Structures of HSF2 reveal mechanisms for differential regulation of human heat-shock factors

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Heat-shock transcription factor (HSF) family members function in stress protection and in human diseases including proteopathies, neurodegeneration and cancer. The mechanisms that drive distinct post-translational modifications, cofactor recruitment and target-gene activation for specific HSF paralogs are unknown. We present crystal structures of the human HSF2 DNA-binding domain (DBD) bound to DNA, revealing an unprecedented view of HSFs that provides insights into their unique biology. The HSF2 DBD structures resolve a new C-terminal helix that directs wrapping of the coiled-coil domain around DNA, thereby exposing paralog-specific sequences of the DBD surface for differential post-translational modifications and cofactor interactions. We further demonstrate a direct interaction between HSF1 and HSF2 through their coiled-coil domains. Together, these features provide a new model for HSF structure as the basis for differential and combinatorial regulation, which influences the transcriptional response to cellular stress.

HSFs are found in eukaryotes including fungi and humans, and they affect diverse aspects of cell biology including stress adaptation, protein folding and quality control, development and disease1–3. The remarkable diversity of HSF-target genes contributes to the influence of HSF in a wide range of cellular processes4–5. Despite the recognition that HSFs have prominent roles in cellular adaptation to stress and in disease, understanding of the mechanisms by which distinct HSF paralogs bind to genomic loci and engage in unique interactions with regulatory factors is limited. A better understanding of HSF structure, interactions and function will benefit the development of therapeutic strategies that modulate HSFs for the treatment of human diseases6,7.

The human HSF family comprises three members: HSF1, HSF2 and HSF4 (ref. 3). HSFs are multidomain transcription factors containing an N-terminal winged helix-turn-helix DBD, an adjacent extended coiled-coil multimerization domain, a central regulatory domain, a C-terminal coiled-coil domain and a transcriptional-activation domain8–12. When activated, HSFs bind to a conserved DNA sequence known as a heat-shock element (HSE). HSEs consist of inverted repeats of 5′-NGAAN-3′ and exhibit variations in sequence and geometry in target genes across the human genome3,11,14. Moreover, HSF family members interpret a diverse array of regulatory inputs that enable sophisticated tuning of the transcriptional response to stress stimuli. Understanding the specific regulatory modalities of HSF family members could allow targeting of specific arms of the HSF-mediated transcriptional response.

HSF1, the most studied member of the human HSF family, has roles in the inducible transcription of genes encoding protein chaperones and the chaperonin TrIC; components of the ubiquitin-proteasome and autophagy pathway for degradation of misfolded proteins; and antiapoptotic proteins and stress adaptation factors upregulated after proteotoxic stress15,16. Under normal conditions, HSF1 is maintained in a repressed monomeric state through functional interactions with the Hsp90 chaperone and direct interactions with the chaperonin TrIC15. In response to proteotoxic stress, HSF1 multimerizes through the extended coiled-coil domain, accumulates in the nucleus and promotes target-gene transcription. In addition to activating the protein quality-control machinery in response to proteotoxic stress, HSF1 activates a large constellation of genes that are influenced by different cellular contexts. For example, in cancer cells, HSF1 promotes the transcription of a subset of prosurvival genes that only partially overlap with genes that are activated in response to heat stress5,17. Moreover, the genomic binding fingerprint of HSF1 in striatal neuronal cells expressing a pathological polyglutamine-expanded Huntingtin protein is distinct from that in cells expressing a nonpathogenic variant of Huntingtin18. These observations highlight the importance of understanding the mechanistic features of HSF1 target-gene recognition and activation in a context-dependent manner.

Like HSF1, HSF2 participates in the transcriptional regulation of genes in response to stress and has a similar overall domain structure, but it exhibits genomic binding-site occupancy and regulatory interactions that are distinct from those of HSF1 (refs. 13,19). One of the most striking contrasts in HSF1 and HSF2 regulation is their relative stability: HSF1 is much longer lived than HSF2 in the presence

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of proteotoxic stimuli\textsuperscript{20,21}. HSF2 is recognized as a critical mediator of brain development, and it has a role in fetal alcohol syndrome\textsuperscript{22}. In addition, both HSF1 and HSF2 contribute to spermatogenesis, and specific mutations in HSF2 have been associated with idiopathic azoospermia\textsuperscript{23–25}. Several studies have identified a functional interaction between HSF1 and HSF2 and have observed that HSF1 and HSF2 are found in a complex \textit{in vivo}\textsuperscript{22,26,27}. However, the nature of the HSF1 and HSF2 interactions and their functional consequences are not well understood. Furthermore, the repertoire of target genes of HSF1 and HSF2 in different cellular contexts has only recently been explored. A study using chromatin immunoprecipitation (ChIP) and massively parallel DNA sequencing has identified the spectrum of HSF1 and HSF2 target genes in human K562 cells and has found that, although they bind similar HSE sequence motifs, HSF1 and HSF2 exhibit only a partially overlapping pattern of genomic occupancy\textsuperscript{11}. This observation raises the interesting question of whether selection of target genes of HSF1 and HSF2 is driven by intrinsic biochemical differences between the two proteins or is mediated by extrinsic factors such as protein-protein interactions and post-translational modifications.

Current knowledge of the HSF structure is very limited. Since the elucidation of the partial structure of the HSF DBD of the yeast \textit{Kluyveromyces lactis}, little structural information has been reported for any HSF\textsuperscript{4}. To gain structural insights with respect to HSF2 DNA binding, we solved two high-resolution crystal structures of the human HSF2 DBD bound to two distinct HSEs at 1.73 Å and 2.10 Å. These structures revealed new structural features that effectively invert current models of HSF DNA binding. Moreover, we demonstrate that the unique structural features identified in this study affect human HSF regulation \textit{in vitro} and \textit{in vivo}. Together, these critical insights into the architecture of HSFs bound to DNA lay a mechanistic groundwork for understanding how HSF structure drives combinatorial regulation that can enable precise control of target-gene transcription at specific loci and in specific physiological and pathophysiological contexts.

RESULTS

Structure of the HSF2 DBD bound to a ‘two-site’ HSE

We solved the crystal structure of the human HSF2 DBD bound to a two-site HSE (5'-CGTTCTAGAACCC-3'; underlines indicate individual binding sites), at 1.73 Å (Table 1 and Supplementary Fig. 1a). The structure shows two HSF2 monomers bound to the two-site HSE, thus creating a dimer interface parallel to the axis of DNA (Fig. 1a). The sequence-specific interaction is largely mediated through a hydrogen-bonding interaction between Arg63 and the guanine of the NGAAN sequence-specific interaction is largely mediated through a hydrogen-bonding interaction between Arg63 and the guanine of the NGAAN sequence, which is conserved and is critical for DNA binding\textsuperscript{8}, is located within HSE motif \textsuperscript{β6,7,8} and is associated with the formation of a complex \textit{in vivo}\textsuperscript{22,26,27}. However, the nature of the HSF1 and HSF2 interactions and their functional consequences are not well understood. Furthermore, the repertoire of target genes of HSF1 and HSF2 in different cellular contexts has only recently been explored. A study using chromatin immunoprecipitation (ChIP) and massively parallel DNA sequencing has identified the spectrum of HSF1 and HSF2 target genes in human K562 cells and has found that, although they bind similar HSE sequence motifs, HSF1 and HSF2 exhibit only a partially overlapping pattern of genomic occupancy\textsuperscript{11}. This observation raises the interesting question of whether selection of target genes of HSF1 and HSF2 is driven by intrinsic biochemical differences between the two proteins or is mediated by extrinsic factors such as protein-protein interactions and post-translational modifications.

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Table 1 Data collection and refinement statistics

| Data collection               | HSF2 DBD–two-site HSE | HSF2 DBD–three-site HSE |
|------------------------------|-----------------------|-------------------------|
| Space group                  | Cl21                  | P1                      |
| Cell dimensions              |                        |                         |
| a, b, c (Å)                  | 84.88, 39.63, 39.93   | 40.17, 47.14, 109.59    |
| α, β, γ (°)                  | 90.00, 91.43, 90.00   | 82.62, 90.03, 64.75     |
| Resolution (Å)              | 50–1.73               | 50–2.1                  |
| Rmerge                       | 0.082 (0.659)*        | 0.116 (0.832)*          |
| Completeness (%)             | 94.3 (89.7)           | 93.9 (89.7)             |
| Redundancy                   | 5.1 (4.1)             | 2.5 (2.3)               |
| Refinement                   |                        |                         |
| Resolution (Å)              | 21.21–1.73            | 24.41–2.069             |
| No. reflections             | 13,023                | 38,940                  |
| Rwork / Rfree               | 0.1479 / 0.1991       | 0.2171 / 0.2730         |
| No. atoms                    | 2,353                 | 9,293                   |
| Protein                      | 1,867                 | 6,827                   |
| Ligand (DNA)                | 380                   | 2,164                   |
| Water                        | 106                   | 302                     |
| r.m.s. deviations            |                        |                         |
| Bond lengths (Å)             | 0.011                 | 0.015                   |
| Bond angles (°)              | 1.396                 | 1.486                   |

One crystal was used for each structure (Supplementary Figs. 1a and 4a). *Values in parentheses are for highest-resolution shell.

HSF2 wing-domain topology

The wing domains of other winged helix-turn-helix transcription factors function in direct DNA binding\textsuperscript{8,28,29}. Although previous structural studies of the \textit{K. lactis} HSF DBD have not resolved the wing domain, our structure of the human HSF2 wing domain showed clear electron density for the entirety of the peptide backbone (Supplementary Fig. 2) and indicated no direct or indirect contacts to DNA (Figs. 1a and 2a). The HSF2 wing domain is formed, in part, by a hydrogen bond between His74 and Glu89 that acts as a wedge between β sheets that ‘pries open’ the wing domain for solvent exposure (Fig. 2b). Additionally, Asp86 engages in a hydrogen bond to Asn56 of the adjacently bound monomer, establishing an HSF2 dimer interface also observed in the \textit{K. lactis} structure (Fig. 2c); formation of this interface has been proposed to be the primary function of the wing domain\textsuperscript{6}. Moreover, the side chain of HSF2 DBD Lys82, identified by \textit{in vivo} proteomic studies to be covalently conjugated by SUMO2 (refs. 30–32), is exposed to solvent (Fig. 2a). The importance of the wing domain is underscored by a report demonstrating that replication protein A subunit 70 kDa (RPA70) binds to the HSF1 wing domain but not to HSF2 (ref. 33). By analogy with the HSF2 DBD structure presented here, the location of the RPA70 interaction with HSF1 has been predicted to occur at the most distal portion of the wing domain, where little steric hindrance would be observed. The structural findings described here, together with earlier published functional studies, suggest that the HSF wing domain has a critical
Figure 1  Structure of the HSF2 DBD bound to a two-site HSE. (a) Overview of the HSF2 DBD dimer. Top, individual monomers (light and dark blue) form a dimer interface parallel to the axis of HSE DNA (red). Bottom, view down the axis of DNA, revealing the recognition helix of the winged helix-turn-helix motif for each monomer inserted into the major groove of the DNA. (b) Ladder diagram of the direct and indirect contacts formed between the HSF2 DBD and HSE DNA, with side chain, peptide backbone and water molecule-mediated contacts indicated. (c) Direct contact between Arg63 of the HSF2 DBD and the guanine of the HSE NGAAN motif. (d) Distance difference matrix of human HSF2 DBD and K. lactis HSF DBD, depicted as a putty diagram. Distances between paired atoms ranging from 0.348 Å (thin, blue) to 3.837 Å (thick, red) indicate structural deviations between HSF2 DBD and the K. lactis HSF DBD. Shown in purple are new HSF2 structural features that are not present in the K. lactis HSF DBD structure.

regulatory role and provides a specificity component for distinct HSF family members. These regulatory events are probably enabled by the unique structural features of the HSF wing domain; these features are strikingly different from those of other members of the winged helix-turn-helix DBD family, which use the wing domain to contact DNA.28,29

HSF2 DBD directs wrapping of the coiled coil around DNA
Current models for the topology of HSFs bound to DNA posit that the coiled-coil multimerization domain sits atop the DBD, thus poising the transactivation region for interactions with the transcriptional machinery1,3,34. We observed that a C-terminal helix, which is present on the outer region of the HSF2 DBD (Fig. 3a), and Phe96, which precedes this helix, are inserted into a deep hydrophobic pocket in the DBD formed by Leu14, Trp15, Trp29 and Phe35 (Fig. 3b). Gly99 enables a hairpin turn into the helix that is formed by the amphipathic sequence LLENI and is directed toward the ‘bottom’ half of the DNA. The hydrophobic surface of this sequence fits into a hydrophobic pocket in the core of the DBD, thereby directing the helix ‘down’ to the opposite side of DNA (Fig. 3a,b). In addition, two conserved residues following the helix, Arg109 and Lys110, contact the DNA phosphate backbone, thus further directionally stabilizing the region preceding the coiled-coil multimerization domain on the opposite side of the DNA (Fig. 3a and Supplementary Fig. 3a,b). Key residues involved in the architecture of this C-terminal helix are conserved throughout HSF species, thus suggesting conservation of the directionality of the coiled-coil multimerization domain (Supplementary Fig. 3c).

HSFs are thought to exist as trimeric proteins, on the basis of the tripartite nature of a canonical HSE (NNGAANNTTNNGAANN), their apparent electrophoretic mobility in heat-shocked cell extracts and the presence of an extended coiled-coil immediately downstream of the DBD.18,35,36 To gain insights into the structural features of the DBD topology in an HSF trimer, we solved the crystal structure of the HSF2 DBD bound to a canonical three-site HSE (Table 1 and Supplementary Fig. 3d). Interestingly, the HSF2 DBD crystallized in the P1 space group with two dimers bound to two three-site HSEs (Fig. 3c), thus demonstrating that even when presented with a tripartite HSE, the HSF2 DBD crystallized as a dimer, with the third
NGAAN of the HSE unoccupied. The independently bound dimers pack against each other, forming pseudo continuous DNA and a ‘dimer of dimers’. Identical direct and indirect DNA contacts are made to those in the HSF2 DBD two-site HSE structure (Supplementary Fig. 3e). Furthermore, all C-terminal helices of the HSF2 DBD are directed to the opposite side of the DNA, similarly to those in to the two-site HSE structure (Fig. 3c). Although it is possible that the dimer structure observed here occurs as a function of crystal packing, HSF oligomeric states may exist outside of the canonical trimer, as has been suggested for HSF2 dimers in K562 cells37.

The HSF2 coiled-coil multimerization domain is located C-terminal to the DBD and is separated by a short linker sequence of 14 amino acids. From both the HSF2 DBD two-site and three-site structures, we propose that the conserved C-terminal amphipathic helix directs the extended coiled coil opposite the DNA, thereby allowing HSFs to wrap around the DNA upon binding. This topology would permit the upper surface of the HSF DBD to be accessible for regulatory interactions that would otherwise be sterically occluded by the multimerization domain, as depicted in current models1,43. This topology presents surfaces for geographically distinct interactions with regulatory proteins via both the DBD and the coiled-coil domain in proximity to DNA. Support for this model is rooted in reports that have identified key interactions and post-translational modifications that occur within both the DNA-binding and coiled-coil domains of transcription factors including HSF1 (refs. 33,40–42). Thus, embracing of DNA by HSF family members would enable geographically separated regulatory interactions at both the DBD and coiled-coil domain that would provide multiple input signals for HSF-mediated regulation. Given this new structural information, we propose a new model for the geometry of HSF bound to DNA; our model is inverted with respect to previous models1,43.

**HSFs exhibit divergent surface characteristics distal to DNA**

A comparison of the structures of the HSF2 DBD bound to DNA with the sequences of the HSF1 and HSF4 DBDs suggests that these proteins interact with DNA in a highly similar manner. To explore the nature of HSF family DBD surface features, we calculated the relative conservation of individual amino acids between HSF paralogs by using the ConSurf Server44. On the HSF2 surface that contacts DNA, it was apparent that amino acid residues such as Arg63 and other residues making direct and indirect DNA contacts are conserved (Fig. 4). This conservation suggests that HSF family members would have few, if any, paralog-specific sequence preferences for DNA and provides support for extrinsic mechanisms driving differential target-gene binding and activation in vivo. Examination of the HSF DBD surface distal to DNA contact sites showed little conservation of sequence or biochemical character (Fig. 4), thus suggesting that HSFs...
have evolved distinct surfaces to accommodate divergent regulatory inputs within the DBD, without altering DNA binding mechanisms. Furthermore, the wrapping of HSF coiled-coils around HSE DNA would expose the wing and other unique surfaces of the HSF1, HSF2 and HSF4 DBDs, thus generating a template for additional paralog-specific interactions with regulatory proteins.

**HSF1 and HSF2 are differentially SUMOylated in vitro**

The HSF1 DBD, but not the HSF2 DBD, has previously been shown to interact with RPA70 in vitro and in vivo, and this interaction contributes to HSF1-mediated target-gene expression. We postulated that a distinct protein–protein interaction or post-translational modification might be specific for the HSF2 DBD. Reports describing the mammalian SUMOylated proteome have found that Lys82 of HSF2 is conjugated by SUMO2 but have not identified the corresponding Lys91 of HSF1 (refs. 30–32). Because HSF2 Lys82 localizes within the exposed wing domain (Fig. 2a), we ascertained whether there is intrinsic SUMOylation specificity between the HSF1 and HSF2 DBDs. SUMOylation is analogous to ubiquitination in that both processes use an E1-E2-E3 enzymatic cascade for covalent modification of lysine residues in substrates. To explore a potential role for the wing domain in differential HSF function in vivo, we expressed triple FLAG (3×FLAG)-tagged derivatives of HSF1 and HSF2 wing-domain chimeras after in vitro SUMOylation and nickel–nitrilotriacetic acid (Ni-NTA) purification. Molecular weight (MW) markers (M) are indicated. Purified HSF1 or HSF2 DBD were added (+) or not (−) as indicated. (b) Anti-His6 immunoblot of HSF DBD wing-domain chimeras after in vitro SUMOylation. ATP was included (+) or not (−) as indicated. S, SUMO. ChIP analysis of HSF wing-domain chimeras expressed in Hsf1−/−Hsf2−/−MEFs by transfection with 3×FLAG-tagged derivatives of HSF1 and HSF2 or empty vector (V). Binding to Hsp70, Hsp25 and IL-6 promoters under control (left) and heat-shock (right) conditions is shown. Specificity is demonstrated by lack of signal in vector-transfected cells (gray). (d) mRNA-expression analysis of Hsp70, Hsp25, IL-6, and GFP in Hsf1−/−Hsf2−/−MEFs transfected with 3×FLAG-tagged derivatives of HSF1 and HSF2 or empty vector (V). Amplification of GFP is used as a transfection and HSF mRNA-expression control. Values for each gene and GFP is used as a transfection and HSF mRNA-expression control. Values for each gene and HSF mutant are normalized to WT HSF1 (red) as 100%. Histograms in e and d depict mean ± s.e.m.; *P < 0.05; NS (not significant), P > 0.05 by unpaired two-tailed t test (statistical analysis in Online Methods). Number of biological replicates from separate cell cultures; n = 3 for ChIP experiments; n = 4 for quantitative reverse-transcription PCR; source data are available online.

(e) Immunoblot analysis of Hsp70 induction in Hsf1−/−Hsf2−/−MEFs transfected with 3×FLAG-tagged HSF1 and HSF2 wing-domain chimera proteins after heat shock or proteasome inhibition with MG132. GAPDH is a loading control.

**The wing domain regulates HSF activity in vivo**

To explore a potential role for the wing domain in differential HSF function in vivo, we expressed triple FLAG (3×FLAG)-tagged derivatives of WT HSF1, HSF1W2, HSF2 or HSF2W1 proteins in mouse embryonic fibroblasts (MEFs) doubly knocked out for HSF1 and
HSF2 (Fig. 5c–e). Under control conditions, in vivo DNA binding activity of WT HSF1 and HSF1W2 were indistinguishable at the Hsp70, Hsp25 and interleukin-6 (IL-6) promoters (Fig. 5c). In contrast, WT HSF2 but not HSF2W1 exhibited basal binding at the Hsp70, Hsp25 and IL-6 promoters, thus suggesting that the endogenous HSF2 wing domain is essential for basal HSF2 DNA binding (Fig. 5c). Interestingly, under heat-shock conditions, HSF1W2 was compromised in binding to the Hsp70 promoter (approximately three-fold reduction) and the Hsp25 promoter (approximately five-fold reduction), whereas DNA binding was unaffected at the IL-6 promoter (Fig. 5c). These results suggest that the HSF wing domains have genomic locus–specific effects on DNA binding. Importantly, differences in the DNA binding of HSF DBD wing-domain chimeras in vivo were not explained by differences in DNA binding to an HSE in vitro (Supplementary Fig. 4c).

Analogously to the DNA binding of HSF wing-domain chimeras, expression of Hsp70, Hsp25 and IL-6 mRNA was unchanged in cells expressing WT HSF1 compared to HSF1W2 under control conditions (Fig. 5d). Upon heat shock, HSF1W2 was compromised in its ability to induce expression of Hsp70 (~37% of WT) and Hsp25 (~21% of WT) but not IL-6, thus suggesting that the HSF wing domain has genomic locus–specific effects on target-gene expression (Fig. 5d). Expression of both WT HSF2 and HSF2W1 did not increase expression of Hsp70, Hsp25 or IL-6 mRNA over that in vector-transfected cells, thus raising the possibility that HSF2 does not exhibit activity at these loci in the absence of HSF1. The analyses of DNA binding and gene expression were further supported by immunoblotting, in which cells expressing HSF1W2, compared to WT HSF1, were strongly compromised for activation of Hsp70 expression after exposure to proteotoxic conditions induced by heat shock or the proteasome inhibitor MG132 (Fig. 5e). Moreover, neither WT nor chimeric HSF2 activated Hsp70 expression after heat shock or proteasome inhibition, although their protein levels were similar to those of the HSF1 variants (Fig. 5e). Together, these results suggest that the HSF wing domain has a locus-specific biological function that does not involve direct DNA contacts, and they lay the foundation for mechanistic studies of critical regulatory events that occur within the HSF wing domains.

HSF1 and HSF2 directly interact and form heterocomplexes

Several reports have documented an interaction between HSF1 and HSF2 in vitro through coimmunoprecipitation and electrophoretic mobility supershift assays, approaches that do not distinguish between direct and indirect interactions.22,26,27 To ascertain whether HSF1 and HSF2 directly interact, we coexpressed Suprepl1-tagged human HSF1 and hexahistidine (His6)-tagged human HSF2 in Escherichia coli from a single plasmid with a bidirectional promoter (Supplementary Fig. 5a). Tandem differential affinity purification resulted in coelution of HSF1 and HSF2, thus suggesting that the two proteins directly interact (Fig. 6a). We fractionated the HSF1–HSF2 heterocomplexes by gel filtration, which resolves monomeric and multimeric HSF1 (ref. 4). The HSF1–HSF2 complex copurified at an elution volume similar to that of the HSF1 monomer, thus demonstrating that HSF1 and HSF2 form heteromultimeric complexes that are stable through three tandem purification steps (Fig. 6b and Supplementary Fig. 5b). Cross-linking and mass spectrometry confirmed that HSF1 and HSF2 were the only nonkeratin proteins detectable in cross-linked complexes (Supplementary Fig. 5c and Supplementary Data Set 1).

We evaluated the possibility that the HSF1–HSF2 interaction is mediated by the extended coiled-coil domain (LZ1–3) contained in both HSF1 and HSF2. We expressed plasmids containing HSF1ΔLZ1–3 and WT HSF2 (Dual Δ1) and the reciprocal HSF1 and HSF2ΔLZ1–3 (Dual Δ2) and purified them as in Figure 6a. WT HSF1 and HSF2 copurified from cell lysates (Dual), but WT HSF2 failed to copurify with HSF1 ΔLZ1–3 (Dual Δ1), and WT HSF1 did not copurify with HSF2 ΔLZ1–3 (Dual Δ2) (Fig. 6c and Supplementary Fig. 5d). These results demonstrate that HSF1 and HSF2 form heterocomplexes directly through their respective extended coiled-coil multimerization domains in a fashion similar to that of other coiled-coil transcription-factor multimers such as c-Fos and c-Jun.40 To validate the direct interaction between HSF1 and HSF2, we performed coimmunoprecipitation experiments in Hsf1−/− Hsf2−/− MEFs expressing hemagglutinin (HA)-tagged HSF1 alone, WT HSF2 or HSF2ΔLZ1–3. HSF1 specifically coprecipitated with WT HSF2 but not HSF2 ΔLZ1–3, thus demonstrating that the HSF2 coiled-coil domain is dispensable for the HSF1 interaction in vitro and in vivo (Fig. 6d). Together, these observations suggest that in the context of the HSF coiled-coil...
modules engaged with the DBD and coiled coil.

wrapping around DNA, HSF1-HSF2 interactions would provide a template for additional differential and combinatorial regulatory events that would not exist in HSF1 or HSF2 homo-oligomeric complexes. Here, we present a new model for HSF DNA binding, using a generic coiled-coil domain, in which the coiled-coil domain of homomultimeric HSF2 embraces DNA (Fig. 7). A similar model for an HSF1–HSF2 heteromultimer bound to DNA enables combinatorial interactions between HSF1 complexes (RPA70 and SSBP1) and HSF2 (SUMO) regulatory modules in the HSF-mediated transcriptional program. The transcriptional outcome of the direct interaction between HSF1 and HSF2, and the consequential regulatory interactions, present an intricate mechanism that could affect a diverse array of normal and pathophysiological cellular processes. Further exploration of surface features of the HSFs, and other transcription factors, may reveal new layers of transcriptional outputs that influence cellular processes and new ways to modulate these complex regulatory interactions.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes PDB 5D8K (HSF2 DBD bound to two-site HSE) and PDB 5D8L (HSF2 DBD bound to three-site HSE).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.M.J. conceived and performed experiments, analyzed data and wrote the manuscript. C.W.P. assisted in crystallographic data analysis and presentation. L.S. and D.J.T. conceived experiments, analyzed data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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in vitro into PyMOL (http://www.pymol.org/). The putty diagram was then generated to an OD600 of ~0.5, and induced with 1 mM IPTG for 5 h. Cell pellets were lysed in 20 mM Ni-NTA lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, and 20 mM imidazole HCl) with a sonic dismembrator in bursts (3 × 30 s). Lysates were then cleared by centrifugation at 20,000g for 30 min. Cleared lysates were incubated with a 5-M bed volume of Ni-NTA agarose (Qiagen) and rotated at 4°C for 2 h.

After addition of samples to a gravity filtration column, the Ni-NTA resin was washed with 2 × 50 mL Ni-NTA wash buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, and 40 mM imidazole HCl) and eluted with 10 mL Ni-NTA elution buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, and 250 mM imidazole HCl). For His₆-tag cleavage, the purified HSFB2 DBD was exchanged with an Amicon Ultra Centricr (MWCO 3 kDa) until the final imidazole concentration was 20 mM in 10 mL. This procedure resulted in a 2-mg protein yield. The buffer-exchanged HSFB2 DBD was then incubated with 3 U of biotinylated thrombin (Novagen) overnight at 15°C. After streptavidin removal of thrombin and Ni-NTA removal of uncleaved HSFB2 DBD, the protein was concentrated to 7 mg/ml before crys-
tallization trials. Expression and purification of HSFB1 DBD, HSFB1W2 DBD, and HSFB2W1 DBD were performed as described for HSFB2 DBD but without removal of the His₆ tag. HSFB2W2 DBD and HSFB2W1 DBD were generated by swapping amino acids 83–97 (HSF1) and 77–89 (HSF2) with Infusion cloning.

Crystalization of the HSFB2 DBD. 7 mg/ml HSFB2 DBD (357 µM) was premixed with 322.2 µM two-site HSE (GGTCTCTAGAAC) or 244.8 µM three-site HSE (GGTCTCTAGATTCCTG) double-stranded oligonucleotide for a final ratio of 1:1.2 protein/DNA-binding site. The resulting complex was then incubated at 25°C for 5 min and subsequently deposited into sitting-drop vapor-diffusion crystallization trays (Intelliplate 3, Robbins). The HSFB2–two-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 approximately 200 µm × 50 µm × 50 µm. Crystals were cryoprotected with 100 mM NaCl, 100 mM Bicine, pH 9, and 30% PEG 400. HSFB2–three-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 d to 50 µm × 50 µm × 25 µm.

Data collection and refinement. Diffraction data were collected on a Rigaku-007HF X-ray generator outfitted with a RaxisIV+ image plate detector, with an incident beam of 1.54 Å in wavelength. Data sets were processed in HKL2000 (ref. 56). Matthews analysis suggested one molecule of protein and one molecule of ssDNA in the asymmetric unit of the structure for the two-site structure and four molecules of protein and four molecules of ssDNA for the three-site structure57. The structures were phased by molecular replacement in PHENIX58 with a modified version of the PDB 3HTS8 structure containing only base pairs 1–4 of the HSE. Rebuilding and real-space refinements were performed in COOT59 with reciprocal space refinements in PHENIX60. Structure validation was performed with the MolProbity server. Ramachandran statistics for the two-site HSE structure were 97% favored, 3% allowed, and 0% outliers. Ramachandran statistics for the three-site HSE structure were 94% favored, 4% allowed, and 2% outliers.

To calculate the distance difference matrix between the HSFB2 and K. lactis DBD, we aligned PDB 3DK5 and PDB 3HTS8 with LSQ superposition in Coot with HSE DNA residues 1–12 as an anchor. The x, y, and z coordinates of the alignment files were then copied into Microsoft Excel. The peptide backbone (C, O, N, S) atoms were paired on the basis of BLAST alignment files, and the distance between paired atoms was calculated with the following equation: distance = sqrt((x₁ - x₂)^2 + (y₁ - y₂)^2 + (z₁ - z₂)^2)). Distances were then copied into the B-factor column of the 3SDK pdb file before import into PyMOL (http://www.pymol.org/). The putty diagram was then generated with the B-factor putty preset in PyMOL.

In vitro SUMOylation assays. SUMO E1 (SAE1/SAE2), SUMO E2 (UbC9), SUMO1, and SUMO2 were purchased from Boston Biochem. SUMO E3 ligases RANBP4F 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 EDTA, 2% Tween 20, 1 µg/ml leupeptin/ pepstatin/aprotinin, 1 mM DTT, and 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 h. Reactions were terminated with 1× SDS loading buffer before SDS-PAGE analysis and subsequent immunoblotting. For colloidal blue–stained samples, 100-µL reactions were incubated with 20 µL Ni-NTA agarose beads for 1 h at 4°C, washed twice with 200 µL Ni-NTA wash buffer, and eluted with 50 µL Ni-NTA elution buffer before SDS-PAGE. SUMO-

Cell culture, transfection, ChIP, and qRT-PCR. HSF1/HSF2 double-knockout mouse embryonic fibroblasts (Hsf1−/− Hsf2−/− MEFs) were a kind gift from I. Benjamin (University of Wisconsin at Madison). Cells were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 0.1 mM nonessential amino acids (Gibco), 1× MycoZap (Lonza), and 50 µg/µl β-mercaptoethanol. For transfection, 4 × 10⁶ cells were transfected with 2 µg of empty pcDNA3.1-ires-GFP, or pcDNA3.1-ires-GFP with human WT HSF1, HSF1W2, HSF2, or HSF2W1 containing a Terminal 3×FLAG tag by use of a 4D-

Nucleofector (Lonza) according to the manufacturer's instructions. Transfected cells were split into 10-cm plates for ChIP or six-well plates for qRT-PCR or immunob-

lingotting. For ChIP analysis, at 36 h after transfection, cells were maintained at 37°C or heat shocked for 30 min at 42°C before being cross-linked with 1% formaldehyde. After being quenched with 125 mM glycine, proteins were extracted with 2 ml SDS lysis buffer (20 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 100 mM KCl, 1% Triton X-100, 0.05% SDS, 1× Halt protease-inhibitor cocktail (Pierce), and 1× phosphatase-inhibitor cocktail (Pierce)) and sonicated in bursts (3 × 30 s). Lysates were cleared by centrifugation at 14,000g for 15 min. FLAG-tagged HSF proteins were then immunoprecipitated with Magnetic M2 FLAG affinity resin (Sigma) for 6 h at 4°C. Beads were then washed twice with lysis buffer, twice with lysis buffer + 0.5 M NaCl, and twice with TE buffer, and eluted with 200 µL TE + 1% SDS. Cross-links were reversed overnight at 65°C before treat-

ment with Proteinase K for 1.5 h at 37°C. Immunoprecipitated genomic DNA was then purified with a PCR cleanup kit (Qiagen) before qRT-PCR analysis. For RNA extraction, 36 h after transfection, cells were maintained at 37°C or heat shocked for 1 h at 42°C with a 1 h recovery. RNA was extracted with RNeasy Mini kits (Qiagen) according to the manufacturer’s instructions and were then DNase treated with Turbo DNase (Ambion). cDNA synthesis was carried out with SuperScript III first-strand kits (Invitrogen) with oligo(dT) primer, and then samples were treated with RNase H for 45 min at 37°C before qRT-PCR analysis. qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad) in 384-well plates with a CFX-384 qRT-PCR machine (Bio-Rad). For immunoblotting,

analyzing transected cells were maintained at 37°C or heat shocked for 1 h at 42°C with a 6-h recovery at 37°C, or treated with 3 µM MG132 for 7 h. Cell lysates were harvested with SDS lysis buffer. Lysates were cleared by centrifuga-
l	
tion at 14,000g for 15 min before quantification with the BCA Assay (Thermo) and subsequent fractionation by SDS-PAGE and transfer to 0.2 µM nitrocellulose membrane. Anti-FLAG (M2, Sigma), anti-Hsp72 (C92, Enzo), and anti-GAPDH (6C5, Santa Cruz) antibodies were used at a dilution of 1:1,000 before incubation with anti-rabbit or anti-mouse antibodies (GE Health Sciences) at a dilution of 1:5,000 (Supplementary Data Set 2). Primary-antibody validation can be found on the manufacturers’ websites.

Statistical analysis. For statistical analysis of ChIP and qRT-PCR data, two-way analysis of variance was performed with GraphPad Prism 5. An interaction between HSF mutant and gene was observed, thus enabling further analysis by unpaired two-tailed t tests for individual mutant and gene pairs, i.e., WT HSF1 versus HSF1W2 for Hsp70.

In vitro DNA binding. Analysis of HSF DBD binding to HSEs was carried out with fluorescence polarization as previously described1. 1 nM fluorescein-labeled HSE was added to 25 nM HEPES and 150 mM NaCl. HSF DBDs were titrated into the binding reaction, and relative polarization was measured after each addi-

tion of DBD. Binding curves were generated in GraphPad Prism 5 with one-site total binding nonlinear-regression curve fitting.

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Purification of HSF1–HSF2 heterocomplexes. Human HSF1 with an N-terminal StrepII tag and human HSF2 with an N-terminal His6 tag were codon optimized for expression in E. coli and cloned into the pET15b expression vector with two divergent lac-operon promoters (Supplementary Fig. 5a). Overnight cultures of E. coli were back-diluted 1:100 into 2 l of LB containing 100 µg/mL ampicillin, grown to an OD600 of 0.5 and induced with IPTG for 5 h. Cell pellets were lysed in 20 mL of Strep binding buffer (50 mM HEPES, pH 7.5, and 300 mM NaCl) and cleared by centrifugation at 20,000g for 30 min at 4 °C. Cleared lysates were then applied to a StrepTrap column (GE Heath Sciences) with an Akta Pure FPLC (GE Health Sciences) at a flow rate of 0.5 mL/min. The column was washed with Strep binding buffer until the A280 reading reached zero at a flow rate of 2 mL/min. The StrepTrap column was then directly attached to a HisTrap column (GE Health Sciences) and reconnected to the FPLC. Bound proteins were eluted with Strep binding buffer supplemented with 5 mM desthiobiotin, which was applied 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 was connected directly to the HisTrap column. The HisTrap was then washed with 50 mL Ni-NTA wash buffer, 20 mL Ni-NTA wash buffer supplemented with 20 mM MgCl2 and 5 mM ATP, and an additional 30 mL Ni-NTA wash buffer at a flow rate of 2 mL/min. Bound proteins were eluted from the HisTrap column with Ni-NTA 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 before being pooled, concentrated to a volume of 10 mL, and loaded onto a Sephacryl S-400 gel-filtration column with an Akta FPLC. The HSF1–HSF2 heterocomplexes were eluted from the S-400 column with 25 mM HEPES, pH 7.5, and 150 mM NaCl, and fractions were analyzed by SDS-PAGE and colloidal blue staining. HSF1-HSF2 cross-linking was performed with disuccinimidyl suberate (DSS, Thermo). 2 µg of HSF1–HSF2 heterocomplexes in 25 mM HEPES, pH 7.5, and 150 mM NaCl were incubated with increasing concentrations of DSS for 30 min at room temperature. Reactions were then quenched with 25 mM Tris for 15 min at room temperature before SDS-PAGE and mass spectrometry analysis.

HSF1–HSF2 co-immunoprecipitation. Hsf1−/− Hsf2−/− MEFs were transfected with HA-tagged HSF1 alone, or with 3×FLAG-tagged WT HSF2 or HSF2∆LZ1–3 for as described above. Cells were cross-linked with 1.5 mM DSP (Pierce) for 30 min at room temperature according to the manufacturer’s protocols. Cells were lysed in SDS lysis buffer before sonication for 1 × 30 s to disrupt chromatin associated HSF complexes. 1.5 mg of total protein was then immunoprecipitated with M2 FLAG affinity beads (Sigma) overnight at 4 °C. Beads were then washed three times in lysis buffer before elution with TE buffer + 1% SDS. HSF1-HA protein was detected with anti-HA antibody (Y-11, Santa Cruz Biotechnology; validation on manufacturer’s website) (Supplementary Data Set 2).

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