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## Regulation of the heat shock response by thiol-reactive compounds in the yeast *Saccharomyces cerevisiae*

Yanyu Wang

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**REGULATION OF THE HEAT SHOCK RESPONSE**  
**BY THIOL-REACTIVE COMPOUNDS**  
**IN THE YEAST *SACCHAROMYCES CEREVISIAE***

by

**Yanyu Wang, B.S., M.S.**

**APPROVED**

---

Supervisory Professor, Kevin A. Morano, Ph.D.

---

Michael C. Lorenz, Ph.D.

---

William Margolin, Ph.D.

---

Ambro van Hoof, Ph.D.

---

Jianping Jin, Ph.D.

**APPROVED:**

---

Dean  
Graduate School of Biomedical Sciences  
University of Texas at Houston

**REGULATION OF THE HEAT SHOCK RESPONSE**  
**BY THIOL-REACTIVE COMPOUNDS**  
**IN THE YEAST *SACCHAROMYCES CEREVISIAE***

A  
DISSERTATION

Presented to the Faculty of  
The University of Texas Health Science Center at Houston and  
the University of Texas M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
In Partial Fulfillment  
Of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY

by

Yanyu Wang, B.S., M.S.

Houston, TX

December 2012

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**Regulation of the heat shock response by thiol-reactive compounds**  
**in the yeast *Saccharomyces cerevisiae***

Publication No. \_\_\_\_\_

Yanyu Wang

Supervisory Professor: Kevin A. Morano, Ph.D.

Cells govern their activities and modulate their interactions with the environment to achieve homeostasis. The heat shock response (HSR) is one of the most well studied fundamental cellular responses to environmental and physiological challenges, resulting in rapid synthesis of heat shock proteins (HSPs), which serve to protect cellular constituents from the deleterious effects of stress. In addition to its role in cytoprotection, the HSR also influences lifespan and is associated with a variety of human diseases including cancer, aging and neurodegenerative disorders. In most eukaryotes, the HSR is primarily mediated by the highly conserved transcription factor HSF1, which recognizes target *hsp* genes by binding to heat shock elements (HSEs) in their promoters. In recent years, significant efforts have been made to identify small molecules as potential pharmacological activators of HSF1 that could be used for therapeutic benefit in the treatment of human diseases relevant to protein conformation. However, the detailed mechanisms through which these molecules drive HSR activation remain unclear.

In this work, I utilized the baker's yeast *Saccharomyces cerevisiae* as a model system to identify a group of thiol-reactive molecules including oxidants, transition metals and metalloids, and electrophiles, as potent activators of yeast Hsf1. Using an artificial HSE-*lacZ* reporter and the glucocorticoid receptor system (GR), these diverse

thiol-reactive compounds are shown to activate Hsf1 and inhibit Hsp90 chaperone complex activity in a reciprocal, dose-dependent manner. To further understand whether cells sense these reactive compounds through accumulation of unfolded proteins, the proline analog azetidine-2-carboxylic acid (AZC) and protein cross-linker dithiobis(succinimidyl propionate) (DSP) were used to force misfolding of nascent polypeptides and existing cytosolic proteins, respectively. Both unfolding reagents display kinetic HSP induction profiles dissimilar to those generated by thiol-reactive compounds. Moreover, AZC treatment leads to significant cytotoxicity, which is not observed in the presence of the thiol-reactive compounds at the concentrations sufficient to induce Hsf1. Additionally, DSP treatment has little to no effect on Hsp90 functions. Together with the ultracentrifugation analysis of cell lysates that detected no insoluble protein aggregates, my data suggest that at concentrations sufficient to induce Hsf1, thiol-reactive compounds do not induce the HSR via a mechanism based on accumulation of unfolded cytosolic proteins. Another possibility is that thiol-reactive compounds may influence aspects of the protein quality control system such as the ubiquitin-proteasome system (UPS). To address this hypothesis,  $\beta$ -galactosidase reporter fusions were used as model substrates to demonstrate that thiol-reactive compounds do not inhibit ubiquitin activating enzymes (E1) or proteasome activity. Therefore, thiol-reactive compounds do not activate the HSR by inhibiting UPS-dependent protein degradation.

I therefore hypothesized that these molecules may directly inactivate protein chaperones, known as repressors of Hsf1. To address this possibility, a thiol-reactive biotin probe was used to demonstrate *in vitro* that the yeast cytosolic Hsp70 Ssa1, which partners with Hsp90 to repress Hsf1, is specifically modified. Strikingly, mutation of

conserved cysteine residues in Ssa1 renders cells insensitive to Hsf1 activation by cadmium and celastrol but not by heat shock. Conversely, substitution with the sulfinic acid and steric bulk mimic aspartic acid led to constitutive activation of Hsf1. Cysteine 303, located in the nucleotide-binding/ATPase domain of Ssa1, was shown to be modified *in vivo* by a model organic electrophile using Click chemistry technology, verifying that Ssa1 is a direct target for thiol-reactive compounds through adduct formation. Consistently, cadmium pretreatment promoted cells thermotolerance, which is abolished in cells carrying *SSA1* cysteine mutant alleles. Taken together, these findings demonstrate that Hsp70 acts as a sensor to induce the cytoprotective heat shock response in response to environmental or endogenously produced thiol-reactive molecules and can discriminate between two distinct environmental stressors.

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**Chapter 1: Introduction to cellular stress response and molecular chaperones in  
the yeast *Saccharomyces cerevisiae***

## INTRODUCTION AND BACKGROUND

### *Cellular stress response to unfavorable environmental conditions*

The natural environment is not static, and most biological processes strive to maintain “homeostasis”, an ideal steady state that is optimal for all organisms to survive. However, a wide range of physiological and environmental stimuli continuously disrupt equilibrium, making the present living condition in constant flux wavering about a homeostatic point. These unfavorable stimuli such as heat, oxidative and osmotic stress and starvation, change the intracellular environments, inhibit cell growth and development, and can eventually lead to cell death. To restore homeostasis under unfavorable conditions, organisms have developed various sophisticated stress response mechanisms at both cellular and tissue-specific levels. The stress responses elicited by cells dictate that the organism adapts, survives, or if beyond repair, undergoes cell death. Response to acute stress is generally transient, including regulations of selective gene expression at both transcriptional and translational levels. Well-studied examples of adaptive and cytoprotective molecules include the inducible heat shock proteins (HSPs) that confer thermal stress protection on cells, and glutathione (GSH) in oxidative stress/antioxidant response.

The fundamental cellular stress program activated in response to a significant increase of temperature is known as the heat shock response (HSR). The goal of the HSR is to rapidly synthesize heat shock proteins (HSPs) during stress conditions, thus preventing aggregation of damaged proteins. This heat shock gene expression pattern is highly consistent throughout evolution, and is primarily governed by the heat shock transcription factor (HSF) in all eukaryotes (1). In the baker's yeast *Saccharomyces*

*cerevisiae*, a second pathway, modulated by two highly related and partially redundant zinc-finger transcription factors Msn2 and Msn4, also responds to the elevated temperature (2). Microarray analysis using conditional and knockout mutants of the *HSF1*, *MSN2*, and *MSN4* genes suggest that the Msn2/4 regulon is broader than that of Hsf1 and includes genes involved in metabolism, oxidative stress, and growth control (3). Therefore, the gene expression program regulated by Msn2/4 is called the "general stress response" or "environmental stress response" (ESR).

In addition to the ESR, recent studies suggest that the HSR is also tightly linked to oxidative stress. Mutants deficient in catalase, superoxidase dismutase (SOD), and cytochrome *c* peroxidase are hypersensitive to heat exposure (4). In *S. cerevisiae*, increased levels of hydrogen peroxide were detected in cells treated at 43 °C for two hours (5). These results indicate that oxidative stress results subsequently from the primary heat stress. Furthermore, many noxious oxidative stressors have been shown to function as Hsf1 modulators. For example, superoxide anion selectively upregulates Hsf1-mediated transcription of the metallothionein gene (*CUP1*) by enhancing DNA-binding activity of the transcription factor (6, 7). Our lab recently demonstrated that celastrol, an active component from Chinese medicine, acts as an activator of both Hsf1 and Yap1 in yeast (8). However, the detailed mechanism behind the coordination of the heat shock and oxidative stress responses remains unclear. For this reason, I have used the model organism, *S. cerevisiae* to understand the cross-protection between the heat shock and oxidative stress responses. Further, I specifically investigated the precise mechanism through which diverse thiol-reactive compounds activate the heat shock transcription factor Hsf1.



### *The heat shock response in yeast*

The heat shock response (HSR) is the predominant response of almost all organisms to the ambient temperature (9, 10). It results in immediate changes in transcriptional activation of heat shock proteins (HSPs), proteases and other proteins essential for protection and recovery from cellular damage. In the yeast *S. cerevisiae*, the optimal growth temperature is between 25 °C and 30 °C. Although the upper temperature limit for most laboratory strains is about 41 °C, *S. cerevisiae* is unable to withstand chronic exposure to higher temperature (11). Therefore, shift from 30 °C to 37 °C is defined as “classic heat shock” in investigation of the HSR induction in yeast (3). In all eukaryotes, the heat shock transcription factor (HSF) family is the major regulator of the HSR. HSF activates transcription of *hsp* genes in response to heat shock by recognizing and binding to arrays of a five base pair heat shock element (HSE), nGAAn, in the promoters of target genes (12). When the HSR is induced, mRNA levels of *hsp* genes increase dramatically within 2-5 min, resulting in a quick burst of synthesis of HSPs (13, 14). Under stress conditions, HSPs such as HSP70 are able to hold and refold denatured proteins, preventing them from aggregation and degradation. In other cases, HSPs such as HSP90 are central to signal transduction, immunity and apoptosis (15-19). Modulation of the HSR, therefore, may be directly beneficial for treatment of a variety of human diseases including those associated with cell growth, such as cancer, and those coupled with damaged and misfolded proteins, such as the neurodegenerative diseases. The benefits of using chaperones and Hsf1 as potential therapeutic targets will be discussed in more detail in Chapter 3.

Importantly, in *S. cerevisiae*, a parallel pathway represented by the *MSN2* and *MSN4* (multicopy suppressor of *snf1*) genes also responds to heat shock, as well as diverse stress conditions (20). The regulatory element of this “general” stress pathway was originally indentified as an Hsf1-independent control element in the promoters of the DNA damage responsive gene *DDR2* (21) and the nutrient stress responsive gene *CTT1* (22). This element, designated stress responsive element (STRE), contains a five base pair sequence functional in both orientations (CCCCT or AGGGG). Most *hsp* genes are regulated exclusively by HSF and contain one or more sets of HSE in their promoters. However, a variety of *hsp* genes such as Hsp26 and Hsp104 also include STRE repeats in their promoter regions, suggesting the potential overlapping between the heat shock and general stress responses (23, 24).

#### *The heat shock transcription factor Hsf1*

Vertebrates and plants have evolved four distinct HSF isoforms, i.e. HSF1-4. HSF1 is the primary mediator of the HSR upon stress stimulation (25, 26). HSF2 and HSF4 are mainly involved in development and differentiation-related gene expression (27-32). Interestingly, recent studies suggest that both HSF2 and HSF4 contribute to inducible expression of select *hsp* genes by functionally interacting with HSF1 (33, 34). HSF3 is specifically found in avian species. However, the roles of HSF3 remain unclear (35). Yeast, invertebrates, nematodes and fruit flies, only express a single, essential HSF equivalent to mammalian HSF1 (34). First reported in 1988, yeast *HSF1* encodes an 833 amino acid protein with a mass of 93,218 daltons (36, 37). Hsf1 is functionally divided into three domains: a DNA-binding domain at N-terminus (DBD), three leucine zipper

repeats (LZ) responsible for trimerization of the factor adjacent to the DNA-binding domain, and two regulatory domains at both N- and C-terminus (NTA and CTA, respectively) (Figure 1-1).

The DNA-binding domain (DBD) is the only functional domain of HSF1 for which detailed structural data are available, and is the most conserved region within the HSF family. The yeast DBD belongs to the 'winged' helix-turn-helix (wHTH) family of DNA-binding proteins. Like other members of the wHTH family, it recognizes target genes by the second helix of the helix-turn-helix motif, called helix  $\alpha 3$  (38, 39).

In addition to the DNA-binding domain, the oligomerization domain is another highly conserved region among all identified *HSF* genes (40, 41). The 91 amino acids located in the center of *S. cerevisiae* *HSF1* form a homotrimer via a triple-stranded coiled coil (42). Circular dichroism (CD) spectroscopy studies demonstrated that this domain contains high  $\alpha$ -helical content and is divided into two subdomains, each containing an amphiphilic helix with the hydrophobic heptad repeats HR-A and HR-B (42, 43). In the trimeric HSF1 complex, the HR-A and HR-B possibly form an anti-parallel and highly elongated structure. Therefore, the oligomerization domain is also called the leucine zipper domain (LZ). Since cells require three HSE repeats for activity *in vivo*, trimerization of Hsf1 might increase the affinity of DNA binding and stabilize the protein-DNA interaction (44). It is also possible that trimerization could be involved in regulation of HSF1 activity in higher eukaryotes. In Vertebrate and *Drosophila* cells, inactive Hsf1 is maintained in the cytoplasm as a monomer (45, 46). Elevated temperature and other Hsf1-activating stresses such as heavy metal and oxidant cause the

**Figure 1-1. Schematic illustration of Hsf1 from *Saccharomyces cerevisiae***

NTA, amino-terminal activation domain; DBD, DNA binding domain; LZ, leucine zipper trimerization domain; CE2, control element 2; CTA, carboxyl-terminal activation domain. The serine-rich region involved in transcriptional regulation is indicated by the sequence "SMSSSSS".

**Figure 1-1. Schematic illustration of Hsf1 from *Saccharomyces cerevisiae***



trimerization of HSF1 to facilitate DNA binding (47). However, this step of activation is not universal. In yeasts such as *S. cerevisiae* and *Kluyveromyces lactis*, Hsf1 appears to constitutively bind DNA as a trimer (48-50).

In most eukaryotes, HSF1 contains a single transactivation domain. However, in *S. cerevisiae* and its closely related yeast *K.lactis*, there are two distinct transcription activation domains at both N-terminus and C-terminus (51). The N-terminal transactivation domain (NTA) is found within the first 170 amino acids (52, 53). The C-terminal transactivation domain (CTA) is located between residues 595 and 783 (51). Studies of a synthetic HSE-*lacZ* reporter suggest that although both transactivation domains are strong activators, the two transactivation domains respond differently to the thermal stress. The NTA appears to mediate "transient" activation of Hsf1; while the CTA is required for "sustained" activity. Deletion of either N- or C-terminal transactivation domain does not affect cell growth under normal growth conditions (52, 53). However, elimination of the CTA, but not the NTA, leads to a temperature-sensitive phenotype and arrest of cell cycle in both G<sub>1</sub>/S and G<sub>2</sub>/M phases due to depletion of Hsp90 at 37 °C (53, 54). Truncated NTA (residues 1-147 or 40-147) leads to constitutive activation of HSF1 in the absence of stress (53, 55). High resolution mapping of the CTA found that point mutations that abolish activation of the heat shock response result in temperature sensitive growth of cells at higher temperature (51). These observations suggest that the NTA functions as a negative regulator by masking the CTA, and that the CTA is not sufficient for Hsf1 activation during stress. Furthermore, the CTA is required for heat- or glucose starvation induced activation of the yeast methallothionein gene, *CUP1*, but is dispensable for transient heat shock induction of Hsp104 and the yeast Hsp70 homologues *SSA1* and

SSA3 (23, 56, 57). Therefore, the presence of two distinct transactivation domains in yeast may provide additional levels of regulation or selection in gene activations.

Hsf1 recognizes target *hsp* genes by inverted repeats of the sequence nGAAn unit, called heat shock transcription elements (HSEs), in their promoters. The perfect-type HSE consists of three continuous inverted repeats of the unit (nTTCnnGAAnnTTCn). The discontinuous gap-type [nTTCnnGAAn(5bp)nGAAn] or step-type [nTTCn(5bp)nTTCn(5bp)nTTCn] HSE contains one or two insertions between the consensus sequence (58-60). Human HSF1 preferentially binds to continuous HSEs rather than discontinuous HSEs. Yeast Hsf1, by contrast, recognizes both continuous and discontinuous repeats of the nGAAn unit (59). This observation is consistent with the fact that vertebrates have four HSFs for diverse cellular processes; while yeast cells only carry one single Hsf1 for constitutive growth and survival in stresses. The active binding form of yeast Hsf1 with *hsp* genes is a homotrimer (50). Although each nGAAn unit in an HSE is a recognition site for a single Hsf1 monomer, a minimum of three pentameric units is required for stable binding *in vitro* (61). Some *hsp* genes contain four to six contiguous units to contact with two Hsf1 trimers, and seven to eight units with three trimers. The cooperative binding between yeast Hsf1 trimers is not as significant as that of vertebrates HSF1. A single yeast Hsf1 trimer is sufficient to activate transcription, and thus, the additional nGAAn units in the natural HSEs possibly function to increase the stability of the Hsf1-DNA interaction (62).

*Activation of the heat shock transcription factor Hsf1*

The stress-dependent conversion of HSF1 into its active form suggests that HSF1 is negatively regulated under normal conditions (63, 64). Upon activation, mammalian HSF1 undergoes a multi-step process including nuclear localization, trimerization, DNA-binding activity, hyper-phosphorylation, and obtaining of transcriptional activity (Figure 1-2A) (65). Monomeric HSF1 is thought to be stabilized by cytosolic HSPs, including HSP40, HSP70 and HSP90, under normal growth temperature. During heat shock, accumulated unfolded proteins may titrate HSPs away from HSF1 and lead to relief of HSF1 repression (1, 66-71). However, it is not clear whether it is the accumulation of existing proteins, newly synthesized proteins, or both that trigger HSF1 activation. Once dissociated from the inhibitory complex, HSF1 trimerizes and acquires DNA binding ability spontaneously, resulting in the increase amount of HSPs in the stressed cells (49, 61, 72). After sufficient amount of HSPs are synthesized to bind all unfolded proteins, the negative regulatory chaperone proteins are theorized to bind to HSF1 trimers, causing them to dissociate and revert to the inactive, monomeric state (73, 74). The HSPs themselves, thus, are proposed to regulate the heat shock gene expression via an autoregulatory loop (45, 64, 66). Interestingly, the behavior of *S. cerevisiae* Hsf1 is very similar to its mammalian homologue, except yeast Hsf1 constitutively binds to DNA as a trimer. Substantial genetic data suggests that under basal conditions, trimerized yeast Hsf1 is likely to be repressed by HSPs including Hsp70, Hsp90 and their co-chaperones (Figure 1-2B) (49, 75-78). This characteristic makes *S. cerevisiae* a suitable simplified model to study chaperone regulation of Hsf1 activation.

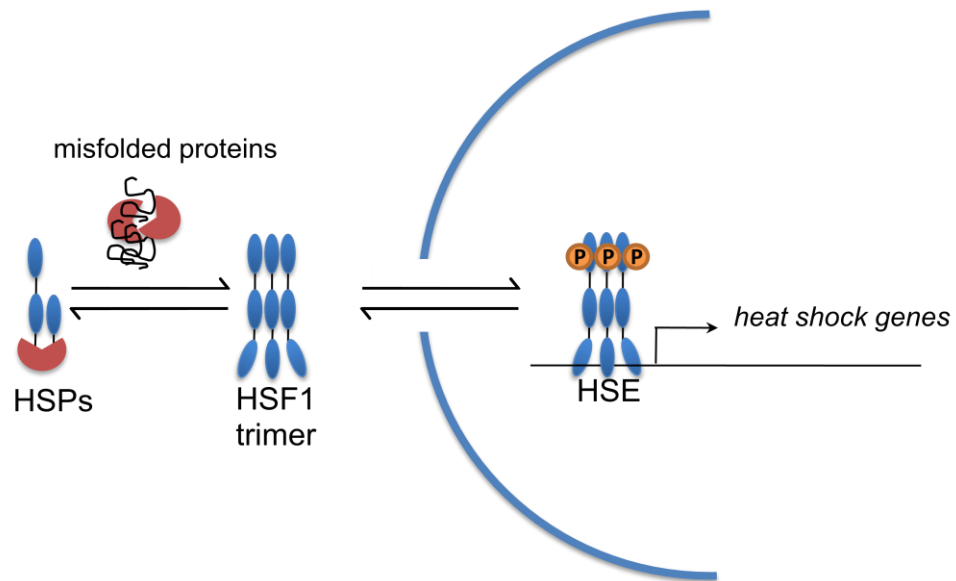


**Figure 1-2. Multistep models of Hsf1 activation in mammalian and yeast cells.**

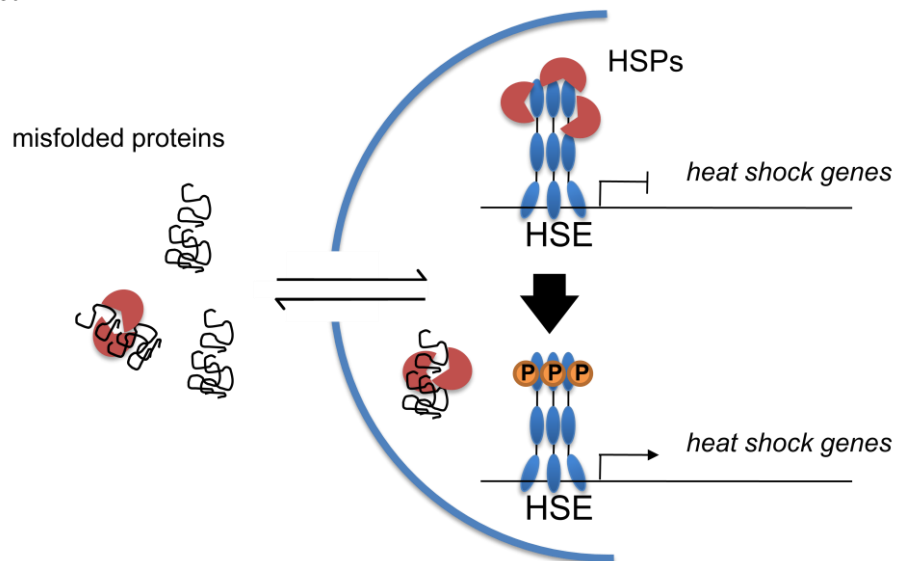
- A. In mammalian cells, HSF1 binds to chaperone proteins and exists primarily as an inactive monomer in the cytoplasm. Upon exposure to protein-damaging stresses (e.g., heat shock), HSF1 dissociates from various negative regulators (including Hsp70 and Hsp90), trimerizes and migrates into the nucleus. The trimeric HSF1 undergoes post-translational modification (e.g., P, phosphorylation) and acquires DNA binding ability, resulting in upregulation of *hsp* gene expression.
- B. In yeast cells, Hsf1 constitutively binds to the HSE in the promoter of *hsp* genes as a trimer during both normal and stress conditions. Chaperone proteins including Hsp70 and Hsp90 are thought to associate with Hsf1 in the absence of stress and repress Hsf1 transcription activation. During stress conditions, chaperones may be recruited away from Hsf1 by accumulated misfolded proteins, allowing activation of Hsf1.

**Figure 1-2. Multistep models of Hsf1 activation in mammalian and yeast cells.**

**A. Mammal**



**B. Yeast**



### *Molecular chaperones of the cytoplasm*

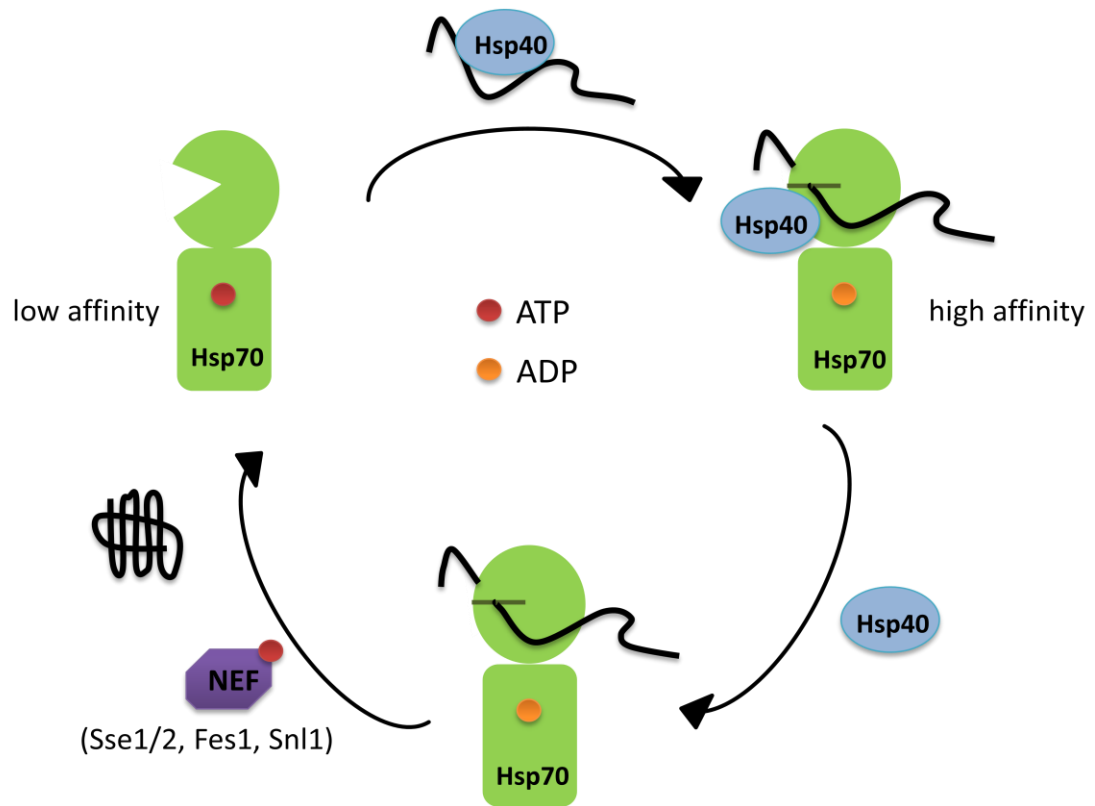
(i) *HSP70 and co-chaperones* - The HSP70s are a family of multifunctional stress proteins involved in protein translocation, folding, assembly and degradation (79). A variety of stressors such as heat and heavy metals induce the expression of HSP70s. The HSP70s have three major functional domains: a 44 kDa N-terminal nucleotide-binding domain (NBD), an 18 kDa C-terminal substrate-binding domain (SBD), and a 10 kDa C-terminal domain (CTD) which forms a lid-like structure in the substrate-binding pocket to help trap substrates bound to the SBD (80-83). Hsp70-dependent folding activity occurs by repeating cycles of binding and release of substrates at the expenditure of ATP. In the ATP-bound state, HSP70 has low substrate binding affinity and a fast exchange rate. ATP hydrolysis induces conformational changes in the NBD, leading to structural changes in the SBD and CTD that increase substrate-binding affinity. Subsequently, the release of ADP and rebinding of ATP restores HSP70 to the low affinity state allowing the folded or partially folded substrate to release and the cycle to repeat (Figure 1-3).

In *S. cerevisiae*, the cytosolic HSP70, include the Ssa and Ssb families and the atypical Ssz1 (stress seventy A, B, Z) (84, 85). The essential Ssa subfamily contains four members. Ssa1 and Ssa2 are constitutively expressed; while Ssa3 and Ssa4 are heat-induced (86). Ssa has been shown to be required for the folding and membrane translocation of nascent peptides, nuclear import, microtubule formation and the transcriptional response to heat shock (87-91). The Ssb1 and Ssb2 isoforms are associated with ribosomes and function in binding nascent peptides during translation

**Figure 1-3. The yeast Hsp70 chaperone cycle**

Hsp70 in the ATP-bound form adopts an open conformation which has low affinity for substrates. Hsp40 promotes substrate binding by catalyzing Hsp70 ATP hydrolysis. The ADP-bound Hsp70 has high affinity for substrates. Bound substrate is subsequently released after a nucleotide exchange factor (NEF) replaces ADP with ATP.

**Figure 1-3. The yeast Hsp70 chaperone cycle**



elongation (75, 92). The Ssb subfamily is not essential, but deletion of both genes leads to a cold-sensitive phenotype (75).

HSP70 itself is characterized as a weak ATPase. The slow rate of ATP hydrolysis and release of ADP limits the rate of HSP70 reaction cycle, but it also provides key regulatory points for fine tuning HSP70 activities by diverse co-chaperones. Two major classes of co-chaperones assist the HSP70 cycle by interacting with its NBD: the HSP40 co-chaperones and the nucleotide exchange factors (NEFs). The Hsp40 co-chaperone family, or “J proteins”, are defined by a conserved four-helix bundle of about 70 amino acids (93-95). As a co-chaperone, HSP40 interacts with the NBD and CTD of HSP70, as well as the substrate proteins. The binding of HSP40 to HSP70 assists the transfer of substrate to the SBD of HSP70 and accelerates its ATP hydrolysis (96-98). The NEFs, unlike J proteins, are a group of proteins with completely unrelated structures. The major functions of NEFs are to enhance the slow intrinsic release of ADP by HSP70, and subsequently reset the HSP70 reaction cycle (99, 100). *S. cerevisiae* has four major NEFs in cytoplasm, i.e. Sse1, Sse2, Fes1 and Snl1 (101-106). A detailed investigation of the shared and unique roles of these cytosolic NEFs is underway in our laboratory (J. Abrams, J. Verghese, unpublished data).

Although *S. cerevisiae* possesses at least 14 HSP70-like genes, only cytosolic Ssa subfamily members have been found to operate within the HSP90 complex (107-109). The understanding of HSP70 functions in HSP90 signal transduction has been limited due to the complexities of gene redundancy. Like HSP90, HSP70 has emerged as an attractive target for human diseases relevant to protein conformation. As a major foldase in the cell, HSP70 ensures rapid response of cells to acute stress. This ability of

cytoprotection extends to pathophysiology and severe trauma in heart and brain, suggesting the therapeutic benefits of HSR activation in the treatment of various tissue trauma and relevant diseases (110, 111). Moreover, HSP70 has been implicated in many neurodegenerative diseases, including Huntington's and Alzheimer's diseases which are shown to associate with accumulation and aggregation of unfolded proteins (112). Overexpression of HSP70 in a mouse model of Huntington's disease suppresses the formation and cellular toxicity of protein aggregates and affects disease progression, suggesting a preventive role of HSP70 in neurodegenerative diseases (113, 114). Additionally, HSP70 also participates in regulation of diverse transcription factors, signaling molecules and kinases such as NF- $\kappa$ B and v-Src (115). Thus, tumor cells are dependent on elevated levels of HSPs (116). Therefore, HSP70 is also relevant for cancer biology due to its important role in tumor cell survival by suppressing various anti-cancer mechanisms (19).

(ii) *HSP90 and co-chaperones* –As one of the most conserved chaperones from bacteria to mammals, the HSP90 functions primarily to hold and fold select proteins involved in signal transduction, protein trafficking, receptor maturation and innate and adaptive immunity (117). These select groups of proteins, whose cellular functions are strictly dependent on HSP90, are also called HSP90 “client” proteins. In yeast, the HSP90 chaperones exist as two isoforms, encoded by *HSC82* and *HSP82*. Hsc82 and Hsp82 share ~97% sequence identity. While Hsc82 is constitutively expressed at high levels (1-2% of the total cytosolic protein) and slightly induced by heat shock, Hsp82 is strongly induced under extreme conditions (118).

HSP90 family proteins all functions as dimers. Each HSP90 monomer consists of a highly conserved N-terminal ATP-binding domain (NTD), a middle substrate-binding domain (M domain), and a C-terminal dimerization domain (CTD) (Figure 1-4). Like HSP70, HSP90 is also defined as a weak ATPase and binds ATP in the NTD (119). The NTD consists of two major functional subdomains: an ATP-binding pocket and a conserved molecular “lid”. The ATP-binding site has a  $\alpha$ - and  $\beta$ -sandwich motif, which is reported as the binding site of a variety of structurally unrelated natural inhibitors such as geldenamycin and radicicol (120). The short “lid” comprised by several amino acids closes over the ATP-binding pocket in the ATP-bound state, but not in the ADP-bound state (121, 122). In the absence of nucleotide, the two NTD in a HSP90 dimer are highly flexible. Binding of one ATP on each NTD results in a pincer movement that dimerizes the ATP-binding site and holds client proteins for stabilization (Figure 1-4A) (123). The NTD is attached to the M domain by a charged linker. Structural and functional analyses indicate that this region has an important role in client recognition and co-chaperone regulation (121, 124). The CTD is responsible for dimerization of HSP90 and binding of HSP90 co-chaperones (125, 126). Although the CTD is less conserved in sequence than the rest of the protein, it contains five highly conserved C-terminal residues (MEEVD motif), which bind tetratricopeptide (TPR) repeats in the co-chaperones (82, 127, 128). Like HSP70s, the chaperone activity of HSP90s is also driven by ATPase activity. Recent structural studies and mutagenesis suggested that both ATP binding and hydrolysis facilitates additional structural rearrangements, which are essential for substrate maturation. In *S. cerevisiae*, both the open and closed conformations can be found in the absence of nucleotide binding (129). When HSP90 binds ATP, the molecular lid folds



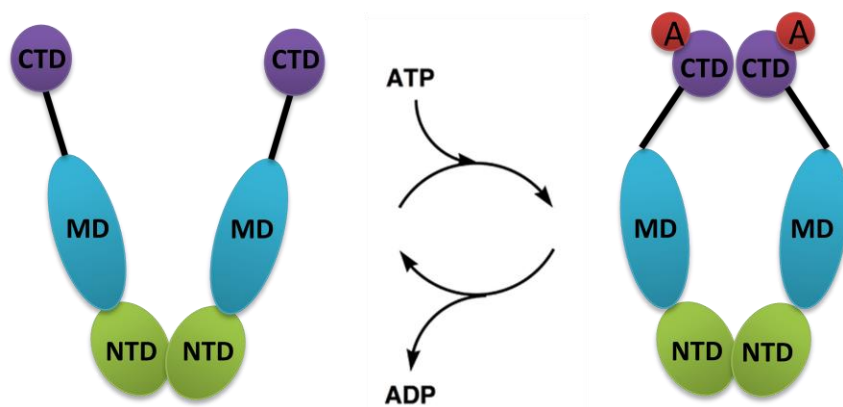
**Figure 1-4. The yeast Hsp90 chaperone cycle**

- A. The conformational change of Hsp90 dimer coupled to ATPase cycle. A, ATP; CTD, C-terminal ATPase domain; MD, middle substrate binding domain; NTD, N-terminal dimerization domain.
- B. The Hsp90 chaperone cycle involved in glucocorticoid receptor (GR) activation. In the "early complex", GR is associated with Hsp70 and its-cochaperone Hsp40. The scaffold protein Sti1 brings the early complex to Hsp90 and forms the "intermediate complex". GR is transferred from Hsp70 to Hsp90 followed by the release of intermediate co-chaperones and binding of late stage cochaperone to form the "late complex". Finally, in the presence of ligand, Hsp90 hydrolyzes ATP and releases the mature ligand-bound GR and co-chaperones. Alternatively, in the absence of ligand, GR will re-enter the cycle.

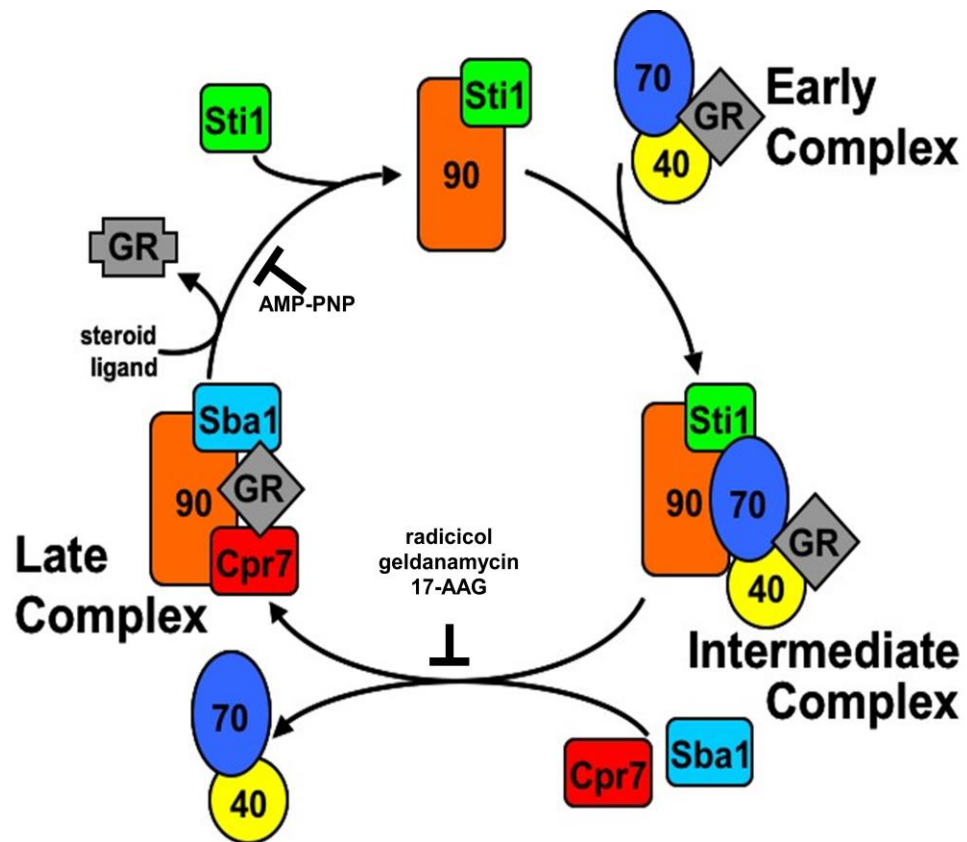
*Figure 1-4B is generated by Dr. Patrick A. Gibney.*

Figure 1-4. The yeast Hsp90 chaperone cycle

A.



B.



over and locks the nucleotide in the binding pocket, leading to a slow transition to a closed state, in which the two monomeric NTDs interact. Dimerization of NTDs leads to twisting and compaction of the M domain, as well as activation of ATP hydrolysis. Recent studies suggested that hydrolysis of ATP may lead to a second closed state, but the detailed structure remains unclear. Finally, release of ADP restores HSP90 to its original open state. Importantly, the conformational rearrangements are the rate limiting steps of the HSP90 reaction cycle and happen much slower than ATP hydrolysis (130-138).

In addition to ATP binding and hydrolysis, HSP90 functions are regulated both positively and negatively by many co-chaperones. More than 20 HSP90 co-chaperones have been identified. In yeast, co-chaperones with known biological roles include Hsp70 (in particular, members of Ssa and Ssb subfamilies), Ydj1 (HSP40), Sse1 (HSP110), Sti1 (HOP), Cdc37 (p50), Sba1 (p23), Aha1, and at least two different cyclophilin 40 isomerases, Cpr6 and Cpr7 (17, 76, 102, 107, 139-144). Studies of HSP90 co-chaperones suggest that these proteins can modulate HSP90 functions in five distinct ways: 1) they coordinate the interaction between HSP90 and other chaperone systems, such as HSP70 cycle; 2) they enhance the ATPase activity of HSP90, such as AHA1; 3) they inhibit the ATPase activity of HSP90, such as Cdc37 and p23; 4) they recruit client proteins to HSP90, like HSP40; and 5) they contribute to different steps of HSP90 chaperone cycle as enzymes such as PP5 (117, 144-151).

HSP90 alone is not sufficient to refold denatured proteins. Rather, the whole Hsp90 complex appears to be adapted to regulatory purposes by displaying a high degree of specificity for particular target proteins. These HSP90 “client proteins” include some

members of the steroid receptor superfamily (*e.g.* glucocorticoid and progesterone receptors) and some kinases (*e.g.* p60<sup>v-src</sup>) (152, 153). Recently, many studies described the role of Hsp90 complex in the human diseases. For example, HSP90 is up-regulated in the choroid plexus (CP) of Alzheimer's disease (AD) patients, resulting in abnormal hormone receptor expression in this secretory tissue (154, 155). In cancer cells, many HSP90 client proteins such as Akt and Her2 are required for tumor growth and/or survival. HSP90 inhibitors (*e.g.* geldenamycin and its derivatives) display dramatic anti-tumor activity by triggering degradation of Hsp90 client proteins (155, 156). HSP90 is also required for maturation and processing of the capsid precursor of picornavirus, a large virus family including rhino-, polio- and coxsackie virus (157, 158). Therefore, HSP90 inhibitors may also prove to be broad spectrum antiviral drugs, in addition to their promising anticancer activity.

*Small molecules as Hsf1 modulators* – Since the HSR plays a key regulatory role in cytoprotection and development, and is relevant to many human diseases including neurodegenerative diseases, cancer, and aging, significant efforts have been made to discover and develop small molecules that acts as potent HSR modulators (56, 159). The early identified Hsf1 activators are mostly protein translation inhibitors such as puromycin and amino acid analogues such as azetidine-2-carboxylic acid (160-163). These compounds generally result in accumulation and aggregation of prematurely terminated or amino acid analog-incorporated nascent proteins, which consequently function as signals to activate Hsf1. This class of molecules also includes inhibitors of protein degradation such as proteasome inhibitors MG132 and lactacystin (164, 165).

Another class of HSR activators is chaperone inhibitors, such as the Hsp90 inhibitors geldanamycin and radicicol. Although structurally distinct, both geldanamycin and radicicol block the ATP binding site and inhibit Hsp90 ATPase activity (166, 167). This class of small molecules is likely to induce the HSR by inhibiting repression of Hsf1 by Hsp90. Some molecules activate the HSR indirectly by inducing a secondary stress signal for the full induction of stress response. A representative of this class is the non-steroidal anti-inflammatory drug (NSAID) sodium salicylate, which facilitates HSF1 DNA binding by enhancing trimerization of HSF1 without triggering HSF1 hyper-phosphorylation (168, 169).

Many thiol-reactive compounds including oxidants, transition metals/metalloids, and organic electrophiles are reported to activate the heat shock response. This class of Hsf1 activators usually react with the side chains on several amino acids including Cys, His, Met, Phe, Tyr, Asp, Glu and Lys. Of these reactive residues, Cys is often the most sensitive, suggesting a potential sensory function (170). Hydrogen peroxide is known to activate and stabilize purified *Drosophila* and human HSF1 by catalyzing the formation of a disulfide bond between the two cysteine residues (C35 and C105) in the DNA binding domain (171). High concentrations of hypochlorous acid (HOCl, ~2.8-3.5 mM), another important cellular oxidative stress, drastically induces chaperone expression, especially HSP70 in mammalian cells (172). Treatment with the sulfhydryl oxidant diamide induces thermotolerance in Chinese hamster ovary (CHO) cells (173). The cyclopentenone prostaglandin, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), is a natural ligand of peroxisome proliferator-activated receptor  $\gamma$  (PPAR  $\gamma$ ) (174). High doses of 15d-PGJ<sub>2</sub> can induce a rapid generation of ROS in mitochondrial of endothelial cells

(175). *In vitro* studies in human endothelial cells and rat paw carrageenin edema show that 15d-PGJ<sub>2</sub> can activate HSF1 and induce HSP70 (175-177). Despite these and other examples, the molecular relationship between ROS generation and HSR activation is unknown. In addition, recent pharmacological studies with celastrol, an active component from Chinese medicine, suggest celastrol acts as an HSF1 activator with kinetics similar to those of thermal stress, as determined by induction of HSF1-DNA binding, hyperphosphorylation of HSF1, and upregulation of chaperone expression (19, 178, 179). Studies in *S. cerevisiae* verify that these same compounds can activate yeast Hsf1. Superoxide anion is known to upregulate the Hsf1-mediated transcription of the yeast metallothionein gene (*CUP1*), which correlates with hyperphosphorylation on serine and threonine residues (6). Our laboratory demonstrated that celastrol treatment activates Hsf1 and confers tolerance to thermal stress in yeast. Unlike mammalian HSF1, yeast Hsf1 lacks cysteine residues, suggesting that Hsf1 is likely not the direct target of thiol-reactive compounds. Recent data from our laboratory show that activation of Hsf1 by celastrol may be based on inhibition of the Hsp90 complex, and inhibition can be reversed by the free thiol, dithiothreitol (DTT) (180). HSP90 was also co-purified with biotinylated-15d PGJ<sub>2</sub> in cultured mesangial cells (181). These data indicate that thiol-reactive compounds may activate HSF1 by modifying key thiols on chaperone complexes, especially HSP90 complex.

Although more and more small molecules have been reported as activators of HSF1, how an individual small molecule is sensed to drive the stress response, whether multiple molecules regulate Hsf1 on different steps, and how the signal is transmitted to the transcription factor Hsf1 are still unsolved questions. Therefore, the work described in

this dissertation seeks to identify a common mechanism of a group of thiol-reactive compounds as potent Hsf1 activators.

## **SIGNIFICANCE OF THE STUDY**

Cells possess dedicated response systems to combat constant challenges from environmental and physiological noxious stimuli. The heat shock response (HSR) is one of the most well studied stress response systems. It results in the immediate induction of genes encoding protein chaperones, also called heat shock proteins (HSPs), to protect against and repair protein damage to the cellular proteome (182). In addition to cytoprotection, proper functions of chaperones are relevant to many human diseases. The disruption of protein folding quality control leads to the accumulation of misfolding proteins that can form oligomers, aggregates, and inclusions. These toxic states of proteins contribute to a variety of neurodegenerative diseases including Alzheimer's and Huntington's diseases (159, 183-185). Furthermore, tumor cells are typically shown to express higher levels of chaperones, which play roles in regulation of many transcription factors, signaling molecules, kinase, and steroid hormone receptors (115, 116, 159). Therefore, understanding regulation of the HSR is an important precursor to the development of therapeutic approaches to these devastating diseases.

In all eukaryotes, the HSR is primarily mediated by the heat shock transcription factor (Hsf1), which binds to a positive control element, the heat shock element (HSE), in the promoters of target gene (63). Recently, numerous efforts have been made to identify Hsf1 modulators. These small molecules include Hsp90 inhibitors such as geldenamycin and radicicol, the proteasome inhibitor MG132, and celastrol, a natural component of Chinese medicine (178, 186-190). Furthermore, a variety of oxidants and electrophiles including hydrogen peroxide, superoxide anion, diamide, cadmium, diethyl maleate (DEM), and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), have been shown to induce the



HSR (6, 171, 177, 191-196). However, the detailed mechanism through which these thiol-reactive compounds induce Hsf1 remains unclear.

In Chapter 3, I report that diverse thiol-reactive compounds, including cadmium sulfate, hydrogen peroxide, diamide, diethylmaleate, and 15d-PGJ<sub>2</sub> all activate Hsf1 and inhibit Hsp90 *in vivo*. Activation of Hsf1 and inhibition of Hsp90 occurs in a reciprocal dose-dependent manner and is prevented by excess free thiol, suggesting a thiol-modification mechanism. Through a series of studies to test models for the observed biological effects, I showed that these compounds do not induce the heat shock response by misfolding cytosolic proteins, or by inhibiting proteasomal protein degradation pathway. Instead, in Chapter 4, I demonstrate that these thiol-reactive compounds specifically modify two cysteine residues, C264 and C303, on the cytosolic Hsp70 Ssa1, to induce the heat shock response. An Ssa1 mutant with both cysteine residues converted into serine was completely resistant to thiol-reactive compounds. Interestingly, these mutants were still heat inducible, suggesting that the cysteine residues of Ssa1 are only involved in sensing thiol-reactive stress, but not heat shock. Furthermore, substitution with aspartic acid to mimic both steric bulk and the oxidized sulfinic acid form of cysteine resulted in derepression of Hsf1 under normal growth conditions. Lastly, C303, located in the ATPase domain of Ssa1, was specifically modified *in vivo* by a model organic electrophile, suggesting that Ssa1 is directly modified by thiol-reactive compounds. Since Hsp70-Hsf1 complexes have not been observed and validated in yeast cells, in Chapter 5, I report on the generation of different fusions of FLAG-Hsf1 to detect Hsf1-Ssa1 interaction to further investigate this regulatory step. Taken together, my results demonstrate that the cytosolic Hsp70 chaperone Ssa1 acts as a sensor for Hsf1

activation by thiol-reactive compounds and can distinguish between two distinct environmental stressors. These findings provide concrete evidence to support the derepression/activation model of Hsf1 regulation in yeast, which could be used to extrapolate a common mechanism for a wide range of biological, pharmacological and environmental Hsf1 activators. The yeast model may then be utilized as a tool to identify additional pharmacological modulators of HSF to combat human diseases which are connected to protein homeostasis and the heat shock response.

## **Chapter 2: Methods and Materials**

## METHODS AND MATERIALS

*Strains, plasmids and growth conditions* - Yeast strains used in this study were of the BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*) or DS10 (*MATa ura3-52 lys1 lys2 trp1-1 his3-11,15 leu2-3,112*) backgrounds. The *ssa1Δssa2Δ* strain (SL314, *MATa ura3-52 lys1 lys2 trp1-1 his3-11,15 leu2-3,112 ssa1::HIS3, ssa2::LEU2*) was kindly provided by Dr. Elizabeth Craig (University of Wisconsin, Madison, WI) and is isogenic with DS10 (197). *SSA1-TAP*, *SSE-TAP*, *FES1-TAP*, *CPR6-TAP* and *CPR7-TAP* (tandem affinity purification tag) strains were purchased from Open Biosystems/ThermoScientific (Huntsville, AL), and are isogenic with BY4741. The *pdr5Δ* and *ubc7Δ* strains are isogenic to BY4741 and were purchased from Open Biosystems/ThermoScientific. The DNY248 strain (*MATa his3Δ leu2Δ lys2Δ ura3Δ, yhsf1::KanMX, pRS316-yHsf1*) was kindly provided by Dr. Dennis Thiele (Duke University, Durham, NC) and is isogenic with BY4741. Yeast expression plasmids utilizing heterologous promoters and terminators were obtained from Dr. Martin Funk (198). Plasmid p414TEF-SSA1 was constructed by PCR amplification of the *SSA1* ORF with primers incorporating 5' *SpeI* and 3' *XhoI* restriction sites. *SSA1*<sup>C264S</sup>, *SSA1*<sup>C303S</sup> and *SSA1*<sup>C264S,C303S</sup> point mutants were generated by the PCR overlap extension method using primers incorporating the appropriate mutations and p414TEF-SSA1 as the template. All mutants were confirmed by DNA sequencing. The HSE-*lacZ* reporter plasmid was described previously (54). The glucocorticoid receptor reporter system consists of plasmids expressing a rat glucocorticoid receptor (p413GPD-rGR) and a transcriptional reporter fusion (pYRPGRE-*lacZ* (*URA3*)) and were used as previously described (54). Plasmid p414TEF-FLAG-SSA1 was constructed by PCR amplification of the *SSA1* ORF with

FLAG insertion and XbaI digestion site in the 5'-end primer and XhoI restriction site in 3'-end primer.

Dithiothreitol (DTT) quenching assays were performed by mixing thiol-reactive compounds with the indicated concentrations of DTT at room temperature for 15 min in prior to addition to cells. BY4741, DS10 and strains containing a chromosomally integrated TAP-tag were grown in rich medium YPD (0.2% Bacto-peptone, 0.1% yeast extract, 2% glucose). Strains carrying plasmids were grown in synthetic complete (SC) media lacking the indicated nutrients. Experiments were performed with cells in logarithmic phase at 30 °C, unless otherwise indicated.

*SDS-PAGE and immunoblotting* - Proteins were separated by sodium dodecyl sulfate-polysacrylamide gel electrophoresis and transferred to either nitrocellulose (Bio-Rad, Hercules, CA) or PVDF (Millipore, Billerica, MA) for Western blot analysis as described (Laemmli, 1970, Towbin 1979). M2 monoclonal antibody (Sigma Aldrich, St. Louis, MA), recognizing the FLAG epitope, was resuspended in a 5% solution of non-fat milk in TBS (0.1 M Tris base, 0.684 M NaCl, pH=7.6) and used at a dilution of 1:1000. Other antibodies were resuspended in a 5% solution of non-fat milk in TBST (0.1 M Tris base, 0.684 M NaCl, 0.1% Tween-20, pH=7.6).

*Hsf1 and glucocorticoid receptor activity assay* - Cells bearing the HSE-*lacZ* reporter were left untreated at 30 °C, heat shocked at 37 °C for 1 h, or treated with 2.5 mM diamide (1,1'-azobis[N,N-dimethylformamide]; Research Organics, Cleveland, OH), 100 µM CdSO<sub>4</sub> (Sigma, St. Louis, MO), 5.6 µM 15d-PGJ<sub>2</sub> (15 deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub>,

Cayman Chemical, Ann Arbor, MI) or 30  $\mu$ M celastrol (Cayman) for 2 h. *In vivo* protein unfolding was carried out using 10 mM AZC (azetidine 2-carboxylic acid; Sigma) 400  $\mu$ M DSP (dithiobis[succinimidyl propionate] ThermoScientific, Rockford, IL) or 400  $\mu$ M DTSSP (3,3'-dithiobis[sulfosuccinimidylpropionate], ThermoScientific). To maintain the solubility of celastrol, experiments were conducted in SC medium supplemented with 50 mM Tris-HCl, pH 7.5.  $\beta$ -galactosidase activity assays utilizing ortho-Nitrophenyl- $\beta$ -galactoside (ONPG) were carried out exactly as described (54). Assays using Beta-Glo assay system (D-luciferin-o- $\beta$ -galactopyranoside, Promega) coupled  $\beta$ -galactosidase activity to a luciferase reaction. The Beta-Glo<sup>®</sup> assay substrate and buffer were mixed and stored at -20 °C as stock reagents. During the assay, the reagent was added into the culture medium as a 1:1 ratio (v/v) in a 96-well plate. Samples were incubated at 30 °C for 30 min and analyzed in a plate reader.

For the GR activity assay, cells transformed with p413-GPDGR and pYRP-GRElacZ were treated with 2.5 mM diamide, 100  $\mu$ M CdSO<sub>4</sub>, 5.6  $\mu$ M 15d-PGJ<sub>2</sub> for 1 h or 400  $\mu$ M DSP for 1, 2 or 3 h, followed by treatment for 1 h with 10  $\mu$ M DOC (deoxycorticosterone; Sigma) or vehicle (ethanol). Cells were harvested by centrifugation and processed for  $\beta$ -galactosidase activity assay as described.

*Heat shock protein expression assay* - To analyze the expression levels of heat shock proteins induced by CdSO<sub>4</sub>, diamide and AZC, protein extracts were prepared using a glass bead lysis procedure, fractionated by SDS-PAGE (10%), and transferred to nitrocellulose as described (77). Polyclonal antibodies recognizing phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GPD) were purchased

from Invitrogen (Carlsbad, CA) and Sigma, respectively. Rabbit polyclonal antibody recognizing Ssa3/Ssa4 was a kind gift from E. Craig. Band intensities were quantified using ImageJ (NIH) and normalized to the load control.

*Protein degradation assay* - To assess the roles of thiol-reactive compounds in the ubiquitin-proteasome system, we measured the stabilities of three reporter proteins expressed from the plasmids YEp13-Deg- $\beta$ Gal, p415GPD-Ub-L- $\beta$ Gal and p415GPD-Ub-P- $\beta$ Gal (kind gifts from Dr. J. Laney, Brown University, Providence, RI) (199, 200). Mid-log phase cells carrying one of the above reporter proteins were treated with DMSO, 100  $\mu$ M MG132 or 100  $\mu$ M Cd<sup>2+</sup> for 1 h, followed by treatment for another 1 h with 100  $\mu$ g/mL cycloheximide to halt new protein synthesis.  $\beta$ -galactosidase activity was measured to determine steady-state levels of the reporter proteins.

*Biotin-BMCC labeling assay* - To investigate the potential targets of thiol modification, protein-TAP fusions were isolated from cells as follows. Protein extracts were prepared by glass bead lysis in TEGN buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl) with a protease inhibitor cocktail (aprotinin, 2  $\mu$ g/mL; pepstatin A, 2  $\mu$ g/mL; leupeptin, 1  $\mu$ g/mL; phenylmethylsulfonyl fluoride, 1 mM; chymostatin, 2  $\mu$ g/mL; Sigma), and protein concentration determined by Bradford assay. 0.5 -1.0 mg of cell extract in a volume less than 300  $\mu$ L was incubated with 75  $\mu$ L IgG-Sepharose (GE HealthCare Lifesciences, Piscataway, NJ) and 625  $\mu$ L TEGN buffer at 4 °C on a rotating wheel for 2 hours. Beads were collected by brief centrifugation. The supernatant was removed, and the beads were washed six times with lysis buffer. The washed beads were

resuspended in 1 mL TEGN buffer and treated with 32  $\mu$ M biotin-BMCC (1-biotinamido-4-(4'-[maleimidoethyl-cyclohexane]-carboxamido)butane) at room temperature for 15 min. The beads then were washed twice with 1 mL TEGN buffer before proteins remaining on beads were eluted by 2X SDS sample buffer (200 mM Tris-HCl, pH=6.8, 20% glycerol, 0.8% SDS, 6 mM  $\beta$ -mercaptoethanol, 0.4% Bromophenol Blue). Immunoblot was performed using streptavidin conjugated to horseradish peroxidase to detect thiol-modified proteins (Bio-Rad, Hercules, CA). Polyclonal antibody recognizing TAP-tag (anti-PtnA, Sigma) was used at 1:1000 dilution. Purified Ssa1 protein was described elsewhere (102) and detected using polyclonal anti-Ssa1 antibody provided by Dr. Mark Ptashne (Memorial Sloan-Kettering Cancer Center, NY) (201). 300 nM Ssa1 was reacted with 32  $\mu$ M biotin-BMCC for the indicated times and the reaction quenched with 2X SDS sample buffer. DTT inactivation of biotin-BMCC was carried out by adding DTT in water to final concentrations sufficient to achieve the indicated molar ratios with biotin-BMCC 15 min prior to incubation with purified Ssa1.

*Heat shock sensitivity assay* - To assay thermotolerance induced by CdSO<sub>4</sub> treatment, *ssa1Δ ssa2Δ* cells bearing an empty vector, *SSA1* wild-type or cysteine mutant alleles were treated with no reagent or 600  $\mu$ M Cd<sup>2+</sup> for 1 h at 30 °C. After treatment, cells were diluted to a density of approximately 100,000 cells/ml in sterile PCR tubes in a volume of 100  $\mu$ L. The diluted cells were heat shocked at 47 °C in a thermocycler for 0, 5, 10, and 20 min before spotting onto solid SC medium, followed by incubation at 30 °C for 2 days.



*Click-it chemistry analysis* - The *ssa1Δssa2Δ* strain carrying empty vector, FLAG-tagged *SSA1* wild-type or cysteine mutant alleles were treated for 1 h with ethanol, or 500  $\mu$ M 4-hydroxyl nonenal alkyne (Cayman Chemical, Ann Arbor, MI). The extract was prepared by glass bead lysis and protein concentration was determined by Bradford assay as described above. 0.5-1.0 mg of cell extract was compensated with TEGN buffer plus a protease inhibitor cocktail to reach a total volume of 700  $\mu$ L and incubated with 20  $\mu$ L FLAG resin (Sigma) at 4  $^{\circ}$ C on a rotating wheel for 2 h. The resin was collected by centrifugation at maximum speed for 30 s. The supernatant was discarded, and the resin was washed eight times with lysis buffer. Proteins tagged by 4-HNE alkyne were detected using the Click-iT<sup>®</sup> reaction buffer kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). In detail, the washed resin was resuspended in 60  $\mu$ L of 50mM Tris-HCl buffer (pH 8.0), 100  $\mu$ L of 40  $\mu$ M Click-iT reaction buffer and 60  $\mu$ L 18 megaOhm water to reach a final volume of 160  $\mu$ L. After vortexing the mixture at room temperature for 5 sec, 10  $\mu$ L of 40 mM CuSO<sub>4</sub> was added to catalyze the reaction. The reaction solution was then incubated with 10  $\mu$ L of additive 1 stock solution on bench for 2-3 min (not longer than 5 min), followed by vortexing with 20  $\mu$ L of additive 2 stock solution for 5 sec. The solution turned bright orange at this step. The tube was then wrapped with foil to prevent from light and rotated for 20 min at room temperature. The resin in the reaction mixture was gently washed three times with 1 mL of 50mM Tris-HCl buffer (pH 8.0). Click-tagged proteins were eluted by incubating with 30  $\mu$ L of 1 x FLAG peptide (Sigma) for 30 min at room temperature, and labeling of Ssa1 was detected by immunoblot with streptavidin-HRP.

*Cell fractionation analysis* - To analyze levels of protein aggregation in the presence of thermal stress and thiolreactive compounds, wild-type (BY4741) cells were fractionated by high-speed centrifugation. Log phase cells were treated with heat shock, 600  $\mu$ M Cd<sup>2+</sup> or 500  $\mu$ M DEM for 1 h. Cell pellets were transferred into 1.5 mL centrifuge tubes containing 300  $\mu$ L dry volume of acid washed glass beads and 600  $\mu$ L ice-cold TEGN with a protease inhibitor cocktail as described above. The cells were lysed by six 45 s rounds of vigorous vortexing followed with 90 s rests of the tubes on ice. The resulting lysate was first subjected to a low-speed spin at 3,000 g for 30 s to remove unbroken cells. 500  $\mu$ L of the supernatant was transferred into new tubes, and incubated with 1% Triton-X for 30 min at 4 °C with gentle rotation to dissolve membranes, followed by centrifugation at 100,000 g for 30 min, yielding supernatant (S) and pellet (P) fractions.

### **Chapter 3: Thiol-reactive compounds do not activate Hsf1 by causing the stress of misfolded proteins**

*NOTE: this chapter is derived from work that has, for the most part, been published in 2012: "The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds." Molecular Biology of the Cell 2012 Sep; 23(17): 3290-8 (<http://www.molbiolcell.org/content/23/17/3290.abstract>, copyright (2012) the American Society of Cell Biology. I am the primary author on this paper and was responsible for preparing the original manuscript. I performed all experiments described in this chapter. The publisher of MBoC, the American Society of Cell Biology, grant authors the right to revise, adapt, prepare derivative works, present, or distribute the manuscript provided that all such distribution is for noncommercial benefit and there appears always the ASCB copyright credit and link to the original publication of the manuscript in MBoC Online. (<http://creativecommons.org/licenses/by-nc-sa/3.0/>)*

## INTRODUCTION

All eukaryotes express so called heat shock transcription factors, mainly HSF1, to regulate transcription of HSP expression in response to stress (12, 202). Upon activation, HSF1 undergoes trimerization, nuclear translocation, DNA binding, and posttranslational modifications. The baker's yeast *Saccharomyces cerevisiae* has a single, essential HSF equivalent to the mammalian HSF1 (3, 34). Although the general structure and functions of HSF1 are conserved among eukaryotes, early structural studies found that yeast Hsf1 constitutively binds to the heat shock transcription element (HSE) in the promoters of *hsp* genes as a homotrimer in the absence of stress (36, 203, 204). This observation strongly suggests that without the two potential control steps – trimerization and nuclear translocation, yeast Hsf1 activity is modulated via a derepression/activation mechanism. Genetic and biochemical investigation indicates that in non-stress conditions, a set of chaperones including Hsp70, Hsp90, and their co-chaperones such as Cpr6/7 and Sse1 associate with Hsf1 and repress its activity (76, 77, 205). During heat shock, the increased temperature leads to massive accumulation of damaged proteins. Since the main functions of chaperones are to hold and re-fold proteins and to prevent them from aggregation and degradation, these chaperones may bind to unfolded proteins and release Hsf1 to its active form. When sufficient chaperones are synthesized to handle protein damage in the cell, the excess chaperones transport back to the nucleus, and bind Hsf1 to terminate transcription. Although concrete evidence for physical association of HSPs and Hsf1 is still missing, this derepression/activation model suggests that Hsf1 is likely to be activated by sensing protein misfolding.

Many neurodegenerative diseases such as Alzheimer's and Huntington's diseases are associated with protein misfolding, and chaperones are known to have an important role in maintaining protein homeostasis (206-209). Recently, many academic and industrial laboratories are actively testing small molecule libraries as HSF1 activators for therapeutic use (34). However, the mechanism through which individual small molecules are initially sensed to activate HSF1, and whether the mechanisms are common for all molecules, remain unclear. Most of the known HSF1 activators, to some extent, affect the protein homeostasis network by either causing proteotoxic stress or inhibiting the chaperone proteins that repress HSF1 activity. Known activators that promote protein misfolding or proteotoxic stress include: 1) protein translation inhibitors, e.g. puromycin; 2) amino acid analogs that result in misfolding of nascent chains, e.g. azetidine 2-carboxylate (AZC); and 3) proteasome inhibitors, e.g. MG132 (159, 170, 209) (Figure 3-9). Some other activators function as pharmacological inhibitors of molecular chaperones. For example, most well-characterized HSP90 inhibitors, such as the benzoquinone ansamycin antibiotic geldenamycin and macrocyclic anti-fungal antibiotic radicicol, inhibit the ATPase activity of HSP90 by binding to its N-terminal ATP-binding pocket (130, 188, 210-212). Several additional compounds including the coumarin antibiotic novobiocin, ITZ-1, and AEG3482, on the contrary, block HSP90 activity by binding to its C-terminal domain (213, 214). The important feature of these HSF1 activators is that they directly target integral parts of the protein homeostasis network to dramatically alter the functions of protein synthesis, folding and degradation machineries. The imbalance of cellular homeostasis probably leads to accumulation of unfolded proteins that aggregate in the cytoplasm, which consequently triggers HSR activation (170, 215, 216).


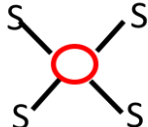
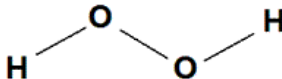
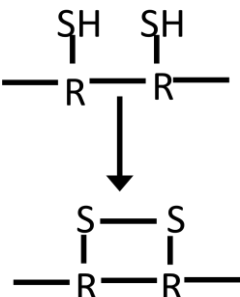
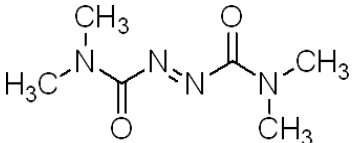
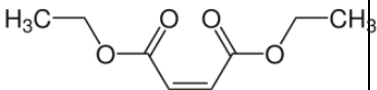
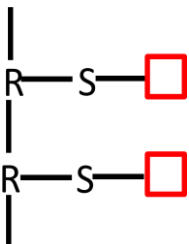
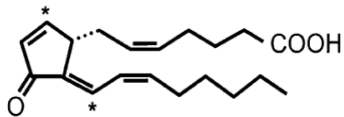
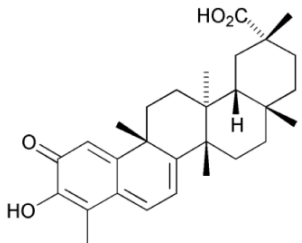
For other HSF1 activators, the molecular targets that are linked with increased expression of chaperones are unknown. Reactive compounds that fall into this category include: 1) oxidants, such as hydrogen peroxide and diamide; 2) transition metals (e.g.,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Co}^{2+}$ ) and metalloids (e.g.,  $\text{AsO}_2^-$ ); and 3) organic electrophiles, for examples, 15-deoxy- $\Delta^{12,14}$ -PGJ2 (15d-PGJ<sub>2</sub>), 4-hydroxy-2-nonenal (4HNE), and celastrol (19, 171, 173, 180, 217-222). Each class of these compounds has the potential to damage proteins directly, often on the side chains of highly reactive cysteine residues (170). Here I discovered that all three classes of thiol-reactive compounds can activate HSR in the yeast *S. cerevisiae*. In addition, these compounds also inhibit Hsp90 signal transduction activity in a reciprocal manner. Hsf1 activation by thiol-reactive compounds is kinetically distinct from activation by protein misfolding induced by an amino acid analog or a membrane-permeable protein cross-linking agent and is also independent of three known pathways of proteasomal degradation.

## RESULTS

### *Thiol-reactive compounds can activate Hsf1 and inhibit Hsp90*

Our lab previously demonstrated that celastrol, a natural compound isolated from a Chinese medicinal herb thundergod vine (雷公藤, lei gong teng), is a potent activator of the heat shock transcription factor Hsf1 and inhibitor of Hsp90 in yeast *Saccharomyces cerevisiae*, as it is in human cell lines (178-180, 223-225). Interestingly, we also found that celastrol is a cellular oxidative stressor. This observation is consistent with the putative identification of at least two electrophilic centers (C<sub>2</sub> on A-ring and C<sub>6</sub> on B-ring) in the molecule that are highly susceptible to nucleophilic attack (226). To test whether Hsf1 is sensitive to activation by thiol-reactive compounds in general, I evaluated a selection of molecules using an HSE-*lacZ* reporter system, which faithfully reports induction of Hsf1 in an easily assayable format (227) (Table 3-1). As shown in Figure 3-1, the thiol oxidants diamide (Figure 3-1A) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Figure 3-1B), the metalloid thiol chelator cadmium (CdSO<sub>4</sub>, Figure 3-1C), and the organic electrophiles diethyl maleate (DEM, Figure 3-1D) and the cyclopentanone prostaglandin 15-deoxy- $\Delta^{12,14}$  - prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>, Figure 3-1E) all activated Hsf1 in a dose-dependent manner. Intriguingly, the effective dose ranges suggested that Hsf1 is exquisitely sensitive to these reactive compounds at low concentrations. This observation is consistent with previous findings that mammalian Hsp90 and Hsp70 are both modified by low concentration (~10  $\mu$ M) of the electrophilic byproduct of lipid peroxidation 4-hydroxynonenal (4-HNE) (228, 229). Early studies demonstrated that Hsf1 is constitutively phosphorylated in the absence of stress, and becomes heterogeneously hyper-phosphorylated during heat shock (6, 36, 37). Therefore, I measured the SDS-

**Table 3-1. Chemical mechanisms for thiol-reactive compounds reacting with cysteine sulfhydryl groups**

Metals/ Metalloids	Cadmium		
Oxidants	Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> )		
	Diamide		
Organic electrophiles	Diethyl maleate (DEM)		
	15-deoxy-Δ <sup>12,14</sup> – prostaglandin J <sub>2</sub> (15d-PGJ <sub>2</sub> )		
	Celastrol		



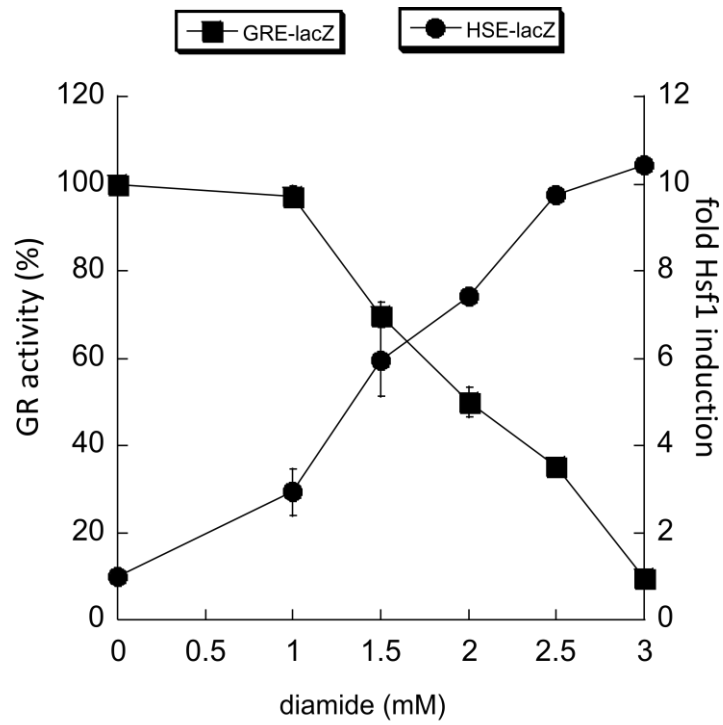
**Figure 3-1. Thiol-reactive compounds activate Hsf1 and inhibit Hsp90 in a dose-dependent manner.**

Wild-type (BY4741) cells carrying the HSE-*lacZ* reporter (●) or the glucocorticoid receptor system (p413GPD-rRG and pYRP-GRE-*lacZ*) (■) were treated with different concentrations of (A) diamide, (B) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), (C) cadmium sulfate (CdSO<sub>4</sub>), (D) diethyl maleate (DEM), or (E) 15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>). For the Hsf1 activation experiments, cells were treated for 2 hr at the indicated concentrations and induction normalized to activity from untreated cells (30 °C). Hsp90 inhibition is measured by GR activity after incubation with the compounds for 1 hr before treatment for another 1 hr with 10 μM deoxycorticosterone (DOC), and plotted as percent of activity in untreated control cells induced with DOC.

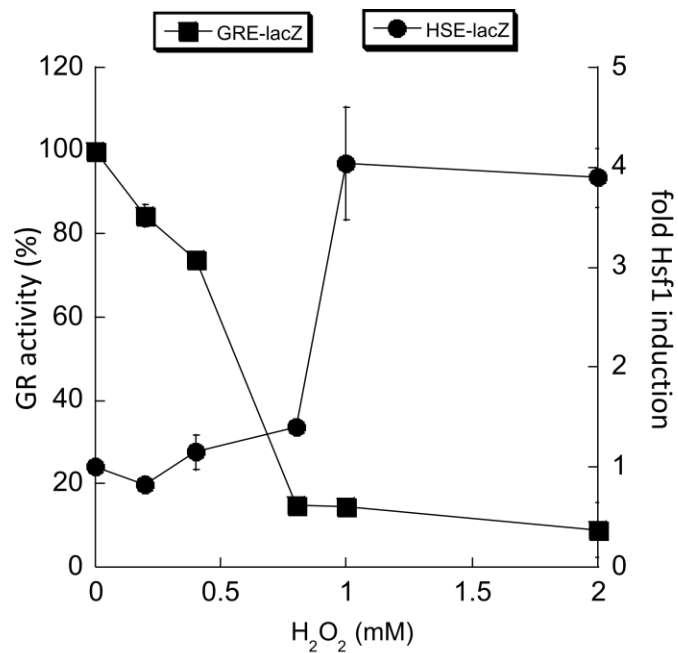
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**Figure 3-1. Thiol-reactive compounds activate Hsf1 and inhibit Hsp90 in a dose-dependent manner.**

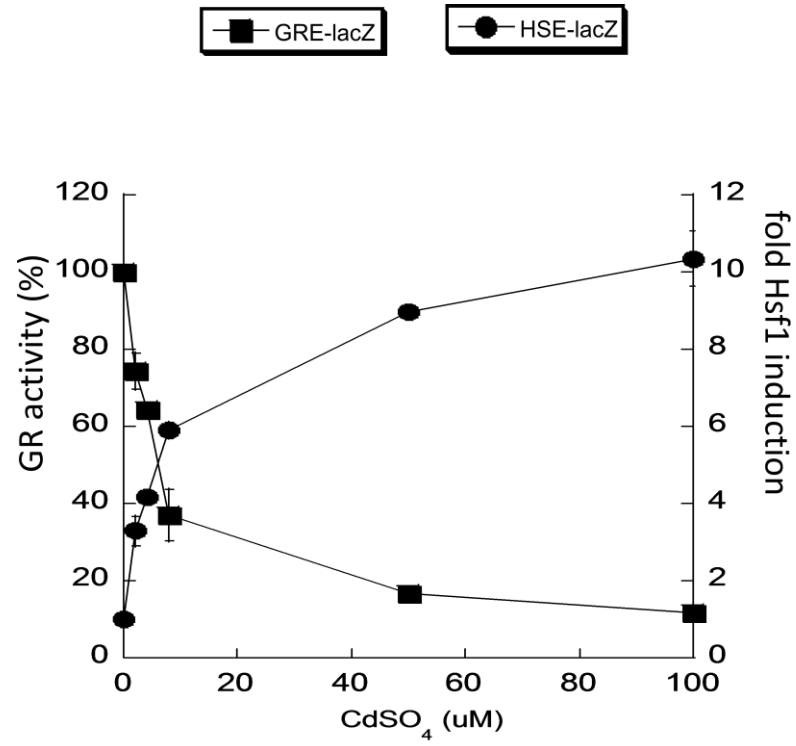
A.



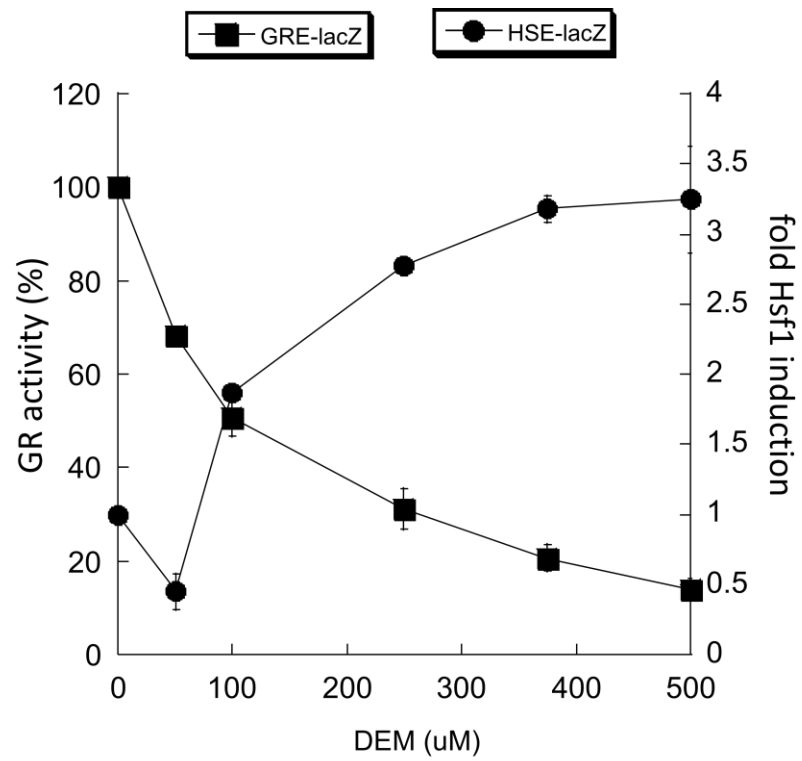
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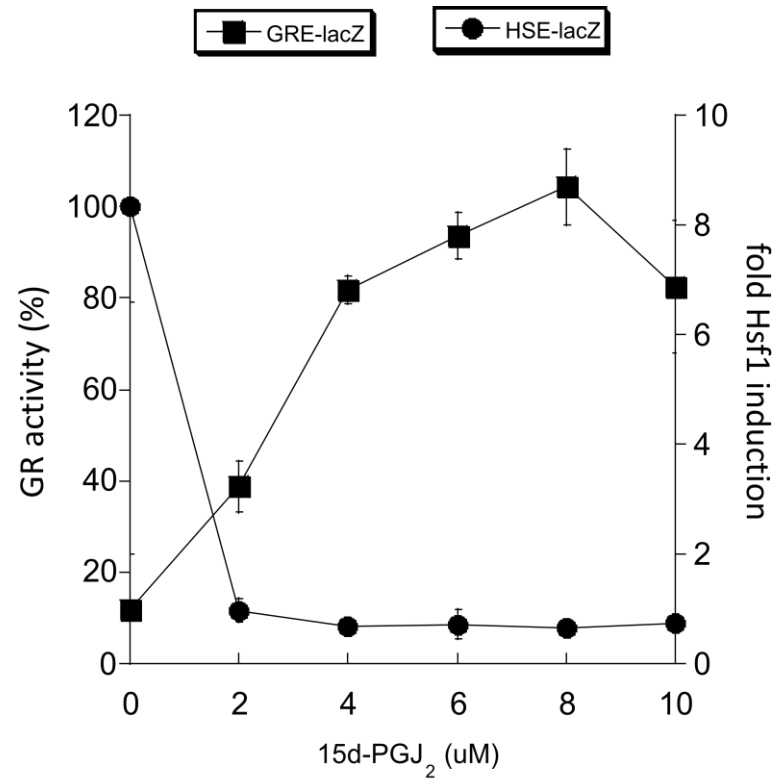
C.



D.



E.



PAGE mobility shift produced by these compounds to verify HSR activation. Since an antibody specific for yeast Hsf1 or phosphor-serine in Hsf1 is not commercially available, I utilized a chromosomal integrated *HSF1*-TAP fusion instead. Due to phosphorylation in the resting state, yeast Hsf1, an 833 amino acid protein, exhibits substantially larger than predicted molecular mass substantially. In addition, the C-terminal TAP insertion, which consists of a calmodulin binding peptide (CBP), a tobacco etch virus protease (TEV protease) cleavage site and two IgG binding domains of protein A increases another 23 kDa of molecular weight to the fusion construct. The large size of *HSF1*-TAP fusion (~140 kDa) limits the mobility of the protein in the gel, making it difficult to detect the shift after treatments. Moreover, little is known about the kinases and phosphatases involved in the phosphorylation/de-phosphorylation of Hsf1 in yeast. Together, it is extremely difficult to demonstrate evidently the changes of phosphorylation states of yeast Hsf1 during stress. The best result I generated is shown in Figure 3-2. Although smeared, the mobility shift of Hsf1 produced by each compound was consistent with phosphorylated Hsf1 observed upon heat shock at 37 °C, confirming Hsf1 activation by these reactive compounds.

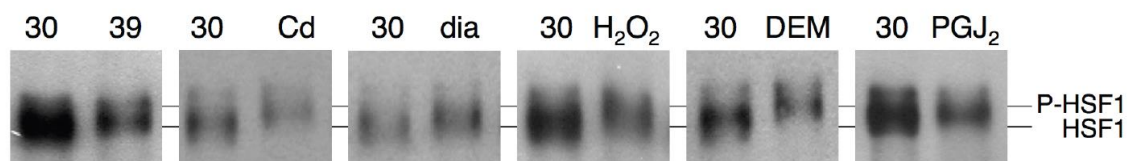
In addition to HSR activation, I also found that these thiol-reactive compounds inhibited Hsp90 activity using a heterologous glucocorticoid receptor (GR) assay (Figure 3-1). The GR is a member of the steroid hormone receptor subfamily of nuclear receptors, which regulates transcription of genes controlling development, metabolism, and immune response. It is normally expressed in all mammalian cell types. In the absence of ligand such as cortisol and other glucocorticoids, the unbound receptor selectively associates with Hsp90 chaperone complex to maintain itself in a stable conformation (230). In order

**Figure 3-2. Thiol-reactive compounds induce hyper-phosphorylation of Hsf1.**

Wild-type cells carrying a chromosome integrated TAP-tagged *HSF1* were grown to mid-log phase and incubated with Cd, diamide, H<sub>2</sub>O<sub>2</sub>, DEM, or 15d-PGJ<sub>2</sub>. Protein extracts were analyzed by 6% SDS-PAGE and immunoblot using antibodies directed against the protein A epitope.

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**Figure 3-2. Thiol-reactive compounds induce hyper-phosphorylation of Hsf1**



to apply the GR system in yeast, the rat GR was expressed under the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (p413-GPD-GR) and transformed with pYRP-GRElacZ into wild-type yeast cells. The Hsp90-dependent GR activity was measured by  $\beta$ -galactosidase activity assay as an indicator of Hsp90 function. In Figure 3-1, I showed that the same doses of thiol-reactive compounds reciprocally activated Hsf1 and inhibited Hsp90 in parallel cell cultures.

Because the GR is a zinc-finger transcription factor with two potentially reactive Cys<sub>2</sub>Cys<sub>2</sub> modules, it is possible that the GR, but not Hsp90 chaperone complex, was impaired by thiol-reactive compounds. To test this possibility, we utilized an Hsp90-independent truncated GR (N525) as a control to test the sensitivity of the GR system to thiol-reactive compounds (231). As shown in Figure 3-3, constitutive activation by GR-N525 was not affected by treatment with Cd<sup>2+</sup>, H<sub>2</sub>O<sub>2</sub>, diamide and DEM, confirming that these reactive compounds inhibited Hsp90 activity rather than function of the GR.

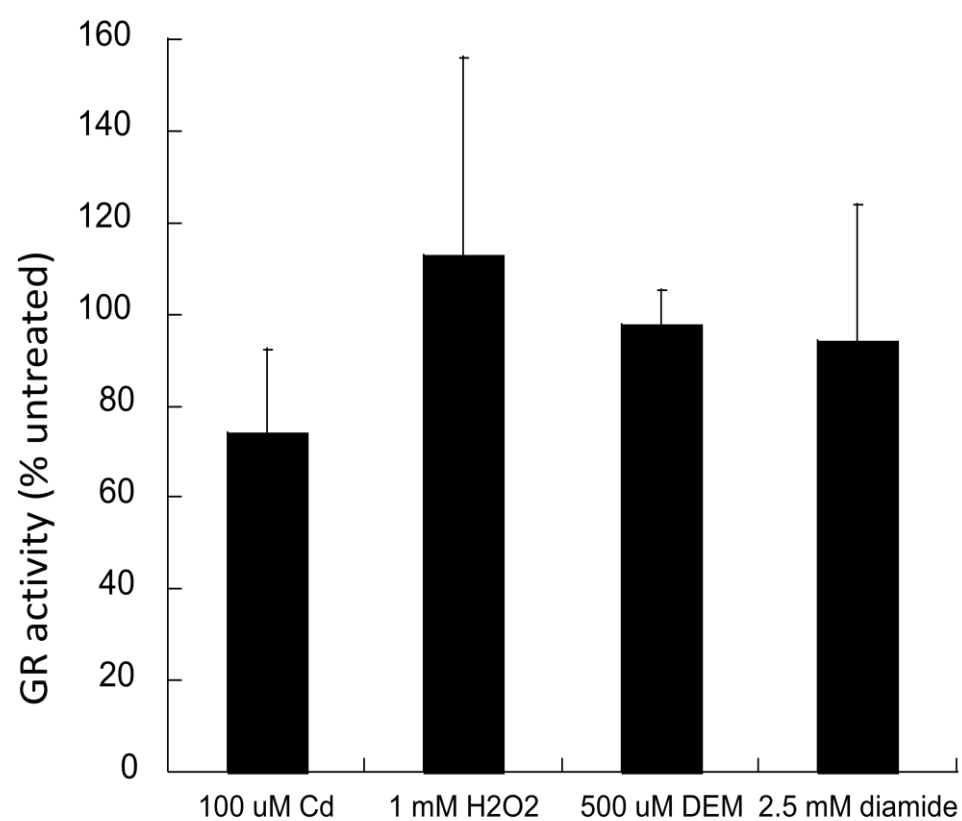
To verify that the observed Hsf1 activation was due to thiol reactivity, we incubated the oxidant diamide or the electrophile 15d-PGJ2 with different concentrations of the reducing agent dithiothreitol (DTT) for 15 min *in vitro* prior to treating cells carrying the HSE-lacZ reporter (Figure 3-4). We observed that five-fold excess of DTT completely abolished activation of Hsf1 by both compounds. These results are consistent with our previous finding that HSR activation and Hsp90 inhibition by celastrol are inhibited by DTT, raising the possibility that different thiol-reactive compounds may activate the HSR via a common mechanism (180).



**Figure 3-3. Thiol-reactive compounds do not inhibit function of the GR client**

BY4741 cells carrying a truncated Hsp90-independent glucocorticoid receptor (N525GR) and the GRE-*lacZ* reporter (p413GPD-rN525GR and pYRPGRElacZ). Cells were grown to mid-log phase and exposed to no reagent, 100  $\mu\text{M}$   $\text{Cd}^{2+}$ , 800  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 2.5 mM diamide or 500  $\mu\text{M}$  DEM for 1hr, followed by treatment with 10  $\mu\text{M}$  deoxycorticosterone (DOC) for 1 hr.  $\beta$ -galactosidase activity was measured and presented relative to untreated cells as percentage of untreated GR activity (% untreated).

**Figure 3-3. Thiol-reactive compounds do not inhibit function of the GR client.**

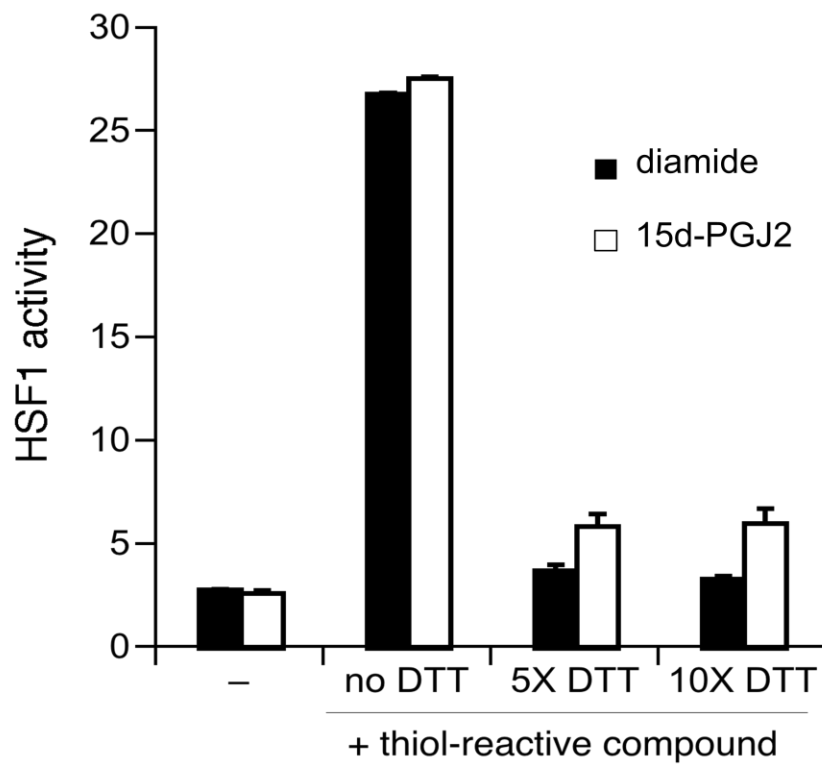


**Figure 3-4. Thiol reactivity is required for the HSR activation by diamide and 15d-PGJ<sub>2</sub>.**

Diamide (2.5 mM, solid bar) or 15d-PGJ<sub>2</sub> (5.6 μM, open bar) was treated with 5X, 10X excess dithiothreitol (DTT) or water alone for 15 min prior to cell treatment. Hsf1 activation was measured as in Figure 3-1, but is plotted as absolute activity in Miller units.

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**Figure 3-4. Thiol reactivity is required for HSR activation by diamide and 15d-PGJ2.**



*Thiol-reactive compounds do not activate Hsf1 by causing accumulation of unfolded proteins*

Hsf1 is generally thought to be activated in response to heat shock through the accumulation of unfolded or damaged proteins. This model is further supported by the observation that treatment with the proline analog azetidine-2-carboxylic acid (AZC) causes misfolding of nascent proteins and induces a set of phenotypic responses that mimic heat shock response in yeast cells. For example, AZC treatment reversibly arrests the cell cycle in G1 phase in a manner similar to heat shock (162, 232). The treatment also leads to expression changes in Hsf1-dependent genes, but fails to induce the general stress response (Msn2/4) in the cytoplasm or the unfolded protein response in the ER (162). Similar gene expression changes have been observed in cells treated with sublethal concentrations of ethanol (4-8%) (233, 234). Furthermore, Hsp70 is shown to bind avidly to AZC-containing proteins *in vivo* (235). Lastly, disruption of protein degradation by the proteasome inhibitor MG132 also results in activation of the HSR (236). Taken together, these studies suggest that the accumulation of misfolded proteins induces the HSR, but none of these data clearly demonstrate whether it is accumulation of nascent chains, existing proteins, or both forms acting as the primary inducer. Therefore, we sought to understand whether the thiol-reactive compounds we identified trigger Hsf1 activation by causing misfolding of cytosolic proteins.

Since nascent proteins are highly susceptible to misfolding, I first tested whether treatment with thiol-reactive compounds damaged newly synthesized proteins by comparing HSE-*lacZ* expression profiles after these treatments with those induced by AZC. The incorporation of AZC was found to inhibit the  $\beta$ -galactosidase activity assay

presumably due to misfolding of the  $\beta$ -galactosidase enzyme. I therefore prepared protein extracts and quantified the expression levels of the inducible Hsp70 Ssa3/4 via immunoblot. Yeast cells were treated with  $\text{Cd}^{2+}$  (100  $\mu\text{M}$ ), diamide (2.5 mM), or AZC (10 mM) for 0.5, 2 and 5 hr. As shown in Figure 3-5A and 3-5B, the activation patterns were distinct.  $\text{Cd}^{2+}$  and diamide demonstrated a typical Hsf1 induction profile with rapid induction of HSP followed by attenuation to a level lower than the maximum but higher than the basal. In contrast, activation of Hsf1 by AZC treatment was slower but persistent, and continued to increase over the 5 hour time course. I noted that in cells treated with thiol-reactive compounds, both Ssa3 and Ssa4 were detected as two separate bands by immunoblot. However, only one band was observed from cells incubated with AZC (Figure 3-5A). The Ssa3/4 antibody was a kind gift from Craig Lab, and was showed to specifically recognize both inducible Hsp70s in yeast (237). The reason why AZC treatment resulted in a different band pattern of Western blot analysis is not perfectly clear. Together, these data suggest that Hsf1 activation by the thiol-reactive compounds is transient and reversible; while activation by AZC is cumulative and irreversible, likely due to continuous production and accumulation of misfolded AZC-containing proteins. Consistent with the kinetic data, we observed that cells treated with  $\text{Cd}^{2+}$  or diamide for 2 hr or heat shocked (37 °C) for 1 hr exhibited no loss in survival compared to untreated control cultures, whereas cells treated with AZC for 2 hr demonstrated significant loss in viability (Figure 3-5C). Together, these data suggest that the thiol-reactive compounds activate Hsf1 by different kinetic induction profiles compared with those of an unfolding reagent, such as AZC. Therefore, thiol-reactive compounds are not likely to induce the heat shock response by causing bulk misfolding of nascent proteins.

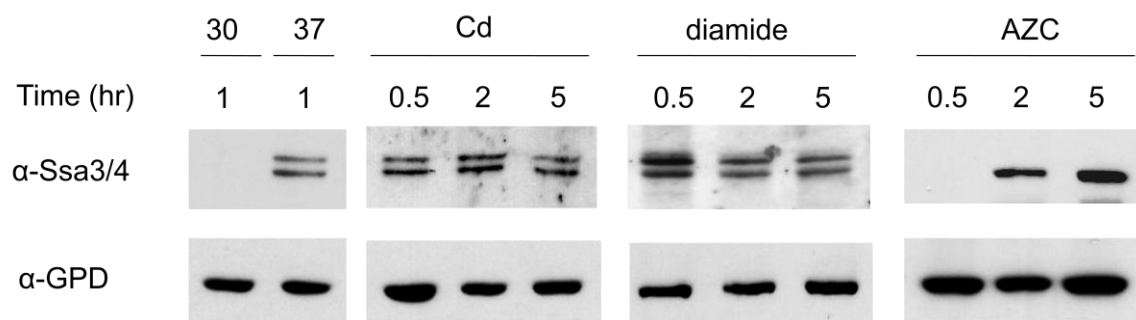
**Figure 3-5. Thiol-reactive compounds do not induce the heat shock response by causing accumulation of nascent proteins.**

- A. Hsf1 activity profiles after treatment with thiol-reactive compounds or the proline analog azetidine 2-carboxylic acid (AZC) are distinct. Wild-type cells (BY4741) were treated with 100  $\mu$ M CdSO<sub>4</sub>, 2.5 mM diamide, or 10 mM AZC for 0.5, 2, and 5 hr. Ssa3/4 levels were detected by anti-Ssa3/4 antibody. glucose 6-phosphate dehydrogenase (GPD) was blotted as the loading control.
- B. Quantification of Ssa3/4 protein levels by immunoblot analysis. Wild-type cells were treated with 100  $\mu$ M CdSO<sub>4</sub> (■), 2.5 mM diamide (▲), or 10 mM AZC (●) as described above. Band intensities were normalized to the loading control GPD, and are plotted relative to cells treated for 5 hr as percentage of maximum HSR induction.
- C. AZC, but not thiol-reactive compounds, are toxic at Hsf1-activating doses. Mid-log phase cells (BY4741) were treated with no reagent (30 °C), heat shock for 1 hr (37 °C), or 100  $\mu$ M CdSO<sub>4</sub>, 2.5 mM diamide, or 10 mM AZC for 2 hr. Cells were then plated on YPD media and grown at 30 °C for 2 days.

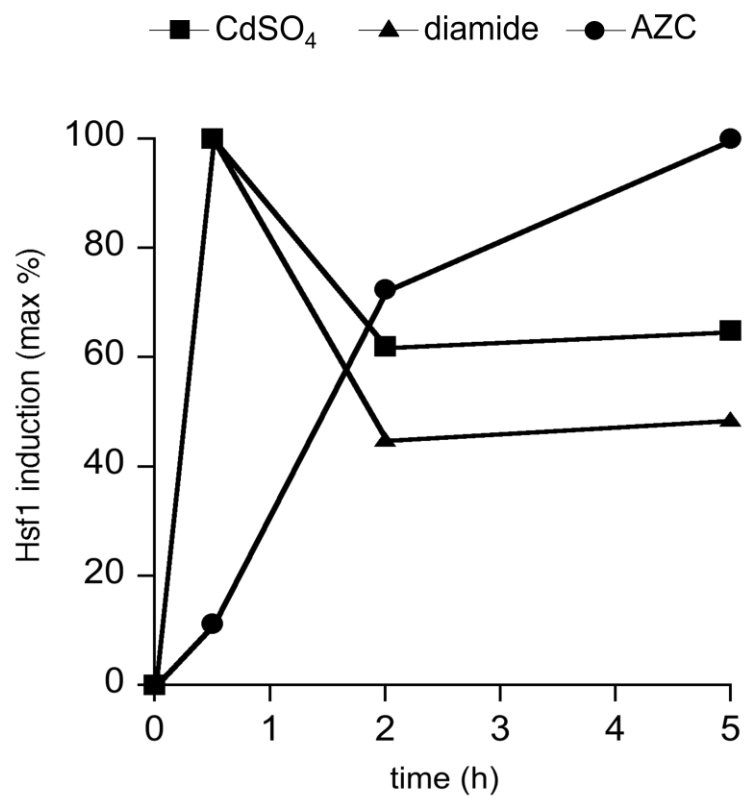
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**Figure 3-5. Thiol-reactive compounds do not induce the heat shock response by causing accumulation of nascent proteins.**

A.



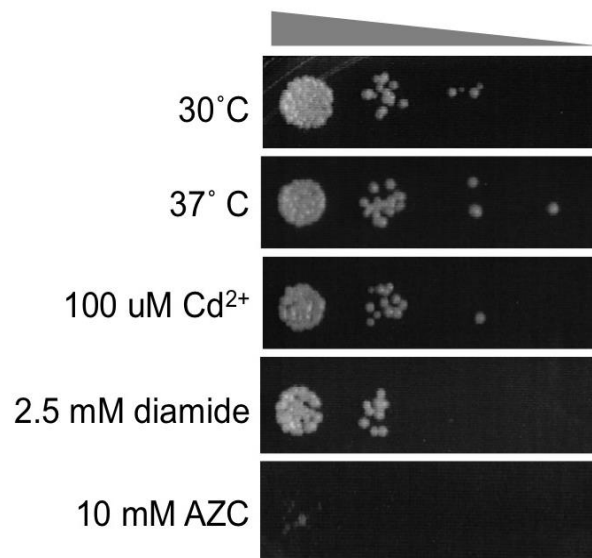
B.





**Figure 3-5. Thiol-reactive compounds do not induce the heat shock response by causing accumulation of nascent proteins.**

C.



Another possible explanation for Hsf1 activation by thiol-reactive compounds could be misfolding of existing proteins caused by non-specific modification of cysteine residues throughout the proteome. To address this, we treated cells with the membrane permeable protein cross-linker dithiobis[succinimidyl propionate] (DSP). DSP, which has amine-reactive *N*-hydroxysuccinimide (NHS) esters at both ends of a cleavable spacer arm, can be used to non-specifically crosslink intracellular proteins and cause misfolding of existing cytosolic proteins. After a 4 hr treatment at normal growth temperature, we observed that DSP, but not the membrane impermeable analog 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), activate Hsf1 in a dose-dependent manner, suggesting that DSP was capable of causing protein misfolding in living cells with maximum efficacy at approximately 400  $\mu$ M (Figure 3-6A). I therefore treated cells bearing the HSE-*lacZ* or GR reporter system with 400  $\mu$ M DSP to study the kinetic profiles of this protein misfolding reagent over time. Figure 3-6B shows that DSP activated Hsf1 in a time-dependent manner, with a continuous induction profile very similar to that observed with AZC treatment. Furthermore, DSP treatment caused minor to no effect on Hsp90 activity over for 4 hours; while thiol-reactive compounds almost completely inhibited Hsp90 in two hours at their maximum effective concentrations (Figure 3-6B and 3-1). These results demonstrated that thiol-reactive compounds and DSP induce Hsf1 by distinct mechanisms, suggesting that bulk protein aggregation or misfolding activates Hsf1 differently from thiol-reactive compounds.

To directly examine whether treatment with thiol-reactive compounds leads to protein aggregation, I fractionated cell lysates after treatment using ultracentrifugation. Protein aggregates segregate into the pellet fraction at 100,000 x g. Whereas heat shock

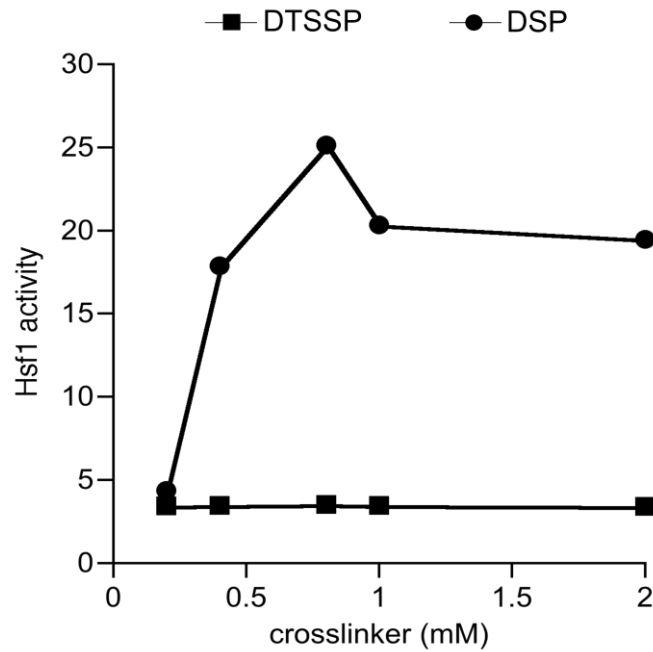
**Figure 3-6. Thiol-reactive compounds do not induce the heat shock response by misfolding existing proteins.**

- A. BY4741 cells carrying the HSE-*lacZ* reporter were treated with the indicated concentrations of DSP (●) or DTSSP (■) for 4 hr and Hsf1 activity measured as described in Figure 3-1.
- B. BY4741 cells carrying the HSE-*lacZ* reporter (●) were treated with 400  $\mu$ M DSP for the indicated times, as were cells bearing the GR reporter system (■) (followed by 1 hr treatment with 10  $\mu$ M DOC). Activities are plotted as described in Figure 3-1.

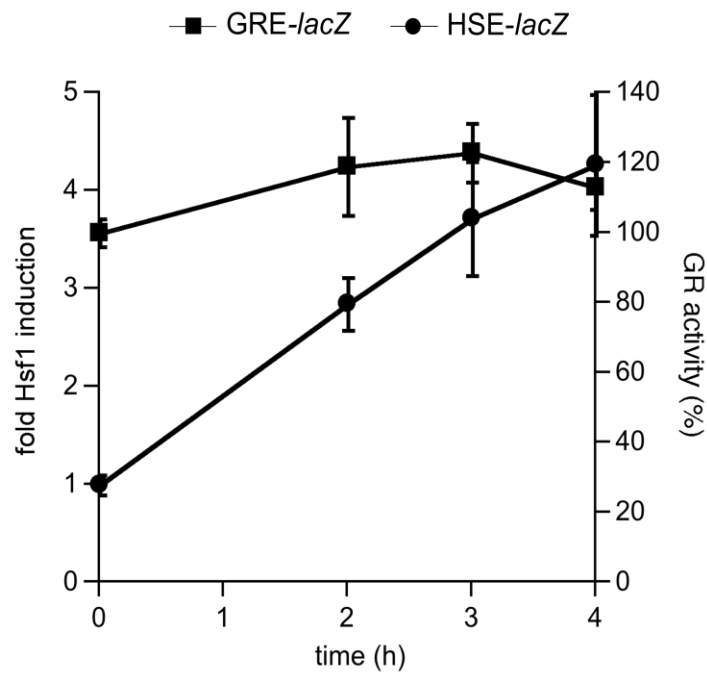
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**Figure 3-6. Thiol-reactive compounds do not induce the heat shock response by misfolding existing proteins.**

A.



B.



led to a significant conversion of proteins from the soluble fraction to the pellet fraction, control cells and those treated with thiol-reactive compounds ( $\text{Cd}^{2+}$  or DEM) maintained solubility of the majority of proteins (Figure 3-7), suggesting that treatment with these compounds does not lead to significant protein aggregation. Taken together, these data indicate that the features of Hsf1 activation by thiol-reactive compounds are not consistent with a model relying on the accumulation of misfolded cytosolic proteins.

*Thiol-reactive compounds do not induce the HSR by inhibiting the ubiquitin-proteasome system (UPS)*

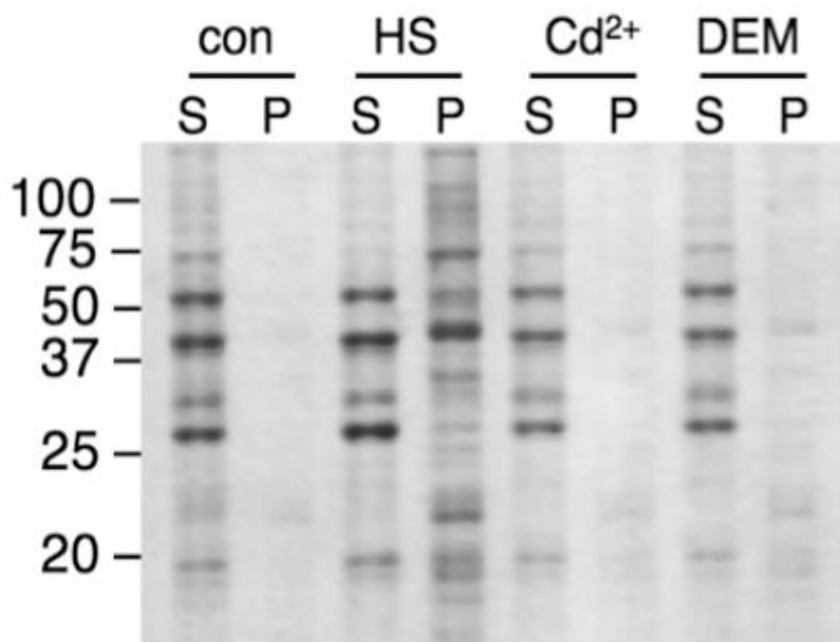
I next asked whether these compounds affect other aspects of the protein quality control system, such as the ubiquitin-proteasome system (UPS). The UPS is responsible for degradation of short-lived and abnormal proteins, and normal protein turnover to provide a pool of free amino acid (238). The yeast *S. cerevisiae* has a single ubiquitin activating enzyme (E1) encoded by the gene *UBA1*, a limited set of ubiquitin conjugating enzymes (E2) and multiple ubiquitin ligases (E3) (239). In the process of ubiquitination, ubiquitin (Ub) is transferred from E1 to the active cysteine of E2 via a thioester linkage (240) (Figure 3-8). I therefore sought to investigate whether thiol-reactive compounds induce Hsf1 by modification and inactivation of E1 or E2 enzymes, which might inhibit UPS-mediated protein degradation, resulting in accumulation of non-ubiquitinated, misfolded proteins. Furthermore, celastrol, first indentified as an Hsf1 activator in yeast and mammalian cells, also inhibits proteasome activity, albeit after extended treatment periods (19, 226). Other proteasome inhibitors including the peptide aldehydes (MG132, MG115, and *N*-acetyl-leucyl-leucyl-norleucinal), and lactacystin are known inducers

**Figure 3-7. Thiol-reactive compounds do not cause protein aggregation at Hsf1-activating dose.**

Cells treated with 100  $\mu\text{M}$   $\text{Cd}^{2+}$ , 500  $\mu\text{M}$  DEM, or heat shock for 1 hr were lysed, and proteins were fractionated by ultracentrifugation. A coomassie-stained SDS-PAGE gel is shown. S, supernatant; P, pellet.

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**Figure 3-7. Thiol-reactive compounds do not cause protein aggregation at Hsf1-activating dose.**

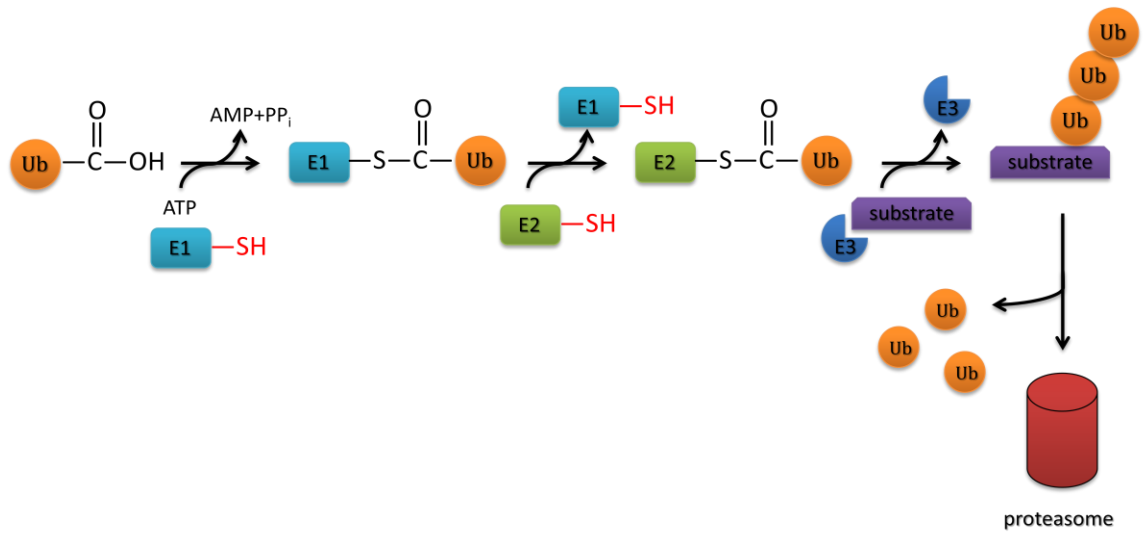


**Figure 3-8. The ubiquitination pathway**

In the first step, a ubiquitin activating enzyme (E1) hydrolyzes ATP to activate free ubiquitin (Ub). A thioester linkage is formed between E1 and the carboxyl terminus of ubiquitin. This adenylylated Ub is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin ligase (E3) recognizes the substrate protein and associates with E2 to transfer Ub from E2 to the substrate. The Ub-labeled substrate protein then is recognized by the proteasome for degradation. The sulfhydryl group of E1 and E2 is highlighted in red.



**Figure 3-8. The ubiquitination pathway**



of the heat shock response as well (190, 236). Recent studies suggest that the E3 ligases are likely to interact with a limited number of E2 proteins, defining distinct pathways for protein ubiquitination and degradation (241). I utilized three  $\beta$ -galactosidase reporter fusions as model substrates to probe pathway specificity: Ub-L- $\beta$ Gal, Ub-P- $\beta$ Gal and *Deg1*- $\beta$ Gal. The linear ubiquitin fusion Ub-L- $\beta$ Gal fusion is recognized by *RAD6/UBC2*, and Ub-P- $\beta$ Gal requires *UBC4* and *UBC5* for degradation. The *Deg1*- $\beta$ Gal fusion requires *UBC6* or *UBC7* for processing (199, 242, 243). Taking advantage of the *pdr5 $\Delta$*  strain (BY4741) with the multidrug resistance (MDR) transporter deleted, I tested stabilization of each substrate after treatment with MG132 or CdSO<sub>4</sub> by  $\beta$ -galactosidase activity assay. *PDR5* encodes a MDR efflux pump that belongs to the family of ATP-binding cassette (ABC) transporters. Deletion of *PDR5* is not lethal, but confers a pleiotropic drug-sensitive phenotype. The *pdr5 $\Delta$*  strain is significantly more sensitive to MG132, and is commonly used to assess protein degradation in combination with proteasome and protease inhibitors (244, 245). The  $\beta$ -galactosidase activity assay revealed that the proteasome inhibitor MG132 significantly stabilized all three substrates, whereas cells treated with Cd<sup>2+</sup> exhibited normal degradation of Ub-L- $\beta$ Gal and Ub-P- $\beta$ Gal, but accumulated *Deg1*- $\beta$ Gal (Figure 3-9A). These results demonstrated that Cd<sup>2+</sup> did not grossly inhibit proteasome or Uba1 (E1) activity as this would have resulted in stabilization of all three reporters. However, stabilization of *Deg1*- $\beta$ Gal suggested that the Ubc6 or Ubc7 E2 enzymes may be compromised by Cd<sup>2+</sup> treatment, which could have led to accumulation of non-ubiquitinated proteins, and consequently activated Hsf1. Alternatively, these events may be correlated, but not causal, in nature. To distinguish between these two possibilities, we measured Hsf1 activity in *ubc7 $\Delta$*  cells, which were

shown to stabilize *Deg1*- $\beta$ Gal (246, 247). As shown in Figure 3-9B, Hsf1 behaved similarly in stressed or non-stressed *ubc7 $\Delta$*  and wild-type cells, suggesting that inhibition of Ubc6/Ubc7 function is not sufficient to activate Hsf1. Cd<sup>2+</sup> treatment may inhibit a specific ubiquitination pathway in the UPS, however, the inhibition appears to be parallel to Hsf1 activation. Moreover, proteasome inhibition caused by MG132 treatment has no effect on Hsp90 activity (Figure 3-10B), suggesting that MG132 and thiol-reactive compounds induce the HSR by different mechanisms. Taken together, these data suggested that thiol-reactive compounds do not induce the HSR by inhibiting the ubiquitin-proteasome system.

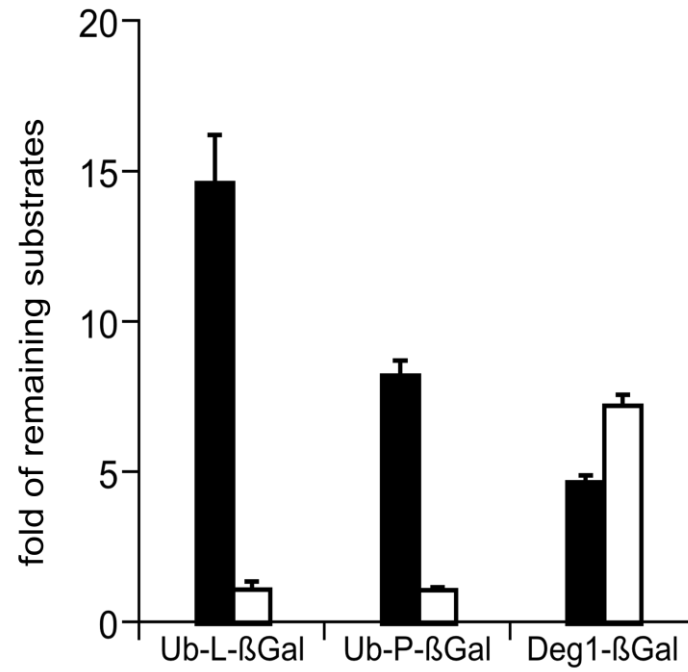
**Figure 3-9. Thiol-reactive compounds do not induce the heat shock response by inhibiting protein degradation.**

- A. *pdr5Δ* cells carrying Ub-L-βGal, Ub-P-βGal, or Deg1-βGal reporter genes were grown to mid-log phase at 30 °C and incubated for 1 hr with 100 μM MG132 (solid bar), 100 μM Cd<sup>2+</sup> (open bar), or vehicle followed by treatment for 1 hr with 100 μg/mL cycloheximide (CHX). β-galactosidase activity was determined and plotted as fold increase relative to cells treated with cycloheximide only.
- B. Wild type (BY4741) and *ubc7Δ* cells carrying the HSE-*lacZ* reporter were grown at control temperature (30 °C, solid bar), or heat shocked at 37 °C (open bar) for 1 hr. β-galactosidase activity was determined and plotted as absolute Miller units.

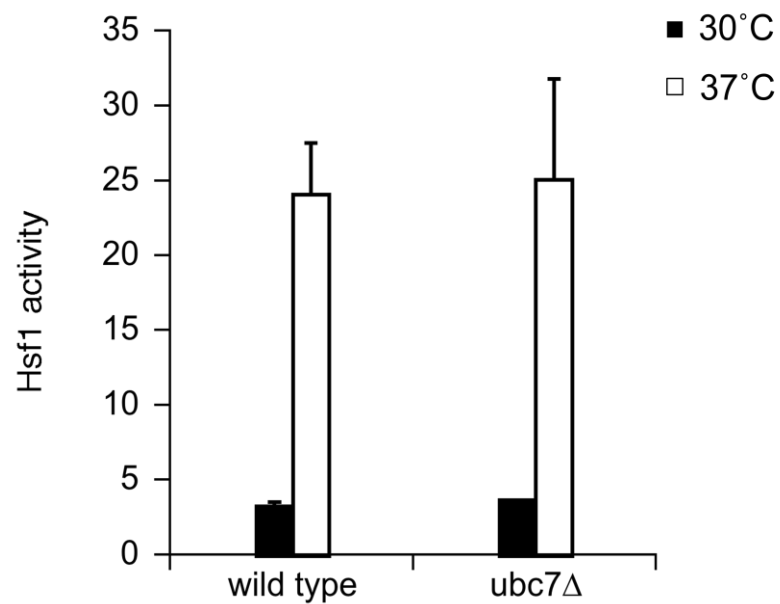
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**Figure 3-9. Thiol-reactive compounds do not induce the heat shock response by inhibiting protein degradation.**

A.



B.

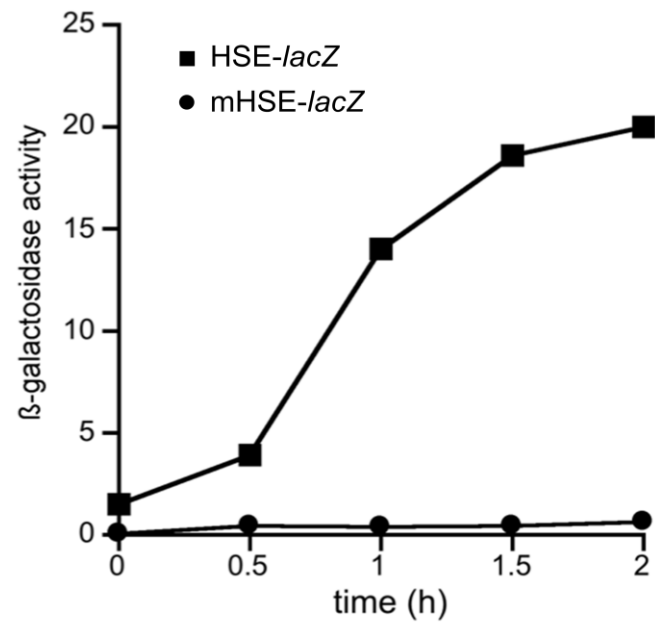


**Figure 3-10. MG132 activates Hsf1, but does not inhibit Hsp90.**

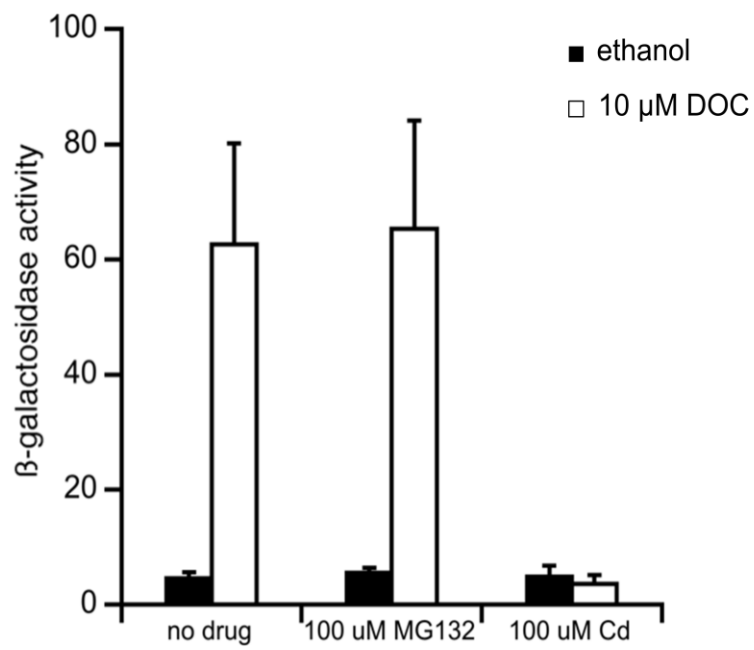
- A. *pdr5Δ* cells were transformed with the *lacZ* reporter constructs containing either wild-type (■) or the mutated HSE (●), and exposed to 100 μM MG132 for 0.5, 1, 1.5 or 2 hr. The β-galactosidase activity was measured to determine Hsf1 activation.
- B. *pdr5Δ* cells bearing the GR reporter system were left untreated, exposed to 100 μM MG132 or 100 μM Cd<sup>2+</sup> for 1 hr, followed by treatment with 10 μM DOC (empty bar) or ethanol (solid bar) for 1 hr. The β-galactosidase activity was measured to determine Hsp90 activity and is plotted as absolute activity in Miller units.

**Figure 3-10. MG132 activates Hsf1, but does not inhibit Hsp90.**

A.



B.



## DISCUSSION

The heat shock response (HSR) is a conserved transcriptional program that results in immediate induction of cytoprotective genes including molecular chaperones to fold, process, and degrade proteins during stress conditions, thereby playing an important role in maintaining cellular protein homeostasis. In almost all eukaryotes, the HSR is regulated by activity of the heat shock transcription factor Hsf1. Deregulation of HSPs can cause many human disorders including cancer and neurodegenerative diseases, offering the HSPs and Hsf1 as potential therapeutic targets (185). Accordingly, numerous efforts have been made to identify small molecules that regulate Hsf1 or modulate chaperone activities. However, the precise mechanisms whereby individual small molecules are sensed to drive the activation of Hsf1 are unclear. In this chapter, I identified a group of small molecules that activated Hsf1 and inhibited Hsp90 complex activity in a reciprocal manner in yeast *Saccharomyces cerevisiae*. Both biological effects were quenched by treatment with excess free thiol. These observations are consistent with previous findings from our laboratory that celastrol, a newly identified Hsf1 activator, also induces the oxidative stress response in both yeast and human cells, confirming a common thiol-modification mechanism (180).

In the absence of stress, Hsf1 binds to Hsp70, Hsp90, and their co-chaperones in a repressive complex (218, 248). It is generally thought that the stress conditions are likely to place increased demands on the molecular chaperones, leading to titration of chaperones away from Hsf1 and activation of the HSR. Consistent with this model, protein misfolding has been observed in mammalian cells after heat shock (235). In addition, the HSR has been shown to be activated by accumulation of nascent proteins,



by inhibition of protein degradation, or by reduced chaperone functions (237, 249, 250). Therefore, the thiol-reactive compounds may activate the HSR by perturbing protein structure and folding. To test this possibility, I used two unfolding reagents, the proline analog azetidine 2-carboxylic acid (AZC) to mimic misfolding of nascent polypeptides and a protein-protein cross-linker DSP to force protein misfolding in cytoplasm. Interestingly, the features of HSR activation by the thiol-reactive compounds were largely distinct from those of unfolding reagents. First of all, the thiol-reactive compounds displayed a typical Hsf1 induction profile of rapid increase of HSP synthesis followed by attenuation and recovery; while Hsf1 induction by both AZC and DSP were slow and persistent. The different time frames suggest that cytosolic protein misfolding caused by AZC incorporation and DSP cross-linking requires time to reach a threshold where chaperones can be recruited from Hsf1. However, I cannot exclude the possibility that the thiol-reactive compounds may cause proteotoxicity more efficiently than the two unfolding reagents. Moreover, I demonstrated that thiol-activation of Hsf1 was transient in nature, whereas the AZC treatment led to persistent induction. I also observed significant loss in viability of cells treated with AZC, but not with thiol-reactive compounds or heat, suggesting that cytotoxicity due to AZC incorporation is irreversible. Together, these results indicate that cells display distinct responses to treatment with AZC, which results in continued production and accumulation of high levels of misfolded nascent chains, and thiol-reactive compounds. Furthermore, only thiol-reactive compounds reciprocally impaired chaperone functions. Using a heterologous glucocorticoid receptor (GR) assay system, I observed that thiol-reactive compounds, but not heat shock or DSP inhibited Hsp90 activity in the same timeframe of Hsf1 activation.

Hsp90 inhibition by these compounds was also abolished in the presence of excess reducing agent DTT, indicating that thiol reactivity was required for the observed biological effects. These observations suggest that Hsf1 activation and Hsp90 inhibition by thiol-reactive compounds are caused by a shared and reciprocally acting biochemical mechanism. Taken together, my results indicate that thiol-reactive compounds do not activate Hsf1 by generating protein misfolding as a primary stress signal.

Cellular protein quality control includes refolding, sequestering or degrading potentially harmful misfolded proteins (251). Inhibition of protein degradation can also generate unfolded proteins, which may accumulate over time. Indeed, numerous studies have shown that proteasome inhibitors, such as MG132 and lactacystin, and protease inhibitors, such as the serine protease inhibitors 3,4-dichloroisocoumarin (DCIC), *N*-tosyl-L-phenylalaninechloromethyl ketone (TPCK) and *N* $\alpha$ -*p*-tosyl-L-ysinechloromethyl ketone (TLCK), all promote *hsp* gene expression by inducing Hsf1 DNA binding activity and hyperphosphorylation (159, 164, 165, 209, 252). In addition, celastrol itself is reported to be a proteasome inhibitor in mammalian cells upon extended treatment (226). Therefore, I asked whether thiol-reactive compounds activate Hsf1 by inhibiting protein degradation. Since thiol-reactivity is a major feature shared among these compounds, the ubiquitin-proteasome system (UPS), which tags substrate proteins with ubiquitin (Ub) via a thioester linkage, becomes a possible target. Thiol-reactive compounds are likely to compromise the UPS by interfering with Ub transfer, leading to the accumulation of non-ubiquitinated, misfolded proteins. Utilizing three  $\beta$ -galactosidase reporter fusions to interrogate the functional status of the major ubiquitin conjugating enzymes (E2), I demonstrated that thiol-reactive compounds did not grossly inhibit the proteasome or the

ubiquitin activating enzyme Uba1 (E1) in yeast. However, cadmium specifically compromised Ubc6/7-mediated ubiquitination. Interestingly, further investigation of Hsf1 activity in *ubc7Δ*, which stabilizes most Ubc6/7-dependent substrate proteins, showed normal Hsf1 repression in non-stressed conditions, indicating that disruption of Ubc6/7 function is not sufficient to activate Hsf1. These results suggest that inhibition of the UPS and activation of Hsf1 by thiol-reactive compounds are parallel, but not causally related events.

Although most proteasomal substrates must be ubiquitinated before being degraded, some proteins are degraded in Ub-independent manner such as ornithine decarboxylase (ODC) in yeast(253). Moreover, Ub conjugation is not required for degradation of oxidized proteins by the proteasome in mammalian cells (254). Therefore, I cannot completely exclude the possibility that thiol-reactive compounds disrupt protein turnover/degradation and consequently accumulate damaged proteins, even though my data suggested that these compounds do not activate Hsf1 through inhibiting the UPS. Instead of testing these distinct degradation pathways individually, I used ultracentrifugation to directly measure the levels of aggregated proteins in the presence of thiol-reactive compounds. Strikingly, cells treated with thiol-reactive compounds maintained solubility of the majority of proteins, while heat shock at proteotoxic levels resulted in accumulation of significant amount of insoluble proteins. Taken together, these results indicate that the features of Hsf1 activation by thiol-reactive compounds, including a rapid but transient induction profile, reciprocal Hsp90 inhibition, and independence from protein degradation, are not consistent with a model relying on the generation and accumulation of misfolded cytosolic proteins. Instead, the inhibition of

Hsp90 activity suggests a potential chaperone-sensing mechanism. This hypothesis is tested in the next chapter.

## **Chapter 4: Hsp70 functions as a sensor of thiol-reactive compounds for Hsf1 activation**

*NOTE: this chapter is derived from work that has, for the most part, been published in 2012: "The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds." Molecular Biology of the Cell 2012 Sep; 23(17): 3290-8 (<http://www.molbiolcell.org/content/23/17/3290.abstract>). I am the primary author on this paper and was responsible for preparing the original manuscript. I performed all experiments described in this chapter. The publisher of MBoC, the American Society of Cell Biology, grant authors the right to revise, adapt, prepare derivative works, present, or distribute the manuscript provided that all such distribution is for noncommercial benefit and there appears always the ASCB copyright credit and link to the original publication of the manuscript in MBoC Online. (<http://creativecommons.org/licenses/by-nc-sa/3.0/>)*

## INTRODUCTION

The stress-dependent conversion of HSF1 into its active form implies that HSF1 is negatively regulated (63). Furthermore, early studies of cultured *Drosophila* cells demonstrated that the synthesis of the heat shock proteins (HSPs) is rapidly induced after a temperature shift from 25 °C to 37 °C, followed by gradual repression on return to normal growth conditions, suggesting that the heat shock response (HSR) is self-regulated by HSPs (255, 256). This hypothesis is further supported by extensive genetic and biochemical evidence that at least two classes of HSPs, HSP70 and HSP90, serve as *trans*-acting HSF1 repressors. In the budding yeast *Saccharomyces cerevisiae*, deletion of the two constitutively expressed cytosolic Hsp70 genes, *SSA1* and *SSA2*, leads to constitutive transcriptional competence of Hsf1 (257-259). The ATPase activity of Hsp70 seems to serve an important role in Hsf1 regulation. Mutants lacking *SSE1* and *FES1*, two genes that encode Hsp70 nucleotide exchange factors in cytoplasm, result in de-repression of Hsf1 at normal growth conditions (77, 100, 260). Moreover, human Hsp70 also stably associates with the HSF1 activation domain *in vivo* and *in vitro* through its C-terminal substrate binding domain (45, 66, 248).

Although Hsp70 has been suggested to be the basis of the feed-back regulatory mechanism of Hsf1 regulation, Hsp70 alone is insufficient to suppress Hsf1 in mammalian cells (261). Instead, Hsp70 may participate as a component of the Hsp90 chaperone complex, which is responsible for maturation and regulation of various client proteins. Hsp90 requires associations with a number of co-chaperones, for example, Hsp70, Sti1, Cpr6/7 and Sba1, to achieve its cellular functions. Some of these functions attributed to Hsp70 and its nucleotide exchange factors in regulation of Hsf1 activity may

reflect a joint effect with Hsp90. In human cells, Hsp90 associates with HSF1 *in vivo* and *in vitro* (71). Direct injection of anti-Hsp90 in *Xenopus* oocyte system activates the heat shock response (70). Deletion of both the constitutively expressed Hsp90 *HSC82* and its co-chaperone, the cyclophilin 40 homolog *CPR7*, results in derepression of Hsf1 at normal growing temperature and constitutive thermotolerance (76, 78). Therefore, the Hsp70/Hsp90 chaperone complex is likely to repress transcriptional activation of Hsf1 under non-stress conditions. During heat shock, the accumulation of unfolded or damaged proteins may recruit the Hsp90 chaperone machinery from Hsf1, allowing derepression of the transcription factor.

Proper function of the HSR is relevant to various human diseases including cancer and neurodegenerative disease. Over the past two decades, numerous efforts have been made to identify and characterize Hsf1 modulators. However, the detailed mechanisms through which individual modulators trigger activation remain largely unresolved. Since substantial evidence supports the model that the chaperone machinery represses Hsf1 activity in the absence of stress, chaperones are generally considered as stress sensors. Indeed, several studies show that some Hsp90-specific inhibitors also function as Hsf1 activators such as geldenamycin and radicicol (186-188). Recently, celastrol, an active component of Chinese medicine, was found to promote *hsp* gene expression and block maturation of an Hsp90-dependent androgen receptor in yeast and human cells, respectively (19, 180, 223). There are at least two potential ways that Hsf1 may be released from chaperone repression. One model posits that chaperones function as sensors of cytosolic protein misfolding and are titrated away from Hsf1 by damaged proteins. This model is supported by the findings that many pharmacological Hsf1

activators have been implicated in disrupting protein translation, folding and degradation (159). A second model is based on direct modification on chaperones by reaction of thiol-reactive compounds with key residues including Cys, His, Lys, Met, Phe, Tyr, Asp, and Glu within these proteins. Of these residues, Cys is reported to be particularly susceptible to thiol modification, likely due to the readily accessible lone pair of electrons in its sulfhydryl group (170). The overall reactivity, or nucleophilicity, of a Cys residue is shown to increase upon thiol deprotonation to the thiolate anion, and therefore a Cys residue with low  $pK_a$  value is considered highly reactive to thiol-reactive compounds (262-264). In mammalian cells, both Hsp70 and Hsp90 have been identified as potential targets of electrophiles and oxidants (228, 265-267). Moreover, *in vitro* activity analysis revealed that Hsp70, Hsp90, and several of their co-chaperones such as Cdc37 and p23 (Sba1 in yeast) are inactivated by electrophiles including celastrol, ethylmaleimide, and 4-hydroxynonenal (133, 228, 267, 268). Taken together, these results suggest that chaperones can function as direct sensors of thiol-reactive molecules.

In Chapter 3, I demonstrated that a group of small molecules including oxidants, transition metals and metalloids, and organic electrophiles activate Hsf1 and inhibit Hsp90 in a reciprocal manner. I reported that these thiol-reactive compounds do not activate Hsf1 by promoting protein misfolding or inhibiting protein degradation. In this chapter, I utilize a thiol-reactive biotin-labeled probe to show *in vitro* that Ssa1, the cytosolic Hsp70 in yeast, possesses highly reactive cysteine residues. Moreover, substitution of C264 or C303 with serine renders cells unresponsive to Hsf1 activation and unable to acquire thermotolerance after treatment with thiol-reactive compounds. Strikingly, substitution with aspartic acid, which increases bulk of the amino acid side



chain as well as mimicking the oxidized sulfinic acid form of the cysteine thiol, leads to constitutively active Hsf1 in non-stress conditions. These same cysteines are shown to be directly modified *in vivo* by the organic electrophile 4-HNE using a Click chemistry approach (269). Together, these data suggest a model wherein the Hsp70 chaperone Ssa1 functions as a direct sensor for activation of Hsf1 and acquisition of cytoprotection by diverse thiol-reactive compounds through reactive cysteine residues.

## RESULTS

### *The yeast Hsp70, Ssa1 is hypersensitive to thiol-modification*

In the previous chapter, I reported that diverse thiol-reactive compounds function as potent Hsf1 activators. Additionally, I demonstrated that these molecules do not induce the HSR by disrupting protein homeostasis. Because incubation of these compounds with excess thiols (e.g., DTT) completely abolished their biological effects on Hsf1 activation (Figure 3-4), I reasoned that the mechanism by which thiol-reactive compounds activate the HSR may involve inactivation of specific regulatory protein(s) through targeting of cysteine residues. In yeast and mammals, HSPs and select co-chaperones are assembled into multi-chaperone complexes that regulate HSF1 activity at different steps (182, 270). Moreover, our lab and others have shown that the Hsf1 activator celastrol functionally inhibits Hsp90, and I observed that the thiol-reactive compounds I identified have the same biological effects (180, 223) (Figure 3-1). These findings are consistent with a model wherein one or more Hsf1-repressing, Hsp90-promoting chaperones are directly modified and perhaps inactivated by thiol-reactive compounds.

Analysis of amino acid sequences from the *Saccharomyces* Genome Database (SGD) revealed that both yeast cytosolic Hsp90s (Hsc82 and Hsp82) and Hsf1 lack cysteine residues; however, several co-chaperones of Hsp90 contain one or more cysteines. Among the four Ssa family members of yeast cytosolic Hsp70, the constitutively expressed Ssa1 and Ssa2 contain three cysteines, while the inducible Ssa3 and Ssa4 contain two (271, 272). Sse1 and Fes1 are Hsp70 nucleotide exchange factors, and contain five and two cysteine residues, respectively (99, 100, 102). The Hsp90-associated peptidyl-prolyl *cis-trans* isomerases (PPIases) Cpr6 and Cpr7 each have seven

cysteines (273). Importantly, previous genetic studies demonstrated that deletion of Ssa1/2, Sse1 or Cpr7 leads to activation of Hsf1 under normal growth conditions, making them prime candidates for targets of thiol-reactive compounds (76, 100, 237, 258). To address whether one or more of these proteins are sensitive to thiol-modification *in vitro*, I used a cysteine biotinylation approach. Tandem affinity tagged (TAP) chaperone proteins were isolated from cell extracts and reacted with the reagent biotin-BMCC (see Methods and Materials). Although five fusions were expressed and detectable using anti-protein A antiserum, only Ssa1 was modified by biotin-BMCC as detected by a streptavidin-horseradish peroxidase conjugate (Figure 4-1A). This result was confirmed by treating purified Ssa1, which was previously generated in the lab, with the same concentration of biotin-BMCC for 0, 1, 2 and 4 h, demonstrating reaction with Ssa1, but not the TAP tag (Figure 4-1B upper). To verify that Ssa1 was labeled due to thiol modification, biotin-BMCC was mixed with the indicated ratios of DTT prior to treatment. Labeling of purified Ssa1 by BMCC was completely abolished by equimolar or greater amounts of free thiol (Figure 4-1B bottom). Since Ssa1 is identified as a potential target with three cysteines in the ATPase domain and the Ssa1 nucleotide exchange factor Sse1 is required for Hsf1 repression, I also tested whether another cytosolic exchange factor, Fes1, also functions as an Hsf1 repressor *in vivo*. Utilizing the HSE-*lacZ* reporter, I found that deletion of Fes1 indeed substantially de-repressed Hsf1 (Figure 4-1C). Together, all these results suggest that of the Hsf1-repressing chaperones and co-chaperones tested, Ssa1 is hypersensitive to thiol-modification and may be a relevant target of Hsf1-activating thiol-reactive compounds.

**Figure 4-1. Ssa1 is a relevant target of Hsf1-activating thiol-reactive compounds.**

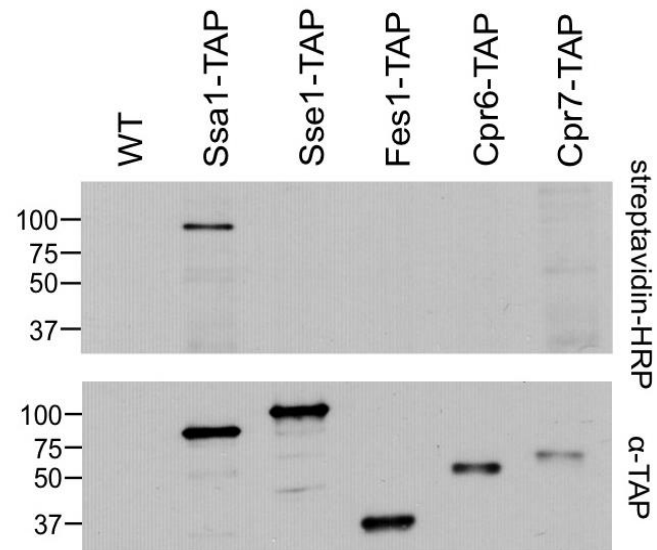
- A. An Ssa1-TAP fusion is hypersensitive to modification *in vitro* by a thiol-reactive biotin probe. The indicated chaperone-TAP fusion proteins were enriched by IgG affinity-purification and were treated with biotin-BMCC.
- B. Ssa1 is rapidly alkylated by biotin-BMCC in a thiol-dependent manner. Purified Ssa1 was incubated with biotin-BMCC for the indicated times, followed by immunoblotting with streptavidin-HRP or anti-Ssa1 (top). Biotin-BMCC was premixed with the indicated concentration ratios of DTT for 15min prior to reaction with purified Ssa1 for 30 min and detected as above (bottom).
- C. The Ssa1 nucleotide exchange factor Fes1 is required for hsf1 repression. Wild-type and *fes1Δ* (BY4741) cells were transformed with the HSE-*lacZ* reporter were grown at 30 °C to midlog phase, and  $\beta$ -galactosidase activity was determined and plotted as absolute Miller units.

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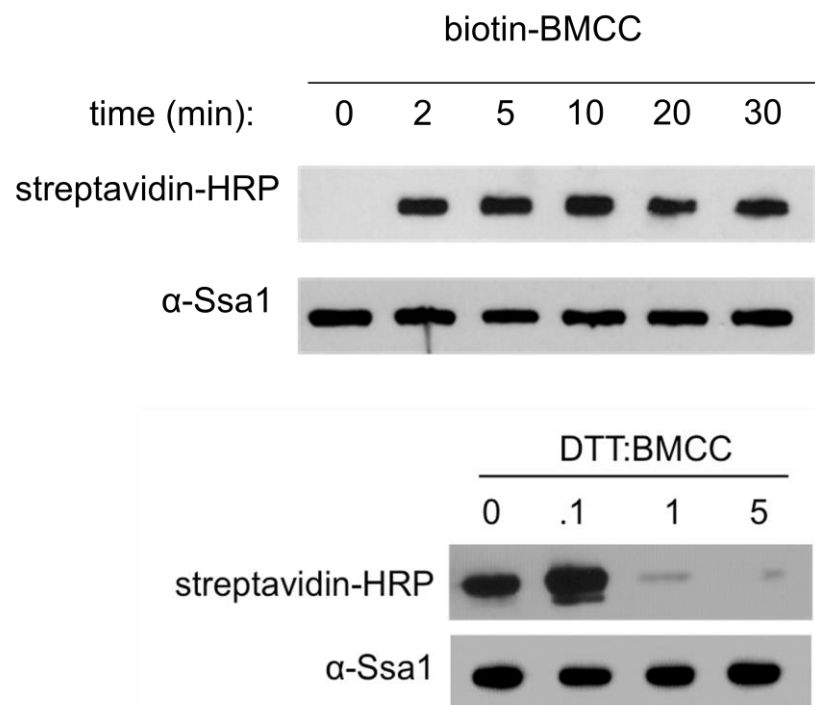
*Figure 4-1C was first generated by Dr. Patrick A. Gibney and was repeated by the author.*

**Figure 4-1. Ssa1 is a relevant target of Hsf1-activating thiol-reactive compounds.**

A.

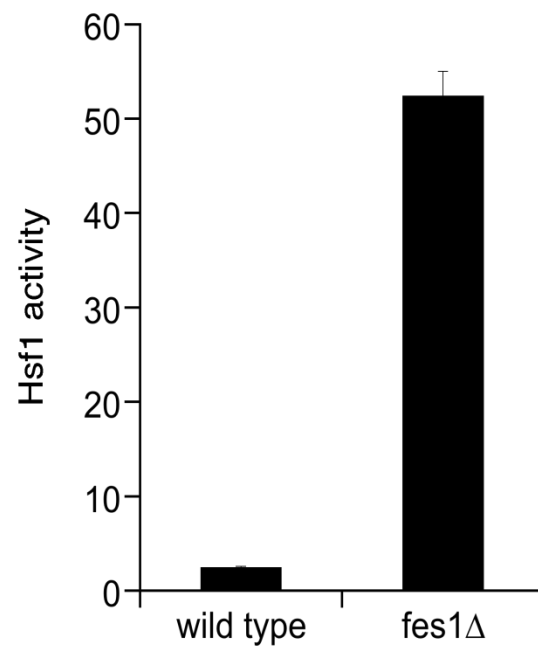


B.



**Figure 4-1. Ssa1 is a relevant target of Hsf1-activating thiol-reactive compounds.**

C.



*Ssa1 is a sensor for Hsf1 activation by thiol-reactive compounds*

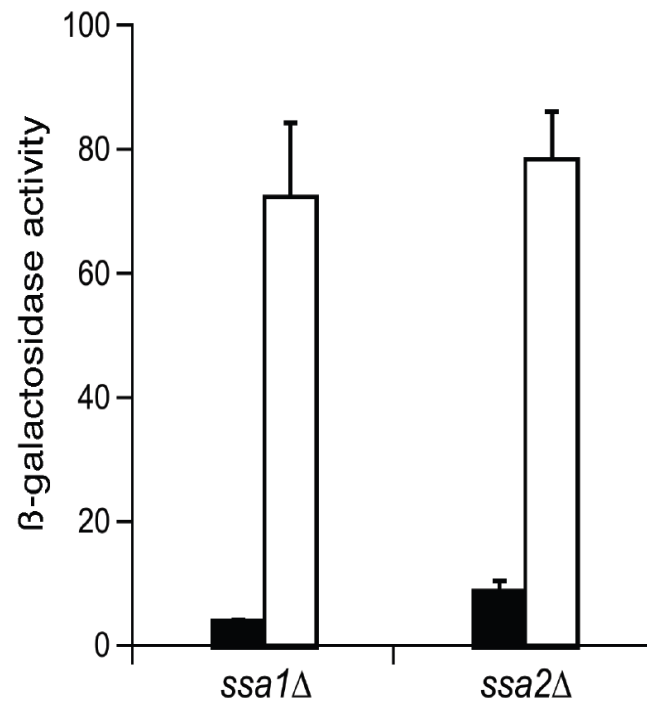
The *in vitro* labeling assay suggests that of the chaperone proteins comprising the Hsp70/Hsp90 multi-chaperone machinery I tested, Ssa1 is specifically modified by a thiol probe. Next I ask whether the modification of Ssa1 on one or more cysteine residues is directly responsible for Hsf1 activation by these thiol-reactive compounds. To address this question, I constructed an experimental system by placing the *SSA1* open reading frame (ORF) under control of the heterologous translation elongation factor 1 alpha (TEF) promoter, and expressed this system in an *ssa1Δssa2Δ* deletion background. *SSA1* and *SSA2* are the constitutively expressed cytosolic Hsp70 isoforms with redundant functions for Hsf1 repression (Figure 4-2). Deletion of both genes leads to reduced growth rates and Hsf1 repression defects at normal temperatures (237). The TEF expression system that produced approximately wild-type levels of Ssa1 was able to complement both defects (Figure 4-3B and C). Interestingly, I noticed that high expression levels of *SSA1* under control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter blunted Hsf1 activation by heat shock or thiol-reactive compounds (Figure 4-3A and C). This observation is consistent with previous findings that Hsp70 is an Hsf1 repressor under normal growth conditions and overexpression of Hsp70 inhibits phosphorylation of Hsf1 in human cells (274). I first attempted to replace all three cysteines, i.e. C15, C264, and C303, with serine. The side chain of serine is the most similar to cysteine, except that serine contains a hydroxyl group instead of a sulfhydryl group. However, substitution of C15 was found to cause instability of Ssa1 and loss of function (Figure 4-4C). I therefore constructed mutant *SSA1* alleles with C264S, C303S, or both mutations. All three

**Figure 4-2. Ssa1 and Ssa2 are functionally redundant for Hsf1 repression.**

Deletion mutants *ssa1Δ::KanMX* and *ssa2Δ::KanMX* (isogenic to BY4741) were transformed with the HSE-*lacZ* reporter, and grown at 30 °C (solid bar) or 37 °C (empty bar) for 1 hr. The  $\beta$ -galactosidase activity was measured to detect Hsf1 induction and is reported as absolute activity in Miller units.



**Figure 4-2. Ssa1 and Ssa2 are functionally redundant for Hsf1 repression.**

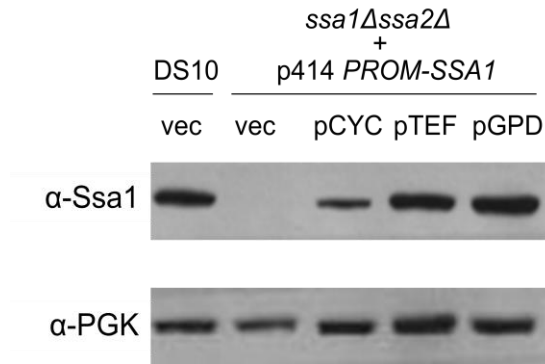


**Figure 4-3. Complementation of *ssa1Δssa2Δ* phenotypes by *SSA1* expressed using heterologous promoters.**

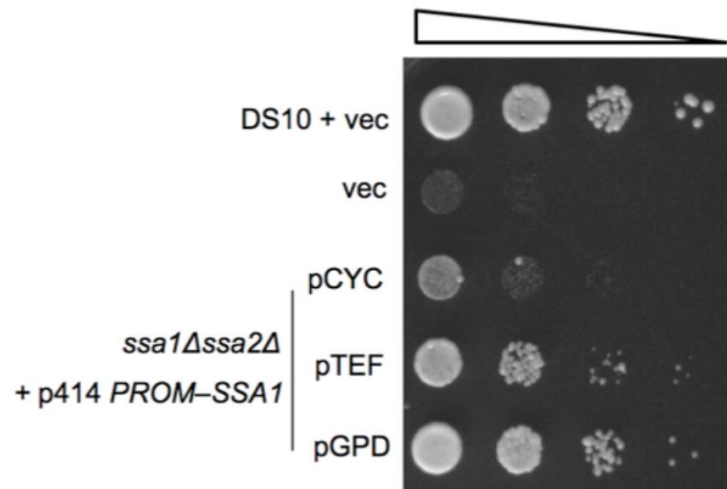
- A. The *ssa1Δssa2Δ* (*ssa1Δ::HIS3*, *ssa2Δ::LEU2*) or the isogenic parent strain DS10 carrying the empty vector or indicated complementation constructs were grown to mid-log phase. Protein-extracts were prepared by glass-bead lysis and analyzed by SDS-PAGE and Western blot analysis. Ssa1 level was detected using anti-Ssa1 antibody. PGK level was used as a loading control
- B. Indicated strains were grown to log phase and spotted on SC media in a 10-fold dilution series and incubated at 30 °C for 3 d.
- C. DS10 cells carrying the empty vector and *ssa1Δssa2Δ* carrying the empty vector or indicated complementation constructs were transformed with the HSE-*lacZ* reporter. Cells were left untreated (solid bar), heat shocked at 37 °C (empty bar), or exposed to 100 μM Cd<sup>2+</sup> (grey bar). The β-galactosidase activity was measured to detect Hsf1 induction and is reported as absolute activity in Miller units.

**Figure 4-3. Complementation of *ssa1Δssa2Δ* phenotypes by SSA1 expressed using heterologous promoters.**

A.

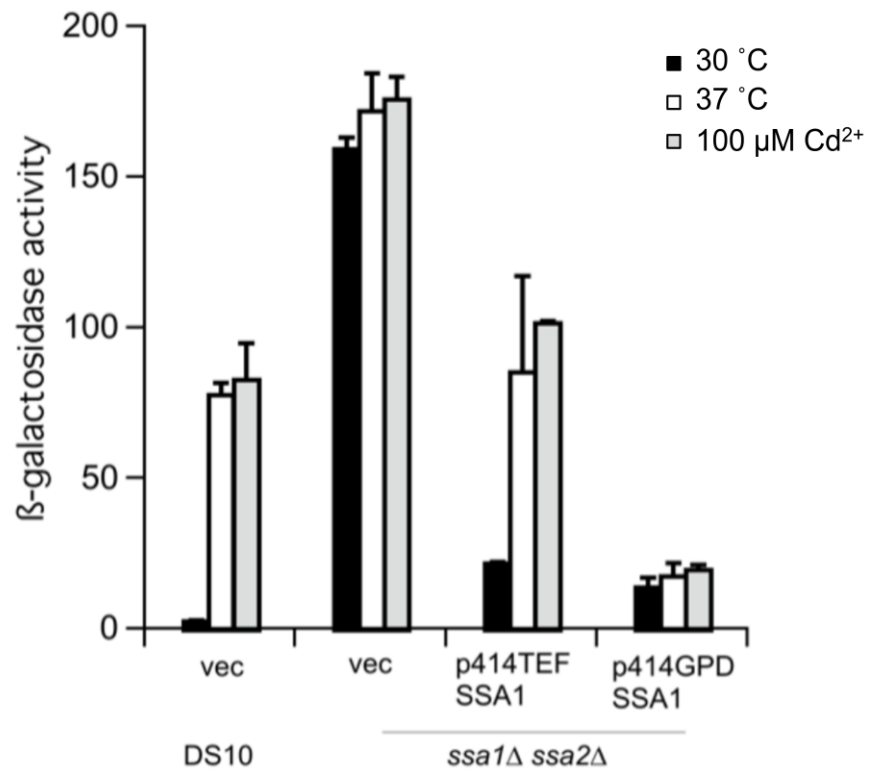


B.



**Figure 4-3. Complementation of *ssa1Δssa2Δ* phenotypes by *SSA1* expressed using heterologous promoters.**

C.



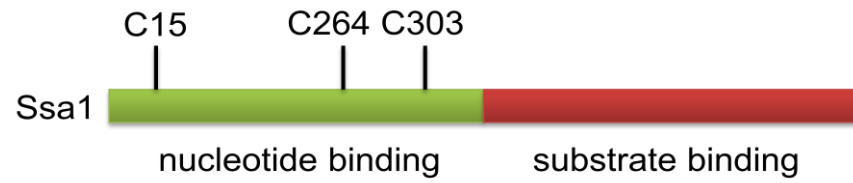
**Figure 4-4. Functional analysis of SSA1 cysteine mutants.**

- A. Diagram depicting the domain architecture of Ssa1 and positions of the three cysteine residues.
- B. The relevant cysteines are mapped in the human Hsc72 nucleotide binding domain (NBD) generated from crystal structure (3D2F) using a protein structure manipulating software PyMOL.
- C. DS10 cells carrying the empty vector and *ssa1Δssa2Δ* carrying the empty vector or indicated complementation constructs were grown to mid-log phase. Protein extracts were prepared by glass-bead lysis and analyzed by SDS-PAGE and immunoblot. Ssa1 level was detected using anti-Ssa1 antibody. GPD levels were determined as a loading control.
- D. Strain *ssa1Δssa2Δ* carrying the empty vector or indicated SSA1 mutant constructs were plated on selective SC plates in a dilution series and grown at 30 °C for 2 d.

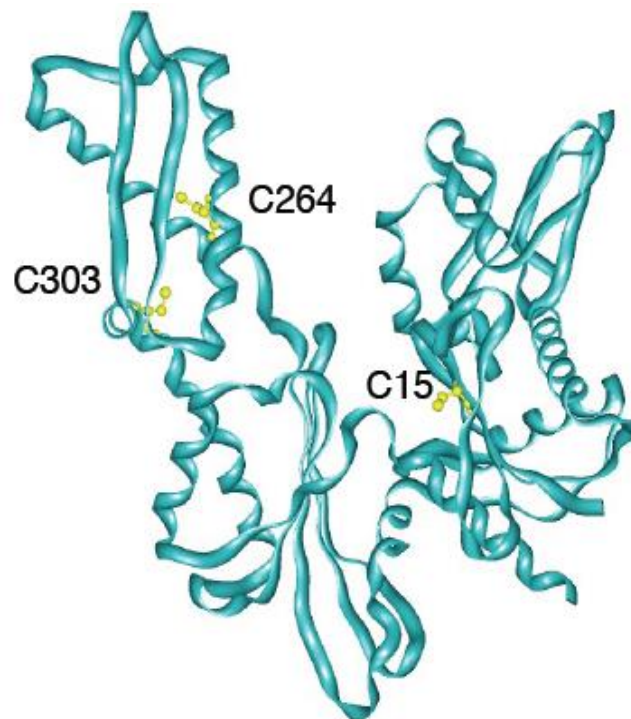
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Figure 4-4. Substitution of cysteine residues in Ssa1 with serine.

A.

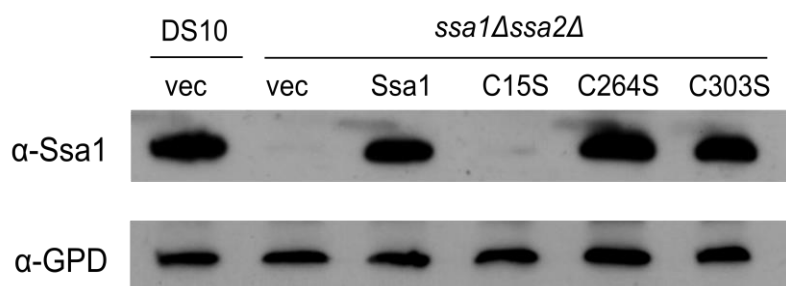


B.

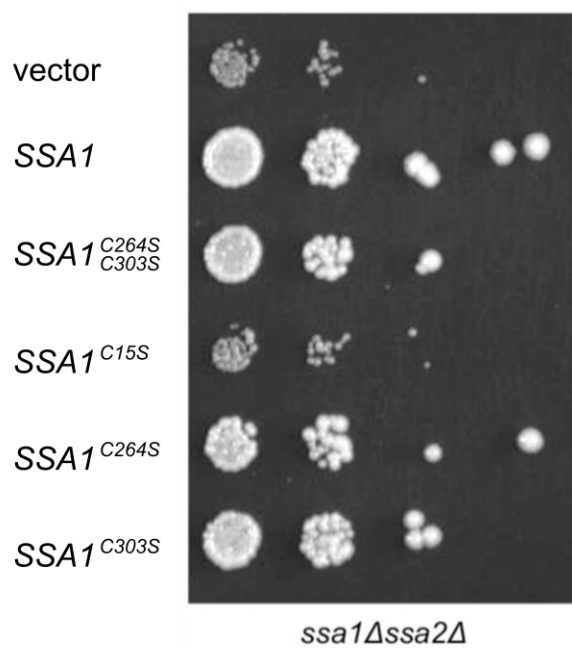


**Figure 4-4. Substitution of cysteine residues in Ssa1 with serine.**

C.



D.



permutations were stable and complemented the slow growth defect of *ssa1Δssa2Δ* (Figure 4-4D).

With the functional *SSA1* mutant alleles, I next asked whether the cysteine residues in Ssa1 are required for sensing thiol-reactive compounds. If this is the case, cells carrying the *SSA1* mutants should not respond to thiol-reactive compounds due to the replacement of reactive cysteines with non-reactive serines. To test this hypothesis, I utilized the HSE-*lacZ* reporter to measure Hsf1 activity in mutant cells in response to heat shock, CdSO<sub>4</sub>, diamide, and the organic electrophile 4-hydroxynonenal (4-HNE) (Figure 4-5). The *ssa1Δssa2Δ* cells carrying an empty vector exhibited Hsf1 derepression, while reintroduction of wild-type *SSA1* under control of the TEF promoter restored Hsf1 repression in non-stress condition (30 °C) and inducibility in response to heat and thiol-reactive compounds. Strikingly, cells complemented with *SSA1-C264S*, *C303S* also retained heat inducibility, but were completely resistant to all three thiol-reactive compounds. To distinguish which of the two cysteine residues is responsible for the thiol-sensing, I generated single-residue mutants of C264 and C303 and assessed HSE-*lacZ* activity as described above. Interestingly, both *SSA1-C264S* and *SSA1-C303S* recapitulated the responses of the double mutant. According to the crystal structure of Hsp70, the distance between C264 and C303 suggests that a disulfide bond is unlikely to form between these two cysteine residues. Alternatively, it is possible that thiol-modification on both cysteines is required for a conformational change of the ATPase domain, which either blocks ATP binding or disrupts ATPase activity of Ssa1, leading to inactivation of Ssa1. Taken together, these results provide genetic evidence that both C264 and C303 of Ssa1 are required to sense thiol-reactive compounds.

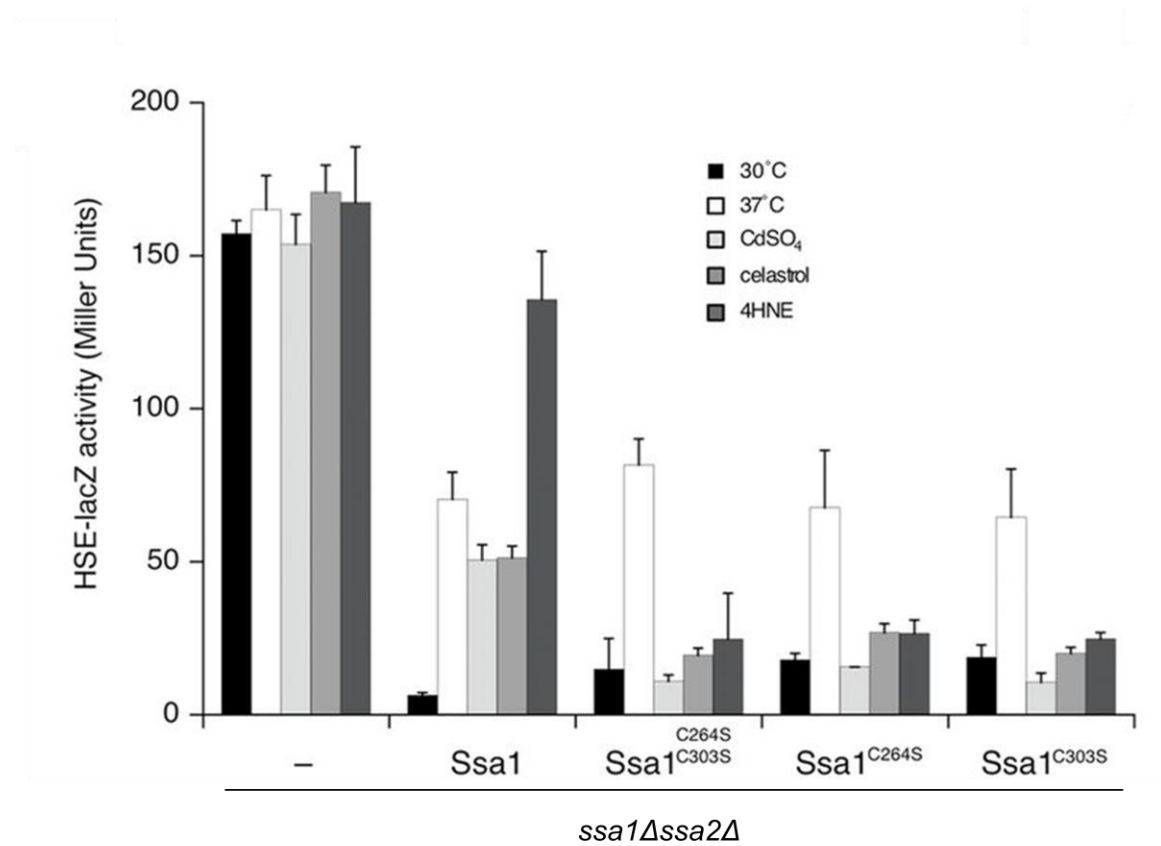


**Figure 4-5. Ssa1 cysteines are required for Hsf1 activation by thiol-reactive compounds but not heat shock.**

Strains carrying wild-type and mutated *SSA1* alleles were grown at the control temperature (30 °C); heat-shocked (37 °C) for 1 hr; or exposed to 600 μM CdSO<sub>4</sub>, 30 μM celastrol, or 400 μM 4-HNE for 2 hr; this was followed by determination of β-galactosidase activity plotted in absolute Miller units. (-), empty vector control.

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Figure 4-5. Ssa1 cysteines are required for Hsf1 activation by thiol-reactive compounds but not heat shock.



The next question is how Ssa1 transmits the thiol-reactive signal to Hsf1. I envisioned two models to explain my observation. In the first model, various thiol-reactive compounds could overwhelm cellular redox buffers such as the thioredoxin and glutathione pathways, which might then fail to maintain Ssa1 in a reduced state. Alternatively, the cysteine residues on Ssa1 could be directly modified, either by oxidation or by formation of transient or stable adducts with thiol-reactive compounds. To distinguish these two possibilities, I utilized Click chemistry to examine thiol-modification of Ssa1 *in vivo*. Click chemistry is a class of copper catalyzed chemical reactions that use the azide and alkyne moieties, which generate a stable triazole derivative, to label and detect a molecule of interest (Figure 4-6A) (269). I used a membrane-permeable alkyne derivative of the 4-HNE that was a potent Hsf1 activator (4-HNE alkyne, Figure 4-5), and FLAG-tagged *SSA1* alleles that could be used for immunopurification. The *ssa1Δssa2Δ* cells bearing the empty vector, wild-type, or mutant *SSA1* alleles were treated with 4-HNE alkyne for 1 hr. After quenching and lysis, purified samples were reacted with biotin-azide in the Click reaction buffer, followed by SDS-PAGE and immunoblot using streptavidin-HRP. As shown in Figure 4-6B, wild-type Ssa1 was modified by 4-HNE alkyne as demonstrated using the Click reaction; whereas labeling was absent in cells lacking FLAG-Ssa1 or missing reaction reagents. The labeling signal was significantly decreased in the *SSA1-C264S*, *C303S* mutant, suggesting that one or more cysteine residues is a direct target of thiol-modification *in vivo*. I speculate that the minor amount of labeling of *SSA1-C264S*, *C303S* may be on C15. Interestingly, *SSA1* single mutants demonstrated different outcomes: the labeling signal was robust in *SSA1-C264S*, but was decreased in *SSA1-C303S*. This observation

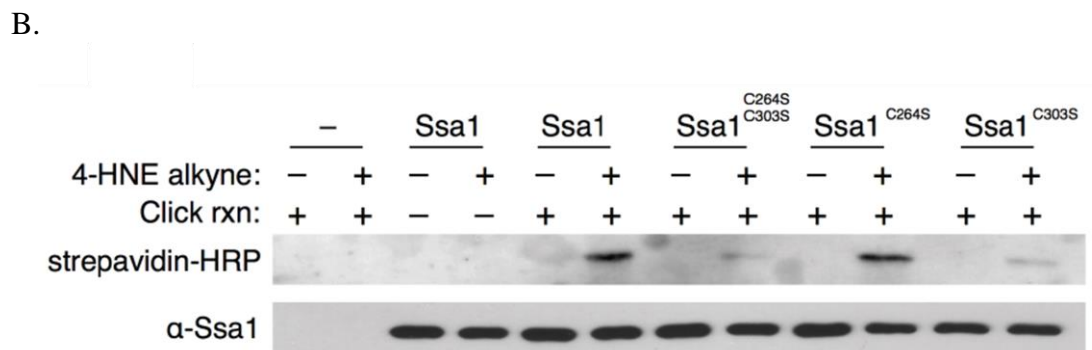
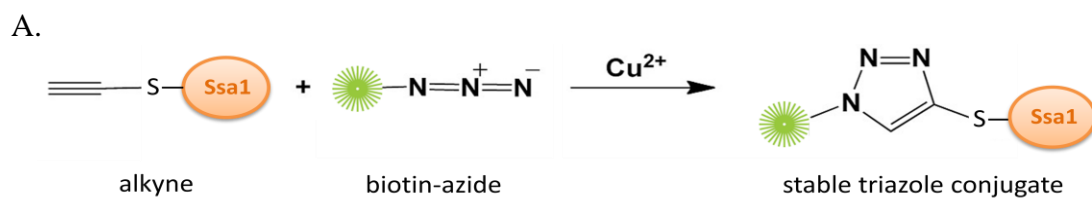
**Figure 4-6. Ssa1 cysteines are directly modified by a thiol-reactive compound.**

A. Click azide/alkyne reaction.

B. Strains containing wild-type and mutated *SSA1* alleles were treated with 4-HNE alkyne. The extract was prepared by glass bead lysis and protein concentration was determined by Bradford assay as described above. 0.5-1.0 mg of cell extract was compensated with TEGN buffer and incubated with FLAG resin at 4 °C on a rotating wheel for 2 h. Proteins tagged by 4-HNE alkyne were detected using the Click-iT® reaction buffer kit following the manufacturer's instructions. Click-tagged proteins were eluted and labeling of Ssa1 was detected by immunoblot with streptavidin-HRP

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**Figure 4-6. Ssa1 cysteines are directly modified by a thiol-reactive compound.**



suggests that C303 may be more reactive compared with C264, and exposure of C264 to thiol-reactive compounds may require prior modification of C303. Although not mutually exclusive, these results support the second model that Ssa1 is directly modified by thiol-reactive compounds *in vivo* in the same time frame (1 - 2 hr) in which I observe induction of Hsf1.

The thiol group (-SH) of cysteine residues can be oxidized to the sulfinic acid form (-SOOH) by oxidants such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Additionally, adduction of a cysteine thiol by small electrophiles such as DEM would add steric bulk which could potentially cause structural or functional perturbations. In order to understand how thiol modifications affect the biological functions of Ssa1, I substituted C264 and C303 with the pseudo-oxidation mimic aspartic acid. As shown in Figure 4-7A, *SSA1-C264D*, *C303D* produced a stable protein. However, it failed to complement the Hsf1 derepression and slow growth phenotypes of *ssa1Δssa2Δ* (Figure 4-7B and C), consistent with the substitution inactivating Ssa1, while serine was tolerated (Figure 4-6). Together, these data support the interpretation that thiol-modification of C264 and C303 result in functional inactivation of Ssa1 in the context of its role as an Hsf1 repressor.

#### *Hsf1 activation by thiol-reactive compounds is distinct from sensing of thermal stress*

Previous work from our lab reported that Hsf1 activation by celastrol leads to acquisition of thermotolerance at sub-lethal temperatures (180). To confirm that sensing of thiol-reactive compounds by Ssa1 is physiologically relevant to cell survival, I tested the *SSA1* mutants for gain of thermotolerance in the presence of cadmium. The *ssa1Δssa2Δ* cells carrying the empty vector, wild-type or mutant *SSA1* alleles were

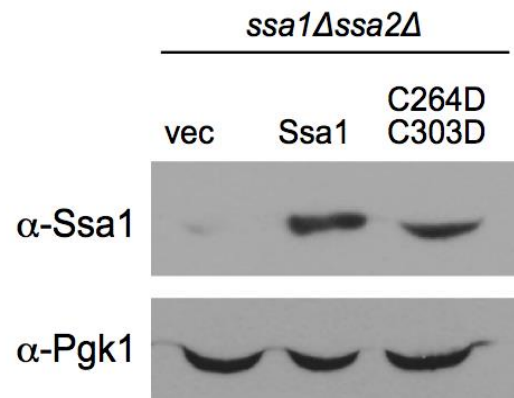
**Figure 4-7. The cysteine oxidation mimic aspartic acid results in Ssa1 inactivation and hsf1 derepression.**

- A. Stability of *SSA1* cys to asp mutants. Strain *ssa1Δssa2Δ* carrying the empty vector or indicated *SSA1* mutant constructs were grown to log-phase. Protein extracts were prepared by glass-bead lysis and analyzed by SDS-PAGE and immunoblot. Ssa1 levels were detected using anti-Ssa1 antibody. PGK levels were used as a loading control.
- B. C264D/C303D combination mutant is non-functional as compared to wild type *SSA1*. Strain *ssa1Δssa2Δ* carrying the empty vector or indicated *SSA1* mutant constructs were plated on selective SC plates in a dilution series and grown at 30 °C for 2 d.
- C. C264D/C303D combination mutant results in Hsf1 derepression. Strains containing wild-type and mutated *SSA1* alleles and the HSE-*lacZ* reporter were grown at 30 °C, and constitutive Hsf1 activity was measured by determination of β-galactosidase activity. (-), empty vector control.

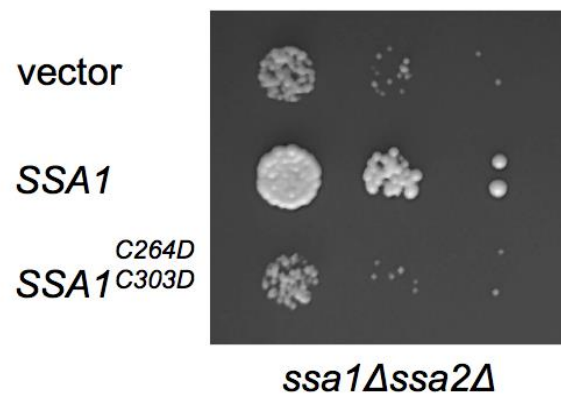
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**Figure 4-7. The cysteine oxidation mimic aspartic acid results in Ssa1 inactivation and Hsf1 derepression.**

A.



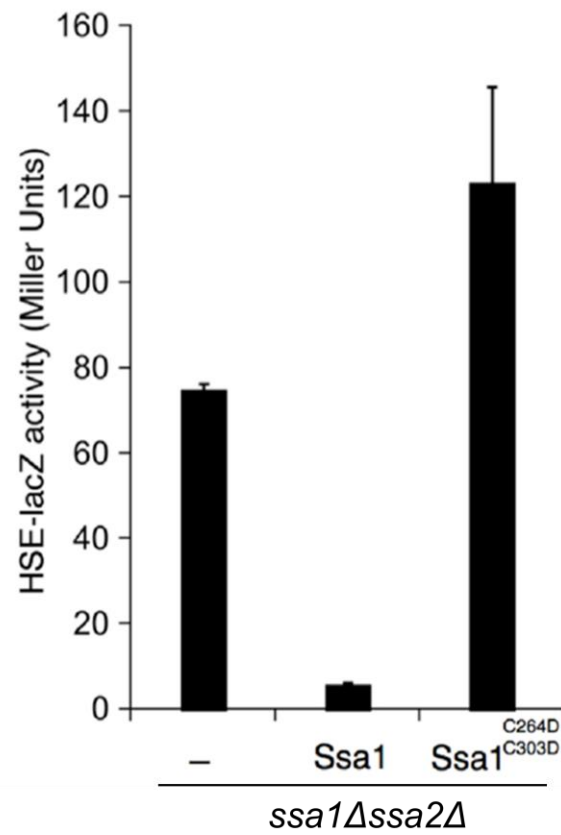
B.





**Figure 4-7. The cysteine oxidation mimic aspartic acid results in Ssa1 inactivation and hsf1 derepression.**

C.



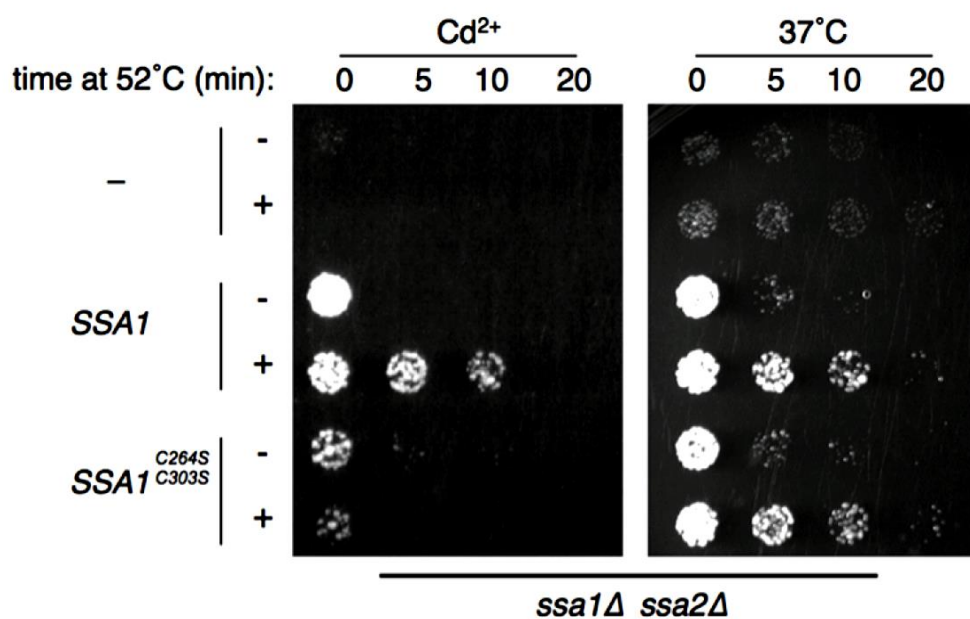
pretreated or not with CdSO<sub>4</sub> and subsequently exposed to a 52 °C heat shock for varying lengths of time. Cells carrying the empty vector demonstrated the characteristic slow-growing phenotype and did not recover within the time course of the experiment (Figure 4-8). However, extended incubation suggests that *ssa1Δssa2Δ* cells are constitutively thermotolerant, consistent with its phenotype of chronic Hsf1 derepression (data not shown). On the other hand, cells complemented with wild-type *SSA1* showed sustained viability only with Cd<sup>2+</sup> treatment and were tolerant up to 10 min severe heat shock. Cells bearing the *SSA1-C264S*, *C303S* mutant allele exhibited equivalent low viability irrelevant of Cd<sup>2+</sup> pretreatment, suggesting that the mutant failed to induce the cytoprotective HSR upon thiol-reactive stress. The previous reporter assay suggested that cells expressing *SSA1-C264S*, *C303S* responded normally to heat shock, even though these cells were resistant to thiol-reactive compounds (Figure 4-5). To confirm that *SSA1-C264S*, *C303S* retained heat-inducibility, I subjected the same strains to mild heat shock (37 °C) before the lethal heat shock (52 °C). Consistent with the reporter assay, *SSA1-C264S*, *C303S* cells displayed the same level of thermotolerance as wild-type cells. Together, these results suggest that thiol-reactive compounds target C264 and C303 of Ssa1 to activate Hsf1, and this process is distinct from how cells sense thermal stress.

**Figure 4-8. Ssa1 cysteines are required for activation of the cytoprotective HSR by thiol-reactive compounds but not by heat shock.**

The *ssa1Δssa2Δ* cells containing wild-type and *SSA1-C264S*, *C303S* mutant allele were treated with either no reagent (-) or 600  $\mu\text{M}$   $\text{Cd}^{2+}$  (+) (left) or were grown at 30 °C (-) or 37 °C (+) for 1 hr (right) before heat shock at 52 °C for the indicated times.

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**Figure 4-8. Ssa1 cysteines are required for activation of the cytoprotective HSR by thiol-reactive compounds but not by heat shock.**



## DISCUSSION

In the previous chapter, I have shown that diverse compounds that share the biochemical property of thiol-reactivity function as potent activators of the heat shock response in yeast cells, consistent with findings in human cell lines. My data suggest that these thiol-reactive compounds do not cause significant aggregation of unfolded proteins in the time frame of HSR activation. Instead, I demonstrate in this chapter that cysteine residues of the cytosolic Hsp70 Ssa1, are specifically modified, which leads to inactivation of Hsp70 and derepression of Hsf1.

Genetic and biochemical evidence suggest that in both yeast and mammals, the Hsp70/Hsp90 multichaperone complex serves an auto-regulatory role in Hsf1 regulation (258). Hsp70 and Hsp90 likely repress Hsf1 by binding to it in an inactive form under non-stress conditions, and release from Hsf1 during stresses (66, 71, 205, 248, 275, 276). Inhibition of the Hsp70/Hsp90 chaperone complex using either pharmacological or genetic means leads to a significant increase of Hsf1 activity, suggesting that chaperones may function as direct stress sensors (19, 91, 159, 277-279). Our lab determined previously that celastrol, an active component of the "thunder god" vine, can activate the heat shock response, as well as the oxidative stress response in both yeast and human cells (180). Additionally, early studies using a powerful sulfhydryl-modifying reagent, *N*-ethyl maleimide (NEM), show that a pair of vicinal cysteines in rat Hsp90 (C589/C590) and all three cysteines in yeast Hsp70 Ssa1 are hypersensitive to thiol modification, leading to inactivation of chaperone functions *in vitro* (6, 280). Similar modification of Hsp70 and Hsp90 are also observed in a rat model using low concentrations of 4-hydroxynonenal (~10  $\mu$ M), a byproduct of lipid peroxidation (228, 267). Moreover,

mammalian cells treated with the A- and J-type cyclopentanone prostaglandins (PGA, PGJ) are shown increase expression levels of Hsp70, and I observed the same induction of Hsp70 production in yeast cells using 15d-PGJ<sub>2</sub>. All these compounds contain an electrophilic  $\alpha,\beta$ -unsaturated carbonyl moiety that can form a transient or permanent adduct with the nucleophilic thiol group of a cysteine residue, suggesting a common chemical mechanism. In addition to organic electrophiles, transition metals and metalloids such as cadmium and arsenic are also reported to induce Hsf1 phosphorylation, trimerization and DNA binding in yeast and mammalian cells (281, 282). Since yeast Hsf1 and Hsp90 lack cysteine residues, the simplest model consistent with the observed Hsf1 activation and Hsp90 inhibition is thiol-modification of Hsp70. Indeed, I observed that among all chaperones comprising the Hsp70/Hsp90 complex, the yeast cytosolic Hsp70 Ssa1 was specifically modified by a thiol-reactive probe, biotin-BMCC at the concentrations used. It is interesting that all five chaperones I tested contain at least two cysteine residues. However, Ssa1 was the only chaperone modified by biotin-BMCC in my experiment. The nucleophilic character of protein cysteines is strongly affected by both surface exposure and the pKa of the thiol functional group. The surface exposure determines the accessibility of a cysteine residue to exogenous compounds; while the pKa is mainly controlled by neighboring side chains in the local microenvironment (283, 284). It is possible that cysteine residues on other chaperones including Sse1, Fes1, Cpr6 and Cpr7 are inaccessible in the Hsp70/Hsp90 complex, making the constitutive expressed Ssa family of Hsp70, Ssa1 and Ssa2, the sole relevant candidate sensor of thiol-reactive compounds for Hsf1. It is worth mentioning that I did not investigate Ssa2 separately, because Ssa1 and Ssa2 are 90% identical with each other and the plasmid

carrying only wild-type *SSA1* has the capacity to complement the Hsf1 derepression and the slow growth defect of the *ssa1Δssa2Δ* mutant, suggesting that Ssa1 and Ssa2 share similar functions (Figure 4-3B and 4-3C) (285).

Hsp70s are known to facilitate protein folding, prevent protein aggregation and assist in the assembly of multi-protein complexes. In this way, Hsp70s can monitor and counteract the accumulation of misfolded proteins to maintain protein homeostasis in the cell (286). The folding function of Hsp70 is highly ATP-dependent: substrate binding activity is regulated by ATP turnover (287). Interestingly, analysis of amino acid sequences from the *Saccharomyces* Genome Database and the crystal structure of human Hsc72 revealed that all three cysteines of Hsp70 are in the N-terminal ATP binding domain. Furthermore, *in vitro* functional assay shows that NEM modification of Ssa1 is inversely related to nucleotide status in the ATPase domain. NEM-modified Ssa1 fails to bind ATP, while nucleotide binding protects Ssa1 from modification and inactivation by NEM (6). These observations are consistent with our finding that substitution of C264 and C303 with aspartic acid completely inactivated Ssa1 as judged by complementation, leading to constitutive activation of Hsf1. Although Ssa1 contains three cysteine residues, our genetic results suggest that only C264 and C303 are primarily responsible to thiol-reactive compounds, suggesting that C15 has little to no effect on sensing of these compounds. Of the three cysteines, C15 is the most conserved among Hsp70s, and may be involved in the direct interaction with ATP. This prediction may explain our finding that substitution of C15 with serine results in instability of Ssa1. C264 is absent from heat inducible Ssa3/4, as well as ribosome-associated Ssa1/2, mitochondrial Ssc1 and ER-localized Kar 2. Therefore, Ssa1/2 chaperones, as the constitutive expressed cytosolic

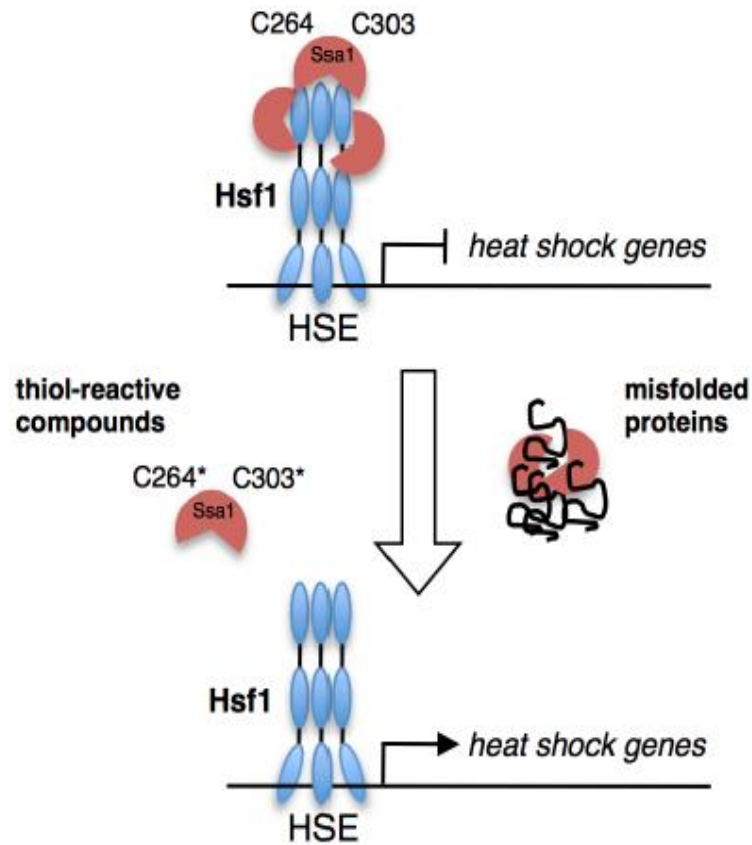
Hsp70 in yeast, are likely to be unique hyper-reactive targets of thiol-reactive compounds with respect to Hsf1 activation. Our genetic assays suggested that both C264 and C303 are required for activation of cytoprotective HSR by thiol-reactive compounds. However, we observed labeling of C303 in the absence of C264 *in vivo*. Since thiol modification of a cysteine residue requires both surface exposure and the pKa of the thiol group, it is possible that modification of C303 potentiates modification of C264 by subtle changes in the microenvironment of the thiol side chain. Strikingly, a recent study of human cell lines reported similar findings that two cysteine residues, C267 and C306, are hypersensitive to a redox-active compound, methylene blue (MB). Consistent with my observation in the yeast system, C306 of human Hsp70 is more reactive compared with C267. Moreover, predictive dynamic modeling of the Hsp70 NBD further revealed that oxidation of C306 may contribute to conformation rearrangements of the ATP-binding domain, and is likely to expose C267, making it more accessible to thiol-modification (288) (and unpublished data from Gestwicki lab). Together, these results provide a crucial functional basis for a mechanism through which Ssa1 modified by thiol-reactive compounds loses its activity, leading to derepression of Hsf1 (Figure 4-9). The correspondence between my finding and the human study suggests that this thiol-sensing system could be conserved in almost all eukaryotes.



**Figure 4-9. Model demonstrating independent activation of hsf1 by misfolded proteins or thiol-reactive compounds in an Ssa1-dependent manner.**

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**Figure 4-9. Model demonstrating independent activation of hsf1 by misfolded proteins or thiol-reactive compounds in an Ssa1-dependent manner.**



**Chapter 5: Characterization of a partially functional FLAG-Hsf1 constructs to detect interaction between Hsf1 and Ssa1**

## INTRODUCTION

In an eukaryotic cell exposed to stress, HSF1, the primary regulator of the heat shock response, is rapidly induced to rescue cellular damage and maintain protein homeostasis. Soon after the discovery of this transcription factor, several lines of evidence suggested that HSF1 is under tight negative regulation. For example, overexpression of HSF1 in human cell lines results in accumulation of DNA-binding trimeric HSF1 under normal growth conditions (17, 289). Constitutive activation of HSR is also observed in human cells with heterologous expression of *Drosophila* HSF1 (290). Early studies in cultured *Drosophila* and human cells showed that HSF1 activity is not modulated at the level of synthesis/degradation of the transcription factor, but mainly posttranslationally (270, 289-294).

Under thermal stress, the expression levels of heat shock proteins (HSPs), or protein chaperones, increase drastically after initiation of heat shock, persist transiently, and attenuate upon recovery. However, exposure to amino acid analogs such as AZC, results in irreversible misfolding of nascent proteins and constitutive activation of the HSR (Figure 3-5) (249, 256, 281). These observations lead to a model that HSF1 is regulated by protein chaperones under a negative feedback mechanism. As discussed in the introduction of Chapter 4, at least two classes of protein chaperones, Hsp70 and Hsp90, are involved in autoregulation of HSF1 activity. In mammalian cells, this model is additionally supported by biochemical identification of Hsp70/Hsp90-Hsf1 interactions. Human Hsp70 associates with both DNA-binding and free monomeric HSF1 (45, 66, 261, 281). More detailed investigation demonstrated that Hsp70 and its co-chaperone Hsp40 interact directly with the C-terminal transactivation domain of HSF1 *in vivo* and *in vitro*

(248). Furthermore, Hsp90 is shown to restrain HSF1 in an inactive monomeric form in a HeLa cell lysate system. This is further supported by in situ crosslinking of Hsp90 with Hsf1 in unstressed HeLa cells (71). Heat shock treatment or depletion of Hsp90 by antibody or pharmacological inhibitors releases HSF1 from the Hsp70/Hsp90 complex and promotes the transcription factor trimerization and DNA-binding activity (70, 275). Consistent with the previous finding that acquisition of HSF1 DNA-binding activity is inseparable from its trimerization, these studies suggest that the Hsp70/Hsp90 complex regulates HSF1 transcription activity by physically interacting with monomeric HSF1 and repressing its trimerization/DNA-binding activity in metazoan cells.

In the yeast *Saccharomyces cerevisiae*, on the other hand, Hsf1 binds constitutively to DNA in the nucleus (203). Additionally, a stable Hsp70/Hsp90-Hsf1 complex has not been observed in the yeast system (295). However, genetic analysis of yeast strains carrying mutations in cytosolic Hsp70, Hsp90, and their co-chaperones Cpr7, Sse1 and Fes1 revealed that same as in higher eukaryotes, the yeast chaperone system also negatively regulates Hsf1 (Figure 4-1) (76, 77, 237, 258). In Chapter 4, I reported that the yeast cytosolic Hsp70 Ssa1 functions as a sensor for Hsf1 activation by thiol-reactive compounds. Although I demonstrated direct thiol-modification of Ssa1 *in vivo* and *in vitro*, I cannot exclude the possibility that Hsp70 inactivation leads to Hsf1 activation through increase of misfolded Hsp70 substrates due to lack of biochemical identification of yeast Hsp70/Hsp90-Hsf1 interactions. Utilizing a chromosomal integrated HSF1-TAP construct, I showed hyper-phosphorylation of Hsf1 in cells treated with heat shock and thiol-reactive compounds. However, detection of Hsp70/Hsp90 interactions with Hsf1 was not successful using this protein fusion. In addition, antibodies

that specifically recognize yeast Hsf1 are not commercially available. Therefore, in this chapter, I reported generation of a partially functional FLAG-Hsf1 fusion for future detection of Ssa1-Hsf1 interactions. Compared with the 21 kDa TAP-tag, the FLAG peptide sequence of DYKDDDK is much smaller (1012 Da). In addition, the tag is more hydrophilic than other common epitope tags such as myc and human influenza hemagglutinin (HA) tag. Therefore, the FLAG-tag is optimized for compatibility with the proteins to which it is appended. Finally, our laboratory has developed reliable protocols to use FLAG immunoprecipitation for biochemical identification of dynamic protein-protein interactions in yeast. In the future, this functional FLAG-Hsf1 construct can be used to answer the question of whether chaperone repression is the primary control mechanism whereby cells sense thiol-stress via unique reactive cysteine residues, and investigate the role of the chaperone-Hsf1 network during diverse stress conditions in yeast system.

## RESULTS

*The N-terminal tagged FLAG-Hsf1 expressed on a centromeric plasmid responds to heat shock in wild-type cells*

One of the advantages working with yeast *S. cerevisiae* is the wide selection of expression plasmids that can be used for different purposes. These plasmid vectors offer diverse choices of vector copy number, promoters of varying strength, and nutrition selection markers. Yeast vectors can be divided into two groups: the low copy centromeric (*CEN*) plasmid and the high copy 2 $\mu$  plasmid. *CEN* plasmids carrying a yeast centromere sequence and a normal yeast origin of replication provide 1-3 copy number per cell, and are stably replicated during mitosis (296). 2 $\mu$  plasmids contain an origin of replication from the endogenous 2 $\mu$  plasmid with a copy number of 20-50 per cell (297). In addition to vector copy number, expression levels of heterologous proteins can also be manipulated by changing the strength of promoters. The constitutive promoters include the weak *CYC1* (derived from gene encoding cytochrome-*c* oxidase), the moderate *TEF* (derived from *TEF2* gene encoding translation elongation factor 1 $\alpha$ ), and the strong *GPD* (derived from gene encoding glyceraldehydes-3-phosphate dehydrogenase) (298-300). The expression system can also be regulated using inducible promoters such as galactokinase promoter *GALI* (198).

To build a functional FLAG-Hsf1 for biochemical identification of Ssa1-Hsf1 interaction, I first fused the FLAG tag on the N-terminus of Hsf1 by PCR amplification of the *HSF1* open reading frame (ORF). The construct was cloned into the p414TEF plasmid, which is a centromeric vector with a moderate strength promoter. Since *S. cerevisiae* contains a single *HSF1* gene, deletion of *HSF1* is lethal and hypomorphic

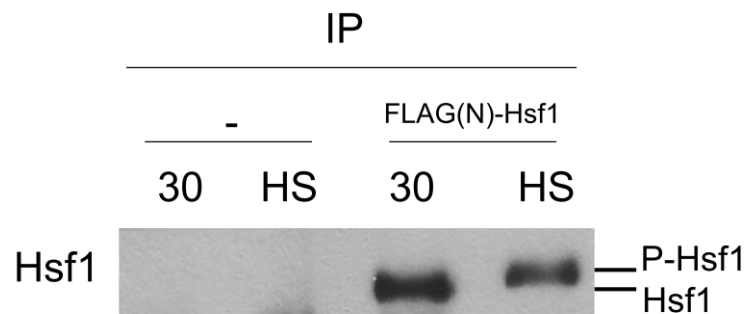
mutants are defective in multiple processes such as cell wall integrity and cell cycle progression at high temperatures (36, 301-303). I transformed the plasmid p413TEF-FLAG(N)-Hsf1 in the wild-type (BY4741) background to test detectability of Hsf1. As shown in Figure 5-1 (upper panel), the FLAG-Hsf1 fusion was expressed at 30 °C and hyperphosphorylated, as detected as the mobility shift, after heat shock, suggesting that the fusion construct can sense and respond to thermal stress. Ssa1 was shown to bind with Hsf1 in non-stressed cells after FLAG co-immunoprecipitation. However, the interaction was mildly diminished by heat shock. The same results were observed after increasing heat shock temperature up to 42 °C and the time of treatment up to 45 min (data not shown). Two possibilities can explain this observation. First, Ssa1 may not dissociate from Hsf1 after heat shock. Instead, a conformation change could occur to release the transcriptional activity of Hsf1. The second possibility is that the FLAG-Hsf1 construct might not be functional, even though hyperphosphorylation of Hsf1 was observed at 37 °C. In human cell lines, overexpression of HSF1 resulted in constitutive trimerization/DNA-binding activities of the transcription factor (289). To test whether Ssa1 constitutively bound to FLAG-Hsf1 as a result of loss of Hsf1 functions, I utilized the HSE-*lacZ* reporter to measure Hsf1 activity in wild-type cells carrying the N-terminal FLAG-Hsf1 fusion at 30 °C and 37 °C. Indeed, Hsf1 was derepressed in these cells, suggesting that the fusion construct did not function properly in the wild-type background, maintaining activity in the absence of stress (Figure 5-2).



**Figure 5-1. The N-terminal FLAG-Hsf1 fusion hyperphosphorylated after heat shock.**

Wild-type (BY4741) cells bearing a empty vector (-) or p413TEF-FLAG(N)-Hsf1 were grown at normal temperature (30 °C), or heat shocked at 37 °C for 20 min (HS). Hsf1 was immunoprecipitated (IP) with anti-FLAG affinity M2 resin. Hsf1 was probed with M2 antibody.

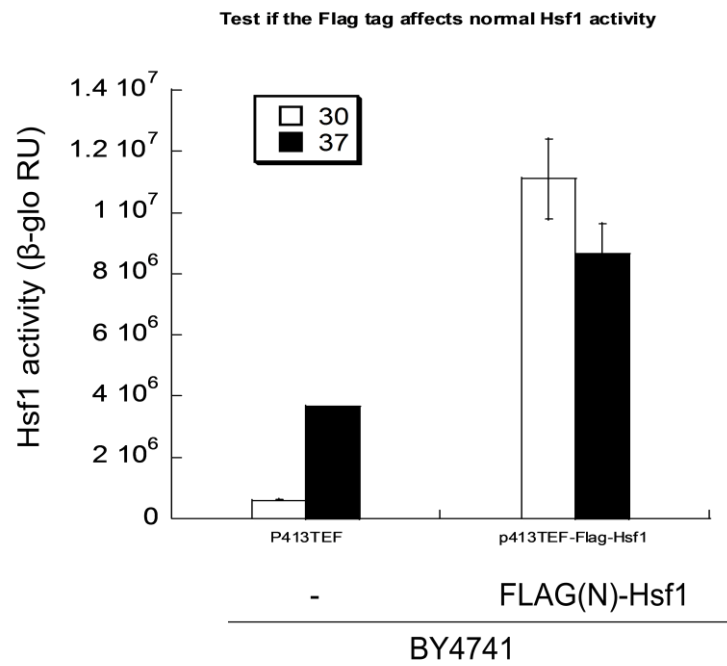
**Figure 5-1. The N-terminal FLAG-Hsf1 fusion hyperphosphorylated after heat shock.**



**Figure 5-2. The N-terminal FLAG-Hsf1 fusion causes derepression of Hsf1 in wild-type cells.**

Wild-type (BY4741) cells carrying the empty vector (-) or p413TEF-FLAG(N)-Hsf1 were transformed with the HSE-*lacZ* reporter, and were grown at the control temperature (30 °C, empty bar) or heat-shocked (37 °C, black bar) for 1 h.  $\beta$ -galactosidase activity was measured by a luminescent  $\beta$ -Glo assay.

**Figure 5-2. The N-terminal FLAG-Hsf1 fusion causes derepression of Hsf1 in wild-type cells.**



*Overexpression of Hsf1 results in constitutive activation of HSR in yeast system*

Two possibilities exist to explain observed derepression of Hsf1 in cells carrying the p413TEF-FLAG(N)-Hsf1 fusion: either the N-terminal FLAG tag interferes with negative regulation of Hsf1 or overexpression of the fusion construct bypasses chaperone repression of Hsf1. Although not mutually exclusive, I sought to distinguish between these two possibilities by expressing Hsf1 under the endogenous promoter on a plasmid in wild-type cells (p314-HSF1), which resulted in ~ 2 folds overexpression of Hsf1. The HSE-*lacZ* reporter then was introduced and  $\beta$ -galactosidase activity assay was applied to determine the Hsf1 activity. As shown in Figure 5-3A, wild-type cells carrying p314-HSF1, without any protein tag, partially restored the chaperone repression upon the HSR with moderately higher basal activity at normal temperature, suggesting that deregulation of Hsf1 may be caused by overexpression.

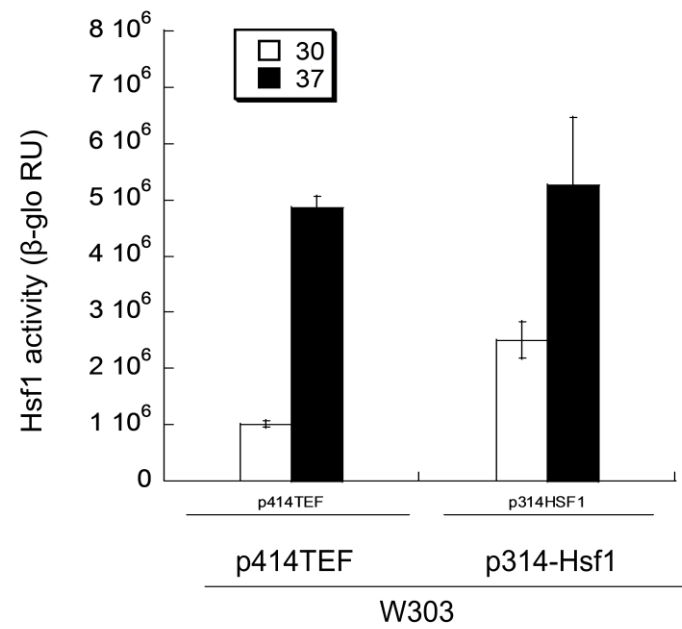
Next I sought to understand whether the N-terminal FLAG tag also contributes to the Hsf1 deregulation. Ideally, this possibility should be tested using FLAG-Hsf1 fusion under control of the endogenous promoter on a plasmid in wild-type cells. However, the insertion of FLAG sequence in the p314-HSF1 plasmid by PCR amplification was not successful. Alternatively, I utilized a yeast strain, DNY248, lacking the endogenous *HSF1* and supported by wild-type *HSF1* on a *URA3* plasmid to eliminate the endogenous expression of Hsf1. The strain was then transformed with the FLAG-Hsf1 construct expressed under a moderate strength promoter TEF (p413TEF-FLAG(N)-HSF1), followed by a plasmid shuffle technique to counterselect the *URA3* plasmid carrying the wild-type *HSF1* on 5-FOA medium. As shown in Figure 5-3B, Hsf1 activity in *hsf1 $\Delta$*  cells bearing p413TEF-FLAG(N)-HSF1 was partially repressed in the absence of stress,

**Figure 5-3. Decrease of Hsf1 expression level partially restores chaperone repression on Hsf1**

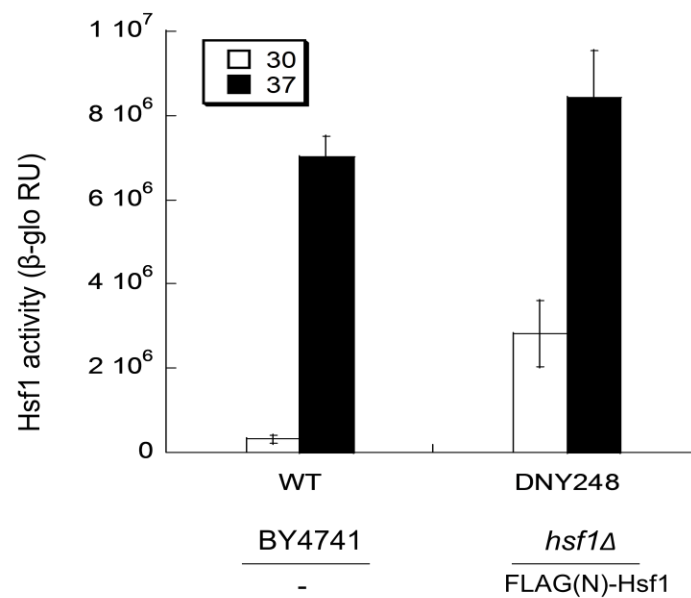
- A. Wild-type (BY4741) cells carrying a empty vector, or p314-HSF1 were transformed with the HSE-*lacZ* reporter, and grown at 30 °C (empty bar) or heat shocked at 37 °C (black bar) for 1 h.  $\beta$ -galactosidase activity was determined using luminescent  $\beta$ -Glo analysis kit.
- B. DNY248 carrying p413TEF-FLAG(N)-Hsf1 was counter selected against the *URA* plasmid expressing wild-type *HSF1* on 5-FOA, followed by transformation with the HSE-*lacZ* reporter. Cells were grown at normal temperature (30 °C, empty bar), or heat shocked at 37 °C (black bar) for 1 hr. The isogenic wild-type BY4741 carrying a empty *HIS* vector and the reporter was used as a control.  $\beta$ -galactosidase activity was determined using luminescent  $\beta$ -Glo analysis kit.

**Figure 5-3. Decrease of Hsf1 expression level partially restores chaperone repression on Hsf1**

A.



B.



and induced normally during heat shock. I noted that the basal Hsf1 activity in DNY248 was also moderately higher compared with wild-type BY4741 cells. It is consistent with the promoter strength of TEF, which probably resulted in higher than endogenous expression level of Hsf1 in DNY248, but lower than that in wild-type cells carrying the fusion construct. However, I cannot completely exclude the possibility that localization and existence of the FLAG tag leads to misfolding of Hsf1, and consequently contributes to Hsf1 derepression. These data suggest that the constitutive activation of Hsf1 in cells carrying the FLAG-Hsf1 fusion is predominantly caused by overexpression of the transcription factor. The total amounts of FLAG-Hsf1 fusion in the cytoplasm possibly overwhelmed chaperone repression. Taken together, I have taken the first step toward generating a functional expression system of FLAG-tagged Hsf1 using a strain lacking the endogenous Hsf1. This expression system then can be used to biochemically identify the dynamic Hsp70/Hsp90-Hsf1 interactions in the presence of thiol-reactive compounds to refine the model I proposed in Chapter 4. It can also be utilized for structural studies of chaperone regulation, screening of Hsf1 targeted genes in various stress conditions, and search of unknown Hsf1 activators and inhibitors such as kinases and phosphatases in the yeast system.



## DISCUSSION

In this chapter, I reported generation of a FLAG-Hsf1 fusion to detect, isolate, and validate Hsp70/Hsp90-Hsf1 complexes in yeast *S. cerevisiae*. Utilizing a heterologous TEF promoter, the FLAG-tagged Hsf1 was successfully expressed and detected. The fusion construct responded to thermal stress and developed hyper-phosphorylation as shown by the SDS-PAGE mobility shift. Genetic studies suggest that the constitutively expressed cytosolic Hsp70, Ssa1 and Ssa2, are Hsf1 repressors in yeast (237, 258). Therefore, I hypothesize that Ssa1 may restrain Hsf1 in inactive form through physical interactions. During stress, Ssa1 may be titrated by accumulation of misfolded proteins, or directly modified to undergo conformation changes, leading to release of Hsf1 into its active form. However, immunoprecipitation assay revealed that heat shock treatment failed to disassociate Ssa1 from Hsf1 using the FLAG-Hsf1 construct. To elucidate this observation, I measured the Hsf1 activity using the HSE-*lacZ* reporter system and demonstrated that Hsf1 was derepressed in the wild-type cells carrying the N-terminal FLAG-tagged Hsf1, suggesting that the fusion construct is not functional. Overexpression of human HSF1 is reported to constitutively activate the HSR (289). Additionally, truncation of the N-terminus of Hsf1 also causes HSR activation in the absence of stress (53, 55). To address whether the expression level or the insertion of N-terminal FLAG tag results in loss of functions of FLAG-Hsf1, I decreased overall expression levels of Hsf1 by transforming it into DNY248, a strain lacking the endogenous *HSF1* gene. Indeed, Hsf1 activity was partially restored in this expression system, suggesting that derepression of Hsf1 in cells carrying FLAG-Hsf1 construct is mainly due to overexpression of Hsf1. However, the possibility that the localization and/or insertion of

the FLAG tag also contribute to the non-functional Hsf1 phenotype is not completely excluded.

HSF1 is a serine-rich, constitutively phosphorylated mediator of the stress response. Phosphorylation events have been observed on 15 serine residues (S121, S216, S230, S292, S303, S307, S314, S319, S320, S326, S344, S363, S368, S419 and S444) and four threonine residues (T142, T323, T367m T369) and show both positive and negative regulation of HSF1 (304-312). On the other hand, HSF1 is negatively regulated by protein chaperones such as Hsp70 and Hsp90. However, little is known about how phosphorylation and chaperone repression collaborate to regulate Hsf1 activity. By overexpressing Hsf1, I observed derepressed, but hyper-phosphorylated Hsf1 during heat shock, suggesting that chaperone regulation may be occurring prior to other post-translational modifications. Interestingly, both phosphorylation events and complex formation of human Hsp70-Hsf1 occur in the transactivation domain of Hsf1 (289, 313). It is unclear how Hsf1 coordinates the multi-layer regulation on the same sites. However, I cannot exclude the possibility that the Hsf1 was not normally phosphorylated due to lack of commercial available antibodies of yeast Hsf1 and knowledge of involved kinases and phosphatases in the yeast system.

Taken together, in this chapter, I report generation of a functional FLAG-Hsf1 fusion construct in the yeast system. In the future, this system can be used to identify biochemical interactions between Hsf1 and chaperone in various stress conditions. According to the genetic and biochemical results I showed in Chapter 4, I predict this construct can be used to observe interaction of Ssa1 and Hsf1 under normal growth conditions. In the presence of thiol-reactive compounds, direct modification may cause

conformation rearrangements of Ssa1, leading to dissociation of Ssa1-Hsf1 complexes. Alternatively, human Hsp70 is observed to associate with either inactive monomeric or active DNA-binding Hsf1, making it is possible that Ssa1 maintains the interaction with Hsf1, but relies on conformation rearrangements to regulate transactivation activity of Hsf1 (45, 66, 261, 281).

## **Chapter 6: Discussion and conclusions**

## SUMMARY AND FUTURE DIRECTIONS

### *Thiol-reactive compounds function as potent Hsf1 activators in yeast*

The heat shock response (HSR) is an ordered genetic response to diverse environmental and physiological stimuli that causes robust induction of genes encoding molecular chaperones, proteases, and other proteins that essential for protection and recovery from cellular damage associated with the accumulation of misfolded proteins (159). Under stress conditions, molecular chaperones, also called heat shock proteins (HSPs) function to hold and refold denatured proteins, prevent them from aggregation, and/or regulate their degradation. In non-stressed cells, HSPs are also central components in signal transduction, immunity and apoptosis (16-19, 215, 314). Proper function of HSPs is relevant to many human diseases, including cancer and neurodegenerative diseases, making it important to understand regulation of the pathway (184, 185).

In all eukaryotes, the HSR is modulated at the transcriptional level by the heat shock transcription factor Hsf1. Interest in the HSR and chaperones as potential targets for therapeutics has spurred investigations on small molecule regulators of Hsf1. These regulators have diverse chemical features: some are non-specifically reactive with a variety of cellular proteins, whereas others have specific targets that disrupt protein homeostasis (or proteostasis) (216). Many known Hsf1 activators influence the proteostasis network by affecting protein translation, such as translation inhibitors (e.g. puromycin) and amino acid analogues (e.g. azetidine 2-carboxylic acid), or by targeting the protein quality control system, such as chaperone (e.g. geldenamycin) and proteasome inhibitors (e.g. MG132) (159, 170, 209). For other Hsf1 activators, the detailed mechanisms and the molecular targets that are linked with Hsf1 activity remain largely

unclear. Interestingly, many Hsf1 activators that fall into this category are capable of reacting with the thiol group of cysteine residues. For example, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) activates mammalian Hsf1 *in vitro* by initiating disulfide bond formation in the DNA binding domain of Hsf1 (171). Transition metals and metalloids such as cadmium and arsenic have been shown to upregulate Hsp70 gene transcription in yeast and mammalian cells (281, 282). Many organic electrophiles including celastrol, the cyclopentenone prostaglandin, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), and 4-hydroxynonenal (4-HNE) are also reported as HSR activators (220-222, 315-317). All of these electrophiles contain an  $\alpha,\beta$ -unsaturated carbonyl group capable of forming a Michael adduct with the thiolate anion. Moreover, previous data from our lab reports that celastrol not only activates the heat shock response, but also induces the oxidative stress response in yeast. These results suggest a common chemical mechanism whereby diverse reactive compounds can activate the HSR by thiol modification of cysteine residues (180). To test this hypothesis, I tested three distinct groups of thiol-reactive compounds, i.e. transition metals and metalloids (e.g., cadmium), oxidants (e.g., diamide and H<sub>2</sub>O<sub>2</sub>), and organic electrophiles (e.g., diethylmaleate and 15d-PGJ<sub>2</sub>). Strikingly, all of these compounds significantly induced Hsf1 activity. The same molecules produced an SDS-PAGE mobility shift consistent with heterogeneously phosphorylated Hsf1 observed during heat shock, providing independent verification of Hsf1 activation. In addition, pretreatment with a reducing agent dithiothreitol (DTT) completely inhibited Hsf1 activation, confirming that these reactive compounds activated Hsf1 due to their thiol-reactivity. Together, my work showed that diverse thiol-reactive compounds can function as potent activators of the HSR in a model eukaryote.

*Thiol-reactive compounds do not activate Hsf1 by accumulation of misfolded cytosolic proteins*

The baker's yeast *S. cerevisiae* contains a single *HSF1* which is essential for cell viability at all temperatures and constitutively binds to the conserved heat shock element (HSE) motif in the promoter of *hsp* genes as a homotrimer (36, 318). Generally, Hsf1 is thought to be stabilized by cytosolic HSPs under normal growth conditions. During heat shock, accumulated misfolded proteins may recruit HSPs away from Hsf1 and relieve Hsf1 repression. Therefore, the accumulation of misfolded proteins can trigger Hsf1 activation.

In this work, I determined that different thiol-reactive compounds activated Hsf1 and inhibited Hsp90 in a reciprocal manner. To understand the mechanism through which cells sense thiol reactive compounds and activate the appropriate transcriptional response, I first asked whether these compounds cause cytosolic protein misfolding, leading to titration of chaperones from the Hsf1. Because it is difficult to label the thiol-reactive compounds I identified, I compared their kinetic induction profiles with those of unfolding reagents and demonstrated that the features of Hsf1 activation by thiol-reactive compounds are not consistent with a model relying on the accumulation of misfolded cytosolic proteins. Two unfolding reagents were used in this work. Azetidine-2-carboxylic acid (AZC) is a toxic analog of proline and is reported to misfold newly synthesized proteins by incorporating into polypeptides competitively with proline, whereas DSP, an amine-reactive protein crosslinker, was used to cross-link existing proteins, predicted to lead to aggregate formation of cytosolic proteins (249). Both AZC and DSP were shown to activate Hsf1 by detecting the expression levels of inducible Hsp70 and using the HSE-*lacZ* reporter assay, respectively. However, these two

unfolding reagents displayed completely different induction profiles when compared with those of thiol-reactive compounds. First of all, the thiol-reactive compounds rapidly induced the HSR. However, Hsf1 activation by AZC and DSP occurred much slower, suggesting that high levels of misfolded proteins are required to activate Hsf1. Second, thiol-reactive compounds induced the HSR transiently, whereas activation due to AZC and DSP treatment is cumulative, likely from continued production and accumulation of high levels of unfolded proteins. Consistent with the cumulative induction of Hsf1, treatment with AZC, but not thiol-reactive compounds, resulted in significant decrease of cell viability. Lastly, thiol-reactive compounds reciprocally inhibited Hsp90 functions using the glucocorticoid receptor analysis system. The inhibition of chaperone functions was not observed in cells treated with DSP. Taken together, I reported that both thiol-reactive compounds and the unfolded reagents were capable of inducing the HSR. However, the thiol-reactive compounds showed distinct kinetic induction profiles from those of AZC and DSP, indicating that these reactive compounds are likely not activating Hsf1 by directly misfolding cytosolic proteins.

Another possible explanation of Hsf1 activation by thiol-reactive compounds could be that these compounds affect other aspects of cellular protein quality control systems. The ubiquitin-proteasome system (UPS) is responsible for degradation of short-lived and abnormal proteins. In the process of ubiquitination, ubiquitin (Ub) is transferred from E1 to the active cysteine residues of E2 via a transthioesterification reaction. It was therefore conceivable that the thiol-reactive compounds induced Hsf1 by modification of the thioester linkage between E1 and E2, leading to the accumulation of non-ubiquitinated, misfolded proteins. Indeed, I used  $\beta$ -galactosidase reporter fusions to probe



distinct pathways for protein ubiquitination and degradation, and demonstrated that cadmium specifically stabilized substrates recognized by *UBC6* and *UBC7*. This observation suggests that cadmium did not inhibit proteasome or Uba1 (E1) activity as this would have led to stabilization of all reporter substrates. Since Ubc6/7-dependent protein degradation was inhibited by cadmium treatment, I asked whether inhibition of this specific degradation pathway leads to activation of Hsf1. Interestingly, inhibition of Ubc6/7 function by deleting the major gene *UBC7* failed to derepress Hsf1 in non-stressed cells, suggesting that the inhibition of a specific ubiquitination pathway and activation of Hsf1 by cadmium are correlated, but not causal, events. Moreover, I demonstrated that the proteasome inhibitor MG132 induced the HSR, but had no effect on Hsp90 activity, suggesting different mechanisms through which MG132 and thiol-reactive compounds activate the HSR. Taken together, my results suggest that thiol-reactive compounds do not induce the HSR by inhibiting the ubiquitin-proteasome system.

Although I successfully demonstrated that thiol-reactive compounds did not non-specifically damage cytosolic proteins or affect the ubiquitin-dependent protein degradation, my results cannot exclude other possibilities relevant to protein homeostasis such as ER-associated degradation and ubiquitin-independent protein degradation. Instead of test these possibilities individually, I utilized high-speed centrifugation to demonstrate that thiol-reactive compounds maintained solubility of the majority of proteins, suggesting that these compounds caused minor to no effect on protein stability. Taken together, I showed in this work that Hsf1 activation by thiol-reactive compounds is not due to generation and accumulation of misfolded cytosolic proteins. Instead, the

reciprocal Hsp90 inhibition by these chemicals suggests an alternative sensing mechanism.

*The yeast Hsp70 Ssa1 is hypersensitive to thiol-reactive compounds*

Genetic and biochemical evidence support a model that at least two classes of chaperones, Hsp70 and Hsp90, play a major role in regulating Hsf1 activity, repressing activation through binding/sequestration under normal growth conditions and promoting transcriptional competence through complex dissociation during stress (45, 66, 71, 76-78, 117, 237, 258). Previous study from our lab reported that celastrol not only activates Hsf1, but also inhibits Hsp90-dependent signal transduction in yeast and mammalian cells (180, 223). Consistently, the thiol-reactive compounds I determined also functionally inactivate Hsp90. Since pretreatment of thiol-reactive compounds with excess free thiol (e.g., DTT) completely abolished their biological effects on Hsf1 activation, I reasoned that Hsf1 induction by thiol-reactive compounds may involve inactivation of specific regulatory proteins through targeting of reactive cysteine residues. Although yeast Hsf1 and Hsp90 proteins (e.g., Hsc82 and Hsp82) lack cysteine residues, several Hsp90 co-chaperones contain one or more cysteines, i.e. all four Ssa family of Hsp70, the Hsp70 nucleotide exchange factors Sse1 and Fes1, and Hsp90-associated peptidyl-prolyl isomerases Cpr6 and Cpr7. Furthermore, mutations of Ssa1/2, Sse1, Fes1 and Cpr7 all lead to significant derepression of Hsf1, suggesting that one or more of these proteins might be sensitive to thiol-modification (76, 77, 237). Indeed, I demonstrated that Ssa1 was hypersensitive to a cysteine biotinylation reagent biotin-BMCC *in vitro*. The labeling of Ssa1 by the thiol-reactive probe was completely abolished after treated with DTT, verifying that Ssa1 was

labeled via thiol modification. It is interesting that other chaperones I tested were completely resistant to the thiol labeling, even though some of them such as Sse1 contain more cysteine residues than Ssa1. This might be because I enriched these chaperones from cell extracts using tandem affinity purification. As important components of the Hsp90 multichaperone machinery, these chaperones probably reacted with the biotin-BMCC as a complex which prevented the exposure of some cysteine residues, suggesting that not every cysteine is equally reactive *in vivo*. These results, together with the genetic evidence from deletion of Ssa1 and the two nucleotide exchange factors, strongly implicate that Ssa1 is a relevant target of Hsf1-activating thiol-reactive compounds in yeast.

*Ssa1 is a sensor of Hsf1 activation by thiol-reactive compounds*

The *in vitro* labeling assay suggested that of the proteins comprising the Hsp70/Hsp90 complex we tested, Ssa1 is specifically modified by a thiol-reactive probe. The Ssa family of cytosolic Hsp70, represented by Ssa1, contain three cysteine residues located in the nucleotide-binding/ATPase domain (271). To test whether modification of Ssa1 on one or more cysteine residues by the thiol-reactive compounds I determined is directly responsible for Hsf1 activation, I substituted the cysteines in Ssa1 with non-reactive serine. Although the C15S substitution leads to instability and loss of functions of Ssa1, cells bearing *SSA1-C264S*, *C303S* complemented the slow growth phenotype of *ssa1Δssa2Δ*, but were unresponsive to thiol-reactive compounds. Interestingly, both single mutants *SSA1-C264S* and *SSA1-C303S* recapitulated the responses of the double

mutant, suggesting that cells required both C264 and C303 of Ssa1 to sense thiol-reactive stress.

Two models can explain the role of Ssa1 in transducing the thiol-reactive signal to Hsf1. First, the thiol-reactive compounds may directly modify the Ssa1 cysteines by either oxidation or by the formation of stable or transient adduct. In the second model, these compounds could deplete cellular redox buffer, i.e. the thioredoxin and glutathione systems, which might fail to maintain Ssa1 in a reduced state. To distinguish between these two possibilities, I used Click chemistry to exam thiol-modification of Ssa1 *in vivo*. Taking advantage of azide-alkyne cycloaddition, I demonstrated that wild type Ssa1 was modified by the alkyne derivative of 4HNE molecule that was a potent Hsf1 activator, whereas the labeling signal was drastically decreased in cells carrying the *SSA1-C264S*, *C303S* mutant allele, suggesting that one or both of these cysteines is a direct target of electrophiles *in vivo*. Interestingly, *SSA1* single mutants responded differently to the *in vivo* labeling. In *SSA1-C264S* with intact C303, the labeling signal was as strong as wild-type, but was significantly decreased in *SSA1-C303S*. This observation is not consistent with our genetic analysis that both cysteine residues are required to sense thiol-reactive compounds. The nucleophilicity of cysteine residues requires both thiol de-protonation to the thiolate anion and surface exposure. Moreover, C306 in human Hsp70 also demonstrates higher reactivity than C267 when treated with an oxidant, methylene blue (MB). Structure analysis further demonstrated that oxidation of C306 results in conformation changes of Hsp70 ATP-binding site, which consequently increases exposure and sensitivity of C267 to thiol-modification. Therefore, C303 may be highly reactive relative to C264 and C303 may need to be modified prior to C264 is exposed. To

verify that thiol modification leads to functional inactivation of Ssa1, I replaced the two cysteines with aspartic acid to add steric bulk and mimic oxidation. Strikingly, *SSA1-C264D*, *C303D* produced a stable, but not functional, Ssa1 protein failed to complement slow-growth and Hsf1 derepression of *ssa1Δssa2Δ* mutant. Taken together, I demonstrated that C264 and C303 of Ssa1 are directly modified by thiol-reactive compounds in living cells in the same time frame in which I observed Hsf1 activation. The thiol-modification leads to inactivation of Ssa1, and consequently derepresses Hsf1 activity.

*Sensing of thiol-reactive compounds by Ssa1 is physiologically relevant for cell survival*

The ability of cells to survive exposure to a sudden lethal temperature is defined as thermotolerance. Wild-type cells shifted from its normal growing temperature at 30 °C to 37 °C, a sub-lethal temperature, before exposure to the lethal condition at 50 °C showed significantly increase of viability compared with cells directly exposed to 50 °C (319). This phenomenon is called acquired thermotolerance. It is generally assumed that the gain of thermotolerance is due to rapid increases of HSP synthesis (320). Deletion of the constitutive cytosolic Hsp70, Ssa1 and Ssa2, leads to derepression of Hsf1 and growth defects at low temperatures. However, this strain is also shown to be more tolerant of extreme temperatures than wild type, probably due to the constitutively active Hsf1 (237).

Yeast cells exposed to sub-lethal stress gain tolerance not only to the higher dose of the same stress, but also to other disparate environmental stimuli. A general microarray dataset indicates that 21 out 37 predicted stress responsive regulators, for

example, Hsf1, Msn2/4 and Yap1, have overlapping functions under at least half of the eight environmental stresses including oxidative stress, heat/cold shock, and osmotic stress (Chen 2009). Mutants deficient in the key antioxidant enzyme catalase, superoxide dismutase (SOD) and cytochrome *c* peroxidase demonstrate thermal-sensitivity at 50 °C; while overexpression of these enzymes lead to acquisition of thermotolerance (4). Superoxide anion ( $O_2^-$ ) not only activates the yeast Yap1 oxidant defense transcription factor, but also selectively induces the Hsf1-dependent expression of the copper metallothionein *CUP1* (6, 7, 171). Mutated yeast Hsf1 exhibiting high basal transcription activity also gains cells cadmium-resistance (321). Our lab showed previously that treatment of celastrol activates both Hsf1 and Yap1, leading to gain of both thermotolerance and oxidant resistance (180). To confirm that sensing of thiol-reactive compounds by Ssa1 is physiologically relevant to cell survival, I tested the *SSA1* mutants for acquired thermotolerance in the presence of thiol-reactive compounds. Indeed, wild-type cells showed increased viability with cadmium treatment, whereas cells expression *SSA1-C264S*, *C303S* mutant allele failed to gain the thermotolerance. All these findings suggest that yeast Hsf1 can sense thiol-reactive stress through the two cysteine residues of Ssa1 and assist in mounting a defensive transcription response.

*Ssa1 cysteines are required for activation of the cytoprotective HSR by thiol-reactive compounds but not by heat*

The heat shock transcription factor Hsf1 plays a central role in cellular protein homeostasis in response to various stress conditions, including heat shock, oxidative stress, heavy metals, infection and inflammation, and pharmacological reagents (182,

322). Previous studies of yeast Hsf1-mediated metallothionein (*CUP1*) gene expression suggest that Hsf1 may regulate transcription of the same gene via genetically separable signaling pathways in response to different stresses (57, 322). Interestingly, my results suggest that cysteine residues of Ssa1 participate in activation of Hsf1 by thiol-reactive compounds, but not by heat. Utilizing a HSE-*lacZ* reporter system, I assessed Hsf1 activity in cells carrying *SSA1-C264S*, *C303S* in response to heat shock and thiol-reactive compounds. Strikingly, the mutant cells, which rendered immunity to the chemicals, retained normal heat inducibility. To confirm this observation, I subjected the mutant cells to mild heat shock prior to the lethal thermal stress. Consistent with the reporter assay, cells bearing *SSA1-C264S*, *C303S* gained thermotolerance in the same manner as wild-type. These results suggest that thiol-reactive compounds target C264 and C303 of yeast Hsp70 Ssa1 to induce the HSR, and that the HSR activation by these compounds is distinct from sensing of acute temperature increase.

Higher eukaryotes such as mice and humans harbor multiple genes encoding HSF isoforms. Among these HSFs, HSF1 is the principal activator of heat shock gene transcription in response to heat shock and other environmental stresses, while HSF2 plays a role in developmental gene expression (323). It is interesting that yeast *S. cerevisiae* and other “lower” eukaryotes express a single essential HSF equivalent to mammalian HSF1, which is capable to activate transcription in response to multiple distinct stresses (34, 36, 37). Furthermore, yeast Hsf1 is unusual that in addition to the C-terminal transactivation domain, it includes an N-terminal extension as a second potent transcriptional activation domain (53). These observations suggest that the higher eukaryotes may have evolved functional specialized HSF isoforms to respond to distinct

stresses, whereas lower eukaryotes like yeast utilize different signal transduction pathways to sense diverse stress by using a single HSF.

*Physical association between Ssa1 and Hsf1 under stress or non-stress conditions*

The heat shock response is self-regulated via repression under normal growth conditions (255, 270). In yeast, numerous genetic evidence suggest that mutations of cytosolic Hsp70, Hsp90 and their co-chaperones including Cpr6/7, Sse1 and Fes1 lead to activation of Hsf1 (76, 77, 237, 260). The role of Hsp90 in Hsf1 repression is also supported by the finding that pharmacological inhibitors of Hsp90, including geldenamycin, radicicol and celastrol, induce the HSR (19, 71, 180, 186-188, 223). These results suggest that Hsp70 and Hsp90 are the major repressors of Hsf1 in non-stressed cells. In this work, I reported that yeast cytosolic Hsp70 is a relevant candidate to regulate Hsf1 activation by thiol-reactive compounds. By using both genetic and biochemical tools, I demonstrated that Ssa1 was directly modified and consequently inactivated by thiol-reactive compounds. However, I cannot yet conclude that inactivation of Ssa1 directly induces Hsf1. It is also possible that inactivation of Ssa1 leads to increase of misfolded Ssa1 substrates, which activates Hsf1. Testing effects of thiol-reactive compounds on the physical interaction of Ssa1 and Hsf1 is necessary to address this possibility. In mammalian cells, Hsp70 associates with the HSF1 transactivation domain *in vivo* and *in vitro* (45, 248, 261, 324). However, the Hsp70/Hsp90-Hsf1 complexes have not been conclusively observed and validated in the yeast system (295). In Chapter 5, I generated a functional FLAG-Hsf1 expression system in a *hsf1Δ* strain DNY248 for detection, isolation, and validation of Hsp70/Hsp90-Hsf1



interactions in yeast system. By utilizing the FLAG-Hsf1 expression system, I expected to observe association of Ssa1 with Hsf1 in non-stressed cells and dissociation of Ssa1 in the presence of thiol-reactive compounds. If this is the case, it would provide direct evidence that Ssa1 retains Hsf1 in an inactive form through physical interactions, and inactivation of Ssa1 derepresses Hsf1. Alternatively, Ssa1 may not dissociate from Hsf1 during stress. Human Hsp70 binds with either free Hsf1 monomers, or active Hsf1 trimers (45, 66, 261, 325). The recent study of human Hsp70 suggested that oxidation of C306 by methylene blue may result in exposure of the nearby C267. These results suggest that conformational rearrangements of Hsp70 also possibly to occur during regulation of Hsf1 activity. Furthermore, the FLAG-Hsf1 fusion can also be used to detect dynamic interactions between Hsf1 and chaperones in various stresses. In chapter 4, I reported that Ssa1 can discriminate between heat shock and thiol-reactive compounds. Therefore, it would be interesting to observe different interactions of Ssa1 and Hsf1. Finally, the FLAG-Hsf1 fusion can be applied to screen downstream target genes in the presence of diverse environmental stressors, and search kinases and phosphatases of yeast Hsf1 which remain unsolved for decades.

#### *The roles of cellular redox buffering in regulating the Hsp70-Hsf1 circuit*

The Click chemistry analysis provided clear evidence that in the presence of thiol-reactive compounds, Ssa1 probably forms a stable adduct with 4-HNE alkyne. However, I cannot completely exclude another possible explanation for our observations that thiol treatment altered the redox balance in cytoplasm, leading to inability to maintain Ssa1 in a reduced and functional state. In response to oxidative stress, the transcription factor

Yap1 in yeast is activated by forming an intramolecular disulfide bond with Gpx3 (glutathione peroxidase-like protein 3) (326). Thioredoxin is required to turn off the pathway by reducing both the sensor Gpx3 and the regulator Yap1 (327). Therefore, it is also possible that Ssa1 requires glutathione and thioredoxin system to maintain C264 and C303 in reduced state. While organic electrophiles can form permanent adducts with reactive cysteine residues, thiol modification by other reactive compounds such as reactive oxygen species (ROS) can be transient and reversible. The redox buffering system thus is likely to affect both repression and attenuation of Hsf1. To address this, the simplest way is to assess Hsf1 activity in mutants lacking redox buffering. The cytoplasm is usually maintained as a reducing environment as a result of high concentrations of glutathione (GSH), a conserved tripeptide with a highly reactive thiol and a very low redox potential (328-330). Levels of reduced glutathione are regulated by a series of enzymes that balance synthesis,  $\gamma$ -glutamylcysteine synthetase (Gsh1) and glutathione synthetase (Gsh2), and reduction, glutathione reductase (Glr1) (331). The cellular factors that regulate redox homeostasis are two small heat-stable oxidoreductases glutaredoxin (Grx1 and Grx2) and thioredoxin (Trx1 and Trx2) (332). Disruption of the redox buffering result in accumulation of oxidized glutathione, lack of reductive glutathione and thioredoxin, hyper-sensitivity to oxidative stressors, and even loss of viability (333). For example, high concentrations of oxidized glutathione are detected in *glr1 $\Delta$ trx1 $\Delta$*  and *glr1 $\Delta$ trx2 $\Delta$*  mutants (334). Deletion of *GRX1* and *GRX2* fails to maintain oxidoreductase activity during heat shock (335). The *glr1 $\Delta$*  mutant is hyper-sensitive to the oxidant diamide, while *trx1 $\Delta$ trx2 $\Delta$*  demonstrates increase sensitivity to free thiol dithiothreitol (DTT) (334, 336). Although *TRX1* and *TRX2* are dispensable during

normal growth conditions, *glr1Δtrx1Δtrx2Δ* and *grx1Δgrx2Δtrx1Δtrx2Δ* mutants are not viable, suggesting that functional redox buffering system is essential for cell viability (334, 337).

Taking advantage of abundant genetic tools in the yeast system, the roles of specific redox buffering pathways in Hsf1 regulation then can be probed using the HSE-*lacZ* reporter system. Alternatively, GSH content can be used as an indicator of redox balance in cytoplasm. Hsf1 activity can be measured along with GSH contents in heat-shocked cells, cells lacking the redox buffering system, or cells bearing *SSA1* cysteine mutant alleles to study cellular redox buffering in regulating the Hsp70-Hsf1 circuit.

#### *Species diversity of cysteine residues in Hsf1 and chaperones*

A database search of HSF1 homologues from a range of experimental organisms revealed the absence of cysteine residues in *Aspergillus*, *Neurospora*, *Kluyveromyces*, and *Candida* genera, whereas *Schizosaccharomyces*, *Caenorhabditis*, *Danio*, and mammalian HSF1 genes all contain at least one reactive cysteine. These findings support a model wherein Ssa1 homologs confer thiol responsiveness in “lower” eukaryotes, while fission yeast and animals express HSF1 that directly senses redox changes. Indeed, early study demonstrated that hydrogen peroxide activates and stabilize purified *Drosophila* and human HSF1 by catalyzing the formation of disulfide bond between the two cysteine residues (C35 and C105) in the DNA binding domain (171). In addition to DNA binding activity, oxidation of cysteines on mammalian HSF1 also affects the trimerization of the protein. More detailed investigation of human HSF1 reveals that thiol-disulfide exchange promotes conformation changes of the protein, leading to both positive and negative

regulation of HSF1 activity (338-341). These observations are consistent with the fact that several steps in the activation process of HSF1 in metazoan cells, including nuclear accumulation, trimerization and acquisition of DNA binding, are missing in *S. cerevisiae*. Moreover, yeast Hsp90s, both Hsc82 and Hsp82, lack cysteine residues, making yeast Hsp70 Ssa1 a potential sole sensor of thiol-reactive compounds. In contrast, human Hsp90 has more than one cysteine residue. My results and earlier studies both showed that the J-type cyclopentanone prostaglandin (15d-PGJ<sub>2</sub>) can activate Hsf1 in yeast and mammalian cells. Interestingly, biotinylated-15dPGJ<sub>2</sub> was shown to modify human Hsp90 *in vitro*, suggesting additional thiol-reactive sensors in higher eukaryotes (181). It remains unclear whether more complex eukaryotes utilize additional cysteine residues for more complicated regulation of HSF1, or primitive eukaryotes "lost" these cysteine residues to simplify the process. Although HSF1 is highly conserved in all eukaryotes, regulation of mammalian HSF1 is more complicated than that of yeast *S. cerevisiae*. Mammalian HSF1 undergoes trimerization, nuclear translocation, DNA-binding, and post-translational modification, while yeast Hsf1 binds constitutively with DNA as a homotrimer in the nucleus. Therefore, it is possible that during evolution, mammals, for example, refined the regulatory process of the heat shock response and developed more cysteine residues to meet the requirements of extra regulatory steps. Alternatively, "lower" eukaryotes such as yeast might have same numbers of reactive cysteines as animals in chaperones and Hsf1. However, these cysteine residues might be seldom used to sense stress due to the relatively simple regulatory process of yeast Hsf1. Moreover, a cellular proteome abundant in reactive cysteines is susceptible to environmental stimuli

including xenobiotics, reaction byproducts, and oxidative stress. Therefore, in the course of time, yeast may lose these reactive residues for adaptation.

#### *Regulation of signal transduction through cysteine oxidation/modification*

In this work, I have demonstrated that diverse compounds that share the biochemical property of thiol-reactivity can potently activate Hsf1. My work suggests that in the time frame of Hsf1 activation, thiol-reactive compounds specifically modify cysteine residues of the general cytosolic Hsp70 family, represented by Ssa1, leading to transient inactivation of chaperones and derepression of Hsf1. Consistent with my finding of Hsf1 activation in yeast, regulation of signal transduction through direct and reversible oxidation of cysteine residues has also been observed in several other pathways. The stress activated protein kinase (SAPK), also known as jun N-terminal kinase (JNK), pathway is induced by various stressors including UV light, osmotic stress and inflammatory cytokines (342). In non-stressed cells, the SAPK/JNK kinase is inhibited by binding with a glutathione-S-transferase, GSTpi. Stimulation with UV light or hydrogen peroxide results in oxidation and oligomerization of GSTpi, releasing the active kinase (343). In contrast, the upstream activator of the SAPK/JNK pathway, MEKK1 protein kinase, is negatively regulated by thiol modification. MEKK1 contains a reactive cysteine residue within the ATP binding domain. Oxidation of the cysteine by menadione or hydrogen peroxide interferes with kinase activity, suggesting that thiol modification may serve a role of feedback inhibition in the SAPK/JNK cascade (344). The cyclic AMP-dependent protein kinase, also known as protein kinase A (PKA), is regulated by fluctuating levels of cAMP within cells and has multiple functions, including regulation

of glycogen, sugar and lipid metabolism. PKA contains a cysteine residue, Cys-199, near its critical phosphorylation site, Thr-197. Oxidation of Cys-199 using the thiol-oxidizing reagent diamide is shown to enhance dephosphorylation and inactivation of PKA (345, 346).

Cysteine oxidation also impacts activities of transcription factors, primarily by affecting DNA binding. In *Drosophila*, activation of HSF1 by hydrogen peroxide requires two cysteines, Cys-35 and Cys-105, in the HSF1 DNA binding domain (171). The *c-jun* transcription factor, however, loses DNA binding activity *in vitro* in response to altered GSH/GSSG ratios (347). These observations suggest that cysteine oxidation can both positively and negatively regulate activities of transcription factors through either redox-dependent disulfide bond formation or sulfenic acid oxidation of critical cysteine(s) in the DNA-binding domain. Although numerous studies have clearly detected that oxidation of cysteine results in structural and catalytic consequences to the targeted proteins in experimental systems, it is still difficult to use current available technology to demonstrate thiol modifications in response to physiologically relevant signaling stimuli and conclusively link these modifications with control of cell signaling.

Theoretically, all protein cysteine residues could be modified with oxidative reagents such as reactive oxygen species (ROS). However, for cysteine oxidation to be important in cell signaling, there must be specificity determinants that allow only a small subset of cysteines to be modified. Two factors appear to contribute to determination of specificity: the accessibility of a cysteine and its inherent reactivity (348). Studies using strong oxidizing reagents such as *N*-ethyl maleimide (NEM) and iodoacetamide (IAA) demonstrate that cysteine residues located at the surface are preferentially modified in a

non-denatured protein (349-351). The inherent reactivity of a cysteine may also be influenced by neighboring amino acids. The thiol ionization state governs cysteine nucleophilicity and redox susceptibility. In the case of hydrogen peroxide, only thiolate anion ( $\text{R-S}^-$ ), but not the sulfhydryl group ( $\text{R-SH}$ ) of a cysteine is capable of reacting directly. Mutagenesis analyses suggest that charged amino acids, such as aspartic acid carboxylate anion and protonated lysine epsilon-amino groups, significantly affect the pKa of a functional cysteine, thereby enhancing the rates of thiol-disulfide reactions at physiological pH (352-354). Alternatively, the local microenvironment of a cysteine, especially the tertiary structure within a properly folded protein, may also contribute to maintain the active site thiol in the reactive thiolate form (355, 356). Taken together, the accessibility and chemical reactivity of a cysteine determine that cysteine oxidation can be a specific, targeted event that affects certain cysteine residues in preference to others. Therefore, cysteine oxidation/modification states are capable of playing physiologically relevant roles in cell signaling.

Several questions remain regarding the role of cysteine oxidation in the control of signal transduction. A current challenge is to define specific events. For example, how general events such as ROS generation are translated into specific events such as cysteine oxidation on a given protein and secondary consequences such as gene expression remain largely unsolved. Another problem is the ability to detect cysteine oxidation in response to physiologically relevant stimuli. The transient and reversible modification of cysteines, as well as the limitation of current available technology, makes the mechanistic dissection of thiol-dependent signal transduction tremendously difficult. Similar to protein phosphorylation, cysteine oxidation can alter protein secondary or tertiary

structure by covalent changes, as well as affect protein charges. Therefore, elucidating the targets of thiol oxidation and the enzymes responsible for these modifications will facilitate understanding of this important regulatory mechanism.

### *HSF1 as a therapeutic target of small molecules in human diseases*

Protein misfolding is associated with many neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), prion diseases and other devastating diseases (183, 357). Recently, many studies have shown that increasing the expression levels of chaperone proteins can suppress the neurotoxicity of misfolded proteins. For example, expression of constitutively active HSF1 in a tissue model and a mouse model of Huntington's disease resulted in reduced polyglutamine protein aggregation (358). Moreover, *hsf1*<sup>-/-</sup> mice with Rocky Mountain Laboratory (RML) prions displayed a shorter lifespan compared with wild-type mice (359). Therefore, small molecules that are capable of modulating HSF1 transcription in human cells would provide a promising avenue for therapeutic intervention in human diseases relevant to protein misfolding. Previous screens in mammalian cells have identified several HSF1 activators (19). However, these screens often result in identification of compounds that induce the HSR through accumulation of unfolded proteins or through the inhibition of Hsp90 functions via competition with ATP (223, 360). In this work, I described that diverse thiol-reactive compounds can modulate Hsf1 activity by thiol-modification on Hsp70, a central chaperone involved in protein folding. My results are consistent with the recent demonstration that compounds with thiol-reactive moieties are highly represented in high-throughput screens of Hsf1



regulators, suggesting that such small molecules may serve as attractive leads for derivatization and clinical evaluation (361).

An obvious challenge of using thiol-reactive compounds as Hsf1 modulators will be to minimize the cross-reactivity of these compounds with other cellular proteins. This task may be aided by rational drug design approaches that can further refine their specificity. In addition, recent studies revealed that the hormetic dose-response model in toxicology and pharmacology has the potential to significantly change importance aspects of drug development (362). Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (363). My results suggest that although high doses of thiol-reactive compounds to some extent result in proteotoxicity, low doses of these compounds selectively modify HSF1-repressing chaperones, such as Hsp70 and Hsp90, to stimulate the cytoprotection of cells by activating HSF1.

## CONCLUDING REMARKS

The heat shock response (HSR) is an ancient and conserved transcriptional program that leads to the immediate induction of a battery of cytoprotective genes, including heat shock proteins (HSPs), also called chaperone proteins, to against acute and chronic stress conditions. In addition to its role in cytoprotection, the HSR also regulates lifespan and protects against misfolding of proteins into toxic states that contributes to a variety of neurodegenerative diseases. Therefore, over the past two decades, interest in the HSR and chaperones as potential targets for therapeutics has stimulated investigations on small molecule regulators of the heat shock transcription factor Hsf1. However, the detailed mechanisms whereby individual small molecules are initially sensed to drive the induction of HSR and whether multiple molecules disrupt on different regulatory steps in Hsf1 activation still remain unclear.

In my dissertation work, I reported that consistent with previous findings, various thiol-reactive compounds activated Hsf1 and inhibited Hsp90 in a reciprocal manner. In Chapter 3, I demonstrated that the biological effects of these compounds required thiol-reactivity, suggesting a common chemical mechanism. Previous screen utilizing Hsf1-dependent reporters to determine Hsf1 activators often result in the identification of compounds that induce the HSR through alteration of protein homeostasis, leading to accumulation of misfolded proteins (170, 209). However, I showed in this work that thiol-reactive compounds I determined did not result in misfolding of cytosolic proteins. Nor did they disrupt the clearance machinery, the ubiquitin-proteasome system, suggesting that these thiol-reactive compounds may activate Hsf1 under a novel mechanism. Indeed, in Chapter 4, I demonstrated that these compounds specifically

modified the yeast cytosolic Hsp70 Ssa1. I provided both genetic and biochemical evidence to show that thiol-modifications of C264 and C303 of Ssa1 resulted in inactivation of Ssa1 and subsequently derepressed Hsf1. Additionally, I specifically demonstrated that between C264 and C303, C303 was more reactive and probably required to facilitate further modification on C264. Moreover, I showed that Ssa1 was only required to sense thiol-reactive compounds. Cells with mutated Ssa1 rendered immunity to thiol-reactive compounds, but responded normally to heat shock, suggesting that Ssa1 can discriminate two different environmental stresses. In Chapter 5, I generated a FLAG-Hsf1 fusion to detect interactions between Hsf1 and chaperones. Since Hsp70/Hsp90-Hsf1 complexes have never been observed in yeast and are very difficult to isolate in mammalian cells, my fusion proteins may help elucidate the detailed mechanisms through which chaperones regulate Hsf1 activities under distinct stress conditions. Taken together, my work demonstrates that disparate compounds that share the biochemical property of thiol-reactivity can potently activate Hsf1 by targeting specific cysteine residues within the general cytosolic Hsp70 family. This novel mechanism of Hsf1 activation by small molecules provides further evidence to support the derepression/activation model of Hsf1 regulation, and new insights of how Hsf1 responds differently to distinct environmental stressors. Finally, my work can provide a template for drug design of human diseases relevant to protein misfolding.

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## **Vita**

Yanyu Wang, 王雁宇, was born in Shanghai, China on June 3, 1982 the daughter of Shaoping Wang, 王绍平, and Yaping Lang, 郎亚屏. After graduating from Shixi High School in Shanghai, China in 2000, she enrolled at Shanghai Jiaotong University. She earned her Bachelor of Science in Environmental Engineering with a minor in Landscape Design from Shanghai Jiaotong University in July 2004. She worked as a Research Assistant in Shanghai Research Institution of Chemical Industry from September 2004 to December 2004. In January of 2005, she enrolled at the University of Houston, Texas. She earned her Master of Science in Civil and Environmental Engineering from the University of Houston in June 2006. In August of 2006 she entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In the summer of 2007, she joined the laboratory of Dr. Kevin A. Morano.