Structural and Functional Evolution of Human Heat Shock Transcription Factors

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Proteotoxic stress is implicated in numerous human diseases including neurodegeneration, cancer, and diabetes. Unfortunately, our mechanistic understanding of the cellular response to proteotoxic stress is limited. A critical feature of the cellular stress response is the activation of Heat Shock Transcription Factors (HSFs) that regulate the expression of numerous genes involved in protein folding, protein degradation, and cellular survival. The studies presented here utilize a diverse array of techniques including yeast genetics, recombinant protein expression and purification, biochemical analysis of protein-DNA interactions, x-ray crystallography, in vitro post-translational modification, and mammalian cell culture to illuminate novel aspects of HSF biology. Critical findings include understanding key principles of HSF-DNA interactions, identification of a novel negative regulator of HSF activity, and identification of structural features of HSF paralogs that enable precise combinatorial regulation. These unique insights lay the foundation for a greater understanding of HSF in specific cellular contexts and disease states.
Dedication

This work is dedicated to my parents, Marlene and Christopher.
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Acknowledgements

I am grateful to all members of the Thiele Laboratory for their insightful and rigorous discussion of this work. I thank Professor Lea Sistonen, who graciously hosted me in her laboratory at Abo Akademi in Turku, Finland. I would also like to acknowledge Dr. Charles W. Pemble IV who patiently taught me crystallography.
1. Protein Homeostasis and Cellular Stress

The central dogma of biology states that “the coded genetic information hard-wired into DNA is transcribed into individual transportable cassettes, composed of messenger RNA (mRNA); each mRNA cassette contains the program for synthesis of a particular protein (or small number of proteins).” While this fundamental concept in biology serves as a foundation for a plethora of biological studies, it does not account for the critical importance of maintaining proteins once they have been synthesized. After a protein is translated from the primary amino acid sequence, it must be correctly folded into specific three-dimensional structures to enable proper function. The assembly of proteins into three-dimensional structures is required for nearly every cellular protein from transmembrane proteins to transcription factors (1). After proteins are properly folded, they must be maintained in a functional and properly folded state through interactions with other proteins, post-translational modifications, and proper subcellular localization. The maintenance of this critical facet of cellular biology is known as “protein homeostasis” (2,3).

At the core of protein homeostasis is maintaining the enormous diversity of cellular proteins in their folded and functional state. Early experiments exploring protein folding are founded in the classic Anfinsen Experiment (4). Christian B. Anfinsen postulated that all of the information necessary for a protein to correctly fold is encoded in the primary amino acid sequence. To test this hypothesis, Anfinsen
denatured the model protein RNase A using urea and reducing agents in vitro. Then, by removing the denaturants, Anfinsen was able to demonstrate that RNase A could refold and retain biological activity in the absence of any other cellular factors. However, as research into protein folding increased, it was soon realized that RNase A was indeed an outlier in this regard, and that the majority of cellular proteins require accessory factors to properly fold into three-dimensional structures required for their biological activity (5,6). While the Anfinsen experiment established a concept that we now know is largely incorrect, it served as a foundation and a critical stimulus for exploring the process of protein folding.

As protein folding is an essential aspect of cellular biology it is important to understand situations where protein folding is disrupted (7). Common stimuli that perturb protein folding are environmental and/or physiological stressors. Environmental stressors include but are not limited to temperature, exposure to toxins, infection, and mechanical stress (8-10). Physiological stress can arise from developmental stimuli, growth signals, or diseased states (11). These diverse stressors result in protein damage from oxidation, degradation, and/or misfolding and aggregation (12,13). Continued investigation into environmental stress led to the discovery of cellular mechanisms that are induced to prevent protein damage and maintain protein homeostasis. We now know that a fine balance of cellular protein homeostasis is required for cellular survival.
and disruptions in this delicate balance can lead to a variety of different disease states (Figure 1).

![Diagram of Protein Homeostasis]

Figure 1. Schematic of Protein Homeostasis. Mediators of Protein Homeostasis (green) assist in properly folding proteins, prevent misfolding of proteins, and also promote the proper degradation of cellular proteins through the proteasome or autophagy. Disruptions in Protein Homeostasis can lead to diseases such as neurodegeneration and cancer.

1.1 Protein Homeostasis and Human Disease

The importance of maintaining protein homeostasis is highlighted by an increasing number of reports implicating protein homeostasis in human diseases including but not limited to neurodegeneration, diabetes and cancer (14-16). For example, a common feature of neurodegenerative diseases such as Parkinsons’ Disease, Huntington’s Disease, Alzheimer’s Disease, and Amyotrophic Lateral Sclerosis is the presence of proteinaceous inclusions in neuronal populations that undergo cell death during disease progression (17). The extensive neuronal cell death observed in these
diseases can result in devastating neurological consequences including loss of
movement control, depression, and psychosis. While the precise mechanisms that cause
neuronal cell death are not known, the common feature of numerous neurodegenerative
diseases is protein aggregation, suggesting that protein homeostasis is at the core of
disease etiology. This concept is further corroborated by numerous studies
demonstrating that amplifying protein homeostasis machinery ameliorates disease
phenotypes in cell culture, fruit fly, and mouse models of neurodegeneration (18-21).

Fundamentally, neurodegenerative disease results from increased neuronal cell
death. In contrast, cancer results from the ability of cells to abnormally prevent cell
death. Interestingly, protein homeostasis mechanisms that are disrupted in
neurodegeneration are enhanced in cancer cells reinforcing the notion that at its core,
protein homeostasis is central to cell survival (22). The amplification of protein
homeostatic mechanisms may enable cancer cells to maintain the hyperproliferative
state and support the rapid and continued synthesis of cellular proteins. Indeed,
disruption of protein homeostasis mechanisms in cancer cells may prove a beneficial
therapeutic strategy for improved phenotypes in a variety of cancers including breast,
lung, colon, and kidney among others (22-24). Taken together, these observations
underscore the importance of understanding key regulators of protein homeostasis. A
greater understanding of these key regulators may eventually lead to the development
of therapeutic strategies that can ameliorate human diseases including neurodegeneration and cancer.

1.2 The Heat Shock Response

A major discovery of mechanisms that regulate protein homeostasis can be attributed to Feruccio Ritossa (25). In his famous 1962 experiment, Ritossa exposed *Drosophila melanogaster* salivary gland cells to elevated temperatures, also known as a “heat shock”. The heat shock resulted in characteristic “puffing” patterns in specific regions of the *Drosophila* chromosomes. This observation provided a foundation for additional experiments by numerous labs that led to the discovery that the puffing pattern was a result of increased transcription occurring at those genomic loci (26). The identification of the genes within these loci led to the discovery of heat shock proteins (Hsps) (5). We now know that heat shock proteins are a family of protein chaperones that assist in the proper folding, function, and degradation of a variety of cellular proteins. The increased transcription of protein chaperone genes, the Hsps, was termed the Heat Shock Response (27).

1.2.1 Protein Chaperones

Protein chaperones comprise a larger family of cellular proteins that functions to assist in the proper folding of the cellular proteome. A major class of protein chaperones is the Heat Shock Proteins (Hsps). Hsps are a highly conserved family of protein chaperones that have diverse functions in maintaining protein homeostasis (28). A
number of Hsp subclasses have been identified and are named according to their molecular weight. The Hsp40, Hsp70, and Hsp90 (40 kDa, 70 kDa, 90 kDa) families of Hsps are perhaps the best studied of the Hsps. In addition, Hsp25 and small Hsps (sHsps) are essential for other cellular processes and can function in concert with Hsp40, 70 and 90 (29). It is also important to note that Hsps function in obligate heterocomplexes with co-chaperones that can facilitate substrate recognition and serve as “receptors” for the folded products of the chaperone machinery (30). The importance of Hsps for the maintenance of protein homeostasis has prompted the discovery of numerous small molecule inhibitors to be used for cancer therapeutics (31, 32).

Hsp90 is among the most abundant proteins in the cell and is estimated to comprise 1-2% of the expressed proteome (33). Such abundance enables Hsp90 to engage in numerous functional complexes with kinases, transcription factors, nuclear hormone receptors, and other members of the protein chaperone family (34-36). Many pro-growth and oncogenic signaling cascades are supported by Hsp90 chaperone activity, which has pinpointed Hsp90 as an attractive therapeutic target in cancer (33). Small molecule inhibitors of Hsp90 are in various stages of clinical development and have shown efficacy in a number of different cancers (37). At the molecular level, treatment with Hsp90 inhibitors results in the degradation of Hsp90 client protein including Akt, Erk, ER, AR, GR, and other pro-oncogenic proteins (23).
The Group I and Group II Chaperonins are protein chaperone complexes that are functionally and structurally distinct from Hsps (38). The Group I Chaperonins are represented by the bacterial GroEL/ES system (39). GroEL/ES is a cage like structure that is formed by a “double donut” structure comprised of 2 rings of seven subunits of the GroEL protein (40). In an ATP and substrate dependent reaction, the GroEL complex interacts with a heptameric GroES lid that forms a cage that facilitates the proper folding of the substrate (41). Upon hydrolysis of ATP, the folded substrate is released from the cage and GroEL and GroES dissociate. In eukaryotes, the Group II chaperonins function in a similar fashion but utilize a heteromeric complex containing between 7 and 9 subunits (42,43). Subunits in Group II Chaperonins contain functionally analogous components of the GroEL and GroES system and instead of the lid attaching to the double donut structure, an ATP dependent conformational change closes the cage for substrate folding (44). In mammals, the cytosolic Group II chaperonin is the Tcp ring complex/chaperonin containing Tcp1 (TRiC/CCT) which has been shown to fold ~10% of all cytosolic proteins (45). In eukaryotes, Hsp60 is a mitochondrial Group I chaperonin that closely resembles GroEL. The precise role of Hsp60 in mitochondrial function is not well understood. In conjunction with Hsps, chaperonins function to maintain protein homeostasis in basal and stressful conditions and as such, are also target of the HSR. The obvious implications for protein chaperones in numerous aspects of biology support the investigation into mechanisms that regulate protein chaperone gene expression.
1.2.2 Heat Shock Transcription Factors

Decades after Ritossa’s initial discovery, the Heat Shock Response (HSR) has been explored in diverse model organisms and has implications in numerous aspects of cellular biology. At its core, the HSR promotes cellular survival during proteotoxic stress by transcriptionally upregulating genes that maintain protein homeostasis. The ubiquitous requirement for protein chaperone expression highlights the importance for understanding the mechanisms by which Hsp genes are transcriptionally upregulated by the HSR. In *E. coli*, the HSR is executed by the sigma factor $\sigma^{32}$ (46). Under non-stressed conditions, $\sigma^{32}$ is maintained in a repressed state by direct interactions with *E. coli* protein chaperones including DnaK, DnaJ, and GroEL/ES (39). Upon induction of proteotoxic stress, protein chaperones are titrated away from $\sigma^{32}$ to bind to the increasing number of misfolded proteins and ameliorate proteotoxicity. As a result, $\sigma^{32}$ is relieved from repression, localizes to the promoters of target genes, recruits RNA Polymerase II, and transcriptionally upregulates a number of genes including those encoding protein chaperones. When the proteotoxic stress is relieved, $\sigma^{32}$ is then degraded through mechanisms that utilize the increased levels of protein chaperones that result from $\sigma^{32}$ activation. This elegant feedback mechanism allows bacteria to finely tune the expression of protein chaperones for a given cellular environment.

In eukaryotic cells, the HSR is executed by the Heat Shock Transcription Factors (HSF) (11). In the baker’s yeast *Saccaromyces cerevisiae*, a single isoform of HSF is
expressed and is essential for viability (47,48). It is thought that the essentiality of HSF in yeast is due to the need for basal expression of protein homeostasis machinery during yeast growth. In mammals, multiple HSF family members have been identified and characterized and the number of HSF paralogs expressed is dependent on the species (49). In humans, three HSFs are expressed and are known as HSF1, HSF2, and HSF4. Similar to E. coli σ^{32}, eukaryotic HSFs sense and respond to proteotoxic stress through direct and indirect interactions with protein chaperones (50,51). The remarkable conservation of sophisticated mechanisms that regulate the HSR underscore the importance of this fundamental cellular response and a greater understanding of these mechanisms in mammalian cells could aid in the discovery of novel therapeutic targets (Figure 2).

Figure 2. Transcriptional responses to proteotoxic stress are evolutionarily conserved. E. coli σ^{32} and eukaryotic HSFs have similar regulatory inputs and regulate gene expression of protein homeostasis machinery.

HSFs are multi-domain transcription factors that are highly conserved from yeast to humans (Figure 3) (27,52,53). Mammalian HSFs contain an amino terminal winged helix-turn-helix DNA binding domain (DBD), a leucine-zipper 1-3 domain (LZ1-3) that
promotes HSF trimerization, a large regulatory domain (RD), a leucine zipper 4 domain (LZ4), and a carboxy-terminal transcriptional activation domain (AD). The HSF DBD recognizes inverted arrays of the DNA sequence nGAA n, which is termed a Heat Shock Element (HSE) (54-57). The HSE sequence is highly conserved in HSF target genes, including those encoding protein chaperones, and as a consequence, the HSF DBD is also highly conserved through eukaryotic HSF family members. The LZ1-3 domain is proposed to form a large coiled-coil interaction with adjacent HSF molecules to facilitate trimerization (47,58,59). The RD of HSFs is predicted to be largely unstructured and undergoes extensive post-translational modification (60,61). LZ4 is thought to form an intramolecular coiled-coil interaction with LZ1-3 to repress trimerization (56,62). While the HSF AD has not been thoroughly characterized, it has been implicated in the recruitment of RNA Pol II to target gene promoters (50).

**Figure 3. Illustration of HSF Domain architecture.** The amino-terminal DNA Binding Domain (DBD) is a member of the “winged” helix-turn-helix family of DNA binding proteins. The Leucine Zipper 1-3 (LZ1-3) domain consists of heptad repeats that form a coiled-coil and facilitate trimerization. The Regulatory Domain (RD) is largely unstructured and is extensively post-translationally modified. Leucine Zipper 4 (LZ4) forms an intramolecular coiled-coil interaction with LZ1-3 to repress trimerization. The Activation Domain (AD) is critical for recruitment of RNA Pol II to target genes.
While a large body of evidence highlights the similarities of HSFs, it is important to note that mammalian HSF family members display a variety of distinct features such as differing propensity to form coiled-coil interactions, differential post-translational modification and distinct protein-protein interactions. The distinct features of mammalian HSF family members may provide the opportunity for context dependent or tissue specific regulation of protein homeostasis.

1.2.3 Heat Shock Transcription Factor 1 (HSF1)

Similar to bacterial σ^32, mammalian HSF1 is maintained in a repressed state under normal conditions (50). This is achieved through a functional interaction with a complex of protein chaperones including the Heat Shock Proteins kDa 90, 70, and 40 (Hsp90, Hsp70, Hsp40). Importantly, a direct interaction between HSF1 and Hsp90, Hsp70, or Hsp40 has not been explicitly demonstrated. Upon exposure to proteotoxic stress, HSF1 multimerizes and is released from the repressive chaperone complex and accumulates in the nucleus to bind Heat Shock Elements (HSEs) in target genes (Figure 4) (63).
Figure 4. Current model of HSF1 Activation. In nonstressed conditions, HSF1 is an inactive monomer. Upon induction of stress, HSF1 trimerizes, accumulates in the nucleus, and binds to Heat Shock Elements in promoter of target genes such as protein chaperones.

The attenuation phase of HSF1 activation is not well understood but it has been proposed that as chaperones accumulate, a negative feedback is initiated to diminish HSF1 activity and restore the basal state. Hsp70 has been demonstrated to interact with the HSF1 AD during attenuation but the specific consequences of this interaction have not been extensively explored (50).

Despite decades of work on the activation and regulation of HSF1 in diverse model systems, we know surprisingly little about HSF1 structure. To date, the only high resolution, crystallographic information of HSFs is from the pioneering work of Littlefield and Nelson on the K. lactis HSF DBD published in 1999 (64). The structure revealed that HSF is a member of the winged helix-turn-helix DBD family. Unlike other members of this family, HSF does not employ the “wing” domain at the DNA binding interface but rather utilizes the wing to form a dimer interface between adjacently bound DBDs. Obtaining high resolution structural data for mammalian HSFs has been difficult.
and a better understanding of the molecular features of mammalian HSFs will be essential for future insights into its function and mechanism of action.

HSF1 has been implicated in numerous human diseases, including neurodegeneration, cancer, diabetes, and traumatic brain injury, among others (65). The importance of HSF1 in neurodegenerative disease is highlighted by two pioneering studies: 1) A small molecule activator of HSF1, HSF1A, was shown to ameliorate disease phenotypes in cell culture and fruit fly models of polyglutamine disease (18) 2) The R6/2 mouse model of Huntington’s Disease phenotype is ameliorated by overexpressing HSF1 (21) (Figure 5).

Figure 5. Evidence for the role of HSF1 in neurodegeneration. On the left is a polyglutamine model of neurodegeneration in D. melanogaster. When the toxic polyglutamine protein is expressed in the photoreceptors of the fly eye, extensive cell death is observed. When flies are fed an activator of HSF1, HSF1A, the cellular death phenotype is ameliorated. Shown on the right are survival curves of the R6/2 Huntington’s Disease Mouse model. Transgenic mice overexpressing HSF1 exhibit increased survival (18,21).

Moreover, an increasing body of evidence suggests that HSF1 activity is reduced during the progression of neurodegenerative disease, albeit through unknown
mechanisms. This may be explained by the fact that HSF1 activity is drastically reduced in aged, post-mitotic neurons when compared to other tissues (66-68). The impairment of HSF1 activation in neuronal populations may render them particularly vulnerable to proteotoxic stress. These studies, and others, have supported the notion that amplification of HSF1 activity would be beneficial for neurodegenerative diseases and underscore the importance of understanding HSF1 regulation.

While HSF1 activation has been proposed to be beneficial for neurodegenerative disease, the opposite is true for HSF1 in cancer. Pioneering studies from the Lindquist Lab have discovered that HSF1 is a multifaceted modifier of carcinogenesis and supports the malignant state (15). This was first demonstrated in a mouse model of melanoma where HSF1 null mice were resistant to tumor formation and exhibited improved survival (Figure 6). Follow up studies to this work demonstrated that HSF1 binds to a unique fingerprint of target genes in cancer cells to regulate a diverse pro-survival transcriptional program that is largely distinct from that activated by proteotoxic stress (69). In addition, HSF1 is activated in the tumor microenvironment in stromal cells to further support growth of the hyperproliferative cancer cells (70). As HSF1 appears to undergo distinct regulation in cancer cells, it will be important to illuminate these mechanisms to inform future therapeutic interventions.
1.2.3.1 HSF1 Post-Translational Modifications

HSF1 is extensively decorated by post-translational modifications. After stress, HSF1 is hyperphosphorylated on at least 15 serine residues in the regulatory domain and a number of serines outside of the RD (71). The hyperphosphorylation of HSF1 is indicated by an electrophoretic mobility shift that has been widely used as an indicator of HSF1 activation. Importantly, both activating and repressive phosphorylation events have been reported for HSF1 (61). Activating phosphorylation events include the modification of Ser230 and Ser326. In contrast, phosphorylation of Ser303 and Ser307 has been characterized as a repressive event that promotes SUMOylation of HSF1 at Lys298 through a phosphorylation dependent SUMOylation motif (PDSM) (72). A recent study mutating all serine residues in the RD found that serine phosphorylation in the RD is dispensable for full HSF1 activation, reinforcing the need for a greater understanding for the role of phosphorylation in HSF1 regulation (73).
The precise role of Small Ubiquitin Like Modification (SUMOylation) of HSF1 on Ser303 is not well understood. The enzymatic cascade that promotes the SUMOylation of proteins is analogous to ubiquitination where an E1 activation enzyme, E2 conjugation enzyme, and E3 ligase function to covalently modify lysine residues (74). SUMO proteins are represented by SUMO1, SUMO2, SUMO3, and SUMO4. SUMO2 and SUMO3 appear to be more similar to each other than SUMO1 and SUMO2/3 impart different functional consequences on recipient proteins than SUMO1. The SUMOylation of HSF1 at Ser303 has been attributed to SUMO2 and is currently thought to reduce HSF1 activity, but empirical evidence for this phenomenon is lacking. Recent reports analyzing the SUMO proteome have identified HSF1, as well as HSF2, to be SUMO2 targets but the regulatory consequences of these SUMOylation events have not extensively been explored (75-77).

HSF1 is also acetylated on numerous lysine residues (78). Perhaps the best studied of the acetylation events is the acetylation of HSF1 on Lys80. Lys80 is within the HSF1 DBD and is predicted to directly contact the phosphate backbone of DNA, an interaction that would be physically blocked through acetylation. The deacetylating enzyme Sirtuin 1 (SIRT1) was also identified to promote HSF1 activity through deacetylation of Lys80. In addition, a recent report suggested that p300 acetylates the RD, which promoted the ubiquitination and degradation of HSF1 (79). These acetylation
events occurred following a transient heat shock, suggesting that this mechanism may contribute to the attenuation phase of HSF1 activity.

1.2.3.2 HSF1 Protein-Protein Interactions

In addition to functional interactions with protein chaperones, HSF1 physically interacts with numerous other proteins with diverse functional categories. A recent study from the Nakai Laboratory identified an interaction between HSF1 and Replication Protein A subunit kDa 70 (RPA70) (80). Interestingly, the interaction between these proteins appears to occur under basal conditions and promotes access of HSF1 to the proximal HSE of the Hsp70 promoter. Furthermore, the interaction was mapped to a specific region of the “wing” domain of the HSF1 DNA binding domain and the single stranded DNA binding domain-A of RPA70. The HSF1-RPA70 interaction was required to recruit “paused” RNA Pol II to the Hsp70 promoter, which is proposed to facilitate the rapid and robust induction of Hsp70 transcription following heat shock. This result provides a foundation for understanding specific protein-protein interactions that occur prior to stress that may prime HSF1 activation to respond during stress. In addition, the unique site of interaction with the HSF1 wing domain highlights the opportunity to explore the wing domain of HSF1 as a potential protein-protein interaction site.

As mentioned earlier, HSF1 attenuation is not well understood but a recent report may provide insight into this important aspect of HSF1 regulation. Aifantis and
colleagues demonstrated that FBXW7α, a component of the SCF ubiquitin ligase complex, interacts with HSF1 and promotes its degradation (81). Phosphorylation of HSF1 on Ser303 and Ser307 by GSK3β and ERK1 was shown to promote the interaction of HSF1 and FBXW7α, enabling a phosphorylation dependent degradation of HSF1. Interestingly, this interaction was only observed in the nucleus, suggesting that a localization component also influences this regulation event. FBXW7α is often mutated or downregulated in melanoma, suggesting that the degradation of HSF1 in impaired in melanoma which may enable HSF1 transcriptional programs to promote cellular survival.

Cells utilize a complex network of protein-protein interactions within the protein homeostasis machinery. A recent report described the extensive array of interactions for heat shock proteins, co-chaperones, and HSFs by adapting the LUMIER assay (82). This study revealed interactions of HSF1 with the karyopherin members of the nuclear pore complex, BAG3, and other co-chaperones of the Hsp70 complex. While this assay does not address direct interactions, it provides a blueprint for identifying key interactions in the HSF1 pathway that are likely to influence HSF1 function and regulation.

1.2.4 Heat Shock Transcription Factor 2 (HSF2)

Similar to HSF1, HSF2 participates in the transcriptional response to stress. However, mounting evidence suggests that HSF2 regulates target genes under different cellular contexts when compared to HSF1 (83). One of the fundamental differences
between HSF1 and HSF2 is their relative induction capability. It has been proposed that HSF2 is constitutively bound to DNA to assist in basal target gene expression (84). This phenomenon suggests that the protein chaperone complex that represses HSF1 in the basal state does not functionally interact with HSF2. Understanding this differential regulation may be instrumental in deciphering the specific roles of HSF1 and HSF2.

Moreover, early studies on HSF2 suggested that it exists as a dimer in unstressed erythroleukemia K562 cells and stimulation of these cells with heme induced the trimerization of HSF2 (84). This observation emphasizes the importance of understanding critical structural features of HSF complexes, as well as deciphering the different mechanisms that HSF1 and HSF2 utilize to integrate stimuli.

Pioneering studies by the Metzger lab have demonstrated a critical role for HSF2 in brain development. HSF2 expression is at its peak during brain development and is highly expressed in neuronal progenitors cells in the ventricular zone as well as the cortical plate (85). Specifically, HSF2 facilitates radial neuronal migration by upregulating the expression of the cyclin dependent kinase 5 (CDK5) activator, p35 and the microtubule associated proteins Dlck and Dcx. Interestingly, the phenotype of HSF2 null mice phenotypically mimics fetal alcohol syndrome (FASD) implicating HSF2 in the pathogenesis of fetal alcohol syndrome (86). It will be imperative to better understand the role of direct and indirect gene targets of HSF2 in cortical development in order to identify mechanisms influencing developmental brain disorders.
The HSR has also been implicated in the progression of the cell cycle and recent studies by the Sistonen laboratory have identified a crucial role for HSF2 in the execution of mitosis (83,87). An unbiased ChIP-seq analysis in freely cycling, and mitotically arrested cells revealed that HSF2 is bound to numerous loci during mitosis, whereas HSF1 is largely excluded from mitotic chromatin (83). Proper execution of the heat shock response during mitosis is required to prevent chromosomal segregation errors, and surprisingly, depletion of HSF2 enhances the ability of cells to correctly execute mitosis. This observation prompts an investigation into the normal physiological role for HSF2 in mitosis. Perhaps HSF2 acts as a “bookmarking” transcription factor to enable restoration of basal chaperone expression following the completion of the cell cycle. Moreover, understanding the mechanism by which HSF1 is excluded from mitotic chromatin, but not HSF2, may reveal insight into differential regulation of HSFs.

A recent study investigating the stress response to febrile temperatures identified HSF2 as a primary mediator of the chaperone gene expression under febrile conditions (88). This observation suggests that HSF family members are engaged at different temperature thresholds and could provide a template for understanding the set point for HSR activation. Furthermore, this study demonstrated that HSF2 is important for the heat shock response under sustained, febrile hyperthermia, which may represent a more physiological stress. HSF2 null cells accumulated polyubiquitinated proteins and exhibited reduced cellular viability in response to sustained, mild temperature stress.
suggesting that HSF2 may be a critical regulator of the heat shock response in physiological temperature ranges. In particular, the alpha B crystallin gene was identified as a primary target for HSF2 in febrile temperatures, suggesting that different temperatures, and different HSFs, may execute specific transcriptional programs.

Studies of HSF2 null mice have revealed a number of interesting, subtle phenotypes. In addition to defects in brain development, HSF2 null mice display severe defects in spermatogenesis specifically during the pachytene stage of sperm maturation (89). HSF2 gene targets include spermiogenesis specific transcript on the Y 2 (Ssty2), Syce3-like Y linked (Sly) and Syce3-like X linked (Slx) which are critical mediators of sperm quality. A recent study also identified a single nucleotide polymorphism in HSF2 that was present in males with idiopathic azoospermia (90). At first glance, the R502H mutation appears relatively benign but genetic and functional studies suggest that the mutation is a dominant negative allele. Thus, the R502H variant of HSF2 may prove a useful tool for understanding HSF2 function in spermatogenesis as well as other cellular contexts.

1.2.5 Heat Shock Transcription Factor 4 (HSF4)

HSF4 has been most well studied in the context of eye development and function. Initial studies on HSF4 identified a transcriptional role for HSF4 in regulating crystallin gene expression in the lens of the eye (91). These studies paved the way for numerous genetic analyses that have identified a number of single nucleotide
polymorphisms in HSF4 that lead to congenital cataract disease, a phenomenon that is especially prevalent in Chinese and Danish families (92,93). A significant majority of these polymorphisms localize to the DBD of HSF4 and are predicted to disrupt DNA binding function, supporting the notion that HSF4 activity is critical for proper eye development and function. The identification of specific loss of function mutations enabled mechanistic investigation into HSF4 function. A recent report identified a role for HSF4 in promoting G1/S arrest in lens epithelial cells (94). The G1/S arrest was dependent on an interaction between HSF4 and p53 and is predicted to promote the differentiation of secondary fiber cells in the eye. The function of HSF4 was also linked to DNA damage repair in senile cataract formation (95). HSF4 binds to an HSE in the promoter of Rad51, which is a critical mediator of homologous recombination following double stranded DNA breaks. Exploring HSF4 regulation in the eye may illuminate analogous regulatory interactions that occur in HSF1 and HSF2 in other tissues.
1.3 Interaction and Cooperation between HSFs

An extensive body of literature has explored the role of HSF heterocomplexes in diverse aspects of cellular function. Initial studies on HSF1 and HSF2 demonstrated an interaction through co-immunoprecipitation experiments from cell extracts (96). This led to a conceptual advance that HSF1 and HSF2 cooperate to fine-tune the transcriptional response to stress. A number of follow up studies have demonstrated similar findings in a variety of cell contexts. For example, treatment of cells with the proteasome inhibitor bortezomib was shown to promote the interaction of HSF1 and HSF2 on the promoter of
arsenite inducible RNA associated protein (AIRAP) (97). Interestingly, knockdown of HSF2 resulted in higher inducibility of the AIRAP gene suggesting that HSF2 may act as a suppressor of HSF1 function. These results present the interesting possibility that interactions between HSF2 and HSF1 function enable cells to precisely regulate the transcriptional output of target genes for a given cellular context.

In fetal alcohol syndrome, abnormal HSF1–HSF2 heterocomplexes were observed and suggested to prevent HSF2 from binding to its normal targets in brain development (86). This interaction demonstrates yet another mechanism by which HSF1 and HSF2 can impact each other’s function in a negative way. Cooperation between HSF1 and HSF2 has also been observed in testes where both proteins are simultaneously bound to developmentally regulated genes related to spermatogenesis (98). Interestingly, the presence of both HSFs is detected at physiological temperatures, whereas only HSF1 is present following hyperthermic shock. It is important to note that while the interaction between HSF1 and HSF2 has been demonstrated in cell extracts, a direct interaction has not been shown. Further mechanistic insight into the nature of the HSF1-HSF2 heteromultimer will lay the foundation for understanding the communication between HSF1 and HSF2 in diverse cellular contexts.

HSF1 and HSF4 were recently shown to have physical and functional interactions in lens epithelial cells (99). Interestingly, HSF4 demonstrated a negative impact on HSF1 function by competing for DNA binding at Hsp promoters. In addition,
HSF4 interacting with HSF1 promoted the cytosolic retention and degradation of HSF1. These functional consequences were proposed to enable HSF4 to upregulate a specific heat shock protein expression program to prevent lens epithelial cell death in response to cisplatin and staurosporine. Further exploration into the physical mechanism by which HSF1 and HSF4 interact could provide a template for understanding HSF1-HSF2 interactions, as well as HSF2-HSF4 interactions.

HSF2 and HSF4 have also been observed to cooperate in the regulation of HIF1α expression by competing for binding to discontinuous HSEs in the promoter of HIF1α (100). Interestingly, RNAi knock-down or overexpression of either HSF alone resulted in increased HIF1α expression suggesting that a specific balance of HSF2 and HSF4 activity is necessary for proper regulation of HIF1α expression. Disrupting the balance of HSF2 and HSF4 activity resulted in aberrant levels of VEGF production and could highlight a role for these HSFs in the vascularization of solid tumors. These results highlight yet another example of functional interactions between HSFs impacting diverse aspects of cellular physiology.

1.4 Heat Shock Transcription Factors: Looking Forward

An extensive amount of research on HSFs over the past three decades has laid the foundation for understanding how HSFs function in diverse cellular contexts. However, as more reports are published on novel aspects of HSFs including newly discovered target genes, protein-protein interactions, post-translational modifications,
and disease specific regulation, it is becoming apparent how little we know about the precise mechanisms that influence HSF activity. HSFs have long been considered an interesting target for therapeutic development for a number of human diseases, but without a greater understanding of HSF structure, function, and regulation, it will be difficult to discover and develop small molecules that directly and specifically modulate HSF activity.

A major limitation in our foundational understanding of HSFs is the lack of structural information. High resolution crystal structures of the *K. lactis* DNA binding domain have provided useful information regarding the interaction of HSF with DNA but high resolution data for mammalian HSFs is lacking. Experimentally determined structural information outside of the DNA binding domain is entirely absent. While computational predictions and modeling have identified coiled-coil regions in HSFs, the precise manner in which these domains come together to form intramolecular repressive interactions or an intermolecular HSF complex is unknown. Moreover, while the RD of HSFs is predicted to be highly unstructured, it will be essential to identify protein-protein interactions and post-translational modifications within this domain that give rise to transient secondary, tertiary, and quaternary structures. Such transient structures may serve as templates for the discovery of small molecule probes that function to promote or disrupt specific regulatory events within the RD. Finally, a surprisingly lack of attention has been paid to the activation domain of HSFs. Similar to the RD, the AD is
predicted to be unstructured but may form transient, structured complexes with key transcriptional co-activators. Understanding the co-activators that cooperate with the HSF AD will further our understanding of stress, tissue, and cell context specific regulation of HSFs.

While an extensive body of work has elucidated the role of HSFs in promoter regions of target genes, much less is known about the role of HSFs in introns, intergenic regions, and enhancers. When bound to promoters, HSFs are thought to promote gene transcription by recruitment of RNA Pol II. However, the recent genome wide binding studies of HSFs have revealed that HSFs bind to numerous regions outside of gene promoters. A growing body of literature suggests that enhancers regulate nearby gene transcription in a hierarchical relationship, highlighting the need for understanding HSF functions at enhancer loci. Furthermore, as we begin to understand that regions of the “silent genome” serve assorted functions in cellular biology, it will be important to identify how HSFs impact these important genomic regions.

Regulation of HSFs at the post-translational level has been at the forefront of stress biology research. However, as expression of HSFs at the transcriptional level is now being realized to drive critical cellular functions in development and disease, it will be essential to understand the biogenesis of HSFs from DNA to RNA to protein. In addition, while a great deal of work has been performed characterizing the steps of HSF activation, much less is known about the attenuation of HSF activity. Finally, HSFs have
been shown to respond to a diverse array of stressors and execute specific responses depending on the stimulus. Understanding how HSF family members can integrate multiple signals, both external and internal, to facilitate the maintenance of protein homeostasis will be paramount to paving the way for HSF based therapeutics. These investigations have the potential to induce specific arms of the stress response that are beneficial in some contexts but not others.

The studies presented here begin to address some of the outstanding fundamental questions in HSF biology. Importantly, these studies utilize a diverse array of model systems including recombinant purified proteins, yeast, and mammalian cells. Chapter 2 details a study utilizing a humanized yeast assay to identify mutations in human HSF1 that allow growth complementation in yeast. These mutations pointed to a role for the coiled-coiled domains in DNA binding in mammalian cells. Chapter 3 explores specific features of human HSF1 that influence the direct interaction with HSE sequences in the human genome. The findings in this study provide insight into how HSF1 prefers certain genomic loci to others. Chapter 4 outlines a study characterizing a novel interaction between HSF1 and the TRiC complex. This interaction enables the cytosolic protein folding machinery to directly communicate with the cytosolic stress responsive transcription factor. Further, a small molecule activator of HSF1A perturbs this interaction and activates HSF1, highlighting a potential therapeutic avenue for HSF1 activation. Chapter 5 utilizes structural, biochemical, and molecular biology techniques
to identify a critical region of the HSF DBD that differentially regulates HSF1 and HSF2. Moreover, the structural data suggest a novel mode of HSF DNA binding that has far reaching implications for HSF biology. Taken together, these observations enlighten numerous aspects of HSFs that have been enigmatic or previously unexplored and pave the way for a better understanding of HSF biology.
2. Genetic selection for constitutively trimerized human HSF1 mutants identifies a role for coiled-coil motifs in DNA binding.

Human heat shock transcription factor 1 (HSF1) promotes the expression of stress-responsive genes and is a critical factor for the cellular protective response to proteotoxic and other stresses. In response to stress, HSF1 undergoes a transition from a repressed cytoplasmic monomer to a homotrimer, accumulates in the nucleus, binds DNA, and activates target gene transcription. Although these steps occur as sequential and highly regulated events, our understanding of the full details of the HSF1 activation pathway remains incomplete. Here we describe a genetic screen in humanized yeast that identifies constitutively trimerized HSF1 mutants. Surprisingly, constitutively trimerized HSF1 mutants do not bind to DNA in vivo in the absence of stress and only become DNA binding competent upon stress exposure, suggesting that an additional level of regulation beyond trimerization and nuclear localization may be required for HSF1 DNA binding. Furthermore, we identified a constitutively trimerized and nuclear-localized HSF1 mutant, HSF1 L189P, located in LZ3 of the HSF1 trimerization domain, which in response to proteotoxic stress is strongly compromised for DNA binding at the Hsp70 and Hsp25 promoters but readily binds to the interleukin-6 promoter, suggesting that HSF1 DNA binding is in part regulated in a locus-dependent manner, perhaps via promoter-specific differences in chromatin architecture. Furthermore, these results
implicate the LZ3 region of the HSF1 trimerization domain in a function beyond its canonical role in HSF1 trimerization.

The work presented in this chapter was performed in collaboration with Daniel W. Neef Ph.D.

2.1 Introduction

All organisms are exposed to stressful conditions that result in the accumulation of misfolded and aggregation-prone proteins that, unless appropriately managed, result in cell dysfunction and death. To respond to these stresses, cells have evolved adaptive mechanisms to protect and stabilize cellular proteins until more favorable conditions for cellular proliferation are encountered. The heat shock transcription factor, HSF, is a conserved, homotrimeric transcription factor that activates gene expression in response to a variety of stresses, including heat shock, oxidative stress, as well as inflammation and infection (11,49). Among HSF target genes are those encoding protein chaperones, which assist in protein folding and protect from stress-induced cell death, and other genes encoding proteins with many distinct functions in cellular homeostasis (6,63). Recent evidence has shown that in Saccharomyces cerevisiae HSF directly activates the expression of genes whose protein products are involved in protein folding and degradation, ion transport, signal transduction, energy generation, carbohydrate metabolism, vesicular transport, cytoskeleton formation, and a broad array of other cellular functions (101). Collectively the stress-dependent activation of target gene
expression by HSF is known as the heat shock response. In S. cerevisiae the heat shock response is mediated by a single HSF that is essential for cell viability under all conditions evaluated (48). Although mammalian cells express four nonessential HSF proteins encoded by separate genes, HSF1 is the primary mammalian heat shock factor responsible for stress responsive gene transcription, with HSF2 also modestly activating protein chaperone gene expression under less acute temperature conditions (88). In the absence of proteotoxic stress the activity of mammalian HSF1 is repressed through a variety of mechanisms that are not fully understood. HSF1 is bound and repressed by the protein chaperones Hsp90 and Hsp70, though the mechanisms for how these chaperones repress HSF1 activity remain unclear (50,102). It is hypothesized that during the initial response to proteotoxic stress, the inactive cytosolic HSF1 monomer dissociates from Hsp90, forms a homotrimer, which is transported to the nucleus to bind to heat shock elements (HSE), found in the promoters of HSF1 target genes and promotes gene activation (103). In response to stress, HSF1 also undergoes several post-translational modifications including sumoylation and hyper-phosphorylation (104). HSF1 is also thought to be maintained in an inactive monomeric state through an intramolecular coiled-coil interaction between a leucine zipper (LZ4) in the carboxyl-terminus of the protein and three leucine zippers (LZ1-3) in the amino-terminus, that are also required for homotrimerization during stress activation (59). The individual helices of a typical coiled-coil domain contain repeats of seven amino acid arrays consisting of
hydrophobic and charged amino acid residues which arrange themselves in such a fashion that the hydrophobic interactions among the helices provide the thermodynamic force for oligomerization, in part guided by the ionic interactions across heptad repeats (105). Although the interaction between LZ4 and the trimerization domain (LZ1-3) of HSF1 is hypothetical and has not yet been described experimentally, this hypothesis suggests a model in which HSF1 exists in an equilibrium between an active trimeric state, mediated by coiled-coil interactions of the trimerization domain, and an inactive state, mediated by coiled-coil interactions between the trimerization domain and LZ4. Whether these coiled-coil domains play other roles in the regulation of HSF1 is not known.

Despite a high level of conservation of both HSF1 and the cognate HSEs from yeast to humans, our previous results demonstrated that expression of the human HSF1 is unable to complement for the loss of the essential yeast HSF (53). This inability to complement appears to be predominantly attributable to an inability of human HSF1 to form a homotrimer in yeast, an essential part of the activation process. Consequently, human HSF1 is unable to bind to and activate HSE-dependent gene expression to support yeast viability. However, the expression of a human HSF1 mutant with amino acid substitutions in LZ4, which is constitutively trimerized in mammalian cells, can function in S. cerevisiae (53). Further studies in yeast also identified an amino-terminal linker-domain, a loop in the DNA binding domain, as well as several phosphorylation
sites as repressive elements that contributed to HSF1 repression in both yeast and mammalian cells (61,106,107). Together, these results suggest that when human HSF1 is expressed in yeast, it is maintained in a constitutively repressed monomeric state through mechanisms that are similar to those of mammalian cells and that the yeast system can serve as a simplified assay system to decipher aspects of the complex mechanisms regulating human HSF1 activity in mammalian cells.

Here we report the use of the humanized yeast assay system to further understand the mechanisms that regulate human HSF1 through a random mutagenesis screen. We identify novel human HSF1 mutants in the leucine zippers of HSF1, which result in the constitutive trimerization, and nuclear localization of HSF1. Interestingly, our results reveal that despite their constitutively trimerized state, the HSF1 mutants we identified are unable to bind DNA in the absence of stress in mammalian cells, suggesting that additional stress responsive events are required to elicit HSF1 DNA binding. Furthermore, we report the identification of a constitutively trimerized HSF1 mutant exhibiting genomic locus-specific DNA binding defects in vivo.

2.2 Yeast screen for constitutively active HSF1 mutants

Our previous studies demonstrated that expression of the human HSF1 protein is unable to complement for the loss of the essential yeast HSF due to an inability to form a homotrimer, even in response to proteotoxic stress conditions such as heat shock (53). However, mutations in the LZ4 coiled-coil domain, or in repressive phosphorylation
sites that render human HSF1 constitutively trimerized in mammalian cells, promote constitutive activation of human HSF1 in yeast and allow for complementation (51,53,61). Together, these data suggest that when human HSF1 is expressed in yeast the protein is maintained in a repressed monomeric state that is unable to respond to proteotoxic stresses and that mutations that enhance the propensity for trimer formation may overcome this repression. This conservation of regulatory mechanisms when human HSF1 is expressed in yeast allowed for the development of a facile assay system to identify human HSF1 mutants that allow human HSF1 complementation in yeast.

Our screen, outlined in Figure 8, used a library of human HSF1 mutant clones generated using the mutagenic XL1-Red E. coli strain. The mutant HSF1 library was transformed into yeast strain PS145, which lacks the genomic copy of yeast HSF and expresses yeast HSF from a plasmid under the control of the galactose-inducible and dextrose-repressible GAL1-10 promoter. After transforming the mutant HSF1 library into yeast strain PS145, recipient cells were selected for mutant human HSF1 clones able to complement for the loss of yeast HSF by growth on dextrose containing medium, which extinguishes expression of yeast HSF and render yeast growth solely dependent on human HSF1 function.
Sequence analysis of those human HSF1 alleles able to complement for the loss of yeast HSF revealed five distinct mutations (Figure 9). Although four of the five mutations identified were located in the three leucine zipper domains (LZ1-3) within the trimerization domain of HSF1 (Figure 9) one mutation was located in the carboxyl-terminal leucine zipper domain (LZ4), a finding that corresponds to the known repressive nature of this domain.
Figure 9. Diagram of active HSF1 Mutants. M161I, K178E, H179Y and L189P localize to the LZ1-3 trimerization domain and L402P is located in the LZ4 domain. Black dots represent every fifth residue and bold indicates key residues for coiled-coil formation.

Specifically, double mutations in LZ4 (M391K and L395P) were previously described by Rabindran et al. to promote the constitutive trimerization of human HSF1 in Hek293 cells and triple mutations in LZ4 (M391K, L395P, L398R) have been shown by our laboratory to promote the activation of human HSF1 in yeast (53,59). The results presented here demonstrate that a single amino acid substitution in a key hydrophobic residue (L402P) of LZ4 can also promote trimerization of human HSF1 and allow for complementation of human HSF1 in yeast.
Two of the human HSF1 mutants we identified in LZ1-3 (M161I, L189P) as well as the mutant we identified in LZ4 (L402P) occur at key hydrophobic residues (Figure 9), and are conserved across a variety of species from yeast to humans (Figure 9). Thus, we predict that these residues are potentially required for the formation of the predicted repressive coiled-coil interactions (Figure 9). However, it is of interest that the isoleucine substitution for Met161 results in the replacement of a hydrophobic amino acid with another hydrophobic amino acid. Thus, we used the Paircoil2 algorithm to analyze the effects of this and the other mutations we identified in LZ1-3 on the propensity for coiled-coil formation. Interestingly, despite its hydrophobic nature, substitution of M161 with isoleucine is predicted to reduce the propensity for coiled-coil formation (Figure 10). Furthermore, we also identified proline substitutions in both LZ1-3 and LZ4 (L189P, L402P), which are well known to disrupt alpha helices and are likely to have detrimental effects on coiled-coil formation. As predicted by the Paircoil2 algorithm, the proline substitution of L189 strongly reduces the propensity for coiled-coil formation (Figure 10). However, it is important to note that the entire trimerization domain is not predicted to be eliminated by this substitution, but rather only a small portion of the coiled-coil domain adjacent to the proline substitution, which corresponds to LZ3, is predicted to be perturbed. Interestingly, LZ3 has previously been linked to HSF1 repression (58,108).
Figure 10. PairCoil2 Analysis of HSF1 Mutants. Lower scores indicate increased propensity for coiled-coil formation.

We also identified mutations in two charged amino acids located between LZ1/2 and LZ3 (K178E, H179Y) which are predicted by the Paircoil2 algorithm to increase the propensity for coiled-coil formation of the trimerization domain and may thereby shift the equilibrium of HSF1 from a monomeric to homotrimeric state (Figure 10). As the tyrosine substitution of His179 substitutes a hydrophobic amino acid for a charged amino acid, it is possible that this mutation simply extends the LZ1/2 domain further and increases the size of the trimerization domain and is consistent with a previous study that identified an arginine substitution of His179 that also promoted constitutive
trimerization of HSF1 (62). However, it remains unclear how the charged glutamate substitution of Lys178 increases coiled-coil propensity.

The ability of the HSF1 mutants we identified to promote human HSF1-dependent yeast growth was confirmed by a qualitative spot assay analysis (Figure 11) as well as quantitative growth curve analysis (Figure 11). More quantitative growth curve analysis revealed that although all of the human HSF1 mutants identified promoted yeast growth, the HSF1 L189P mutant was somewhat more potent at activating yeast growth in comparison to the other mutants we identified. The remaining mutants displayed essentially similar efficacy in their ability to promote yeast growth in this assay (Figure 11).

![Figure 11. Human HSF1 Mutants enable yeast growth on plates and in liquid culture. (Left) Spot assay for yeast growth on galactose and dextrose media. (Right) Quantitative liquid culture growth curves in dextrose media.](image)

Our lab previously showed that constitutively trimerized human HSF1 mutants are detected at higher steady state levels in comparison to wild-type human HSF1 in yeast (61). In accordance with these results, all of the human HSF1 mutants identified
here except for the M161I mutant were detected at increased steady state levels when compared to the wild-type protein (Figure 12), indicating that an elevation in human HSF1 steady state protein levels is not required to complement for loss of yeast HSF.

![Figure 12. Steady state levels of human HSF1 mutants in yeast. Protein extracts from yeast grown in dextrose media were analyzed by immunoblotting with anti-human HSF1 antibody and anti-Pgk1 as loading control.](image)

As predicted by our previous findings, the ability of HSF1 mutants we identified to promote yeast growth should be dependent on their ability to homotrimerize. In support of this hypothesis, EGS crosslinking experiments reveal that while wild-type HSF1 exists predominantly in the monomeric form, the active HSF1 mutants identified were all enriched in the trimer form (Figure 13).

![Figure 13. EGS Crosslinking of human HSF1 mutants in yeast. Black dots represent predicted MW of monomer and trimer.](image)
2.3 Differential activation of gene expression by HSF1 mutants in mammalian cells

To test how the human HSF1 mutants identified in yeast function in mammalian cells, wild-type or mutant HSF1 alleles were transiently transfected into hsf12/2 MEFs and their ability to promote stressresponsive transactivation of gene expression was assessed by assaying Hsp70 protein expression by both immunoblotting and ELISA experiments. Consistent with the notion that regulation of HSF1 transactivation is a complex process involving homotrimerization, nuclear localization, DNA binding and stress-responsive hyperphosphorylation, none of the HSF1 mutants tested promoted Hsp70 expression in the absence of stress beyond that observed by wild type HSF1 (Figure 14). Interestingly, the HSF1 L189P mutant, which was constitutively trimerized and functional in yeast cells, was strongly compromised in its ability to promote HSF1-dependent Hsp70 expression under stress conditions (Figure 14).
Figure 14. Human HSF1 mutants except for L189P induce Hsp70 similar to WT. (Top) MEF protein extracts were analyzed by immunoblotting against Hsp70, HSF1, and GAPDH. (Bottom) Quantitative ELISA analysis of Hsp70 expression in MEF cells expressing HSF1 mutants.

Because the L189P HSF1 mutant was compromised in its ability to promote Hsp70 expression in mammalian cells, we ascertained if the human HSF1 mutants we identified in our yeast screen were also constitutively trimerized in mammalian cells. In correlation with the results obtained in yeast, EGS crosslinking experiments in mammalian cells revealed that in the absence of stress, wild-type HSF1 exists predominantly as a monomer, which is converted to the trimeric form in response to heat stress.
Figure 15. EGS Crosslinking of HSF1 mutants in mammalian cells. Cell extracts from MEF cells expressing HSF1 mutants were crosslinked with EGS and analyzed by immunoblotting against HSF1. Black dots represent predicted MW of monomer and trimer.

In contrast, each of the HSF1 mutants exist predominantly in the trimeric form and stress exposure only modestly enhanced trimerization (Figure 15). In previous experiments authors have shown that mammalian HSF1 trimerization is a pre-requisite for nuclear accumulation (52). Therefore, we hypothesized that these mutants should also be localized to the nucleus even in the absence of stress. This hypothesis was supported by indirect immunofluorescence microscopy and cell-fractionation experiments, which demonstrated that while the wild-type protein exists predominantly in the cytoplasm, the mutants we identified, are localized to the nucleus in the absence of stress and remained nuclear in response to a heat shock (Figure 16).
Figure 16. HSF1 Mutants are localized to the nucleus. (Top) Indirect immunofluorescence of MEFs expressing HSF1 mutants using anti-HSF1 antibody under control and heat shock conditions. (Bottom) Cytoplasmic (C) and Nuclear (N) subcellular fractionation of MEFs expressing HSF1 mutants under control conditions. c-Fos and Sod1 serve as nuclear and cytoplasmic controls respectively.

Our observation that the L189P HSF1 mutant is constitutively trimerized and constitutively localized to the nucleus, but is deficient in its ability to promote heat shock-dependent Hsp70 expression suggests that this mutant is defective in an important aspect of the HSF1 activation pathway downstream of nuclear localization.
Although a definitive role for HSF1 hyperphosphorylation has not been elucidated, it is well established that HSF1 is hyperphosphorylated as part of the stress-dependent HSF1 activation pathway. Our data show that, similar to the wild-type HSF1 protein, the L189P mutant is readily hyperphosphorylated in response to heat stress-exposure and suggest that a different aspect of the HSF1 activation pathway is affected by the L189P mutation (Figure 17).

![Figure 17. WT HSF1 and HSF1 L189P are hyperphosphorylated at similar levels following heat shock. Hyperphosphorylation is indicated by a change in electrophoretic mobility upon heat shock.](image)

### 2.4 Constitutively trimerized HSF1 mutants are not constitutively bound to DNA

On the basis of conventional model for the mammalian HSF1 activation pathway, once HSF1 is trimerized and accumulates in the nucleus, it would be competent for DNA binding to promoter HSEs (104). Because the human HSF1 mutants isolated in this study are constitutively trimerized and nuclear-localized we hypothesized that these mutants should be constitutively bound to DNA in vivo. To address this hypothesis we performed chromatin immunoprecipitation experiments using MEF cells transfected with plasmids expressing wild-type HSF1 or the HSF1
mutants we identified in this study. Because previous in vitro DNA binding studies have shown that constitutively trimerized HSF1 mutants readily bind DNA (59), it was surprising and contradictory to the current model of the HSF1 activation pathway to find that the constitutively trimerized HSF1 mutants did not bind to the Hsp70 or Hsp25 promoters in the absence of stress beyond that observed for the monomeric wild-type human HSF1 (Figure 18).

Figure 18. L189P HSF1 has reduced binding to Hsp Promoters. Chromatin Immunoprecipitation analysis of Hsp70, Hsp25, and IL-6 promoters under control and heat shock measured by enrichment over GAPDH. Data is presented at the mean ± SEM.

These observations suggest that the ability of HSF1 to bind DNA in vivo is more complex than what is observed in vitro and may not be solely dependent on HSF1 trimer formation, perhaps requiring additional stress-responsive factors. However, upon exposure to heat stress, inducible binding to the Hsp70 and Hsp25 promoters was
observed for wild-type HSF1 as well as most of the mutant HSF1 proteins (Figure 18).

Interestingly the HSF1 L189P mutant was strongly compromised for binding to both the Hsp70 and Hsp25 promoters even under stress conditions, consistent with the compromised ability of this mutant protein to induce Hsp70 expression.

The reduced ability of the HSF1 L189P mutant protein to bind to promoter DNA in vivo and promote Hsp70 expression even in response to heat stress was surprising, as the HSF1 trimerization domain has not previously been linked to DNA binding. To further evaluate the DNA binding propensity of these mutant proteins, we assayed HSF1 DNA binding at the IL-6 promoter where HSF1 has previously been shown to bind constitutively, even in the absence of stress (109). In concert with previously published findings, wild-type HSF1 was bound to the IL-6 promoter in the absence of stress and binding increased modestly upon exposure to heat shock (Figure 18).

Similar levels of HSF1 DNA binding were observed for all the HSF1 mutants including the L189P mutant, suggesting that this mutation does not directly impinge on the function of the HSF1 DNA binding domain but rather imparts a genomic locus specific DNA binding defect, perhaps by precluding the interaction with auxiliary factors that are required for modulating loci-specific chromatin landscapes.

2.5 The L189P HSF1 mutant fails to protect from heat stress induced cytotoxicity

The inability of the HSF1 L189P mutant to bind DNA and promote heat shock-dependent Hsp70 or Hsp25 expression in vivo suggested that cells expressing this
mutant isoform of HSF1 might be severely compromised in their ability to protect from cytotoxic stress. To test this hypothesis we assayed the viability of hsf12/2 MEFs transfected with an empty vector, wild-type HSF1 or the M161I, K178E, H179Y, L189P or L402P HSF1 mutants after exposure to a heat shock for 2 h. The results of these experiments show that although the wild-type HSF1 as well as the M161I, K178E, H179Y and L402P HSF1 proteins were able to protect cells from heat stress-induced cell death, the HSF1 L189P mutant was strongly compromised in its ability to provide cytoprotection (Figure 19).

![Cell viability following heat shock](image)

Figure 19. Cell viability following heat shock is compromised in cells expressing HSF1 L189P. Viability was measured using Cell-Titer Glo following a 2-hour heat shock at 42 °C. Data are represented as the mean ± SEM.

However, the protection from cytotoxic stress achieved by the L189P HSF1 mutant is greater than what we observed for cells transfected with an empty vector, consistent with observations presented here that this mutant retains a low level of DNA binding activity (Figure 18) and does promote low levels of Hsp70 expression in response to heat shock (Figure 14).
2.6 Discussion

Many studies during the past two decades have demonstrated that the activation of mammalian HSF1 is a complex, multistep process involving the conversion of an inactive cytosolic monomer to a homotrimer, nuclear localization, DNA binding, and gene trans-activation as sequential and highly regulated events. When human HSF1 is expressed in yeast, its ability to homotrimerize, bind DNA and promote gene activation is repressed suggesting that the mechanisms regulating HSF1 activity are at least partially conserved between yeast and mammalian cells. Moreover, consistent with this notion, we previously demonstrated that small molecules that activate human HSF1 in yeast cells also activate HSF1 in cultured mammalian cells and in fruit flies (18). We have used this repression of human HSF1 trimerization in yeast to create and utilize a screen aimed at identifying novel mutations that promote the constitutive trimerization of human HSF1 as a means to further decipher this complex regulatory pathway. Here we report the identification of mutations that lead to constitutive trimerization of human HSF1 in yeast and therefore allow human HSF1-dependent yeast growth. Our identification of these mutations, which are located in key hydrophobic residues of the amino-terminal and carboxyl-terminal leucine zippers, lend support to the hypothesis that these domains engage in coiled-coil interactions that repress the overall activity of the HSF1 protein and suggest that future modeling studies of this hypothetical interaction should consider these residues. Although the intra-molecular interaction
between LZ1-3 and LZ4 has been widely accepted, definitive biochemical evidence of this interaction has not been reported. As such, it remains formally possible that these domains may repress HSF1 function by mediating a trans-acting interaction with other coiled-coil proteins. Because mutation of these coiled-coil domains results in the nuclear accumulation of the HSF1 trimer, we speculate that any such putative HSF1 interacting protein should be predominantly cytoplasmic, perhaps acting as a cytoplasmic anchor for HSF1.

One candidate would be TRiC, a cytosolic chaperone complex that is conserved in yeast and mammalian cells which was previously shown to interact with a small molecule activator of human HSF1 trimerization and may be a tissue specific regulator of HSF1 in *C. elegans* (110). Alternatively, it remains formally possible that the coiled-coil regions of HSF1 mediate its repressive interactions with known HSF1-repressors such as Hsp70 and Hsp90. The hydrophobic nature of the coiled-coil domains makes this hypothesis potentially intriguing as protein chaperones often recognize exposed hydrophobic patches within proteins. Although the authors of previous studies have established the carboxyl-terminal activation domain of HSF1 as the primary binding site of Hsp70, the specific HSF1 domains bound by Hsp70 or Hsp90 have not yet been identified (50).

Interestingly, although all of the mutations we identified in this screen result in the constitutive trimerization of human HSF1 both in yeast and mouse hsf1 null cells, as
well as the nuclear localization of HSF1, these mutant HSF1 proteins are deficient in DNA binding until the cells are exposed to stress. These results were surprising as extensive evidence has shown that constitutively trimerized mammalian HSF1 mutants are competent to bind DNA in vitro using gel shift analysis (59). Although the precise defect underlying this inability to bind DNA remains unclear, it is possible that condensed chromatin architecture at specific loci precludes HSF1 DNA binding and an additional heat shock responsive regulatory event is required before trimerized HSF1 can access the promoter. Based on our data showing that a constitutively trimerized HSF1 protein is deficient in binding to the Hsp70 and Hsp25 promoter in the absence of stress, but able to bind to the IL-6 promoter, this hypothesis would predict that the Hsp70 promoter may have a very condensed chromatin architecture requiring chromatin remodeling factors for HSF1 DNA binding while HSF1 binding sites in the IL-6 promoter would be predicted to be unobstructed by chromatin and allow for unrestricted HSF1 binding even in the absence of stress. Unfortunately, detailed information describing the chromatin architecture of these promoters is lacking. Nevertheless, several previous reports suggest that condensed chromatin can preclude HSF1 DNA binding and the recruitment of many distinct chromatin-remodeling factors is required for HSF1 DNA binding at the Hsp70 promoter in vivo (80,111,112). An alternative hypothesis to explain the differential binding of HSF1 to the IL-6 and Hsp70 promoters could be the architecture of the HSE. Although a consensus HSE
(GAAnnTTCnnGAA) has been defined as the ideal high affinity-binding site for HSF1, many variations of this consensus sequence are bound by HSF1 at reduced affinities in vivo. Based on the constitutive binding of HSF1 to the IL-6 promoter but not to the Hsp70 promoter, one might predict that greater affinity HSEs are found in the IL-6 promoter compared with the Hsp70 promoter. However, the authors of a recent study have identified several putative HSEs in the IL-6 promoter, which vary significantly from the consensus HSE, and are bound by HSF1 at sevenfold lower affinity than the near-ideal HSE (GGAnnTTCnnGAC) found in the Hsp70 promoter (113). As such, our observation that both wild-type HSF1 as well as the constitutively trimerized HSF1 mutants can bind constitutively to the IL-6 promoter but are unable to bind to the Hsp70 promoter supports the notion that binding of HSF1 to the Hsp70 promoter is being precluded by a compacted chromatin architecture. Although the idea of loci-specific chromatin architecture dictating HSF1 DNA binding remains hypothetical, we propose that the stress responsive acquisition of DNA binding competency, at least in the context of the Hsp70 and Hsp25 promoter, serves as an additional level of regulation in the overall model of the HSF1 activation pathway. To this extent we report the identification of the HSF1 L189P mutant which despite its constitutively trimerized and nuclear state, remains largely defective in its ability to bind to the Hsp70 and Hsp25 promoters even upon heat stress stimulation and may in fact uncouple this additional layer of regulation. Because the L189P mutation is predicted to disrupt the carboxyl-terminal
portion of the LZ3 domain, it is tempting to speculate that this portion of the trimerization domain may be indirectly important for HSF1 DNA binding in vivo. The importance of this domain being linked to DNA binding is striking as this small region of the protein is significantly removed from the DNA binding domain and is unlikely to make direct contact with the DNA. Although it is formally possible that the L189P mutation alters the overall structure of HSF1 in such a fashion that it affects the structure and function of the DNA binding domain, our findings that this mutant is fully functional in yeast, binds to the IL-6 promoter similar to the wild-type protein and is still able to provide some level of protection from cytotoxic stress suggests that this is not the case. However, it is difficult to effectively test this hypothesis without a crystal structure of HSF1 outside of the previously determined DNA binding domain in complex with DNA (64). We favor an alternative explanation, in that the LZ3 domain is important for the recruitment of additional factors required for DNA binding in response to stress.

Previous evidence has shown that a significant group of additional factors assist in stress induced binding of HSF1 to the DNA. Several of these include the general transcription factor TFIID as well as the chromatin remodeling complexes NURF, Swi/Snf, ASC-2, HMGN1 and TAC1 (114-117). The idea that LZ3 is important for recruitment of chromatin remodeling factors suggests that these factors might also have coiled-coil domains and that interactions between this domain and LZ3 mediates the stress responsive recruitment.
2.7 Materials and Methods

Random mutagenesis screen and yeast cell growth: A library of human HSF1 mutants was generated by transforming the plasmid pRS423GPD-hHSF1 (61) into the Escherichia coli XL-1 Red strain deficient in three primary DNA repair pathways (mutS, mutD, mutT) resulting in a spontaneous mutagenesis rate ~5000-fold greater than wild-type cells. Plasmid transformed bacterial colonies were scraped off plates, pooled, and inoculated into liquid growth medium for plasmid maxiprep. The resulting library of mutant HSF1 plasmids were transformed into the previously described yeast strain PS145 (MAT a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 hsf1Δ::LEU2 pGAL-HSF:URA3) (REF), and yeast cells were plated on medium containing 2% dextrose to extinguish expression of yeast HSF and select for those human HSF1 mutants able to promote human HSF1-dependent yeast growth. Plasmids were rescued from the resulting yeast colonies, re-transformed to confirm activity and HSF1 sequence analyzed by DNA sequencing. Yeast strain PS145, transformed with either a wild-type or mutant HSF1 expressing plasmid, was analyzed in liquid growth curve assays and spot assays as previously described (61).

Immunobloting, trimerization, and subcellular localization experiments: Total protein was extracted from 1·108 yeast cells by incubation in 200 mL of NaOH lysis buffer (0.1 M NaOH, 0.05 M ethylenediaminetetraacetic acid [EDTA], 2% sodium dodecyl sulfate [SDS], 2% beta-mercaptoethanol) for 10 min at 90. The NaOH was
neutralized by the addition of 5 mL of 4 M acetic acid and after the addition of Laemmli buffer proteins were fractionated by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting using anti-HSF1 (18) and anti-PGK1 (22C5D8; Abcam) antibodies.

For protein analysis from mammalian cells wild-type and mutant HSF1-expressing plasmids were transfected into hsf1 null mouse embryonic fibroblasts (MEF), previously described (118), using a 4D Nucleofector (Lonza). Mammalian protein extracts were prepared and assayed for expression of Hsp70 by immunoblotting and enzyme-linked immunoassay (ELISA) as previously described (18). The antibodies used in this study were anti-Hsp70 (W27; Santa Cruz Biotechnology) and anti-GAPDH (6C5; Santa Cruz Biotechnology). Analysis of HSF1 trimerization and cellular localization were performed as previously described (18). Indirect immunofluorescence utilizing an Axio Imager was performed as described (119) using an anti-HSF1 antibody (10H8, Enzo)

DNA binding studies: MEF cells transfected with wild-type or mutant HSF1 proteins were heat shocked at 42 for 20 min followed by crosslinking via the addition of 1% formaldehyde for 5 min on ice. The formaldehyde crosslinker was quenched on ice by the addition of 375 mM glycine for 5 min. The cross-linked cells were washed twice in phosphatebuffered saline, harvested, and suspended in cell lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS) supplemented with protease
inhibitors. Cell lysates were diluted twofold by the addition of IP Buffer (50 mM Tris, 150 mM NaCl, 1 mM Triton X-100), and the chromatin was sheared by sonication three times for 30 sec using a Sonic Dismembrator 550 on setting 3 and then incubated with 5 µL of anti-HSF1 antibody for 18 hr at 4. Protein2antibody complexes were purified using 50 mL of Protein A agarose beads for 3 hr at 4 and washed twice in IP-buffer, twice in IP-buffer + 0.5 M NaCl, and twice in 1X TE buffer at 24.

Protein-DNA complexes were eluted from beads by incubating in TES (50 mM Tris, 10 mM EDTA, 1% SDS) at 65 for 10 min and crosslinks were reversed overnight at 65. The purified proteins were digested by the addition of Proteinase K, and the DNA was purified using the GFX PCR DNA and Gel Purification kit (GE Healthcare). DNA binding of HSF1 was assessed at the Hsp70, Hsp25, and interleukin (IL)-6 promoters as well as the GAPDH open reading frame (negative control) by qPCR using the following primers: Hsp70, forward CACCAGCAGTTCCCCA, Hsp70 reverse CGCCCTGCGCCTTTAAG; Hsp25, forward CCGTCATTTGTTTTCTTCAACAAG, Hsp25 reverse GCACCCCGAAAGCTTGATC; IL-6, forward GCAACTCTCACAGAGACTAAAGG, IL-6 reverse GGACAACAGACAGTAATGTGC; GAPDH forward AGAGAGGGAGGAGGGGAATG, GAPDH reverse AACAGGGAGGAGCAGAGCAC.
3. Genomic heat shock element sequences drive cooperative human heat shock factor 1 DNA binding and selectivity

The Heat Shock Transcription Factor 1 (HSF1) activates expression of a variety of genes involved in cell survival including protein chaperones, the protein degradation machinery, anti-apoptotic proteins and transcription factors. While HSF1 activation has been linked to amelioration of neurodegenerative disease, cancer cells exhibit a dependence on HSF1 for survival. Indeed, HSF1 drives a program of gene expression in cancer cells that is distinct from that activated in response to proteotoxic stress, and HSF1 DNA binding activity is elevated in cycling cells as compared to arrested cells. Active HSF1 homo-trimerizes and binds to a DNA sequence consisting of inverted repeats of the pentameric sequence nGAAAn, known as Heat Shock Elements (HSEs). Recent ChIP-seq experiments demonstrate that the architecture of HSEs is very diverse in the genome, with deviations from the consensus sequence in the spacing, orientation and extent of HSE repeats that could influence HSF1 DNA binding efficacy and the kinetics and magnitude of target gene expression. To understand the mechanisms that dictate binding specificity, HSF1 purified as either a monomer or trimer was used to evaluate DNA binding site preferences in vitro using fluorescence polarization and thermal denaturation profiling. These results were compared with quantitative chromatin immunoprecipitation assays in vivo. We demonstrate a role for extended HSE sequences in specific sequence orientation and in driving preferential HSF1 DNA
binding to specific target loci in vivo. These studies provide a biochemical basis for understanding differential HSF1 target gene recognition and transcription in neurodegenerative disease and cancer.

The work presented in this chapter was performed in collaboration with Leah Makley Ph.D. and Professor Jason E. Gestwicki at the University of California San Francisco.

3.1 Introduction

All organisms encounter a diverse array of stresses that originate from external (e.g. temperature, toxins and infection) or physiological (e.g. developmental signals or disease states) stimuli. These stresses damage the proteome through oxidation, misfolding and/or aggregation, so organisms have developed sophisticated mechanisms to repair this damage and limit accumulation of damaged proteins (2,11,65,120,121). For example, a major cellular response to stress is to increase expression of genes encoding protein chaperones, the protein degradation machinery and anti-apoptotic proteins that prevent protein misfolding-induced cell death (122,123). This stress response system is critical in protecting cells from proteotoxicity by accelerating the turnover of damaged proteins and favoring refolding (124,125). Imbalance in the stress response system is associated with many diseases, including neurodegeneration and cancer, highlighting the need to better understand the regulation of stress gene expression.
Heat Shock Transcription Factor 1 (HSF1) is a primary mediator of stress-responsive transcription that regulates the expression of many pro-survival genes, including those encoding protein chaperones (101). HSF1 is a multi-domain stress-activated transcription factor consisting of an amino-terminal helix winged-loop helix DNA binding domain (DBD), three leucine zipper domains, (LZ1-3) that form coiled-coil interactions to facilitate HSF1 multimerization, a central regulatory domain (RD) that is extensively modified by phosphorylation, acetylation, and sumoylation, an additional leucine zipper domain (LZ4), and a carboxyl-terminal transcription activation domain (AD) (11). While a number of post-translation modifications are thought to either activate or repress HSF1, the role of specific post-translational modifications on HSF1 activity is not well understood. Under normal conditions, HSF1 largely exists as a repressed monomer in the cytoplasm and is thought to be bound, directly or indirectly, by the protein chaperones Hsp90, Hsp70, and Hsp40 (126). The LZ4 domain has also been proposed to function as an auto-inhibitory module through intra-molecular interactions with LZ1-3 that repress multimerization (59,62). In response to proteotoxic stress, HSF1 trimerizes, accumulates in the nucleus and binds a DNA sequence known as a Heat Shock Element (HSE), which consists of inverted repeats of the consensus sequence nGAAAn (11,55). Importantly, while the consensus HSE sequence is well characterized, an enormous variety of HSEs exists throughout the human genome. These HSEs vary in their primary sequence, length, and orientation of nGAAAn repeats
A striking example of this diversity is the satellite III repeat region of chromosome 9 in human cells, which contains the non-coding satellite III RNA involved in the stress response. HSF1 binds to this genomic region consisting of hundreds of GAA repeats in numerous orientations (127). While substantial work has been done to understand the HSE sequence for a single trimer binding site, the features that influence the variety of binding modes for multiple trimers are not well understood.

Increasing evidence suggests that numerous stressors of environmental and physiological origin activate HSF1 (96,110,113,128,129). Importantly, activation of HSF1 in different cellular contexts has been shown to result in remarkably varied gene expression patterns and genomic binding fingerprints (69,110,130). While decades of research have been devoted to understanding the activation cycle of HSF1, recent work also points to the enormous constellation of HSF1 target genes (69,101). This observation is highlighted by ChIP-seq studies in cell culture models of polyglutamine disease and cancer. Immortalized mouse striatal neurons expressing an expanded poly-glutamine huntingtin protein demonstrate a unique HSF1 binding profile when compared to cells expressing a non-pathological form of huntingtin (130). Moreover, genome-wide binding of HSF1 is drastically altered in malignant transformed cells, compared to benign-heat shocked cells (69). This striking diversity of HSF1 target genes reinforces the importance of understanding how HSF1 recognizes HSE sequences.
The genome-wide binding studies of HSF1, coupled with global sequence analyses, revealed that genes bound by HSF1 contain sequences that resemble the canonical nGAAn repeat. How differences in binding site preference between individual HSEs could account for differences in the transcriptional output for specific target genes has not been elucidated. Further, HSEs containing extended sequences such as those in the satellite III region have been proposed to promote cooperative HSF1 binding, but the precise DNA target site features, or HSF1 structural domains, that drive cooperative binding have not been elucidated.

While relatively little structural information on HSF1 is available, the structure of the HSF1 DNA binding domain from the yeast *K. lactis* presented the interesting finding that HSF makes a single direct contact with the major groove of the nGAAn sequence while the rest of the protein-DNA interaction occurs through water-mediated hydrogen bonds to the phosphate backbone (64). This observation supports the notion that there is substantial flexibility in HSF1 target sequences that may drive binding site preferences. Here we characterize DNA binding site preferences *in vitro* using fluorescence polarization and differential scanning fluorimetry (ThermoFluor), coupled with chromatin immunoprecipitation assays. We demonstrate a role for extended HSE sequences in specific sequence orientation and in driving preferential HSF1 DNA binding. This binding site preference is dictated by contributions from HSF1 homo-trimerization and via the HSF1 DNA binding domain. Together, these studies provide a
biochemical basis for understanding differential HSF1 target gene recognition and transcription in neurodegenerative disease and cancer.

3.2 Expression and purification of Human HSF1 Derivatives from E. coli

To examine the DNA binding site characteristics for human HSF1 in vitro, three derivatives of a synthetic 6xHis tagged human HSF1 gene were codon optimized for expression in E. coli and then purified: 1) Wild type Human HSF1 (WT) 2) Trimeric HSF1 harboring three point mutations (L391M, L395P, L398P) in the LZ4 domain that disrupt the proposed auto-inhibitory intramolecular coiled-coil interaction (LZ4m) and 3) Monomeric HSF1 with the LZ1-3 deleted (ΔLZ1-3) (Figure 20).

![Figure 20. Schematic of HSF1 derivative expression constructs in the pET15b vector. All HSF1 derivatives contain an amino-terminal 6xHis tag for affinity purification.](image)

The three proteins were purified using Ni-NTA affinity chromatography and further purified by Sephacryl s400 size exclusion chromatography. As shown in Figure 21, wild type HSF1 elutes as two distinct peaks, indicative of equilibrium between monomer and trimer. The LZ4m HSF1 derivative elutes as a single peak corresponding
to the HSF1 homotrimer and the HSF1 ΔLZ1-3 derivative elutes as a single peak corresponding to the monomeric protein. These results confirm the long-standing hypothesis that the LZ4 domain represses trimerization by demonstrating that the HSF1 LZ4m protein is constitutively trimerized in the absence of eukaryotic regulation (58). Colloidal blue staining of the purified proteins demonstrates that the HSF1 monomer, trimer, ΔLZ1-3, and LZ4m proteins are highly purified (Figure 22).

Figure 21. Size Exclusion Chromatograms of HSF1 Derivatives using Sephacryl s400 column. WT HSF1 elutes as two distinct peaks corresponding to trimer (160 mL elution) and monomer (220 mL elution). LZ4m and ΔLZ1-3 elute as single peaks corresponding to trimer and monomer respectively.
Figure 22. Colloidal blue staining of purified HSF1 derivatives confirms high purity of HSF1 proteins. Minor band is a carboxy-terminal truncation product of HSF1 confirmed by mass spectrometry.

3.3 HSF1 DNA binding in vitro correlates with multimeric state

Fluorescence polarization assays were used to quantitatively characterize the DNA binding features of the HSF1 derivatives in vitro, using a fluorescein-labeled double stranded oligonucleotide containing a canonical HSE sequence (HSE), and a derivative containing a mutated HSE sequence (mHSE) as negative control (Figure 23).

![Figure 23](image)

Figure 23. HSE and mHSE sequences used for HSF1 DNA binding analyses. Key changes in the HSE sequence in the mHSE are underlined.

As shown in Figure 24, specific DNA binding was detected for all HSF1 constructs, with a range of affinity for the specific HSE sequence.
Figure 24. Fluorescence polarization binding curves for HSF1 derivatives. Binding of HSF1 to the HSE is indicated by an increased in relative sample polarization (mP: millipolarization units).

Both HSF1 homo-trimeric species demonstrated low nanomolar affinity for the specific HSE while the HSF1 monomer exhibited an approximately 10-fold lower affinity than the HSF1 trimer (Table 1). On the other hand, the constitutive monomer (ΔLZ1-3) bound DNA with ~70 fold lower affinity than the HSF1 trimer.

Table 1. Affinity of HSF1 Derivatives for an HSE.

<table>
<thead>
<tr>
<th>HSF1 Derivative</th>
<th>WT Monomer</th>
<th>WT Trimer</th>
<th>LZ4m</th>
<th>ΔLZ1-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_d (nM)</td>
<td>122.9 ± 25.1</td>
<td>12.1 ± 2.1</td>
<td>35 ± 7.1</td>
<td>609.3 ± 160.5</td>
</tr>
</tbody>
</table>
The affinity difference between constitutive monomer and monomeric WT HSF1 may reflect HSE-binding inducing trimerization. Collectively, these results demonstrate that the purified HSF1 derivatives specifically bind to an HSE sequence in vitro.

3.4 An HSE sequence element enhances HSF1 thermal stability

To comparatively evaluate HSF1 binding to multiple HSEs, we utilized thermal denaturation profiling. In this method, fluorescent dyes, such as 1,8 ANS or SYPRO Orange, are used to measure the melting temperature of a target protein and prospective ligands can be identified by an apparent shift in that melting curve (131-133). As shown in Figure 25, a DNA oligonucleotide containing a functional HSE consensus sequence significantly stabilized all of the HSF1s tested, validating this method.

![Thermal denaturation curves for HSF1 derivatives. In the presence of HSE DNA, the melting curve is shifted to the right for all HSF1 derivatives demonstrating that DNA increases the thermal stability of HSF1. Melting is indicated by an increase in the relative fluorescence (RFU) of the sample.](image)

Figure 25. Thermal denaturation curves for HSF1 derivatives. In the presence of HSE DNA, the melting curve is shifted to the right for all HSF1 derivatives demonstrating that DNA increases the thermal stability of HSF1. Melting is indicated by an increase in the relative fluorescence (RFU) of the sample.
Next, we optimized the concentration of HSE oligonucleotides in the presence of 5 μM HSF1 to achieve the largest increase in melting temperature. Under these conditions, the mutated HSE did not cause a shift, confirming a specific protein-DNA interaction (Figure 26).

**Figure 26.** HSE increases HSF1 melting temperature in a dose dependent manner for monomer and trimer. Upon reaching stoichiometric levels of HSE:HSF1 (1.56 – 3.125 μM) an apparent increasing in melting temperature is observed (blue). At the same concentration of mHSE (red) no increase in melting temperature is observed. Data are represented as mean ± SEM, n=3.

This control was essential because DNA can change the ionic strength of a buffer, potentially influencing the apparent melting curve. Using these conditions, we determined that the HSF1 monomer, trimer, and LZ4m species experience a 17 - 19.5 °C shift in thermal stability in the presence of an HSE sequence, while the thermal stability of the ΔLZ1-3 variant was shifted by 5 °C (Figure 27).
3.5 Extended HSEs in tandem orientations facilitate cooperative HSF1 binding

HSEs throughout the mammalian genome have varied lengths, orientations, and sequences. To understand the influence of these factors, we measured the thermal shifts caused by a panel of oligonucleotides containing distinct binding sites that are
representative of genomic sequences. It has been previously shown that HSF1 makes critical polar contacts with the guanine of the nGAAAn sequence within the major groove (64). To understand the importance of the orientation of the critical guanine, we constructed an oligonucleotide consisting of tandem repeats of the nGAAAn sequence with no inversion (head to tail oligo, H2T; Figure 28). This construct maintains the contact site for the HSF1 interaction, but alters its three dimensional structure. We also analyzed DNA sequences containing three HSEs in tandem, with different orientations between the HSEs. These extended HSEs can theoretically accommodate three HSF1 trimers, but their orientations will dictate the possible interactions between trimers. A parallel oriented HSE (II HSE) contains three HSE binding sites but the orientation of sequence between trimers forces a head to tail interaction between adjacently bound trimers similar to the orientation in the H2T oligonucleotide (Figure 28). Finally, a triple HSE (triHSE) contains three HSEs, but allows for head-to-head and tail-to-tail interactions between trimers.
Using this collection of designed HSE oligos, we tested differential HSF1 binding to the panel by thermal stability profiling. As shown in Figure 29, inverting the middle nGAAAn (H2T HSE) results in a significant reduction in HSF1 binding (-12 °C for WT Monomer, WT Trimer, and LZ4m, -3 °C for ΔLZ1-3) suggesting that the interface present in the nGAAAnnTTCn HSE DNA binding site contributes to binding affinity. These data confirm the importance of sequence orientation in driving HSF1-HSE interactions. The II HSE, containing binding sites fostering head to tail interactions between trimers, does not differ from a single HSE. This suggests that although multiple trimers can be adjacently bound, they do not bind cooperatively under these conditions. However, when HSF1 binds the triHSE that contains continuous inversions of the nGAAAn sequence and head-to-head and tail-to-tail interactions between trimers, the thermal shift is increased by 7-10 °C. We hypothesize that this additional thermal shift
may be due to cooperative interactions between trimers, which enhance the thermal stability of the HSF1-HSE complex.

**Figure 29.** Melting temperature shifts of HSF1 derivatives in the presence of representative HSEs. Head to tail HSEs have reduced binding, the II HSE demonstrates no difference in binding compared to a single HSE, and the triHSE imparts a significant increase in thermal stability for all HSF1 derivatives. Data are represented as the mean ± SEM, n=3.

The importance of HSE orientation in thermal stabilization of HSF1 is further supported by data shown in Figure 30, with DNA molecules containing two HSE binding sites. The oligos shown are derivatives of the II HSE and triHSE, where the third HSE is mutated to a non-specific binding site. This allows for two trimers to be bound adjacent to each other, without changing the length of the DNA sequence to eliminate the possibility of nonspecific DNA length effects. The two adjacent HSEs are present in cooperative (green check) or non-cooperative (red X) orientations. Finally, a DNA site containing two HSEs separated by a mutant HSE is shown to accommodate two trimers, but not at adjacent HSEs. Using the WT HSF1 homotrimer, we demonstrate that when
one cooperative site is available between trimers, an additional thermal shift is observed that is greater than a single HSE but less than the triHSE that contains two cooperative interfaces.

Figure 30. Binding of WT HSF1 trimer to HSEs with varied cooperative binding sites. Continued inversion of nGAAn for an extended HSE results in higher melting temperature shifts. Data are represented as mean ± SEM, n=3.

The increased thermal shift is not observed when the two adjacent HSEs do not contain a head-to-head orientation or if the HSEs are separated by a non-functional HSE. Together, these results support the notion that specific HSE sequences and orientations can facilitate cooperative HSF1 binding as measured by an increase in HSF1 thermal stability.
3.6 The HSF1 DNA Binding domain facilitates cooperative HSE binding

It was unexpected that an increase in thermal stability would be observed with HSF1 ΔLZ1-3 because it cannot trimerize. To test whether HSF1 domains in addition to the trimerization domain mediate this effect, we tested whether interactions between DNA binding domains might be involved. We favored this possibility because DNA Binding Domains (DBDs) of adjacent HSF1 monomers might be predicted to assemble in an orientation resembling that found within a trimer.

To determine if the DBD contributes to the increase in thermal stability, we purified the HSF1 DBD and analyzed the thermal shifts elicited by different HSEs. Figure 31 shows that the HSF1 DBD is purified to homogeneity using a strategy similar to that of the other HSF1 derivatives.

**Figure 31. Purification of amino-terminal 6xHis tagged human HSF1 DBD.**
As shown in Figure 32, the thermal shifts imparted by an HSE, H2T HSE, II HSE, and triHSE have a similar relationship for the HSF1 DBD as compared to the full length HSF1 homo-trimer. Specifically, the DBD exhibits an increase in melting temperature when the HSE contains continuously inverted nGAAAn repeats – triHSE vs HSE/II HSE (Figure 32).

Figure 32. Melting temperature shifts of the HSF1 DBD and full-length HSF1 trimer. The relationship between melting temperature shifts imparted by the HSEs is similar for the DBD and the WT trimer. Data are represented as mean ± SEM, n=3.

The observation that HSF1 DNA binding domain interactions stabilize HSF1 binding to the triHSE is supported by the structural characterization of the *K. lactis* HSF1 DBD in the HSF-DNA co-crystal structure (Figure 33).
Figure 33. Structural rendering of the K. lactis HSF DBD bound to a tail-tail HSE. An extensive dimer interface (green) may contribute to cooperative binding to DNA containing continuous inversions of nGAAAn.

The crystal structure was solved with two adjacent DBDs in a tail-to-tail orientation. Importantly, this orientation is present between DBDs within a trimer when bound to an HSE but also between adjacently bound trimers with properly oriented extended HSEs, such as that found in the triHSE sequence. The observed inter-DBD interaction interface described in this structure is quite extensive, with numerous hydrogen bonds between the “wing” and “turn” of the adjacent DNA binding domains. We propose that this interface may be important not only for the high affinity HSE DNA binding of an individual trimer, but also for cooperative binding of multiple HSF1 trimers. While the initial publication of the HSF-DNA co-crystal modeled and speculated about cooperative interactions between trimers, our data represent the first quantitative measurement of cooperativity between human HSF1 trimers for HSEs.
oriented in such a manner as to permit cooperative binding (57). Moreover, we propose that this putative DBD interaction surface may underlie an additional layer of complexity for HSF1-DNA interactions when present in extended genomic HSE sequences.

3.7 Cooperative orientations in physiological HSEs influence HSF1 binding in vitro and in vivo

It is imperative to ascertain whether the cooperative HSF1-DNA interactions observed in vitro have consequences for HSF1 DNA binding in vivo. To address this question, a set of genomic HSEs recently identified in K562 cells by ChIP seq experiments was evaluated for HSF1 binding (83). In the previous study, some genomic HSE sequences were bound by HSF1, while others were bound only by the homologous transcription factor HSF2. Interestingly, the sequences that were bound by HSF1 in vivo generally contain extended and/or cooperative HSE sequences. Table 1 illustrates a panel of genomic sequences and highlights the diversity of HSEs in length and orientation of the critical guanine (red). We tested a panel of DNA oligonucleotides containing these HSE sequences in thermal shift assays. Interestingly, a range of HSF1 thermal shifts was elicited by the distinct genomic HSEs (Figure 34). The sequence and orientation of the MLL HSE resembled the H2T HSE and it elicited little increase in HSF1 thermal stability. In contrast, the prototype HSE from the HspA1A (Hsp70) promoter, or that from the UBB ubiquitin gene promoter, elicit thermal shifts similar to a “perfect” HSE. Importantly, the genomic HSEs associated with the greatest shift in HSF1
thermal stability have orientations that resemble the cooperative HSEs shown in Figure 34.

Figure 34. HSF1 HSE binding in vitro and in vivo. (Left) In vitro binding to genomic HSE sequences measured by thermal denaturation. (Right) In vivo binding to genomic HSEs measured by chromatin immunoprecipitation in HEK293 cells. Data are represented as mean ± SEM, n=3.

To evaluate whether the HSF1 thermal shifts caused by specific HSEs in vitro correlate with the strength of HSF1 DNA binding in vivo, binding was evaluated by Chromatin Immunoprecipitation experiments in HEK293T cells grown at control temperature (37°C) or heat shocked for one hour at 42°C. As shown in Figure 34, a strong correlation exists between HSE sequences driving strong HSF1 thermal shifts in vitro and HSF1 binding to HSEs in human cells. Further, the strongest binding observed in vivo was seen in HSEs with cooperative orientations. Together, these results support the hypothesis that HSF1 prefers extended HSE sequences with specifically oriented GAA repeats.
To further test the importance of cooperativity in genomic HSE binding, a panel of mutated genomic HSEs was generated (Figure 35). To test the influence of cooperative binding to the MLL HSE, a non-cooperative guanine was substituted with thymine to allow binding to a potentially cooperative guanine located on the opposing strand. Similarly, an ARHGEF1 HSE mutant predicted to accommodate cooperative binding was created by inverting an internal TTC sequence to facilitate cooperative binding throughout the HSE. In both cases, HSF1-HSE binding was enhanced (+1°C for MLL and +5°C for ARHGEF1) as measured by thermal shifts (Figure 36). To ascertain whether cooperative sites can be disrupted in genomic sequences, a variant was constructed in the HSPA1A HSE that is predicted to lack cooperative sequences between trimer binding sites without disrupting either trimer binding site individually (Figure 36). This sequence imparts a significantly lower thermal shift, -8°C compared to WT, suggesting that inter-trimer interactions are important for binding at the Hsp70 promoter. Moreover, a UBB HSE lacking the signatures of a predicted cooperative orientation results in significantly reduced HSF1 binding affinity, -7°C compared to WT. Collectively, these data demonstrate that the orientation of extended HSE sequences dictates the DNA binding affinity of HSF1 to genomic loci in human cells.
Figure 35. Illustration of WT and mutants genomic HSEs for cooperativity analysis. Blue boxes highlight regions of the sequence that were altered to increase (MLL and ARHGEF1) or decrease (HSPA1A and UBB) cooperativity.

**Table:**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Mut</th>
</tr>
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<tbody>
<tr>
<td>MLL</td>
<td>GAGAGGGCCCCGACAAGCTA CTCTCCGGGCTGTTCGAT</td>
<td>GAGAGGGCCCTACAAAGCCTA CTCTCCGGGAGATGTTCGAT</td>
</tr>
<tr>
<td>ARHGEF1</td>
<td>GAGCCCGAGCCGGAGGGCTGGGTCCGGGGCTGAGGCAACCCCGCCGAT</td>
<td>GAGCCCGAGCCGGAGGGCTGGGTCCGGGGCTGAGGCAACCCCGCCGAT</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>TCACTGAATCCCGAGAAGACTCTGAGAGTGCTACG</td>
<td>TCACTGAATCCCGAGAAGACTCTGAGAGTGCTACG</td>
</tr>
<tr>
<td>UBB</td>
<td>AAGGAAGGTTTCCGAGATCTCGAGGAAAGG</td>
<td>AAGGAAGGTTTCCGAGATCTCGAGGAAAGG</td>
</tr>
</tbody>
</table>

Figure 36. Thermal denaturation of HSF1 bound to WT and mutant genomic HSEs. Mutant HSEs increase thermal stability of HSF1 trimer (MLL, ARHGEF1) by increasing cooperativity and decrease thermal stability (HSPA1A, UBB) when decreasing cooperativity. Data are represented as mean ± SEM, n=3.
3.8 Discussion

Increasing evidence demonstrates that the human Heat Shock Transcription Factor 1 targets a diverse array of genomic loci for activation, which can have far reaching consequences for normal cellular physiology during stress and in disease. Elucidating the mechanism by which HSF1 selects different genomic loci is imperative for understanding the consequences of HSF1 activation in the diverse chromatin environment of different physiological and disease states.

The purification of HSF1 using tandem affinity and gel filtration revealed that recombinant human HSF1 exists in an equilibrium between monomer and trimer \textit{in vitro}, and that the two species can be purified by size exclusion chromatography. In this work, we also developed a method for purifying HSF1 LZ4m and ΔLZ1-3, derivatives of HSF1 that should be important tools for studying HSF1 binding to DNA. For example, the results obtained with purified HSF1 LZ4m support the idea that the carboxyl-terminal coiled-coil, LZ4, auto-inhibits trimer formation because we found this derivative to be exclusively trimeric.

One limitation to our knowledge of HSF1-DNA interactions has been the relatively cumbersome assays that are commonly used, such as electrophoretic mobility shift assays (EMSA). Initial analysis of recombinant HSF1 DNA binding with fluorescence polarization rapidly revealed quantitative differences in the affinity of HSF1 derivatives for a consensus HSE sequence. Moreover, we found that thermal
denaturation profiling was amenable to high throughput, enabling studies of DNA binding to a wide array of putative interaction sites.

Using these platforms, we studied binding of genomic HSE sequences to HSF1 variants and correlated these results with binding in the context of chromatin in HEK293T cells. Importantly, we found that the thermal shift profiling method accurately reported on the relative affinity of the genomic interactions, further validating this experimental platform. Furthermore, the results suggested that HSF1 prefers extended, cooperative HSEs, supporting the notion that this mechanism for DNA binding contributes to selectivity. It is important to note that the correlation between in vitro and in vivo binding is not perfect, particularly for HSEs found in the promoters of genes such as DARS and Hsp70. This observation suggests that another layer of complexity exists in HSF1 binding in vivo. Additional layers of complexity could be attributed to the previously reported hetero-multimerization of HSF1 with HSF2, chromatin environments, post-translational modifications and other factors (96,134-136). However, our results suggest that when HSF1 is presented with a given array of available HSEs, it will prefer cooperatively oriented HSEs. These distinct binding preferences are likely to impact the kinetics and amplitude of HSF1 transcription activation at different loci and dictate the function of HSF1 under diverse physiological and disease contexts.
3.9 Materials and Methods

Expression and Purification of human HSF1 Derivatives: A DNA cassette encoding codon optimized human HSF1 (GenScript) was cloned into the pET15b expression vector containing an amino-terminal 6x-His tag using NdeI and XhoI to generate hHSF1-pET15b. HSF1-LZ4m was generated using mutagenic PCR on the hHSF1-pET15b plasmid to introduce the L391M, L395P, and L398P mutations in the codon-optimized gene (59). ΔLZ1-3 was generated using overlapping PCR to join fragments encoding amino acids 1-137 and 199-529 of human HSF1 and subsequent cloning into pET15b with NdeI and XhoI. The HSF1 DBD was generated using PCR to amplify the sequence encoding aa 1-123. The resulting plasmids were transformed into E. coli strain BL21(DE3). Overnight cultures were diluted 1:100 and grown to OD₆₀₀ = 0.6 at 37 °C. Cultures were then transferred to 15 °C, induced with 1 mM IPTG, and grown for 16 hrs. Cell pellets were lysed in NiNTA Buffer (NB; 50 mM HEPES pH 7.5, 300 mM NaCl) supplemented with 20 mM Imidazole HCl (ImHCl), using sonication for 3 x 30 sec bursts. Lysates were cleared by centrifugation at 20,000 g for 30 min. The cleared lysate was incubated with 2 mL (bed volume) of NiNTA-Agarose beads (Qiagen) per L of culture. Beads were washed 2x with NB + 40 mM ImHCl, 2x with NB supplemented with 40 mM ImHCl, 5 mM ATP, and 20 mM MgCl₂, then 1x with NB + 40 mM ImHCl. Bound protein was eluted with NB + 250 mM ImHCl. Eluted proteins were then separated on a Sephacryl s400 (GE Healthcare) gel filtration column using an Akta FPLC
(GE Healthcare) at a flow rate of 1.3 mL/min in 25 mM pH 7.5 HEPES and 150 mM NaCl. Fractions were collected, pooled, concentrated and aliquoted at 10 μM (~0.6 mg/mL), flash frozen in N₂ and stored at -80°C.

Fluorescence Polarization Assays (FP): Fluorescently labeled HSE and mutant HSE (mHSE) oligonucleotides with a 5’ fluorescein amidie (5’FAM) modification (IDT) were used for FP. The labeled oligos were annealed to complementary sequences by heating to 95 °C and slow cooling to room temperature. FP experiments were performed in 25 mM HEPES pH 7.5, 75 mM NaCl. 1 nM labeled oligo was added to FP Buffer and a baseline milli-polarization (mP) value was taken. Human HSF1 derivatives were titrated into the reaction and mP values were taken at each concentration of HSF1. Curves shown are representative and Kₐ values are calculated from 3 independent experiments using a one-site binding fit of the curves in Graph Pad Prism 5.

Thermal Denaturation Profiling: Differential scanning fluorimetry (DSF) experiments were performed in a BioRad CFX384 RT-PCR thermocycler. A temperature gradient that started at 25 and increased to 95 °C at a rate of 0.5 °C per 30 sec was used to generate melting curves. Fluorescence readings were taken using the FRET channel. Each reaction was composed of: 25 mM HEPES pH 7.5, 75 mM NaCl, 5 μM HSF1, 5x SYPRO Orange Dye (Invitrogen), 1 mM TCEP, and DNA to a final volume of 10 μL. The reaction was plated into Hard-Shell PCR Plates 384-Well CLR/WHT (Bio-Rad). This solution was equilibrated for at least 5 minutes prior to initiation of the DSF experiment.
DNA concentration was used at a 3-fold molar excess over HSF1, as calculated using 3 HSF1 molecules binding to a single HSE. This ratio was chosen because we found that a 3-fold molar excess of DNA saturated HSF1 without significant non-specific binding to mHSE. For clarity, the resulting data sets were trimmed to the 25 – 75 °C and then normalized as a percentage of the highest and lowest RFU values within individual wells. The T\textsubscript{m} was then calculated from the maximum of the first derivative plot of the normalized RFU melting curves. Finally, ΔT\textsubscript{m} was calculated by subtracting the T\textsubscript{m} for a control mHSE from the T\textsubscript{m} of each individual HSE. The final data are presented as the mean ΔT\textsubscript{m} and SEM. Statistical analyses of WT and mutant genomic HSEs was performed by One-Way ANOVA followed by Newman-Keuls Multiple Comparison Test using Graph Pad Prism 5.

Quantitative Chromatin Immunoprecipitation: HEK293T cells were grown in 15 cm dishes to 75% confluency and maintained at 37 °C or heat shocked at 42 °C for 20 min. Cells were crosslinked with 1% Formaldehyde on ice for 5 min, quenched with 125 mM glycine for 5 min, washed 2x with cold PBS, then lysed in 2 mL Lysis Buffer (25 mM HEPES, 150 mM NaCl, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 1% Triton X-100, 0.05% SDS, 0.03% NP40, 1 mM EDTA, pH 7.4). Lysates were then sonicated 3 x 30 sec bursts and cleared by centrifugation at 14,000 g for 15 min. Lysates were then split into 2 x 1 mL aliquots (30 uL saved for input): 1 mL incubated with 5 uL affinity purified rabbit anti-HSF1 antibody overnight and 1 mL incubated without antibody. The next day, 50 uL
DynaBeads Protein G (Invitrogen) were added for 4 hrs at 4 °C. Beads were washed 2x with Lysis Buffer, 2x with Lysis Buffer + 0.5 M NaCl, and then 2x with TE pH 7.5. Proteins were eluted with TE+1% SDS for 10 min at 65°C. Beads were spun at 14k rpm for 10 min and the supernatant was incubated over night at 65°C to reverse crosslinks. The next day samples were treated with Proteinase K for 1.5 hr and purified using Qiagen PCR-cleanup kit prior to quantitative PCR analysis.
4. A direct regulatory interaction between chaperonin TRiC and stress responsive transcription factor HSF1

Heat Shock Transcription Factor 1 (HSF1) is an evolutionarily conserved transcription factor that protects cells from protein misfolding-induced stress and apoptosis. The mechanisms by which cytosolic protein misfolding leads to HSF1 activation have not been elucidated. Here we demonstrate that HSF1 is directly regulated by TRiC/CCT, a central ATP-dependent chaperonin complex that folds cytosolic proteins. A small molecule activator of HSF1, HSF1A, protects cells from stress-induced apoptosis, binds TRiC subunits in vivo and in vitro and inhibits TRiC activity without perturbation of ATP hydrolysis. Genetic inactivation or depletion of the TRiC complex results in human HSF1 activation and HSF1A inhibits the direct interaction between purified TRiC and HSF1 in vitro. These results demonstrate a direct regulatory interaction between the cytosolic chaperone machine and a critical transcription factor that protects cells from proteo-toxicity, providing a mechanistic basis for signaling perturbations in protein folding to a stress-protective transcription factor.

The work presented in this chapter was a direct collaboration with Daniel W. Neef Ph.D. and Rocio Gomez-Pastor Ph.D. In addition, Felix Willmund Ph.D. and Judith Frydman contributed through a generous gift of purified bovine TRiC as well as performing in vitro TRiC refolding and ATPase assays.
4.1 Introduction

Protein misfolding is a biochemical hallmark of diseases that include Alzheimer’s, Parkinson’s and Huntington’s, cardiomyopathy, cataract formation, lysosomal storage disease, Cystic Fibrosis, sickle cell disease and diabetes (8). Specific protein quality control mechanisms operate to both sense and respond to protein misfolding in the endoplasmic reticulum, nucleus, mitochondria and cytosol, resulting in increased folding capacity or degradation of irreversibly damaged proteins. Heat Shock Transcription Factor 1 (HSF1) is a eukaryotic transcription factor that protects cells from cytoplasmic proteo-toxicity and stress-induced apoptosis and is a promising target for neurodegenerative disease therapy (11,65). HSF1 serves as a primary mediator of cellular stress responses, facilitating the expression of genes encoding proteins involved in protecting the proteome from stress, including proteins that function in protein folding and degradation as well as transcription, transport, signal transduction, metabolism and a broad array of other adaptive and survival functions in yeast, somatic cells and neurons (63,101,137).

HSF1 is activated in response to a diverse set of environmental conditions associated with cytoplasmic protein misfolding including elevated temperatures, oxidant exposure, metals and bacterial and viral infection. Under normal cell growth conditions HSF1 is largely present as an inactive monomer, where it is thought to be bound and repressed by Hsp40, Hsp70 and Hsp90, abundant protein chaperones that
are also involved in the folding and maturation of many cellular proteins including hormone receptors and protein kinases (50,138). In response to proteo-toxic stress, HSF1 assembles as a homo-trimer, accumulates in the nucleus and binds cis-elements, termed Heat Shock Elements (HSEs) in the promoters of target genes (11). HSF1 is post-translationally modified by phosphorylation, sumoylation, ubiquitinylation and acetylation reactions that are proposed to either activate or repress HSF1 function during the regulatory cycle (72,78,104). Both HSF1 and Hsp70 possess redox-regulated thiols that also allow intrinsic HSF1 stress-sensing and stress-sensing by repressive protein chaperones, respectively (10,139). However, the mechanism by which cytoplasmic proteo-toxicity is sensed and transmitted to HSF1 is not well understood.

Sigma 32 ($\sigma^{32}$) is a bacterial proteotoxic stress-responsive transcription initiation factor that directs RNA Polymerase to the promoters of protein chaperone genes and other stress-protective target genes. $\sigma^{32}$ is regulated by feedback control via direct binding of the DnaK, DnaJ and GroE/L protein folding machinery, functionally analogous to the Hsp70 and Hsp40 chaperones and the TRiC/CCT chaperonin complex of eukaryotic cells, respectively (39,110). These chaperone interactions provide a direct mechanism for the protein folding apparatus to sense and integrate aberrant cellular protein folding with stress-protective responses by modulating $\sigma^{32}$ activity and abundance. Here we demonstrate that a chemical activator of HSF1, HSF1A, directly binds the TRiC/CCT chaperonin and modulates TRiC-dependent protein folding.
Furthermore, TRiC directly interacts with HSF1 in vitro and represses HSF1-dependent gene activation in vivo. HSF1A antagonizes the repressive HSF1-TRiC interaction, promoting the expression of protein chaperones and other HSF1 target genes that protect cells from protein-misfolding and stress-induced apoptosis. This work establishes a direct regulatory connection between the cytoplasmic protein folding nanomachine TRiC/CCT, and HSF1, a critical transcription factor activated in response to cytoplasmic proteotoxicity.

4.2 HSF1A protects against ER stress-induced apoptosis

Previous studies using a humanized HSF1-based yeast screen identified HSF1A, a benzyl-pyrazole-based small molecule, as an activator of human HSF1 (18). HSF1A activates HSF1-dependent target gene expression in mammalian and Drosophila cells and ameliorates protein aggregation-mediated toxicity and cell death in neuronal precursor cells and Drosophila models of poly-glutamine (polyQ) mediated protein-misfolding disease (18). Since HSF1 activates transcription of target genes that prevent stress-induced apoptosis such as Bag-3, which binds and stabilizes the anti-apoptotic factor McI1 (140), HSF1A was evaluated for protection of cells from apoptosis induced by other cell stressors. The endoplasmic reticulum (ER) stress agent, tunicamycin, causes the accumulation of misfolded proteins in the ER and promotes activation of the Unfolded Protein Response (UPR), which, upon prolonged activation, induces apoptotic cell death (121,141). The proline analogue azetidine (AZC) incorporates into nascent polypeptide
chains and promotes their misfolding, causing widespread misfolding of both ER and cytoplasmic proteins. Pre-treatment of cells with HSF1A, followed by tunicamycin or AZC exposure, ameliorated stress-induced cell death (Figure 37). Furthermore, HSF1A treatment reduced tunicamycin-induced expression of Bip and Erdj3, two UPR-dependent ER chaperone genes, and blunted activation of caspase-3, the primary mediator of apoptotic cell death (141) (Figure 37).

Figure 37. HSF1A protects cells from apoptotic cell death induced by tunicamycin. (Left) Cell viability of MEFs following tunicamycin treatment measured by Cell Titer Glo. (Right) Immunoblot analysis of MEF protein extracts for Hsp70 ER stress markers (Bip, Erdj3) and the apoptosis marker Caspase-3. Cell viability data is represented as mean ± SEM, n=3.

The HSF1 targets Hsp70 and Bag-3, have been shown to ameliorate ER-stress induced apoptosis by enhancing UPR signaling and stabilizing anti-apoptotic proteins, respectively (140,142). As ER-stress induced apoptosis contributes to the pathology of diabetes and cardiovascular disease (143,144), we tested whether HSF1A protects cells in disease-relevant models of ER-stress. Previous studies showed that exposure of the...
pancreatic beta-cell line INS 832/13 to free fatty acids or high levels of glucose promotes UPR activation and activates apoptosis (145). While palmitate caused a marked reduction in INS 832/13 cell viability (Figure 38) and promoted the expression of the UPR and caspases-3 activation (Figure 38) both phenotypes were ameliorated by HSF1A pretreatment.

![Graph showing cell viability](image)

**Figure 38. HSF1A Protects cells from INS 832/13 cells from Palmitic Acid (PA) induced apoptosis.** (Left) Cell viability of INS 832/13 cells exposed to PA measured by Cell Titer Glo. (Right) Immunoblot analysis of INS 832/13 protein extracts for Hsp70, ER stress markers (Bip, Erdj3) and apoptosis marker Caspase-3. Cell viability data is represented as mean ± SEM, n=3.

Elevated levels of homocysteine induce inflammatory toxicity and is a risk factor in cardiovascular disease, particularly in endothelial cells which fail to express cystathionine β-synthase (CBS) (146). Since mouse NIH3T3 cells are also deficient in the expression of CBS (147) homocysteine exposure results in a reduction in both NIH3T3 cell viability and basal Hsp70 levels, which promotes activation of the UPR and caspase-3 cleavage (Figure 39), phenotypes that were reduced by HSF1A administration.
Figure 39. HSF1A Protects NIH3T3 cells from homocysteine (Hcy) toxicity. (Left) Cell viability analysis of 3T3 cells exposed to Hcy measured by Cell Titer Glo. (Right) Immunoblot analysis of 3T3 cells for Hsp70, ER stress marker Erdj3, and apoptosis marker Caspase-3. Cell viability data is represented as mean ± SEM, n=3.

Taken together, the HSF1 activator HSF1A protects cells from a range of disease relevant proteotoxic conditions that induce apoptosis, supporting further investigation into the mechanism by which HSF1A activates HSF1.

4.3 HSF1A directly interacts with TRiC/CCT subunits

Pull-down experiments using a biotinylated form of HSF1A (HSF1A-Biotin) resulted in the enrichment of all subunits of the TRiC/CCT chaperonin in lysates from both yeast and mammalian cells (18). As several protein chaperones including Hsp/Hsc70 and Hsp90 have been linked to the regulation of HSF1, experiments were carried out to determine if HSF1A-Biotin associates with these protein chaperones, or with HSF1. While HSF1A-Biotin associated with the TRiC complex as shown by immunoblotting for two TRiC subunits, Tcp1 and Cct3, it did not bind Hsp90, Hsp70, Hsc70, Hsp27, nor did it associate with HSF1 itself (Figure 40).
HSF1A-Biotin associates with the TRiC complex but not with HSF1 protein chaperones known to regulate HSF1. HSF1A bound proteins were captured by Neutravidin-agarose purification.

To ascertain if HSF1A directly binds TRiC, HSF1A-Biotin was incubated with purified bovine TRiC and purified recombinant Hsp70 and captured proteins analyzed by immunoblotting (Figure 41).

Figure 40. HSF1A-Biotin associates with the TRiC complex but not with HSF1 protein chaperones known to regulate HSF1. HSF1A bound proteins were captured by Neutravidin-agarose purification.

Figure 41. HSF1A-Biotin interacts with purified TRiC but not purified Hsp70. Either TRiC or Hsp70 was incubated with HSF1A-Biotin prior to purification with Neutravidin-agarose.
Using antibodies against TRiC subunits Cct2 and Cct3 demonstrates that HSF1A directly interacts with TRiC but not with Hsp70. The TRiC complex is composed of eight independently expressed protein subunits that assemble into a dual-ringed hetero-oligomeric structure (148). While HSF1A-Biotin directly binds to TRiC, these experiments do not distinguish whether HSF1A-Biotin binds to individual TRiC subunits or only interacts with the assembled TRiC complex. As shown in Figure 42, HSF1A-Biotin binds to the Tcp1, Cct2, Cct5 and Cct8 subunits of yeast TRiC (44) when independently expressed in E. coli.

![Image of Figure 42](image.png)

**Figure 42.** HSF1A-Biotin interacts with yeast TRiC subunits when individually expressed in E. coli. HSF1A-Biotin interacting proteins were captured using Neutravidin-agarose purification from E. coli lysate.

A direct interaction between HSF1A and TRiC is further supported by the observation that the thermal stability of purified bovine TRiC is reduced in a dose-dependent manner in the presence of HSF1A-Biotin, but not Biotin (Figure 43).
Figure 43. HSF1A-Biotin decreases the thermal stability of purified TRiC. Data are represented as mean ± SEM, n=3.

Moreover, fluorescence anisotropy experiments using FITC coupled to HSF1A demonstrated that HSF1A-FITC bound to a purified Tcp1 subunit of TRiC with an affinity of approximately 600 nM (Figure 44).

Figure 44. Fluorescence polarization of HSF1A-FITC binding to purified yeast Tcp1. Purified 6xHis tagged Tcp1 was titrated into a solution containing 1 nM HSF1A-FITC to generate binding curves. Data points are represented as the mean ± SEM, and curves generated using a one-site binding fit with GraphPad Prism, n=3.
This was validated qualitatively via titration of purified Tcp1 into binding reactions containing 500 nM Biotin or HSF1A-Biotin (Figure 45). Taken together, these data demonstrate that HSF1A associates with TRiC in vivo and in vitro and can engage in interactions with individual TRiC subunits. These results suggest that HSF1A stimulation of HSF1 activity is mediated through the modulation of TRiC upon direct binding.

![Figure 45. HSF1A-Biotin pulldown of yeast Tcp1. Increasing concentrations of purified 6xHis tagged Tcp1 was incubated with HSF1A-Biotin and captured using Neutravidin-agarose.](Image)

The ability of HSF1A to modulate TRiC-dependent protein folding activity was assessed by monitoring TRiC-mediated refolding of denatured $^{35}$S-labeled Actin in vitro (44). Addition of 200 µM HSF1A reduced TRiC-mediated actin folding by approximately 50% (Figure 46), while only mildly inhibiting TRiC-dependent ATP-hydrolysis (Figure 47).
Figure 46. HSF1A reduces TRiC mediated refolding of actin in vitro. $^{35}$S-Actin refolding was measured by binding to immobilized DNAse1 and subsequent elution and SDS-PAGE and radiography. (Top) quantification of radiograph (bottom).

Figure 47. HSF1A does not impact TRiC ATPase activity. ATP hydrolysis is indicated by the relative ratio of ADP/ATP measured by thin layer chromatography. Consistent with the observation that HSF1A is not a potent inhibitor of ATP hydrolysis, ATP, but not HSF1A eluted a human Cct4-GFP fusion protein pre-bound to a gamma phosphate-linked ATP-sepharose resin (149) (Figure 48, Figure 49).
Figure 48. HSF1A does not compete for CCT4-GFP binding to ATP-sepharose resin. Immunoblot analysis of CCT4 following elution of HEK293T extracts bound to ATP-sepharose with either ATP (top) or HSF1A-Biotin (bottom).
Figure 49. GFP Fluorescence analysis of CCT4-GFP elution from ATP-Sepharose. ATP efficiently elutes CCT-GFP from ATP-Sepharose (top), whereas CCT4-GFP is retained in the presence of HSF1A-Biotin (bottom).

As shown by example in Figure 50 for Tcp1, all eight distinct TRiC subunits are composed of two equatorial domains that form the ATP binding domain, two hinge regions and a central apical domain that binds substrates.
Figure 50. Schematic of Tcp1 domain architecture. D1 contains the equatorial domain A of Tcp1, D2 contains hinge 1 and the substrate binding domain, and D3 contains hinge 2 and equatorial domain B.

While full-length purified Tcp1 is bound by HSF1A-Biotin, a Tcp1 fragment (designated D3) containing only the second hinge and equatorial domain (B) is sufficient for HSF1A-Biotin binding (Figure 51, Figure 52).

Figure 51. HSF1A-Biotin interacts with Tcp1 Domain 3. Immunoblot analysis of full length 6xHis tagged Tcp1 (left) or Tcp1 domain fragments (right) following HSF1A-Biotin pulldown with Neutravidin-agarose.
Moreover, the presence of the second hinge region is important for HSF1A-Biotin binding, and mutation of three amino acids within this hinge (LDE to AAA), within the context of the full D3 fragment, abrogated HSF1A-Biotin binding (Figure 53).

Figure 52. HSF1A interacts with the Hinge region of Tcp1 D3. A truncated derivative of Tcp1 D3 containing amino acids 404-559 does not interact with HSF1A-Biotin as measured by Tcp1 immunoblot following Neutravidin-agarose pull-down.

Figure 53. Mutation of a conserved sequence of the Hinge region in Tcp1 D3 disrupts interaction with HSF1A. Mutation of LDE395 to AAA disrupts the HSF1A-Tcp1 interaction. Mutation of ATPase catalytic residues DSL404 or ATP binding residues GGG423 does not impact the Tcp1 HSF1A interaction.
These results demonstrate that HSF1A binds to TRiC and perturbs its folding activity, but that this interaction does not require the bipartite ATP binding pocket on the TRiC Tcp1 subunit.

**4.4 Compromising TRiC function activates human HSF1 in yeast and mammalian cells**

TRiC is essential for *S. cerevisiae* and mammalian cell viability (150). As HSF1A-Biotin interacts with both yeast and mammalian TRiC, and modulates mammalian TRiC activity *in vitro*, experiments were conducted to assess whether HSF1A modulates TRiC activity *in vivo*. Yeast DAmP strains, in which disruption of the *TCP1* and *CCT8* 3′UTR destabilizes their corresponding mRNA (151), were exposed to HSF1A or DMSO. Low concentrations of HSF1A (10 µM) did not affect the growth rate of a wild-type yeast strain at 30°C, but reduced the growth rate of a tcp1-DAmP strain by ~50% (Figure 54), a phenotype that was exacerbated when the tcp1-DAmP strain was grown under a mild thermal stress of 37 °C. While 10 µM HSF1A did not inhibit growth of the cct8-DAmP strain at 30 °C, growth was significantly reduced at 37 °C. Collectively, these data suggest that HSF1A inhibits yeast TRiC function *in vivo*. 
Figure 54. Growth of yeast containing hypomorphic TRiC alleles are sensitized to HSF1A treatment. Growth rate was determined in liquid culture and measured by OD$_{600}$ of cultures exposed to HSF1A normalized to cultures grown in DMSO. Data are represented as mean ± SEM, n=3.

Hsp90 is required for the folding and stability of a number of client proteins and genetic or pharmacological inhibition of Hsp90 promotes the degradation of client proteins (152). While cells exposed to HSF1A exhibited increased Hsp70 levels as expected due to HSF1 activation, the steady state levels of actin and α-tubulin, two TRiC-client proteins, were not altered (Figure 55).
Figure 55. Immunoblot analysis of TRiC substrates following HSF1A treatment. TRiC substrates actin, tubulin, and VHL are not significantly reduced with HSF1A treatment.

A modest reduction in von-Hippel-Lindau tumor suppressor protein (VHL) levels was observed in response to HSF1A, though this reduction was dramatic in response to heat shock and may result from cell stress rather than inhibition of TRiC activity. Modest increases in Cct2 and Cct3 levels were also observed in response to HSF1A (Figure 55), consistent with the mammalian TRiC genes being direct HSF1 targets (153).

VHL requires both the TRiC complex and Hsp70 for correct folding and function and association of VHL with TRiC/Hsp70 can be detected by co-immunoprecipitation (154). To test whether HSF1A inhibits TRiC function in vivo, the interaction between TRiC and VHL was assessed in extracts from cells treated with HSF1A or DMSO solvent after HA-tagged-VHL immunoprecipitation. In control cells HA-VHL was
immunoprecipitated with Hsp70 and the TRiC complex. HSF1A treatment reduced the association of Cct3 and Cct8 with VHL approximately 50% and 80% respectively, while association of Hsp70 with HA-VHL was unaffected (Figure 56).

Figure 56. Co-immunoprecipitation of VHL interacting proteins following HSF1A treatment. Immunoblots (left) and densitometry (right) demonstrate that while TRiC interaction with VHL is reduced with HSF1A treatment, VHL interaction with Hsp70 in unaffected.

As HSF1A was identified as an activator of human HSF1 expressed in yeast, and HSF1A-Biotin binds both mammalian and yeast TRiC, humanized HSF1 yeast cells were used to ascertain whether yeast TRiC represses human HSF1. Assembly of the functional TRiC chaperonin is dependent on the correct stoichiometry of the individual subunits. Disruption of the stoichiometry of the TRiC subunits, by overexpression of one subunit, reduces yeast cell viability (155). To ascertain if reduced yeast TRiC activity promotes human HSF1 activation in yeast, a strain expressing yeast HSF from a URA3 plasmid, and human HSF1 from a LEU2 plasmid, was used as recipient to over-express individual TRiC subunit genes. Activation of human HSF1 is demonstrated by the ability of cells to grow on medium containing 5-FOA, indicative of the ability of cells to
lose the *URA3*-based plasmid carrying the yeast HSF gene. Over-expression of all five individual TRiC subunits tested promoted human HSF1-dependent yeast growth, suggesting that yeast TRiC represses human HSF1 function in yeast (Figure 57).

![Figure 57](image)

**Figure 57.** Spot assay analyzing hHSF1 dependent yeast growth. Under normal conditions, hHSF1 cannot complement for the loss of essential yHSF, but when TRiC activity is compromised through overexpression of individual TRiC subunits yeast can grow under conditions where *URA3* yHSF expression plasmid is lost (5-FOA).

Neither HSF1A, nor TRiC subunit over-expression activated expression from a yeast HSF-*lacZ* fusion reporter gene in a wild type yeast strain, indicating that these conditions did not cause global protein misfolding (Figure 58).
Figure 58. Analysis of global protein misfolding measured by SSA3–lacZ reporter assay. Treatment with HSF1A (left) or overexpression of TRiC subunits (right) did not induce SSA3 promoter activity. Data are represented as mean ± SEM, n=3.

Moreover, consistent with the low conservation of primary structure between yeast and human HSF1 (Figure 59), and the constitutive trimerization state of yeast HSF, these results suggest that yeast HSF is not regulated by TRiC.

Figure 59. Conservation of yHSF and hHSF1 is primarily localized to the DBD and LZ1-3 highlighting the possible regions of differential regulation by TRiC.
In addition, the high sequence identity between yeast and mammalian TRiC of ~47 to 65% (Figure 60) is consistent with both yeast and mammalian TRiC being able to bind to human HSF1.

![Molecular structure with sequence identities](image)

**Figure 60. Yeast and Human TRiC subunits are highly conserved.**

While partial loss of function mutations in TRiC subunits have been described, it is difficult to ascertain the specific contribution of these mutations on the activation of human HSF1 in yeast, since both human HSF1 and yeast HSF bind to similar promoter elements. To circumvent this complexity, yeast were transformed with a plasmid encoding a human HSF1 protein that lacks the DNA binding domain but is fused to the DNA binding domain of the prokaryotic LexA repressor (HSF1-LexA) (Figure 61).
Figure 61. Illustration of the hHSF1-LexA-LacZ Reporter Assay. Fusion of the LexA DNA binding domain (DBD) to hHSF1 lacking a DBD enables monitoring of hHSF1 regulation outside of the DBD without competing for yHSF DNA binding.

HSF1-LexA binds the LexA operator and lack of activity of HSF1 persists in the HSF1-LexA fusion, as the wild-type fusion protein does not promote activation of a LexA operator, β-galactosidase reporter, (Figure 62). Expression of HSF1-LexA in a strain expressing a CCT6-D89E mutant allele, which partially disrupts Cct6 function (156), resulted in activation of the LexA Op-lacZ reporter as compared to the wild type strain. No activation of the yeast HSF- specific SSA3-lacZ reporter was observed in the CCT6-D89E mutant strain (Figure 62). These results demonstrate that TRiC functions to repress human HSF1, but not yeast HSF, in yeast. Moreover, these results suggest that TRiC represses human HSF1 independently of its DNA binding function.
Figure 62. Measurement of hHSF1-LexA fusion activity using lacZ reporter assay. Yeast cells expressing a hypomorphic allele of CCT6 (D89E) exhibit increased hHSF1 activity (left). Expression of CCT6 D89E does not induce global protein misfolding measured by SSA3-lacZ. Data are represented as mean ± SEM, n=3.

To ascertain if TRiC represses mammalian HSF1 in mammalian cells, expression of TRiC subunits was reduced by RNAi and Hsp70 protein and mRNA levels were assessed. As previously reported, knock-down of either TCP1 or CCT3 in HeLa cells (Figure 63) resulted in significantly diminished expression of the RNAi-targeted gene and other TRiC subunits (157). Knock-down of either TCP1 or CCT3 resulted in a ~2-fold increase in Hsp70 expression in unstressed cells (Figure 63).
Figure 63. Knockdown of Tcp1 or Cct3 in HeLA cells results in increased Hsp70 expression measured by immunoblot (left) or ELISA (right). ELISA measurements are represented as mean ± SEM, n=3.

Similarly, over-expression of TCP1 in 3T3 cells resulted in significant elevation of both Hsp70 protein and mRNA levels, as measured by immunoblotting and qRT-PCR, respectively (Figure 64).

Figure 64. Overexpression of Tcp1 results in increased Hsp70 protein levels measured by immunoblot (left) and mRNA levels measured by qRT-PCR (right), which is represented as the mean of three experiments ± SEM.

These results demonstrate that TRiC represses human HSF1 activity in both yeast and mammalian cells.
4.5 HSF1A antagonizes direct inhibition of HSF1 by TRiC

To ascertain if repression of HSF1 by TRiC occurs via TRiC-HSF1 interactions, co-immuno-precipitation experiments were conducted by transfecting HEK293T cells with plasmids to express FLAG-tagged HSF1 protein and cells were treated with or without the membrane permeable cross linker DSP, as interactions between HSF1 and Hsp90 are stabilized by the addition of a cross linker (138). FLAG-HSF1 was immunoprecipitated and associated proteins analyzed by immunoblotting. Hsp70 co-purified with HSF1, with some enrichment after treatment with DSP, while Hsp90 was highly enriched upon addition of the cross linker (Figure 65).

![Figure 65. Co-immunoprecipitation of HSF1-FLAG. Addition of DSP crosslinker enriches the pulldown of HSF1 interacting proteins Hsp90, Hsp70 and TRiC subunits Cct2 and Cct3.](image-url)
Co-purification of the TRiC complex, visualized through immunoblotting for the Cct2 and Cct3 subunits, was preferentially observed in the presence of cross linker, suggesting that, like Hsp90, the TRiC-HSF1 interaction is labile. The DSP-stabilized TRiC-HSF1 interaction was independently validated utilizing the dual carboxyl-terminal TAP-GFP-tagged mouse HSF1 allele expressed in HEK293T cells (Figure 66). No interaction between HSF1 and the abundant protein tubulin was detected.

Figure 66. Co-immunoprecipitation of HSF1 interacting proteins using Tandem-affinity-purification (TAP) with GFP. HSF1 co-precipitates with Hsp90, Hsp70 and TRiC subunit Cct3.

To test whether TRiC and HSF1 directly interact, purified His\textsubscript{6}-tagged HSF1 was incubated either alone, or with purified bovine TRiC, and HSF1 was affinity captured by cobalt-resin and analyzed by immunoblotting. While very low levels of TRiC (ascertained by immunoblotting for Cct2 and Cct3) bound to the cobalt-resin (Figure 67, lane 4), TRiC was enriched when co-incubated with HSF1 (Figure 67, lane 8). As TRiC interacts with the N17 domain of the Huntingtin protein that is known to form coiled-
coils (158) the possibility that the TRiC-HSF1 interaction is mediated via the HSF1 coiled-coil trimerization domain was investigated. However, as shown in Figure 67 (lane 7) the HSF1-TRiC interaction was not abrogated by deletion of the HSF1 trimerization domain (HSF1ΔLZ1-3). To ascertain if HSF1A influences the HSF1-TRiC interaction in vitro, TRiC was pre-incubated with HSF1A-Biotin (AB), or Biotin (B) alone, before the addition of HSF1.

Figure 67. In vitro co-purification of HSF1 and TRiC. Purified TRiC co-precipitates with 6xHis tagged WT HSF1 and HSF1ΔLZ1-3 using Cobalt-agarose beads.

While Biotin alone had no effect on the interaction, HSF1A-Biotin inhibited TRiC-HSF1 complex formation (Figure 68).
Figure 68. HSF1A perturbs the direct interaction of HSF1 and TRiC by measured by Cobalt-agarose pulldown.

Similarly, treatment of NIH3T3 and HEK293T cells with HSF1A, prior to DSP crosslinking, reduced the interaction between TRiC and HSF1 in vivo though this was more variable in vivo (Figure 69).

Figure 69. HSF1A treatment perturbs the interaction between TRiC and HSF1 in NIH 3T3 cells (left) and HEK293T cells (right). Immunoprecipitation of HSF1-FLAG results in co-purification of Hsp90, Hsp70 and Cct3. Upon HSF1A treatment, the precipitation of Cct3 is reduced but pulldown of Hsp90 and Hsp70 is unchanged.
Taken together, these data suggest a model that HSF1A directly binds to TRiC, perhaps destabilizing the interaction between TRiC and HSF1 resulting in the amelioration of TRiC-mediated repression of HSF1 (Figure 70).

Figure 70. Model for TRiC Mediated Repression of HSF1 Activity and HSF1A Mechanism of Action.

4.6 Discussion

Cytoplasmic proteotoxic stress causes the generation of misfolded proteins that lead to cellular dysfunction and apoptosis. While HSF1 is a central stress-responsive
transcription factor that undergoes a switch from an inactive monomer to a DNA binding active homo-trimer, little is known about the mechanisms that regulate this transition, and downstream activation steps, in response to cytosolic protein misfolding. Previous studies reported that the Hsp90 and Hsp70 chaperones associate with HSF1 in cell extracts and play an inhibitory role in HSF1 regulation. However, neither purified Hsp90, nor Hsp70, has been reconstituted into complexes with HSF1 in vitro to distinguish between a direct, versus indirect regulatory role on HSF1. In contrast, here we reconstitute a direct interaction between TRiC and HSF1 that can be targeted pharmacologically to activate the stress-protective response.

Our previous studies identified HSF1A as an activator of HSF1 in a humanized yeast screen that is also active in diverse metazoan cell types. While HSF1A neither inhibits nor binds Hsp90, HSF1A-Biotin and HSF1A-FITC interact with the TRiC/CCT chaperonin complex in yeast and mammalian cell lysates and as purified subunits or in the fully assembled, active complex. HSF1A binds to the TRiC complex directly and modulates TRiC-dependent chaperone activity in vivo and in vitro. While small molecule inhibitors have been described for Hsp90 that result in the destabilization of Hsp90 client proteins and the concomitant activation of HSF1, no direct small molecule inhibitors of the TRiC/CCT complex have been previously reported.

The precise mechanism by which HSF1A modulates TRiC activity is not yet understood. HSF1A may associate with client-bound TRiC or with the apo-form of
TRiC, but in either case, HSF1A interacts with the highly conserved TRiC subunits from both *S. cerevisiae* and mammalian cells (Figure 60). Given that HSF1A binds directly to all four of individual CCT subunits evaluated, the chaperonin ATPase domain was a candidate binding site for HSF1A. However, our experiments suggest that HSF1A neither strongly inhibits TRiC ATPase activity nor competes effectively for ATP binding. Furthermore, mutagenesis experiments suggest that a distinct chaperonin domain, involving the hinge 2 region, may constitute the site to which HSF1A binds. Indeed, it has been described that TRiC activity and stability can be regulated by Vaccinia-related kinase 2 (VRK2) through a mechanism that requires an interaction with the carboxyl-terminal region of the TRiC equatorial domain, without affecting ATP hydrolysis (159). Further analysis will be required to characterize the HSF1A binding site and to understand how HSF1A binding alters TRiC structure, function and regulatory interactions with HSF1.

As TRiC directly interacts with HSF1, our data support a direct repressor role for TRiC in regulating HSF1 activity. Perhaps the binding of HSF1A to TRiC competes for a TRiC-HSF1 interaction surface, or initiates a conformational change in TRiC that reduces the affinity for the HSF1-TRiC interaction. An alternative explanation for HSF1A-dependent HSF1 activation via TRiC is that HSF1A may inhibit TRiC activity, leading to the accumulation of misfolded TRiC client proteins that, in turn, stimulate the HSF1-mediated heat shock response. However, neither HSF1A exposure, nor genetic
inhibition of TRiC, promotes the activation of yeast HSF which, like its metazoan
counterparts, is activated in response to conditions that cause protein misfolding (160). This suggests that the negative regulation of HSF1 by TRiC is a metazoan feature of the stress response. Unlike the high degree of conservation between the yeast and human TRiC subunits, yeast and human HSF1 show little overall protein sequence homology outside of their DNA binding domains (Figure 59). Furthermore, given that yeast HSF has been reported to be a constitutive homo-trimer, the lack of conservation of the HSF-TRiC regulatory interaction in yeast is not surprising. While data presented herein suggest that HSF1A antagonizes TRiC-dependent HSF1 repression, our data demonstrating the co-purification of TRiC and HSF1 in vivo and in vitro strongly support a model that TRiC acts directly on HSF1. Furthermore, our data suggest that the highly conserved TRiC subunits from both S. cerevisiae and human (Figure 60) are able to engage in repressive interactions with human HSF1. The TRiC interactome encompasses many functional classes of cytoplasmic proteins including those involved in the function of the cytoskeleton, DNA replication and repair, cell cycle progression, RNA processing and protein trafficking (42,45).

Studies in E. coli demonstrated that the chaperonin GroEL directly binds to and represses the activity of σ32, the prokaryotic proteotoxic stress-responsive transcription initiation factor (39,46). Indeed, σ32 regulation by DnaK, DnaJ and GroE/L provides a sophisticated chaperone-mediated regulatory circuit in which the protein quality control
pathway directly integrates and communicates with the stress-responsive transcription machinery. Our work suggests that this direct regulatory relationship between a chaperonin complex and stress-activated transcription factor is conserved in mammals and provides an additional level for regulating stress activation of HSF1. Recent work demonstrating specific thiol oxidation in Hsp70 as an activating signal for HSF1 highlights the sophisticated mechanisms built into chaperone-mediated regulation of HSF1 (139). As HSF1 is activated by a plethora of proteotoxic stress conditions, perhaps distinct stresses integrate HSF1 activation via the modulation of distinct sets of chaperone repressors. As arsenic is an HSF1 activator that also inhibits (161), it will be interesting to evaluate whether chaperonin inhibition is a key pathway for HSF1 activation by arsenic. Indeed, Pan et al observed that TRiC activity is very sensitive to thiol oxidation in vivo and in vitro, which may resemble the thiol-dependent regulation of HSF1 observed through the action of other chaperones such as Hsp70 (161).

Human HSF1 is regulated by Hsp70 and Hsp90, either directly or indirectly and our data show that the TRiC complex directly binds and represses HSF1. HSF1 may exist in sub-populations regulated by different protein chaperones, perhaps in a tissue or cell type dependent manner or in response to diverse proteotoxic stimuli. This hypothesis is supported by the finding that RNAi mediated knock down of TRiC subunits in C. elegans elicits muscle-specific activation of HSF1 (110) and suggests that TRiC may be a primary regulator of HSF1 in myocytes. Interestingly, tissue specific regulation of HSF1 by
different protein chaperones may have direct physiological disease relevance. Evidence has shown that the expression of protein chaperones such as Hsp70, as well as the activity of HSF1, is strongly repressed in insulin-resistant tissues in Type 2 diabetes (162). While the mechanisms underlying the repression of HSF1 and Hsp70 are not understood, proteomics analysis of muscle biopsies revealed that Hsp70 levels were dramatically reduced, yet levels of individual TRiC subunits were significantly elevated, in diabetes. The levels of specific chaperones in different tissues or in disease states may determine their contribution toward HSF1 regulation and allow for sophisticated integration of diverse stressful stimuli. A greater understanding of the mechanisms regulating HSF1 activity in tissues and disease states could lead to development of pharmacological interventions targeting HSF1 for specific human conditions.

4.7 Materials and Methods

Yeast and mammalian cells, transfections, siRNA: Yeast cell growth conditions are detailed in figure legends. Mammalian cell lines used in this study were human HeLa and HEK293T cells, mouse NIH3T3, wild-type and hsf1+/− MEFs (REF) stably transfected with either pcDNA3.1(+)/Zeo or pcDNA3.1(+)/Zeo-hHSF1 and rat INS 832/13 cells. Plasmids used in this study were transfected into cells using Lipofectamine LTX (Invitrogen) following the manufactures guidelines. siRNA against TCP1 and CCT3 was purchased from Dhharmacon and 2 nmoles of each siRNA were transfected into HeLa or 3T3 cells using Dharmafect 1.
HSF1A-Biotin affinity-capture experiments: Protein extracts were generated from mammalian, yeast and *E. coli* cultures using biotin-binding buffer (20 mM HEPES, 5 mM MgCl2, 1 mM EDTA, 100 mM KCl, 0.03% NP-40) supplemented with 1% Triton-X100 and protease inhibitors. Approximately 0.5 mg of protein extract was incubated with 100 μM HSF1A-Biotin for 4 h at 4 °C and HSF1A-Biotin associated proteins captured by with NeutrAvidin Agarose Resin (Pierce). After washing in biotin binding buffer proteins were eluted using 50 μL biotin elution buffer (100 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 2 mM D-biotin), resolved on a 4-20% SDS- PAGE, and immunoblotted. For purified TRiC and Hsp70 analyses, 5 nM protein was incubated in biotin-binding buffer + 0.5% Triton X-100 with 100 μM biotin or 100 μM HSF1A-Biotin for 4 h at 4 °C and captured with NeutrAvidin Resin. For NiNTA purified yeast Tcp1, different concentrations of Tcp1 0.5 mM, 1 mM, 2 mM, 3 mM and 4 mM in 25 mM Hepes pH 7.5, 150 mM NaCl were incubated with 0.5 mM Biotin or HSF1A-Biotin for 4 h at 4 °C and captured with NeutrAvidin Resin.

TRiC subunit expression in *E. coli*: ORFs of yeast TRiC subunits were PCR amplified and cloned into the *E. coli* expression vector pT7-FLAG-4 (Sigma), transformed into BL21(DE3) cells and protein expression induced via the addition of 1 mM IPTG for 3 h at 37 °C. Total protein extracts were generated by cell lysis in biotin binding buffer supplemented with 1% Triton X-100 and protease inhibitors.
HSF1-TRiC co-immunoprecipitation assay: HEK293T cells transfected with the indicated plasmid were crosslinked with DSP, lysed in IP buffer, and 0.5 mg of protein was immunoprecipitated with anti-FLAG-M2 affinity gel (Sigma) for FLAG-HSF1 or anti-GFP agarose resin (Santa Cruz) for mHSF1-TAP. Captured proteins were eluted with Laemmli buffer and analyzed by immunoblotting.

*In vitro* TRiC – HSF1 binding assays: Recombinant human HSF1 was purified as previously described (56). Purified HSF1 and purified TRiC (5 nM) were incubated either alone or together in biotin binding buffer for 1 h at RT and captured using a cobalt-agarose resin for 90 min at 4 °C. After washing, bound proteins were eluted with buffer supplemented with 500 mM imidazole and analyzed by immunoblotting.

Mammalian Cell Culture conditions: HeLa, HEK293T and 3T3 cells were maintained on DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. MEF cells were maintained on DMEM medium supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol. INS 832/13 cells were maintained on RPMI-1640 supplemented with 10% FBS, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate and 55 μM 2-mercaptoethanol.

Endoplasmic Reticulum stress experiments: NIH3T3 cells were treated for 15 hours with or without pre-incubation with 50 μM HSF1A and 5 mM homocysteine. In separate experiments, NIH3T3 cells were treated with or without HSF1A in the presence
or absence of 0.4 µg/ml tunicamycin or azetidine (AZC). For palmitic acid (PA) experiments, INS1 cells were pre-incubated in the absence or presence (20 uM) of HSF1A followed by incubation in the absence or presence of 0.5 mM PA in bovine serum albumin. INS1 cells were treated with 30 mM glucose for 3 days following HSF1A pre-incubation as described above. Cell Titer Glo was used to assess cell viability.

Thermal denaturation profiling: Thermal denaturation experiments were performed in a BioRad CFX384 qPCR apparatus with standard 384 well qPCR plates. Melting curves were generated using a temperature gradient of 25 – 95 °C with 1°C/min increments and fluorescence was quantified with the FRET channel every 30 seconds. Well components included 20 mM HEPES pH 7.4, 100 mM KCl, 1 mM EDTA, 5 mM MgCl2, 0.1 mg/ml purified bovine TRiC, 5X SYPRO orange dye (Invitrogen), 0.4 % DMSO, and increasing concentrations of HSF1A-Biotin or Biotin. First derivative curves were generated using Graph Pad Prism. The maximum value from the derivative curve is determined to be the melting point. Melting temperature values in histograms were calculated from the mean of three derivative curves from independent experiments. Error bars represent the standard error of the mean (SEM).

Fluorescence Polarization: Fluorescence polarization experiments were carried out using a fluorescein conjugated HSF1A (HSF1A-FITC). The FITC moiety was attached to HSF1A at the same location as Biotin in HSF1A-Biotin. 1 nM HSF1A FITC was added to 1 mL of buffer containing 20 mM HEPES pH 7.4, 100 mM KCl, 5 mM MgCl2, 1 mM
EDTA, 1 mM DTT, and 5% glycerol and basal FITC polarization was measured (mP, millipolarization units). Purified Tcp1 was titrated into the reaction and polarization readings were taken at each indicated concentration. mP values were normalized as a percentage of the highest value (100%). Kd values were calculated using One-Site Binding in Graph Pad Prism.

NiNTA protein purification of Tcp1: A one Liter culture of E. coli expressing yeast His6-Tcp1 grown at 37 ºC and induced with IPTG 0.1 mM for 7 h was harvested and extracted in 25 mL Lysis buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 20 mM Imidazol HCl). Protein extract was incubated with Ni-NTA agarose beads (Quiagen) for 4 h at 4ºC, beads washed three times in (50 mM Hepes pH 7.5, 300 mM NaCl, 40 mM Imidazol HCl) and protein eluate was obtained using (50 mM Hepes pH 7.5, 300 mM NaCl, 250 mM Imidazol HCl). The eluate was subjected to FPLC purification using Superdex 75 10/300 GL column (GE Healthcare) in 25 mM Hepes pH 7.5, 150 mM NaCl. Aliquots of 10 µM His6-Tcp1 protein were stored at -80ºC for further analysis.

Actin folding and ATPase assays: TRiC folding activity was measured by monitoring folding of denatured Actin as described before (44). 35S-actin was diluted 1:100 into a master mix containing 0.2 µM purified TRiC and folding buffer (100 mM KCl, 30 mM Tris-HCl [pH 7.4], 5 mM MgCl2, 10% Glycerol, and 1 mM DTT). The reaction was incubated for 10 min at 30ºC and split into equal fractions. DMSO or various concentrations of HSF1A were added in a 1:50 dilution and incubated for 10 min
at 30°C. The reaction was supplemented with 1 mM ATP and incubated for 0, 30 and 60 min at 30°C. 1 mg/ml DNasel was added and generation of native 35S-actin was determined by nondenaturing gel electrophoresis as described. The gel was exposed on a phosphorimaging screen (Kodak), which was scanned in a Typhoon 9410 imager (GE Healthcare). The radioactive signal was quantified using ImageQuant 5.2 (Molecular Dynamics). DNasel binding of folded, radiolabeled Actin was monitored according to (REF). In brief, master mixes containing 0.2 μM purified TRiC and folding buffer were split into fractions and incubated with various concentrations of HSF1A or DMSO for 10 min at 30°C. Denatured, radiolabeled 35SActin was added in a 1:100 dilution and incubated for 15 min at 30°C. To start the folding reaction, ATP was added to a final concentration of 1 mM and incubated for 45 min at 30°C. As negative control, water was added. After the folding reaction, DNasel bound resins were incubated with samples for 1h at 4°C. Resins were subsequently washed two times with DNase binding buffer (DB; 10 mM Tris-HCl, pH 7.4, 1 mM CaCl2, 10% glycerol, 1 mM dithiothreitol (DTT), 0.2 mM ATP, and 10% formamide (v/v)), once with DB buffer plus 300 mM NaCl and again twice with DB buffer. Eluted samples were analyzed by SDS-PAGE, and autoradiography. TRiC ATPase activity was measured in folding buffer at 30°C in the presence of α-32P-ATP. Reactions containing 0.25 μM TRiC and 200 μM HSF1A or DMSO were pre-incubated for 5 min at 30°C. Steady-state ATPase was measured after addition of 1000 μM α-32P-ATP. At times indicated, samples were transferred to PEI-
cellulose F TLC plastic sheets (EMD Chemicals Inc.). Plates were developed in TLC chamber containing 1 M LiCl and 0.5 M formic acid in H2O. Plates were exposed to a phosphor screen (Kodak), and analyzed as described before.

ATP-sepharose purification of Cct4-GFP: The open reading frame encoding human CCT4 was PCR amplified from human cDNA (OpenBiosystems) and cloned into pcDNA3.1 and pEGFP-N1 using KpnI/BamHI and XhoI/BamHI restriction sites respectively. Hek293T cells were transfected with 10 µg pcDNA3.1-CCT4, pEGFP-N1-CCT4 or the corresponding empty vector controls and cell lysates were generated in Cell Lysis Buffer (50 mM Tris pH 7.4, 0.1% Tritonx-100, 0.15 M NaCl, 1mM DTT) supplemented with protease inhibitors. Total protein extracts were incubated with an ATP resin slurry in LSWB buffer (25 mM Tris pH 7.4, 0.15 M NaCl, 60 mM MgCl2, 1mM DTT) at a ratio 1:1. The mixture was incubated on ice for 1 h with gently mixing, transferred to a gravity feed column and washed three times with 3 column volumes of HSWB buffer (25 mM Tris pH 7.4, 1.15 M NaCl, 60 mM MgCl2, 1 mM DTT) followed by 3 times with 3 column volumes of LSWB. The resin was resuspended in 1/2 volume ATP slurry of LSWB buffer and transferred in equal amounts to a 96 well filter plates (Corning 3504). Proteins bound to the ATP-resin were eluted by the addition of increasing amounts of ATP (2 mM – 200 mM), HSF1A-Biotin or biotin alone (0.1 mM – 10 mM) and analyzed by immunoblotting analysis of measuring GFP fluorescence using a VictorTM X2 multilabel reader (Perkin Elmer).
HSF1-LexA fusion protein assay: Plasmid pHSF1-LexA(Trp1) or pHSF1S303A-LexA were generated by PCR amplification of the human HSF1 open-reading frame encoding amino acids 79-529 and cloning the fragment into pLexA (Addgene). pHSF1-LexA and the reporter plasmid pSH18-34 expressing β-galactosidase under control of eight LexA operator sites were transformed into yeast strain BJ2168 and isogenic MA6 (CCT6-D89E). Ten-milliliter yeast cultures were grown in SC-trp-ura to mid-log phase and total protein was isolated as described above. Reporter gene expression was analyzed by β-galactosidase assays.

VHL-TRiC co-immunoprecipitation assay: HeLa cells growing in a 10 cm plate were transfected with 10 µg pRc/CMV-HA-VHL (Addgene) and after 24 h split into two 10 cm plates. After overnight recovery the cells were shifted to Opti-MEM medium (Invitrogen) and treated with DMSO or 80 µM HSF1A for 1 h upon which time the cells were lysed in IP buffer (50 mM Tris, 150 mM NaCl, 1 mM Triton X-100). HA-VHL was immunopurified using an anti HA-affinity resin (Sigma) and VHL and associated proteins were analyzed by immunoblotting.

TRiC-HSF1 co-immunoprecipitation: HEK293T cells growing in a 10 cm plate were transfected with 10 µg pcDNA3.1 FLAG-HSF1, pcDNA3.1-mHSF1-TAP-GFP, or pcDNA3.1. After 24 h each 10 cm plate was split into two new 10 cm plates. After overnight recovery, the cells were washed twice in 10 ml PBS + CaCl2 + MgCl2, resuspended in 4 ml PBS + CaCl2 + MgCl2 and crosslinked with 2 mM
Dithiobis[succinimidyl propionate] (DSP) for 30 min at RT. The crosslinker was quenched by the addition of Tris/Glycine for 15 min at RT. Cells were lysed in IP buffer supplemented with protease inhibitors and 0.5 mg of protein extract was incubated with an anti-FLAG-M2-Affinity Gel (Sigma) to purify FLAG-HSF1 or a antiGFP agarose resin (Santa Cruz) to purify mHSF1-TAP-GFP. As Protein A is part of the TAP-tag (REF), native TAP-tagged proteins are readily bound by all IgG antibodies. Captured proteins were eluted from the beads by incubating in Laemmli Sample Buffer at 95°C for 5 min and analyzed by immunoblotting.
5. Structural Features of Heat Shock Transcription Factor 1 and 2 Drive Distinct Regulation and Function

Heat Shock Transcription Factors are known to serve important functions in numerous human diseases including neurodegeneration, cancer, diabetes, and infection. However, despite decades of elegant experiments characterizing HSF function in many model systems, our understanding of HSF structure is surprisingly limited. Moreover, the mechanisms regulating differential activity of HSF family members in distinct cellular contexts are unknown. Here we present two high-resolution crystal structures of the Human Heat Shock Factor 2 DNA binding domain (DBD) bound to DNA. The structures provide an unprecedented view of the winged helix-turn-helix HSF DBD that provide novel insights into the differential function of HSF family members. The structures reveal a helix that suggests a mode of DNA binding that not been previously described. In addition, we identify critical features of the “wing” domain of the HSF2 DBD structure that are predicted to be strikingly different than analogous regions of the HSF1 DBD. These observations provide evidence for the mechanisms by which HSF1 and HSF2 are differentially regulated specifically pertaining to SUMOylation and interaction with Replication Protein A (RPA). Using HSF wing domain chimeras, we demonstrate differential regulation with *in vitro* biochemical assays, and establish a functional and specific role for the HSF DBD wing domain in cell culture. Taken together, these results greatly improve our understanding of HSF structure and identify the wing domain as a critical regulator that differentiates the activity of HSF1 and HSF2.
The work presented in this chapter was performed in collaboration with Charles W. Pemble IV Ph.D. who assisted with crystallography data analysis. In addition, Professor Lea Sistonen contributed reagents and critical comments with regard to the SUMOylation assays.

5.1 Introduction

Heat Shock Transcription Factors (HSFs) are highly conserved transcription factors that impact numerous aspects of cellular biology including but not limited to stress responses, development, and infection (11). The diverse functions of HSFs have prompted investigations of their roles in human disease and a number of recent studies have highlighted the role of HSFs in neurodegeneration, cancer, diabetes, and cataract formation (22,86,93,163). Despite the recognition that HSFs play a significant role in disease states, our mechanistic understanding of HSF structure and function is limited. The design and development of therapeutic strategies targeting HSFs for the treatment of human disease will benefit from a greater understanding of HSF structure and function (65,164).

The human HSF family is comprised of three members, HSF1, HSF2, and HSF4 (11). HSFs are multi domain transcription factors containing an amino-terminal winged helix-turn-helix domain, a leucine zipper trimerization domain, a large regulatory domain, a carboxyl terminal leucine zipper and a transcriptional activation domain. When active, HSFs bind to a conserved DNA sequence element known as a Heat Shock
Element (HSE), which consists of inverted repeats of nGAAn (56). Importantly, HSF family members experience a diverse array of regulatory inputs that enable sophisticated tuning of the transcriptional response to stress and understanding the specific regulation of HSF family members could empower the ability to target specific arms of the stress response.

HSF1 is perhaps the most well studied HSFs for its role in the inducible transcriptional activation of Heat Shock Proteins (Hsps) such as Hsp70, Hsp90 and Hsp40, which function as protein chaperones following proteotoxic stress (165). Under normal conditions, HSF1 is maintained in a repressed monomeric state through functional interactions with the Hsp90 chaperone and direct interactions with the chaperonin TRiC, and upon proteotoxic stress, HSF1 accumulates in the nucleus and promotes target gene transcription (11,51). In addition to regulating Hsp gene expression, growing evidence demonstrates that HSF1 regulates a large constellation of target genes that are influenced by different cellular contexts. For example, in malignant cancer cells, HSF1 promotes the transcription of a subset of pro-survival genes that only partially overlap genes that are up-regulated by HSF1 during heat shock (69,70). Also, the genomic binding fingerprint of HSF1 in striatal cells expressing a pathological polyglutamine expanded Huntingtin protein is different than in cells expressing a non-pathogenic variant of Huntingtin (130). A recent report also demonstrated that HSF1 maintains distinct binding fingerprints in cycling cells versus mitotically arrested cells.
These observations highlight the importance of understanding the mechanistic features of HSF1 target gene recognition in a context dependent manner.

Similar to HSF1, HSF2 participates in the transcriptional regulation of genes in response to stress. HSF2 is similar to HSF1 in domain structure but also exhibits specific regulatory interactions distinct from HSF1. One of the most striking contrasts in HSF1 and HSF2 regulation is their relative stability, where HSF1 is much longer lived than HSF2 following stressful stimuli (87,96). HSF2 has been recognized as a critical mediator of brain development and suggested to play a role in the development of fetal alcohol syndrome (86). In addition, both HSF1 and HSF2 contribute to spermatogenesis and HSF2 mutations have been associated with idiopathic azoospermia (90).

A number of studies have identified a functional interaction between HSF1 and HSF2 and have observed that HSF1 and HSF2 can be found in a complex in immunoprecipitation experiments (86,96,98,166). However, the mechanisms and functional consequences of HSF1 and HSF2 interactions are not well understood. Furthermore, the repertoire of HSF1 and HSF2 target genes under different cellular contexts has only recently been explored. A recent ChIP-seq study identified the spectrum of HSF1 and HSF2 gene targets in K562 cells and found that despite binding similar sequence motifs, HSF1 and HSF2 exhibited distinct genomic targets (83). While global analysis revealed little difference in sequence preference, subtle differences may be present in individual binding sites. This observation presents the interesting question.
of whether HSF1 and HSF2 target gene selection is driven by intrinsic biochemical differences in HSF1 and HSF2 or mediated by extrinsic factors.

Our current knowledge of HSF structure is very limited. Since the pivotal publications by Littlefield and Nelson elucidating the structure of the *K. lactis* HSF DNA binding domain, little structural information about HSFs has been published (64). Here we present two high-resolution crystal structures of the human HSF2 DNA binding domain (DBD) bound to two distinct HSEs. The structures of these HSF2-DNA complexes provide unprecedented insight into structural features of HSFs. In addition, the structures provide evidence that HSF1 and HSF2 interact with DNA using identical direct and indirect contacts, suggesting that differential HSF1 and HSF2 target gene recognition is mediated through extrinsic factors. Furthermore, a newly observed carboxy-terminal helix in the HSF2 DBD suggests that the coiled-coil trimerization domain of HSFs is directed opposite the DNA from the DBD, exposing the DBD and the coiled-coil domain for regulatory interactions. We demonstrate that the HSF1 and HSF2 DBD are differentially SUMOylated *in vitro* in the “wing” domain and propose that the wing domain is an important mediator of differential activity of HSF1 and HSF2. Finally, we demonstrate that HSF1 and HSF2 directly interact through the trimerization domain. This study lays the foundation for understanding how the structure of HSF DBDs enables complex regulation for HSF family members. Moreover, interaction between
HSFs could provide a template for combinatorial regulation that can enable precise control of target gene transcription.

5.2 Structure of the HSF2 DBD bound to a “2-site” HSE

To understand how human HSFs specifically interact with HSE DNA, we solved the crystal structure of human HSF2 DBD bound to a 2-site HSE (GGTTCTAGAACC). The structure has a similar overall topology to the K. lactis HSF DBD bound to DNA where two HSF2 monomers are bound to the 2-site HSE creating a dimer interface parallel to the axis of DNA (Figure 71).

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<th>Table 2. Data Collection and Refinement Statistics</th>
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Figure 71. Overall Structure of HSF2 DBD bound to 2-site DNA. The HSF2 DBD (blue) forms a dimer interface parallel to the axis of DNA (red) and the recognition helix of the “winged” helix turn helix motif inserts into the major groove of DNA.

The sequence specific interaction is largely mediated through a hydrogen bonding interaction between Arg63 and the “G” of the nGAA\textsubscript{n} HSE motif (Figure 72). This critical arginine is located within the SFVRQ recognition helix that is present within HSFs throughout eukaryotic life. In addition, numerous direct and indirect contacts are made to the phosphate backbone throughout the length of DNA (Figure 73, Figure 74). We propose that Arg63 provides sequence specificity for HSEs, while the numerous phosphate backbone contacts drive the high affinity interaction with DNA.
Figure 72. Arg63 makes direct contact to the guanine in the nGAA{n} HSE motif.

A particularly interesting non-sequence specific contact is the interaction of Lys72 with the phosphate backbone. This lysine is analogous to Lys80 in HSF1, which was identified by the Morimoto Laboratory to be acetylated in cell culture. This acetylation event results in reduced HSF1 DNA binding and our structure reveals the first empirical evidence for this lysine directly contacting the phosphate backbone of DNA (Figure 73).

Figure 73. Lys72 directly contacts the phosphate backbone. The primary amine and the peptide backbone contact two adjacent phosphate groups. The lysine is located on the surface of the protein where it is accessible to modification by acetyltransferases.
Figure 74. Ladder diagram of the HSF2DBD-DNA interactions. Interactions depicted are predicted to be identical to those in HSF1 based on a threaded structure of HSF1.

Perhaps the most intriguing aspect of the structure is a newly observed structure for a carboxy-terminal helix present on the outer portion of the DBD (Figure 159). A Phe95 preceding this helix inserts into a deep hydrophobic pocket in the DBD formed by Leu14, Trp15, Trp29, and Phe35 (Figure 76). A glycine residue at position 99 then allows for a hairpin turn into a helix that is formed by a conserved amphipathic sequence “LLENI” and is directed toward the “bottom” half of the DNA. The hydrophobic surface of this sequence fits into a pocket of hydrophobic residues in the core of the DBD and
directs the polypeptide “down” to the opposite side of DNA (Figure 76). In addition, two conserved residues following the helix, Arg102, and Lys103 directly contact the phosphate backbone in the major groove that is opposite of the one bound by the SFVRQ recognition helix (Figure 75).

![Figure 75. Illustration of the carboxy-terminal helix in the HSF2 DBD. The green box is highlighting the carboxy-terminal LLENI helix packing against the hydrophobic surface of the DBD. Arginine 109 and Lysine 110 are shown directed towards the phosphate backbone. Dotted lines depict the predicted continuation of the polypeptide into the coiled-coil trimerization domain.](image)

![Figure 76. Surface representation of the hydrophobic pocket and carboxy-terminal helix interaction. On the left is shown the hydrophobic groove (white) present between negatively charged (red) and positively charged (blue) surfaces. Shown in](image)
sticks on the right are the Phe and LLENI residues packing into the hydrophobic pocket (gray).

Genetic and biochemical evidence have demonstrated that the HSF coiled-coil trimerization domain is located carboxy-terminal to the DBD and they are separated by a short linker sequence. We propose that the newly observed amphipathic helix present in the DBD directs the coiled coil opposite the DNA so that HSFs can “wrap-around” the DNA upon binding. This topology would leave the upper surface of the DBD exposed for interactions as opposed to being sterically occluded by the trimerization domain. This topology also provides geographically distinct opportunities for other proteins to interact with the coiled-coil domain juxtaposed to DNA. Support for this hypothesis is rooted in the increasing number of reports that have identified key interactions and post-translational modifications that occur within the DNA binding domain and coiled-coil domain (80,167).

5.3 Structure of the HSF2 DBD bound to a 3-site HSE

HSFs are thought to exist as trimeric proteins largely based on the tripartite nature of a canonical HSE (nnGAAAnnTTCnnGAAAnn) and their apparent MW after crosslinking. However, determining empirical evidence for trimer formation has been elusive. To address this, we solved the crystal structure of the HSF2 DBD bound to a 3-site HSE (Figure 77). Interestingly and unexpectedly, the protein crystallized in the P1 space group with 2 dimers bound to two-3 site HSEs. The independently bound dimers packed against each other to form pseudo continuous DNA and form a “dimer of
dimers”. Identical direct and indirect contacts are made when compared to the 2-site HSE structure.

Figure 77. Overview of the 2 DBD-3site HSE structure. Depicted is the asymmetric unit present in the P1 space group. Two dimers each bound to a single 3-site HSE are packed against each other to form pseudocontinuous DNA. All four monomers make identical direct and indirect contacts to DNA as in the 2-site structure.

Further, the carboxyl-terminal helices are all directed to the opposite side of the DNA, similar to the 2-site HSE structure.
It remains to be determined whether the biological unit of HSF2 is a dimer, or whether this packing topology is a crystallographic artifact. Nevertheless, this structure confirms the helical conformation of the carboxyl terminal portion of the DBD.

5.4 HSF “wing” domain topology

Despite the relative proximity of the wing domain to DNA, no direct or indirect contacts between the wing domain and DNA are observed, suggesting that the wing domain serves a different function (Figure 79). This finding is in concordance with Littlefield and Nelson’s original suggestion in the *K. lactis* HSF DBD, however, we demonstrate clear electron density for the entirety of wing domain peptide backbone. The wing domain appears to be formed in part by a hydrogen bond between His74 and
Glu89 that acts as a wedge between beta sheets to “open” the wing domain into solvent exposure.

Figure 79. Topology of the HSF2 DBD wing domain. Shown on the left is the overall wing domain structure with Lys82 shown in a stick representation. Importantly, the wing domain does not contact DNA. Shown on the right are His74 and Glu89 represented as sticks wedging the wing domain open into solvent exposure.

Additionally, Asp85 engages in a hydrogen bond to the adjacently bound monomer, establishing a dimer interface that was also observed in the *K. lactis* structure (Figure 80).
Asp85 engages in a hydrogen bond across the dimer interface. A similar interaction was observed in the *K. lactis* HSF DBD.

Finally, the side chain of Lys82 is predicted to be exposed to solvent space (Figure 80). Lys82 has recently been identified by a number of *in vivo* proteomic studies to be covalently SUMOylated by SUMO2 (75-77). Additionally, a recent study by Nakai and colleagues identified Replication Protein A subunit kDa 70 (RPA70) as a protein that interacts with the HSF1 wing domain but not with HSF2. The location of the RPA70 in HSF1 is predicted to occur at the most distal portion of the wing domain where little steric hindrance would be observed.

Taken together, our structural data and functional data from the literature suggest that the HSF wing domain may play a critical regulatory role as well as a role for differentiating between HSF family members. These regulatory events are enabled by the unique structural features of the HSF “wing” domain that are strikingly different that other members of the winged helix-turn-helix DBD family.
Importantly, the topology of the carboxy-terminal helix in the DBD that may
direct the coiled coil domain opposite the DNA would allow for the top of the DBD to be
fully exposed for regulatory interactions. This orientation would also allow the wing
domain to be fully accessible for interaction with RPA70, the SUMO ligase Ubc9, and/or
HSF interacting proteins that have yet to be identified (168).

5.5 HSF Family Members share similar DNA binding surfaces but
distinct wing domain surfaces

As discussed earlier, residues of HSF2 that interact with DNA are highly
conserved with HSF1. To further explore surface features of HSF family members, we
generated HSF1 and HSF4 DBD structures by threading their sequences over the newly
solved human HSF2 structure. As shown in Figure 81, the surfaces of HSF1, HSF2, and
HSF4 that are in contact with DNA (in close proximity to Arg63 shown in yellow) are
100% identical between all three family members. This indirectly suggests that HSF
family members would have little, if any, paralog specific sequence preferences for DNA
and provides support for external regulatory mechanisms driving differential target
binding in vivo.
Figure 81. Surface representations of HSF DBDs generated from the HSF2 structure and threaded sequences of HSF1 and HSF4. Shown in blue are regions of 100% identity between HSF paralogs. Shown in red, green and orange are regions where HSF1, HSF2, and HSF4 contain divergent sequences. Highlighted in yellow is Arg63 which is critical for contacting the guanine in the nGAA motif.

Interestingly, when examining the surfaces of the DBDs distal to DNA contact sites, the HSF1, HSF2, and HSF4 contain highly divergent sequences. This observation suggests that HSFs have evolved differential surfaces to accommodate distinct regulatory mechanisms without perturbing the DNA sequence preferences. A similar evolutionary phenomenon was recently observed to occur in the MADS-box family of
transcription factors in yeast (169) and was demonstrated to have significant functional consequences in gene transcription.

### 5.6 HSF1 and HSF2 are differentially SUMOylated in vitro

Covalent modification of proteins by the small ubiquitin like modifier (SUMO) results in diverse functional consequences including subcellular localization, protein degradation, and protein-protein interactions (77,170). Importantly, SUMOylation of HSFs has not been extensively explored outside of SUMOylation of Lys298 in HSF1. SUMOylation is analogous to ubiquitination in that both utilize an E1, E2, E3 enzymatic cascade for covalent modification of lysines on substrate proteins. Three recent proteomic experiments identifying the SUMOylated proteome found that Lys82 of HSF2 is SUMOylated by SUMO2 in cell culture (75-77). Interestingly, the corresponding lysine in HSF1 was not identified in any of the three studies. As this lysine is part of the wing domain, we sought to determine if there is an intrinsic SUMO specificity between HSF1 and HSF2. Using recombinant SUMO E1 (SAE1/SAE2), SUMO E2 (Ubc9) and SUMO E3 (RANBPΔFG) enzymes, we examined whether SUMO1 or SUMO2 could be covalently attached to the HSF1 or HSF2 DBD in vitro. As shown in Figure 82, the HSF1 DBD shows little to no modification by SUMO1 or SUMO2, whereas the HSF2 DBD is efficiently modified by both SUMO isoforms in vitro.
Figure 82. *In vitro* SUMOylation of HSF DBDs. The HSF1 and HSF2 DBD were purified using NiNTA resin following completion of the SUMOylation reaction and analyzed by SDS-PAGE and Colloidal Blue staining. SUMO modified HSF2 is observed by the appearance of a band at the MW of ~26 kDa (HSF2DBD (14 kDa) and SUMO (12 kDa)).

This result was also recapitulated when performing the SUMO reactions with the SUMO E3 ligase PIAS1 and in the absence of E3 ligase (Figure 83, Figure 84).

Figure 83. HSF2 DBD is efficiently SUMOylated using both RANBPΔFG and PIAS1 E3 ligases. HSF DBDs were detected by immunoblotting using anti 6xHis tag antibody.
**Figure 84.** HSF2 DBD is SUMOylated in the absence of E3 ligase. DBD proteins were detected by immunoblotting using anti 6xHis antibody.

This suggests that the HSF2 DBD has intrinsic properties that enable modification by SUMO that are not present in HSF1 DBD. Since SUMO proteomics studies identified Lys82 in the HSF2 wing domain as a SUMO substrate, we constructed wing domain chimeras where the HSF1 DBD contains the HSF2 wing domain (HSF1W2) and vice versa (HSF2W1) (Figure 85).

**HSF1 -** 73LNMYGFRKVVHIEQGGLVKPERDDTEFQHPFCLR106

**HSF2 -** 64LNMYGFRKVVHIDSGIVKQERDGPEFQHPFQK98

**HSF4 -** 75LNMYGFRKVVSEEQGGLLPERDHVEFQHPFVPSFVR108

**Figure 85.** Sequence comparison of HSF DBDs. The wing domain sequences are underlined and highlighted in red are potential SUMOylation motifs.

The HSF1W2 and HSF2W1 chimeras display opposite SUMOylation phenotypes compared with their WT counterparts suggesting that the HSF1 and HSF2 wing domain maintain distinct biochemical features that enable differential SUMOylation (Figure 86).
Importantly, the HSF4 DBD does not contain a lysine in the wing domain, which would render the wing domain of the HSF4 DBD incompetent for SUMO modification.

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Figure 86. *In vitro* SUMOylation of HSF DBD wing domain chimeras. HSF1W2 (1W2) and HSF2W1 (2W1) exhibit opposite SUMOylation phenotypes as compared to their WT HSF DBD counterparts.

The distinct SUMOylation profiles of HSF1 and HSF2 at the DBD wing domain could account for regulatory differences that impart target gene selectivity *in vivo*.

### 5.7 The wing domain is a critical mediator of HSF function in cells

To further explore a potential role for the wing domain in differential HSF function, we expressed FLAG tagged variants of WT HSF1, HSF1W2, HSF2, or HSF2W1 in mouse embryonic fibroblasts doubly knocked out for HSF1 and HSF2. Basal expression of HSF1 WT and HSF1W2 exhibits no significant difference, and a similar result is shown for HSF2 and HSF2W1. Interestingly, cells expressing HSF1W2 demonstrate significantly less induction of Hsp70 compared to WT HSF1 following a heat shock or treatment with the proteasome inhibitor MG132 (Figure 87). This suggests that the HSF2 wing domain imparts negative regulation on HSF1, despite the lack of evidence for a direct DNA interaction of the HSF wing domains. Moreover, both HSF2
constructs demonstrate no induction of Hsp70 following heat shock or proteasome inhibition, despite having similar protein levels to the HSF1 variants (Figure 87). This result may suggest that HSF2 is a much weaker transcriptional activator or that HSF2 requires an interaction with HSF1 for its full activity.

Figure 87. The HSF2 wing domain reduces HSF1 mediated expression of Hsp70. HSF1/2 double knockout MEFs were transfected with empty vector (V), WT HSF1 (1), HSF1wing2 (1W2), WT HSF2 (2), or HSF2wing1 (2W1) and exposed to a 1 hour heat shock at 42 °C followed by a 6 hour recovery at 37 °C, treated with MG132 for 7 hours, or maintained at 37 °C.

These results establish functional importance for the wing domain in target gene transcription and lay the foundation for mechanistic studies into regulatory events that occur within the wing domain.

5.8 HSF1 and HSF2 Directly Interact and form Heterocomplexes

Numerous labs have reported an interaction between HSF1 and HSF2 in cell extracts. Importantly, these observations do not distinguish between a direct interaction or an indirect interaction between HSF1 and HSF2. To address this, we co-expressed
human HSF1 and HSF2 in *E. coli* from a single plasmid using a bi-directional promoter (Figure 88).

![Diagram of plasmid](Image)

**Figure 88. Illustration of the HSF1-HSF2 co-expression plasmid.**

To enable co-purification, HSF1 was epitope tagged with Strep-Tag II and HSF2 was tagged with 6xHis. Tandem affinity purification with Streptactin resin followed by NiNTA resin, results in co-elution of HSF1 and HSF2 suggesting that the two proteins are directly interacting (Figure 89).

![Affinity purification gel](Image)

**Figure 89. Affinity purification of HSF1-HSF2 heterocomplexes.** P- pellet; CL; cleared lysate; SF-Strep flow through; SW- strep wash; SE- strep elution; NF – nickel flow through; NW – nickel wash; NE – nickel elution. The top band corresponds to HSF2 and the bottom band corresponds to HSF1 (confirmed by mass spectrometry).
The HSF1-HSF2 heterocomplexes were then fractionated using a Sephacryl s400 gel filtration column, which we previously reported is able to resolve monomeric and trimeric HSF1 (56). The HSF1-HSF2 complex co-purified sample eluted from size exclusion at a volume similar to the HSF1 trimer, suggesting that these complexes are indeed heteromultimeric complexes that are stable through three subsequent purification steps (Figure 90).

![Abs280 (mAU)](image)

**Figure 90.** Size Exclusion Chromatography of HSF1-HSF2 heterocomplexes. The elution profile of HSF1-HSF2 heterocomplexes (red) is similar to the trimer elution profile of WT HSF1 (first peak, blue). Colloidal blue staining of 10 fractions between 150 and 200 mL demonstrate the purity of the HSF1-HSF2 complexes.

Crosslinking followed by mass-spectrometry demonstrated that no contaminating protein was present in the sample, confirming the direct interaction between HSF1 and HSF2.
To further demonstrate the specificity and understand the biochemical nature of this interaction, we generated co-expression plasmids containing WT HSF1 and HSF2ΔLZ1-3 and the corresponding HSF1ΔLZ1-3 and HSF2 construct. As shown in Figure 91, WT HSF1 and HSF2 co-purify from cell lysates, but WT HSF1 does not co-purify with HSF2 ΔLZ1-3 and WT HSF2 does not co-purify with HSF1 ΔLZ1-3.

Figure 91. Purification of HSF1-HSF2 Complexes lacking ΔLZ1-3. Co-expression of WT HSF1 and WT HSF2 results in co-purification following affinity chromatography (red arrow). When HSF1 ΔLZ1-3 is co-expressed with WT HSF2 (DUAL Δ1), the two proteins do not co-purify following tandem affinity purification (green arrow). When HSF2 ΔLZ1-3 is co-expressed with WT HSF1 (DUAL Δ2), the two proteins do not co-purify (blue arrow).

These results demonstrate that HSF1 and HSF2 form heterocomplexes through their respective coiled-coil multimerization domains in a similar fashion to other coiled-coil heteromers such as c-Fos and c-Jun. Taken together, these results unequivocally demonstrate, that HSF1 and HSF2 directly interact in heterocomplexes and may provide
a template for complex, differential regulatory events that would not exist in HSF1 or HSF2 homo-oligomeric complexes (Figure 92).

![Diagram of HSF1, HSF1-HSF2, and HSF2 complexes]

**Figure 92.** A model for combinatorial regulation of HSFs. The interaction of HSF1 and HSF2 may enable the recruitment of a diverse spectrum of interaction proteins that would not be present within either homomultimer.

**5.9 Discussion**

The evolution of transcriptional regulatory networks has predominantly been proposed to occur through variation in cis-regulatory elements upstream of target genes. This was thought to occur because mutation in the transcriptional regulators themselves would have the potential for widespread consequences in comparison to single mutations in regulatory sequences for specific genes. However, a growing body of evidence suggests that variation in transcriptional regulators themselves can give rise to complex regulation in transcriptional networks (169). Here we present an example of this variation in the Heat Shock Factor Family of transcription factors.
High-resolution structures of the human HSF2 DNA binding domain reveal an extensive protein-DNA interaction interface with sequence specific and non-specific interactions. Interestingly, all contacts with DNA, both sequence specific and non-specific, are predicted to be highly conserved through the HSF family suggesting that little, if any, intrinsic sequence preference exists between HSF paralogs. Moreover, examination of DNA binding domain surfaces outside of the protein-DNA interface illuminated highly divergent sequences between HSFs that may be available for differential regulatory interactions.

We demonstrate that SUMOylation occurs specifically on the HSF2 DBD “wing” domain and not the HSF1 DBD (and also cannot occur on the HSF4 DBD due to a missing lysine). Taken together with previous reports demonstrating an HSF1 specific interaction with RPA70 at this location, HSF family members may be exhibiting differential and combinatorial regulation that is enabled surface evolutions distal to DNA binding. Further exploration into the divergent surfaces of HSF DBDs may yet reveal additional paralog-specific regulatory events that enable complex transcriptional regulation that would not be achieved with cis-regulatory element variation alone.

The conserved and unique surfaces of human HSF paralogs resemble the evolution of MADS-box proteins in yeast. A recent report detailed how a gene duplication event in the MADS-box protein enabled the sub-functionalization of MADS-box paralogs (Mcm1 and Arg80 in S. cerevisiae) (169). The authors demonstrate that
Mcm1 and Arg80, paralogs arising from a gene duplication event, maintain similar DNA binding preferences but exhibit diversity in co-factor binding pockets. Further, interactions between Mcm1 and Arg80 exhibited interference with the function of the individual paralogs. A similar phenomenon could be observed with HSFs where a direct interaction between HSF1 and HSF2 could exhibit “paralog interference”. For example, an HSF1-HSF2 interaction would enable HSF2 DBD specific SUMOylation to occur within an HSF1 containing heterocomplex that would not exist within an HSF1 homocomplex. On the other hand, this interaction would allow for HSF2 to exist in proximity to the HSF1-RPA70 interaction, facilitating localization at certain genomic loci that would not be achieved with an HSF2 homomultimer. The transcriptional outcome of the HSF1 and HSF2 interaction and regulatory events will be the focus of future investigations.

HSF1 and HSF2 are increasingly realized to play a role in numerous cellular processes and pathologies. The cooperation of HSF1 and HSF2 add an additional layer of complexity for the response to stress but may provide cells with a precise rheostat for transcriptional output at HSF target genes. HSF1 and HSF2 specific regulatory events add another layer of regulatory complexity and understanding HSF specific regulation will aid in our understanding our cellular stress responses. Finally, paralog interaction and paralog specific regulation may be present in numerous mammalian transcription factor families and can explain complex regulation of transcriptional networks.
5.10 Materials and Methods

Expression and Purification of HSF DNA Binding Domains: The HSF2 DNA Binding Domain (8-115) was cloned into the pET15b expression plasmid containing an amino-terminal 6xHis tag using InFusion (Clontech) and the plasmid was transformed into BL21(DE3) E. coli. Overnight LB cultures were back diluted 1:100 into 4 L flasks containing 2 L of LB + 100 μg/mL ampicillin, grown to an OD$_{600}$~ 0.5 and induced with 1 mM IPTG for 5 hours. Cell pellets were lysed in 20 mL NiNTA Lysis Buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 20 mM Imadazole HCl) using a sonic dismembrator for 3 x 30 sec bursts. Lysates were then cleared by centrifugation at 20,000 g for 30 min. Cleared lysates were incubated with 5 mL bed volume of NiNTA Agarose (Qiagen) and rotated at 4 °C for 2 hours. Following addition to a gravity filtration column, the NiNTA resin was washed with 2 x 50 mL NiNTA wash buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 40 mM Imidazole HCl) and eluted with 10 mL NiNTA elution buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 250 mM Imidazole HCl). For 6xHis tag cleavage, the purified HSF2 DBD was buffer exchanged using a Amicon Ultra Centricon (MWCO 3kDa) until the final Imidazole concentration was 20 mM in 10 mL. This procedure resulted in 2 mg protein yield. The buffer exchanged HSF2 DBD was then incubated with 3 units of biotinylated-thrombin (Novagen) overnight at 15 °C. Following streptavidin removal of thrombin and NiNTA removal of uncleaved HSF2DBD, the protein was concentrated to 7 mg/ml prior to crystallization trials.
Expression on purification of HSF1 DBD, HSF1W2 DBD, and HSF2 DBD were performed as described for HSF2 DBD but without removal of the 6xHis tag. HSF1W2 DBD and HSF2W1 DBD were generated by swapping amino acids 85-97 (HSF1) and 77-89 (HSF2) using Infusion cloning.

Crystallization of HSF2 DBD: 7 mg/ml HSF2 DBD (537 µM) was premixed with 322.2 µM 2-site HSE (GGTTCTAGAACC) or 214.8 µM 3-site HSE (GGTTCTAGAATATTCCG) for a final ratio of 1:1.2 protein:DNA binding site. The resulting complex was then incubated at 25 °C for 5 min prior to depositing into sitting drop vapor diffusion crystallization trays (Intelliplate 3). The HSF2 DBD:2-site HSE complex crystallized at 25 °C against 100 mM NaCl, 100 mM Bicine pH 9, and 30% PEG 550 MME. Parallelogram shaped crystals grew overnight to 200 µm x 50 µm x µM.

Crystals were cryoprotected with 100 mM NaCl, 100 mM Bicine pH 9, and 30% PEG 400. HSF2DBD:3-site HSE complex crystallized against 170 mM ammonium-acetate, 85 mM sodium acetate pH 4.6, 25.5% PEG 4000, and 15% Glycerol. Parallelogram shaped crystals grew in 3-4 days to 50 µM x 50 µm x 25 µm.

Data Collection and Refinement: Diffraction data were collected on a Rigaku 007 HF x-ray instrument with an incident beam 1.54 Å in wavelength. Datasets were reduced in HKL-2000 (Otwinowski and Minor, 1997). Matthews analysis suggested 1 molecule of protein and ssDNA in the asymmetric unit of the structure for the 2-site structure and 4 molecules of protein and 4 molecules of ssDNA for the 3-site structure.
(Matthews, 1968). The structures were phased by molecular replacement in PHENIX (Terwilliger et al., 2008) using a slightly modified version of the 3HTS structure containing only base pairs 1-4 of the HSE. Rebuilding and real-space refinements were done in Coot (Emsley et al., 2010) with reciprocal space refinements in PHENIX (Adams et al., 2010).

*Generation of threaded HSF1 and HSF4 DBD structures: HSF1 amino acids 16-132 and HSF4 amino acids 18-125 were threaded over the HSF2DBD protein structure generated from the 2-site HSE complex. Threading was performed using the Phyre2 server using the intensive modeling setting. Surface renderings of HSF1, HSF2, and HSF4 DBD were generated using PyMOL.*

*In vitro SUMOylation assay: SUMO E1 (SAE1/SAE2), SUMO E2 (Ubc9), SUMO1, and SUMO2 were purchased from Boston Biochem. SUMO E3 ligases RANBPΔFG and PIAS1 were purchased from Enzo Life Sciences. Reactions were performed in SUMOylation Assay Buffer (SAB; 20 mM HEPES pH 7.3, 110 mM KOAc, 2 mM Mg(Oac)₂, 1 mM EGTA, 2% Tween 20, 1 μg/mL Leupeptin/Pepstatin/Aprotinin, 1 mM DTT 10 mM ATP). 20 μL reactions containing 150 nM E1, 150 nM E2, 150 nM E3, 10 μM SUMO1/2, and 1 μM HSF DBD in SAB were incubated at 30 °C for 1 hour. Reactions were terminated with 1X SDS loading buffer prior to SDS-PAGE analysis followed by immunoblotting. For colloidal blue stained samples, 100 μL reactions were incubated
with 20 µL NiNTA agarose beads for 1 hour at 4 °C, washed 2x with 200 µL NiNTA wash buffer, and eluted with 50 µL NiNTA elution buffer prior to SDS-PAGE.

Cell Culture and Transfection: HSF1/HSF2 double knock out Mouse embryonic fibroblasts (dKO MEFS) were a kind gift from Dr. Ivor Benjamin. dKO MEFs were maintained in DMEM (Gibco) supplemented with 10% heat inactivated FBS (Gibco), 0.1 mM non-essential amino acids (Gibco), 1X MycoZap (Lonza), and 55 µM beta-mercaptoethanol. For transfection, 1 x 10^6 cells were electroporated with 1 µg of either empty pcDNA3.1, or pcDNA3.1 with human WT HSF1, HSF1W2, HSF2, or HSF2W1 containing a 3xFLAG tag using a 4D Nucleofector (Lonza). 24 hours post transfection, cells were maintai at 37 °C, heat shocked for 1 hr at 42 °C with a 6 hour recovery at 37 °C, or treated with 3 µM MG132 for 7 hours. Cell lysates were harvested using SDS lysis buffer (20 mM HEPES, 5 mM MgCl2, 1 mM EDTA, 100 mM KCl, 0.03% NP-40, 0.1% SDS, 1x halt protease inhibitor cocktail (Pierce), 1x phosphatase inhibitor cocktail (Pierce). Lysates were cleared by centrifugation at 14,000 x g for 15 min prior to quantification with BCA Assay (Thermo) and SDS-PAGE and transfer to 0.2 µM nitrocellulose membrane. Anti-FLAG (Sigma), Anti-Hsp72 (Enzo), Anti-GAPDH (Santa Cruz) were used at a dilution of 1:1000 prior to incubation with anti-rabbit or anti-mouse antibody (GE Health Sciences) at a dilution of 1:5000.

Purification of HSF1-HSF2 Heterocomplexes: Human HSF1 with an amino-terminal StrepII Tag and human HSF2 with an amino-terminal 6xHis tag were codon
optimized for expression in *E. coli* and cloned into the pET15b expression vector using two divergent lac-operon promoters (Supplementary Figure). Overnight cultures of *E. coli* were backdiluted 1:100 into 2 L of LB containing 100 µg/mL ampicillin, grown to an OD$_{600}$ = 0.5 and induced with IPTG for 5 hours. Cell pellets were lysed in 20 mL of Strep Binding Buffer (50 mM HEPES pH 7.5, 300 mM NaCl) and cleared by centrifugation at 20,000 x g for 30 min at 4°C. Cleared lysates were then applied to a StrepTrap column (GE Heath Sciences) using an Akta Pure FPLC (GE Health Sciences) at a flow rate of 0.5 mL/min. The column was then washed with Strep Binding Buffer until the Abs$_{280}$ reading reached zero at a flow rate of 2 mL/min. The Strep Trap column was then directly attached to a HisTrap column (GE Health Sciences) and reconnected to the FPLC. Bound proteins were then eluted with Strep Binding Buffer supplemented with 5 mM desthiobiotin directly onto the HisTrap column at a flow rate of 1 mL/min for 50 mL. The StrepTrap column was then removed from the assembly and the FPLC connected directly to the HisTrap column. The HisTrap was then washed with 50 mL NiNTA wash buffer, 20 mL NiNTA wash buffer supplemented with 20 mM MgCl$_2$ and 5 mM ATP, and an additional 50 mL NiNTA wash buffer at a flow rate of 2 mL/min. Bound proteins were eluted from the HisTrap column with NiNTA elution buffer at a flow rate of 1 mL/min. The eluate fractions containing HSF1 and HSF2 were analyzed by SDS-PAGE and Colloidal Blue staining prior to combining, concentrating to a volume of 10 mL and loading onto a Sephacryl s400 Gel Filtration column using the Akta FPLC.
The HSF1-HSF2 heterocomplexes were eluted from the s400 column using 25 mM HEPES pH 7.5 and 150 mM NaCl and fractions analyzed by SDS-PAGE and colloidal blue staining.
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Biography

I was born in Cincinnati, Ohio on April 18, 1988. In Cincinnati, I attended Archbishop Moeller High School where I graduated with honors in June 2006. I completed my undergraduate degree in Biomedical Science at The Ohio State University. I graduated in June 2010 with Honors and Research Distinction in Allied Medical Professions. I participated in the Duke University Scholars in Molecular Medicine and am a member of the American Association for the Advancement of Science.