Published in final edited form as:
Chem Res Toxicol. 2012 October 15; 25(10): 2036–2053. doi:10.1021/tx300264x.

Small Molecule Activators of the Heat Shock Response: Chemical Properties, Molecular Targets, and Therapeutic Promise

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Abstract

All cells have developed various mechanisms to respond and adapt to a variety of environmental challenges, including stresses that damage cellular proteins. One such response, the heat shock response (HSR), leads to the transcriptional activation of a family of molecular chaperone proteins that promote proper folding or clearance of damaged proteins within the cytosol. In addition to its role in protection against acute insults, the HSR also regulates lifespan and protects against protein misfolding that is associated with degenerative diseases of aging. As a result, identifying pharmacological regulators of the HSR has become an active area of research in recent years. Here, we review progress made in identifying small molecule activators of the HSR, what cellular targets these compounds interact with to drive response activation, and how such molecules may ultimately be employed to delay or reverse protein misfolding events that contribute to a number of diseases.

1. Cellular Stress Responses to Adverse Environmental Conditions

The environment in which organisms exist is not static, and most biological systems can adapt to maintain homeostasis, an ideal steady state that promotes growth, replication, and/or survival. However, a wide range of physiological and environmental stimuli continuously disrupt this equilibrium, resulting in constant flux around a homeostatic point. Unfavorable conditions such as elevated temperature, oxidant accumulation, abrupt alterations in osmolarity, and nutrient deprivation change the intracellular environment, restrict cell growth and development, and can lead to cell death. To restore homeostasis under such unfavorable conditions, organisms have developed sophisticated stress response mechanisms that increase expression of protective genes within a cell. A cell's response to acute stress is generally transient and can involve a number of transcriptional regulators, some of which are activated simultaneously by particular stressors. Many of these stress responses are in place to prevent, repair, or clear damage to proteins caused by harsh environmental conditions, thereby influencing protein homeostasis. Examples of transcriptional responses to stress that are often simultaneously induced include the antioxidant response, ER stress responses, and the heat shock response (HSR) (Fig. 1).

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1.1. Antioxidant Response

In response to certain stresses (e.g., oxidative stress, proteasome inhibition), many organisms induce the expression of genes involved in detoxification. In mammalian cells, the antioxidant response is mediated by the transcription factor Nrf2 and its negative regulator, Keap1. Normally, Nrf2 undergoes rapid degradation in cells, a process that is facilitated by Keap1; upon exposure to stress, Nrf2 accumulates and migrates into the nucleus to promote the expression of its target genes, including many involved glutathione metabolism and scavenging of free radicals.[1] Transcription factors related to mammalian Nrf2 regulate similar responses in multicellular eukaryotes.[2] However, in unicellular eukaryotes, the response is controlled by unrelated transcription factors with different mechanisms of activation.[3] Despite the lack of conserved antioxidant response transcription factors in all eukaryotes, the genes that are induced by these disparate factors universally protect cells against excessive protein and DNA damage caused by reactive species (e.g., oxidants, electrophiles) and are commonly induced by sub-cytotoxic concentrations of these molecules.[4]

1.2. ER Stress Responses

Eukaryotic cells have one or more transcriptional regulators that respond to protein misfolding in the endoplasmic reticulum.[5] In mammalian cells, the transcription factors XBP1, ATF6, and ATF4 are activated following treatment with protein reductants, protein glycosylation inhibitors, molecules that influence Ca^{2+} stores, and other conditions that perturb ER homeostasis.[6,7] Collectively, XBP1, ATF6, and ATF4 control the expression of multiple protective proteins including molecular chaperones that reside within the ER.[7] These chaperone proteins, in turn, allow for proper folding and function of many membrane and secreted proteins. In addition, the ER chaperones can assist with targeting misfolded or damaged ER proteins for export into the cytosol where they are degraded by the proteasome.[8]

1.3. Heat Shock Response

In contrast to ER stress responses, which predominantly serve the secretory pathway, the heat shock response (HSR) primarily responds to protein misfolding conditions in the cytoplasm.[9,10] It results in the transcriptional activation of genes encoding cytosolic molecular chaperones, proteases, and other proteins essential for protection and recovery from cellular damage. Under stress conditions, many heat shock proteins (Hsps) (e.g., Hsp70) hold and refold denatured proteins, prevent them from aggregating, and/or regulate their degradation. Some Hsps (e.g., Hsp90) play integral roles in signal transduction, immunity, and apoptosis.[11–16] Modulation of the HSR, therefore, may be directly beneficial for a variety of human diseases associated with cell growth (e.g., cancer) and accumulation of damaged and misfolded proteins (e.g., neurodegenerative diseases).

2. HSF1: A Key Regulator of Heat Shock Genes

In all eukaryotes, the HSR is primarily mediated by heat shock transcription factors (HSFs), which bind to heat shock elements (HSEs) in the promoters of target genes.[17,18] A functional HSE contains a series of pentameric units arranged as inverted adjacent arrays of the sequence 5′-nGAAn. At least three pentamers are required for optimal interaction between HSF and HSE.[19,20] Whereas a single HSF has been identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, multiple HSF isoforms are encoded in the genomes of vertebrates and many plants.[21–24] Among these HSFs, HSF1 is the principal activator of heat shock gene transcription in response to elevated temperature and other environmental stresses. Despite variation in size and homology, HSFs share several conserved features: a conserved DNA-binding domain composed of a winged helix-

turn-helix motif located near the *N*-terminus, three leucine zipper repeats that regulate trimerization of the factor, and an activation domain that undergoes many inducible post-translational modifications during stress (Fig. 2).[24–28]

In many organisms, HSF1 activation is a multi-step process that includes nuclear accumulation, trimerization, acquisition of DNA binding activity, and transactivation of genes encoding Hsps (Fig. 3).[29] The stress-dependent conversion of HSF1 into its active form implies that HSF1 is negatively regulated under normal conditions.[22,30] The Hsps themselves are proposed to inhibit heat shock gene expression via an autoregulatory loop.[24,30,31] Under normal growth conditions, monomeric HSF1 is purportedly stabilized by cytosolic Hsps such as Hsp70 and Hsp90.[32] During heat shock, accumulated unfolded proteins may titrate Hsps away from HSF1 and relieve HSF1 repression. Ultimately, HSF1 trimerizes and acquires DNA binding ability, resulting in an increased amount of Hsps in the stressed cells.

2.1. Subcellular Localization

HSF1 undergoes changes in its cellular localization in response to stress in mammalian cells. Under homeostatic conditions, it is distributed in a diffuse pattern in both the cytoplasm and/or the nucleus, depending on the cell type. However, in heat-shocked cells, HSF1 is found primarily in the nucleus in punctate granules that form in various cell lines and are correlated with the active form of HSF1.[33–35] During attenuation and recovery, the granules disappear and HSF1 redistributes to the cytoplasm, thus suggesting a role for HSF1 granules as a temporal nuclear compartment for spatial regulation of HSF1 activity.[35]

2.2. Trimerization

In vertebrate and *Drosophila* cells, HSF1 is expressed as an inert monomer with both DNA binding and transcription activity repressed through intramolecular interaction and constitutive phosphorylation.[31,36] Upon activation, HSF1 trimerizes to acquire DNA binding activity.[37] The trimerization domain in HSF1 contains high α-helical content and can be divided into two subdomains, each containing an amphipathic helix with hydrophobic heptad repeats HR-A and HR-B.[28,38] In trimeric HSF1, HR-A and HR-B possibly form a highly elongated structure. Intermolecular hydrophobic interactions between HR-A and HR-B mediate the formation of a triple-stranded coiled-coil configuration.[38] Spontaneous trimerization of HSF1 is inhibited by another hydrophobic heptad repeat HR-C between the trans-activation and regulatory domains near the *C*-terminus. It is thought that HR-C interacts with the HR-A and HR-B domains and maintains HSF1 in monomeric form under normal growth conditions.[36]

2.3. Post-Translational Modifications

HSF1 can undergo numerous post-translational modifications, including phosphorylation, sumoylation, and acetylation. Of these, phosphorylation has been the most extensively studied. For human HSF1, phosphorylation occurs on at least 15 Ser residues (S121, S216, S230, S292, S303, S307, S314, S319, S320, S326, S344, S363, S368, S419 and S444) and four Thr residues (T142, T323, T367 and T369) and has both positive and negative effects on HSF1 activity.[39–45] Inducible phosphorylation of S230 by the calcium/calmodulin-dependent kinase CaMKII induces HSF1 activation.[46] In addition, phosphorylation of S326 facilitates the association between HSF1 and the coactivator DAXX.[47] However, other phosphorylation events repress the activity of HSF1. For instance, mutation of S303 and S307 to Ala derepresses mammalian HSF1 activity under non-stress conditions.[48,49]

Phosphorylation at certain sites may influence additional post-translational modification events. In support of this, phosphorylation at S303 correlates with sumoylation of HSF1 on

K298. Phosphorylation mapping and analysis further established that HSF1 is modified by SUMO-1 and SUMO-2 in a stress-inducible and phosphorylation-dependent manner at S303.[50,51] Similar to S303 phosphorylation, sumoylation of HSF1 is thought to repress HSF1 transcriptional activity. HSF1 also undergoes stress-inducible acetylation on several different lysine residues. Activation of the deacetylase sirtuin 1 (SIRT1) promotes maintenance of HSF1 in a DNA-binding competent state.[52] Unlike phosphorylation events that primarily occur in the transactivation domain, lysines prone to acetylation are found in the DNA binding, oligomerization, and transactivation domains of HSF1, suggesting potentially broader regulation of acetylation upon its transcriptional activity.[43]

2.4. Binding to Negative Regulators

The heat shock response is self-regulated under thermal stress.[53,54] In yeast, deletion of the two constitutively expressed cytosolic Hsp70 genes results in activation of the HSR at normal growth temperature.[55,56] Hsp70 associates with the HSF1 transactivation domain *in vivo* and *in vitro*.[31,57–59] However, Hsp70 alone is not sufficient to deactivate HSF1 *in vitro*.[59] Regulation of HSF1 by Hsp90 and associated co-chaperones represents another plausible means of repressing HSF1 activity, as Hsp90 associates with HSF1 *in vivo* and *in vitro*.[60] In support of this, direct injection of anti-Hsp90 in a *Xenopus* oocytes induces the HSR.[61] A role of Hsp90 in HSF1 repression is also supported by the finding that Hsp90 inhibitors (e.g., geldanamycin, radicicol, and celastrol) also activate HSF1.[16,62–66] Therefore, the likely model is that HSF1 remains inactive due to binding with Hsp70 and Hsp90 under normal growth conditions.

3. Small Molecular Activators of the Heat Shock Response

In recent years, there has been growing interest in identifying small molecule regulators of the HSR. These molecules have a variety of chemical features–some are highly reactive with a diverse array of cellular proteins, whereas others have specific targets that impair protein homeostasis. Below, we will highlight recent studies aimed at identifying small molecule HSF1 activators and their mechanisms of action.

3.1. Pharmacological Activators with Known Targets in the Protein Homeostasis Network

Many known HSR activators influence parts of the protein homeostasis network, a collection of proteins that provide surveillance for the synthesis, maturation, and turnover of proteins. Known activators of the HSR that play roles in this capacity include protein translation inhibitors (e.g., puromycin), amino acid analogs that prevent proper protein folding (e.g., azetidine 2-carboxylate, canavanine), several different pharmacological inhibitors of molecular chaperones (e.g., geldanamycin and radicicol), and proteasome and protease inhibitors (e.g., MG132).[67,68] A defining feature for these molecules is that their molecular targets are integral parts of the protein homeostasis network.[12,69] Drastically altering the function of the protein synthesis, folding, and clearance machineries imbalances cellular homeostasis and is likely to lead to accumulation of misfolded polypeptides that oligomerize and aggregate in the cytosol,[70,71] thereby necessitating an increase in molecular chaperone expression.

3.2. Thiol-Reactive Molecules Activating the Heat Shock Response

For other HSF1 activators, the molecular targets that are linked with HSR activation are less clearly defined. Three distinct classes of reactive molecules–oxidants, certain transition metals/metalloids, and organic electrophiles–fall into this category (Fig. 4).[32,67,72] Each class of molecules has the propensity to damage proteins directly, often on the side chains of highly reactive Cys residues in target proteins (Fig. 5).[73–75] Below, we explore the reactions that each of these classes of thiol-reactive HSF1 activators undergo with proteins.

3.2.1. Oxidants—A variety of oxidants are capable of activating HSF1 in mammalian cells, including hydrogen peroxide, hypochlorous acid, and diamide.[76–78] Depending on its strength, an oxidant can cause breaks within the peptide backbone or react with the side chains on several amino acids, including Cys, His, Met, Phe, and Tyr.[79] Of these residues, highly reactive Cys residues (i.e., those with lower pK_a values) in specific target proteins are often the most susceptible to oxidation (Fig. 5), with the residue's thiol group initially forming a sulfenic acid (Fig. 5).[74,80] There are several possible fates of the sulfenic acid form of Cys. It can undergo a condensation reaction with a nearby thiol (intramolecularly, with a Cys residue in another protein, or with free thiols within the cell (e.g., GSH)) to form a disulfide bond.[74,80] Alternatively, the Cys sulfenic acid can undergo further oxidation to a sulfinic or sulfonic acid.[74,80] Oxidative modifications of Cys can play a regulatory role in protein function, as Cys residues are often incorporated into sensor proteins to detect the accumulation of oxidants and promote expression of various oxidant defense genes.[74,81–83] Such sensory Cys residues may be present in HSF1 or particular Hsps which are implicated in HSF1 regulation, as will be described later.

3.2.2. Transition metals and metalloids—Exposure to many metal ions (e.g., Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Co^{2+}) and some metalloid-containing anions (e.g., AsO_3^{3-}) also promotes HSF1 activation.[84–86] In terms of their reactions with proteins, these molecules function as Lewis acids and form coordinate covalent bonds with amino acid side chains bearing readily accessible lone pairs of electrons.[87] Clusters of nearby Cys, His, Asp, or Glu residues in proteins commonly chelate the metal or metalloid ions (Fig. 5), and such reactions may perturb protein structure and function by displacing normal metal centers or by forming non-natural metal binding sites.[75,87] These molecules typically are slower to activate the HSR compared with other thiol-reactive compounds or acute heat stress,[33,88] which may be because they require increased time to cross the cell membrane through a transporter and/or that they react more slowly with protein targets responsible for bringing about the response. Nonetheless, increased levels of metal chelators (e.g., GSH, *N*-acetyl Cys) decrease overall Hsp expression by these molecules,[89–91] suggesting that their reaction with protein targets is required for HSR activation.

3.2.3. Organic electrophiles—Many organic electrophiles induce Hsp expression, including the cyclopentenone prostaglandins (PGs) 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and PGA_1,[92–95] the lipid peroxidation products 4-hydroxy-2-nonenal (HNE), acrolein, and crotonaldehyde,[96–99] the lipid nitration product nitrooleic acid,[100] the phytochemicals celastrol, withaferin A, and sulforaphane,[16,101,102] and numerous synthetic chemicals including iodoacetamide and cyclopentenone.[103–105] All of these molecules contain one (or more) electrophilic center(s) (Fig. 6), but they vary widely in their physical and chemical properties (Fig. 1, Table 1). Below, we will highlight several features within these molecules that may contribute to their potency as HSF1 activators.

Like oxidants and transition metals, electrophilic molecules react predominantly with the thiol group in Cys, although the nucleophilic amine groups of Lys and His residues may become modified in some target proteins.[73,82,106–108] Since nucleophilicity (and hence, overall reactivity) of Cys residues is increased upon thiol deprotonation to the thiolate anion, Cys residues with low pK_a values are particularly susceptible to modification by electrophilic molecules in target proteins.[109] Reactivity of these molecules with proteins is closely linked with HSF1 activation. Analogs of cyclopentenone PGs, cyclopentenone, withaferin A, gedunin, and dehydrocurvularin that are saturated at their electrophilic centers cannot react with proteins and no longer activate HSF1.[92,93,102,104] Similarly, the addition of DTT to celastrol or iodoacetamide prevents these electrophiles from increasing the expression of HSR genes.[66,103] The overall differences in potency between these molecules

may be due, in part, to differences in their specificity and rates of reactivity with target proteins, but a comprehensive study to explore these possibilities has not been performed.

Other properties of these electrophiles, including their hydrophobicity, adduct reversibility, and the ability to form intermolecular cross-links between target proteins, may contribute to HSF1 activation as well. Studies on the cyclopentenone PGs suggest that hydrophobicity alters the potency of HSR activation. 15d-PGJ_2 is considerably more hydrophobic (estimated logP ~ 4.1) and more potent than cyclopentenone (estimated logP ~ 0.3).[94,95,104] PGA_1, which has an estimated logP value between that of 15d-PGJ_2 and cyclopentenone, activates HSF1 at doses between that of the other molecules.[92,93,104] Conversely, converting the natural product celastrol into more hydrophobic derivatives by esterifying its carboxylate group decreases its potency,[16] perhaps due to poor solubility in aqueous solution.

In addition to an electrophile's hydrophobicity, reversibility of the thiol adducts they form may contribute to HSR activation by these molecules. Several of the most potent HSF1 activators–nitrooleic acid, celastrol, and sulforaphane–carry out reversible reactions with thiol-containing molecules, with each molecule activating HSF1 in the low micromolar range.[110–112] The equilibrium between the adducted form and the free form of the electrophile may be influenced by the relative concentrations of the electrophile and various target proteins (and nucleophilic small molecules), by pH changes, and by the degree of protein turnover. Therefore, it is likely that protein damage by these molecules is transient in treated cells.

The ability of an electrophile to form protein cross-links may also enhance HSR activation. Consistent with this idea, 15d-PGJ_2 can cause cross-links to form between two proteins,[113,114] suggesting that both its hydrophobicity and ability to form intermolecular cross-links may contribute to its increased potency in comparison with analogous electrophiles. In addition, HNE, acrolein, and crotonaldehyde are heterobifunctional electrophiles that are capable of causing unstable intermolecular cross-links between Cys and Lys residues in proteins,[115–118] although the extent to which protein cross-linking contributes to HSF1 activation has not been explored with these molecules. Likewise, two recently identified and relatively potent HSF1 activators, TBE-31 and *bis*(2-hydroxybenzylidene) acetone, contain two Michael acceptor sites,[119] through which they presumably cause protein cross-linking.[120,121] Protein cross-linking may greatly enhance activation of cytoprotective response genes, as our recent studies indicate that protein cross-linkers impact cellular physiology at much lower concentrations than corresponding monofunctional alkylating agents.[122,123]

4. Models for HSF1 Activation by Pharmacological Activators

Over the past two decades, much has been learned about the properties and targets of the molecules that activate HSF1 and the specific molecular events occurring most proximal to HSF1 activation. However, the mechanism through which individual small molecule stressors are initially sensed to drive activation of the heat shock response, whether multiple sensors exert influence on different regulatory steps in HSF1 activation, and whether these mechanisms are common for all stresses remain largely unresolved. Below, we highlight several potential models that have been proposed for HSF1 activation by the pharmacological agents described earlier. Our focus will be on how certain stresses are sensed to promote activation of the response.

4.1. HSF1 as a Direct Stress Sensor

HSF1 may directly sense multiple types of stress, including elevated temperature, high levels of oxidants, altered pH, and exposure to hydrophobic molecules.[24,76,124–126] This

causes HSF1 to trimerize and acquire DNA binding activity. Consistent with these observations, HSF1 undergoes secondary and tertiary structural changes in its *C*-terminal regulatory domain, thereby promoting its trimerization *in vitro*.[125] Structural changes that occur in HSF1 following exposure to increased temperature are readily reversible when the stress is removed, causing the protein return to its inactive, monomeric state.[124] The nature of the reversibility for other stressors and whether this sensing mechanism occurs in a cellular context remain unresolved.

Several groups have carried out additional *in vitro* studies on the oxidation of mammalian HSF1 to explore if and how it senses peroxides. Following exposure to peroxides, HSF1 is oxidized *in vitro* on a number of Cys residues located within the DNA binding and/or the trimerization domains of the protein, leading to the formation of disulfide bonds.[76,126–129] Conversely, exposure of HSF1 to dithiothreitol (DTT) prevents activation in these instances, an effect that mirrors the inhibitory effects of DTT on endogenous HSF1 activation.[103] While these reports indicate that direct redox regulation of HSF1 is possible, there is some discrepancy regarding the Cys residues in HSF1 that are involved in oxidant sensing and whether disulfides are formed intra- or inter-molecularly. In addition, a limitation of this work is that disulfide formation has not been definitively shown to occur in endogenous HSF1 exposed to oxidants. Moreover, HSF1 in certain organisms (including *S. cerevisiae*) lacks Cys residues at proposed sites of disulfide formation,[128] implying that direct oxidation may be a contributing factor but is not a conserved mechanism of HSF1 activation.

4.2. Chaperones as Stress Sensors

In the absence of stress, the chaperones Hsp70 and Hsp90 repress HSF1 trimerization and/or activation directly by binding to it in an inactive form.[24,57,60,130] In the case of Hsp70, these binding interactions are mediated through its *C*-terminal substrate binding domain and the activation domain of HSF1.[57] Following exposure to some stresses, interactions between HSF1 and these chaperones decrease.[131,132] In addition, when chaperone function is inhibited through either pharmacological or genetic means,[67,133] HSF1 activity increases significantly in a variety of organisms. There are at least two potential ways that HSF1 may be released from repression by these chaperones, depending on the type of pharmacological agent being added. One of these models involves titration of chaperones away from HSF1 to deal with protein folding problems elsewhere, whereas the other model posits that Hsp70 and Hsp90 function as direct sensors of many thiol-reactive HSR inducers.

4.2.1. Chaperones as Sensors of Cytosolic Protein Misfolding—Chaperones monitor all aspects of a protein's 'life cycle,' from its emergence as a nascent peptide from the ribosome to its degradation within the cell.[12,69] Many different pharmacological agents that activate HSF1 have been implicated in disrupting translation, post-translational protein processing and modification, protein folding, and protein degradation (Fig. 4).[67] The end result of perturbing these points in the protein homeostasis network is the generation of peptide fragments or polypeptides that are prone to self-association.[70,71] Indeed, several studies using genetic and pharmacological approaches have shown that inhibition of protein synthesis by the ribosome or protein degradation by the proteasome leads to enhanced protein aggregation in the cytosol.[71,134–136] Such misfolding events are likely to place increased demands on the cytosolic chaperones, recruiting them away from HSF1 (Fig. 8).

Likewise, protein modification by oxidants, metals, and electrophiles may cause misfolding of some target proteins, leading to their self-association and aggregation.[137–139] Oligomerization of aggregation-prone proteins is accelerated by thiol-reactive molecules in many instances,[140,141] perhaps through the molecules having a 'seeding effect' to promote further oligomerization. Since many different proteins are susceptible to modification by

thiol-reactive molecules, protein folding problems may occur when cellular concentrations exceed levels that can be readily detoxified. In support of this, proteins form aggregates in cells treated with high concentrations of the oxidants hydrogen peroxide and hypochlorous acid.[142,143] Taken together, these results indicate that protein damage caused by cellular exposure to thiol-reactive molecules may perturb the structures of proteins in their native state, creating a burden on the chaperones that normally repress HSF1 activity (Fig. 8).

In addition, several studies have highlighted that thiol-reactive molecules can impair efficient protein degradation by the proteasome. This inhibition may involve the covalent inactivation of multiple enzymatic activities within the proteasome, including those involved in ubiquitin chain removal and proteolysis of the targeted substrate.[144–147] Similar to the effects of specific proteasome inhibitors,[71] thiol-reactive molecules may cause increased levels of misfolded oligomers and protein aggregates in the cytosol through their inhibitory effects on proteasome activity and may thereby promote HSF1 activation.

4.2.2. Chaperones as Direct Sensors of Thiol-Reactive Molecules—Thiol-reactive molecules can modify the chaperones that repress HSF1 by directly reacting with key Cys residues within these proteins. Specifically, both Hsp70 and Hsp90 have been identified as potential targets of electrophiles and oxidants in multiple proteome-wide profiling studies.[148–152] Hsp70 association with HSF1 decreases upon treatment with electrophiles,[132] implying that alkylation may alter its ability to bind HSF1. Indeed, *in vitro* chaperone activity assays reveal that Hsp70, Hsp90, and several of their co-chaperones are inactivated by thiol-reactive molecules.[112,139,148,149,153,154] Taken together, these results imply that chaperones are targets for inactivation by thiol-reactive molecules.

Using a yeast model system, we have found that HSR activation by thiol-reactive molecules depends on the presence of Cys residues 264 and 303 in the Hsp70 Ssa1.[155] Several lines of evidence suggest that modification of these residues in Hsp70 may be a universal sensing mechanism for activation of the HSR by thiol-reactive molecules. First, one of these Cys residues (C264 in *S. cerevisiae*) is highly conserved among eukaryotic Hsp70s (Fig. 9A), whereas other potential sites of modification in Hsp70 and Hsp90 are not as highly conserved. The conserved Cys residues in Hsp70 are alkylated by several different electrophiles *in vitro*, leading to inactivation of the chaperone function of the protein.[148,153,156] Conversely, DnaK, an Hsp70 from *E. coli* that lacks C264, is not sensitive to inactivation by the electrophile HNE.[148] Mutation of C264 to Ser in Ssa1 significantly decreases the magnitude of the HSR in response to thiol-reactive molecules, but not heat.[155] Since it is widely thought that Hsp70 forms a repressive, multichaperone complex with HSF1, then it is likely that modification of this key thiol group at C264 disrupts the interactions between these proteins, thereby derepressing HSF1 activity (Fig. 9B). A similar mechanism of derepression has been proposed for electrophilic modification of Hsp90;[119,157] however, the yeast Hsp90 lacks conservation of reactive amino acids most likely to be modified by thiol-modifying agents in mammalian Hsp90.[149,158]

4.3. HSF1-Modifying Enzymes as Potential Sensors of Stress

As described earlier, HSF1 undergoes numerous constitutive and inducible post-translational modifications.[43] Many of the enzymes that attach or remove these modifications may exhibit altered cellular activities in response to cellular stress. If so, these HSF1-regulating enzymes or upstream proteins may function as stress sensors and thereby influence the magnitude or duration of the HSR (Fig. 10). In support of this, treatment of cells with Ser/Thr phosphatase inhibitors or overexpression of specific phosphatases prolongs HSF1 phosphorylation and DNA binding,[159–162] whereas enhancing acetylation of HSF1 through inhibition of deacetylase activity or increased expression of acetyl transferases results in

reduced HSF1 DNA binding.[52] Likewise, stress-induced alterations in HSF1 sumoylation may play a key role in altering its activity,[50,163,164] with prolonged sumoylation presumably blocking HSF1 DNA binding. While the sensory mechanisms that allow for altered post-translational modification of HSF1 are still being investigated, several of the enzymes that play a direct role in carrying out in post-translational modifications are modified by thiol-reactive molecules.[165–168] Accordingly, stress-responsive modification of these enzymatic activities may significantly influence the overall HSR in stressed cells.

4.4. Future Work to Explore the Models of HSF1 Activation

Because HSF1 regulation is a complex, multi-step process, determining the upstream molecular events leading to its activation and the order of steps in its inactivation has represented a challenge, leading to a variety of models for how the process occurs in cells. While many of these models are referenced herein, it is important to note that none of them fully addresses the complexity of HSF1 regulation. For instance, we still know relatively little about the enzymes responsible for post-translational modification of HSF1, the timing of these events in relation to transcription of Hsp-encoding genes, and their overall effects on HSF1 activity. To assess the importance of these and other events, validating each effect in a cellular context (to complement *in vitro* biochemical work), performing detailed time-course experiments to tease out the timing of specific molecular events, and utilizing new biological and chemical tools to explore these problems will be essential in moving the field forward.

5. Ramifications of Influencing the Heat Shock Response

With the recent advances in understanding activation of the HSR at a biological and chemical level, we are approaching the point where pharmacological manipulation of this cytoprotective response is a distinct possibility. A number of pathophysiological conditions have been strongly linked to dysregulation of the HSR, including several debilitating diseases. For many of these disorders, there are few, if any, therapies available, making the possibility of HSR-targeted approaches highly attractive.

5.1. Acute Stress Tolerance

Defense against acute insult, most notably thermal stress, is perhaps the most appreciated cellular role of the HSR. The ability to tolerate severe stress is directly dependent on the capacity to produce Hsps in all organisms studied. This is true for survival of an immediate acute stress, dependent on sufficient basal Hsp expression, as well as acquired stress tolerance, the phenomenon whereby challenge with a sub-lethal stress potentiates survival of a subsequent lethal stress. Yeast cells lacking the Hsp70 and Hsp104 protein chaperones, or deficient in HSF1 function, are hypersensitive to stress.[169–171] Correspondingly, $HSF1^{-/-}$ mice are compromised in their ability to withstand environmental insult.[172] While severe heat stress (45–55°C) would be expected to perturb cellular ultrastructure, including membranes and cytoskeleton, the elevated expression of Hsp104 in yeast is sufficient to confer enhanced thermotolerance, suggesting that proteotoxicity is the primary cause of cell death.[173] In contrast to the situation in unicellular eukaryotes, a careful balance is struck between Hsp-mediated cell survival and apoptosis in mammalian cells.

Because the regulation of apoptosis involves Hsps, it is possible that they play a major role in a cell's decision to repair damaged proteins or undergo cell death. Hsp70, Hsp90, and Hsp27 inhibit apoptosome formation and caspase activation.[174,175] In addition, Hsp90 is required for activity of a number of pro-survival proteins, including Akt and proteins in the Ras-Raf cascade.[176] It is currently thought that when challenged with lethal stress, HSF1-mediated HSR induction is the first line of response. If protein homeostasis is restored,

growth may resume. If not, an irreversible commitment to apoptosis is triggered to eliminate the terminally damaged cell. In support of this conjecture, pharmacological inhibition of Hsp90 enhances apoptosis, and this effect is amplified when combined with the compound KNK437, which inhibits the HSR through an unknown mechanism.[177] Conversely, activation of the HSR can delay or block the cell death response. Thus, pharmacological induction of the HSR provides protection against cell death in tissues immediately after cytotoxic stresses. Indeed, thermal induction of Hsps correlates with an increase in the success rate of organ transplant, a prime example of physiological stress.[178]

5.2. The HSR and Human Disease

There is growing evidence that the HSR may play both positive and negative roles in initiation and progression of human disease. At the heart of this involvement is the ability of Hsps to fold and promote maturation of substrate proteins. Because Hsps are generally considered non-selective in their targets and interact with a number of proteins, abrogation of their function can alter fundamental aspects of cell physiology, leading to pathology and disease. In contrast, the known dependence of select signaling and regulatory molecules such as cyclin dependent kinases, Akt, growth-promoting tyrosine kinases, and telomerase as client of the Hsp90 chaperone system means that upregulation of the HSR favors cancer progression and metastasis. In addition to the HSR, the degradative capacity of the cell provides the counterweight to protein repair functions, together defining protein homeostasis from cradle to grave. We examine these features and their relevance to human health below.

5.2.1. Aging—All cells must replenish and recycle their components over time to maintain viability. This is especially true of many terminally differentiated cells, which are not renewed as organisms age. Mounting evidence suggests that the ability of cells to maintain proper protein homeostasis also deteriorates over time. Aged rats (20+ months) display marked reduction in their capacity to mount a functional cardiac HSR when subjected to thermal stress relative to adults (~ 6 months), which correlates with reduced tolerance to ischemia.[179] HSF1 levels remain unchanged in aged versus younger rats, but the transcription factor exhibits poor DNA binding activity that may account for diminished HSR and stress sensitivity.[180] This phenomenon extends to other mammalian cell types, as human lymphocytes and fibroblasts from aged donors exhibit the same loss of HSF1 activity, as do serially passaged cells of both types from younger donors.[180,181]

Non-mammalian model systems have been instrumental in probing the molecular details of the links between aging and the HSR. Longevity in *C. elegans* is dependent on HSF-1, an effect more pronounced in worms with a genetic deficiency in the insulin-like growth factor pathway.[182] The insulin-like growth factor pathway also regulates lifespan in mice and other organisms, underscoring its central role in aging. In addition to HSF-1, the FOXO-like transcription factor DAF-16 also plays a role in slowing aging.[183] Both factors contribute to the expression of chaperones of the small Hsp (sHsp) family, whose deletion (namely Hsp22) leads to decreased lifespan in *Drosophila*.[184] In contrast, overexpression of Hsp22 in motor neurons extends lifespan, with specific benefits in tolerating oxidative and thermal stresses.[185] Gradual loss of protein homeostasis in early adulthood occurs in *C. elegans*, and this can be slowed by enhancing cytoplasmic- and endoplasmic reticulum-based stress response pathways.[186] These results are consistent with the observation that soluble polyglutamine (polyQ)-fluorescent protein chimeras aggregate within muscle tissue in aged, but not young, worms.[187]

Further studies have yielded mechanistic explanations for the observed decline in protein quality control with age. Caloric restriction extends lifespan in many organisms, and this effect requires HSF1 in the worm model.[188] The age-related decline in HSF1 DNA binding

affinity and accompanying loss in Hsp70 production is also reversed by caloric restriction in rat hepatocytes.[189] Many of the benefits of caloric restriction are purportedly mediated by the sirtuin family of protein deacetylases, and both HSF1 and FOXO are regulated by SIRT1 in mammalian cells.[52,190] In the case of HSF1, SIRT1 promotes prolonged DNA binding and Hsp gene expression, providing a possible direct link between nutritional status, the HSR, and protein homeostasis in the aged cell.[52] Pharmacological SIRT1 activators are under development, based on initial indications that the natural product resveratrol mimics caloric restriction in a SIRT1-dependent manner. However, recent findings suggest the mechanisms favoring prolonged lifespan may be more complex than anticipated.[191]

5.2.2. Neurodegenerative disease—A major advance in our understanding of a wide range of neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's, and Lou Gehrig's diseases is the realization that all are fundamentally pathologies caused by the misfolding or altered processing/localization of key proteins. For example, the etiology of Alzheimer's disease is strongly linked with production of the Aβ1–42 peptide and aggregation of the cytoskeletal protein tau. Many of these maladies are diseases associated with aging, and their inception may correlate with the aforementioned decline in protein quality control. Genetic factors also play a role, with familial forms of some neurodegenerative diseases linked to polymorphisms in the causal protein, such as Cu/Zn-superoxide dismutase (SOD-1) mutants that predispose individuals to Lou Gehrig's disease (amyotrophic lateral sclerosis).[192] The preponderance of known protein misfolding pathologies are neuronal in nature, prompting the speculation that neurons are relatively deficient in protein quality control. This hypothesis is supported by evidence that HSF1 is inactive or weakly active in cultured motor neurons, resulting in diminished Hsp production.[193,194] This phenomenon is ameliorated by introduction of constitutively active HSF1, demonstrating that these cells are competent to mount an HSF1-mediated response but fail to do so for reasons that have yet to be determined.[193]

A growing body of evidence indicates that the HSR may play a role in preventing or delaying the onset of age-associated degenerative diseases. Cultured $HSF1^{-/-}$ cells accumulate ubiquitinated proteins and polyQ-fluorescent protein fusions, demonstrating a requirement for mediating clearance of damaged proteins.[195] A recent human genetics study identified an allele of the HspB1 gene that produces lower levels of the sHsp Hsp27 as a contributing factor in a form of Charcot-Marie-Tooth disease, a hereditary neuromuscular disorder.[196] Aβ toxicity was also decreased in an Alzheimer's disease mouse model when lifespan was extended through inhibition of the IGF signaling pathway, which correlates with heightened HSR activity.[197] Balance in the protein homeostasis network appears to be delicately maintained, as the inclusion of metastable folding variants of select proteins enhances the aggregation of SOD-1 and aggregation-prone polyQ fusion proteins in *C. elegans* muscle cells.[198] The converse is also true; overexpression of polyQ proteins causes aggregation and loss of function of the same metastable proteins.[199]

5.2.3. Cancer development and progression—Cells regulate growth and proliferation at a sustainable rate that does not exceed their biosynthetic or degradative capacity. Transformation into the malignant state deregulates this control and inhibits apoptosis, resulting in a highly active, proliferative and physiologically robust cell. Among other diagnostic and prognostic indicators, elevated Hsp levels have been observed in a variety of cancers including those of the prostate and breast.[200] Hsp90, Hsp70, and Hsp27 levels are routinely found to be two to fourfold higher in tumor tissue and still higher in metastatic variants.[201] Hsps may be limiting for cancer transformation, as overexpression of the kinase chaperone Cdc37 is sufficient to induce mammary gland tumors in a transgenic mouse model.[202] Indeed, Hsp90 and Cdc37 promote the activity of a number of pro-growth kinases such as the cyclin-dependent kinases, receptor tyrosine kinases, and Akt.[203] Hsp90

is also required for signaling by steroid hormone receptors, and a chemical genomic search for novel compounds capable of blocking elevated androgen receptor activity linked to prostate cancer identified celastrol and the structurally related compound gedunin as potent inhibitors, highlighting this chaperone as a primary pharmacological target.[65]

The observations that chaperones promote cancer growth and progression extend to global control of the HSR. A recent study revealed elevated levels and constitutive nuclear localization of HSF1 in *in situ* and invasive breast carcinomas, and statistical analysis supports a strong correlation between these characteristics and poor prognosis.[204] These and other results have led to the "Hsp addiction" hypothesis, wherein cancer cells depend on elevated Hsp levels to support their rapid growth and metabolism. This idea is bolstered by the finding that HSF1 is required for tumor formation. $HSF1^{-/-}$ mice exhibit a marked reduction in tumor formation induced by either oncogenic Ras or p53 mutations in mice, and HSF1 knockdown by siRNA technology decreases proliferation of human cancer cell lines.[205] Chaperone-mediated autophagy is an important aspect of cellular recycling and degradation, and was recently shown to be required for tumorigenesis and metastasis, underscoring the degree to which Hsps and chaperones are involved in cancer.[206]

A corollary to the apparently constitutive activation of the HSR is that the resultant Hsp production levels render cancer cells highly resistant to cytotoxic compounds typically used in chemotherapy. Overexpression of Hsp27 increased resistance of a bladder cancer cell line to paclitaxel, while siRNA knockdown had the opposite effect, leading to enhanced apoptosis upon chemotherapeutic treatment.[207,208] Pancreatic cancers are highly resistant to known chemotherapies and are thus extremely difficult to treat. Hsp27 knockdown in a pancreatic cell line resulted in sensitivity to one of the few partially effective drugs, gemcitabine.[209] Similarly, pharmacologic inhibition of Hsp90 with 17-allyamino 17-demethoxygeldanamycin synergizes with suberanoylanilide hydroxamic acid (a histone deacetylase inhibitor) to promote apoptosis in leukemia cell lines.[210] Together these findings suggest that inhibiting HSR activation at the level of HSF1, or selective inhibition of one or more Hsps, may be a promising avenue to explore in the fight against cancer.

5.2.4. Other protein folding maladies—In contrast to the involvement of the HSR in cancer, which appears to be a "gain-of-function" relationship, many human diseases are caused by deficiencies in critical metabolic enzymes due to misfolding and/or degradation caused by spontaneous or inherited polymorphisms. Two lysosomal storage disorders, Gaucher's and Tay-Sachs disease, result from ER-associated degradation of glucocerebrosidase and β-hexosaminidase, respectively. Lysosomal accumulation of glucosylceramide in Gaucher's and GM2 gangliosides in Tay-Sachs leads to the accompanying pathologies, fostering the idea that disease symptoms could be reversed by increasing the amount of enzyme that escapes degradation to be successfully delivered to the lysosome.[211] This has been achieved at the cellular level through the combined use of the proteasome inhibitor MG132 and celastrol to upregulate the HSR and ER stress responses in parallel.[212] A similar effect on multiple lysosomal storage disorders has been observed using Ca^{2+} channel blockers. These agents presumably enhance ER chaperone production through ER stress response induction and activation of Ca^{2+}-dependent chaperones.[213,214]

The role of the protein quality control machinery in regulating the abundance and targeting of the cystic fibrosis transmembrane conductance regulator (CFTR) and known disease-inducing mutants has been heavily investigated. The CFTRΔ508 mutant is a naturally occurring variant that is misfolded within the ER and selected for ER-associated degradation, greatly reducing the amount of functional channel at the cell surface, which ultimately leads to altered chloride and water transport across the airway epithelium. The synthesis of CFTR is regulated by ER and cytosolic chaperones as well as the ER-associated

degradation and proteasomal machinery, providing multiple opportunities for pharmacologic intervention. In support of this notion, *S*-nitrosation of the Hsp70/Hsp90 organizing protein (Hop) increases cell surface expression of CFTRΔ508, as does downregulation of other Hsp90 co-chaperones including Aha1.[215,216] Induction of Hsp70 expression via treatment with 4-phenylbutyrate rescues CFTRΔ508 processing, raising the possibility that the next generation of Hsp70 activators may have significant therapeutic potential in this and other diseases.[217]

5.3. Efficacy of HSR Activators in Disease Models

Pharmacological HSR activators show promise for slowing the onset of age-related phenotypes and degenerative diseases in various animal models. For instance, Hsp90 inhibitors that activate HSF1 are capable of ameliorating cytotoxicity in an Alzheimer's disease model with only moderate induction of Hsps.[218] Another recently described HSR activator, the benzyl pyrazole derivative HSF1A, abrogates polyQ-dependent toxicity in neuronal cells.[68,219] Similarly, several HSR activators with Michael acceptor properties decrease overall protein aggregation and motility defects in worms expressing expanded polyQ proteins.[105] In addition, administration of geranylgeranylacetone, an HSR activator that interacts with the substrate binding domain of Hsp70, protects against degenerative cell death in mouse models of spinal and bulbar muscular atrophy, age-related hearing loss, and ischemia-reperfusion tissue injury.[220–223] Collectively, these findings suggest that pharmacological manipulation of the HSR will be of benefit in a variety of disorders of both an acute and chronic nature.

However, several issues must be resolved before HSR activators may be of clinical use in humans. A number of current HSR activators show moderate to significant cytotoxicity, including hepatotoxicity, at the higher concentrations that would be required in clinical trials. In addition, many of these compounds are reactive and therefore have numerous targets and signaling effects that occur independently of HSR activation. Another challenge to consider is that prolonged HSR activation may not always be desirable, as it could lead to the onset of other conditions (e.g., cancer) later on. Therefore, current efforts in many laboratories are focused on generating derivatives of HSR activators with increased potency and specificity while limiting toxicity and additional complications that may arise from long-term administration.

5.4. Future Work

Because of their ability to fundamentally alter the stability, processing, or localization of multiple substrates, compounds that modulate aspects of protein quality control including the HSR, ER stress responses, and the degradation machinery have been collectively dubbed "proteostasis regulators." As described earlier in this review, these compounds, both natural and synthetic, have the capacity to significantly impact human disease. Most exciting is the apparent synergy with pharmacologic chaperones, small molecules that stabilize protein folding states or otherwise promote the activity of targets expressed at low levels. Current work has revealed a number of relatively broad-acting compounds whose therapeutic utility is compromised by frequent cytotoxicity or bioavailability issues. Therefore, an important goal for the immediate future will be the rational design of molecules that build on these findings to provide a much higher degree of specificity, while retaining efficacy, either alone or as adjuvants to increase the success of traditional therapies. An additional area that needs exploration is determining whether altering the HSR to treat one disorder leads to the onset of others, given that the HSR can play opposing roles in specific diseases (Fig. 11). Determining appropriate dosing for HSR activators and inhibitors will be key in validating their efficacy in treating a given disorder without triggering another. Nonetheless, by pharmacologically targeting the HSR, we can envision enhancing the protein homeostasis

network as a general means to ameliorate loss of function problems in proteins in aging, metabolic disease, and neurodegenerative disease or selectively interrupting it to slow the development and spread of cancer (Fig. 11). Given the recent advances and success of *in vitro* models, it is conceivable that treatments of protein folding disorders may be among the first to realize the potential of personalized medicine.

Acknowledgments

Funding Support. Work in the authors' laboratories is sponsored by a Cottrell College Science Award from Research Corporation for Science Advancement (to J.D.W.), the Luce Fund for Distinguished Scholarship from The College of Wooster (to J.D.W.), and NIH grant GM074696 from NIGMS (to K.A.M.). Y.W. was supported by the Cameron Foundation.

We thank Sandy Westerheide and Aaron Jacobs for critically reading the manuscript.

Abbreviations

HSR	heat shock response
HSF1	heat shock factor 1
HSE	heat shock element
Hsp	heat shock protein
UPR	unfolded protein response
SUMO	small ubiquitin-like modifier
HR	heptad repeat
SIRT1	sirtuin 1
ROS	reactive oxygen species
PG	prostaglandin
15d-PGJ$_2$	15-deoxy-$\Delta^{12,14}$-PGJ$_2$
HNE	4-hydroxy-2-nonenal
sHsp	small Hsp
CFTR	cystic fibrosis transmembrane conductance regulator
Hop	Hsp70/Hsp90 organizing protein

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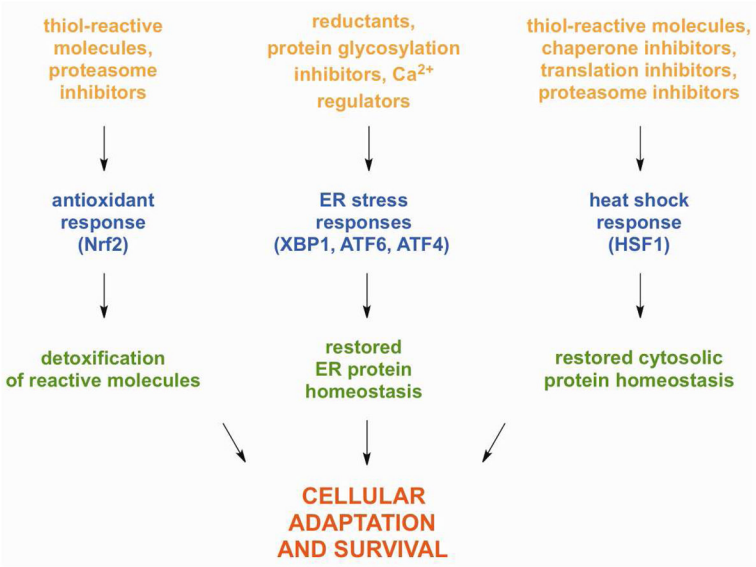

Figure 1. Parallel Stress Pathways

The antioxidant response, ER stress response, and HSR pathways help maintain cellular homeostasis following exposure to different environmental stresses.

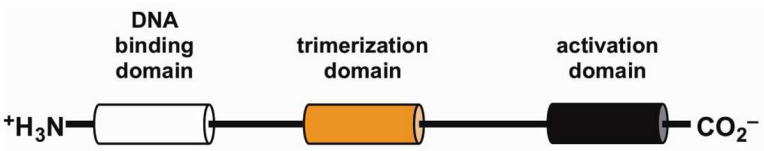

Figure 2. HSF1 Domain Structure

HSF1 has three principal domains that facilitate its regulation of Hsp genes in response to stress. The DNA binding domain can bind inverted 5′-nGAA-3′ repeats upon trimerization of monomeric HSF1 through its trimerization domain. The activation domain near the *C*-terminus is the site of many inducible post-translational modification following exposure to various stresses.

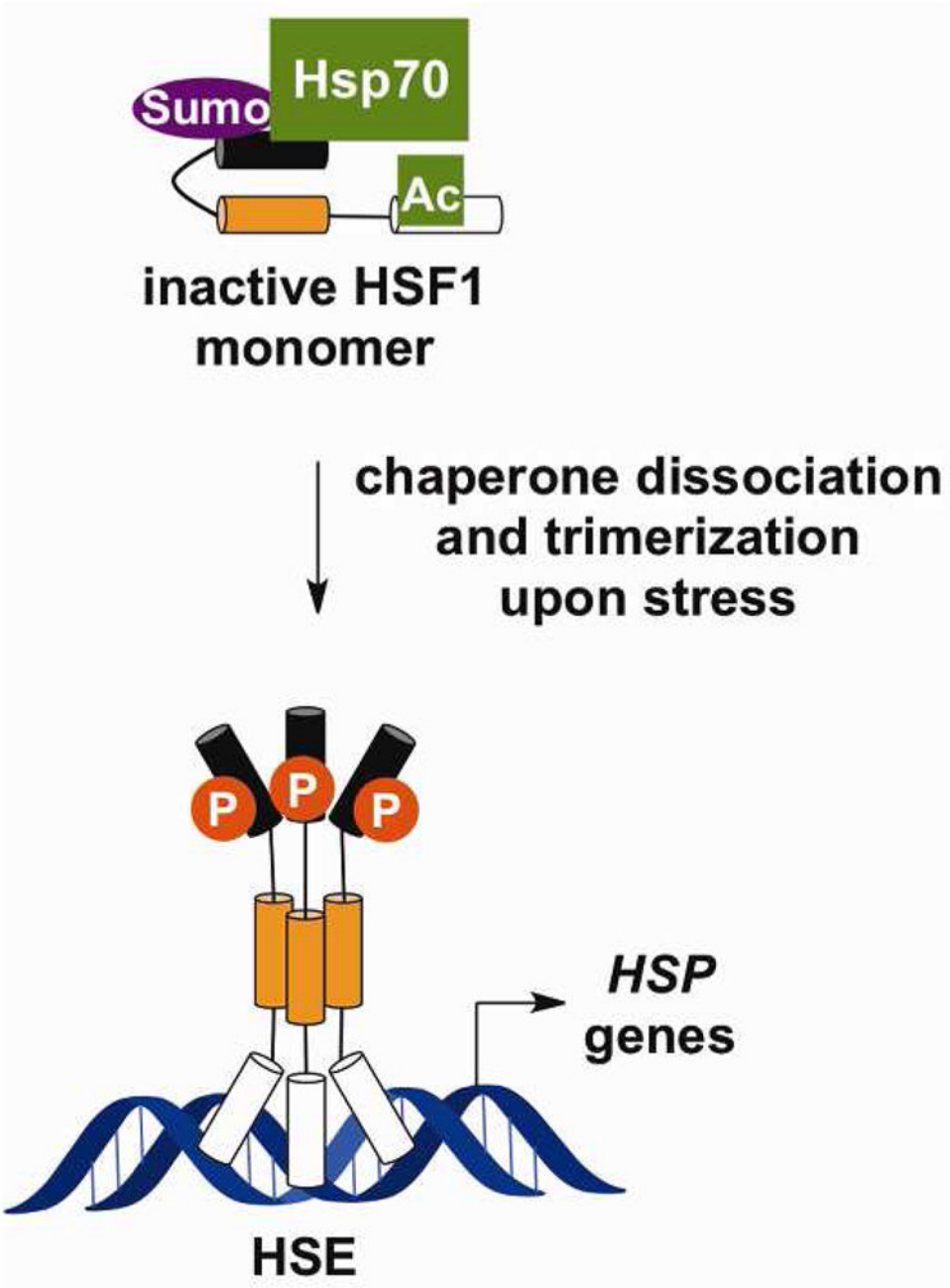

Figure 3. Steps in HSF1 Activation

HSF1 activation following exposure to protein-damaging stresses often involves its dissociation from various negative regulators (including Hsp70 and Hsp90), its trimerization, and its inducible post-translational modification. Post-transalational modifications to HSF1 include sumoylation (SUMO), acetylation (Ac), and phosphorylation (P).

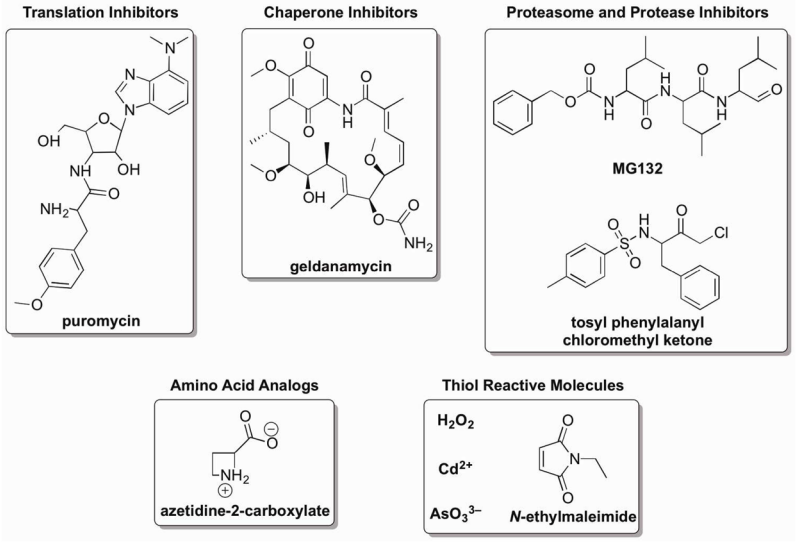

Figure 4. Families of Molecules That Activate HSF1

HSF1 activators can perturb various aspects of protein homeostasis including translation, protein folding, processing, and degradation.

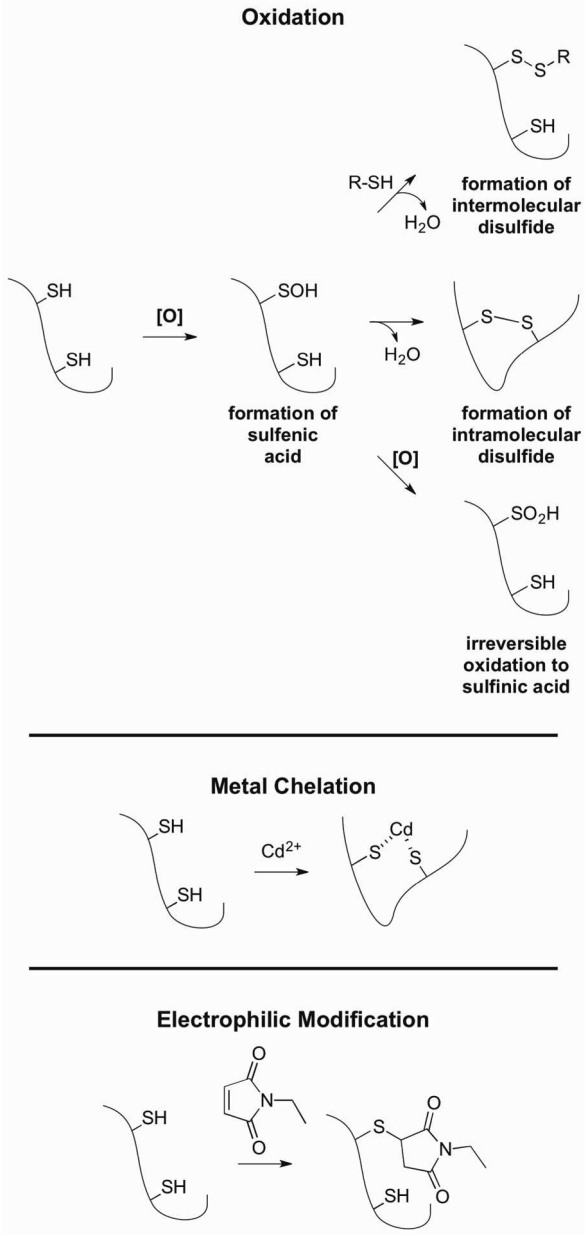

Figure 5. Thiol-Modifications by Oxidants, Transition Metals, and Organic Electrophiles

Oxidants, metals and metalloids, and organic electrophiles carry out different modifications on reactive Cys residues in target proteins.

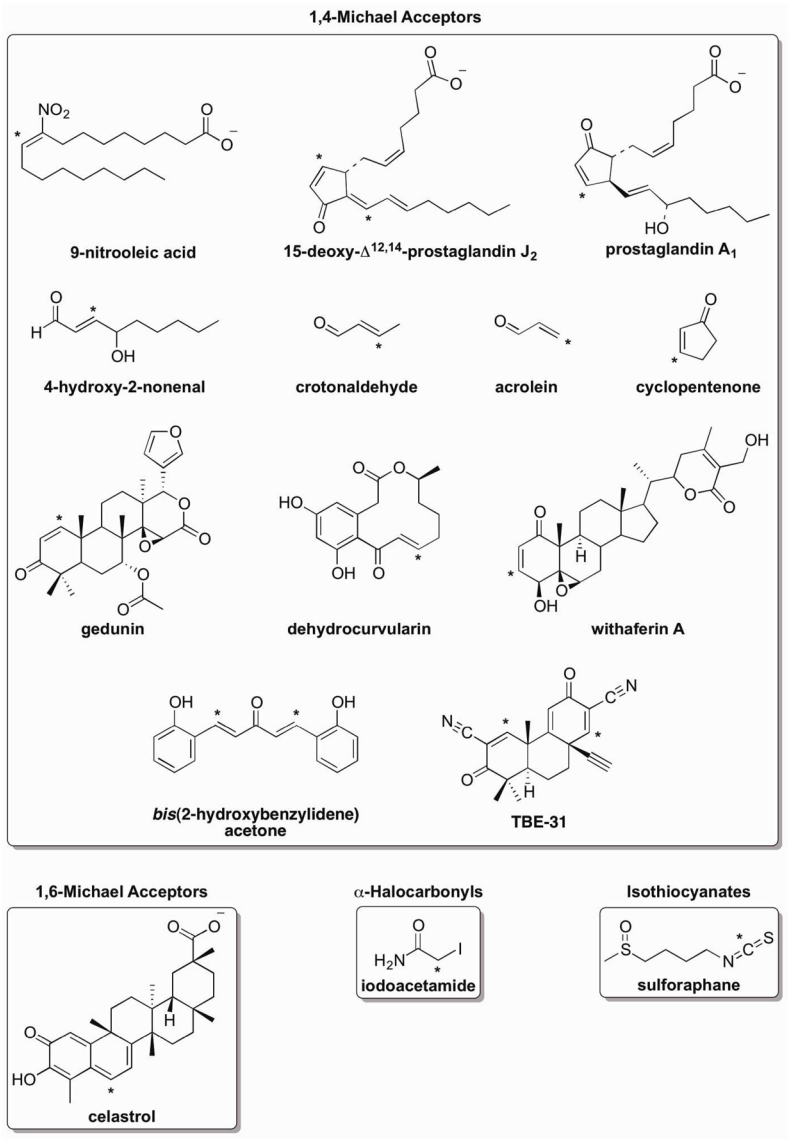

Figure 6. Classes of Organic Electrophiles That Activate HSF1

Electrophiles that activate HSF1 are grouped according to the type of addition reaction that they carry out with thiol groups. An asterisk (*) indicates where thiol groups are likely to add within these molecules. More information about the physicochemical properties of these molecules and their thiol adducts is provided in Table 1.

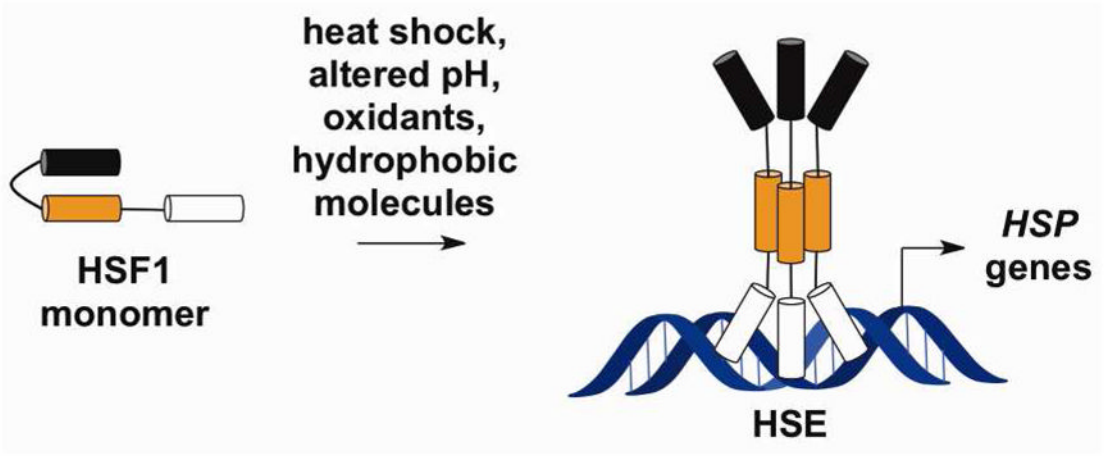

Figure 7. Direct Regulation of HSF1 Under Stress Conditions

Upon exposure to several stress conditions *in vitro*, HSF1 undergoes trimerization and shows increased DNA binding.

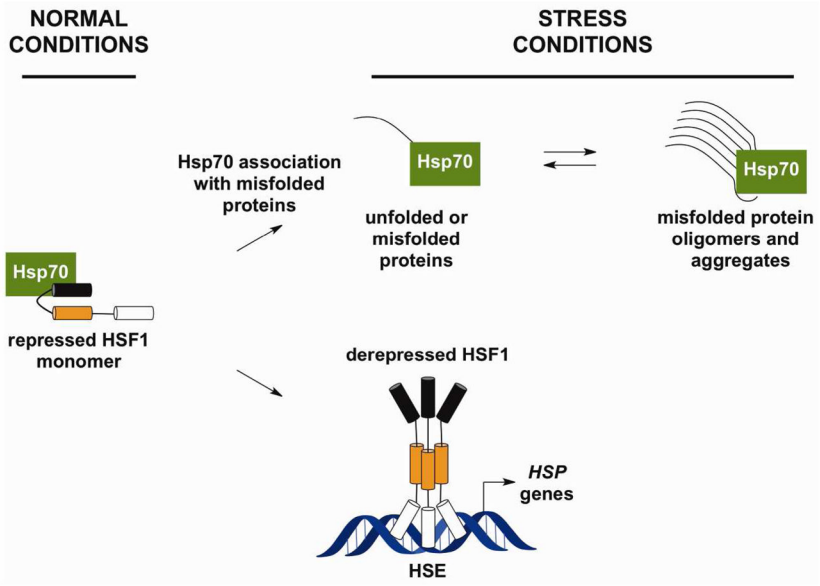

Figure 8. Stress-Induced Derepression of HSF1 Activity

Molecular chaperones (e.g., Hsp70 and Hsp90) that bind to and repress HSF1 activity are recruited to sites of misfolded polypetides and aggregated proteins that accumulate upon exposure to many stresses, allowing HSF1 to trimerize and bind DNA. For simplicity, only Hsp70 is shown as a negative regulator in complex with HSF1.

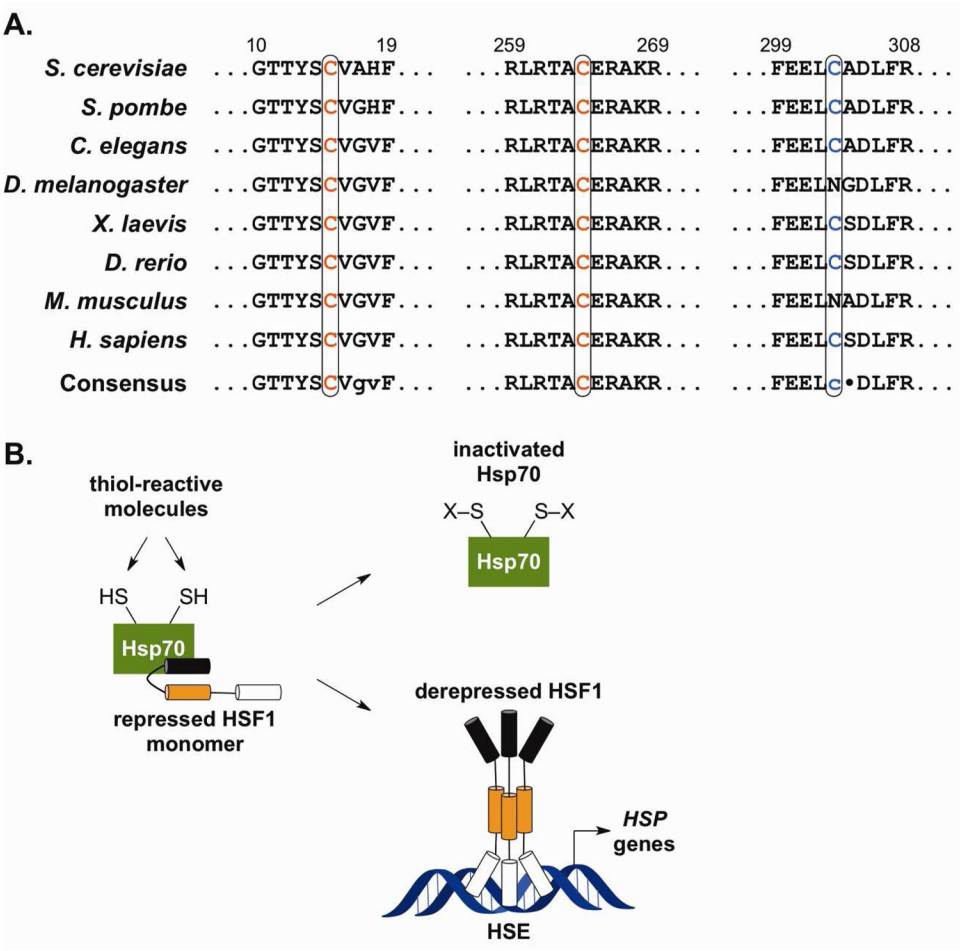

Figure 9. Direct Sensing of Thiol-Reactive Compounds by Hsp70

(A) Conservation of Cys residues in Hsp70 homologs among various eukaryotic species. C264 and C303 are sites of alkylation in the *S. cerevisiae* Hsp70 Ssa1. (B) Hypothetical model for activation of HSF1 by thiol-reactive molecules. Hsp70 is modified on conserved Cys residues by reactive molecules, leading to its dissociation from HSF1 and subsequent steps in HSF1 activation.

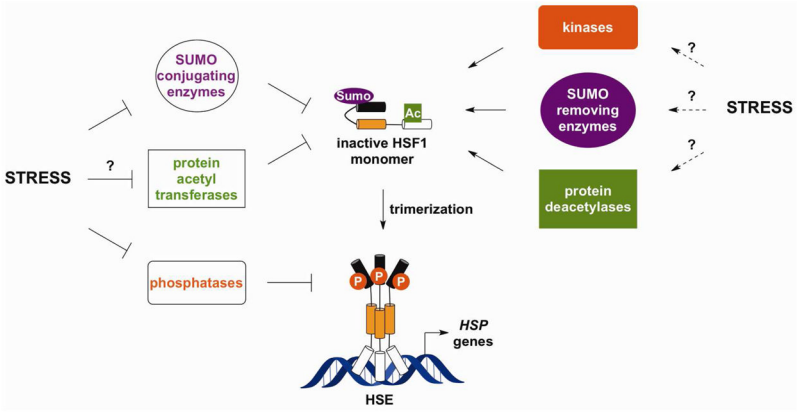

Figure 10. Sensing by Enzymes Involved in Post-Translational Modification of HSF1

Stresses that activate the HSR may influence the activities of multiple enzymes that facilitate HSF1 post-translational modification.

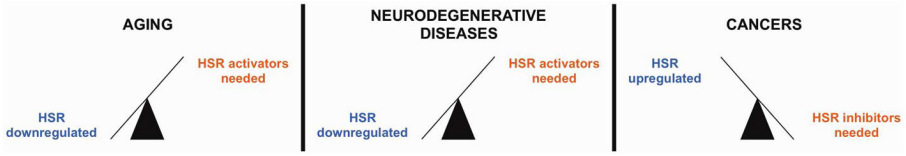

Figure 11. Model for Therapeutic Utility of Pharmacological HSR Regulators

The HSR is altered in aging and several disease states. As a possible way of intervening in each of these physiological states, pharmacological regulators of HSF1 activity may be used to restore the HSR to normal activity levels.

Table 1

Electrophilic Activators of the Heat Shock Response in Cultured Mammalian Cells.

Class	Electrophile	Origin	Active Concentrations[a]	Predicted logP[b]	Adduct Properties
1,4-Michael Acceptors					
	nitrooleic acid	lipid nitration	1–5 μM[100]	6.6	reversibility[111]
	15d-PGJ_2	lipid metabolism	~5–15 μM[94,95]	4.1	cross-linking[113,114]
	PGA_2	lipid metabolism	20–100 μM[92,93]	2.6	–[c]
	HNE	lipid peroxidation	20–60 μM[97,132]	1.5	hemiacetal formation; cross-linking[116–118]
	crotonaldehyde	lipid peroxidation; combustion	40–80 μM[99]	0.5	cross-linking[d]
	acrolein	lipid peroxidation; combustion	~100 μM[98]	0.1	cross-linking[115]
	cyclopentenone	synthetic	250–1000 μM[104]	0.3	–
	gedunin	natural product	1–5 μM[102]	2.9	–
	dehydrocurvularin	natural product	1–5 μM[102]	2.0	–
	withaferin A	natural product	1–5 μM[102]	2.6	–
	bis(2-hydroxybenzylidene) acetone	synthetic	1–5 μM[119]	3.2	cross-linking[120],[d]
	TBE-31	synthetic	0.1–0.5 μM[119]	2.5	cross-linking,[d] reversible[121],[d]
1,6-Michael Acceptors	celastrol	natural product	1–10 μM[16]	6.9	reversibility[112],[d]
α-Halocarbonyls	iodoacetamide	synthetic	50–100 μM[103]	0.1	–
Isothiocyanates	sulforaphane	natural product	5–10 μM[101]	−0.5	reversibility[110]

[a] Values are estimated from literature reports cited in various mammalian cell lines.

[b] logP values were predicted using ChemDraw.

[c] Dashes (−) indicate predicted lack of reversibility, cross-linking, or other adduct rearrangement.

[d] Predicted based on structure.