

Regulation of the members of the mammalian heat shock factor family

Johanna K. Björk^{1,2} and Lea Sistonen^{1,2}

¹ Department of Biosciences, Åbo Akademi University, Turku, Finland

² Turku Centre for Biotechnology, University of Turku, Finland

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Correspondence

L. Sistonen, Department of Biosciences,
Åbo Akademi University, BioCity,
Tykistökatu 6, 20520 Turku, Finland
Fax: +358 2 333 8000
Tel: +358 2 215 3311
E-mail: lea.sistonen@abo.fi

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Regulation of gene expression is fundamental in all living organisms and is facilitated by transcription factors, the single largest group of proteins in humans. For cell- and stimulus-specific gene regulation, strict control of the transcription factors themselves is crucial. Heat shock factors are a family of transcription factors best known as master regulators of induced gene expression during the heat shock response. This evolutionary conserved cellular stress response is characterized by massive production of heat shock proteins, which function as cytoprotective molecular chaperones against various proteotoxic stresses. In addition to promoting cell survival under stressful conditions, heat shock factors are involved in the regulation of life span and progression of cancer and they are also important for developmental processes such as gametogenesis, neurogenesis and maintenance of sensory organs. Here, we review the regulatory mechanisms steering the activities of the mammalian heat shock factors 1–4.

Introduction

Heat shock factors (HSFs) are master transcriptional regulators activated by various proteotoxic stress stimuli. This cellular stress response, which is called the heat shock response after the original discovery in *Drosophila* larvae exposed to elevated temperatures [1], is a well-conserved defence mechanism existing in all organisms from bacteria to mammals [2]. By inducing transcription of the genes encoding heat shock proteins (HSPs) that function as molecular chaperones, the HSFs protect the cell from the deleterious consequences of protein-damaging insults. In invertebrates, such as yeasts, nematodes and insects, a single HSF has been found, whereas mammals possess a whole HSF family consisting of four members: HSF1–4 [2–4].

Besides regulating a multitude of stress-responsive genes, the HSFs have been implicated in a variety of processes beyond the heat shock response, including murine gametogenesis in both genders, corticogenesis, maintenance of sensory organs and aging [5–14]. Similarly, the target genes of the HSFs under nonstress conditions represent a capricious group, ranging from cytokines and chemokines to fibroblast growth factors in the lens and sex-chromosomal multicopy genes in the testis [14,15]. Interestingly, the HSFs are able to act as both activators and repressors in a target gene-dependent manner [16–18]. Because HSFs control the transcription of genes that are involved in such a multitude of biological processes, understanding the regulatory mechanisms specific for distinct HSFs is of great importance.

Abbreviations

DBD, DNA-binding domain; FGF, fibroblast growth factor; HR, hydrophobic heptad repeat; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock protein; HSR1, heat shock RNA-1; miRNA, micro RNA; nSB, nuclear stress body; PDSM, phosphorylation-dependent sumoylation motif; SIRT1, sirtuin 1; SWI/SNF, switch/non-fermentable; SUMO, Small Ubiquitin-like Modifier protein.

Common features among the HSF family members

Similarly to most transcription factors, the members of the HSF family are modular proteins composed of functional domains (see figure 2 in [4]). The most conserved domain is the amino-terminal helix-turn-helix DNA-binding domain (DBD). Upon activation, the HSFs assemble as trimers, mediated by the oligomerization domain composed of hydrophobic heptad repeats (HR-A/B). Although unusual for helical coiled-coil structures, they form a triple-stranded configuration [19]. The trimerization process is repressed by another, more carboxy-terminal, heptad repeat (HR-C), the deletion of which renders HSF1 constitutively trimeric [19,20].

All HSFs bind DNA sequences that are called heat shock elements (HSEs) and are composed of an array of inverted repeats of the pentamer nGAAn. Each DBD recognizes one nGAAn, and thus an HSE typically contains three pentameric repeats [19,21]. However, many target promoters contain more than three repeats and it has been shown that HSF trimers bind to DNA in a cooperative manner, and that the number of trimers bound is reflected in the transactivation capacity [22–24]. Although all HSFs bind HSEs, HSF family members display certain binding-site preferences concerning the architecture of the HSEs [23,25]. The precise composition of an HSE can also determine the state of activation required of a specific HSF to induce transcription of its target genes [21,24]. This flexibility in HSE design and HSF binding provides great diversity in the control of target gene transcription. An additional regulatory level to control gene expression is potentially mediated by the distinct HSF isoforms, as alternative splicing appears to be another common feature among the family members [3,26].

Despite common structural features, especially in the DBD and HR-A/B domains, the HSFs have been considered functionally distinct: HSF1 and the recently discovered murine HSF3 are the main regulators of the heat shock response, whereas HSF2 and HSF4 are better known as developmental factors. Lately, however, interactions between HSF family members have been reported, and will be discussed here in detail.

Differentially regulated expression patterns and activities of HSF1, HSF2 and HSF4

As the functions of the HSF family members differ, so also do the molecular mechanisms by which they are regulated. Albeit they all recognize and bind HSEs, the

HSFs regulate different types of target genes that are involved in a broad range of cellular processes. Therefore, the expression and activity of HSFs need to be under strict regulatory control in their specific physiological contexts.

HSF1: regulation through intra- and intermolecular interactions and post-translational modifications

HSF1 is the prototype of all HSFs and the mammalian counterpart of the single HSF of yeasts, nematodes and fruit flies [3,27–29]. Deletion experiments of the *Drosophila Hsf* demonstrated that HSF1 is a developmental factor, and subsequent studies in mice showed that lack of HSF1 leads to increased prenatal lethality, growth retardation and female infertility [5,30]. In eukaryotes, HSF1 is expressed in most tissues and cell types, and no other HSF can replace its function in the heat shock response, as revealed by studies on HSF1-deficient mice [5,31]. Because of its constitutive expression, HSF1 is, under normal growth conditions, kept inactive through intra- and intermolecular interactions and various post-translational modifications [32,33]. In the inactive state, HSF1 prevails as a monomer, and it is thought that the C-terminal heptad repeat domain, HR-C, folds back to interact with the HR-A/B domain, thereby preventing oligomerization [19]. Indeed, yeast HSF and mammalian HSF4, both lacking the HR-C, exist as constitutively DNA-bound trimers [34,35].

Activation of HSF1 in response to diverse environmental and physiological stress stimuli is a multistep process, involving a monomer-to-trimer conversion, nuclear accumulation, increased phosphorylation, and acquisition of DNA-binding and transactivation capacity (Fig. 1). Although HSF1 can be activated by diverse stimuli, a common denominator might be misfolded or aggregated proteins disturbing the protein homeostasis. As a defence mechanism, HSFs induce the synthesis of HSPs that act as molecular chaperones through binding to the hydrophobic surfaces of unfolded proteins, thereby facilitating refolding of peptides and preventing protein aggregation [32]. The discovery of an interaction between HSF1 and HSPs, such as Hsp70/Hsp40 and HSP90, led to the hypothesis of a negative-feedback loop, where excess HSPs under nonstress conditions keep HSF1 inactive [32,36–39]. Upon exposure to stress, the HSPs are sequestered to denatured proteins and HSF1 is released from the chaperone complexes to induce transcription of the genes encoding additional HSPs. Once the pools of HSPs are saturated, they can again bind HSF1 and

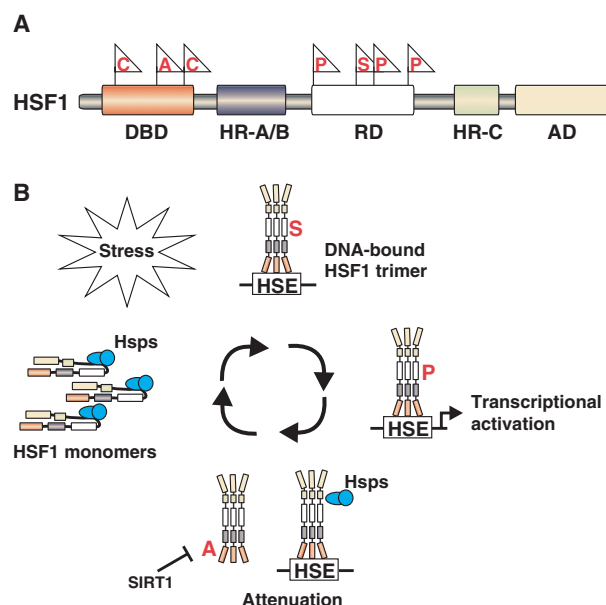


Fig. 1. (A) Schematic presentation of HSF1 with its functional domains. Some of the sites subjected to stress-induced post-translational modifications are marked with flags. (B) The activation cycle of HSF1. In its resting state, HSF1 exists in the nucleus or cytosol as an inert monomer that is negatively regulated by interactions with Hsps. Stress induces relocalization to the nucleus and conversion to a DNA-bound trimer. Stress also induces a dramatic increase in sumoylation, without affecting the DNA-binding capacity, but the sumoylation is diminished upon more severe stress, when profound and sustained. The stress-inducible hyperphosphorylation that follows correlates with target gene induction. During the attenuation phase the transactivation capacity of HSF1 is repressed through a negative-feedback loop via binding of HSPs. The DNA-binding activity of HSF1 is inhibited by acetylation of several lysines, including K80, within the DBD. The attenuation phase is regulated by the deacetylase SIRT1, which prevents HSF1 acetylation. A, acetylation; AD, transactivation domain; C, cysteine residues subjected to disulfide bond formation; DBD, DNA-binding domain; HR-A/B and HR-C, hydrophobic heptad repeats; P, phosphorylation; RD, regulatory domain; S, sumoylation.

inhibit its function [32,40]. In support of this hypothesis, denatured, but non-native proteins injected into *Xenopus* oocytes are capable of activating HSF1 [41].

Alternatively, kinetic studies on HSF activation upon exposure to stress favours a model where HSF can also be activated directly [42]. Both *Drosophila* HSF and mammalian HSF1 have been demonstrated to exhibit intrinsic stress-sensing capability as the recombinant proteins undergo a monomer-to-trimer conversion and bind DNA in response to different stress stimuli such as heat shock, H_2O_2 , low pH and increased calcium levels *in vitro* [43–47]. In accordance, mammalian HSF1 was shown to directly sense heat and oxidative stress *in vitro*, which was mediated

through two conserved cysteine residues, C35 and C105, located in the DBD (Fig. 1A). This redox-dependent activation requires the formation of disulfide bonds, leading to trimerization and subsequent target gene activation. Furthermore, mutation of the cysteine residues rendered HSF1 refractory to stress [48].

In response to stress, HSF1 undergoes post-translational modifications, such as a massive increase in phosphorylation. At least 12 serine residues have been identified to be phosphorylated upon heat stress, most of which reside in the regulatory domain located between the HR-A/B and HR-C domains [49] (Fig. 1A). Interestingly, this domain is, under normal conditions, required for repressing the transactivation domain that encompasses the last 150 carboxy-terminal residues of HSF1 [50,51]. Thus, stress-induced phosphorylation of the key serines within the regulatory domain could function as a trigger, relieving the inhibition of the transactivation domain to enable transcription of the target genes.

Despite numerous studies conducted in different laboratories, the impact of multisite phosphorylation on HSF1 functions has remained elusive. Nevertheless, in the light of current knowledge, most of the phosphorylation events seem to repress the transactivation capacity of HSF1 [52–57]. Another post-translational modification, suggested to affect HSF1 activity, is the stress-inducible covalent attachment of the Small Ubiquitin-like Modifier protein (SUMO) [58,59] (Fig. 1). Interestingly, SUMO conjugation to lysine 298 is directly linked to phosphorylation, because phosphorylation of serine 303 is a prerequisite for sumoylation, which inhibits the transactivation capacity of HSF1 [59–61]. The phosphorylation-dependent sumoylation of HSF1 provided the first example of an extended motif combining a SUMO consensus site to an adjacent proline-directed phosphorylation site, $\psi KxExxSP$ (where ψ is a hydrophobic amino acid, K is the lysine to which SUMO is attached and x is any amino acid). This motif is called a phosphorylation-dependent sumoylation motif (PDSM) and is frequently found in proteins associated with transcriptional regulation [60,61].

Although the mode of HSF1 activation follows the same principle upon various stresses, there are stimulus-specific differences, arguing against a single common signal pathway to activate HSF1. HSF1 itself could act as a hub for stress-induced gene activation, providing a relay point for downstream signalling of different stress stimuli. For example, yeast HSF is differently phosphorylated when exposed to either oxidative stress or heat stress [62]. Phosphorylation of HSF

probably also specifies the subset of target genes that are activated, because a mutation inhibiting oligomerization and hyperphosphorylation impairs the transcription of target genes whose promoters contain an HSE composed of three nGAAn units, but not those composed of four or more [24]. Other examples come from studies in mammalian cells, where transcriptional induction of the well-known HSF1 target gene *Hsp70.1* depends both on the chromatin remodelling activity of the SWI/SNF complex and the p38 mitogen-activated protein kinase pathway in response to arsenite, but not in response to heat shock [63,64].

An intriguing feature of HSF1 activation is that its threshold temperature is determined by the cell type or organism in which it is expressed: when human HSF1 was transfected into *Drosophila* cells, the threshold temperature of HSF1 activation was lowered to that normally occurring in *Drosophila* [65]. This finding points to additional regulatory mechanisms. One such mechanism involves an RNA molecule termed heat shock RNA-1 (HSR1), which could act as a thermosensor [66]. According to the proposed model, HSR1 undergoes a conformational change in response to heat shock, and together with the translation elongation factor eEF1A, it facilitates HSF1 trimerization and activation. The model is supported by *in vitro* experiments where physiological concentrations of purified HSR1 and eEF1A proteins were capable of activating HSF1 [66]. Another possible stress-sensory mechanism is provided by cellular membranes. Stress-induced perturbations, such as altered compositions of lipids and proteins, which affect the cell-membrane fluidity, are known to activate *Hsp* genes, although the precise signalling pathway originating from the membrane is unclear [67]. Furthermore, an impact of re-organization of membrane microdomains has been demonstrated both *in vitro* and *in vivo* using the membrane fluidizer benzyl alcohol, which changes the microdomain structure in a way similar to that induced by heat stress and induces HSF1 DNA-binding and transcriptional activity [68].

To induce transcription, direct interactions between HSF1 and components of the transcriptional machinery have been reported (Fig. 2). At the mammalian *Hsp70* promoter, Brahma-related gene 1 (BRG1), the ATPase subunit of the chromatin-remodelling complex SWI/SNF, interacts with the transactivation domain of HSF1, which stimulates RNA polymerase II release and elongation [69,70]. HSF1 also recruits the Mediator co-activator complex through interacting directly with the dTRAP80 subunit in fruit fly [71]. Human HSF1, in turn, has been shown to interact with the co-activator activating signal co-integrator 2 (ASC-2),

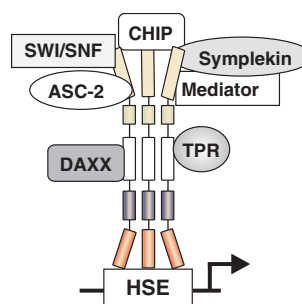


Fig. 2. Hypothetical model of proteins interacting with HSF1 at the onset of, or during, transcription. SWI/SNF, ASC-2, Symplekin and Mediator are thought to interact with the transactivation domain of HSF1, whereas the interaction site for TPR is still unknown. The interaction between HSF1 and CHIP probably occurs via Hsp70. DAXX interacts with trimeric HSF1 and mediates its activation. For details see the text. DAXX.

promoting HSF1-mediated transcription [72]. Interestingly, HSF1 may also be involved in co-transcriptional mRNA processing, as direct interaction with symplekin, a scaffold for polyadenylation factors, has been reported to mediate polyadenylation of *Hsp70i* transcripts [73]. Furthermore, through interacting with the nuclear pore-associating translocated promoter region (TPR) protein, HSF1 is suggested to participate in nuclear export of mRNAs transcribed from the *Hsp70i* promoter [74].

Although interactions with Hsps function in the negative-feedback loop, thereby inhibiting HSF1 transactivation competence, it seems possible that the regulatory functions are affected by the precise composition of the chaperone complexes. Thus, C-terminus of Hsp70-interacting protein (CHIP), a co-chaperone of Hsp70, has been shown to interact with HSF1 and to activate HSF1-mediated transcription [75]. Another mediator of HSF1 activation is the nuclear protein FAS death domain-associated protein (DAXX), which directly interacts with trimeric HSF1 and thereby opposes repression by the multichaperone complexes [76] (Fig. 2).

To complete the activation cycle of HSF1, both DNA-binding and transcriptional activities must be attenuated (Fig. 1B). The attenuation mechanism cannot be explained solely by the negative-feedback loop, because an increase in the concentration of Hsps does not result in the release of HSF1 from its target promoters [77,78]. Instead, it was recently reported that HSF1 undergoes stress-inducible acetylation, which negatively regulates its DNA-binding activity. Interestingly, deacetylation of HSF1 is mediated by the longevity factor sirtuin 1 (SIRT1), leading to prolonged binding of HSF1 to the *Hsp* promoters [79]. Previous studies have shown that HSF1 affects the life span of

Caenorhabditis elegans and that the heat shock response is impaired during aging [9,13,80]. Accordingly, recent cell-based aging experiments indicate that the age-related decline in HSF1 activity and the heat shock response are connected to progressive loss of SIRT1 expression and activity [79]. These results raise questions about the impact of SIRT1-mediated regulation of HSF1 activity on various age-dependent and protein folding-associated diseases, such as neurodegenerative and metabolic disorders.

HSF2: regulation through the expression level is critical for proper activity

Unlike HSF1, whose activity is induced by external stimuli and regulated through multiple post-translational modifications, the regulation of HSF2 is less well characterized. Nevertheless, both factors acquire DNA-binding competence only as trimers; HSF1 undergoes transition from a monomer to a trimer, whereas inactive HSF2 exists predominantly as a dimer [81]. This difference in the control state implies different regulatory mechanisms for HSF1 and HSF2.

HSF2 has first and foremost been associated with developmental and differentiation-related processes, and HSF2-deficient mice display neurological and reproductive abnormalities in both genders [15]. When compared with HSF1, which is evenly expressed in most tissues, HSF2 shows a highly specific expression pattern in different types of tissues and cells [82]. How this spatiotemporal expression pattern of HSF2 is achieved is largely enigmatic, although it is likely to result from multiple steps in the pathway from DNA to RNA to protein, such as control of transcription and mRNA stability, and the relative rate of protein synthesis and degradation. Moreover, the mechanisms by which HSF2 is activated and recruited to its target promoters are not well understood. Previously, it was suggested that HSF2 exists in an active DNA-binding form in the testis, where HSF2 shows the most abundant expression in comparison to other tissues [82,83]. Embryonic stem cells and embryonic carcinoma cells also contain constitutively active HSF2, as elucidated by electrophoretic mobility shift assays [84,85]. During embryogenesis, HSF2 exhibits a stage-specific expression pattern, and its DNA-binding activity coincides temporally with the increased expression level [86,87]. In line with earlier studies, it was recently demonstrated that the amount of HSF2 is directly linked to its activity; by merely increasing the expression of HSF2, it translocates to the nucleus and induces transcription of target genes, suggesting that HSF2 is activated by its elevated concentration [18].

The question of the molecular basis behind the dynamic expression pattern of HSF2, and thereby its activity, was addressed using mouse spermatogenesis as the model system [88]. In the seminiferous epithelial cycle, where the male germ cells mature from spermatogonia through spermatocytes, elongated and round spermatids to mature sperm, HSF2 displays a characteristic cell- and stage-specific expression in a wave-like manner [83,88]. The expression pattern of HSF2 correlates inversely with that of a specific micro RNA (miRNA), miR-18, which is a member of the Oncomir-1/miR-17~92 cluster [89]. Intriguingly, miR-18 was found to repress the expression of HSF2 by directly targeting its 3'-UTR [88]. For the entire spermatogenic process to succeed, correct cell type- and stage-specific gene expression is a prerequisite, and is therefore strictly controlled at multiple levels [90]. The significance of functional HSF2 in the testis is demonstrated by the phenotype of HSF2 null mice, exhibiting reduced sizes of testis and epididymis, altered morphology of the seminiferous tubules and lowered numbers of spermatids [7,10]. Mature sperm in *Hsf2*^{-/-} mice also display defective chromatin compaction, increased sperm head abnormalities and impaired quality [17]. Under normal conditions in the testis, HSF2 binds to a number of target genes and regulates the transcription of sex chromosomal multicopy genes, such as *Ssty* and *Slx* [17]. Considering the hypothesis that the activity of HSF2 is dependent on its amount, strict regulation becomes necessary for the correct expression of HSF2 target genes. Indeed, when miR18-mediated regulation of HSF2 was disrupted in male germ cells *in vivo*, expression of HSF2 target genes was altered [88]. These results shed light on the regulatory mechanisms steering the developmental expression pattern of HSF2, and they also provide the first example of involvement of miRNAs in the HSF biology.

HSF2 is a short-lived protein and ubiquitination-mediated degradation has been proposed to regulate its abundance [91–93]. Recently, Cullin3, a subunit of a Cullin-RING E3 ubiquitin ligase, was reported to interact with the enriched in proline, glutamate, serine and threonine (PEST) sequence of HSF2, which could direct it to the ubiquitin/proteasome-degradation pathway [94]. Another study showed that HSF2 interacts with Cdc20, Cdh1 and Cdc27, all co-activators or subunits of the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C). This interaction was enhanced during the acute phase of exposure to heat stress, coincident with degradation and clearance of HSF2 from the *Hsp70.1* gene promoter. As Cdc20 and the proteasome 20S core $\alpha 2$ subunit were also recruited to the *Hsp70.1* promoter in a stress-inducible manner,

the results imply that, in particular, the promoter-bound pool of HSF2 proteins is subjected to degradation (J.K. Ahlskog, J.K. Björk, A.N. Elsing, M. Kallio, P. Roos-Mattjus, L. Sistonen, unpublished work). Viewing the ubiquitination of HSF2 from another angle, it has long been known that when the ubiquitin/proteasome pathway is repressed using the proteasome inhibitors hemin, lactacystin or MG132, HSF2 is activated and the same set of Hsps are induced as during heat stress [91,92,95]. This finding was interpreted as a consequence of increased abundance of non-native proteins generating a stress signal. However, in light of the more recent data on concentration-dependent activation of HSF2 (discussed above), the enhanced activity of HSF2 could be caused by preventing its degradation. A model of the importance of HSF2 levels for its function and activity under various circumstances is presented in Fig. 3.

Besides ubiquitination, sumoylation is another post-translational modification that affects HSF2. The SUMO protein is covalently conjugated to lysine 82, which is located in a flexible loop within the DBD [96,97]. Sumoylation at this site has been suggested to influence bookmarking of the stress-inducible *Hsp70i* gene during mitosis and to enhance the DNA-binding capacity of HSF2 [96,98]. However, another report showed that the modification rather hinders the DNA-binding activity of HSF2, without interfering with its

trimerization [97]. A subsequent study further strengthened the molecular basis for sumoylation-dependent regulation by showing that SUMO conjugation negatively affects the HSF2–DNA interaction through a randomly distributed steric interference [99]. It remains to be established whether sumoylation and ubiquitination are involved in the regulation of HSF2 in developmental processes, perhaps in a similar way as in cell-based experimental settings or in synergy with the miRNA-mediated regulation that occurs during the maturation of male germ cells (Fig. 3).

HSF4: a constitutively trimeric complex displaying tissue-specific expression

The expression of HSF4, the third member of the mammalian HSF family to be identified, is restricted to only a few tissues [35,100]. It differs from the other mammalian HSFs in that it lacks the HR-C domain and hence is a constitutively DNA-bound trimer [100]. Similarly to both HSF1 and HSF2, HSF4 is expressed as two isoforms, HSF4a and HSF4b, as a result of alternative splicing, leaving HSF4b with an isoform-specific region composed of 30 amino acid residues. HSF4b displays transactivation capacity and can substitute for yeast HSF, whereas HSF4a is transcriptionally inactive and functions as a repressor [100]. HSF4 is a phosphoprotein under physiological growth conditions, although

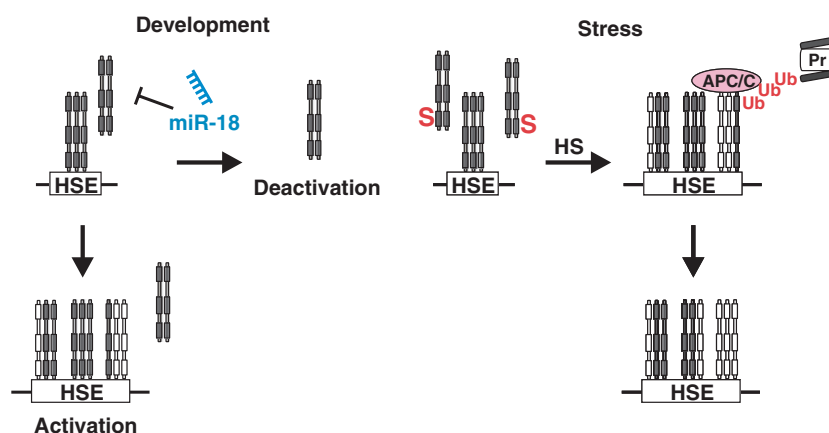


Fig. 3. Regulatory mechanisms affecting the expression and activity of HSF2 during development and in response to cellular stress. In certain developmental processes, a high level of expression of HSF2 correlates with active DNA-binding, indicating that the activity of HSF2 depends on its amount. In spermatogenesis, a decrease in HSF2 is mediated by miR-18 targeting the 3'-UTR of the HSF2 mRNA. Importantly, the down-regulation of HSF2 is needed for correct target gene expression during male germ-cell maturation. Further investigations are warranted to elucidate whether miR-18-mediated regulation of HSF2 also applies to other developmental processes. In control situations, HSF2 exists mostly in a dimeric form and sumoylation negatively affects its DNA-binding capacity. Upon stress, the DNA-binding activity is increased, but the amount of HSF2 protein simultaneously decreases, at least in part, because of enhanced ubiquitination by the E3 ligase APC/C followed by degradation by the proteasome. HSF2 is also regulated by interactions with HSF1; for example, the DNA-binding activity of HSF2 upon stress and hemin-induced differentiation of human K562 erythroleukemia cells is dependent on intact HSF1. HSF1 and HSF2 form heterotrimers when bound to DNA, as seen on the *clusterin* and *Hsp70.1* promoters and on *satellite III* repeats in NBs. In the figure, HSF2 is depicted in black and HSF1 is depicted in white. HS, heat shock; Pr, proteasome; S, sumoylation; Ub, ubiquitin.

the functional consequences of the modification are still not fully elucidated [60,101]. HSF4b also contains the extended consensus motif PDSM, and consequently, phosphorylation-dependent sumoylation represses its transactivation capacity. Yet, the conjugation of SUMO differs between HSF1 and HSF4b; HSF1 undergoes sumoylation in a stress-inducible manner, whereas HSF4b is constitutively sumoylated [60,101]. Depending on the target genes and cellular circumstances, HSF4b acts either as a transcriptional activator or as a repressor [11]. It has therefore been proposed that sumoylation could mediate the transition of HSF4b from an activating form to a repressing form [60].

The constitutively trimeric state of HSF4 suggests that it may have physiological roles during development. Indeed, HSF4 is crucial for development of the lens and maintenance of the olfactory epithelium [102]. The first evidence for a developmental function of HSF4 was provided by population genetic studies where mutations of the *Hsf4* gene were found to be associated with autosomal-dominant lamellar and Marner cataract occurring in certain Chinese and Danish families [103]. Subsequently, three research groups demonstrated that HSF4-deficient mice develop cataracts early during postnatal life [11,12,104]. In the lens, the level of HSF4 protein is particularly high compared with other tissues and, interestingly, the level of expression changes during development. HSF4 can be detected in the fetal lens but its expression peaks during the postnatal period and then declines. This maximal postnatal expression pattern also corresponds to the appearance of an HSE/HSF4 trimeric complex on several promoters, such as rat α B-crystallin, rat *Hsp70* and *Drosophila melanogaster* *Hsp82* [11,105,106]. The question of the regulatory mechanisms underlying the spatiotemporal expression of HSF4 in development remains to be solved. Considering that HSF4 exists as a constitutively DNA-bound trimer that possesses major HSE-binding activity in the lens and induces demethylation of histone H3K9 within its binding regions [11,105,106], strict regulation can be assumed as prerequisite for proper expression of its target genes.

Interactions between distinct HSFs as a regulatory mechanism for functional diversity

HSF1–HSF2: interplay during the heat shock response and in development

HSFs have long been considered as individual factors functioning in normal physiology, development and cellular stress responses. However, a number of recent

studies have revealed that distinct HSFs co-exist in many cells and under different circumstances and that they are capable of interacting with each other. The physical and functional interactions may therefore provide another layer of control for HSF-mediated transcription (Fig. 3).

In a chromatin immunoprecipitation-based study on heat shock gene promoter occupancy, both HSF1 and HSF2 were found to bind numerous promoters upon heat shock or hemin-induced differentiation of K562 erythroleukemia cells [107]. Many known target gene promoters contain several HSEs, enabling the simultaneous binding of different HSF homotrimers to the same promoter. However, experimental evidence has accumulated and other possibilities have been raised. One of the first indications for a physical interaction between HSF1 and HSF2 was the finding that HSF1 and HSF2 directly bind each other, and that this interaction is mediated through their HR-A/B oligomerization domains [108,109]. The factors also co-localize in the nuclear stress bodies (nSBs) that are formed on specific chromosomal loci upon stress, where they bind *satellite III* repeats [108,110,111]. Another study focused on the *Hsp70.1* promoter and found that both HSF1 and HSF2 were present on the promoter upon heat stress and hemin-induced differentiation [16]. The *Hsp70.1* promoter contains two HSEs – a proximal HSE and a distal HSE separated by 100 nucleotides – which would allow binding of at least two homotrimers composed of either HSF1 or HSF2. However, maximal binding of HSF2 required the presence of HSF1 with an intact DBD, arguing for a closer interaction between the factors. Furthermore, the target genes, such as several major *Hsps*, were differently expressed in the presence or absence of HSF2 [16]. Binding of both HSF1 and HSF2 was also detected on the *clusterin* promoter after proteotoxic stress. Interestingly, this promoter contains only one minimal HSE corresponding to the binding site for one HSF trimer, which suggests that the site is bound by a heterocomplex of HSF1 and HSF2. This assumption was supported by co-immunoprecipitation, supershift and gel-filtration experiments, indicating an interaction between HSF1 and HSF2 and the presence of both factors in the same HSF–HSE complex, equivalent in size to an HSF trimer [112]. The formation of HSF1–HSF2 heterotrimers was confirmed in a subsequent study using structural modelling, as well as fluorescence resonance energy transfer (FRET) microscopy and fluorescence-activated cell sorter–FRET, to demonstrate that HSF1 and HSF2 bind as a complex to *satellite III* DNA in nSBs [18]. To establish the functional relevance of heterotrimerization, depletion of

HSF1 prevented localization of HSF2 to nSBs and abrogated stress-induced synthesis of the noncoding satellite III transcripts. Conversely, elevated expression of HSF2 led to its activation and to the subsequent localization of both HSF1 and HSF2 to nSBs, where transcription was induced spontaneously in the absence of stress stimuli, indicating that HSF2 can incorporate HSF1 into a transcriptionally competent heterotrimer [18]. Taken together, these studies have revealed how HSF1 and HSF2 influence each other and how heterotrimerization relays the inputs originating from activation of either HSF1 or HSF2 to transcriptional regulation of target genes.

Interaction between HSF1 and HSF2 is not restricted to the heat shock response. For example, both factors are involved in male and female gametogenesis of mice [6,7,10,15,113–115]. In spermatogenesis, disruption of both *Hsf1* and *Hsf2* leads to a more pronounced phenotype (i.e. male sterility) than disruption of either factor alone. The phenotype of the double knockout suggests that compensatory functions exist between the factors, or, alternatively, that additive or synergistic transcriptional activity of HSF1 and HSF2 is needed for normal spermatogenesis and male fertility [115]. The finding that HSF1 and HSF2 physically interact in lysates of whole testis provides further evidence for their cooperation [18].

Hsf2 gene-inactivation studies from two laboratories revealed brain defects in both embryonic and adult mice deficient in HSF2 [7,10], whereas a third laboratory did not report any brain defects in their mouse model [8]. Based on the phenotypic analyses of the developing brain where disruption of *Hsf2* was shown to have an effect, HSF2 was concluded to regulate the proper migration of neurons in the cerebral cortex. Interestingly, the HSF2-deficient phenotype resembles that of mice lacking cyclin-dependent kinase 5, or its activator, p35, and it was found that HSF2 indeed controls neuronal migration in the cerebral cortex through the direct regulation of p35 expression [116]. The function of HSF1 in brain development is less well elucidated, although a role in maintenance of the postnatal brain under nonstress conditions has been suggested [117]. *Hsf1* disruption also results in a phenotype exhibiting enlarged ventricles, astrogliosis, neurodegeneration and accumulation of ubiquitinated proteins in specific areas [117,118]. Similarly to the testis, the double knockout of both *Hsf1* and *Hsf2* causes a more severe phenotype than observed in mice deficient in HSF1 alone [118]. It is thus possible that HSF1 and HSF2 together influence certain aspects of the neural development although, to date, no lucid co-localization has been reported.

HSF1–HSF4: competitors and collaborators

The first example of interplay between two members of the HSF family stems from studies on mouse lens epithelial cells, where HSF4 regulates proliferation and differentiation by suppressing the expression of fibroblast growth factor 7 (FGF-7) [11]. Both HSF4 and HSF1 directly bind the *Fgf-7* promoter, but this results in different effects: the expression of FGF-7 is increased in *Hsf4*^{−/−} mice but reduced in *Hsf1*^{−/−} mice. In a double knockout of *Hsf1* and *Hsf4*, the abnormal levels of FGF-7 returned to normal, and proliferation and differentiation of the epithelial cells were stabilized. These findings indicate that HSF1 and HSF4 compete for common targets that regulate the expression of growth factor genes [11]. HSF1 and HSF4 seem to have opposing effects also in olfactory neurogenesis. In *Hsf1*^{−/−} mice, the olfactory epithelium is atrophied, resulting in increased cell death of olfactory sensory neurons, which is accompanied by an increase in the expression of leukemia inhibitory factor. Interestingly, HSF4 shows the opposite effects on olfactory neurogenesis and leukemia inhibitory factor expression [119].

An important question is how the activities of HSF1 and HSF4 are coordinated in different developmental processes. For instance, during lens development, the trimeric form of HSF4 increases, while the levels of HSF1 and HSF2 are reduced [102]. In the olfactory epithelium of 3–6-week-old mice, the expression profile of HSF1 remains constant. However, a significant increase in the DNA-binding activity of HSF1 can be detected during the same time period [119]. Although it is well documented that HSF4 and HSF2 are regulated during development [7,11,86,88,120], little is known on how the developmental activity of HSF1 is regulated. The identity of the developmental signal that promotes a monomer-to-trimer transition of HSF1 in the olfactory epithelium warrants further investigations. In accordance with the fundamental role of HSF1 in the heat shock response, the requirement of HSF1 and HSF4 in development of the lens and olfactory epithelium is limited to the postnatal period, coinciding with exposure to environmental stimuli of the sensory organs [119,121,122]. Thus, it remains to be shown whether the common denominator could be stress stimuli, or whether the activation is genetically programmed.

A recent study on the genome-wide DNA binding of mammalian HSFs in the lens revealed that HSF4 occupied various regions, including introns and distal parts of genes [106]. Interestingly, a substantial number of the genes (70%) were co-occupied by HSF1 and/or

HSF2. Heat stress surprisingly induced a large set of HSF4 targets, although the constitutive expression of most genes was not affected by HSF4 binding. Instead, HSF4 occupancy induced demethylation of histone H3K9 within the binding regions. Lack of HSF4 led to increased H3K9 methylation, which is associated with the generation of heterochromatin, and reduced the binding of HSF1. These results show that HSF4 promotes the DNA-binding activity of another member of the HSF family, through modulating the chromatin status [106].

Conclusions and perspectives

Nearly 10% of the genes in the human genome encode transcription factors, and a defining characteristic for this group of proteins is the DBD, providing specificity in target-gene recognition [123,124]. In the HSF family, the most prominent common feature is the DBD, which is composed of a looped helix-turn-helix and is highly conserved between the different members of the family [19]. Although distinct HSFs share similar DBDs and other structural features, their biological roles are highly diverse and are implemented in a broad range of biological processes. Here, we have focused on describing the regulatory mechanisms steering the different members of the mammalian HSF family. The family provides an excellent example of how proteins that share common functional domains and bind similar DNA sequences (HSEs) can be under different regulatory control, as the current knowledge points towards HSF-specific regulatory mechanisms. The results currently available are, however, not yet conclusive and should be interpreted with caution, because the regulatory differences found for the individual factors might just be variations on the same theme. Further investigations, using more sophisticated methods that are particularly suitable for *in vivo* studies, are warranted. For example, although HSFs are known to undergo various post-translational modifications that influence their subcellular localization and transactivating capacity, little is known about the specific modifications of HSFs in different tissues and organs during development of an organism or differentiation of certain cell types. This lack of knowledge severely hampers the understanding of the physiological consequences of various post-translational modifications. One of the most challenging objects for future studies is to develop tools and techniques to be able to follow individual molecules as they become modified in biologically relevant experimental settings.

Apart from the regulatory mechanisms acting directly on individual HSFs, as discussed above, a fascinating

topic is raised by the recent findings that different members of the HSF family are able to interact, both structurally and functionally, thereby impacting the actions of their partners. The interplay among the distinct HSFs obviously expands their functional diversity. One factor can be steered by a specific set of regulatory events, but in cooperation with another factor, also subjected to a specific regulation, the combinatorial regulation generates a plethora of control modalities. Interaction with different partners further broadens the cell- and stimulus-specific regulation, in particular because both synergistic and antagonistic effects have been observed on the expression of target genes, which are also being discovered with increasing pace.

Because of the roles of HSFs in protein-misfolding disorders such as neurodegenerative diseases, but also in aging and cancer progression, much effort has been focused on finding molecules that affect the activity of HSFs. These studies have mostly concentrated on HSF1, and several molecules acting either as activators or inhibitors have already been found, although none is yet in clinical use [125–127]. It is, however, important to take into consideration the existence of multiple HSFs and the interactions between them, such as the formation of heterocomplexes, when searching for potential drugs. Preferably, molecules that target a specific regulatory step, instead of simply activating or inhibiting the HSFs, would allow more sophisticated manipulation of only a certain pathway or desirable process. Therefore, despite all the recent progress in this active research field, further efforts are required to explore the regulatory mechanisms of HSFs and to develop therapeutic HSF-targeted interventions.

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