The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds

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ABSTRACT **The heat shock transcription factor HSF1 governs the response to heat shock, oxidative stresses, and xenobiotics through unknown mechanisms. We demonstrate that diverse thiol-reactive molecules potently activate budding yeast Hsf1. Hsf1 activation by thiol-reactive compounds is not consistent with the stresses of misfolding of cytoplasmic proteins or cytotoxicity. Instead, we demonstrate that the Hsp70 chaperone Ssa1, which represses Hsf1 in the absence of stress, is hypersensitive to modification by a thiol-reactive probe. Strikingly, mutation of two conserved cysteine residues to serine in Ssa1 rendered cells insensitive to Hsf1 activation and subsequently induced thermotolerance by thiol-reactive compounds, but not by heat shock. Conversely, substitution with the sulfinic acid mimic aspartic acid resulted in constitutive Hsf1 activation. Cysteine 303, located within the nucleotide-binding domain, was found to be modified in vivo by a model organic electrophile, demonstrating that Ssa1 is a direct target for thiol-reactive molecules through adduct formation. These findings demonstrate that Hsp70 is a proximal sensor for Hsf1-mediated cytoprotection and can discriminate between two distinct environmental stressors.**

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INTRODUCTION

Cells elaborate dedicated response systems to combat environmental and physiological noxious stimuli. The heat shock response (HSR) is an ancient and conserved transcriptional program that results in the immediate induction of a battery of cytoprotective genes, including protein chaperones, also called heat shock proteins (HSPs), to protect against and repair damage to the cellular proteome (Morimoto, 2008). In all eukaryotes, the HSR is primarily mediated by HSF1, a member of the heat shock transcription factor family that binds to heat shock elements (HSEs) in the promoters of target genes. On exposure to diverse stress conditions, monomeric HSF1 undergoes a multistep activation process that includes trimerization, phosphorylation, localization to the nucleus, and DNA binding in mammalian cells (Akerfelt *et al.*, 2010). The budding yeast homologue Hsf1 is constitutively trimerized, nuclear, and associated with high-affinity HSEs under normal conditions and additional HSEs during stress (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988; Hahn *et al.*, 2004). These properties suggest multiple mechanisms exist to control heat shock factor activity in the absence of stress.

Genetic and biochemical evidence supports a model whereby at least two classes of HSPs serve an autoregulatory role in HSF1 regulation (Craig and Gross, 1991). Deletion of *SSA1* and *SSA2*, the two constitutively expressed cytosolic Hsp70 isoforms in *Saccharomyces cerevisiae*, derepresses Hsf1 transcriptional activity, as does deletion of the Hsp70 nucleotide exchange factor *SSE1* (Craig and Jacobsen, 1984; Liu *et al.*, 1999). Hsp70 also stably associates in vivo and in

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Abbreviations used: 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; 4HNE, 4-hydroxynonenal; AZC, azetidine 2-carboxylic acid; BMCC, (1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]-carboxamido)butane); DEM, diethyl maleate; diamide, 1,1′-azobis[*N*,*N*-dimethylformamide]; DTT, dithiothreitol; GPD, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; HSE, heat shock element; HSF1, heat shock transcription factor 1; HSP, heat shock protein; HSR, heat shock response; IgG, immunoglobulin G; ORF, open reading frame; SC, synthetic complete; TAP, tandem affinity purification.

vitro with the transactivation domain of human HSF1 (Abravaya *et al.*, 1992; Baler *et al.*, 1992). However, Hsp70 alone is not sufficient to suppress HSF1 in mammalian cells. Hsp70 operates within the Hsp90 chaperone system, which is responsible for maturation and regulation of a wide range of cellular clients (Taipale *et al.*, 2010). Hsp90 partners with a number of cochaperones, including Hsp70, Hop, cyclophilins, and p23, at different stages in client-specific folding cycles. An Hsp90-Hsf1 complex can be detected in unstressed human cells using protein cross-linking approaches, and this complex dissociates upon thermal stress (Zou *et al.*, 1998). Yeast harboring mutations in Hsp90 or a subset of its cochaperones, including the cyclophilin *CPR7*, exhibit high levels of Hsf1 activity (Duina *et al.*, 1998; Harris *et al.*, 2001). The Hsp70/Hsp90 chaperone machines therefore play a major role in regulating HSF1, repressing activation through binding/sequestration under normal growth conditions and promoting transcriptional competence through complex dissociation during stress.

Proper function of the HSR is relevant to many human disorders, including cancer and neurodegenerative disease (Dai *et al.*, 2007; Morimoto, 2008). Accordingly, numerous efforts have been made to identify and characterize Hsf1 modulators. These include molecules of known function, such as the Hsp90-specific inhibitors geldanamycin and radicicol (Winklhofer *et al.*, 2001; Piper *et al.*, 2003; Griffin *et al.*, 2004), as well as the novel compounds HSF1A and celastrol (Westerheide *et al.*, 2004; Neef *et al.*, 2010). Celastrol, an active component of a traditional Chinese medicinal herb, promotes HSP gene expression in mammalian cells by inducing HSF1 DNA binding and hyperphosphorylation (Westerheide *et al.*, 2004). It also blocks maturation of androgen receptor by inhibiting Hsp90 activity in a manner distinct from geldanamycin (Hieronymus *et al.*, 2006). Celastrol has the same biological effects in budding yeast: activation of the HSR through Hsf1 and inhibition of Hsp90-mediated maturation of the glucocorticoid receptor, indicating that its mechanism of action is fundamentally conserved (Trott *et al.*, 2008). Both activities are prevented by incubation with excess reduced thiol, and celastrol induces the yeast oxidant-defense regulon controlled by Yap1, suggesting that celastrol may induce Hsf1 through a thiol-modification mechanism (Trott *et al.*, 2008). Interestingly, a number of molecules previously shown to induce the HSR are also thiol-reactive. For example, the thiol chelator cadmium has long been known to induce HSP expression (Mosser *et al.*, 1988). In vitro studies in human and rat cultured cells have shown that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which contains an electrophilic α,β-unsaturated ketone group, can activate HSF1 and induce the synthesis of Hsp70 (Amici *et al.*, 1992). Indeed, a recent high-throughput screen for HSF1 activators revealed a strong bias for thiol reactivity in the most promising molecules (Santagata *et al.*, 2011). In the absence of a relevant target molecule, the precise mechanism through which diverse thiol-reactive compounds induce Hsf1 therefore remains unclear.

Here we report that thiol-reactive compounds, including oxidants and organic electrophiles, activate Hsf1 in the yeast *S. cerevisiae* in a manner distinct from activation by protein misfolding caused by an amino acid analogue. A subset of HSPs are genetically implicated in Hsf1 repression and contain cysteine residues; of these, only Ssa1 was labeled with a thiol-reactive biotin-labeled probe in cell extracts, suggesting that this Hsp70 chaperone possesses highly reactive cysteines. Strikingly, substitution of C264 or C303 of Ssa1 renders cells unresponsive to Hsf1 activation and unable to acquire thermotolerance by thiol-reactive compounds but does not affect activation by heat shock. Moreover, substitution with aspartic acid, which adds steric bulk and mimics the oxidized sulfinic acid form of the cysteine thiol, resulted in Hsf1 derepression in the absence of exogenous stress. C303 is shown to be directly modified in vivo by the organic electrophile 4-hydroxynonenal using a Click chemistry approach. The Hsp70 chaperone Ssa1 therefore functions as a direct sensor for Hsf1 activation by diverse thiol-reactive compounds through reactive cysteine residues. Moreover, these results establish that this sensing mechanism is functionally distinct from the ability to respond to thermal stress.

RESULTS

Thiol-reactive compounds activate Hsf1

We previously demonstrated that celastrol is a potent activator of the Hsf1-mediated HSR in yeast, as it is in human cell lines (Trott *et al.*, 2008). The biological effects of celastrol are consistent with the putative identification of at least two electrophilic centers in the molecule that are capable of reacting with cellular nucleophiles, such as the thiol group in protein cysteines (Yang *et al.*, 2006). To test whether Hsf1 is broadly responsive to thiol-reactive compounds, we evaluated activation by a selection of compounds utilizing an HSE-*lacZ* reporter system. This transcriptional fusion faithfully reports induction of Hsf1 (Duina *et al.*, 1998; Morano and Thiele, 1999). The metalloid thiol chelator cadmium, the thiol oxidants 1,1′-azobis[*N*,*N*-dimethylformamide] (diamide) and hydrogen peroxide (H_2O_2), and the organic electrophiles diethyl maleate (DEM) and 15d-PGJ_2 all activated Hsf1 between four- to 10-fold (Figure 1A). The same molecules produced an SDS–PAGE mobility shift consistent with heterogenously phosphorylated Hsf1 observed upon heat shock, providing independent verification of Hsf1 activation (Figure 1B; Sorger and Pelham, 1988). To verify that thiol reactivity was required for the observed biological effects, we reacted diamide or 15d-PGJ_2 with different concentrations of the reducing agent dithiothreitol (DTT) for 15 min in vitro prior to treating cells carrying the HSE-*lacZ* reporter (Figure 1C). We observed that fivefold excess or greater DTT completely abolished Hsf1 activation by both compounds. These results are consistent with our previous finding that the biological effects of celastrol are inhibited by DTT and raise the possibility that disparate thiol-reactive compounds may activate Hsf1 via a common mechanism (Trott *et al.*, 2008).

Thiol-reactive compounds do not induce the HSR by causing accumulation of unfolded proteins

Hsf1 is thought to be activated in response to heat shock through the accumulation of misfolded cellular proteins that titrate Hsp70 and Hsp90 chaperones, thereby releasing repression of the transcription factor. We therefore sought to understand whether two of the thiol-reactive compounds we identified as Hsf1 activators, cadmium and diamide, cause misfolding of cytosolic proteins by comparing their Hsf1 induction profiles with those of a well-described unfolding agent. Newly synthesized polypeptides are highly susceptible to misfolding due to environmental stress. The proline analogue azetidine 2-carboxylic acid (AZC) is incorporated into nascent chains, in which it leads to misfolding and ubiquitination of newly synthesized proteins (Trotter *et al.*, 2002; Mandal *et al.*, 2010). AZC treatment was found to inhibit ß-galactosidase activity; we therefore prepared protein extracts from treated cells and quantitated the levels of the stress-induced Hsp70 Ssa3 via immunoblot over time (Figure 2A). The activation patterns were clearly distinct, with the thiol-reactive compounds displaying a characteristic Hsf1 induction profile of rapidly increased HSP production (within 30–60 min), which was followed by attenuation to a level lower than the maximum but higher than before treatment (Slater and Craig, 1987; Liu and Thiele, 1996). In contrast, Hsf1 induction by AZC was persistent over the 5-h time course. These data suggest that Hsf1 activation caused by cadmium

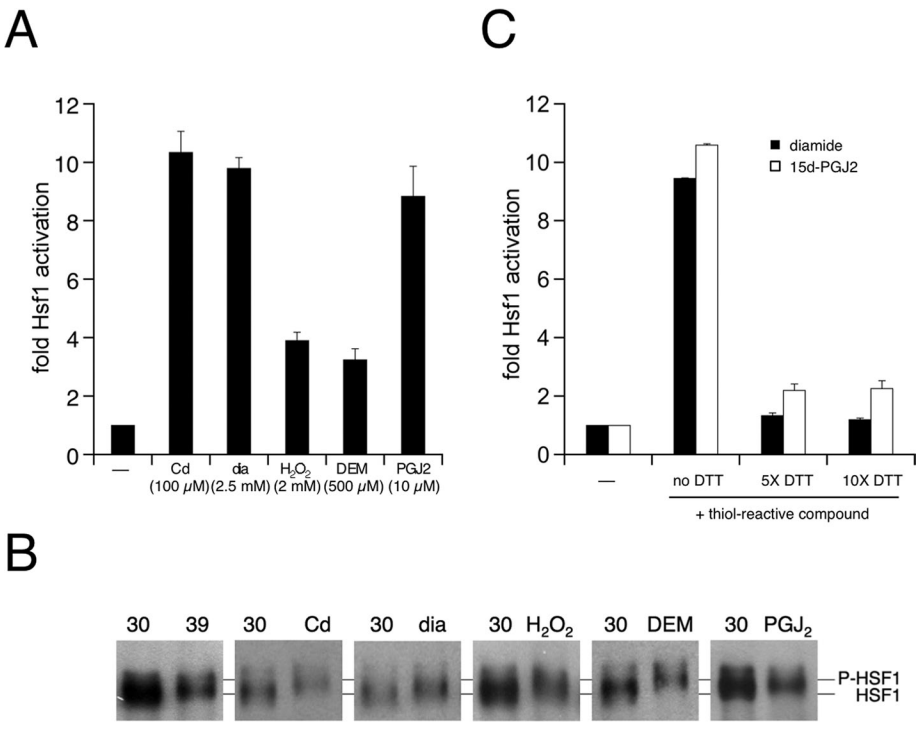

FIGURE 1: Thiol-reactive compounds activate Hsf1. (A) Wild-type (BY4741) cells carrying the HSE-*lacZ* reporter were treated with different concentrations of cadmium sulfate (Cd), diamide (dia), H_2O_2, DEM, or 15d-PGJ_2 at the indicated concentrations and induction-normalized to activity from untreated cells (30°C). (B) Thiol-reactive compounds induce hyperphosphorylation of Hsf1. BY4741 cells carrying an integrated functional TAP-tagged *HSF1* were grown to midlog phase and exposed to the indicated compounds as in (A). Protein extracts were analyzed by 6% SDS–PAGE and immunoblotting using antibodies directed against the protein A epitope. (C) Quenching of diamide and 15d-PGJ_2 activation of Hsf1 by DTT. HSE-lacZ activity was measured after addition of diamide (2.5 mM, solid bar) or 15d-PGJ2 (5.6 μM, open bar) in the presence of 5× or 10× excess dithiothreitol (DTT) or water alone for 15 min prior to cell treatment. Hsf1 activity is reported as in (A).

and diamide is transient in nature, while activation due to AZC treatment is cumulative, likely from continued production and accumulation of high levels of misfolded proteins. Consistent with this model, we observed that cells treated with cadmium or diamide for 2 h or heat-shocked for 1 h (37°C) remained viable, with little to no loss in survival relative to an untreated control culture (30°C), whereas AZC treatment resulted in near-total loss in viability (Figure 2B).

To directly examine whether treatment with thiol-reactive compounds caused protein aggregation, we fractionated lysates prepared from treated cells by ultracentrifugation. Whereas heat-shocked cells accumulated significant amounts of protein in the high-speed pellet fraction, control cells and those treated with thiol-reactive compounds (cadmium or DEM) maintained solubility of the majority of proteins, suggesting that treatment with these chemicals caused little to no effect on protein folding and stability (Figure 2C). Taken together, the features of Hsf1 activation by thiol-reactive compounds are not consistent with a model relying on the generation and accumulation of misfolded cytosolic proteins and instead suggest an alternate sensing mechanism.

Ssa1 is hypersensitive to thiol modification

Because incubation of thiol-reactive compounds with excess thiol (e.g., DTT) completely abolished their effects on Hsf1

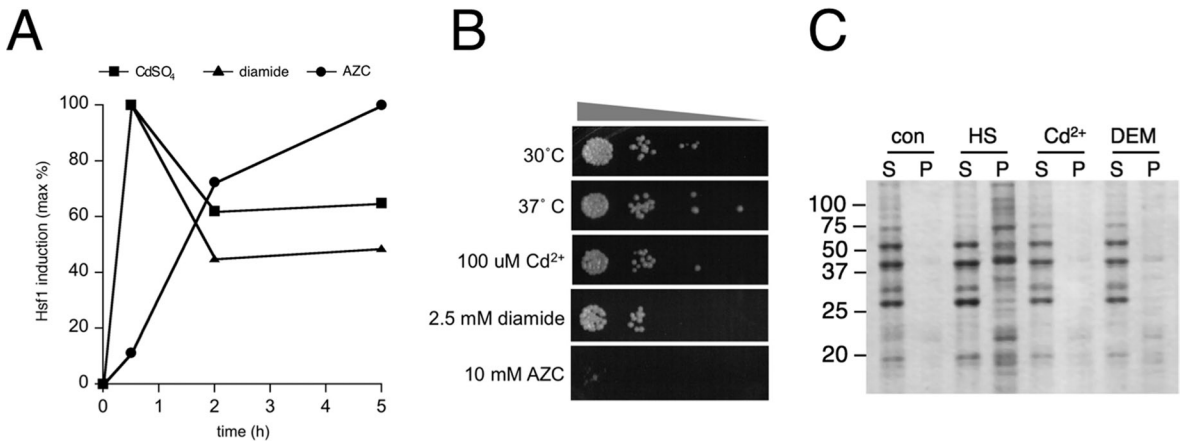

FIGURE 2: Thiol-reactive compounds do not induce the HSR by causing accumulation of unfolded proteins. (A) Hsf1 activity profiles after treatment with thiol-reactive compounds or the proline analogue AZC are distinct. Quantitation of Ssa3/4 protein levels by immunoblot analysis after cells (BY4741) were treated with 100 μM $CdSO_4$ (■), 2.5 mM diamide (▶), or 10 mM AZC (●). Band intensities were normalized to glucose 6-phosphate dehydrogenase as a loading control and are plotted relative to cells treated for 5 h as percentage of maximum HSR induction. (B) AZC, but not thiol-reactive compounds, are toxic at Hsf1-activating doses. Logarithmic-phase cells (BY4741) were treated with no reagent (30°C), heat-shocked for 1 h at 37°C, or treated for 2 h with 100 μM Cd^{2+}, 2.5 mM diamide, or 10 mM AZC. Cells were plated in a 10-fold dilution series on YPD media and grown at 30°C for 2 d. (C) Thiol-reactive compounds do not cause protein aggregation at Hsf1-activating doses. Cells treated with the indicated compounds at the same concentrations as in Figure 1 or heat-shocked were lysed, and proteins were fractionated by ultracentrifugation. A Coomassie-stained SDS–PAGE gel is shown.

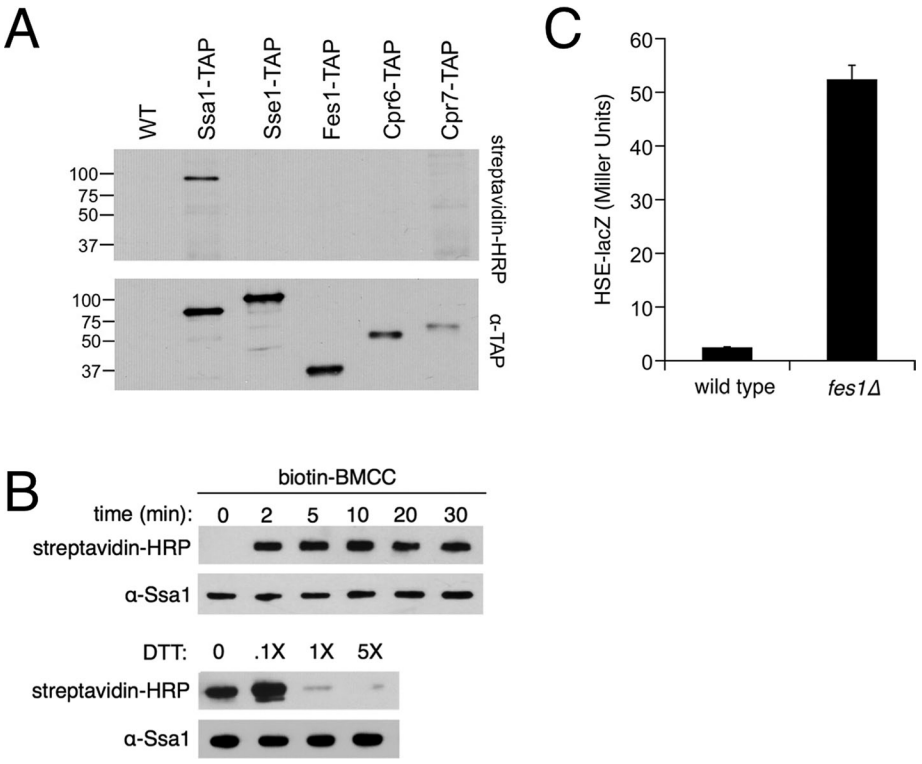

FIGURE 3: Ssa1 is a biologically relevant target of thiol-reactive compounds. (A) An Ssa1-TAP fusion is hypersensitive to modification in vitro by a thiol-reactive biotin probe. The indicated chaperone-TAP fusion proteins were enriched by IgG affinity-purification and were treated with biotin-BMCC. (B) Ssa1 is rapidly alkylated by biotin-BMCC in a thiol-dependent manner. Purified Ssa1 was incubated with biotin-BMCC for the indicated times; this was followed by immunoblotting with streptavidin-HRP or anti-Ssa1 (top). Biotin-BMCC was premixed with the indicated concentration ratios of DTT for 15 min 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 *fes1*Δ (BY4741) cells carrying the HSE-*lacZ* reporter were grown at 30°C to midlog phase, and β-galactosidase activity was determined and plotted as absolute Miller units.

activation, we reasoned that the mechanism by which thiol-reactive compounds induce the HSR may involve inactivation of specific regulatory protein(s) through targeting of cysteine residues. In both yeast and metazoans, HSPs and cochaperones organize into multichaperone complexes and regulate HSF1 activity at different stages (Shi *et al.*, 1998). We and others have shown that the electrophile celastrol functionally inactivates Hsp90-dependent signal transduction in yeast and mammalian cells (Hieronymus *et al.*, 2006; Trott *et al.*, 2008). 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 molecules.

Analysis of amino acid sequences obtained from the *Saccharomyces* Genome Database revealed that both yeast Hsp90 proteins (Hsc82 and Hsp82) lack cysteine residues; however, several Hsp90 cochaperones possess one or more cysteines (Cherry *et al.*, 1997). All four Ssa cytosolic Hsp70 chaperones contain three cysteine residues located within the nucleotide-binding/ATPase domain (Slater and Craig, 1987). The nucleotide exchange factors Sse1 and Fes1 contain five and two cysteines, respectively (Kabani *et al.*, 2002; Dragovic *et al.*, 2006; Raviol *et al.*, 2006; Shaner *et al.*, 2006). Cpr6 and Cpr7 are Hsp90-associated peptidyl-prolyl isomerases and each contains seven cysteines (Duina *et al.*, 1996). Additionally, previous studies have shown that mutation of Ssa1/2, Sse1, or Cpr7 results in Hsf1 activation in the absence of thermal stress (Craig and Jacobsen, 1984; Duina *et al.*, 1998; Liu *et al.*, 1999). To address whether one or more of these proteins might be sensitive to modification in vitro, we reacted the cysteine biotinylation reagent 1-biotinamido-4-(4′-[maleimidomethyl-cyclohexane]-carboxamido)butane (biotin-BMCC) with tandem affinity purification (TAP)-tagged chaperone proteins enriched from cell extracts using immunoglobulin G (IgG)-Sepharose (Figure 3A). All five fusions were expressed and detectable using anti–protein A antiserum. In contrast, only Ssa1 appeared to be modified by biotin-BMCC as detected by a streptavidin–horseradish peroxidase (HRP) conjugate. This result was confirmed by treating purified Ssa1 protein with the same concentration of biotin-BMCC and demonstrating reaction with Ssa1 and not the TAP tag (Figure 3B, top). For verifying that Ssa1 was labeled via thiol modification, biotin-BMCC and Ssa1 were mixed with the indicated ratios of DTT. Labeling of purified Ssa1 by BMCC was completely abolished by equimolar or greater free thiol (Figure 3B, bottom). Finally, given the identification of Ssa1 as a potential target for thiol-reactive compounds and the previous finding that the Ssa1 exchange factor Sse1 is required for Hsf1 repression, we tested whether another cytosolic exchange factor, Fes1, acted as an Hsf1 repressor in vivo (Liu *et al.*, 1999). Indeed, Hsf1 exhibited substantial derepression in a *fes1*Δ strain (Figure 3C). Together these results suggest that of the Hsf1-repressing chaperones and cochaperones tested, Ssa1 alone is hypersensitive to thiol modification, and based on genetic evidence from deletion of Ssa1 nucleotide exchange factors, is strongly implicated as a relevant target of Hsf1-activating thiol-reactive compounds in yeast.

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

The in vitro labeling experiments demonstrated that of the proteins comprising the Hsp70/Hsp90 multichaperone complex we tested, Ssa1 is specifically modified by a thiol-reactive probe. However, these results do not establish that modification of Ssa1 on one or more cysteine residues by the various thiol-reactive compounds we have used is directly responsible for Hsf1 activation. We reasoned that if that were indeed the case, then replacement of the cysteines in Ssa1 with nonreactive residues would render Hsf1 immune to activation by the same compounds. We constructed an experimental system to address this question by placing the *SSA1* open reading frame (ORF) under control of the heterologous *transcription elongation factor 1 alpha* (*TEF*) promoter, expressed in a strain background lacking both *SSA1* and *SSA2*, the constitutively expressed Hsp70 isoforms. *SSA1* and *SSA2* are functionally redundant for Hsf1 repression, necessitating deletion of both alleles (Supplemental Figure S1). The expression system produced Ssa1 at levels approximating wild-type that also complemented the *ssa1*Δ *ssa2*Δ slow-growth and Hsf1 repression defects (Figure S2; Craig and Jacobsen, 1984). Interestingly, we noted that high levels of SSA1 expression from

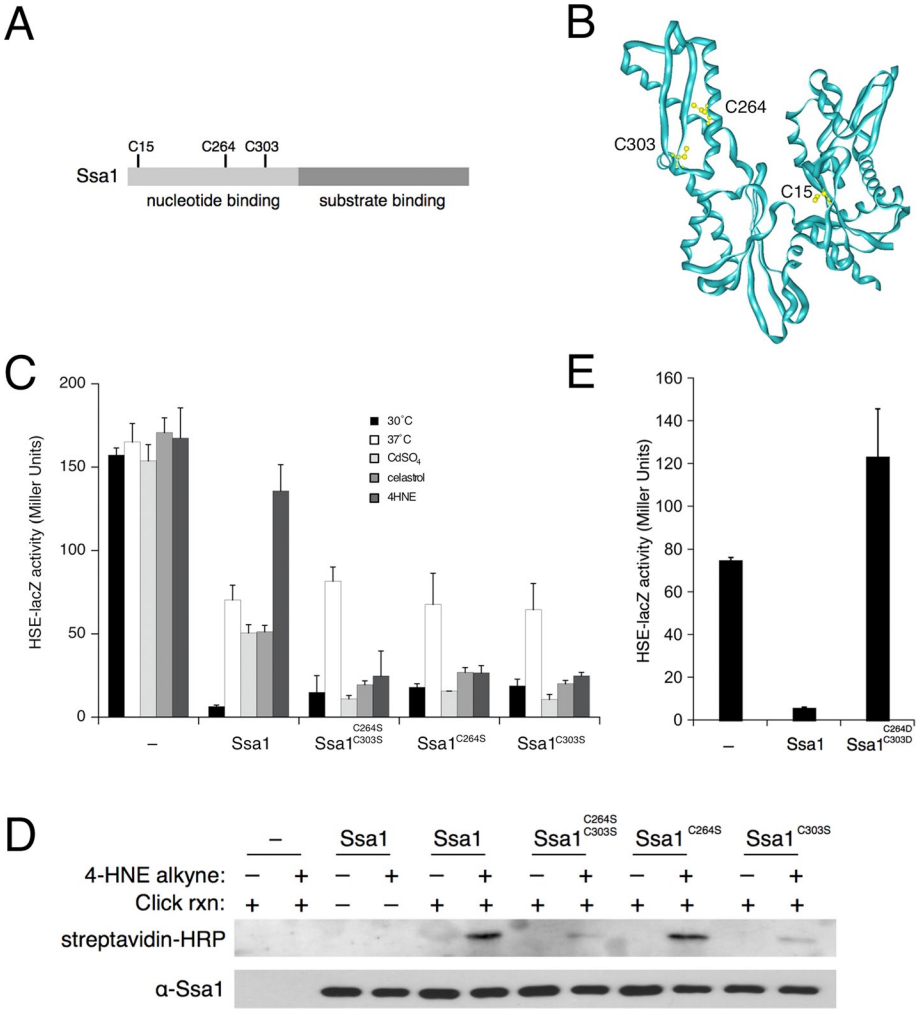

FIGURE 4: Ssa1 is a sensor for activation of Hsf1 by thiol-reactive compounds. (A) Diagram depicting the domain architecture of Ssa1 and positions of the three cysteine residues. (B) The relevant cysteines are mapped in the human Hsc70 nucleotide binding domain (NBD) generated from crystal structure (3D2F). (C) Ssa1 cysteines are required for Hsf1 activation by thiol-reactive compounds but not heat shock. Strains containing wild-type and mutated *SSA1* alleles were grown at the control temperature (30°C; black bar); heat-shocked (37°C; white bar) for 1 h; or exposed to 600 μM $CdSO_4$ (light gray bar), 30 μM celastrol (medium gray bar), or 400 μM 4-HNE (dark gray bar) for 2 h; this was followed by determination of β-galactosidase activity plotted in absolute Miller units. (–), empty vector control. (D) Ssa1 cysteines are directly modified by a thiol-reactive compound. The same strains as in (C) were treated with 4HNE-alkyne and detected via Click chemistry as described in *Materials and Methods*. (E) The cysteine oxidation mimic aspartic acid results in Ssa1 inactivation and 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.

the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter blocked Hsf1 activation by heat shock or thiol-reactive compounds (Figure S2), consistent with this chaperone acting as an Hsf1 repressor. We first attempted to replace all three cysteines (C15, C264, and C303; Figure 4, A and B) with serine, however the C15S substitution was found to cause instability of Ssa1 and loss of function (Figure S3). We therefore constructed mutant *SSA1* alleles with C264S, C303S, or both mutations and found that all three permutations were stable and complemented the *ssa1*Δ *ssa2*Δ slow-growth phenotype (Figure S3). To determine whether mutation of Ssa1 cysteine residues resulted in loss of Hsf1 response to thiol-reactive compounds, we assessed HSE-*lacZ* activity in the mutant cells in response to heat shock, cadmium, celastrol, and the organic electrophile 4-hydroxynonenal (4HNE; Figure 4C). Control cells carrying an empty vector exhibited derepression of Hsf1, while reintroduction of wild-type *SSA1* restored repression at 30°C and heat inducibility at 37°C. Strikingly, cells bearing *SSA1-C264S, C303S* also retained heat inducibility, but Hsf1 was unresponsive to all three thiol-reactive compounds. To distinguish which of the two cysteine residues was responsible for sensing these molecules, we analyzed *SSA1* single mutants. Interestingly, both *SSA1-C264S* and *SSA1-C303S* recapitulated the responses of the double mutant. These results provide genetic evidence that cells require both C264 and C303 of Ssa1 to sense thiol-reactive compounds.

We envisioned two models to explain the role of Ssa1 in transducing the thiol-reactive signal to Hsf1. In the first model, diverse thiol-reactive compounds could deplete cellular redox buffers, such as the thioredoxin and glutathione pathways, which might then fail to maintain Ssa1 in a reduced state. Alternatively, the Ssa1 cysteines could be modified directly, either via oxidation or by the formation of stable adducts with thiol-reactive molecules. Although not mutually exclusive, we sought to distinguish between these two possibilities using Click chemistry to examine thiol-modification of Ssa1 in vivo. This approach used a membrane-permeable alkyne derivative of the 4HNE molecule that was a potent Hsf1 activator (4HNE-alkyne; Figure 4C), as well as FLAG-tagged *SSA1* alleles that could be used for affinity-purification. Cells were treated or not with 4-HNE-alkyne; this was followed by quenching, lysis, and purification of FLAG-Ssa1 proteins. Samples were subsequently reacted with biotin azide in the Click reaction, which was followed by SDS–PAGE and immunoblot using streptavidin-HRP. As shown in Figure 4D, wild-type Ssa1 was modified via the Click reaction, whereas the labeling signal was absent in cells lacking FLAG-Ssa1 or in which the Click reaction was omitted. Labeling was significantly decreased in cells bearing *SSA1-C264S,C303S*, demonstrating that one or both of these cysteines is a direct target of electrophiles in vivo. It is possible that the minor amount of labeling observed in *SSA1-C264S,C303S* may be due to reaction with C15. Interestingly, *SSA1* single mutants demonstrated slightly different outcomes: the labeling signal was robust in *SSA1-C264S*, but was decreased in *SSA1-C303S*, suggesting that C303 may be highly reactive relative to C264 and that C303 may need to be modified before C264 is exposed. Regardless, these data demonstrate that Ssa1 is directly modified by a thiol-reactive compound in living cells in the same time frame in which we observe induction of Hsf1.

Modification of a cysteine residue with a small electrophile like DEM would be expected to add steric bulk that may result in structural and functional perturbations. Likewise, cysteine can be oxidized in several steps by oxidants such as H_2O_2. We sought to

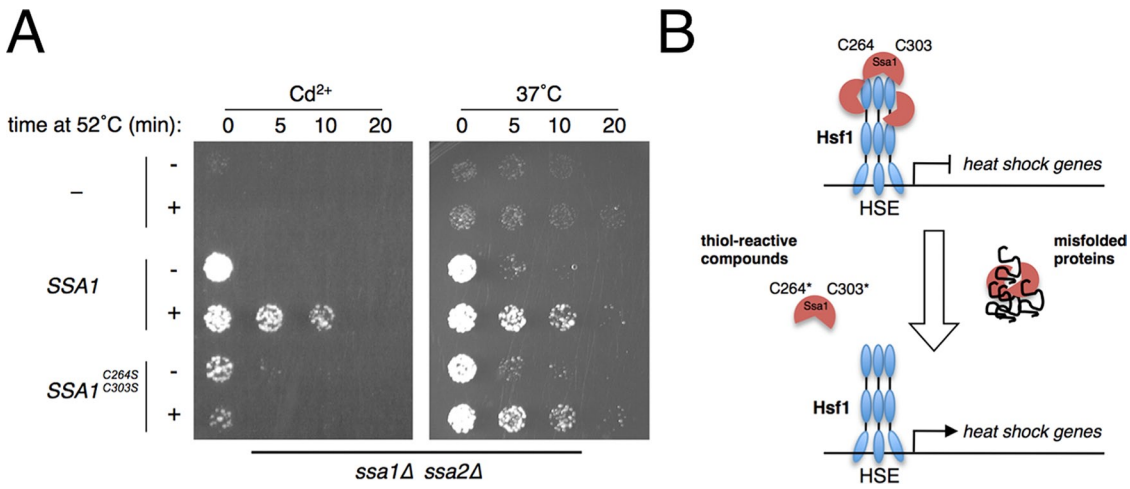

FIGURE 5: Ssa1 cysteines are required for activation of the cytoprotective HSR by thiol-reactive compounds but not by heat. (A) Strains described in Figure 4 were treated with either no reagent (–) or 600 μM Cd^{2+} (+) (left) or were grown at 30°C (–) or 37°C (+) for 1 h (right) before heat shock at 52°C for the indicated times. (B) Model demonstrating independent activation of Hsf1 by misfolded proteins or thiol-reactive compounds in an Ssa1-dependent manner.

model these effects by substituting C264 and C303 with the pseudooxidation mimic aspartic acid, which most closely resembles the sulfinic acid form of cysteine. Whereas wild-type *SSA1* complemented the Hsf1 derepression phenotype of *ssa1Δ ssa2Δ* cells, *SSA1-C264D,C303D,* which produced a stable but nonfunctional Ssa1 protein (Figure S4) failed to do so, phenocopying the null mutant (Figure 4E). These results suggest that modification of C264 and C303 result in functional inactivation of Ssa1 in the context of its role as an Hsf1 repressor.

We have shown previously that Hsf1 activation by celastrol results in increased thermotolerance (Trott *et al.*, 2008). To confirm that sensing of thiol-reactive compounds by Ssa1 is physiologically relevant for cell survival, we tested the *SSA1* mutants for acquired thermotolerance using cadmium as the thiol-reactive stress. Cells were pretreated or not with cadmium and subsequently exposed to a lethal 52°C heat shock for varying lengths of time (Figure 5A). Wild-type *SSA1* cells showed increased thermotolerance only with cadmium pretreatment, tolerating up to 10 min of severe heat shock. However, cells expressing *SSA1-C264S, C303S* failed to acquire thermotolerance, exhibiting equivalent low levels of viability irrelevant of cadmium pretreatment. Cells transformed with the empty vector exhibited the characteristic slow-growth phenotype of *ssa1Δ ssa2Δ* mutants and did not recover within the time course of the experiment. However, *ssa1Δssa2Δ* cells are constitutively thermotolerant upon extended incubation, consistent with chronic Hsf1 derepression (Craig and Jacobsen, 1984). To confirm that *SSA1-C264S, C303S* retained heat-inducible thermotolerance, we subjected the same strains to mild heat shock prior to the lethal heat stress. Consistent with the reporter assay, cells bearing *SSA1-C264S, C303S* gained thermotolerance in a manner indistinguishable from wild-type. Together these genetic and biochemical results support the model that thiol-reactive compounds target C264 and C303 of Ssa1, leading to Hsf1 activation, and that this process is distinct from sensing of thermal stress.

DISCUSSION

We determined previously that celastrol, a natural component of Chinese medicine, can induce the heat shock and antioxidant responses in yeast and human cells, leading us to propose that celastrol may cause these biological effects by thiol modification of cysteine residues (Trott *et al.*, 2008). In this study, we have demonstrated that in addition to celastrol, disparate compounds that share the biochemical property of thiol-reactivity can potently activate Hsf1. Our findings suggest that in the time frame of Hsf1 activation, thiol-reactive compounds do not cause significant aggregation of unfolded proteins. Instead, we propose that specific cysteine residues within the general cytosolic Hsp70 family, represented by Ssa1, are modified leading to transient inactivation of the chaperone and derepression of Hsf1 (Figure 5B).

The nucleophilic character of protein cysteines is strongly influenced by both surface exposure, which dictates accessibility to exogenous compounds, and the pKa of the thiol functional group, largely controlled by neighboring side chains in the local microenvironment of the residue (Li *et al.*, 1993; Srinivasan *et al.*, 1997). Mammalian Hsp90 and Hsp70 are both reported to be exquisitely sensitive to modification by low concentrations (~10 μM) of 4-hydroxynonenal, an electrophilic byproduct of lipid peroxidation (Carbone *et al.*, 2004, 2005). Intriguingly, this concentration range is consistent with the effective doses of celastrol and 15d-PGJ_2 we observed. All three compounds contain an α,β-unsaturated carbonyl moiety capable of forming a Michael adduct with the thiolate anion, suggestive of a common chemical mechanism. In vitro studies have also demonstrated that a pair of vicinal cysteines in rat Hsp90 (C589/C590), and all three cysteines in yeast Hsp70, react with the powerful alkylating agent *N*-ethyl maleimide (NEM; Liu *et al.*, 1996; Nardai *et al.*, 2000). NEM modification of Ssa1 is inversely related to nucleotide status in the ATPase domain: NEM-Ssa1 does not bind ATP, nor is ATP-Ssa1 as reactive toward NEM as apo-Ssa1 (Liu *et al.*, 1996). NEM-Ssa1 is incompetent for protein folding and competitively inhibits folding by unmodified Ssa1 (Hermawan and Chirico, 1999). These results provide biochemical support for our findings that substitution of C264 and C303 with aspartic acid caused apparent inactivation of Ssa1 as judged by complementation, resulting in constitutive Hsf1 derepression. Together these data provide a crucial functional basis for a mechanism whereby Ssa1 modified by thiol-reactive molecules in vivo not only would lose activity but may also act in a dominant negative manner to interfere with the activities of unmodified Ssa1, including Hsf1 regulation. Although Ssa1 contains three reactive cysteines, responsiveness to thiol-reactive molecules in our experiments was largely abolished in the absence of C264 and C303, suggesting that C15 plays little to no role in sensing of these molecules. Of the three cysteines, C15 is the most conserved among Hsp70 proteins. C264

in contrast is absent from most yeast Hsp70s, including heat inducible Ssa3/4, ribosome-associated Ssb1/2, ER-localized Kar2 and mitochondrial Ssc1, and C303 is absent from all but Ssa3/4 (Liu *et al.*, 1996). The Ssa1/2 chaperones are therefore uniquely poised to act as sensors for thiol-reactive molecules with respect to Hsf1 activation: they are constitutively expressed, contain unique susceptible cysteine residues sensitive to inactivation, and are absolutely required for Hsf1 repression. We observed labeling of C303 in the absence of C264, but not the converse; it is possible that C303 may be exquisitely reactive, and C264 less so, or that modification of C303 potentiates modification of C264 via subtle changes in the microenvironment of the thiol side chain. While the Ssa1 cysteines are clearly subject to direct modification and oxidation, another possible explanation for our observations is that some treatments may alter the cellular redox status, resulting in inability to maintain Ssa1 in a reduced and functional state. Indeed, the Yap1 redox-activated transcription factor in budding yeast requires the thioredoxin system to reduce cysteines oxidized via an intermolecular disulfide bridge with the Gpx3/Orp1 protein. Thioredoxin mutant strains exhibit constitutive activation of Yap1 and are defective in attenuation of the oxidant response after insult (Delaunay *et al.*, 2002). A parallel scenario can be envisioned for Ssa1 whereby glutathione- or thioredoxin-based systems serve to reduce oxidized C264 and C303, impairment of which results in derepression of Hsf1. The role of cellular redox buffering in regulating the Hsp70-Hsf1 circuit is currently under investigation.

HSF1 has long been known to respond to a diverse range of physiological and environmental stressors. Cadmium and other metalloids such as arsenic are potent activators, inducing phosphorylation, trimerization and DNA binding in yeast and mammalian cells (Mosser *et al.*, 1988; Chang *et al.*, 1989). The A- and J-type cyclopentanone prostaglandins (PGA, PGJ) are the only members of the prostaglandin family to include an α,β-unsaturated ketone group and the only ones capable of inducing Hsp70 production in mammalian cells (Rossi *et al.*, 1996). Our discovery that a human PGJ potently activates Hsf1 in yeast, which lacks any known prostaglandin receptors, in a thiol-reversible manner, is consistent with our proposed mechanism of cysteine modification. Recent work has established that mammalian HSF1 senses both heat and H_2O_2 through a pair of cysteines in the DNA-binding domain, obviating the need for additional sensors of thiol-perturbing stress (Ahn and Thiele, 2003). However, yeast Hsf1 lacks cysteine residues, implicating one or more ancillary factors to explain the observed responsiveness to thiol-reactive stressors. The most parsimonious model consistent with our results involves Hsp70 (Ssa1) and Hsp90 directly repressing Hsf1 through a physical interaction that is relieved upon modification and inactivation of Ssa1 by thiol-reactive molecules. A recent study identified Hsp90 as a target of a thiol-reactive compound capable of inducing the HSR in mammalian cells (Zhang *et al.*, 2011). While intriguing, Hsp90 cannot be considered a viable candidate for an Hsf1 sensor for thiol-reactive molecules, because both Hsp90 genes in yeast lack cysteines, and we did not observe reaction of biotin-BMCC with other Hsp90 cochaperones required for Hsf1 repression. Ssa1 therefore appears to be the sole relevant candidate to regulate Hsf1 responsiveness in budding yeast. Hsp70/Hsp90-HSF1 complexes are difficult to detect or isolate in mammalian cells and in vitro reconstitution experiments, and they have not been conclusively observed and validated in the yeast system (Voellmy, 2004). Therefore, at this time, we cannot exclude the possibility that chaperone inactivation indirectly leads to Hsf1 activation through yet another intermediate, perhaps due to an increase in misfolded substrates of Ssa1. While HSF1 clearly is subject to multiple layers of regulation, especially in metazoan cells, chaperone repression is a primordial control mechanism that has the potential to sense both heat through titration by unfolded proteins and thiol oxidants via unique reactive cysteine residues. A database search of HSF1 homologues from a range of experimental organisms revealed an absence of cysteines in *Aspergillus, Neurospora, Kluyveromyces*, and *Candida* genera, whereas *Schizosaccharomyces*, *Caenorhabditis*, *Danio*, and mammalian HSF1 genes all contain at least one cysteine residue. These findings support a model wherein Ssa homologues confer thiol responsiveness in primitive eukaryotes, while fission yeast and higher genera express HSF1 proteins that directly sense redox changes and, potentially, cytotoxic thiol-reactive xenobiotic compounds.

The realization that HSF1 is a nexus for protein homeostasis has fueled the search for small molecules capable of modulating HSF1 transcriptional activity in human cells. Such compounds would provide therapeutic control over the cytoprotective aspects of the HSR, including HSP production and its concomitant antiapoptotic effects. Compounds such as the ansamycins (geldanamycin, macbecin) and radicicol are the best-described HSF1 activators to date. These molecules inhibit Hsp90 via competition with ATP, and physiologically active derivatives are currently in clinical trials. Our results are consistent with the recent demonstration that compounds with thiol-reactive moieties are highly represented in high-throughput screens to identify HSF1 modulators and suggest that such molecules may serve as attractive leads for derivatization and clinical evaluation (Santagata *et al.*, 2011). An obvious challenge will be to minimize the cross-reactivity of these molecules with other cellular proteins. This task may be aided by the findings that multiple HSF1-repressing chaperones, including Hsp70 and Hsp90, appear to be highly sensitive to thiol modification. In addition, the fact that one or more electrophilic centers confers HSP-inhibitory properties on molecules with diverse structural architectures (celastrol, 15d-PGJ_2) suggests that rational drug design approaches can exploit steric constraints to further refine specificity.

MATERIALS AND METHODS

Yeast strains, plasmids, and procedures

Yeast strains used in this study were of the BY4741 (MATa his3Δ leu2Δ met15Δ ura3Δ) or DS10 (MAT**a** ura3-52 lys1 lys2 trp1-1 his3-11,15 leu2-3112) backgrounds. The ssa1Δ ssa2Δ strain (SL314, MAT**a** ura3-52 lys1 lys2 trp1-1 his3-11,15 leu2-3112 ssa1::HIS3, ssa2::LEU2) was kindly provided by Elizabeth Craig (University of Wisconsin, Madison, WI) and is isogenic with DS10 (Craig and Jacobsen, 1985). SSA1-TAP, SSE1-TAP, FES1-TAP, CPR6-TAP, and CPR7-TAP (TAP-tagged) strains were purchased from Open Biosystems/ThermoScientific (Huntsville, AL) and are isogenic with BY4741. Yeast expression plasmids utilizing heterologous promoters and terminators were obtained from Martin Funk (Mumberg *et al.*, 1995). Plasmid p414TEF-SSA1 was constructed by PCR amplification of the SSA1 ORF with primers incorporating 5′ *Spe*I and 3′ *Xho*I restriction sites. $SSA1^{C264S}$, $SSA1^{C303S}$, $SSA1^{C264S,C303S}$, and $SSA1^{C264D,C303D}$ 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 (Morano *et al.*, 1999). DTT quenching assays were performed by incubating compounds with the indicated concentrations of DTT at room temperature for 15 min before adding them to cells. Rich-medium YPD (0.2% Bacto-peptone, 0.1% yeast extract, 2% glucose) was used for growth of BY4741, DS10, and strains containing a chromosomally integrated TAP tag. 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. All chemicals were ACS grade and purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Hsf1 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 (Research Organics, Cleveland, OH), 100 μM $CdSO_4$, 10 μM 15d-PGJ_2 (Cayman Chemical, Ann Arbor, MI), 2 mM H_2O_2, or 500 μM DEM for 2 h at 30°C. In vivo protein unfolding was carried out using 10 mM azetidine 2-carboxylic acid (AZC). To maintain the solubility of celastrol, we conducted experiments in SC medium supplemented with 50 mM Tris-HCl (pH 7.5). β-Galactosidase activity assays were carried out exactly as described in duplicate with error bars representing SD of the mean (Morano *et al.*, 1999).

HSP expression analysis

For analysis of the expression levels of HSPs induced by $CdSO_4$, diamide, and AZC, protein extracts were prepared using a glass bead lysis procedure, fractionated by SDS–PAGE (10%), and transferred to nitrocellulose as described (Liu *et al.*, 1999). Polyclonal antibodies recognizing phosphoglycerate kinase and GPD were purchased from Invitrogen (Carlsbad, CA) and Sigma-Aldrich, respectively. Rabbit polyclonal antibody recognizing Ssa3/Ssa4 was a kind gift from E. Craig. Band intensities were quantified using ImageJ (National Institutes of Health) and normalized to the load control.

Cell fractionation

For analysis of levels of protein aggregation in the presence of thermal stress and thiol-reactive compounds, wild-type (BY4741) cells were fractionated by high-speed centrifugation. Log-phase cells were treated with heat shock, 600 μM Cd^{2+}, or 500 μM DEM for 1 h. Cell pellets were transferred into 1.5-ml centrifuge tubes containing 300 μl dry volume of acid-washed glass beads and 600 μl ice-cold TEGN buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl) with a protease inhibitor cocktail. The cells were lysed by six 45-s rounds of vigorous vortexing, each of which was followed with 90-s rests of the tubes on ice. The resulting lysate was first subjected to a low-speed spin at 3000 × *g* for 30 s to remove unbroken cells. The supernatant (500 μl) was transferred into new tubes and incubated with 1% Triton-X for 30 min at 4°C with gentle rotation to dissolve membranes; this was followed by centrifugation at 100,000 × *g* for 30 min, which yielded supernatant and pellet fractions.

Biotin-BMCC labeling

To investigate the potential targets of thiol modification, we isolated protein-TAP fusions from cells as follows. Protein extracts were prepared by glass bead lysis in TEGN buffer with a protease inhibitor cocktail (aprotinin, 2 μg/ml; pepstatin A, 2 μg/ml; leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 1 mM; chymostatin, 2 μg/ml; Sigma-Aldrich), and protein concentration was determined by Bradford assay. Cell extract (0.5–1.0 mg) in a volume < 300 μl was incubated with 75 μl IgG-Sepharose (GE Healthcare Life Sciences, Piscataway, NJ) and 625 μl TEGN buffer at 4°C on a rotating wheel for 2 h. 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 μM biotin-BMCC 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 β-mercaptoethanol, 0.4% bromophenol blue). Immunoblotting was performed using streptavidin-HRP to detect thiol-modified proteins (Bio-Rad, Hercules, CA). Polyclonal antibody recognizing TAP-tag (anti-PtnA; Sigma-Aldrich) was used at 1:1000 dilution. Purified Ssa1 protein has been described elsewhere (Shaner *et al.*, 2005) and was detected using polyclonal anti-Ssa1 antibody provided by Mark Ptashne (Memorial Sloan-Kettering Cancer Center, New York, NY; Floer *et al.*, 2008). Ssa1 (300 nM) was reacted with 32 μM biotin-BMCC for the indicated times, and the reaction was quenched with 2X SDS sample buffer. DTT inactivation of biotin-BMCC was carried out by adding DTT in distilled water to final concentrations sufficient to achieve the indicated molar ratios with biotin-BMCC 15 min prior to incubation with purified Ssa1.

In vivo labeling of Ssa1

The *ssa1*Δ *ssa2*Δ strains carrying empty vector, FLAG-tagged *SSA1* wild-type, or cysteine mutant alleles were treated for 1 h with ethanol or 500 μM 4HNE-alkyne (Cayman Chemical, Ann Arbor, MI). The extract was prepared by glass bead lysis, and protein concentration was determined by Bradford assay. Cell extract (0.5–1.0 mg) was compensated with TEGN buffer plus a protease inhibitor cocktail to reach a total volume of 700 μl and incubated with 20 μl FLAG resin (Sigma-Aldrich) at 4°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. The washed resin was resuspended in 60 μl of 50 mM Tris-HCl buffer (pH 8.0). Proteins tagged by 4HNE-alkyne were detected using the Click-iT reaction buffer kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Click-tagged proteins were eluted by incubation with 30 μl of 1× FLAG peptide (Sigma-Aldrich) for 30 min at room temperature, and labeling of Ssa1 was detected by immunoblot with streptavidin-HRP.

Heat shock sensitivity assay

For assay of thermotolerance induced by $CdSO_4$ treatment, *ssa1*Δ *ssa2*Δ cells bearing an empty vector, *SSA1* wild-type, or cysteine mutant alleles were treated with no reagent or 600 μM Cd^{2+} for 1 h at 30°C. After treatment, cells were diluted to a density of ~100,000 cells/ml in sterile PCR tubes in a volume of 100 μl. The diluted cells were heat-shocked at 47°C in a thermocycler for 0, 5, 10, and 20 min before being spotted onto solid SC medium; this was followed by incubation at 30°C for 2 d. For measurement of thermotolerance induced by mild heat shock, strains were incubated at 37°C for 1 h, diluted, and heat-shocked at 47°C in a thermocycler for 0, 5, 10, and 20 min before being spotted onto solid SC medium.

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