Versatile Functions of Heat Shock Factors: It Is Not All about Stress

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Abstract

Organisms are constantly exposed to acute and chronic stress conditions, which challenge the maintenance of protein homeostasis. Heat Shock Proteins (HSPs) function as molecular chaperones to stabilize protein structures, facilitate refolding of misfolded proteins, and prevent uncontrolled protein aggregation. Therefore, HSPs serve as the first and last line of defense in the events of proteotoxic stresses. The stress-inducible expression of HSPs, which is a hallmark of the heat shock response, is under strict control of evolutionary conserved transcription factors, known as Heat Shock Factors (HSFs). Invertebrates have only a single HSF, whereas the HSF family in vertebrates consists of multiple members. Direct interactions of HSFs with various proteins, including HSPs, chromatin-associated proteins, and other HSF family members as well as their complex post-translational modifications, allow these transcription factors to function not only in stress responses but also in many other biological processes. For example, mammalian HSF1, HSF2 and HSF4 are fundamental for normal organismal development and healthy aging. Moreover, recent discoveries have highlighted the importance of HSFs in tumorigenesis, neurodegeneration, and metabolic disorders, which positions them as promising therapeutic targets in multiple human diseases. In this review, we focus on recent advances in the HSF biology and discuss the functional impact of HSFs on stress responses, development, aging, and age-related pathologies.

INTRODUCTION

In 1962, an Italian geneticist Ferruccio Ritossa, discovered a puffing pattern in the polytene chromosomes of *Drosophila busckii* larvae exposed to elevated temperatures, which later became the foundation of what is today called the Heat Shock Response (HSR) [1]. Subsequently, it was shown that the puffing pattern correlates with the increased production of mRNAs coding for Heat Shock Proteins (HSPs) that are now known to function as molecular chaperones [2]. HSPs help proteins to fold properly and maintain their conformation under conditions that otherwise would cause protein damage, formation of protein aggregates and eventually cell death [3]. The HSR, defined as the inducible expression of HSPs, is conserved among all living organisms and is essential for organismal survival during exposure to a vast variety of protein-damaging stresses, such as heat, oxidative stress, heavy metals, alcohol, and microbial infections.

In eukaryotes, the HSR is mediated by a transcription factor family called the Heat Shock Factors (HSFs). After the initial discovery of HSFs as master regulators of the HSR [4, 5], their biological significance has greatly expanded. Tremendous progress in the genome-wide sequencing techniques has allowed for detailed characterization of target *loci* bound by HSFs, and HSF-driven transcriptional programs in various organisms under different conditions (for summary of genome-wide studies involving HSFs, see Table **1**). HSFs are essential for proper development, including gametogenesis, and maintenance of protein homeostasis throughout the lifespan of organisms. HSFs are also considered as key regulatory factors in progression of human pathologies, such as different types of cancers and neurodegenerative disorders (Fig. **1)**. In this review, we will summarize the recent advances on the knowledge of HSFs structure and regulation. We will also discuss the involvement of HSFs in normal development, healthy aging, and human pathologies. However, the control mechanisms and implications of HSFs in metabolic disorders are presented in detail by Su & Dai [89] and are therefore excluded from this review.

STRUCTURE AND DOMAIN ORGANIZATION OF HSFS

Heat Shock Elements and HSF DNA-binding domain

Invertebrates express only a single HSF, whereas vertebrates and plants have multiple HSFs [6–8]. The mammalian family of HSFs consists of six members, HSF1, HSF2, HSF3, HSF4, HSFY, and HSFX. Since HSF3 protein has been found only in mouse [9], and HSFX [10] HSFY [11, 12] are sex chromosome-specific transcription factors, which are still poorly characterized, they are not discussed in this review. The family of plant HSFs, consisting of more than thirty members, are comprehensively reviewed by Scharf and co-workers [8]. The classification into the HSF family is based on the high amino acid sequence homology of the DNA-binding domain (DBD) which belongs to the winged helix-turn-helix family and is shared by all HSFs (Fig. **2**) [13–17]. Another functional domain that is structurally conserved throughout evolution is the oligomerization domain (HR-A/B), composed of leucin-zipper like heptad repeats. Intact HR-A/B is required for formation of HSF trimers that are capable of binding to DNA [18, 19]. All the other domains of HSFs, such as regulatory domain and activatory domain are defined based on their function instead of sequence similarity.

The DBD of HSFs binds to a *cis*-acting sequence, the Heat Shock Element (HSE), consisting of inverted pentameric nGAAn repeats, where "n" can be any nucleotide [20]. The consensus HSE found in the promoters of HSPs has at least three continuous inverted repeats of nGAAn [21–23]. HSE architecture facilitating HSF binding can vary in sequence, spacing, orientation, and number of repeats [24–26]. However, the genome-wide analyses of HSF binding motifs in yeast, round worm, fly, mouse, and human have revealed a striking similarity in the core HSE sequence (Fig. **3**) (Table **1**) [27–30].

The crystal structure of yeast HSF DBD bound to HSE was obtained already in 90's, and showed that unlike many other winged helix-turn-helix proteins, the HSF wing domain does not contact the DNA [31]. Two recent structural studies increased our understanding of how HSF trimers interact with HSEs [16, 17]. HSF proteins embrace the DNA, which positions the three DBDs and HR-A/B domains on the opposite sites of the DNA strand (Fig. **3**). This unique conformation results in stable HSF binding to DNA and exposes the surfaces of wing domains as well as the DBDs for different post-translational modifications (PTMs) and other regulatory inputs. Moreover, the structural data by Jaeger and co-workers provide an explanation how the subtle differences in the amino acid sequence within the DBD result in differential and combinatorial regulation by PTMs and interacting proteins [17].

Oligomerization, Regulatory and Activatory Domains of HSFs

In the light of current evidence, active HSFs function as trimers [32]. The trimerization of HSFs occurs *via* intermolecular interactions between the HR-A/B domains of adjacent HSF monomers [33]. The spontaneous trimerization of HSFs under normal conditions is suppressed by another heptad repeat domain, called HR-C (Fig. **2**). Under non-stressed conditions, the HR-C interacts with the HR-A/B and is considered to be responsible for the maintenance of monomer state, as introduction of mutations to HR-C results in trimerization and DNA-binding of HSF in the absence of stress [34, 35]. Furthermore, eukaryotic HSFs that do not contain HR-C, such as mammalian HSF4 and yeast HSF (yHSF) (Fig. **2**), have been shown to exist as trimers [36, 37]. The functional consequences of constitutively active HSF4 and yHSF are discussed in section "HSFs in Development".

The activatory domain (AD) that is required for the *trans*-activating capacity of HSFs is found in their C-terminal regions, except for yHSF that harbors an activatory domain both in the N- and C-terminus (Fig. **2**) [38–40]. HSF4 was thought to lack an AD, but a recent study identified AD in the C-terminus [41]. The AD is largely unfolded, enriched in hydrophobic and acidic amino acid residues [42, 43], creating "acid blobs" [44], which facilitate interactions with other transcription factors and co-factors and ensure rapid and accurate activation to stress stimuli [45–47]. In the absence of stress, another region of HSF called the regulatory domain (RD) (Fig. **2**), represses the AD under normal conditions and allows stress inducible activation [42]. To date, the mammalian HSF1 RD is extensively characterized [43, 48, 49], but the corresponding regions have been found also in HSF2 and HSF4 (Fig. **2A**) [41, 50].

POST-TRANSLATIONAL MODIFICATIONS OF HSFS

In cells exposed to various stress stimuli and growth conditions, HSFs undergo numerous PTMs, such as acetylation, phosphorylation, sumoylation, and ubiquitination [49, 51–55]. Phosphorylation is a hallmark

modification of HSFs, since the single HSF in yeast and fly (Fig. 2**B**) and the mammalian HSF1 (Fig. 2**A**) are all heavily phosphorylated [49, 54, 56]. Other PTMs to which HSFs are subjected are best characterized in mammalian cells (Fig. **2A)** [52]. Remarkably, 73 phosphorylation sites have been identified in yHSF [54], and 23 in human HSF1 (hHSF1) [49, 52, 57]. hHSF1 can be phosphorylated both *in vitro* and *in vivo* by many kinases, including MAPKAPK2 [58, 59], CKII [60], PLK1 [61], CamKII [62], MAPK [58], GSK3β [63, 64], PKC [64] and PLK1 [65]. Moreover, a recent study showed that hHSF1 is phosphorylated directly by MEK but not by the downstream MEK target ERK [57]. Because a majority of the phosphorylation sites in hHSF1 reside within the RD and hHFS1 activation coincides with stress-inducible hyperphosphorylation, it was hypothesized that phosphorylation acts as a switch from transcriptionally inactive to active form of HSF [48, 59]. Recently, however, it was demonstrated that a change in the phosphorylation status is not required for HSF activation, whereas it rather fine-tunes the magnitude of the stress response [49, 54].

HSFs are ubiquitinated, which affects their stability [53, 66, 67], but specific ubiquitination sites have been reported only on HSF2 and HSF4 (Fig. **2A**) [67–69]. HSFs undergo also sumoylation [17, 51, 70, 71]. Both, hHSF1 and hHSF4 are sumoylated on a lysine located within the specific ΨKxExxSP motif, named phosphorylation-dependent sumoylation motif (PDSM), where Ψ denotes a branched hydrophobic amino acid (usually a leucin, isoleucin, or valine) [70]. Within the PDSM, the phosphorylation of a serine is required for sumoylation to occur. The PDSM was first discovered in HSF1 and HSF4, but later it has been found in many other proteins, especially transcription factors [70]. It was proposed that sumoylation of transcription factors, including hHSF1 and hHSF4 would restrict their *trans*-activating capacity, but the mechanisms have remained unknown [70]. HSF2 is sumoylated on two sites (K82 and K139), and sumoylation of K82 within the DBD has been shown to prevent its binding to DNA [17, 71].

To date, only hHSF1 has been reported to be subjected to acetylation. Acetylation of hHSF1 plays a dual role, as acetylation within the DBD, such as at K80 and K118 inhibits DNA binding during the attenuation phase [53, 72], whereas acetylation of K208 and K298, located within the HR-A/B and RD, stabilizes hHSF1 (Fig. **2A**) [53]. We will discuss the functional impact of these modifications in the section "Stress-Induced HSF1 Activation-Attenuation Pathway". Interestingly, K298 of HSF1 is a target for both acetylation and sumoylation (Fig. **2A**), and as these modifications have distinct effects on HSF1 activity, it is tempting to speculate that they function as a molecular switch regulating HSF1 activity. Taken together, complex interplay between different PTMs provides a fine-tuning mechanism for controlling the activity of HSFs. This enables HSFs to operate in a broad spectrum of biological processes, such as stress responses, development, aging, and human pathologies (Fig. **1**). However, the exact PTM-signatures of HSFs during these processes remain to be established.

REGULATORY MECHANISMS OF HSF ACTIVATION

HSF-mediated transcriptional response is conserved throughout evolution, yet the molecular mechanisms regulating HSF activation are not fully understood. Several studies have demonstrated that purified HSFs are capable of sensing various proteotoxic conditions, such as elevated temperature, increased calcium concentration, H_2O_2, and low pH [73–77]. Notion that an intact RD is critical for stress-inducible activation of HSF1, and that it harbors the majority of HSF1 phosphorylation sites, led to a hypothesis that stress-inducible phosphorylation would drive activation of HSF1. However, the finding that the disruption of the known phosphorylation sites within the RD, neither resulted in spontaneous activation of HSF1 under normal conditions, nor abolished the capacity of

RD to sense heat stress [49], did not support the original hypothesis. Similarly, the genome-wide RNAi-based screen did not find kinases among the modulators of HSF1 activity [53]. These results indicate that the stress-inducible phosphorylation of HSF1 is not required for its activation.

A common nominator of conditions leading to HSF activation is that they are protein-damaging, cause accumulation of misfolded proteins, and eventually lead to formation of protein aggregates in cells [78]. The inducible expression of HSPs counteracts the toxic effects of damaged proteins. HSPs bind to mature proteins and prevent their denaturation and aggregation, which is essential for preserving the protein activity during stress [79]. Based on multiple observations, a feedback model for HSPs and HSF was presented [3]. Under normal conditions, HSF interacts with HSPs, which prevents HSF trimerization, DNA-binding and transcriptional activity [80–83]. In the event of proteotoxic stress, accumulation of misfolded proteins titrates away HSPs from HSF monomers, which allows formation of active HSF trimers. HSP90 was proposed to be a main chaperone restricting HSF activity, with the auxiliary function of HSP70 and its co-chaperone HSP40. In the light of recent results, the evidence for HSP90 keeping HSF inactive is not conclusive. Albeit the depletion of HSP90 or HSP70 activates the HSR in round worms, depletion of HSP90 results in a lower magnitude of response when compared to the HSP70 depletion [84]. In another report, yHSF, under control conditions, was found to interact with HSP70 but not with HSP90 [54]. Moreover, titration of only HSP70 and HSP40 had an effect on HSF-mediated gene expression, whereas HSP90 overexpression did not inhibit HSF. Intriguingly, in fungus C. *albicans*, depletion of HSP90 resulted in increased binding of HSF to DNA already under control conditions, whereas upon stress it delayed the onset of HSF1-mediated gene expression, suggesting that HSP90 acts as a positive regulator of the HSF1-mediated HSR [85]. Finally, the *in vitro* studies on the thermosensory function of hHSF1 revealed that instead of inhibiting HSF1 activation, addition of purified HSP90 lowered the response threshold, which indicates that HSP90 does not prevent, but rather enhances trimerization and DNA-binding of HSF1 [77].

In multi-cellular organisms, another layer of HSF regulation, besides an HSP-mediated feedback loop, has been reported. In round worms, the activation of HSR is under strict control of thermosensory neurons [86]. These neurons control the thermotactic behavior helping round worms finding the optimal temperature for growth and reproduction [87]. Mutations affecting the thermosensory neurons and their post-synaptic partner cells, greatly abolished the HSR. However, the same mutation did not have an effect on the HSR induced by exposures to heavy metals, such as cadmium. A subsequent study showed that the HSR can be activated without stress, through an optogenetic stimulation of thermosensory neurons, which enhanced serotonin release and activated HSF in distal tissues [88]. Future studies need to elucidate whether a similar neuronal control is present in other metazoans, including mammals, and what types of proteotoxic insults trigger the neuronal HSF activation.

STRESS-INDUCED HSF1 ACTIVATION-ATTENUATION PATHWAY

Activation Phase

For decades, fly HSP70 gene activation by HSF in response to stress served as a model for studies on how inducible transcription is regulated. Although invertebrates and vertebrates share many fundamental mechanisms of transcription, the stress-inducible transcription is less understood in mammals due to high complexity (for a comprehensive review of HSF-mediated transcriptional regulation in fly see [89]). Similarly to invertebrates, the

mammalian HSP genes are predominantly primed with the transcriptionally engaged but paused RNA polymerase II (Pol II) (Fig. **4**) [90, 91]. Under normal growth conditions, HSF1 is kept in an inactive state by intramolecular and intermolecular interactions [77, 82]. Inactive HSF1 shuttles between the cytoplasm and nucleus, and under non-stressed conditions it can be found diffusely distributed in both compartments [18]. However, a small portion of HSF1 is trimerized and bound to the promotor regions of certain target genes already in the absence of stress stimuli [29]. The DNA-bound HSF1 interacts with replication protein A (RPA) that is involved in the replication and DNA repair [92]. HSF1-RPA interaction recruits the histone chaperone FACT and a chromatin remodeling complex containing Brahma-related gene 1 (BRG1), which increases chromatin accessibility at the HSP70 gene promoter region by nucleosome eviction [92]. The poly(ADP-ribose) polymerase 1 (PARP1) has been found to interact with the HSP70 promoter under control conditions both in human [93] and fly [94]. In fly exposed to heat shock, HSF stimulates PARP1 activity and redistribution, which is essential for the rapid nucleosome clearance from the HSP70 *loci* [94]. It remains to be determined, whether the mammalian PARP1 has a similar function.

In the event of proteotoxic stress, HSF1 forms trimers, accumulates in the nucleus and binds to the HSEs in the promoters of target genes [18, 29, 82]. DNA-bound HSF1 trimers recruit other transcriptional activators, such as the mediator complex [95], activating transcription factor 1 (ATF1) [96], SWI/SNF chromatin remodeling complex containing BRG1[45, 96, 97], histone methyltransferase mixed-lineage leukemia 1 (MLL1) [98], death associated protein-6 (Daxx) [46], and co-activator activating signal co-integrator 2 (ASC-2) [99]. While HSF1 has been shown to interact *in vitro* with the basal transcription component TAF-9 [100], it remains unclear whether it directly or indirectly recruits general transcription factors (GTFs) to the HSPs promoters. The HSF1-initiated changes in the target gene promoters result in the release of Pol II from the paused state into elongation (Fig. **4**) [91]. Screens in mammalian cells for proteins involved in the transcriptional regulation by HSF1 have revealed that chromatin modifiers and nuclear proteasomes are involved in inducible HSP expression [53, 92], but the exact functions of these factors need to be established.

Attenuation Phase

Signals that initiate shutting down the HSF1 activity are not known. Negative feedback from newly produced HSPs could provide a mechanism forcing HSF1 attenuation. Indeed, in the course of stress HSP70 interacts with HSF1, thereby recruiting transcriptional co-repressor CoREST, which represses the HSF1-mediated transcription [101]. Interestingly, the overexpression of HSP70 in CoREST-deficient cells had only a little effect on HSF1 activity, suggesting that HSP70 alone is not capable of inhibiting HSF1. Another mechanism that enforces the attenuation of HSF1 from its target promoters is acetylation of the HSF1 DBD which prevents HSF1 from binding to DNA [72]. The acetyltransferase complex CBP/p300 has been shown to acetylate HSF1. Interestingly, ATF1 which enhances the *trans*-activating capacity of HSF1 during stress [96], has an opposite effect during the attenuation phase, by recruiting CBP/p300 to HSF1. Acetylation of HSF1 is counteracted by histone deacetylases SIRT1, HDAC7, and HDAC9, thereby prolonging the HSR [72, 102]. It is, however, plausible that multiple acetyltransferases and deacetylases are involved in the stress-induced HSF1 activation-attenuation pathway.

Previously, it was postulated that inactivated HSF1 timers, when released from DNA, would dissociate back to monomers. New evidence highlights the importance of regulating the stability of HSF1 during its activation-attenuation pathway. The turnover of HSF1 protein is accelerated in cells exposed to heat stress and hyperphosphorylated HSF1 trimers undergo proteasomal degradation upon attenuation [53]. FBXW7α, a substrate-targeting subunit of the SCF (Skp1–Cul1–F box) ubiquitin E3 ligase complex, ubiquitinates HSF1 and promotes its degradation upon stress [103]. Loss of FBXW7α in cells results in the accumulation of nuclear HSF1 after stress, prolongs HSF1 binding to its target promotors and extends the HSR (Table **1**). These observations support the model, where trimerized HSF1 would not revert to monomers, but rather be actively degraded during the attenuation phase (Fig. **4**).

Interplay of HSF1 and HSF2 in the Heat Shock Response

HSF2 shares a ~70% identity of the amino acid sequence in the DBD and HR-A/B domains, and ~35% identity in the rest of the protein with HSF1 [104]. Both HSF1 and HSF2 are co-expressed in a wide range of mammalian tissues [105], and are able to bind to the same HSEs [29, 106, 107]. These findings have raised a question about their possible interplay during stress responses. However, HSF2 is not able to compensate for the loss of HSF1, since cells devoid of HSF1 do not mount the HSR [91, 108]. Furthermore, in cells exposed to elevated temperatures, HSF1 stability is not affected, whereas HSF2 is ubiquitinated by ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C), which leads to a rapid decrease in HSF2 protein levels [66]. Nevertheless, HSF1 and HSF2 interact and form heterotrimers during stress responses (Fig. **5A,B,D**) [109]. HSF1-HSF2 heterotrimers are thought to regulate the magnitude of stress responses, since many HSPs are differentially expressed in *hsf2*$^{-/-}$ cells when compared to wild-type cells (Fig. **5A**) [110]. Moreover, a recent study that examined the transcriptional response in a genome-wide manner, using PRO-seq to directly measure synthesis of nascent transcripts, found that the depletion of HSF2 in the *hsf1*$^{-/-}$ background decreased the number of downregulated genes under stress conditions (Table **1**) [91]. This finding indicates that HSF2 can function as a repressor during stress responses.

HSF2 binds to a large number of target *loci* both in unstressed and stressed mitotic cells [29]. Previously, it was suggested that HSF2 binding during mitosis "bookmarks" the target *loci*, thereby enabling rapid transcription right after cell division [111, 112]. However, it appears that HSF2 binding has also another function, where HSF2 represses the HSR in mitotic cells exposed to stress [113]. In cells with high levels of HSF2 during mitosis, HSF2 can be found on the promoter of HSP70, where it decreases the chromatin accessibility and prevents the stress-inducible binding of HSF1 and Pol II. This impairs the HSR, and eventually leads to cell death (Fig. **5B**). In contrast, in mitotic cells with reduced levels of HSF2, both HSF1 and Pol II are able to bind to the HSP70 promoter upon stress, increasing the HSP70 expression and allowing cells to progress through mitosis. These results suggest that HSF2 activity is regulated in a concentration-dependent manner, and that HSF1 and HSF2 have opposite effects in mitotic cells exposed to heat stress, where HSF1 acts as a transcriptional activator and HSF2 is a repressor.

HSFS IN DEVELOPMENT

HSF as a Developmental Factor in Yeast and Round Worm

Studies of HSF-*null* organisms have revealed that functions of HSF are not limited only to stress responses. In yeast, yHSF is essential under normal growth conditions, and lack of yHSF results in loss of cell wall integrity and arrests the cell cycle [114–116]. In a recent study by Solís and co-workers, depletion of yHSF from the nucleus decreased expression of 18 genes, encoding mainly molecular chaperones, which subsequently led to cell cycle arrest and cell death [117]. Surprisingly, the cell growth was rescued in yHSF-depleted yeast by ectopically expressing only two genes, *i.e.* HSP70 and HSP90, which demonstrates that the essential function of yHSF is to drive expression of molecular chaperones, and that overexpression of HSP70 and HSP90 is sufficient to complement for loss of yHSF.

In round worm, amorphic HSF mutations result in developmental arrest in the L1/L2 larva stage [118]. During development, HSF in cooperation with transcription factor E2F, drives the expression of a specific set of genes, different from those activated upon stress (Table **1**) [30]. Genes involved in RNA biogenesis and translation, protein folding, processing and transport, and metabolism, which are all required for proper larva development, are directly regulated by HSF [30]. It is still unclear how E2F directs HSF to its target genes; E2F might directly interact with HSF or act as a co-activator of HSF1 through chromatin remodeling.

HSF has also been shown to regulate lifespan of round worm [119, 120]. HSF and transcriptional factor Daf-16 drive the expression of specific genes, including HSPs, which extends the lifespan. Intriguingly, the magnitude of several stress responses, such as HSR, unfolded protein response, and oxidative stress response are repressed in adult worms which coincides with the onset of reproductive maturity [121–123]. This repression has been shown to be mediated on the chromatin level [123]. In adult worms, the levels of demethylase jmjd-3.1 decrease, which increases tri-methylation of K27 of histone H3 and forces chromatin to the closed state, making it inaccessible to the transcriptional machinery [123]. These findings suggest that the programmed collapse of stress responses plays a key role in maximizing the reproduction success of the round worms. Based on the current evidence, HSF in round worms contributes to the larval development and long-term health of adult organisms by driving distinct transcriptional programs.

HSFs as Mammalian Developmental Factors

Single knock-out of HSF1, HSF2 or HSF4, or double knock-out of HSF1&HSF2 or HSF1&HSF4 is not lethal in mouse [124, 125]. However, each of these knock-out animals display different phenotypes, indicating that all mammalian HSFs have their specific functions in development. *hsf1*$^{-/-}$ mice are not able to induce the HSR, and they display several developmental defects, such as increased prenatal lethality, growth retardation, complete female infertility, abnormalities of the olfactory epithelium, and aberrant development of neurons [108, 126, 127]. *hsf2*$^{-/-}$ mice have abnormalities in the central nervous system and decreased male and female fertility [128, 129]. *hsf4*$^{-/-}$ mice display improper lens development and develop post-natal cataract [125, 130]. Interestingly, the double knockout of HSF1&HSF2 has an additive deficiency phenotype [131], including complete arrest of spermatogenesis, while the lens development is more severely impaired in double knock-out of HSF1&HSF4 mice

than in *hsf4*$^{-/-}$ mice [130]. The transcriptional programs driven by HSFs during development will be discussed below.

HSF1 also contributes to the post-natal development of mice in a transcription-independent manner. The mechanistic target of rapamycin complex 1 (mTORC1) is a prominent regulator of translation which also acts as a sensor for environmental stresses [132]. c-JUN N-terminal kinase (JNK) interacts with mTORC1 thereby inhibiting protein translation [133]. In cells exposed to stress, HSF1 directly interacts and suppresses JNK to promote mTORC1-mediated translation, which is critical for maintaining protein synthesis [133]. Intriguingly, *hsf1*$^{-/-}$ mice which suffer from growth retardation also showed increased JNK but diminished mTORC1 activity. The double knock-out of HSF1&JNK restored the cell, organ and body size, indicating that the HSF1-JNK-mTORC1 axis controls post-natal organismal growth [133]. This is the first example of HSF1 functioning independently of its canonical transcriptional action.

Gametogenesis

Fertilized oocytes originating from *hsf1*$^{-/-}$ mice are not able to develop past the zygotic stage, even when mice are mated with wild-type males, which indicates that HSF1 is an maternal factor [134]. It has been shown that HSF1 is required for the expression of HSP90α in maturing oocytes, to promote proper meiotic maturation and asymmetrical division [135]. These findings suggest that HSF1 through regulating HSPs, specifically HSP90, is essential for the reproductive success of pre-implantation embryos.

Interplay of HSF1 and HSF2 is required for spermatogenesis. Mice deficient of either HSF1 or HSF2 display defects in spermatogenesis [128, 136], whereas the double HSF1&HSF2 knock-out male mice are completely sterile [131]. Among all murine tissues, HSF2 is most abundantly expressed in testis and its levels vary during different stages of spermatogenesis [105, 137]. In developing spermatocytes, HSF2 was found to occupy multiple genomic *loci*, and in addition to HSPs, HSF2 regulates expression of genes that are specifically important for sperm quality (Table **1**) [106, 107]. Examples of these genes are Y chromosomal multi-copy genes *e.g.* spermiogenesis specific transcript on the Y 2 (Ssty2), Sycp3-like Y-linked (Sly), and Sycp3-like X-linked (Slx) [106]. During spermatogenesis, the levels of HSF2 are strictly regulated by a microRNA, mir-18 [138], which supports the notion that HSF2 activity is mainly regulated through its concentration. Since loss of both HSF1 and HSF2 in mice results in complete male infertility, it emphasizes the requirement for synergistic cooperation of these factors for spermatogenesis. Indeed, HSF1 and HSF2 can be found occupying the same genomic regions, including HSPs and Y chromosomal multi-copy genes, in spermatocytes (Fig. **5C**) [107, 109].

Brain Development

Both HSF1 and HSF2 are key factors in the development of brain and maintenance of central nervous system, and they are ubiquitously expressed in the developing brain until birth [139]. HSF1 is required for proper myelin formation, neuron maturation, lateral ventricles and hippocampal development [127, 140], but the exact mechanism of action is still poorly understood. HSF2, on the other hand, plays an important role in the development of cerebral cortex, as *hsf2*$^{-/-}$ mice show defective neuronal migration [141]. Lack of HSF2 caused

differential expression of genes involved in normal neuronal migration, such as p35, p39, which are the activators of Cdk5, a kinase essential for neuronal migration [141]. Furthermore, loss of HSF2 reduced the expression of T-box brain gene 1 (Tbr1), a protein expressed by the Cajal-Retzius cells which are reelin-secreting neurons found in the marginal zone of the developing mammalian cortex [129]. Hence, it is feasible to consider that the *trans*-activating capacity of HSF2 is required for proper cortex development *via* controlling neuronal migration.

Another example of the interplay between HSF1 and HSF2 was recently reported in migrating cortical neurons exposed to stress. Alcohol exposure of developing brain leads to constitutive activation of HSF1 [139]. Activated HSF1 and HSF2 form heterotrimers, which compete with or prevent binding of HSF2 homotrimers to target gene promoters (Fig. **5D**). HSF1-HSF2 heterotrimers reduce the expression of genes required for proper neuronal migration, such as mitogen-activated proteins (MAPs), and promote defects characteristic for Fetal Alcohol Syndrome (FAS). Intriguingly, in the absence of HSF2, defects in neuronal migration caused by exposure to alcohol are less severe, which suggests that HSF2 has a detrimental role in the developing cortex chronically exposed to alcohol [139]. Therefore, HSF2 as a regulator of the MAPs genes plays a dual role, as it is beneficial under normal conditions but has adverse effects under alcohol exposure.

Lens Development

The loss-of-function mutation or deletion of HSF4 results in abnormal lens development in post-natal mice, which leads to cataract [130]. HSF4, due to the lack of HR-C (Fig. **2A**) is constitutively trimeric and active transcription factor [37]. It is plausible that activity of HSF4 is regulated similarly to HSF2 by its expression levels in cells, since HSF4 undergoes ubiquitination on a single site (K206), followed by rapid degradation in human lens epithelial cells [67]. In lens tissue, HSF4 binds to the discontinued HSEs in the promoter of γ-crystallin genes and drives their expression [130, 142]. γ-crystallins are the major structural proteins in lens [143]. Since the initial discovery of the involvement of HSF4 in cataract [130, 144], multiple mutations altering HSF4 function have been identified in human cataract patients [41, 144–147]. During lens development, differentiation from proliferating epithelial cells into secondary fiber cells is crucial [148]. In *hsf4*$^{-/-}$ mice, lens epithelial cells showed abnormal proliferation and premature differentiation [130]. It has been demonstrated that HSF1 and HSF4 compete in binding to the promoter of fibroblast growth factor 7 (FGF7) and have an opposite effect on its expression. HSF4 also counteracts the transcriptional activity of HSF1, by promoting its degradation in lens tissue [149]. Furthermore, HSF4 contributes to normal lens development, by interacting, stabilizing and activating the cell cycle suppressor protein p53 [150]. Specific mutations in the HSF4 protein have also been associated with age-related cataract [151, 152], indicating that HSF4 function is required for maintaining lens homeostasis during aging. Although HSF4 expression is not limited to lens tissue, cataract patients with HSF4 mutations have no other symptoms, suggesting that HSF4 function is especially critical in lens, but possibly not in other tissues.

HSFS IN AGE-RELATED PATHOLOGIES

HSFs in Cancer

In 2007, two articles reported an impact of HSF1 on tumorigenesis [153, 154]. Dai and co-workers showed that mice lacking HSF1 have less tumors and increased survival when exposed to chemical skin carcinogenesis using a clinically relevant p53 mutation mouse model [154]. Similarly, Min and co-workers presented that in *p53*$^{-/-}$ mice, which normally develop lymphomas, the loss of HSF1 resulted in a marked reduction of lymphomas [153]. Subsequently, a great number of studies have shown the involvement of HSF1 in various forms of cancer [28, 155–160]. For example, transcriptional activation of HSF1 in breast cancer cells is associated with poor prognosis [160]. Malignant cells carry multiple genetic and epigenetic alterations and are exposed to more stresses than healthy cells. This has been postulated to promote HSF1 activity, which in turn elevates the levels of HSPs to maintain protein homeostasis in cancer [161]. However, it was found that HSF1 in cancer drives a transcriptional program different from that in stress responses, and thus directly promotes tumorigenesis [28]. In addition to HSPs, HSF1 regulates transcription of genes involved in cell cycle regulation, chromatin remodeling, translation, signaling pathways, and metabolism (Fig. **5E**). This cancer-specific transcriptional program has been found in breast and colon cancer patient samples as well as in cell lines derived from breast, colon, and lung cancers (Table **1)**. Importantly, cancer cells have been shown to depend on HSF1 activation in tumor stroma, to support their growth [162]. In cancer-associated fibroblasts representing the stroma, HSF1 drives a distinct transcriptional program, where it activates genes involved in angiogenesis, extracellular matrix (ECM) organization, adhesion, and migration [162]. It is, however, unknown by which mechanism cancer cells active HSF1. To date, it has been proposed that cancer-specific alterations in signaling pathways, such as epidermal growth factor receptor (EGFR)/HER2 [163], MAPK [164], and the insulin growth factor system [165], would result in HSF1 activation through a specific PTM signature [166], but compelling experimental evidence is still missing.

In addition to HSF1, HSF2 is also involved in tumorigenesis. Meta-analyses of human malignancies indicated that HSF2 expression is frequently decreased in a variety of cancer patient samples [167]. Moreover, it was shown that in prostate cancer cells grown in three-dimensional organotypic cell cultures, HSF2 levels decreased during prostate cancer organoid formation, acinar differentiation and invasion, and that silencing of HSF2 further stimulated these processes (Fig. **5E**) [167]. Transcriptome analyses revealed that HSF2 regulates expression of genes involved in GTPase activity, adhesion, and ECM organization, thereby controlling cell movement and invasiveness. These findings support the hypothesis that HSF2 acts as a tumor suppressor. Thus, both HSF1 and HSF2 play important but opposite roles in tumor progression and invasion, which suggests that these factors could serve as attractive pharmacological targets for development of novel cancer therapies.

HSFs in Neurodegenerative Disorders

Protein aggregation is considered to be a hallmark of neurodegeneration. Neurodegenerative diseases are pathologically associated with aggregates of specific proteins, such as α-synuclein in Parkinson's disease, huntingtin in Huntington's disease, TDP-43 and SOD1 in amyotrophic lateral sclerosis (ALS), and amyloid precursor protein (APP) in Alzheimer's disease (review in [168] and [169]). Since HSPs function as molecular chaperones, it has been proposed that they may inhibit formation of toxic protein aggregates. Indeed, certain HSPs are able to prevent the initiation of protein aggregation, facilitate the removal of aggregates and ameliorate their toxic effects in various models of neurodegenerative disorders [170–175]. Majority of HSPs are under the

transcriptional control of HSF1, and therefore, exogenous activation of HSF1 could serve as a promising strategy to combat neurodegeneration. To this end, overexpression or chemical activation of HSF1 has been shown to reduce protein aggregation and ameliorate cytotoxicity in multiple cellular and organismal models [176–181].

A recent study revealed that in neuronal cells exposed to aggregation-prone proteins, such as α-synuclein or a mutant form of huntingtin, HSF1 protein was degraded by the ubiquitin-proteasome system [182]. α-synuclein aggregation resulted in increased ubiquitination of HSF1, mediated by a specific ubiquitin E3 ligase, NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4), but the targets sites for this PTM remained unknown. Intriguingly, treatment of cells with resveratrol, an activator of deacetylase SIRT1, or by overexpressing SIRT1, in the presence of α-synuclein aggregates, prevented HSF1 degradation [182]. Since SIRT1 counteracts the stress-induced acetylation of HSF1 [72] and prevents NEDD4-mediated ubiquitination [182], it is tempting to speculate that there is a functional interplay between acetylation and ubiquitination of HSF1. Future studies should explore the significance of specific PTM signatures in the regulation of HSF1 functions under various physiological and pathological conditions.

HSF2 has also been indicated as an important protective factor in protein aggregation diseases. Loss of HSF2 shortens the lifespan of Huntington's disease mice due to the accelerated accumulation of misfolded huntingtin proteins [183]. In this study, HSF2 protected cells against protein aggregation through the expression of a specific small HSP, αB-crystallin. Interestingly, HSF2 bound to the αB-crystallin promoter only in the presence of HSF1. However, HSF1 alone was not capable of expressing αB-crystallin, which indicates a unique requirement of cooperativity between HSF1 and HSF2 [183]. It remains to be determined, whether these factors occupy the αB-crystallin promoter as heterotrimers. Taken together the currently available knowledge of HSFs in neurodegeneration, HSF1 and HSF2 could serve as viable targets for the development of drugs aimed at reducing pathogenic protein aggregates.

CONCLUDING REMARKS

Since the initial discovery of HSFs as specific transcriptional regulators of the heat shock response, it has become increasingly evident that their biological functions reach far beyond stress responses. The spectrum of HSF-mediated actions spans all the way from development and lifespan to metabolism and age-related pathologies. Despite the extensive efforts of numerous laboratories focusing on HSF biology, we still lack comprehensive understanding of the structure, regulation and physiological impact of distinct members of the HSF family. The most fundamental questions include how different types of cells and tissues sense stress, and by which molecular mechanisms HSFs are strictly regulated in a spatiotemporal manner in organisms. Given that the mammalian genome is composed of thousands of *loci* which contain HSE-like sequences, and yet HSFs bind to only a specific set of targets, it remains to be established how specific binding of HSFs to DNA is achieved in the context of complex chromatin architecture. Our knowledge of the functional impact of PTMs that HSFs are subjected to is still scarce. It is plausible that multiple PTMs of HSFs provide a sophisticated mechanism for fine-tuning the activity of HSFs across tissues and under various conditions. Thus, more emphasis should be laid on characterizing the specific PTM-signatures of HSFs during development, cancer and protein aggregation disorders.

The crosstalk between HSF family members was detected almost a decade ago and has subsequently been observed in numerous biological processes, such as stress responses, lens and brain development, gametogenesis, and cancer. Invertebrates as model organisms have been invaluable in discovering a plethora of HSF functions, but since they express only a single HSF, they are not suitable for investigating the interplay of multiple mammalian HSFs. Future studies are warrantied to unravel the complex interaction networks involving distinct members of the HSF family, especially during age-related pathologies such as cancer and neurodegenerative disorders.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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FIGURE LEGENDS

Figure 1. Versatile functions of Heat Shock Factors (HSFs). An overview of biological functions of the HSF family members. HSF1 (blue), HSF2 (yellow) and HSF4 (green) are important for normal development and age-related pathologies. HSF1 and HSF2, but not HSF4, have been shown to regulate gene expression in response to stress stimuli. Both HSF1 and HSF2 are required for proper oogenesis and spermatogenesis. However, HSF1 and HSF2 have opposite effects in cancer, where HSF1 promotes tumorigenesis and HSF2 inhibits the invasive and metastatic capacity of cancer cells. In mitotic cells exposed to stress, high levels of HSF2 prevent the HSF1-mediated Heat Shock Response leading to cell death. HSF1 and HSF2 have also been shown to be able to prevent formation of pathological protein aggregates. To date, only HSF1 has been reported as a downstream target of signaling pathways involved in RNA biogenesis and translation. Original references are indicated in the text.

Figure 2. HSFs share similar functional domains and undergo extensive post-translational modifications. A) Schematic illustration of human HSF1 (hHSF1), human HSF2 (hHSF2), and human HSF4 (hHSF4) with the known post-translationally modified amino acids indicated. **B)** Schematic illustration of fly HSF (fHSF) and yeast HSF (yHSF). fHSF is phosphorylated both in control and stress conditions, but individual sites have not been mapped [56]. Recently, 73 phosphorylation sites were reported throughout yHSF [54]. HR-A/B/C – leucin-zipper-like heptad-repeat domains. The last amino acid of each protein is indicated. Note that the figure is not drawn to scale.

Figure 3. Model of HSF trimer bound to DNA. Activated HSF forms a trimer through the oligomerization domain HR-A/B (in blue) and binds to DNA with its DNA-binding domain (DBD, in yellow). DBD recognizes the *cis*-acting elements, Heat Shock Elements, containing at least three inverted 5'-nGAAn-3' repeats. HSF trimer wraps around DNA, which positions DBD and HR-A/B on the opposite sites of DNA, stabilizes HSF1-DNA interaction and exposes surfaces of both DBD and HR-A/B for regulatory inputs. Model is based on the recently published structure of HSF2 DBD bound to DNA [17].

Figure 4. Model of stress-induced HSF1 activation-attenuation pathway. Under normal conditions, a majority of HSF1 is present in cells as inactive monomers interacting with HSPs. A small fraction of HSF1 is binding to the promotors of HSPs, where it interacts with replication protein A (RPA) and histone chaperone FACT. This interaction keeps the promotor regions of HSP genes open. HSP genes are preloaded with the paused RNA polymerase II (Pol II). Upon activation, HSF1 trimerizes, accumulates in the nucleus and binds to DNA. The DNA-bound HSF1 interacts with other transcriptional regulators, such as general transcription factors (GTFs), activating transcription factor 1 (ATF1), transcription activator BRG1, death associated protein-6 (Daxx), and Mediator complex, which subsequently leads to release of the paused Pol II to elongation. Active HSF1 is heavily phosphorylated, mainly within the regulatory domain, which fine-tunes the magnitude of the heat shock response. During the attenuation phase, HSF1 interacts with CBP/p300 in an ATF1-dependent manner. CBP/p300 acetylates the HSF1 DNA-binding domain, preventing its binding to DNA. Attenuation of HSF1 DNA-binding keeps Pol II in the paused state and halts transcription. Furthermore, HSF1 interacts with ubiquitin ligase FBXW7α, resulting in degradation of the nuclear HSF1 (dashed lines). Functional domains of HSF1 are color-coded as in Figure 1. Original references are indicated in the text.

Figure 5. Interplay of HSF1 and HSF2 in a variety of biological processes. A) In freely cycling cells exposed to stress, HSF1 and HSF2 form heterotrimers that bind to HSP genes, thereby inducing their expression. While HSF2 is not required for inducible expression of HSPs, it fine-tunes the magnitude of the transcriptional response. **B)** In mitotic cells exposed to stress, HSF2 occupies the HSP70 promotor, preventing the HSF1-mediated HSP70 expression, which leads to cell death. **C)** Both HSF1 and HSF2 are required for proper male germ cell development. In spermatogenesis, HSF1 and HSF2 heterotrimers regulate expression of a myriad of genes, mainly HSPs and multi-copy genes found in the X and Y chromosomes, such as Sly, Ssty2, and Slx. **D)** During corticogenesis, alcohol exposure causes the formation of DNA-binding competent HSF1 and HSF2 heterotrimers. These heterotrimers compete with HSF2 homotrimers in binding to the promoters of microtubule-associated proteins (MAPs) genes, thereby reducing their expression. Decreased levels of MAPs disturb radial neuronal positioning, which is the hallmark of the Fetal Alcohol Syndrome (FAS). **E)** In cancer cells, HSF1 and HSF2 have opposite functions. HSF1 promotes tumorigenesis and cancer progression, by directly driving the expression of genes involved in protein folding, cell cycle, and energy metabolism. In contrast, HSF2 inhibits invasiveness by controlling expression of genes involved in GTPase activity, adhesion, and extracellular matrix (ECM). However, it remains unclear whether HSF2 directly regulates the expression of these genes.