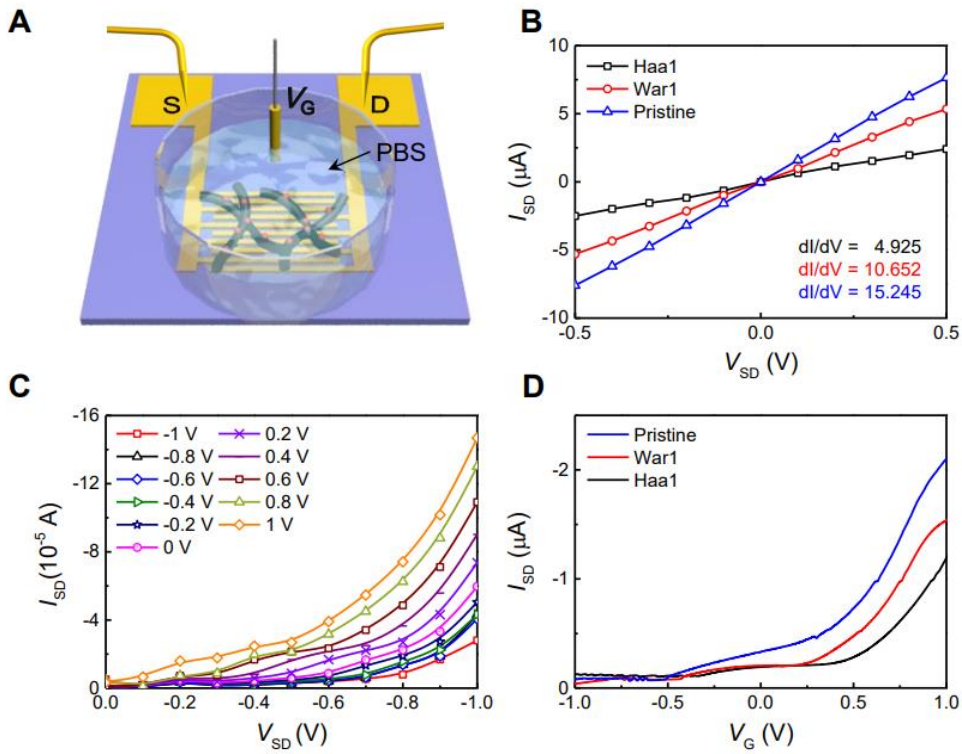

A
S
D
$V_G$
PBS
B
Haa1
War1
Pristine
dI/dV = 4.925
dI/dV = 10.652
dI/dV = 15.245
$I_{SD}$ (μA)
$V_{SD}$ (V)
C
-1 V
-0.8 V
-0.6 V
-0.4 V
-0.2 V
0 V
0.2 V
0.4 V
0.6 V
0.8 V
1 V
$I_{SD}$ ($10^{-5}$ A)
$V_{SD}$ (V)
D
Pristine
War1
Haa1
$I_{SD}$ (μA)
$V_G$ (V)

**Figure 4.16. Schematic illustration of CNF-FET biosensor with liquid-ion gate for solution measurements.**

(A) Schematic illustration of CNF-FET biosensor with liquid ion gate for solution measurements.

(B) Current-voltage (I-V) curves of CNFs on the electrode substrate before and after protein immobilization over a range of -0.5 to +0.5 V. Although dI/dV value slightly decreased after anchoring proteins on CNF, the I-V graphs maintained linear slope, indicating that stable ohmic contact was preserved during the protein attachment. It can be assumed that the contact resistance is negligible and signal change occurs only by electrostatic gating.

(C) $I_{SD}$-$V_{SD}$ output curves of CNF-FET biosensor ($V_G$ from -1.0 to +1.0 V in a step of 0.1 V at $V_{SD}$ scan rate of -100 mV). The source-drain current ($I_{SD}$) negatively increase as $V_G$ positively increases.

(D) Transfer curves of CNF-FETs with or without proteins. The transistor characteristics of protein immobilized CNT-FET device was evaluated by $I_{SD}$-$V_G$ transfer curve.

Characterization of the Haa1-functionalized CNF-FET electrodes are described (Figure 4.16 B ~ D). Both output and transfer curves show that the device exhibits n-type (electron-transporting) semiconducting characteristics. Given these results, the CNF-FET device can detect signals measure when the charge carrier density is changed by the interaction between the proteins (Haa1) and particular target molecules (acid anions). CNF-FET biosensors allowed real-time sensing measurements to confirm if Haa1 makes direct interaction with weak acids. Real-time responses of the FET type device was observed by monitoring current ($I_{SD}$) changes upon exposures of various concentrations of acetic acid solution adjusted to pH 7.0. Since p*K*a of acetic acid is 4.76, acetic acid mainly present as dissociated acetate anions in the experimental solution of pH 7.4. As a result, full-length Haa1 functionalized biosensors cleared shoed instant current rises after adding acetate solutions with concentration varying from 1 fM to 1 nM (Figure 4.17).

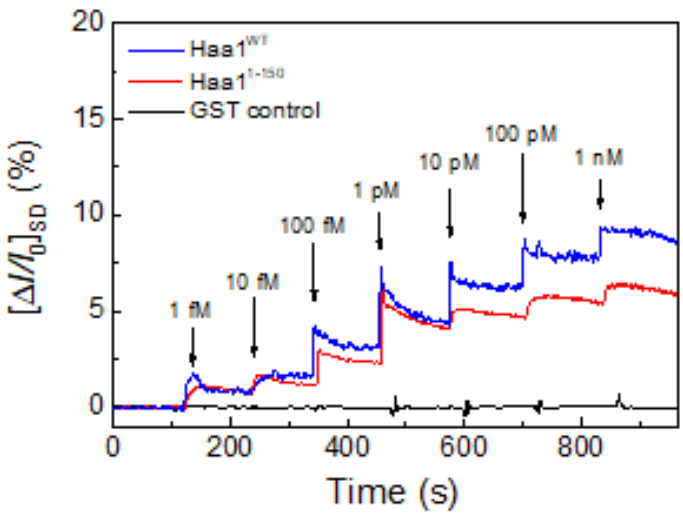



**Figure 4.17. Direct binding of acetate with full-length Haa1 or Haa1$^{1-150}$ with GST tagging.**

Receptor (Haa1$^{WT}$, Haa1$^{1-150}$, and GST control)-dependent sensing responses of CNF-FET devices to various concentrations of acetic acids (1 fM to 1 nM).

Owing to the high aspect ratio of CNFs, the response time was very short (3 s) and remarkably low concentration (1 fM) of acetate was detected in this experiment. However, the addition of acetate to the electrode functionalized with GST tag as a control did not show any perceptible changes in currents. These results confirm that direct interaction between Haa1 and acetate is the main reasons for current changes (Figure 4.17). The underlying mechanism of the current increase followed by acid injection into the CNF-FET electrode can be explained by charge carrier accumulation theory (110,111). Since CNF-FET displays the n-type semiconducting behavior, accumulated electrons on the surface of the CNF are the charge carriers. The interaction between protein (Haa1) and acid anion enhanced positive point charges near the CNF surfaces. This indirect gating effect increases the number of electrons in the CNF and thus increased $I_{SD}$.

Haa1$^{1\text{-}150}$ was also functionalized on CNF-FET electrode to test for the direct binding with acetate. Although Haa1$^{1\text{-}150}$ fused with VP16 did not show acetic acid-dependent transcriptional activation of *TPO2* (Figure 4.11), direct binding between Haa1$^{1\text{-}150}$ and acetate was observed with comparable extent of Haa1 wild-type (Figure 4.17). These results N-terminal 1-150 amino acids region of Haa1 might be

responsible for the direct binding with acetate, suggesting that Haa1 might be activated by direct sensing of weak acid anions.

## 4.9. Differential binding of weak acid anions to Haa1 and War1

To discover the different binding affinity of Haa1 with various weak acids, weak acids based on different lipophilicity were tested with CNF-FET sensor electrode. Lactic acid, sorbic acid and benzoic acid are further considered. Resembling acetate addition, when lactate, sorbate, and benzoate solutions were added, current changes were observed in a concentration-dependent manner (Figure 4.18 A and B). These results suggest that Haa1 directly binds with acid anions tested. Acetate elicited strongest sensitivity of the CNF-FET followed by relatively lowest sensitivity of lactate, benzoate, and sorbate. (Figure 4.18 A and B). These results confirm that Haa1 plays important roles towards less lipophilic acids such as acetic acid and lactic acid. By using CNF-FET sensor functionalized with War1 protein, we further examined if weak acid anions may directly bind to War1.

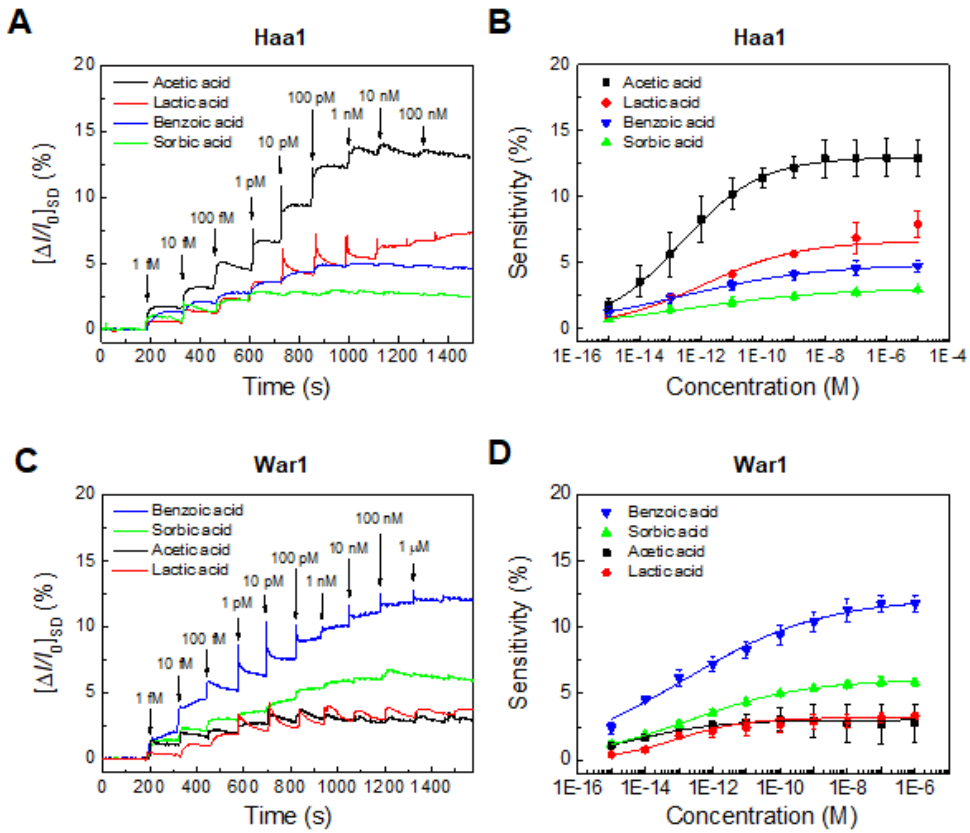

A
Haa1
Acetic acid
Lactic acid
Benzoic acid
Sorbic acid
1 fM
10 fM
100 fM
1 pM
10 pM
100 pM
1 nM
10 nM
100 nM
[ΔI/I0]SD (%)
Time (s)
B
Haa1
Acetic acid
Lactic acid
Benzoic acid
Sorbic acid
Sensitivity (%)
Concentration (M)
C
War1
Benzoic acid
Sorbic acid
Acetic acid
Lactic acid
1 fM
10 fM
100 fM
1 pM
10 pM
100 pM
1 nM
10 nM
100 nM
1 μM
[ΔI/I0]SD (%)
Time (s)
D
War1
Benzoic acid
Sorbic acid
Acetic acid
Lactic acid
Sensitivity (%)
Concentration (M)

**Figure 4.18. Real-time response of Haa1- or War1-functionalized CNF-FET biosensor.**

**(A)** Real-time response of Haa1-functionalized CNF-FET biosensors to various concentrations of acetic acid, lactic acid, sorbic acid, and benzoic acid (1 fM to 100 nM).

**(B)** Concentration-dependent responses to acetic acid, lactic acid, sorbic acid, and benzoic acid with normalized sensitivity. Normalized sensitivity indicates the saturation level of $[I/I_0]_{SD} \times 100$ measured after exposure to the acid analyte. The real time response measurement was repeated three times.

**(C)** Real-time response of War1 functionalized CNF-FET biosensor to various concentrations of acetic acid, lactic acid, sorbic acid, and benzoic acid (1 fM to 100 nM).

**(D)** Concentration-dependent responses to acetic acid, lactic acid, sorbic acid, and benzoic acid with normalized sensitivity. The real time response measurement was repeated three times.

Like the Haa1-funtionalized CNF-FET sensor, concentration-dependent changes in currents ($I_{SD}$) were observed when weak acid anions were applied to the War1-funtionalized CNF-FET sensor, suggesting their direct binding to War1 (Figure 4.18 C and D). Contrary to Haa1, War1 displayed the strongest sensitivity towards benzoate followed by sorbate. However, acetate and lactate exhibited much lower sensitivities towards War1. These results suggest that War1 may prefer more lipophilic weak acid anions. Even if acetic acid failed to elicit transcriptional activation of War1 target genes *in vivo* (Figure 4.11), War1 was still able to show weak current changes ($I_{SD}$) in response to acetate (Figure 4.18 C and D). Discrepancy shown between transcriptional activation and CNF-FET may suggest that binding between acetate and War1 is relatively less significant compared to the binding of other weak acids. Nevertheless, it is still possible that War1 might bind with acetate with low binding affinity. However, this binding is not able to cause significant conformational changes required for the activation of War1. Taken together, direct binding of weak acid anions might activate Haa1 and War1 with different sensitivity due to different structures of weak acid anions. This differential activation caused by weak acid anions may lead to proper

cellular responses against the weak acids.

## 4.10. Conclusions

In this chapter, it has been shown that transcription factors Haa1 and War1 in *S. cerevisiae* are shown to be critical for cellular adaptation against weak organic acids. Haa1 is known to play protective roles for more hydrophilic weak acids, whereas War1 is for more lipophilic acids. Nevertheless, regulatory mechanisms where Haa1 and War1 are activated in response to weak acids have remained largely unknown. By using filed-effect transistor (FET) type biosensor based on carbon nanofibers, we show that Haa1 and War1 can directly bind to various weak acid anions with varying affinities. It has been revealed that Haa1 shows highest sensitivity towards acetate followed by lactate. In contrast, War1 shows highest sensitivity towards benzoate followed by sorbate (Figure 4.19). We also show that the N-terminal Zn-binding domain of Haa1 is critical for its DNA binding affinity in acetic acid stress dependent manner. Haa1 N-terminal region up to the 150-amino acid residue is responsible for its direct binding with acid anions, but additional 150-180 amino acids are required to elicit transcriptional

activation of Haa1 target genes. For proper expression of target genes, the activation domain of Haa1 spanning amino acids 230 through 483 is necessary. Therefore, direct binding of acetate with Haa1 may cause conformational changes to convert Haa1 from inactive Haa1 to an active form that is capable of DNA binding and transcriptional activation.

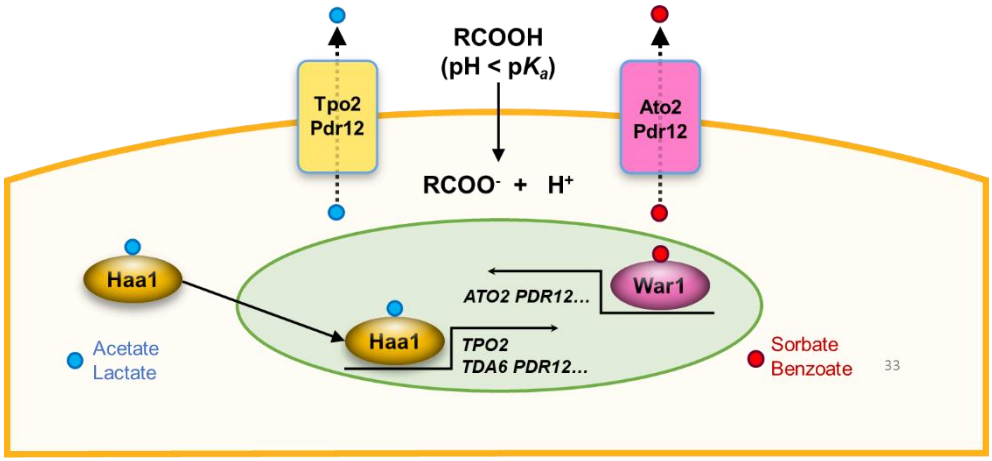



**Figure 4.19. Graphical summary.**

Haa1 makes direct contact with less lipophilic acids such as acetic acid or lactic acid to induce transcriptional activation of its target genes including *TPO2*, *TPO3*, and *PDR12*. On the contrary, War1 directly interacts with more lipophilic acids such as propionic acid, sorbic acid, and benzoic acid to induce transcriptional activation of its target genes including *ATO2* and *PDR12*. Transcription of these genes contributes to adaptation and survival of yeast cells in response to weak organic acids.

# Chapter 5.

# Role of CK2-dependent phosphorylation of Ifh1 and Crf1 in transcriptional regulation of ribosomal protein genes in *S. cerevisiae*

## 5.1. Introduction

A miniscule cellular machine that is comprised of RNA and ribosomal proteins found in cytoplasm brings mRNA and tRNA together to synthesize proteins. In *S. cerevisiae,* a single ribosome is composed of 4 rRNAs, 5S, 5.8S, 18S, and 25S, and 79 ribosomal proteins (45-47). rRNAs are transcribed by RNA polymerase I and III followed by extensive cleavages by endo-and exonucleases (48,51,52). Furthermore, ribosomal protein genes are transcribed by RNA polymerase II. It is well known that ribosomal proteins play important roles such as trimming of rRNAs, exports of premature ribosomes, and surveillance of malfunctioning ribosomes (53). Generation of ribosome is very complex and energy consuming processes. More than 76 small nucleolar RNAs and 200 assembly factor are required to make ribosome and yeast cells generate about 2,000 ribosomes per minute to maintain robust cell growth.

Transcription of ribosomal protein genes are mainly dependent on TORC1, target of rapamycin complex 1, and PKA, protein kinase A. TORC1 and PKA are nutrient-sensing master kinases and well known for the regulation in response to nutrient availability and cellular

stresses (56,57). TORC1 promotes cell growth and proliferation including ribosome biosynthesis. However, upon inhibition of TORC1, transcription of ribosomal protein genes is completely abolished (58). Down-regulation of ribosomal protein gene expression caused by inhibition of TORC1 can be rescued by hyperactivation of PKA (36,57,112).

In this chapter, regulatory mechanisms involving RP gene transcription factors such as Ifh1 and Crf1 are examined. Protein kinase CK2, which is closely related with cell proliferation is known to phosphorylate Ifh1 *in vitro* (65). We propose evidences that CK2 phosphorylates Ifh1 and Crf1 at T681 and T348, respectively. These phosphorylation is important for interaction with the FHA domain of Fhl1 followed by regulation of RP gene expression in response to nutrient availability. We also propose that regulatory subunits of CK2 mediate interaction between CK2 subunits and target proteins. Absence of regulatory subunits cause significantly reduced CK2 dependent phosphorylation of Ifh1, which led to reduced interaction with Fhl1.

## 5.2. CK2 phosphorylates Ifh1 and Crf1

The FHA domain of Fhl1 is well known as a phosphopeptide-binding module. This module highly prefers phosphothreonine followed by acidic amino acid residues at +3 position on its interaction partners (74,75,113). Furthermore, Ifh1 is phosphorylated by CK2 *in vitro* (72), whose consensus phosphorylation site (S/T x x D/E) overlaps with the recognition site of the FHA domain (114). These results suggested that CK2 phosphorylated Ifh1 might bind to the FHA domain of Fhl1. Therefore, possible phosphorylation sites in the FHB domain of Ifh1 were closely examined. To this end, protein sequences of Ifh1s from different yeast species were compared. Well conserved protein sequences that satisfies criteria for both the phosphopeptide-binding module and the CK2 consensus site were centered on Ifh1 T681 of *S. cerevisiae* (Figure 5.1). Although Ifh1 orthologs are present in all *Ascomycota* fungi, Crf1 are found only in species after whole genome duplication (WGD) event (115).

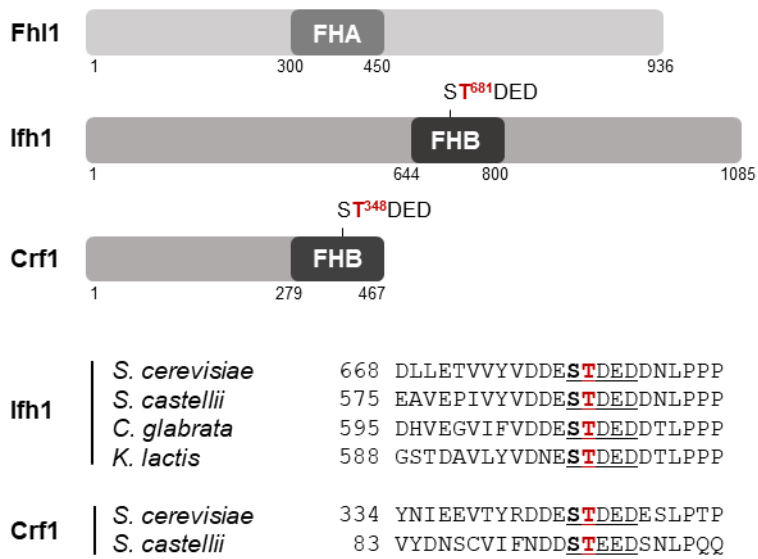



**Figure 5.1. Protein sequences of Ifh1 and Crf1 around FHB domains**.

Organization of the FHA domain in Fhl1 and its interaction partner, FHB domains, in Ifh1 and Crf1. Multiple amino acid sequence alignment of the FHB domains of Ifh1 and Crf1 is shown, where the highly conserved STD/EED motif, which satisfies both FHA recognition site (pT x x D/E) and CK2 consensus site (S/T x x D/E) is underlined.

In yeast post-WGD species such as *S. cerevisiae* and *S. castellii*, the CK2 consensus site is also conserved in the FHB domain of Crf1. T681 of Ifh1 and T348 of Crf1 were highly expected to be phosphorylated by CK2 and recognized by the FHA domain of Fhl1 (Figure. 5.1). We further examined T681 and T348 can be actually phosphorylated by the catalytic subunit of CK2 *in vitro*. Recombinant proteins purified from *E. coli*, GST-Ifh1 (644-710) and GST-Crf1 (280-370), were phosphorylated by CK2 (Figure. 5.2).

The phosphorylation status of GST-Ifh1 (644-710) showed no significant reduction with single mutation, S680A or T681A. However, double mutation, S680A T681A, completely failed to be phosphorylated by CK2 (Figure. 5.2). Ifh1 fragments besides the FHB domain of Ifh1 were also phosphorylated by CK2, suggesting the numerous CK2 phosphorylation sites in Ifh1. Phosphorylation of GST-Crf1 was also observed and double mutations, S347A T348A, failed to show significant phosphorylation levels as expected (Figure 5.2). We next examined the phosphorylation of Ifh1 and Crf1 by CK2 using antibodies specific against CK2-phosphorylated substrates (pTDxE). This antibody still recognized Ifh1 (644-

710)$^{S680A}$ and Crf1 (280-370)$^{S374A}$ mutants that had been phosphorylated by CK2, but not Ifh1 (644-710)$^{T681A}$ and Crf1 (280-370)$^{T378A}$ mutants, indicating that this antibody can specifically recognize CK2-dependent phosphorylation of Ifh1 T681 and Crf1 T348 (Figure. 5.3).

CK2 dependent phosphorylation of Ifh1 and Crf1 confirmed by phospho-CK2 substrate antibody was completely abolished when a CK2-specific inhibitor, tetrabromobenzotriazole (TBBt), was treated with Cka2, confirming the CK2-specific phosphorylation events (Figure. 5.4).

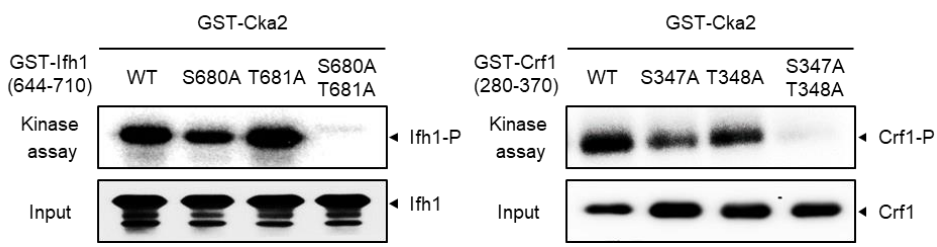



**Figure 5.2. FHB domains of Ifh1 and Crf1 are phosphorylated by CK2.**

Identification of CK2-dependent phosphorylation sties in Ifh1 and Crf1. Wild-type or mutant GST-Ifh1 (644–710) and GST-Crf1 (280–370) proteins purified from *E. coli* were phosphorylated by GST-Cka2 purified from *S. cerevisiae* in the presence of [γ32P]-ATP.

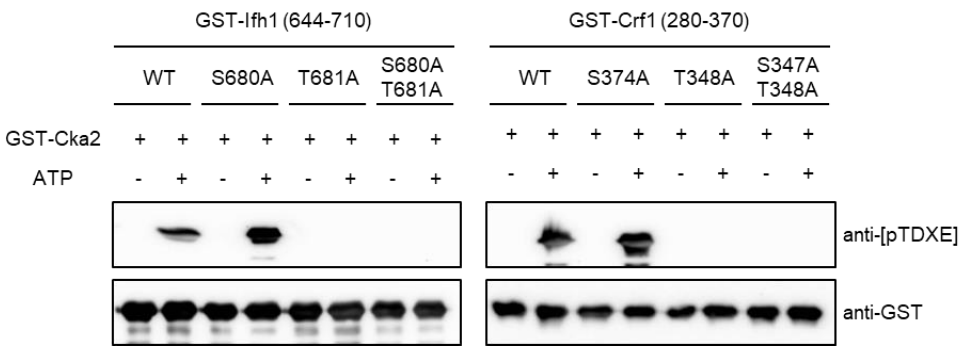



**Figure 5.3. CK2 phosphorylation of Ifh1 and Crf1 confirmed by phospho-CK2 substrate antibodies.**

Detection of *in vitro* phosphorylation of Ifh1 and Crf1 by using *anti*-phospho-CK2 substrate antibody. After *in vitro* phosphorylation of the indicated GST-Ifh1 (644–710) and GST-Crf1 (280–370) proteins by GST-Cka2, input and phosphorylated proteins were detected by Western blotting with *anti*-GST antibody and *anti*-phospho-CK2 substrate antibody raised against pTDxE, respectively.

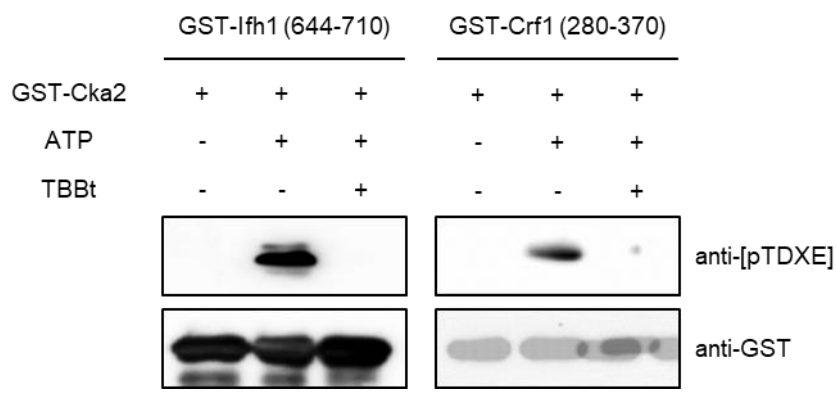



**Figure 5.4. CK2-specific inhibitor, TBBt, abolishes phosphorylation of Ifh1 and Crf1 by CK2.**

*in vitro* phosphorylation was performed with GST-Cka2 in the presence of 10 μM TBBt or an equal volume of DMSO as a control, and input and phosphorylated proteins were detected by Western blotting.

## 5.3. Phosphorylation of Ifh1 T681 and Crf1 T348 is required for interaction with Fhl1

We next tested if CK2 dependent phosphorylation of the FHB domain of Ifh1 is required for its interaction with the FHA domain of Fhl1 *in vitro.* T7-Fhl1 (270-570) and GST-Ifh1 (400-800) proteins were purified from *E. coli* lacking CK2 activity. GST pull-down assay only with prior phosphorylation by CK2 successfully showed interaction between GST-Ifh1 (400-800) and T7-Fhl1 (270-570), demonstrating the role played by the CK2-dependent phosphorylation of Ifh1 in Fhl1 binding (Figure 5.5). Furthermore, CK2 dependent interaction between Fhl1 and Ifh1 or Crf1 *in vivo* was examined along with mutations on Ifh1 T681 or Crf1 T348. Cell lysates were prepared from *S. cerevisiae* cells co-expressing T7-Fhl1 (270-570) with wild-type or mutant GST-Ifh1 (400-800), and GST pull-down experiments were carried out. Unlike GST-Ifh1 (400-800) purified from *E. coli* (Figure. 5.6), GST-Ifh1 (400-800) from yeast cell lysates showed a specific interaction with T7-Fhl1 (270-570) (Figure. 5.7).

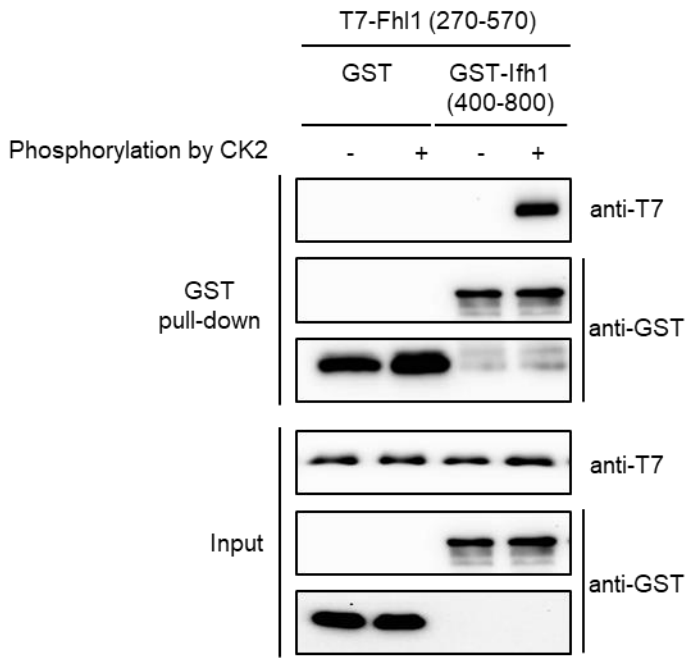



**Figure 5.5. Interaction between GST-Ifh1 (400-800) and T7-Fhl1 (270-570) *in vitro*.**

GST-Ifh1 (400–800) and T7-Fhl1 (270–570) proteins were purified from *E. coli*, and GST pull-down assay was performed in the absence or presence of prior *in vitro* phosphorylation of GST-Ifh1 (400–800) by CK2. GST protein was used as a control.

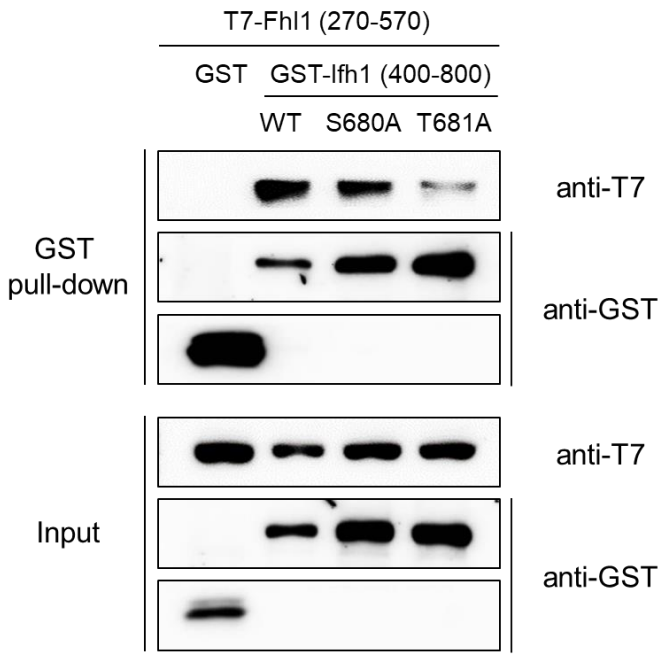



**Figure 5.6. Interaction between GST-Ifh1 (400-800) and T7-Fhl1 (270-570) *in vivo*.**

*S. cerevisiae* BY4741 cells co-expressing T7-Fhl1 (270–570) and the indicated form of GST-Ifh1 (400–800) were grown in SC-His-Ura medium to exponential phase, and the cell lysates were subjected to GST pull-down assay. Cells expressing GST and T7-Fhl1 (270–570) were used as a control.

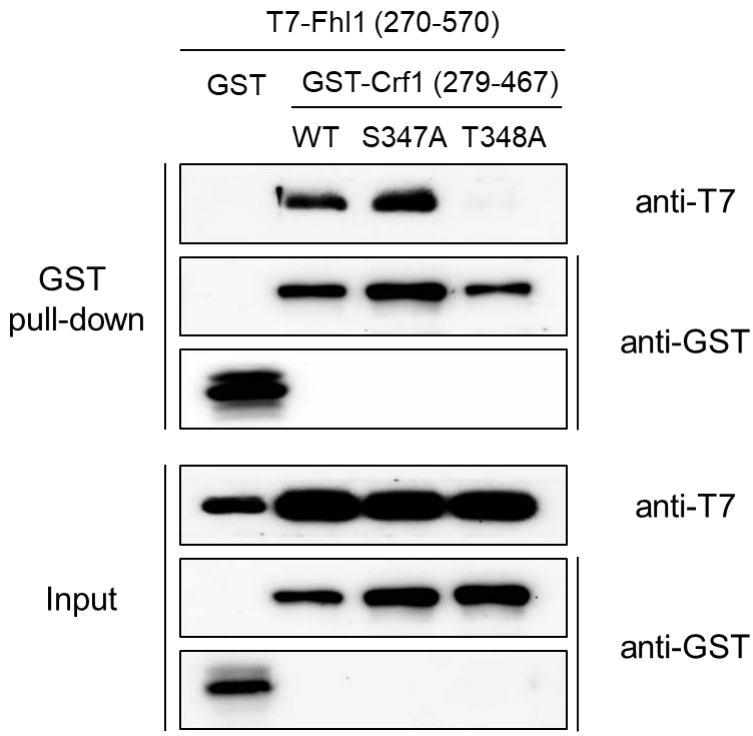



**Figure 5.7. Interaction between GST-Crf1 (279-467) and T7-Fhl1 (270-570) *in vivo*.**

GST pull-down assay was performed with cells co-expressing T7-Fhl1 (270–570) and the indicated form of GST-Crf1 (279–467).

When the phosphorylation site mutants were examined, GST-Ifh1 (400-800)$^{T681A}$, but not GST-Ifh1 (400-800)$^{S680A}$, showed significantly reduced interaction with T7-Fhl1 (270-570) (Figure 5.6). These results indicate that CK2-dependent phosphorylation site T681 of Ifh1 play important roles for interaction with Fhl1, even though both S680 and T681A can be phosphorylated by CK2. Remaining weak interaction signals between Fhl1 and Ifh1$^{T681A}$ might be caused by non-specific interaction, but we cannot rule out the possibility that Fhl1 still interacts with Ifh1 through sites other than T681. The same results were also observed for Crf1-Fhl1 interaction. Only T348A mutation, and not S347A mutation, of GST-Crf1 (279-467) significantly reduced the interaction with T7-Fhl1 (270-570) (Figure 5.7). These results support our hypothesis that CK2-dependent phosphorylation of Ifh1 T681 and Crf1 T348 provides the binding sites for Fhl1.

## 5.4. Regulatory subunits of CK2 regulate the interaction between Fhl1 and Ifh1

It is well known that CK2 consists of two catalytic subunits and two

regulatory subunits. These subunits form a heterotetramer. *S. cerevisiae* has *CKA1* and *CKA2* as the catalytic subunits and *CKB1* and *CKB2* as the regulatory subunits (114). Since CK2 phosphorylates Ifh1, we examined the interaction between Ifh1 and subunits of CK2 by yeast two-hybrid assay. The FHB domain of Ifh1, Ifh1 (644-800), clearly showed interaction with regulatory subunits of CK2. This assay showed stronger interaction with Ckb1 rather than CKb2. This result indicate that the regulatory subunits might play important roles bringing CK2 targets, in this case Ifh1, to phosphorylate (Figure 5.8).

This result is in agreement with the proposed role of the CK2 regulatory subunit as a multi-substrate docking platform regulating substrate selectivity as well as catalytic activity and stability of CK2 tetramer (116,117). Subunits of CK2 for this assay were expressed at comparable levels, but interaction between catalytic subunits and Ifh1 was not detected, but interaction between the catalytic subunits and Ifh1 was not detected in our yeast two-hybrid assay system (Figure 5.9).

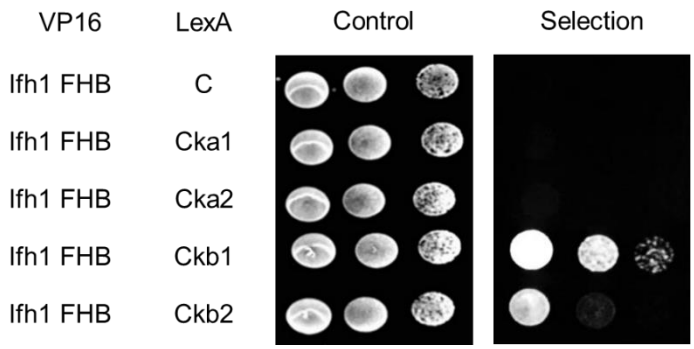



**Figure 5.8. Interaction between regulatory subunits of CK2 and Ifh1 by yeast two-hybrid assay.**

Interaction between the FHB domain of Ifh1 and CK2 subunits detected by yeast two-hybrid assay. *S. cerevisiae* strain L40 expressing VP16-fused Ifh1 FHB domain (644–800) and LexA-fused subunits of CK2 (Cka1, Cka2, Ckb1, and Ckb2) were serially diluted and spotted on a control plate medium containing histidine and a selection plate medium containing 1 mM 3-aminotriazole (3-AT) but lacking histidine to detect protein-protein interaction.

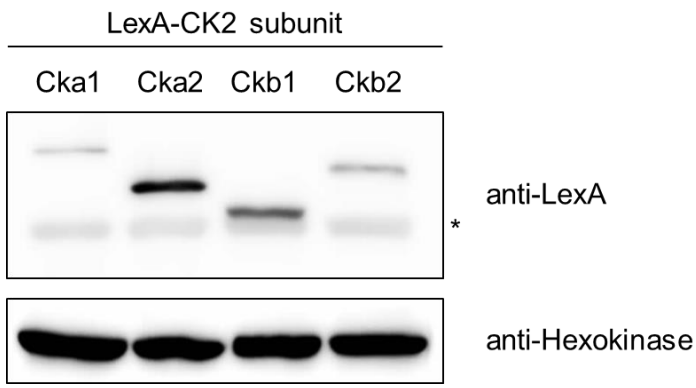



**Figure 5.9. Expression levels of CK2 subunits for yeast two-hybrid assay.**

Protein levels of LexA-CK2 fusion proteins were examined by immunoprecipitation followed by Western blotting with *anti*-LexA antibody. The asterisk denotes the heavy chain of the antibody that was used for immunoprecipitation.

In agreement with yeast two-hybrid assay, Ifh1 immunoprecipitated from cells lacking regulatory subunits clearly showed severely reduced CK2-dependent phosphorylation detected by phospho-CK2 substrate antibody when compared that of wild-type Ifh1 (Figure 5.10). Furthermore, interaction between Ifh1 and Fhl1 was reduced significantly in *ckb1Δckb2Δ* compared to that in wild-type cells (Figure 5.11). Reduced interaction between Fhl1 and Ifh1 caused by abolishment of CK2-dependent phosphorylation of Ifh1 resulted in reduced DNA binding affinity of Ifh1, but not Fhl1, to the RP gene promoters (Figure 5.12 A and B). Protein levels of both Ifh1 and Fhl1 were also checked and they are expressed in comparable levels (Figure 5.12 C). Taken together, these results suggest that binding of the regulatory subunits of CK2 to Ifh1 might be responsible for CK2-dependent phosphorylation of Ifh1, and its subsequent binding to Fhl1.

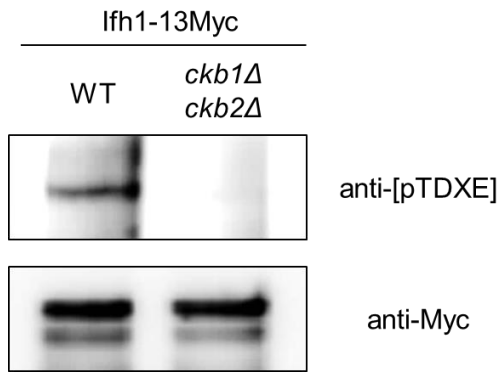



**Figure 5.10. Phosphorylation levels of Ifh1 confirmed by phospho-CK2 substrate antibody.**

Reduced phosphorylation levels of Ifh1 in *ckb1Δckb2Δ* strain. Wild-type and *ckb1Δckb2Δ* strains where Ifh1 is tagged with 13Myc and Fhl1 is tagged with 3HA in the genome were grown in YPD medium to exponential phase, and the cell lysates were immunoprecipitated with anti-Myc antibody. Total and phosphorylated levels of Ifh1-13Myc protein were detected by Western blotting with *anti*-Myc and *anti*-phospho-CK2 substrate antibody, respectively.

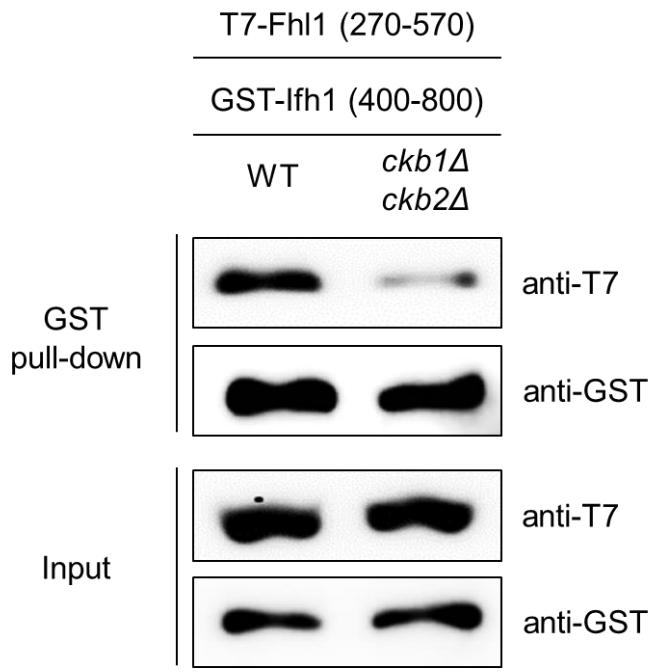
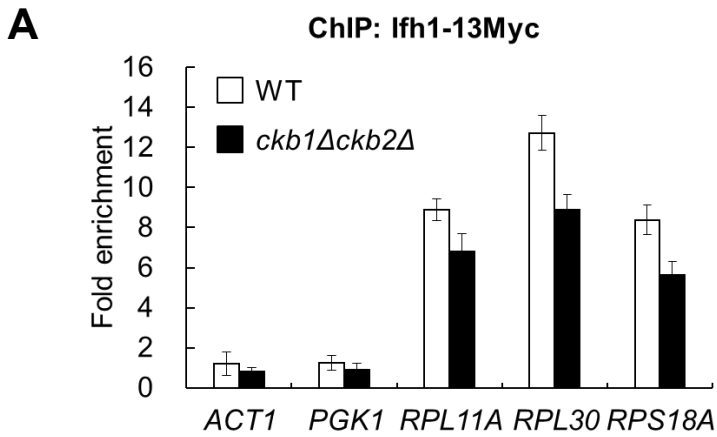
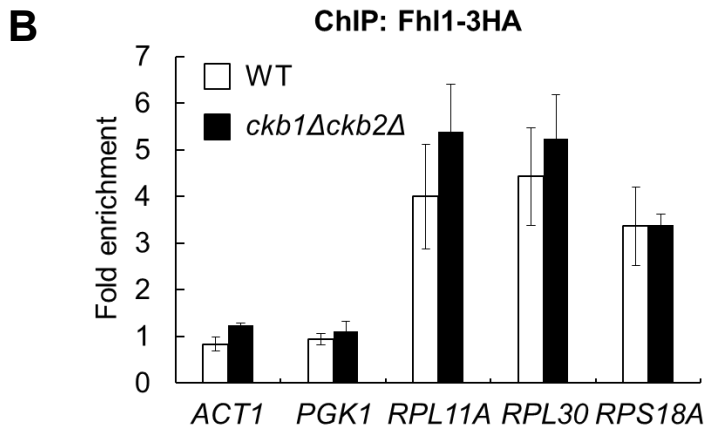



**Figure 5.11. Interaction between Fhl1 and Ifh1 in wild-type or *ckb1Δckb2Δ*.**

Reduced interaction between Ifh1 and Fhl1 in *ckb1Δckb2Δ* strain. Wild-type and *ckb1Δckb2Δ* cells expressing GST-Ifh1 (400–800) and T7-Fhl1 (270–570) were grown in SC-His-Ura medium, and cell lysates were subjected to GST pull-down assay.


A
ChIP: Ifh1-13Myc
WT
ckb1Δckb2Δ
Fold enrichment
16
14
12
10
8
6
4
2
0
ACT1
PGK1
RPL11A
RPL30
RPS18A


B
ChIP: Fhl1-3HA
WT
ckb1Δckb2Δ
Fold enrichment
7
6
5
4
3
2
1
0
ACT1
PGK1
RPL11A
RPL30
RPS18A

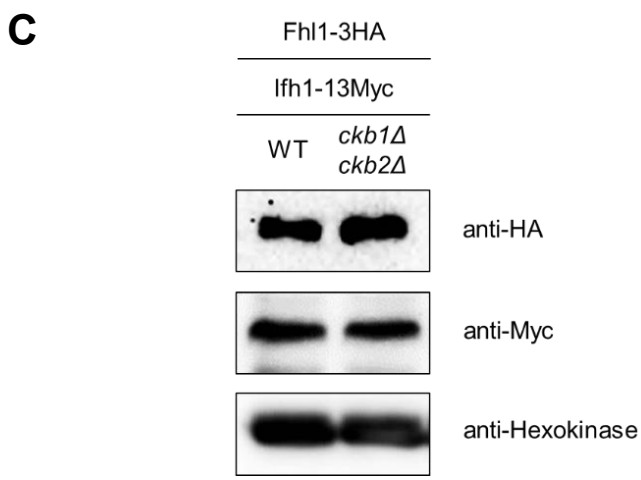

C
Fhl1-3HA
Ifh1-13Myc
WT
ckb1Δ
ckb2Δ
anti-HA
anti-Myc
anti-Hexokinase

**Figure 5.12. DNA binding affinity of Fhl1 and Ifh1 in wild-type or *ckb1Δckb2Δ* to the RP gene promoters.**

**(A and B)** Reduced binding of Ifh1 at the RP gene promoters in *ckb1Δckb2Δ* strain. Wild-type and *ckb1Δckb2Δ* strains were grown in YPD medium, and ChIP assay was performed with *anti*-Myc and *anti*-HA antibodies. Ifh1-13Myc and Fhl1-3HA occupancies at the promoters are indicated as fold enrichment relative to the untagged control from three independent experiments. Error bars indicate SD. The *ACT1* and *PGK1*promoters were used as control promoters.

**(C)** Protein levels of Ifh1-13Myc and Fhl1-3HA in wild-type and *ckb1Δckb2Δ* strains were detected by Western blotting. Hexokinase levels were used as a loading control.

## 5.5. Impaired activation of RP gene transcription by Ifh1 T681A

Having established that Ifh1$^{T681}$ can be phosphorylated by CK2 and this phosphorylation play critical roles for interaction with Fhl1, we next examined whether Ifh1$^{T681}$ causes any changes in transcriptional activation of RP genes. To this end, strains expressing either wild-type or T681A mutant of Ifh1-13Myc under the control of its own promoter were generated followed by deletion of endogenous *IFH1*. In addition, Fhl1 was chromosomally tagged with 3HA. These two strains exhibited significantly different growth rates. Cells expressing Ifh1$^{T681A}$-13Myc showed significantly lower specific growth rates rate than did cells expressing wild-type Ifh1-13Myc at 30°C ($\mu_{WT}$ = 0.306 ± 0.003 $h^{-1}$, $\mu_{T681A}$ = 0.247 ± 0.001 $h^{-1}$) and 37°C ($\mu_{WT}$ = 0.423 ± 0.003 $h^{-1}$, $\mu_{T681A}$ = 0.358 ± 0.009 $h^{-1}$) (Figure 5.13). DNA binding affinity detected by chromatin immunoprecipitation showed significantly reduced binding levels of Ifh1$^{T681A}$-13Myc to RP gene promoters compared to those of wild-type Ifh1-13Myc (Figure 5.14 A).

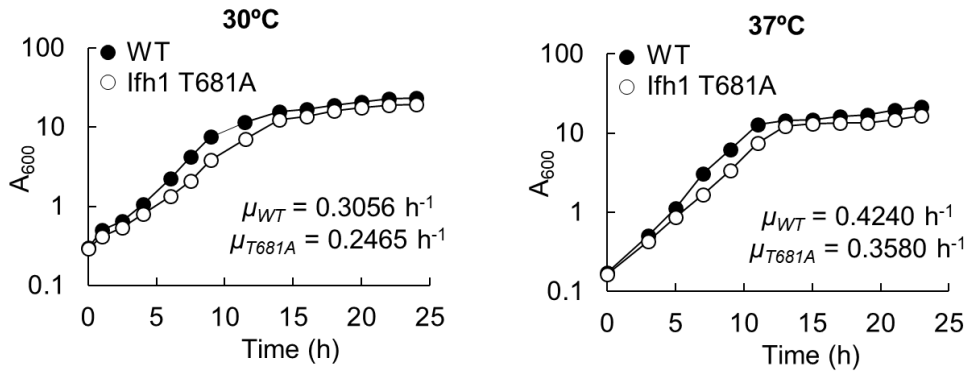



**Figure 5.13. Comparison of growth rates between Ifh1$^{T681}$ and Ifh1$^{T681A}$.**

Cells expressing Ifh1-13Myc and Ifh1$^{T681A}$-13Myc were grown in YPD medium and monitored for cell growth at 30 °C, 37 °C. Specific growth rates were indicated.

About half of RP genes are constitutively occupied by chromatin associated high mobility group family member, Hmo1. For this region, three Hmo1-independent genes (*RPS31*, *RPL11A*, and *RPS2*), and 4 Hmo1-dependent genes (*RPS18A*, *RPS22A*, *RPL30*, and *RPL2A*) were examined to clarify the relationship with Hmo1 and Fhl1/Ifh1. Although the fold enrichment of Ifh1 occupancy to the promoters were variable from 3.8 to 11.5 relative to the untagged control, Ifh1$^{T681A}$ mutant showed 2.1- to 3.0-fold decrease in binding at the promoters compared to Ifh1 wild type (Figure 5.14 B). DNA binding of Fhl1 was neither dependent of Ifh1 mutation nor Hmo1 presence. The remaining DNA affinity of Ifh1$^{T681A}$ might be caused by residual interaction between Ifh1 and other transcription regulators such as Rap1 (65). Furthermore, there is still possibility that Ifh1 interacts with Fhl1 through regions other that involving T681. On the contrary, DNA binding affinities of Fhl1 was not changed in cells expressing Ifh1$^{T681A}$-13Myc, suggesting that reduced interaction between Ifh1$^{T681}$ and Fhl1 is the main reason for the decreased DNA binding affinities for Ifh1T681 to RP gene promoters (Figure 5.14 A).

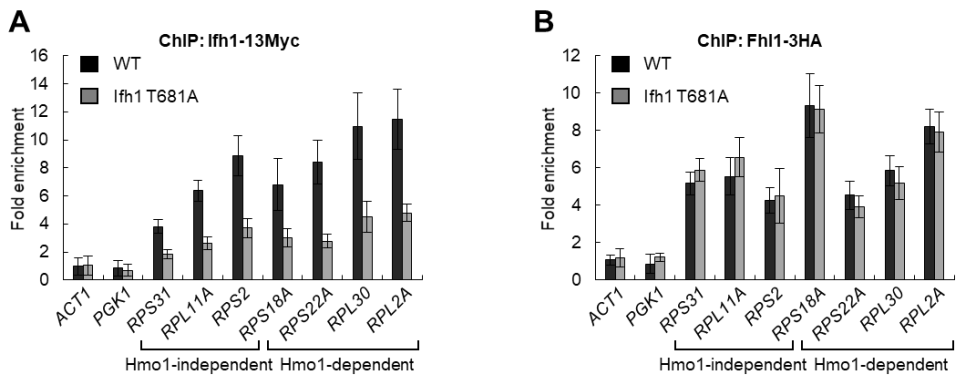



**Figure 5.14. DNA binding affinities of Fhl1 and Ifh1$^{WT/T681A}$.**

**(A)** Association of Ifh1-13Myc and Ifh1$^{T681A}$-13Myc at the RP gene promoters was detected by ChIP with *anti*-Myc antibody.

**(B)** Association of Fhl1-3HA at the RP gene promoters was detected by ChIP with *anti*-HA antibody in strains JHY805 and JHY806.

We further inspected whether the reduced DNA binding affinities Ifh1$^{T681A}$ to RP gene promoters led to reduced transcriptional activation of RP genes. As expected from the growth defects (Figure 5.12), cells expressing Ifh1$^{T681A}$ exhibited significant decreases in transcriptional activation of RP genes when compared to wild-type control (Figure 5.15 A). The residual transcription levels of RP genes in Ifh1$^{T681A}$ might be mediated by other transcription factors such as Sfp1 and remaining interaction between Fhl1 and Ifh1$^{T681A}$. Cells expressing Ifh1$^{T681A}$ showed reduced RP gene mRNA levels by 1.2- to 2.6-fold relative to wild type. The Ifh1$^{T681A}$-dependent fold reduction in RP gene transcription levels showed a correlation with the fold reduction in DNA binding of Ifh1 at RP gene promoters, meaning a significant contribution of Ifh1 binding to Fhl1 in RP gene transcription (Figure 5.15 B).

Upon inhibition of TORC1 by treatment of rapamycin, expressions of *RPL11A* and *RPL30* were reduced in both wild-type and Ifh1$^{T681A}$ mutant strains (Figure 5.16), reflecting the contribution of other transcription factors such as Sfp1 in transcriptional regulation of RP genes (60,118).

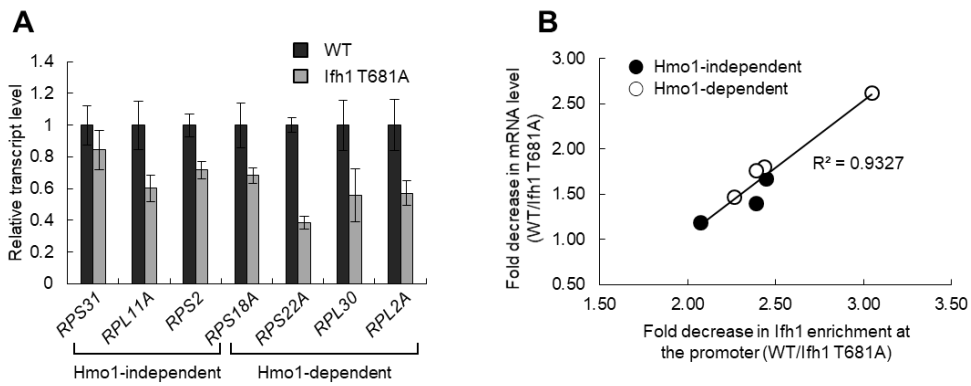



**Figure 5.15. RP gene expression caused by either Ifh1 T681 or T681A.**

**(A)** Transcription levels of RP genes for Ifh1 T681 and T681A were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*. Relative transcription levels were shown for each RP genes. Each value indicates the average ± SD of triplicate experiments.

**(B)** The fold decrease in Ifh1 binding at the RP gene promoter (WT/T681A) was plotted *versus* the fold decrease in RP gene transcription level (WT/T681A).

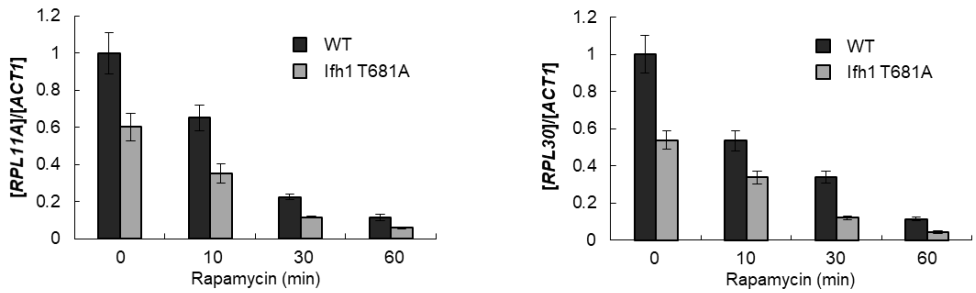



**Figure 5.16. Reduction of RP gene expression caused by rapamycin in cells expressing either Ifh1 T681 or T681A.** Cells expression Ifh1 T681 or T681A were grown in YPD medium to exponential phases and treated with 200 ng/ml of rapamycin for the indicated times. mRNA levels of the RP genes were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*

## 5.6. Impaired repression of RP gene transcription by Crf1 T348A

Crf1 is known to be dispensable in some yeast strains and competes with Ifh1 to bind to Fhl1. For this reason, deletion of *CRF1* results in no alterations in RP gene transcription in BY4741 strain. However, it has been shown that Crf1 mediates RP gene transcription in TB50 strains (72). Therefore, TB50 strain was exploited for this reason to examine the role of Crf1 in the repression of RP gene transcription in response to TORC1 inhibition. *CRF1* deletion strain was generated followed by complementation of either $Crf1^{T348}$ or $Crf1^{T348A}$. Defects caused by *CRF1* deletion in response to rapamycin treatment was repaired by introducing a plasmid expressing wild-type Crf1 from its own promoter into *crf1Δ*. However, expression of $Crf1^{T348A}$ was not as efficient as that of wild-type Crf1 in rescuing the defects of *crf1Δ* in RP gene repression upon rapamycin treatment (Figure 5.17). These results demonstrate that CK2-dependent phosphorylation of Crf1 T348 is necessary for its Fhl1 binding to repress RP gene transcription.

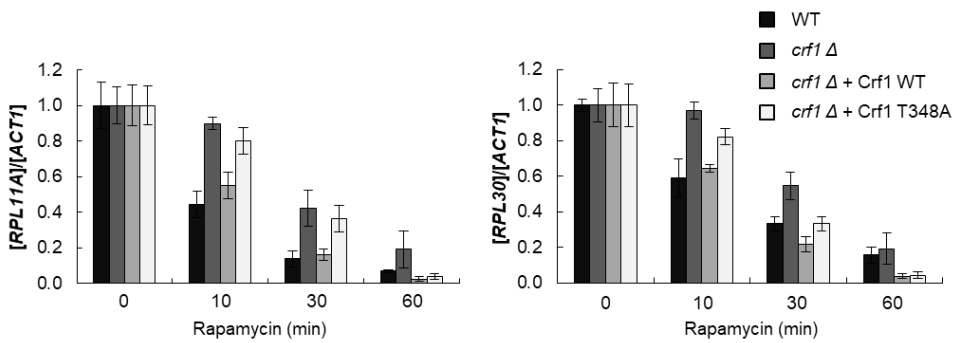



**Figure 5.17. Impaired repression of RP gene transcription by Crf1$^{T348A}$.**

TB50 wild type and *crf1*Δ expressing empty vector, and *crf1*Δ expressing wild type or T348A mutant of Crf1 were grown in YPD medium to exponential phases and treated with 200 ng/ml of rapamycin for the indicated times. mRNA levels of RP genes were measured by qRT-PCR and normalized to the mRNA levels of *ACT1*.

## 5.7. Conclusion

In this chapter, it has been shown that transcription co-activator Ifh1 and co-repressor Crf1 are involved in the regulation of ribosomal protein (RP) genes in *S. cerevisiae*. Ifh1 and Crf1 interact with transcription regulator Fhl1, which is constitutively bound at the RP gene promoters. Fhl1 interacts with Ifh1 in nutrient rich conditions, whereas Fhl1 interacts with Crf1, which translocates into the nucleus in nutrient limiting conditions. Interaction of Fhl1 is achieved through a forkhead-associated (FHA) domain of Fhl1 with FHB domains of either Ifh1 or Crf1. Furthermore, the FHA domain is known as phosphopeptide-binding module and binds with phosphothreonine followed by acidic amino acid residues at +3 position on its interaction partners. CK2–dependent phosphorylation of Ifh1 T681 and Crf1 T348 plays critical roles for interaction with the FHA domain of Fhl1. Cells expressing Ifh1 T681A exhibited significant reduction of association of Ifh1 at the RP gene promoters. Reduction of Ifh1 recruitment onto RP gene promoter led to the reduced transcriptional activation of RP genes. On the contrary, cells expressing Crf1$^{T348A}$ failed to repress RP gene transcription upon inhibition of target of rapamycin complex 1

(TORC1) caused by rapamycin treatment. Taken together, these results suggest the mechanisms underlying RP gene regulation in *S. cerevisiae through* CK2-dependent phosphorylation of Ifh1 and Crf1 and interaction with Fhl1 in response to nutrient availability and stresses (Figure 5.18).

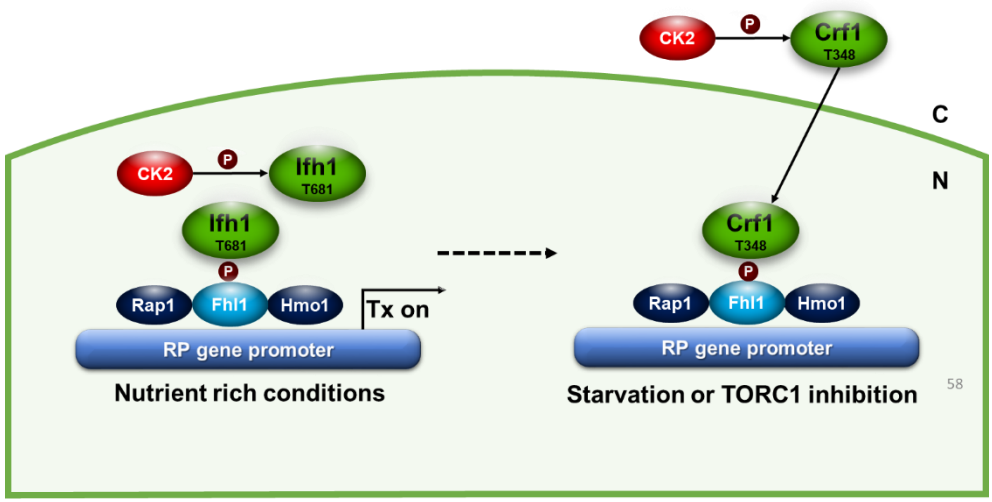



**Figure 5.18. Graphical summary**

Transcription regulators such as Rap1, Fhl1, and Hmo1 are constitutively bound to RP gene promoters providing a platform for other transcription factors. In nutrient rich conditions, Ifh1, which is constitutively localized in the nucleus, is phosphorylated by CK2 in the nucleus. Phosphorylation of Ifh1 T681 by CK2 in the nucleus promotes interaction with Fhl1 followed by transcriptional activation of RP genes. In response to starvation or inhibition of TORC1, cytoplasmic Crf1 translocates into the nucleus promotes by phosphorylation of stress response kinase Yak1. Further phosphorylation of Crf1 T348 by CK2

promotes interaction with Fhl1 at the RP gene promoters leading to the repression of RP gene transcription. It is not clear whether phosphorylation of Crf1 by CK2 occurs in the cytoplasm prior to Yak1 phosphorylation or in the nucleus after translocation.

# Chapter 6.

# Overall discussion and recommendations

## Regulation mechanisms of Haa1 and War1 by direct and differential binding of weak acid anions

How cells sense and adopt to weak organic acid stresses is directly related to cell survival and proliferation. Cells inevitably change metabolisms and signaling pathways to develop resistance to such stresses. However, detailed mechanisms how cells sense such insults remain elusive. Transcription factors such as Haa1 and War1 are revealing their roles involved in cellular protection against less lipophilic and more lipophilic weak acids, respectively.

Haa1 shares great extent of protein similarity with Ace1, especially over N-terminal regions. Cysteine residues responsible for binding with either zinc or copper ions are well conserved. Nevertheless, these two transcription factors have evolved to function differently. Ace1 is known to be activated in response to cellular concentration of copper ions, whereas Haa1 is activated by weak acids. Ancestral Haa1 before the whole genome duplication event in *Z. cerevisiae* shows dual functions. *Zb*Haa1 can be activated by both copper and acetic acid (95). *Zb*Haa1 is functionally separated into two

completely different transcription factors, Haa1 and Ace1 in *S. cerevisiae*. N-terminal zinc binding domains are well conserved in both Haa1 and Ace1 as well as another copper regulated transcription factor Mac1 (119,120). We showed that zinc binding domain of Haa1 is very critical for DNA binding affinity in response to acetic acids. However, DNA binding domain of Haa1 greatly differs from that of Ace1, suggesting other that N-terminal region of Haa1 is required to determine such distinction (121). In fact, the 40-residue Zn-binding domain fused to VP16 activation domain failed to induce expression of the Haa1 target genes. Furthermore, Ace1 is constitutively present in the nucleus but addition of copper suddenly changes inactive Ace1 to active Ace1 state that can bind to DNA (98,122,123). On the contrary, Haa1 is cytoplasmic in the absence of stresses but translocates into the nucleus in response to weak acid stresses by unknown mechanisms.

The activation domain of Haa1 is shown to be between 230 and 483 amino acid residues. Furthermore, the acetate binding region might be located within 1 to 150 amino acid residues. However, VP16-fused $Haa^{1-150}$ was not able to induce target gene expression in response to acetic acid, whereas VP16-fused $Haa^{1-180}$ showed acetic

acid-dependent transcriptional activation of target genes. Therefore, 150-180 of Haa1 may represent the regulatory region of Haa1 activation triggered by conformation changes in response to acetic acid. More detailed identification of acetate-binding sites and structural changes induced by acetate-binding might be necessary to further understand the activation mechanisms of Haa1.

Haa1 and War1 activating signaling pathways are extensively studied, but are mostly unknown. Failure to fine evidences for upstream activating signaling pathways lead to the idea that the direct binding of weak acid may cause the activation of these transcription factors and the expression of target genes in response to weak acid stresses. Furthermore, War1 is proposed to change its structure upon propionic acid treatment (42). However, direct binding of weak acids to either Haa1 or War1 has not been proved. To this end, CNF-FET biosensor, which allows sensitive real-time detection of protein-small molecule interaction (124), was exploited to present the evidence that Haa1 and War1 are activated by direct binding of acid anions with different binding affinities. Since CNF-FET biosensor device is a label-free sensing system, it is convenient to construct and gives good electric signals without compromising the protein activity. Furthermore,

experiments are performed in liquid phase so that it is possible to detect microscopic binding between receptors and analytes (125,126). In agreement with previous studies, Haa1 showed strongest sensitivity towards acetate, whereas War1 towards benzoate. However, both transcription factors further showed binding affinities by a wide range of weak acids. These unexpected bindings of acid anions are resulted in transcriptional activation of appropriate target genes. Haa1 is known to possibly regulate more and 80% of genes induced by acetic acid stress (87). However, the Haa1 induced target genes rarely overlap with War1 induced target genes, suggesting that Haa1 and War1 have specific roles against different weak acid stresses. Therefore, Haa1 is responsible for protection towards more hydrophilic acids, especially acetic acid. However, weak interaction between Haa1 and more lipophilic acid anions such as sorbate and benzoate results in transcriptional activation of Haa1 target genes. Haa1 preferentially bound with acid anions in the decreasing order of acetate, lactate, benzoate, and sorbate. However, the hydrophobic constant (logP) reflecting hydrophobicity increases in the order of lactic acid (-0.72), acetic acid (-0.17), sorbic acid (1.33), and benzoic acid (1.87). Therefore, hydrophobicity might not be the major factor determining

binding specificity of weak acid anions to Haa1. On the contrary, War1 showed highest sensitivity towards benzoate followed by sorbate. War1 is a member of Zn(II)2Cys6 family of transcription factors. Amongst these members, several transcription factors are known to make direct contact with their ligands for transcriptional activation. For examples, Pdr1 and Pdr3, involved in multi drug responses, are activated by direct interaction with structurally diverse xenobiotics (127), and Put3, involved in proline utilization, is activated by direct interaction with proline to adapt to nitrogen starvation (128). Leu3, involved in branched-chain amino acid synthesis (129), is regulated by direct binding of alpha-isopropylmalate (IPM), which accumulates as the first product in leucine biosynthesis during leucine starvation (130-132). The direct binding of IPM with the middle region of Leu3 stimulates activation of Leu3 by interfering intramolecular interaction between middle region and activation domain of Leu3 (132,133). Therefore, our results demonstrated by CNF-FET further support the idea that many Zn(II)2Cys6 family member of transcription factors are activated by direct binding with small molecule ligands.

While sensor experiments do not reveal what exact reactions occur when proteins and analytes meet, it can be sure that the two

have caused a direct interaction, resulting in a current change. Future investigations will be required to clarify the exact binding site of the Haa1 and War1, and precise binding mechanisms between the proteins and weak acid anions.

**Transcriptional activation of ribosomal protein genes by CK2-phosphorylated Ifh1**

Ribosome biogenesis is one of the fundamental processes for cell growth and proliferation and RP gene transcription lies at the heart of ribosome biogenesis. However, much remains elusive regarding how RP gene transcription is mediated in response to environmental changes (54). CK2-phosphorylated RP gene transcription activator Ifh1 and repressor Crf1 interact with the FHA domain of Fhl1. Fhl1 is found constitutively bound to RP gene promoters along with other transcriptional regulators such as Rap1 and Hmo1 (57,72). It has demonstrated that Sir2, an $NAD^+$-dependent protein deacetylase, represses RP gene transcription through deacetylating K16 of histone H4 (112). It was also found that phosphorylation of Sir2 by CK2 can

inactivate Sir2 activity in a PKA-dependent manner (112). These results suggest that CK2 is deeply involved in the regulation of RP gene transcription by phosphorylating diverse proteins. Furthermore, other than phosphorylation of Ifh1, Ifh1 is acetylated and activated by Gcn5, histone acetyltransferase, and deacetylated by Sir2 in response to TORC1 inhibition (134). It is possible that CK2 might regulate Ifh1 activity by mediating the acetylation events of Ifh1 via Sir2. Furthermore, phosphorylation of Ifh1S$^{969}$ by PKA resulted in Sir2-dependent replicative lifespan (RLS) extension (134). In addition, CK2 is involved in countless biological processes (117). It has been recently shown that CK2 plays critical roles by phosphorylating all three RNA polymerases for ribosome biogenesis. For example, mammalian CK2 is known to phosphorylate UBF and SL1 subunit TAF$_I$110, subunits of the RNA polymerase I, to activate rRNA transcription (135,136). In addition, CK2 is known to phosphorylate Maf1, a negative regulator of RNA polymerase III, and regulates transcription of tRNA in yeast and human (137). Much remains to elucidate the complex interconnection among TORC1, PKA, CK2, Sir2, and Ifh1 for the regulation of RP gene transcription.

It has been long known that Ifh1 is detached from the RP gene

promoters upon TORC1 inhibition (59). However, regulatory mechanisms underlying this behavior remain unanswered. Crf1 is known to be phosphorylated by stress responsive kinase Yak1. And this phosphorylation induces translocation of Crf1 into the nucleus to interact with Fhl1 instead of Ifh1 upon TORC1 inhibition (57), thus resulting in clearance of Ifh1 from the RP gene promoters. However, Crf1 is known to be non-essential for some yeast species and many species do not have Crf1, suggesting that there exists more prominent mechanisms how Ifh1 is detached from the RP gene promoters. Further studies might be necessary to elucidate the TORC1-dependent regulatory mechanisms of Ifh1-Fhl1 interaction depending on nutrient conditions.

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# Abstract in Korean

## 국 문 초 록

*S. cerevisiae*는 분자 및 세포 생물학 연구에 있어 연구가 활발히 진행된 단세포 진핵 생물로서 다른 미생물들과 비교하여 조작 및 성장이 용이하여 고차원의 진핵 생물 연구에 많은 이바지를 하고 있다. 영양분이 풍부한 상황에서 효모는 활발히 성장을 하는데 이를 위하여 많은 수의 리보좀을 필요로 하게 된다. 하지만 영양분이 부족한 상황이나 스트레스가 발생하면 다양한 종류의 전사조절인자를 이용하여 해당 상황에 맞게 세포의 반응을 조절하여 생존을 도모하게 된다.

첫 번째로, 약산성의 유기산 스트레스가 처리되면 효모는 Haa1과 War1이라고 하는 전사조절인자를 이용하여 해당 스트레스에 적응하게 된다. Haa1은 조금 더 아세트산이나 젖산 같은 수용성 약 유기산에 의해 작동한다고 알려져 있는 반면 War1은 좀 더 프로피온산, 소르브산, 또는 벤조산과 같은 지용성 약 유기산에 대항하여 세포를 보호 하는 역할을 하는 것으로 잘 알려져 있다. 하지만 두 전사조절인자가 어떤 방법으로 해당 유기산에 의해 작동하게 되는지는 전혀 알려진 바가 없다. 따라서 해당 연구에서는 탄소 나노파이버를 이용한 biosensor를 제작하여 약 유기산의 음이온과 직접 접촉

을 하여 앞서 언급한 두 전사조절인자가 작동하게 되는 원리를 처음으로 밝혀 내었다. Haa1은 아세트산 염과 가장 강한 접촉을 하였고 War1은 벤조염에 의해서 가장 강한 반응을 보였다. 또한 Haa1 같은 경우는 아세트산 염과 접촉을 하면 아미노 말단의 아연이 붙을 수 있는 단백질 부분을 이용하여 Haa1의 대상 유전자의 전사를 조절하고 이에는 Haa1의 카르복시기 말단을 추가적으로 필요로 한다는 것이 밝혀졌다. 따라서 Haa1은 아세트산 염과 직접적인 접촉을 하게 되면 단백질의 구조에 큰 변화가 생겨 DNA에 붙을 수 있는 활성화된 모습으로 바뀌는 것으로 추측된다.

두 번째로, 리보조말 단백질의 발현을 위해서 효모는 Ifh1 또는 Crf1이라고 하는 전사조절인자를 이용한다. 이 두 전사조절인자는 Fhl1이라고 하는 forkhead-associated (FHA) domain을 가진 전사조절인자와 상호작용을 하게 된다. Ifh1은 영양분이 충분한 상황에서 FHB domain을 이용하여 Fhl1과 상호작용하여 리보조말 단백질의 발현을 촉진하고 Crf1은 스트레스 상황에서 Fhl1과 상호작용하여 리보조말 단백질의 발현을 저해한다. 하지만 Crf1같은 경우는 모든 효모 종에 보존 되어 있지 않거나 보존 되어 있더라도 그 역할이 미미한 것으로 알려져 있다. Ifh1$^{T681}$ 또는 Crf1$^{T348}$ 는 FHB domain에 위치한 아미노산으로 CK2라고 불리는 kinase에 의해 인산화 된다. 이 인산화는 Fhl1과의 상호작용에 매우 중요한 역할을 한다. Ifh1$^{T681A}$ 돌연변이 같은 경우 CK2에 의해 인산화가 되지 못 하여 Fhl1과의

상호작용이 현저히 줄게 들게 되고 그 결과 리보조말 단백질의 발현이 줄어들게 된다. 그 결과 효모 세포의 성장이 매우 느려지게 되는 결과를 초래하게 된다. 반면 $Crf1^{T348A}$ 돌연변이의 경우 스트레스 상황에서 리보조말 단백질의 발현을 저해 하지 못 하고는 것으로 밝혀졌다. 결과를 종합해 보면 CK2에 의해 인산화된 Ifh1과 Crf1의 Fhl1과의 상호작용을 통하여 리보조말 단백질의 발현을 조절 하는 것으로 판단된다.

주요어 : Haa1, War1, CNF-FET, 약 유기산,
Fhl1, Ifh1, Crf1, 리보조말 단백질,
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