Akira Nakai *Editor*

Heat Shock Factor

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Preface

All organisms sense varying conditions in the environment that they live in, and respond and ultimately adapt to them through sophisticated mechanisms. These adaptive mechanisms play pivotal roles for homeostasis and biological defense; therefore, the elucidation of the adaptive mechanisms has grown to be major research field in molecular biology. A prominent system among them is a primitive and evolutionally conserved adaptive mechanism called heat shock response, which induces the heat shock proteins against proteotoxic stress including high temperature.

It has been 53 years since the discovery of the heat shock response (in 1962), and 27 years after the molecular cloning of the key regulator, heat shock factor (HSF) (isolated in 1988). This adaptive response to high temperature or protein misfolding is a fundamental mechanism to maintain the capacity of protein homeostasis, or proteostasis, and is evolutionally conserved among all living organisms, including bacteria and humans, on the earth. Furthermore, physiological and pathological roles of HSF have been extensively studied in fruit fly, worm, and mouse models for the last 18 years (starting in 1997). It has been revealed that HSF plays roles in development of the brain, reproductive and sensory organs, and in ageing, inflammation, and circadian rhythm. Analysis of the mechanisms have uncovered that HSF exerts a wide range of effects on gene expression and epigenetic status on the whole genome. Moreover, loss or gain of HSF function is also closely related to protein-misfolding diseases including neurodegenerative diseases, psychiatric diseases, heart diseases, and cancers. Therefore, HSF is now thought to be a promising therapeutic target for treatment of these refractory diseases.

At this point (2015), we should bring a large amount of HSF-related information, describe core observations about molecular mechanisms and pathophysiological roles, and provide fundamental concepts on the basis of information from diverse aspects in one book. The aim of this publication is to provide a resource on the heat shock response and HSF for undergraduate students. This book will not only be a guide of the heat shock response and HSF to be understandable to students and

young researchers in other fields, but will also be a cornerstone for future works in the field related to the heat shock response and HSF. I would like to take this opportunity to thank all the authors for their contributions and the staff members at Springer Japan for their enthusiasm and effort.

Yamaguchi, Japan Akira Nakai

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Part I
The Basis of HSF Biology

Chapter 1
Proteostasis and Adaptation to High Temperature Stress

Akira Nakai

Abstract All living organisms respond to elevated temperatures by rapidly producing a set of highly conserved proteins, known as heat shock proteins (HSPs) or chaperones, which facilitate proper protein folding. Simultaneously, they produce non-HSP proteins with diverse functions including protein degradation. This adaptive response to elevated temperature or proteotoxic stress is called as the heat shock response and is mainly regulated at the transcription level by heat shock factor (HSF). Thus, HSF regulates the capacity of protein homeostasis (proteostasis) or buffering capacity against protein misfolding. This review describes history of the discovery of the heat shock response, chaperones, and HSF and explains mechanisms by which proteostasis capacity is regulated in cells. Furthermore, it discusses the fundamental function of the heat shock response, which is required for adaptation in all organisms, including those living in extreme high-temperature environments.

Keywords Chaperone • Energy landscape • Hyperthermia • Protein folding • Thermotolerance • Transcription

1.1 Introduction

It is assumed that all living organisms on the earth come from a common ancestor. This is because organisms possess common properties, e.g., they transcribe RNA from DNA and synthesize proteins based on the code written in the RNA (Barton et al. 2007). In particular, the nucleotide sequences of the genes encoding ribosomal proteins and RNAs have been highly conserved during evolution, and those of genes encoding a group of heat shock proteins (HSPs), which facilitate protein folding, are equally well conserved (Craig 1985). Organisms each live at an optimal temperature for them and dramatically induce HSPs when they are exposed to elevated temperatures (Lindquist 1986). This adaptive response to heat stress or

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proteotoxic stress is called the heat shock response and is present in all organisms including those living in extreme environments from cold areas in Antarctica to hot hydrothermal vents at the bottom of the deep sea (Conway de Macario and Macario 1994). Thus, the heat shock response is a primitive adaptive mechanism to respond to proteotoxic stresses including heat shock, which had been acquired by a common ancient ancestor of all organisms that exist today.

The focus of this review is the fundamental function of the heat shock response and transcription factors that regulate this adaptive response. After reprising the history of the discovery of the heat shock response and functions of HSPs, I describe the processes of protein folding and mechanisms that maintain protein homeostasis. Furthermore, I review heat shock transcription factors and their requirement for cell growth and survival of various organisms in normal and proteotoxic stress conditions.

1.2 Discovery of the Heat Shock Response

In the early 1960s, gene expression in the polytene chromosomes of *Drosophila* was monitored as chromosome puffing under the light microscope. Different puffing patterns were observed in various tissues and at particular developmental stages. Ferruccio Ritossa was studying the nucleic acid produced in puffs of the salivary gland of *Drosophila busckii*, as these were thought to represent gene activation. One day, he noticed a different puffing pattern in larvae that were accidentally incubated at a high temperature (Ritossa 1996). He found that a unique puffing pattern was observed when larvae grown at 25 °C were subjected to a temperature shock at 30 °C for 30 min and the induced puffs receded during recovery at 25 °C (Ritossa 1962). It is worth noticing that the regression of some normal puffs was also observed during the temperature shock. Thereafter, several laboratories showed that a similar pattern of puffing appeared in different ages and strains of *Drosophila* during temperature shock. In *D. melanogaster*, there were nine heat-inducible puffs (33B, 63C, 64F, 67B, 70A, 87A, 87C, 93D, and 95D) (Ashburner 1970).

Meanwhile, it was established that the puffs were the sites of RNA synthesis, and the RNA synthesized in a particular puff was transported to the cytoplasm and translated into proteins (Daneholt and Hosick 1973). In 1974, Tissières and Mitchell examined proteins synthesized after heat shock (Tissières et al. 1974). Larvae of *D. melanogaster* were exposed to 37.5 °C for 20 min, and the isolated salivary glands were cultured for 20 min in medium containing [^{35}S]methionine. The labeled proteins were separated on a polyacrylamide gel and detected as bands on the autoradiograph. They found the rapid appearance of six strong bands after heat shock and suggested that the heat-induced puffs were associated with the synthesis of these proteins. Parallel changes in puffing activity and the induced synthesis of new proteins after heat shock were further confirmed by using an inhibitor of puffing (Lewis et al. 1975). The synthesis of the small set of proteins

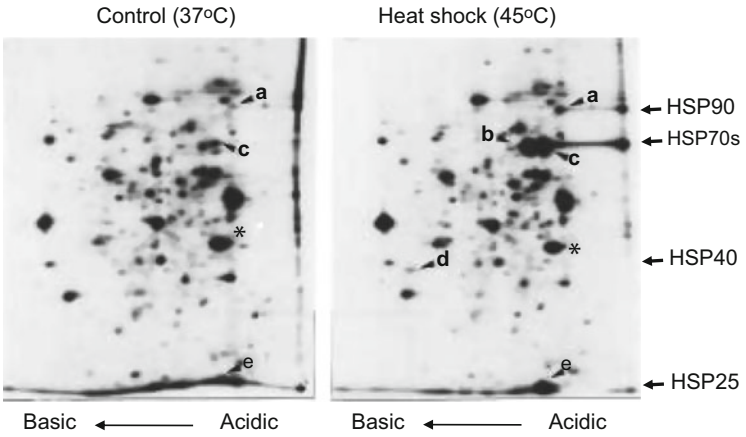

Fig. 1.1 Synthesis of major heat shock proteins (*HSPs*) was induced in avian cells upon upshift of growth temperature. Chicken B lymphocyte DT40 cells were maintained at 37 °C. Cells were metabolically labeled with [^{35}S]methionine for 60 min at 37 °C (control), or heat shocked at 45 °C for 30 min, allowed to recover at 37 °C for 3 h, and metabolically labeled with [^{35}S]methionine during the last 60 min of the recovery period (heat shock). Cell extracts were subjected to two-dimensional gel electrophoresis consisting of 10 % SDS-PAGE and nonequilibrium pH gradient gel electrophoresis (NEPHGE). *Arrowheads a* to *e* indicate the bands for HSP90, HSP70, HSC70, HSP40, and HSP25, respectively, which are also marked on the *right*. *Asterisks* indicate β-actin bands. Acidic and basic sides are indicated at the *bottom* (Tanabe et al. 1998)

was also induced in different tissues and various *Drosophila* species (Daneholt and Hosick 1973; Lewis et al. 1975). The apparent molecular weights of these proteins in *D. melanogaster* were 82,000; 70,000; 68,000; 36,000; 27,000; 26,000; 23,000; and 22,000 daltons. These heat shock polypeptides or proteins (HSPs) were called as HSP82, HSP70, and so on (Ashburner and Bonner 1979).

The selective induction of HSPs by heat shock was thought to be unique to flies for about 15 years after the discovery of induced puffing after heat shock. In 1978, Kelly and Schlesinger found that heat shock induced the synthesis of similar proteins in cultured avian and mammalian cells, suggesting that this response was a conserved system of gene regulation between species (Kelly and Schlesinger 1978) (Fig. 1.1). It was also reported at almost the same time that the synthesis rate of four to five proteins was induced in bacteria and yeast upon upshift of the growth temperature (Lemaux et al. 1978; Yamamori et al. 1978; Miller et al. 1979). Therefore, it was assumed that this response, termed the heat shock response, was a general phenomenon of cellular adaptation to high temperature.

The potential benefit of hyperthermia in the treatment of human cancers had been recognized (Bronk 1976). The exposure of mammalian cells maintained at 37 °C to temperatures above 40 °C led to reproductive death, which was progressive with increasing time at the elevated temperature (Hahn 1974). The plating

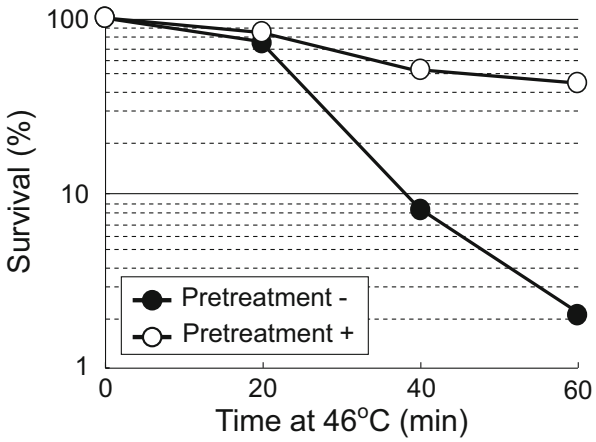

Fig. 1.2 Avian cells pretreated with sublethal heat shock acquire thermotolerance. Chicken B lymphocyte DT40 cells, maintained at 37 °C, were then incubated at 46 °C for the indicated periods (*closed circles*; pretreatment −). Some cells were pretreated with sublethal heat shock at 45 °C for 20 min and allowed to recover at 37 °C for 2 h (open circles; pretreatment +). The numbers of surviving cells were counted using a colony formation assay. Survival percentages are shown (Tanabe et al. 1998)

efficiency or proliferative capacity, which was monitored as colony-forming ability in vitro, was used as a measure of cell survival. It was revealed that a single hyperthermic pretreatment to human carcinoma HeLa cells induced a transient resistance to heat, and this induced heat resistance required the return of the culture temperature at 37 °C for 2–3 h (Gerner and Schneider 1975; Gerner et al. 1976). This phenomenon is universal and has been termed thermotolerance (Henle and Dethlefsen 1978) (Fig. 1.2). As was expected, the acquisition of a transient thermotolerance was tightly correlated with the induced synthesis of a set of HSPs (Li and Werb 1982). Agents that elevated the synthesis of HSPs also induced thermotolerance.

1.3 Heat Shock Proteins as Molecular Chaperones

The heat shock response was thus characterized originally by the induction of the synthesis of a set of HSPs upon stress conditions. This response was induced by many kinds of treatments, which had the common property of causing the accumulation of denatured or damaged proteins within the cells. First, the synthesis of abnormal proteins in avian and mammalian cells treated with amino acid analogs led to this response (Kelly and Schlesinger 1978; Hightower 1980). Second, HSPs

were strongly induced in mouse ts85 cells, which exhibited temperature sensitivity in ubiquitin-protein conjugation, at the nonpermissive temperature (Finley et al. 1984). In these cells, abnormal proteins would be accumulated. Third, the heat shock response was induced by the production of abnormal proteins in *Escherichia coli* (Goff and Goldberg 1985) or the microinjection of a denatured protein into *Xenopus* oocytes (Ananthan et al. 1986). These observations suggested that HSPs might prevent denaturation of cellular proteins in stressed conditions.

Numerous studies examining the function of HSPs have focused on the major heat shock protein, HSP70. DnaK, an *E. coli* homologue of mammalian HSP70, had ATPase activity (Zylicz et al. 1983) and mammalian HSP70 bound to ATP (Welch and Feramisco 1985). Upon heat shock, HSP70 was immobilized in the nuclei and nucleoli (Lewis and Pelham 1985), probably owing to its binding to hydrophobic protein precipitates (Evan and Hancock 1985). HSP70 was released from heat-shocked nuclei and nucleoli by treatment with ATP, but not with non-hydrolysable ATP analogues (Lewis and Pelham 1985). Pelham speculated that HSP70 had a general affinity for denatured or abnormal proteins and proposed in 1986 that HSP70 directly prevented aggregate formation of denatured proteins which expose hydrophobic regions during heat shock and promoted disaggregation by using energy from ATP hydrolysis (Pelham 1986). The latter idea was supported by the function of another isoform of HSP70, which was initially known as uncoating ATPase. Rothman and colleagues purified uncoating ATPase, which bound to and released clathrin trimers from clathrin cages in coated vesicles (Schlossman et al. 1984). This protein hydrolyzed ATP in this uncoating process. Uncoating ATPase was subsequently renamed HSC70 (Ungewickell 1985; Chappell et al. 1986). Therefore, it was speculated that HSP70 family proteins catalyzed the assembly and disassembly of a variety of proteins by coupling to the energy of ATP hydrolysis (Rothman and Schmid 1986). The mechanism of substrate binding and the release cycle of HSP70 have more recently been elucidated in much greater detail (Mayer and Bukau 2005) (Fig. 1.3). Because HSC70 was abundantly expressed in unstressed cells, Pelham and colleagues further proposed that it recognized newly synthesized proteins, which by definition were denatured, and was involved in protein folding (Pelham 1986).

In 1987, Ellis coined the term “molecular chaperone” to describe the new class of proteins whose function was to ensure that the folding of other proteins and their assembly into oligomeric structures occurred correctly (Ellis 1987). The term molecular chaperone was used first by Lasky et al. to describe nucleoplasmin, an acidic nuclear protein required for the assembly of nucleosomes from DNA and histones (Laskey et al. 1978). It was later extended by Ellis to include an abundant chloroplast protein (now known as chloroplast chaperonin) that functions to keen nascent Rubisco large subunits from forming insoluble aggregates and was further refined after the discoveries of the roles of HSPs in protein folding and assembly as described above (Ellis 1987). Molecular chaperones were originally defined as proteins whose role was to mediate the folding of certain polypeptides and, in

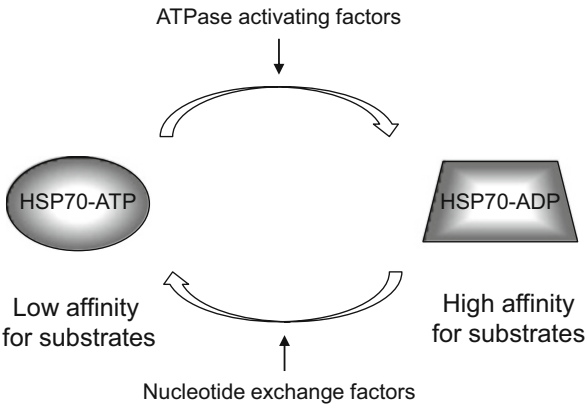

Fig. 1.3 The substrate binding and release cycle of HSP70. This cycle consists of the HSP70-ATP state with low affinity and fast exchange rates for substrates and the HSP70-ADP state with high affinity and low exchange rates for substrates. The rate of ATP hydrolysis of HSP70 is very low by itself. ATP hydrolysis is stimulated after HSP70-ATP binds to substrate and ATPase-activating factors such as HSP40. The stable complex of HSP70-ADP and substrate is then released by the exchange of ADP with ATP, which is mediated by nucleotide exchange factors such as BAG1 (Mayer and Bukau 2005)

some instances, their assembly into oligomeric structures, but which were not components of these final structures (Ellis and Hemmingsen 1989).

1.4 Protein Folding

Proteins are constructed by the polymerization of 20 different types of amino acids (Lodish et al. 2008; Alberts et al. 2008). Amino acids are composed of amino and carboxyl groups and various side-chain groups extending from the α-carbon atom (Fig. 1.4a). Individual amino acids are linked together in linear, unbranched chains by covalent amide bonds, called peptide bonds. The repeated amide N, α-carbon, and carboxyl C atoms of each amino acid residue form the backbone of protein, from which the various side-chain groups project (Fig. 1.4b). The primary structure of a protein is the linear arrangement of amino acids (Fig. 1.5a). The secondary structures form as stable spatial arrangements of segments of a polypeptide chain held together by hydrogen bonds between the backbone amide and carboxyl groups. The principal secondary structures are the α helix and β sheet (Fig. 1.5b). Within an α helix, all the backbone amino and carboxyl groups are hydrogen-bonded to one another, and there is a complete turn of the spiral every 3.6 residues. The β sheet consists of laterally packed β strands, which are short, fully extended polypeptide segments. Hydrogen bonding in the β sheet occurs between backbone atoms in separate, but adjacent β strands. The tertiary structure refers to the overall conformation of a polypeptide chain or the three-dimensional arrangement of all amino

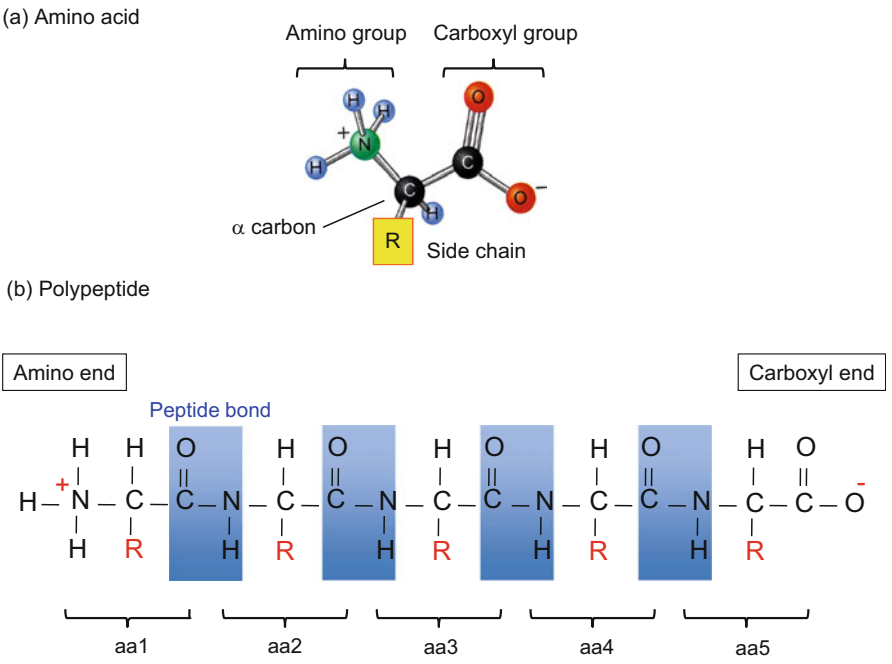

Fig. 1.4 Structure of a polypeptide. (**a**) A ball-and-stick model of an amino acid composed of amino ($-NH_2$) and carboxyl ($-COOH$) groups, along with a side chain (R) extending from the α carbon atom. (**b**) Individual amino acids are linked together by peptide bonds (*blue*). The polypeptide, which is a linear polymer of peptide bound-linked amino acids, has free amino nitrogen and carboxyl carbon atoms

acid residues (Fig. 1.5c). This structure is stabilized by hydrophobic interactions between nonpolar side chains, together with hydrogen bonds between polar side chains and peptide bonds. These stabilizing forces compactly hold together elements of secondary structures such as the α helix and β sheet. Chemical properties of the side chains help define the tertiary structure. Amino acids with charged hydrophilic polar side chains tend to be on the outer surface of proteins in aqueous solutions, whereas amino acids with hydrophobic nonpolar side chains usually form a water-insoluble central core. The quaternary structure, which is a fourth level of structural organization, describes the number and relative positions of subunits in multimeric proteins (Fig. 1.5d).

Proteins function properly only when they fold into the proper three-dimensional conformation with the appropriate secondary, tertiary, and sometimes quaternary structures (Dill and MacCallum 2012). In general, any protein adopts only one or just a few closely related characteristic functional conformations called native state, which is the most stable form of the molecule. In thermodynamic terms, the native

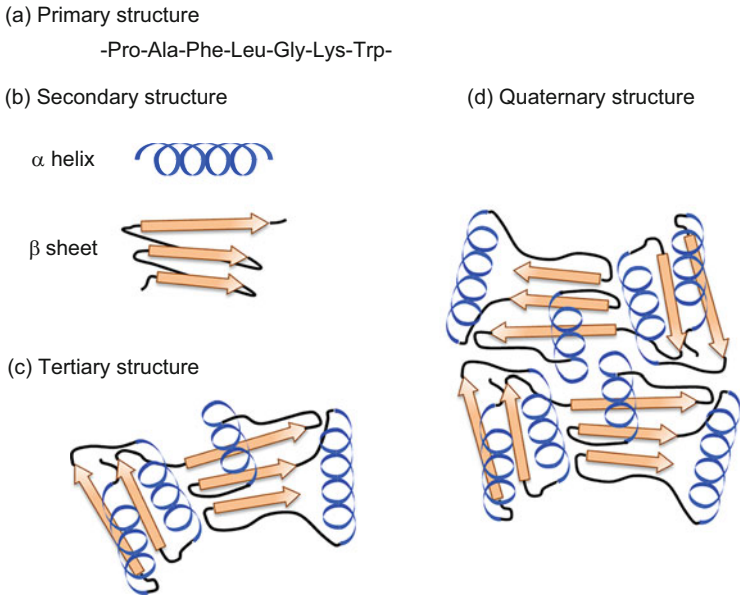

Fig. 1.5 Four levels of protein hierarchy. (**a**) The primary structure is the linear sequence of amino acids. (**b**) The secondary structure is formed of the local α helices and β sheets. (**c**) The tertiary structure refers to the three-dimensional arrangement of all amino acid residues in a protein. (**d**) The quaternary structure is the number and relative positions of the subunits in multimeric proteins

state is usually the conformation with the lowest free energy. Information directing the native state of each protein is encoded in its amino acid sequence (Anfinsen 1973). The process of protein folding can be understood based on statistical characterization of the energy landscape of folding proteins (Bryngelson and Wolynes 1987; Leopold et al. 1992; Bryngelson et al. 1995) (Fig. 1.6). Each protein has a funnel-shaped energy landscape with many high energy, unfolded structures and only a few low-energy, folded structures. Folding of a newly synthesized, unfolded polypeptide is the progressive organization of an ensemble of partially folded structures, through which the polypeptide passes on its way to the natively folded structure (Onuchic and Wolynes 2004). Specifically, the formation of secondary structure occurs early, which is followed by assembly of more compact and complex domains and then formation of tertiary and quaternary structures.

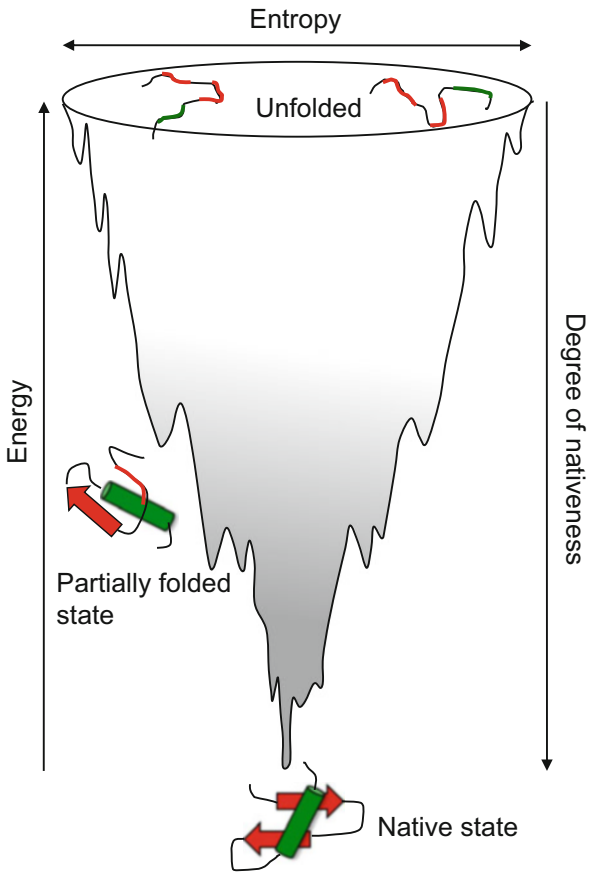

Fig. 1.6 The energy landscape of folding proteins. The free-energy landscape represents the configuration space of energy (y-axis) and entropy (x-axis) available to a protein, from its unfolded to folded structure. The protein does not have to follow a specific path through the configuration space, but instead it may travel any number of ways, taking on various partially formed structures that form the energy landscape

1.5 Proteotoxic Stress Responses Maintain the Balance of Quality and Quantity of the Proteins

Cellular functions are carried out by proteins, which are encoded in the genome. Mammalian cells contain more than 10,000 different proteins at concentrations ranging from 200 to 300 mg/ml. The extremely high concentration of cellular proteins results in an excluded-volume effect (also known as macromolecular crowding), which increases the tendency of nonnative proteins to aggregate (Ellis 2001). To maintain the proper conformations and physiological concentrations of numerous proteins, whose size, stability, and chemical modification are heterogeneous, cells must maintain a balance between the synthesis, folding, and clearance of individual proteins in a wide range of environmental and metabolic conditions (Wolff et al. 2014; Hipp et al. 2014) (Fig. 1.7). Newly synthesized proteins are correctly folded by HSP or chaperone networks. Simultaneously, a substantial proportion (about 30 %) of these proteins are misfolded and degraded in

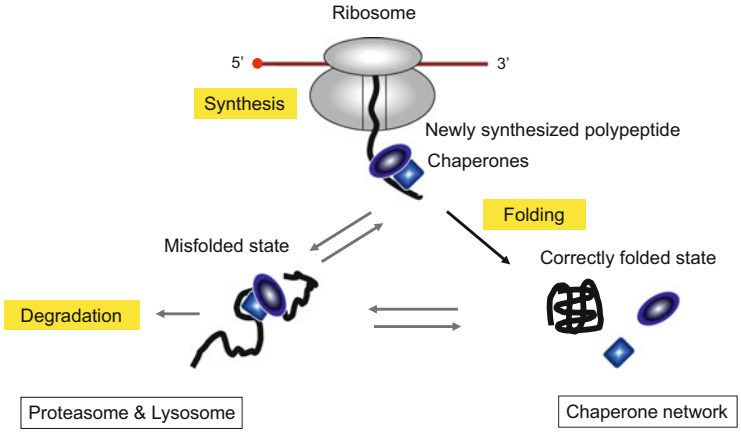

Fig. 1.7 Regulation of proteostasis capacity. Cells maintain a balance between the synthesis, folding, and clearance of individual proteins. Proteostasis capacity or buffering capacity against protein misfolding is regulated mainly at the level of protein synthesis, folding, and degradation. The chaperone network facilitates correct folding of newly synthesized proteins, and misfolded proteins are degraded by proteasomes and lysosomes

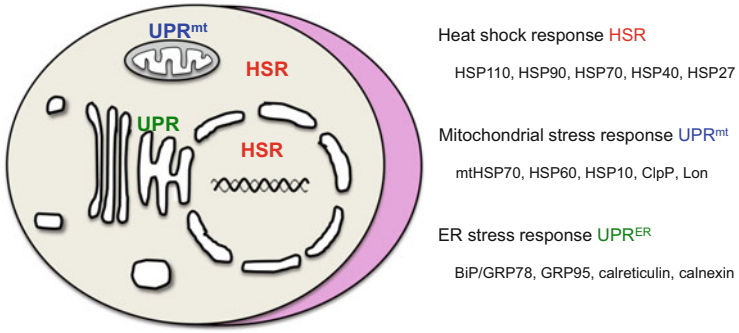

Fig. 1.8 Adaptive responses to protein misfolding in various organelles. The heat shock response (*HSR*) and unfolded protein response in the ER (UPR^{ER}) are characterized by drastic induction of chaperones localized in the cytoplasm and nucleus and in the ER, respectively. The UPR in the mitochondria (UPR^{mt}) is accompanied by the induction of mitochondrial chaperones and proteases including Lon and ClpP

proteasomes or lysosomes. This state of dynamic equilibrium, in which protein synthesis and folding are balanced with degradation, is referred to as protein homeostasis or proteostasis (Balch et al. 2008). Cells have evolved sophisticated mechanisms accompanied by changes in gene expression, which adjust proteostasis capacity or buffering capacity against protein misfolding, at the level of protein synthesis, folding, and degradation. They include the heat shock response in the cytoplasm/nucleus and the unfolded protein response (UPR) in the endoplasmic reticulum and mitochondria (Ron and Walter 2007; Morimoto 2011) (Fig. 1.8). The

Table 1.1 Transcription factors (TFs) responsible for proteotoxic stress responses

Stress response	Location	TFs in yeast	TFs in worm	TFs in mammal
Heat shock response (HSR)	Cytoplasm/ nucleus/ mitochondria	HSF	HSF-1	HSF1, HSF2, HSF3, HSF4
Mitochondrial stress response (UPR^{mt})	Mitochondria	Unknown	ATFS-1	Unknown[a]
ER stress response (UPR^{ER})	ER	Hac1 (XBP1)	XBP1, ATF6, ATF4*(atf-5)*	XBP1, ATF6, ATF4

[a]HSF1 is in part involved in the mitochondrial stress response (Tan et al. 2015)

major inducible proteins in these proteotoxic stress responses are chaperones (HSPs, GRPs, calreticulin, calnexin) and proteases (Lon, ClpP). Recent genome-wide analysis revealed that heat shock also induces the synthesis of factors related to the ubiquitin-proteasome system and lysosome (autophagy) system in mammalian cells (see below). The transcription factors that regulate these responses in yeast, worm, and mammals are shown in Table 1.1. The heat shock response is conserved in both prokaryotes and eukaryotes, which suggests that it may be the prototype of proteotoxic stress response mechanisms in organelles.

1.6 Heat Shock-Specific Transcription Factors

The expression of heat shock genes that encode HSPs is tightly regulated at the level of transcription. Therefore, heat shock-specific transcription factors have been studied intensively. In *E. coli*, *HSP* genes are under the control of the specific transcription factor σ^{32} that directs the core RNA polymerase to the heat shock promoter (Grossman et al. 1984; Yura et al. 1984) (Fig. 1.9). σ^{32} is an unstable protein under normal growth conditions. The concentration of σ^{32} increases rapidly in response to changes in translational efficiency and protein stability during heat shock, and the increased concentration is sufficient for the induction of HSPs such as DnaK (a bacterial homologue of HSP70) and GroEL (HSP60) (Grossman et al. 1987). Furthermore, the products of σ^{32} target genes, DnaK/DnaJ (HSP70/HSP40) and GroEL/GroES (HSP60/HSP10), negatively regulate its activity (Guisbert et al. 2004; Tomoyasu et al. 1998).

Regulation of eukaryotic *HSP* genes was studied first in *Drosophila*. Clones of several *HSP* genes were isolated from *D. melanogaster*, and comparison of these sequences revealed that their 5′ upstream regions from the transcription start site were closely homologous to each other (Ingolia et al. 1980; Karch et al. 1981; Holmgren et al. 1981). This sequence was able to induce *HSP70* gene transcription during heat shock when the *HSP70* gene included a 1.1 kb fragment of the 5′ upstream region was incorporated into the genome of mouse cells (Corces et al. 1981). It was shown in monkey cells that the upstream element within −48

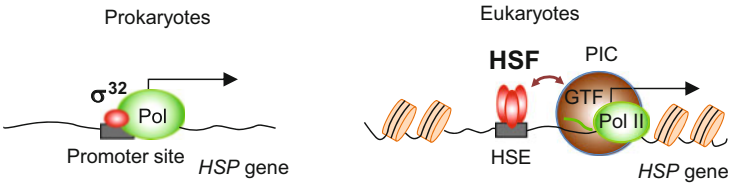

Fig. 1.9 Mechanism of the heat shock response. In *E. coli*, heat shock transcription factor σ^{32} directs the core RNA polymerase to the heat shock promoter (the promoter site) of *HSP* gene. In eukaryotes, the heat shock response element (*HSE*) is recognized by heat shock factor (*HSF*), which facilitates assembly of the pre-initiation complex (*PIC*) including general transcription factors (*GTF*) and RNA polymerase II (*Pol II*)

to −62 from the transcription start site of the *Drosophila HSP70* gene was necessary for heat-induced transcription, and its sequence features were common to those of other heat shock genes (Pelham 1982; Mirault et al. 1982). Furthermore, this element contained a consensus 14-bp element, CTnGAAnnTTCnAG, which was found upstream of heat shock genes from a wide variety of organisms (see Nover 1987 for review), and this is referred to as the heat shock response element (HSE) (Pelham 1985) (Fig. 1.9). The HSE is now defined as at least three inverted repeats of an nGAAn unit (Xiao and Lis 1988; Amin et al. 1988; Perisic et al. 1989).

The HSE could be a binding site of the heat shock-specific transcription factor or heat shock factor (HSF). The DNA around the *HSP70* and *HSP83* gene promoters is exposed in the chromatin of isolated *Drosophila* nuclei and is hypersensitive to DNase I digestion (Wu 1980). Furthermore, the TATA boxes within the promoters are protected from an exonuclease both before and during heat shock, whereas the HSEs were protected only in heat-shocked cells (Wu 1984a). The HSE-binding protein, presumably HSF, was detected only in heat-shocked *Drosophila* cells or embryos (Parker and Topol 1984; Wu 1984a) and could be purified from *Drosophila* and yeast cell extracts by sequence-specific DNA affinity chromatography (Wu et al. 1987; Wiederrecht et al. 1987; Sorger and Pelham 1987). In yeast, HSF binds to the HSE constitutively and its transcriptional activity is induced during heat shock, whereas in *Drosophila* and human cells HSE binding and the transcriptional activity of HSF are both induced by heat shock (Sorger et al. 1987; Zimarino and Wu 1987; Kingston et al. 1987; Larson et al. 1988; Mosser et al. 1988). HSF is phosphorylated during heat shock in yeast and human cells (Sorger et al. 1987; Larson et al. 1988), suggesting that the phosphorylation modulates the transcriptional activity of HSF. Thus, it has been proposed that HSF1 activation in metazoan species is controlled by two steps: the acquisition of DNA-binding activity and transcriptional activity (see Chap. 3).

A single-copy *HSF* gene from *Saccharomyces cerevisiae* was first cloned using antibodies against HSF (Sorger and Pelham 1988; Wiederrecht et al. 1988). Thereafter, *HSF* genes were isolated from another budding yeast *Kluyveromyces lactis* and from the fission yeast *Schizosaccharomyces pombe* by cross-hybridization (Jakobsen and Pelham 1991; Gallo et al. 1993). A *Drosophila HSF* was isolated

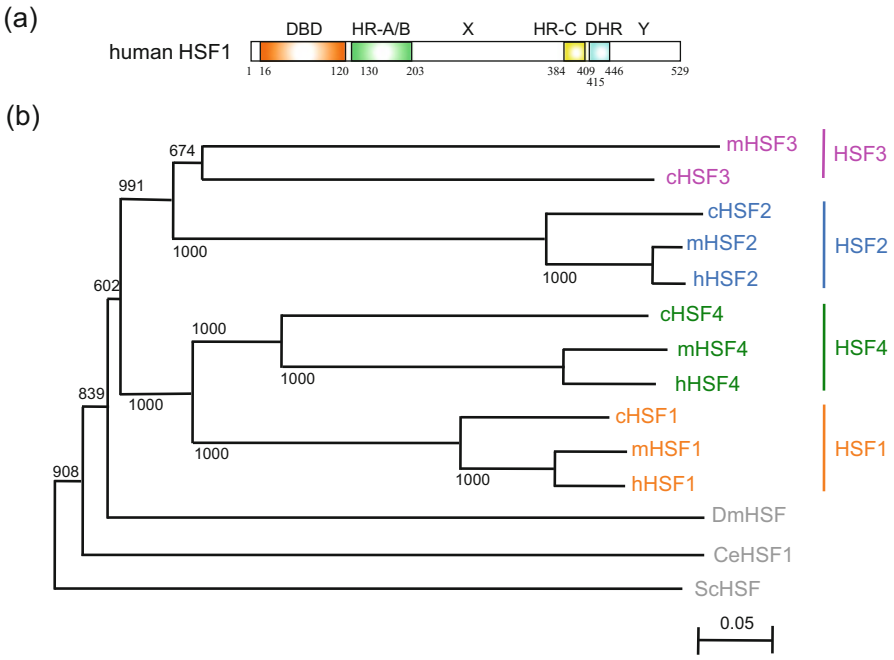

Fig. 1.10 HSF family members. (**a**) Structure of human HSF1. The numbers of amino acids are shown. *DBD* DNA-binding domain; *HR-A/B* hydrophobic heptad repeats A and B or oligomerization domain; *DHR* downstream of HR-C; *Region X* an upstream region of the HR-C domain; *Region Y* a C-terminal region downstream of the DHR domain. (**b**) Phylogenetic tree generated in CLUSTAL W (Thompson et al. 1994) for members of the HSF family. HSFs from human (*h*), mouse (*m*), chicken (*c*), *D. melanogaster* (*Dm*), *C. elegans* (*Ce*), and *S. cerevisiae* (*Sc*) are shown. Gaps were excluded from all phylogenetic analyses. The numerals represent bootstrap values (1000 bootstrap replicates were performed). The unrooted tree was drawn with the program TREEVIEW (Page 1996). Bar represents 0.05 substitutions per site

by screening a library with oligonucleotide probes derived from HSF peptide sequencing (Clos et al. 1990). Human *HSF1* and *HSF2* genes were isolated by screening a library with degenerate oligonucleotide probes (Rabindran et al. 1991; Schuetz et al. 1991) and the mouse *HSF1* and *HSF2* genes by cross-hybridization (Sarge et al. 1991). Thereafter, chicken *HSF3* and human *HSF4* genes were also isolated by cross-hybridization (Nakai and Morimoto 1993; Nakai et al. 1997). Mouse HSF3 is expressed in cells, whereas in human the *HSF3* gene is present as a pseudogene (Fujimoto et al. 2010). Thus, vertebrate cells have three or four HSFs (HSF1 to HSF4) that bind specifically to the HSE (Table 1.1). In contrast, multiple HSFs are expressed in plant cells (Scharf et al. 2012). HSFs in various species are composed of an evolutionally conserved DNA-binding domain (DBD) and an oligomerization domain (HR-A/B) (Fig. 1.10) (see Chap. 2).

1.7 HSF Is Essential for Normal Growth in Yeast

In budding yeast *S. cerevisiae*, neither the amount of HSF DNA-binding activity nor the affinity of HSF for DNA was altered by heat shock (Sorger et al. 1987). Remarkably, the *HSF* gene was essential for normal growth (Sorger and Pelham 1988; Wiederrecht et al. 1988). In contrast to *S. cerevisiae* HSF, fission yeast *S. pombe* HSF not only bound to the HSE constitutively (Zimarino et al. 1990) but also exhibited a heat-inducible HSE-binding activity (Gallo et al. 1991). Surprisingly, S. pombe HSF was also required for growth at normal temperatures (Gallo et al. 1993). These observations indicated that yeast HSF regulates gene expression not only upon heat shock but also under normal growth conditions. The expression of these genes may be regulated through the HSE, because human HSF2 functionally substituted for yeast HSF (Liu et al. 1997). Genome-wide analysis showed that the yeast HSF target genes encoded proteins that have a broad range of biological functions including protein folding and degradation, energy generation, protein trafficking, maintenance of cell integrity, small molecule transport, cell signaling, and transcription (Hahn et al. 2004).

The *S. cerevisiae* HSF has two distinct transcriptional activation domains, and the C-terminal domain (amino acids 584–783) is required for a sustained increase in HSF activity at elevated temperatures (Sorger 1990). Yeast cells harboring HSF (1–583), a truncated form of the C-terminal domain, could grow at a normal temperature, but were unable to grow at temperatures above 33 °C. This activation domain upregulated the expression of HSP82 (an orthologue of mammalian HSP90) at both normal and elevated temperatures and was required for cell cycle progression at elevated temperatures (Morano et al. 1999). The overexpression of HSP82 complemented the defect of HSF(1–583). Yeast cells harboring another HSF mutant, hsf1-82, were also defective in HSP82 synthesis and cell cycle progression at 37 °C (Zarzov et al. 1997). The HSP90 chaperone complex is involved in the regulation of cell cycle regulators such as Wee1 kinase (Richter and Buchner 2001). These observations showed a pivotal role of the HSF-HSP90 pathway in yeast growth at elevated temperatures.

1.8 HSFs Are Dispensable for Normal Growth in Metazoan Species

In contrast to the essential role of yeast HSF, a single HSF was dispensable for cell growth and survival under normal conditions in *Drosophila* (Jedlicka et al. 1997). However, HSF was required for oogenesis and early larval development under normal growth conditions. These developmental functions were not mediated through the induction of major HSPs, suggesting unique developmental functions of HSF (Jedlicka et al. 1997).

HSF1, HSF2, HSF3, and HSF4 are expressed in mouse and chicken cells, whereas HSF1, HSF2, and HSF4 are expressed in human cells (Fujimoto et al. 2010). HSF1-, HSF2-, and HSF4-deficient mice have been generated and show defects in some developmental processes. Meiosis was impaired in the absence of HSF1 and HSF2 in female (Christians et al. 2000; Metchat et al. 2009) and male germ cells (Nakai et al. 2000; Kallio et al. 2002; Wang et al. 2004). Neurogenesis was affected in HSF1-null and HSF2-null brains (Santos and Saraiva 2004; Chang et al. 2006; Homma et al. 2007). Furthermore, differentiation and maintenance of sensory placodes were impaired in HSF1-null and HSF4-null mice (Fujimoto et al. 2004; Min et al. 2004; Takaki et al. 2006). Thus, members of the HSF family exert some essential functions during development. However, mouse HSFs, including HSF3, are not necessary for cell growth and survival under normal conditions (McMillan et al. 1998; Fujimoto et al. 2008; Fujimoto et al. 2010; Shinkawa et al. 2011). Chicken B lymphocyte DT40 cells deficient in HSF1, HSF2, and HSF3 have been generated, and they are also dispensable for cell growth and survival under normal conditions (Tanabe et al. 1998; Nakai and Ishikawa 2001; Shinkawa et al. 2011).

Cancer cells proliferate and survive in different ways from normal cells, and many of the signaling pathways and transcription factors displayed a striking dependence on the chaperone machinery (Whitesell and Lindquist 2005; Calderwood et al. 2006). Furthermore, HSF1 expression was elevated in human cancer cells (Hoang et al. 2000) (see Chap. 13). Surprisingly, HSF1 was required for the proliferation and survival of various human cancer cell lines, but not in normal or immortalized cells (Dai et al. 2007). For example, HSF1 deficiency suppressed chemical skin carcinogenesis and lymphomagenesis in a p53-deficient mouse model (Dai et al. 2007; Min et al. 2007). This function was mediated through the regulation of HSF1-target gene expression in normal growth conditions (Fujimoto et al. 2012; Mendillo et al. 2012). These observations might suggest a common HSF-mediated mechanism for cell proliferation and survival in cancer cells and yeast cells.

1.9 Vertebrate HSFs Play Roles in Adaptation to High Temperature

Heat shock induces both apoptotic and necrotic cell death, but the pathways of cell death and factors that are primarily impaired are not clear, because it causes various types of stress including a proteotoxic stress. Although HSPs should not be the only proteins that protect against cell death, they are recognized as major players in the protection of cells from heat shock. HSPs prevent the denaturation and aggregation of cellular proteins and support their renaturation when cells are recovering. Alternatively, HSPs inhibit the activity of mitochondria-mediated apoptotic factors such as Apaf-1 and cytochrome c (Xanthoudakis and Nicholson 2000).

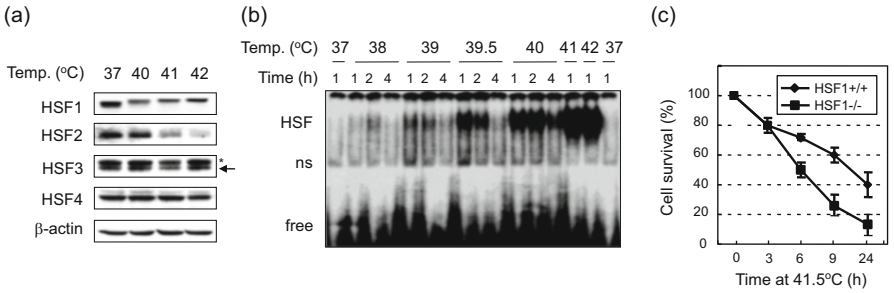

Fig. 1.11 Characterization of mouse HSFs in control and heat shock conditions. (**a**) Mouse NIH3T3 cells maintained at 37 °C were heat shocked at 40, 41, or 42 °C for 1 h. Protein levels of HSF1, HSF2, HSF3, and HSF4 are shown by Western blotting (Shinkawa et al. 2011). An arrow indicates the position of the HSF3 bands, and a star indicates nonspecific bands. (**b**) Induction of the HSE-binding activity. MEF cells, maintained at 37 °C, were heat shocked at the indicated temperatures for the indicated periods. Whole cell extracts were prepared and subjected to EMSA using an HSE oligonucleotide as a probe (Takii et al. 2010). *HSF* complex of HSFs and the HSE oligonucleotide; *ns* nonspecific band; *free* free probe. (**c**) Resistance to a high temperature. Primary MEF cultures from HSF1+/+ and HSF1−/− mice were incubated at 41.5 °C for the indicated periods. Surviving cells were counted, and percentages of cell survival are shown (Inouye et al. 2003)

HSF1, HSF2, HSF3, and HSF4 are expressed in mouse NIH3T3 cells (Fig. 1.11a) and in chicken DT40 cells (Shinkawa et al. 2011). Mouse HSF1 was hyperphosphorylated during heat shock even at a febrile-range temperature of 40 °C. HSF2 was stable during heat shock at 40 °C, but rapidly degraded at 41 °C (Mathew et al. 2001). HSF3 and HSF4 were stable even at 42 °C (Fig. 1.11a). The HSE-binding activity in extracts of mouse embryonic fibroblasts (MEF) was faintly detected at a normal growth temperature of 37 °C and was induced to a maximal level at 41 °C (Fig. 1.11b). This activity was mainly composed of HSF1. HSF1 is a master regulator of HSP expression in mammalian cells (McMillan et al. 1998), as is HSF3 in avian cells (Tanabe et al. 1998). Therefore, mouse HSF1, as well as chicken HSF3, was required for the acquisition of thermotolerance (McMillan et al. 1998; Tanabe et al. 1998), which was tightly correlated with the induced synthesis of HSPs (Li and Werb 1982).

Analysis of avian HSFs revealed that HSFs are also required for cell growth and survival at elevated temperatures. Chicken DT40 cells deficient in both HSF1 and HSF3 expressed reduced levels of HSP90α (Nakai and Ishikawa 2001). These mutant cells were highly sensitive to continuous exposure to high temperature, like *S. cerevisiae* harboring a mutant HSF as described above, and the cell cycle was blocked at G_2 phase (Nakai and Ishikawa 2001). Mouse HSF1 also regulated the expression of some HSPs including HSP90 in various mouse tissues (Yan et al. 2002; Takaki et al. 2006, 2007; Metchat et al. 2009). Therefore, HSFs could be involved in the determination of temperature, at which cells can grow and survive by regulating the constitutive expression of HSPs such as HSP90.

Chicken HSF1 hardly upregulated the expression of HSPs during heat shock, but had a significant effect on the growth and survival at high temperatures (Inouye et al. 2003). This effect was mediated through neither the induction of HSP nor regulation of the constitutive expression of HSPs such as HSP90. In mouse cells, HSF1-null MEF cells, which lacked the induced expression of HSPs, were more sensitive to continuous exposure to high temperature than wild-type cells (Fig. 1.11c) (Inouye et al. 2003). These phenotypes may be in part caused by the regulation of non-HSP proteins, because HSF1 regulates the expression of not only HSPs but also non-HSP proteins such as PDZK3 and CRYAB, which suppress protein aggregation through degradation (Hayashida et al. 2010; 2011). Mouse HSF3 was also able to protect HSF1-null MEF cells from heat shock without inducing the expression of HSPs (Fujimoto et al. 2010), and mouse HSF2 was required for resistance to a febrile-range thermal stress (Shinkawa et al. 2011). Furthermore, HSF4 regulated the expression of non-HSP genes during heat shock (Fujimoto et al. 2008). These observations suggested that each member of the HSF family supported cell growth and survival at high temperatures, probably by regulating the expression of HSPs as well as non-HSP proteins (Fig. 1.12).

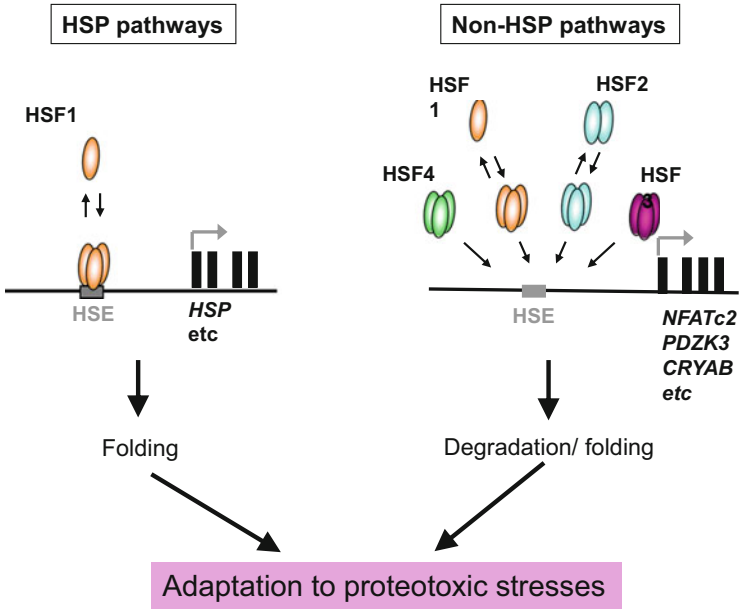

Fig. 1.12 Pathways of HSF-mediated adaptation to proteotoxic stress. HSF1 remains mostly as an inert monomer in unstressed cells. It is converted to an active trimer that binds to the promoters of *HSP* genes and induces the expression of HSPs (HSP pathways). In addition to HSF1, HSF2, HSF3, and HSF4 bind to the promoters of *non-HSP* target genes under normal or proteotoxic stress conditions and elevate the expression of non-HSP proteins involved in protein degradation and folding (Non-HSP pathways). These pathways are required for adaptation to proteotoxic stresses

1.10 The Heat Shock Response in Organisms Living at Various Temperatures

The induction of HSPs in response to elevated temperature has been studied in various organisms living at various temperatures, in addition to major model organisms such as human, mouse, chicken, *D. melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae*, and *E. coli* (Table 1.1.). These included ectothermic animals, such as lizard, frog, and fish (Ulmasov et al. 1992; Bienz and Gurdon 1982; Mosser et al. 1986), and thermophiles that grow at temperatures above 25–40 °C that sustain most life forms (Conway de Macario and Macario 1994; Stetter 1999) (Table 1.2.). For example, the hyperthermophilic archaeon Pyrodictium occultum grew optimally at 100°C, and HSP60 (GroEL) was induced by heat shock at 100 °C for 4 h (Phipps et al. 1991). Furthermore, HSPs were induced in the thermophilic bacterium *Bacillus caldolyticus* (grown at 60 °C and heat shocked at 69 °C for 10 min), the hyperthermophilic archaeon *Sulfolobus shibatae* (grown at 70 °C and heat shocked at 88 °C for 60 min), and the thermophilic eukaryote *Thermomyces lanuginosus* (grown at 50 °C and heat shocked at 55 °C for 60 min), and acquired thermotolerance is correlated with the induction of HSPs (Trent et al. 1994). Thus, the heat shock response is conserved during evolution.

The mechanism of the heat shock response is also evolutionary conserved in eukaryotes. Lower animals such as fly and worm possess a single HSF gene. Two rounds of whole-genome duplication may have occurred in vertebrate ancestral cells more than 440 million years ago (Sidow 1996; Meyer and Schartl 1999), which resulted in polyploidization. The expression and function of the duplicated HSF genes have been conserved or diverged during evolution (Semon and Wolfe 2007). Functional analyses of vertebrate HSFs have been conducted extensively in endothermic animals including human, mouse, and chicken cells so far, which implied that HSF1 is a master regulator of HSP expression during heat shock, whereas HSF3 is its master regulator uniquely in birds (Fujimoto and Nakai 2010). However, the functions of HSF1 and HSF3 in other ectothermic animals such as lizards and frogs should be examined in the future, because these animals also seem to express both HSF1 and HSF3 (see Chap. 2).

In the eubacterium *E. coli*, σ^{32} mediates the heat shock response by directing RNA polymerase to the promoters of HSP genes (Guisbert et al. 2008) and also mediates this in other proteobacteria, which are Gram-negative and comprise a major group within the eubacteria (Nakahigashi et al. 1995). The single RpoH locus encoding the σ^{32} factor may be replaced by two or more differentially regulated stress response σ factors, and multiple RpoH σ factors are common in alphaproteobacterial genomes (Barnett et al. 2012). However, heat shock σ factors do not appear to be utilized for the heat shock response in other bacteria such as the eubacterium *Bacillus subtilis* (Zuber and Schumann 1994). Archaea have a eukaryotic type of transcriptional machinery containing homologues of the transcription factor TATA-binding protein, TFIIB, and RNA polymerase II and also use histones that assemble their chromatin and suppress transcription (Geiduschek and

Table 1.2 Temperatures at which HSPs are induced in cells isolated from various organism

Species	Scientific name	Cell line	Optimal temp. (°C)	Induced temp. (°C)	References
Human	*Homo sapiens*	HeLa	37	42	Gerner et al. (1976)
Mouse	*Mus musculus*	MEF	37	42	McMillan et al. (1998)
Chicken	*Gallus gallus domesticus*	CEF	37	45	Kelley and Schlesinger (1978)
		DT40	39.5	45	Tanabe et al. (1998)
Lizard	*Gekko gecko*	GL-1	30	42	Clark et al. (1970)[a]
Frog	*Xenopus laevis*	A6	21	33	Mercier et al. (1997)
	Xenopus tropicalis	Speedy	28	37	Sinzelle et al. (2012)[a]
Fish	*Danio rerio* (zebrafish)	ZF4	28	37	Airaksinen et al. (2003)
	Oncorhyhnchus mykiss (rainbow trout)	RTG-2	22	28	Mosser et al. (1986)
Worm	*Caenorhabditis elegans*	(aminal)	20	30	Snutch and Baillie (1983)
Fly	*Drosophila melanogaster*	SL2	25	37	Zimarino and Wu (1987)
Mould	*Thermomyces lanuginosus*		50	55	Trent et al. (1994)
Yeast	*Saccharomyces cerevisiae*		22	37	Miller et al. (1979)
	Schizosaccharomyces pombe		28	43	Gallo et al. (1991)
Eubacteria	*Escherichia coli*		30	42	Yamamori et al. (1978)
	Bacillus subtilis		37	48	Zuber and Schumann (1994)
	Bacillus caldolyticus		60	69	Trent et al. (1994)
Archaea	*Sulfolobus shibatae*		70	88	Trent et al. (1994)
	Pyrodictium occultum		100	108	Phipps et al. (1991)

[a]Heat shock experiments were conducted in our laboratory

Ouhammouch 2005). In the hyperthermophilic archaea *Pyrococcus furiosus* and *Sulfolobus solfataricus*, HSPs seem to be under the control of negative regulators, which bind to these promoters and inhibit RNA polymerase recruitment, in control conditions (Vierke et al. 2003; Iqbal and Qureshi 2010). A positive regulator of the heat shock response has not been identified yet.

1.11 Conclusions and Perspectives

In summary, the heat shock response is a universal, adaptive response to protein misfolding in cells and is regulated mainly at the transcription level by heat shock transcription factors, such as HSF in eukaryotes and σ^{32} in the eubacterium *E. coli*. HSF maintains proteostasis capacity in cells by regulating the expression of HSPs, which facilitate protein folding, and non-HSP proteins including proteins involved in protein clearance.

Genetic screening has been performed extensively using fly and worm models to identify genes involved in proteostasis, and it has been shown that a variety of genes play roles in regulating proteostasis capacity (Fernandez-Funez et al. 2000; Kazemi-Esfarjani et al. 2000; Nollen et al. 2004; Silva et al. 2011). On the other hand, hundreds of HSF target genes have been identified in yeast and human cells (Hahn et al. 2004; Trinklein et al. 2004), and substantial numbers of HSF1 targets elevate proteostasis capacity in mammalian cells (Hayashida et al. 2010). However, the same set of genes may not be regulated by HSF1 in other cell types, and another set of genes may be under the control of HSF2, HSF3, or HSF4 in vertebrate cells. To understand the pathways controlled by HSF family members and the biological significance of these pathways, it would be necessary to resolve the common targets and cell type-specific targets of each HSF involved in the regulation of proteostasis capacity.

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Chapter 2
Structure and Function of the HSF Family Members

Ryosuke Takii and Mitsuaki Fujimoto

Abstract The heat shock response is a fundamental mechanism to adapt against various proteotoxic stresses in all living organisms. This response is characterized by the induction of heat shock proteins (HSPs) and regulated mainly at the level of transcription by heat shock factor (HSF). Vertebrate cells possess four *HSF* genes, which are located in the conserved syntenic regions among species. The amino acid sequences of the DNA-binding domain (DBD) and oligomerization domain (HR-A/B) located in the N-terminal region are highly conserved. The DBD interacts with genomic DNA, and HR-A/B is required for the formation of an HSF trimer that binds to DNA with high affinity. The HR-A/B is flanked by two nuclear localization signals. There are some activation or regulatory domains in the C-terminal region. Among HSF family members, HSF1 is a master regulator of the expression of *HSP* gene in mammalian cells, while that of *non-HSP* genes is also regulated by HSF2, HSF3, and HSF4. Furthermore, the HSF family members cooperatively or competitively regulate the expression of some common targets.

Keywords Amino acid • Consensus sequence • DNA binding • Evolution • Hydrophobic heptad repeat • Structure • Transcriptional activity

2.1 Introduction

When cells are exposed to elevated temperatures, cellular proteins are denatured and aggregated. Cells have the ability to adapt to this proteotoxic stress by inducing the expression of heat shock proteins (HSPs) or molecular chaperones, which facilitate protein folding and suppress protein aggregation. This response is called the heat shock response and is a universal mechanism to maintain protein homeostasis and to protect cell from protein-damaging insults (Lindquist 1986). This response is well conserved from bacteria to human. In eukaryotes, the heat shock response is regulated mainly at the level of transcription by heat shock factor (HSF).

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HSF stays as an inert monomer in normal growth condition. In response to heat shock, HSF is quickly converted to a trimer that binds to the heat shock response element (HSE) located in the promoters of heat shock genes encoding HSPs including HSP70. The consensus sequence of the HSE is at least three inverted repeats of a pentanucleotide nGAAn (Fernandes et al. 1994). As a result, the expression of HSPs is rapidly induced.

HSF is required not only for the heat shock response but also cell growth and differentiation and normal lifespan in yeast, *C. elegans*, and *Drosophila* (Hsu et al. 2003; Morano et al. 1999; Morley and Morimoto 2004). There is a single HSF in these invertebrates, whereas four *HSF* genes (*HSF1* to *HSF4*) exist in higher animals (Nakai et al. 1997; Nakai and Morimoto 1993; Rabindran et al. 1991; Schuetz et al. 1991). In mammals, HSF1 is required for the heat shock response, whereas HSF3 is required for this response in birds (McMillan et al. 1998; Tanabe et al. 1998). Both mammal HSF1 and chicken HSF3 are necessary for acquisition of the thermotolerance, which is correlated with the induction of HSPs. In addition, members of HSF family are involved in various developmental processes including gametogenesis and neurogenesis and maintenance of sensory organs (Chang et al. 2006; Kallio et al. 2002; Takaki et al. 2006; Wang et al. 2004). Thus, the *HSF* gene has duplicated and acquired multifaceted functions during evolution. In this review, we describe the structure of HSF family members; e.g., the DNA-binding domain, oligomerization domain, and activation domain. We also review the transcriptional activity, biochemical characters, and target sequences of HSF family members and discuss about evolution of the *HSF* gene family.

2.2 Structure of the HSF Family Members

The sizes and amino acid sequences of eukaryotic HSF family members are different, except for some conserved domains (Fig. 2.1) (Clos et al. 1993). Especially, amino acid sequences of the DNA-binding domain (DBD) and oligomerization domain (HR-A/B, hydrophobic heptad repeat-A/B) are highly conserved in eukaryotic species (Nakai et al. 1997; Nakai and Morimoto 1993; Sarge et al. 1991). In addition, there are several short sequences including the nuclear localization signal, which are conserved in all or parts of the HSF family members. The location and sequences of the transcriptional activation domain are different in the HSF family members.

2.2.1 DNA-Binding Domain

The DBD is located near the N-terminus of all HSFs. Amino acid sequence of the DBD of human HSF1 (hHSF1) is 46 % identical to that of yeast HSF (ScHSF) (Fig. 2.1). The crystal structure and NMR solution structure of the DBD in ScHSF

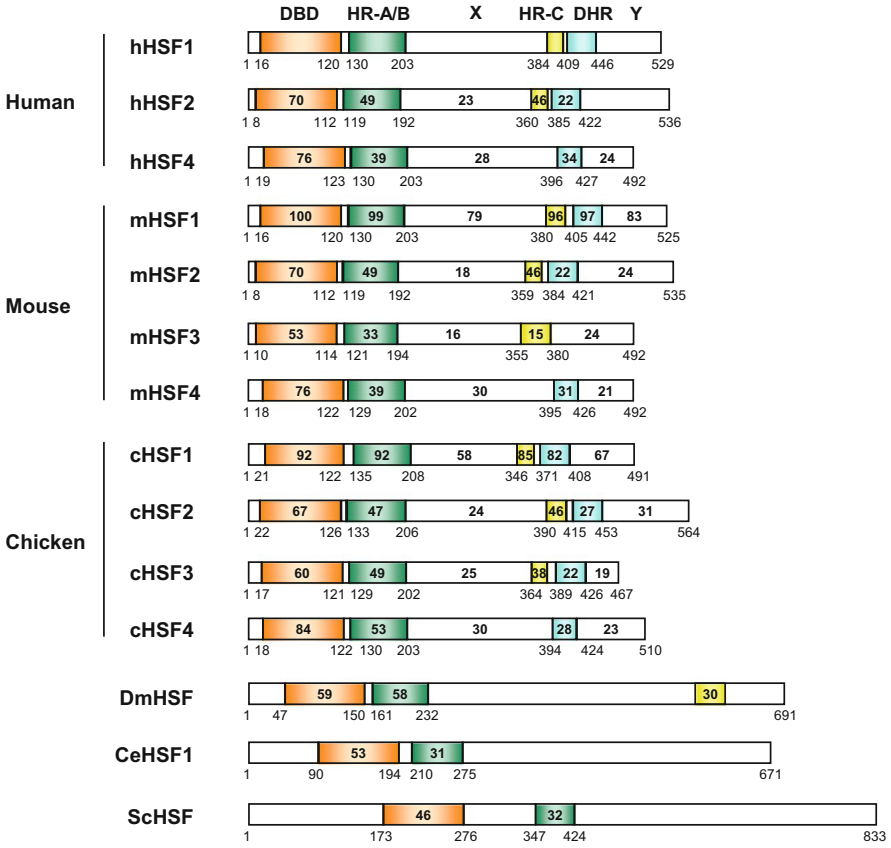

Fig. 2.1 Structures of the HSF family members. Diagrammatic representation of structures of the HSF family members. The percentage identities between amino acid sequences in regions of human HSF1 and those corresponding regions in other HSFs are established using the computer program GENETYX-WIN. The number of amino acids of each HSF is shown at the N-terminus. *DBD* DNA-binding domain; *HR* hydrophobic heptad repeat; *DHR* downstream of HR-C; *h* human; *m* mouse; *c* chicken; *Dm Drosophila melanogaster*; *Ce C. elegans*; *Sc Saccharomyces cerevisiae*. hHSF1 (an isoform hHSF1α) (Rabindran et al. 1991); hHSF2 (hHSF2α) (Schuetz et al. 1991); hHSF4 (hHSF4b) (Nakai et al. 1997); mHSF1 (mHSF1α) and mHSF2 (mHSF2α) (Sarge et al. 1993); mHSF3 (mHSF3a) (Fujimoto et al. 2010); mHSF4 (mHSF4b) (Tanabe et al. 1999); cHSF1, cHSF2, and cHSF3 (Nakai and Morimoto 1993); cHSF4 (cHSF4b) (Fujimoto et al. 2010); DmHSF (Clos et al. 1990); CeHSF1 (Swiss-P accession no Q9XW45); ScHSF (Wiederrecht et al. 1988). The HR-C is not conserved in HSF4, CeHSF1, and ScHSF

show that HSF belongs to winged helix-turn-helix DNA-binding proteins containing mammalian ETS and HNF-3/forkhead proteins (Gajiwala and Burley 2000) and consisted of three helical bundles (H1, H2, and H3) and four-stranded, antiparallel β-sheets (Fig. 2.2) (Damberger et al. 1995; Harrison et al. 1994; Vuister et al. 1994a, b). The central α-helix H3 interacts with the major groove of DNA. In contrast, the α-helix H1 and wing (loop) region are exposed outside and interact

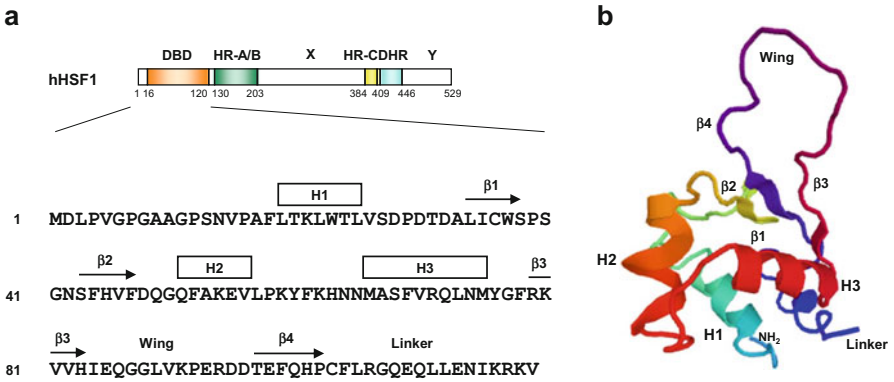

Fig. 2.2 Structure of the DNA-binding domain in human HSF1. (**a**) Sequences of the DBD in hHSF1. *Boxes* indicate α-helices and *arrows* indicate β-sheets. (**b**) A model to predict the structure of the DBD in hHSF1 by comparison with that in *Drosophila* HSF1 using SWISS-MODEL (http://swissmodel.expasy.org/). Each absolute structure is indicated by rainbow color. It shows a winged helix-turn-helix motif, which consisted of H2 (*orange*) and H3 (*red*). The α-helix H3 interacts with the major groove of DNA. The α-helix H1 and wing (loop) region are exposed outside

with regulatory factors such as RPA1 and ATF1 (Fujimoto et al. 2012; Takii et al. 2015). The wing is not necessary for the trimer formation of HSF but is required for full activity of HSF (Cicero et al. 2001; Littlefield and Nelson 1999).

2.2.2 *Oligomerization Domain*

The HR-A/B is connected to the DBD by a flexible linker of 10–20 amino acids (Flick et al. 1994). It contains two α-helices, HR-A and HR-B, which consist of a repeating pattern of seven amino acids. Especially, amino acids at positions a and d of the heptad repeat, a-b-c-d-e-f-g, are hydrophobic amino acids such as leucine (L), isoleucine (I), and valine (V) (Fig. 2.3a). HSF uniquely forms a trimer that binds to DNA with a high affinity through the hydrophobic interaction between three HR-A/B domains located in parallel when HSF is activated (Clos et al. 1990; Peteranderl et al. 1999) (Fig. 2.3a). The electrostatic interactions also occur primarily between positions e and g, which are often charged residues such as arginine (R), lysine (K), aspartic acid (D), and glutamic acid (E) (Fig. 2.3b) (Creighton 1993). The HR-A/B is required for the formation of not only a trimer but also a dimer (Nakai et al. 1995; Sistonen et al. 1994). Therefore, the HR-A/B is also called the trimerization domain or oligomerization domain. The linker domain modulates the trimerization of HSF (Liu and Thiele 1996). There is another hydrophobic heptad repeat HR-C near the C-terminus of HSF. The HR-C inhibits the oligomerization of the HR-A/B by forming an intramolecular coiled coil with the HR-A/B and keeps HSF as a monomer that cannot bind to DNA in control conditions. Therefore, the point mutation of hydrophobic amino acids in the HR-C of hHSF1

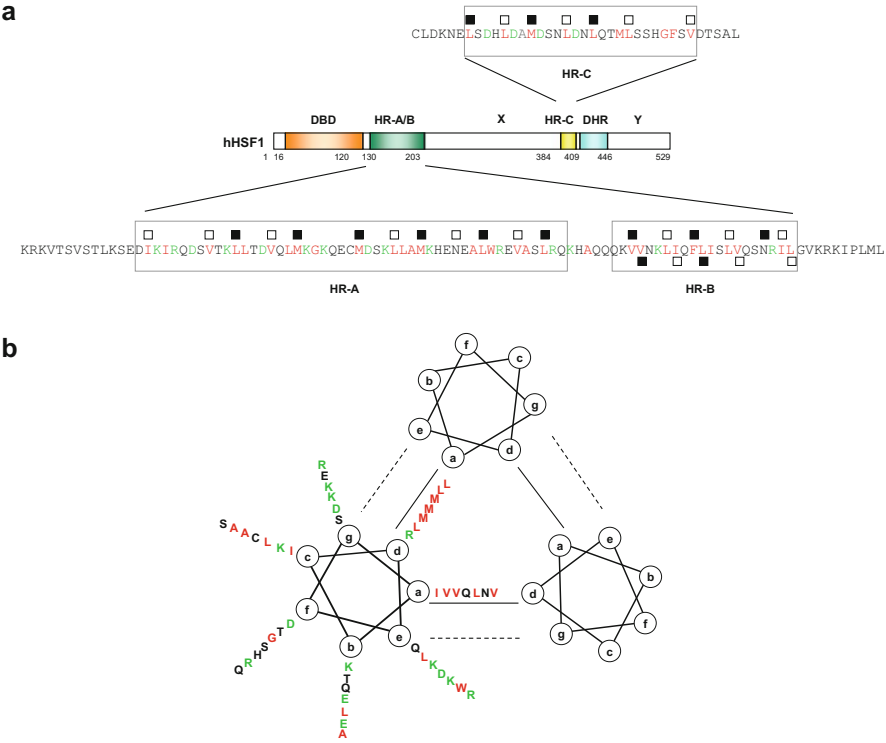

Fig. 2.3 Trimer formation through interaction between the hydrophobic heptad repeats. (**a**) Amino acid sequences of the HR-A/B and HR-C in hHSF1. The *open* and *solid squares* indicate amino acids at positions a and d, respectively, in the repeating seven amino acids. Hydrophobic amino acids are marked by *red* and charged amino acids by *green*. (**b**). Trimer formation of the HR-A/B. The HR-A/B forms a trimer through the interaction between hydrophobic amino acids at positions a and d (*solid lines*) in the HR-A/B. The electrostatic interactions also occur primarily between positions e and g (*dotted lines*), which are often charged residues

results in the formation of a DNA-binding trimer (Rabindran et al. 1993; Zuo et al. 1995). Because HSF in yeasts and HSF4 lack the HR-C, they exist mostly as DNA-binding trimers in control conditions (Giardina et al. 1995; Jakobsen and Pelham 1988; Nakai et al. 1997; Sorger and Nelson 1989; Sorger and Pelham 1988; Wiederrecht et al. 1988). Another hydrophobic heptad repeat DHR exists downstream of the HR-C in most vertebrate HSFs (Nakai et al. 1997), but its function is unknown yet.

2.2.3 Nuclear Localization Signal

The HR-A/B domains in the HSF family members are surrounded by two putative nuclear localization signals (NLSs), NLS1 and NLS2 (Fig. 2.4). These sequences fit

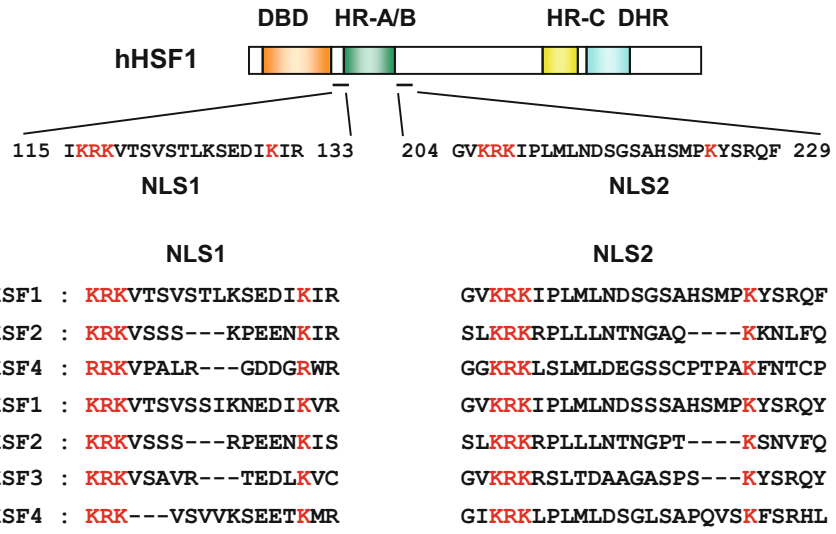

Fig. 2.4 Nuclear localization signals in vertebrate HSFs. Sequences of the NLS1 and NLS2 in human and chicken members of the HSF family are shown. These sequences fit the consensus for a bipartite NLS, which consisted of two clusters of basic amino acids (*red*), separated by about ten amino acids

the consensus for a bipartite NLS, which consisted of two clusters of basic amino acids, separated by a spacer of about ten amino acids (Sheldon and Kingston 1993). Human HSF2 (hHSF2) partly accumulates to the nucleus upon heat shock (Shinkawa et al. 2011), but a mutated hHSF2 lacking a cluster of the basic amino acids in the NLS1 or NLS2 does not translocate to the nucleus (Sheldon and Kingston 1993). Thus, both NLS1 and NLS2 are required for the nuclear translocation of HSF2. Human HSF1 (hHSF1) is a master regulator of the HSP expression during heat shock. The NLS2 of hHSF1 is required for the nuclear translocation, whereas the NLS1 is dispensable for that (Vujanac et al. 2005). Lysine amino acids in the NLSs are acetylated and related with the formation of nuclear stress bodies in response to heat shock (Raychaudhuri et al. 2014). Chicken HSF3 (cHSF3) is a master regulator of the HSP expression in birds, and the NLS2, but not the NLS1, is required and sufficient for the nuclear translocation of cHSF3 upon heat shock (Nakai and Ishikawa 2000). *Drosophila* HSF also has a bipartite NLS located downstream of the HR-A/B which is required for its nuclear translocation in response to heat shock and during development (Fang et al. 2001; Orosz et al. 1996). In plants, HSFs have a nuclear export signal (NES) in the C-terminal activation domain (Kotak et al. 2004), but there is no report about the NES in vertebrate HSFs.

In addition to the NLS, there are at least seven conserved sequences, termed sites a to g in the regions X and Y, in vertebrate HSF members (Fujimoto et al. 2010; Nakai et al. 1997; Nakai and Morimoto 1993; Tanabe et al. 1999). The function of these conserved sequences should be revealed in the future.

2.2.4 *Transcriptional Activation Domain*

HSF from a budding yeast *Saccharomyces cerevisiae* possesses the N-terminal and C-terminal transcriptional activation domains (Sorger 1990). In contrast, HSF from another budding yeast *Kluyveromyces lactis* does not have the N-terminal activation domain but has the C-terminal activation domain, whose sequence is completely divergent from that of *S. cerevisiae* HSF (Jakobsen and Pelham 1991). Thus, the sequences of transcriptional activation domains in HSFs are not evolutionally conserved. However, transcriptional activity of the activation domain is repressed in unstressed condition by a conserved motif CE2, which is located near the activation domain.

Mammalian HSF1 is functionally analogous to yeast HSF and robustly induces the expression of HSPs during heat shock. It has a potent transcriptional activation domain (AD) in the C-terminus, which is divided into two regions (Green et al. 1995; Shi et al. 1995; Zuo et al. 1995) (Fig. 2.5a). The AD1 (a.a. 372–431)

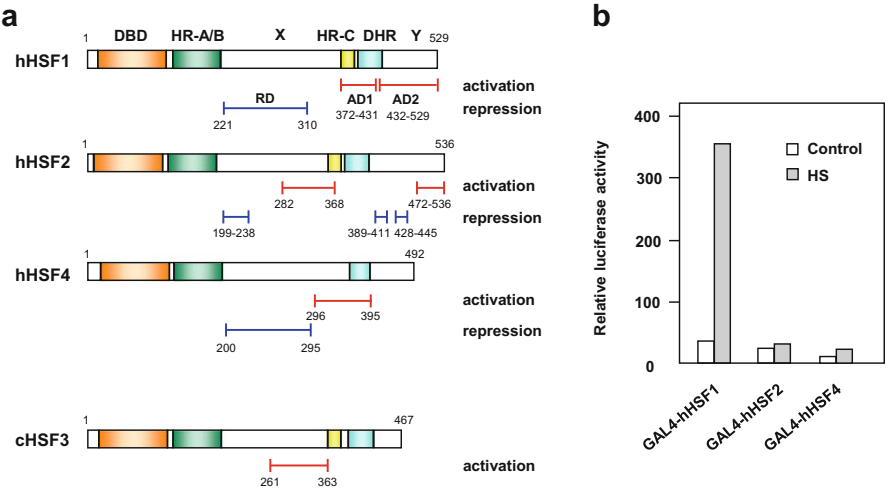

Fig. 2.5 Transcriptional activation domains of human HSFs. (**a**) Localization of activation domains in human HSF1, HSF2, and HSF4 and chicken HSF3. *Red bars* indicate transcriptional activation domains (*ADs*), and *blue bars* indicate repression domains of the activation domains. Human HSF1 possesses two activation domains in the C-terminus (AD1, a.a. 372–431; AD2, a.a. 432–529) (Green et al. 1995). The AD1 is predicted to form an α-helix, while AD2 is rich in proline and glycine. The regulatory domain (*RD*, a.a. 221–310) represses the two activation domains (Green et al. 1995). In human HSF2, two activation domains (a.a. 282–386 and a.a. 472–536) and three negative regulatory domains (a.a. 199–238, a.a. 389–411, and a.a. 428–445) are present (Yoshima et al. 1998b). Human HSF4 also has an activation domain (a.a. 296–395) and a negative regulatory domain (a.a. 200–295) (Nakai et al. 1997). Chicken HSF3 has at least a strong activation domain (a.a. 261–363) (Tanabe et al. 1997). (**b**) Human HSF1, hHSF2, and HSF4 have different potential to activate transcription. Reporter analysis in human cells treated without (control) and with heat shock (*HS*) (42 °C for 1 h and then recovery at 37 °C for 6 h) (Nakai et al. 1997; Yoshima et al. 1998a)

contains the HR-C and is predicted to form an α-helix, while AD2 (a.a. 432–529) is rich in proline (13 %) and glycine (8 %). Hydrophobic residues in the AD1 and AD2 are involved in the elongation step of the transcription processes, and acidic residues are in the initiation steps (Brown et al. 1998). The regulatory domain (RD, a.a. 221–310) located downstream of the HR-A/B represses the two activation domains in unstressed condition and confers heat shock inducibility of the transcriptional activity upon heat shock (Green et al. 1995; Newton et al. 1996).

In contrast to the strong potential of mammalian HSF1 to activate transcription upon heat shock, the potential of HSF2 and HSF4 is weak in both unstressed and heat-shocked conditions (Tanabe et al. 1999; Yoshima et al. 1998a) (Fig. 2.5b). Nevertheless, HSF2 and HSF4 have a potential to activate the reporter gene constitutively, and HSF4 induces the gene expression in response to proteotoxic stresses including heat shock. In human HSF2, two activation domains (a.a. 282–386 and a.a. 472–536) and three negative regulatory domains (a.a. 199–238, a.a. 389–411, and a.a. 428–445) are present (Yoshima et al. 1998b). Human HSF4 also has an activation domain (a.a. 296–395) and a negative regulatory domain (a.a. 200–295) (Nakai et al. 1997). Chicken HSF3, like human HSF1, has a strong potential to activate transcription upon heat shock and possesses at least a strong activation domain (a.a. 261–363) (Tanabe et al. 1997).

2.3 Vertebrate *HSF* Gene Family

In contrast to invertebrate cells, vertebrate cells have four *HSF* genes. The human, mouse, and chicken genome sequences have become available (International Chicken Genome Sequencing Consortium 2004; International Human Genome Sequencing Consortium 2001; Mouse Genome Sequencing Consortium 2002); therefore, it is possible to compare the syntenic regions, where the same genes occur in a similar order along the chromosomes of different organisms (Koonin et al. 2000). The orthologues of each *HSF* gene are located in the same syntenic regions, which are derived from the same ancestral genomic region (Fujimoto et al. 2010) (Fig. 2.6). For example, *HSF2* is flanked by the *SERINCI* gene in human, mouse, and chicken orthologous segments. *HSF4* was located in a region between the *TRADD-FBXL8* and *NoL3* in these genomes, and *HSF3* was located between the *Vsig4* and *HEPH*. The exon-intron structures of each *HSF* gene are also evolutionally conserved in three species (Fujimoto et al. 2010).

Four HSF family members are expressed in mouse and chicken cells, whereas human HSF3 is not expressed since human *HSF3* is a pseudogene (Fujimoto et al. 2010). Because all of human, mouse, and chicken *HSF* genes have been molecularly cloned and functionally characterized, phylogenetic tree is generated from predicted amino acid sequences of the HSF family members in these endotherms (Fig. 2.7). It shows that the sequence of each HSF (HSF1, HSF2, HSF3, or HSF4) in a species is more related with those of orthologous ones in other species than those of paralogous ones. Furthermore, the sequences of HSF1 orthologues are

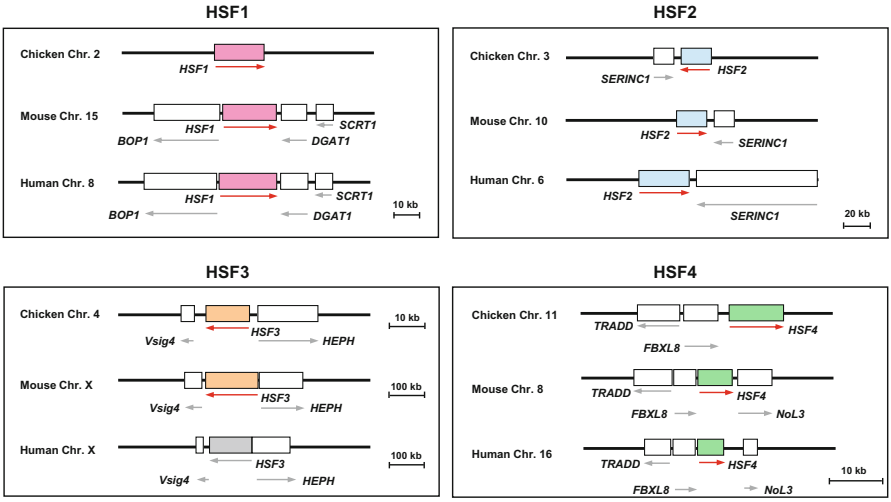

Fig. 2.6 Syntenic regions containing the vertebrate *HSF* genes. The location of each segment is as follows: *HSF1*, human Chr. 8 q24.3 and mouse Chr. 15 D3; *HSF2*, human Chr. 6 q22.31, mouse Chr. 10 B4, and chicken Chr. 3 63.95–63.98 Mb; *HSF3*, human Chr. X q12, mouse Chr. X B4, and chicken 0.252–0.265 Mb; *HSF4*, human Chr. 16 q22.1, mouse Chr. 8 D3, and chicken 2.44–2.45 Mb. A genomic sequence corresponding to chicken HSF1 cDNA has not yet been identified. *Arrows* indicate the 5′ to 3′ orientation of each gene. *Colored boxes* indicate the *HSF* genes, which are flanked by genes shown as *white boxes*. Human *HSF3* is a pseudogene. Note that size markers differ in human, mouse, and chicken HSF3 loci

more related with those of HSF4 orthologues than those of HSF2 or HSF3 orthologues, whereas those of HSF2 orthologues are more related with those of HSF3 orthologues. It has been suggested that two rounds of whole-genome duplication have occurred in vertebrate ancestral cells more than 440 million years (Myr) ago (Holland et al. 1994; Ohno 1970; Putnam et al. 2008), which resulted in polyploidization. Thereafter, avian and mammalian cells evolved differently from an ancestral cell 310 Myr ago, and human and mouse cells did 75 Myr ago. Taken together, four *HSF* genes may have been generated through the two rounds of whole-genome duplication, and thereafter the sequences and functions have been conserved or diverged during vertebrate evolution (Semon and Wolfe 2007). For example, mammal HSF1 is required for the induction of HSPs during heat shock, whereas HSF3, but not HSF1, is required for this response in birds (McMillan et al. 1998; Tanabe et al. 1998). In contrast, HSF4 is dominantly expressed in the lenses of the human, mouse, and chicken eyes (Bu et al. 2002; Fujimoto et al. 2004; Fujimoto et al. 2010). The phylogenetic tree also shows that the relatedness of chicken HSF3 with mammalian HSF3 is much weaker than that of chicken HSF1 with mammalian HSF1, that of chicken HSF2 with mammalian HSF2, or even that of chicken HSF4 with mammalian HSF4. These estimations indicate that nucleic acid sequences of HSF3 diverged most quickly during evolution, whereas those of

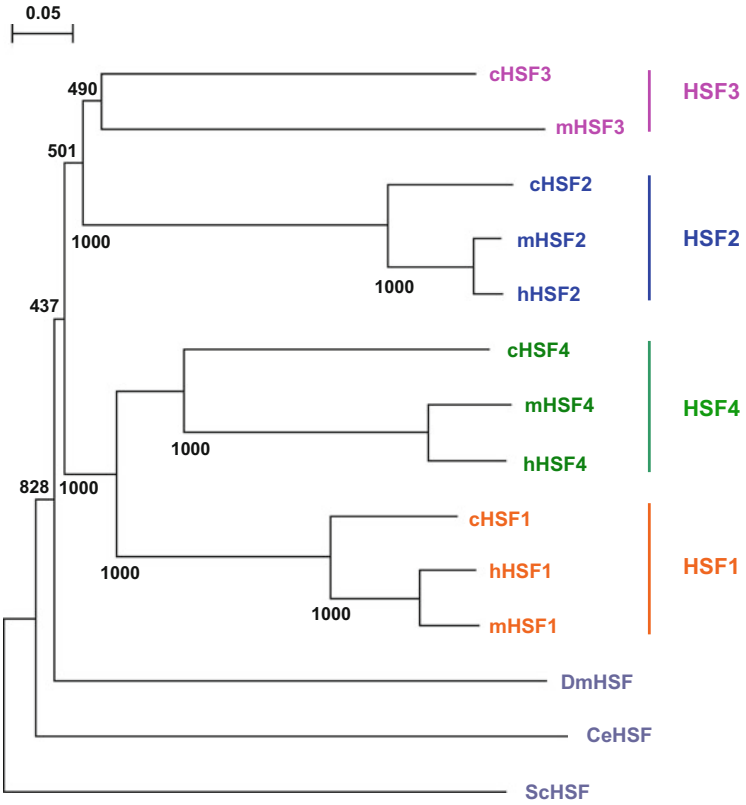

Fig. 2.7 The phylogenetic tree of HSF family members. Phylogenetic tree generated in CLUSTAL X (Thompson et al. 1997), using amino acid sequences of human (*h*), mouse (*m*), and chicken (*c*) HSF family members as well as *Drosophila melanogaster* HSF (*DmHSF*), *C. elegans* HSF1 (*CeHSF1*), and *Saccharomyces cerevisiae* HSF (*ScHSF*) (Fujimoto et al. 2010; Inouye et al. 2003). The number represents bootstrap values (1000 bootstrap replicates were performed). The unrooted tree was drawn using program TreeView (Page 1996). The bar represents 0.05 substitutions per site

HSF1 and HSF2 were similarly conserved. The divergence rates of the HSF4 sequence might also be relatively high.

In contrast to the HSF family members in the endotherms, genes of the HSF members in ectotherms, such as frog, lizard, and fish, have been molecularly cloned and functionally characterized only partially (Hilgarth et al. 2004; Råbergh et al. 2000; Stump et al. 1995; Swan et al. 2012; Yeh et al. 2006; Zatsepina et al. 2000) (Fig. 2.8). Analysis using the gene database (http://www.ensembl.org/index.html) suggests that *HSF1*, *HSF2*, and *HSF4* genes are evolutionally conserved in all vertebrate species. In contrast, *HSF3* is conserved in rodents, avians, reptiles, and amphibians, while *HSF3* seems to be lost in some mammalian and fish species. In plants, 252 HSF family members, which have similar structures and

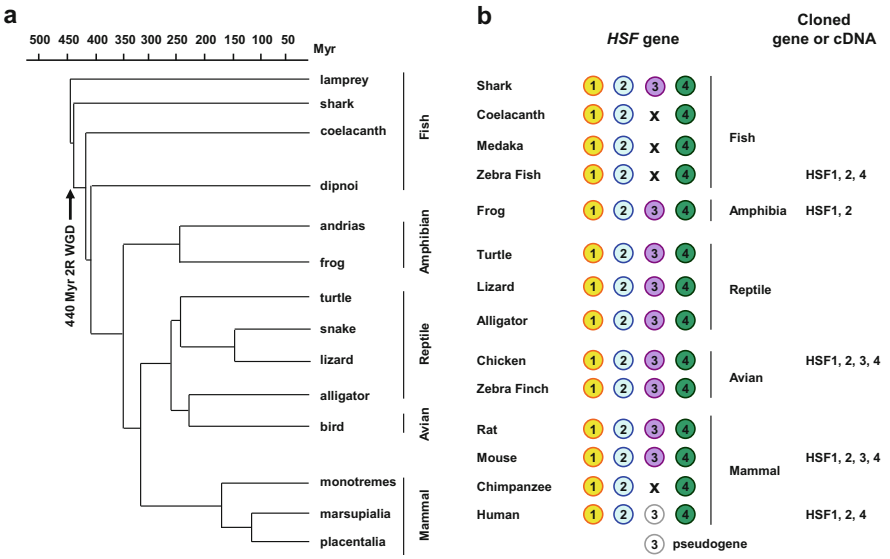

Fig. 2.8 Members of vertebrate *HSF* gene family. (**a**). Evolution of the vertebrates. Phylogenetic tree among main groups of the vertebrates is shown (Patterson 2001). Two rounds (*2R*) of whole-genome duplication (*WGD*) may have occurred in vertebrate ancestral cells more than 440 million years (Myr) ago. (**b**) *HSF* genes in vertebrate species. HSF genes, whose products are registered in NCBI database (http://www.ncbi.nlm.nih.gov/), are shown. *1 HSF1*; *2 HSF2*; *3 HSF3*; *4 HSF4*. HSF3 protein is not registered in chimpanzee, zebra fish, medaka, and coelacanth (x). Human *HSF3* is a pseudogene (*white circle*). Cloned cDNAs are zebra fish HSF1 (Hsu and Yeh 2002; Råbergh et al. 2000), HSF2 (Yeh et al. 2006), and HSF4 (Swan et al. 2012); frog HSF1 (Mercier et al. 1997) and HSF2 (Hilgarth et al. 2004); chicken HSF1, HSF2, HSF3 (Nakai and Morimoto 1993), and HSF4 (Fujimoto et al. 2010); mouse HSF1, HSF2 (Sarge et al. 1991), HSF3 (Fujimoto et al. 2010), and HSF4 (Tanabe et al. 1999); and human HSF1 (Rabindran et al. 1991), HSF2 (Schuetz et al. 1991), HSF3 (Fujimoto et al. 2010), and HSF4 (Nakai et al. 1997). The genes for shark HSF3 (XP_007891631), frog HSF3 (NP_001039053), turtle HSF3 (XP_006136197), lizard HSF3 (XP_008118582), alligator HSF3 (XP_006258300), and zebra finch HSF3 (XP_004177297.1) are located on the same syntenic regions in the genomes of these species

features, are identified from nine plant species (Scharf et al. 2012). It is necessary to examine the function of each HSF member in ectotherms in the future.

2.4 Potential to Activate *HSP* and *Non-HSP* Genes During Heat Shock

The heat shock response is characterized by the induction of *HSP* gene expression during heat shock. After the identification of multiple *HSF* genes in vertebrate genomes (Fujimoto et al. 2010; Nakai et al. 1997; Nakai and Morimoto 1993; Rabindran et al. 1991; Sarge et al. 1991; Schuetz et al. 1991), researchers have

investigated which HSF activates the *HSP* genes. Biochemical analyses showed that human and mouse HSF1, but not HSF2, acquires DNA-binding activity and translocated to the nucleus in response to heat shock, suggesting that HSF1 is involved in the induction of *HSP* genes (Baler et al. 1993; Sarge et al. 1993). Subsequently, disruption of *HSF* genes in mouse embryonic fibroblasts (MEFs) demonstrated that mouse HSF1 is required for the HSP induction during heat shock (McMillan et al. 1998; Zhang et al. 2002), while HSF2 is not (McMillan et al. 2002). Overexpression of human HSF1 restored the induction of HSPs during heat shock (Inouye et al. 2003). HSF1 is also responsible for the induction of the HSP expression in response to other proteotoxic stresses including a blockade of proteasome and an incorporation of amino acid analogues. Thus, HSF1 is a master regulator of the HSP expression in mammalian cells. HSF2 modulates the expression of HSPs to some extent, probably through the direct interaction with HSF1 (Ostling et al. 2007).

In addition to the expression of *HSP* genes, the expression of many non-*HSP* genes is induced during heat shock in mammalian cells (Trinklein et al. 2004). HSF1 plays a major role in the induction of these non-*HSP* genes, but HSF2, HSF3, and HSF4 are also involved in the induced expression of some non-*HSP* genes in MEF cells (Fujimoto et al. 2008; Fujimoto et al. 2010; Shinkawa et al. 2011).

Avian *HSF* genes have functionally diverged during evolution. Both HSF1 and HSF3 acquired the DNA-binding activity during heat shock in chicken cells (Nakai et al. 1995). Unexpectedly, disruption of chicken *HSF3* in chicken B-lymphocyte DT40 cells resulted in a severe reduction in the induction of HSP70 expression during heat shock, and the expression of HSP110, HSP90α, HSP90β, and HSP40 was not induced at all (Tanabe et al. 1998). The disruption of chicken *HSF1* had no effect on the induction of HSP expression during heat shock in DT40 cells, and overexpression of chicken HSF1 did not restore the induction of HSPs during heat shock in HSF1-null MEF cells (Inouye et al. 2003). These results demonstrate that HSF3, but not HSF1, is a master regulator of the HSP expression in chicken cells.

2.5 Oligomeric State

HSFs are defined by its ability to bind to the heat shock response element (HSE) that is composed of three inverted repeats of an nGGAn pentanucleotide. Therefore, activated HSFs form homotrimers that bind to the HSE with high affinity, through the interaction between the HR-A/B domains (Fig. 2.9). In budding yeast *Saccharomyces cerevisiae*, HSF is constitutively a DNA-binding trimer because it does not have the HR-C domain that inhibits oligomerization of the HR-A/B (Sorger and Pelham 1988; Wiederrecht et al. 1988). It acquires elevated potential to activate transcription via phosphorylation upon heat shock (Sorger 1990). In contrast, HSF from fission yeast *Schizosaccharomyces pombe* or *Drosophila melanogaster* stays an inert monomer in unstressed condition and is converted to a DNA-binding trimer in heat shock condition (Clos et al. 1990; Gallo et al. 1993). Thus, a monomer-to-

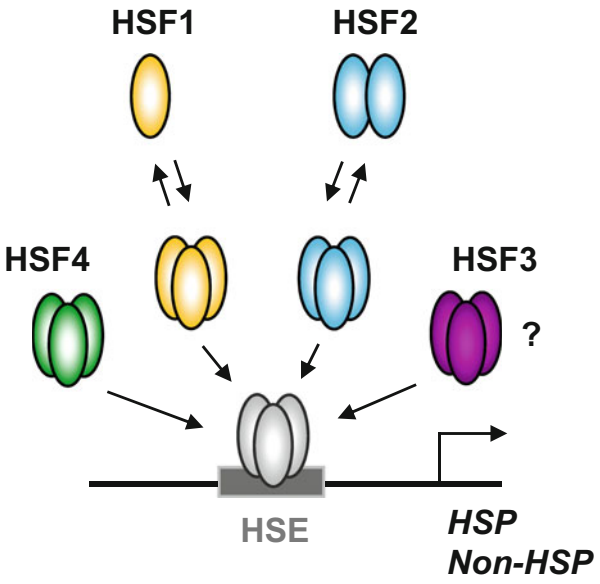

Fig. 2.9 Oligomeric states of mammalian HSFs. HSF1 remains mostly as an inert monomer in unstressed condition and is converted to a DNA-binding trimer. The activated HSF1 binds to the HSE located in the promoters of *HSP* and non-*HSP* genes. HSF2 stays as a dimer and a trimer and is converted to a trimer in response to proteasome inhibition or mild heat shock. HSF4 exists as a DNA-binding trimer constitutively because it lacks the HR-C. HSF3 may be a trimer in unstressed mouse cells. Mouse HSF3 may exist in trimer in unstressed condition

trimer transition of HSF is a fundamental mechanism of HSF activation in response to heat shock (Rabindran et al. 1993).

Oligomeric status of vertebrate HSFs involves a monomer, dimer, and trimer (Fig. 2.9). Mammalian HSF1, like *Drosophila* HSF, mostly stays as an inert monomer in unstressed condition and is converted to a DNA-binding trimer (Baler et al. 1993; Sarge et al. 1993). Mammalian HSF2 stays as both an inert dimer and a DNA-binding trimer in unstressed condition, and the latter increased during the treatment with cells with proteasome inhibitors such as MG132, lactacystin, and hemin (Sistonen et al. 1994; Mathew et al. 1998). Although HSF2 is unstable in severe heat shock conditions such as 42 °C (Sistonen et al. 1994), it is stable and acquires a DNA-binding activity through trimerization in febrile-range, mild heat shock conditions such as 40 °C (Shinkawa et al. 2011). Mammalian HSF4 lacks the HR-A/B domain and therefore forms a DNA-binding trimer constitutively in the lens and other tissues including the brain and lung (Fujimoto et al. 2004; Tanabe et al. 1999). HSF3 may exist as a trimer in unstressed mouse cells, because sequences of the heptad repeats of hydrophobic amino acids in the HR-C domain are not well conserved (Fujimoto et al. 2010). It is worth noticing that chicken HSF3, a master regulator of HSP expression, stays mostly as an inert dimer in control condition and is converted to a DNA-binding trimer upon heat

shock (Nakai et al. 1995). Therefore, the dimer-to-trimer transition of HSF2 and HSF3 is also one of HSF activation mechanisms in vertebrates.

2.6 Recognition Sequences

Extensive studies have revealed that *HSP* genes in *Drosophila* and other eukaryotic species have a 14 bp conserved promoter element, CnnGAAnnTTCnnG (n is any nucleic acid), termed the heat shock response element (HSE) (Pelham 1985). Furthermore, inverted repeats of a 5 bp sequence nGAAn are shown to be key features of the HSE (Xiao and Lis 1988; Amin et al. 1998). In fact, *Drosophila* HSF trimer stably binds to the HSE composed of three contiguous inverted 5 bp units (Perisic et al. 1989; Xiao et al. 1991). Analysis of crystal structure of the DBD of yeast HSF reveals that the second G and ninth C of 5′-nGAAnnTTCn-3′ in major groove of DNA are essential for the interaction with the α-helix H3 of the winged helix-turn-helix motif (Littlefield and Nelson 1999).

Recognition sequences of mammalian HSFs have been analyzed using in vitro random oligonucleotide selection. As is expected from the fact that sequences of the DBD in all of eukaryotic HSFs are highly conserved, both mouse HSF1 and HSF2 recognize inverted repeats of a pentameric consensus nGAAn (Kroeger and Morimoto 1994) (Fig. 2.10). Each HSF has some preference for the nucleotides

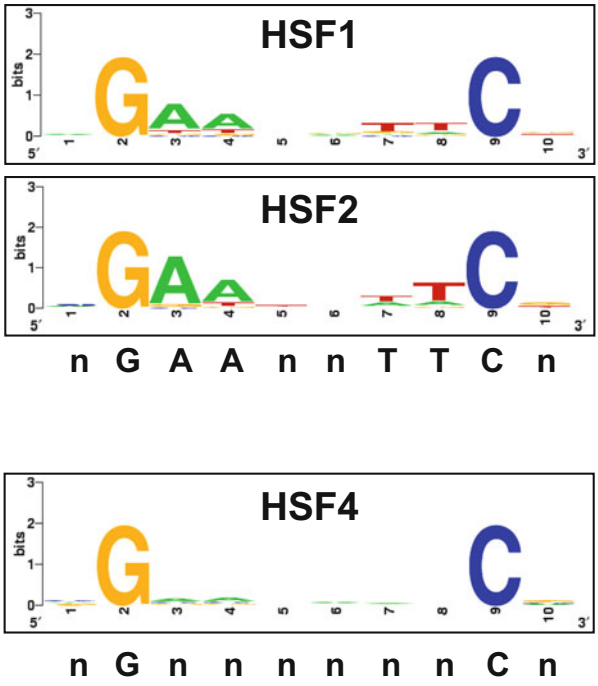

Fig. 2.10 In vitro consensus sequence of mammalian HSF binding sites. Consensus recognition sequences for mammalian HSF1, HSF2, and HSF4 binding in vitro were generated with WebLogo (http://weblogo.berkeley.edu/) by using DNA sequences of HSF1, HSF2 and HSF4 binding fragments (Fujimoto et al. 2008; Kroeger and Morimoto 1994). The height of each letter represents the relative frequency of nucleotides at different positions in the consensus

flanking the core GAA motif. In contrast, human HSF4 uniquely recognizes inverted repeats of an ambiguous nGnnn sequence in vitro (Fujimoto et al. 2008). Furthermore, most of the HSF4 binding region contains none or only one GAA sequence even in vivo. The numbers of consensus pentamers per binding sites are high (four to five pentamers) in only HSF1-selected oligonucleotide, suggesting that cooperative binding between trimers could affect the binding of HSF1 (Bonner et al. 1994; Kroeger and Morimoto 1994).

2.7 Cooperativity and Competition Between Distinct HSFs

Vertebrate HSF family members typically form a homotrimer when they are activated and bind to the HSEs in the promoters of target genes. Cooperativity of HSF1 and HSF2 has been shown in spermatogenesis (Wang et al. 2004). Spermatogenesis is normal in HSF1-null mice (Izu et al. 2004) and is partially impaired in HSF2-null mice (Kallio et al. 2002; Wang et al. 2003). These male mice are still fertile, although the number of sperm is markedly reduced in HSF2-null mice. In marked contrast, spermatogenesis is completely blocked in mice lacking both HSF1 and HSF2, and the double-null mice are infertile (Wang et al. 2004). Analysis of target genes shows that the expression of some genes, including *HSP70-2*, *HSC70t*, and *acrosin*, is significantly reduced only in double-null testis. Thus, HSF1 and HSF2 cooperatively upregulate target genes and support spermatogenesis. In addition, both factors are required for the constitutive and inducible expression of CRYAB in mouse embryonic fibroblasts (Shinkawa et al. 2011), and HSF2 enhances the HSF1-mediated inducible expression of HSP70 (Östling et al. 2007). Mechanisms of these cooperativities are unclear yet. HSF1 and HSF2 trimers may simultaneously occupy the target gene promoters, or HSF1 trimer may interact with HSF2 trimer. Alternatively, HSF1 and HSF2 may form heterotrimers (Sandqvist et al. 2009).

On the contrary, there is evidence that HSF1 and HSF4 compete with each other. In lens fiber cells of the mouse lens, both HSF1 and HSF4 cooperatively upregulate the expression of *γ-crystallin* genes. In contrast, in lens epithelial cells, HSF4 downregulates the expression of *FGF* genes, while HSF1 upregulates it (Fujimoto et al. 2004). Therefore, increased proliferation and premature differentiation due to the elevated expression of FGFs in the HSF4-null lens are alleviated in the lens lacking both HSF4 and HSF1. Furthermore, HSF1 and HSF4 have opposing effects on the expression of *LIF* gene in the mouse olfactory epithelium (Takaki et al. 2006). Thus, HSF4 competes with HSF1 for the expression of genes such as cytokine genes.

In chicken B-lymphocyte DT40 cells, disruption of *HSF1* or *HSF3* gene does not affect the expression of HSP90α (Nakai and Ishikawa 2001). However, disruption of both genes results in the marked reduction of the constitutive expression of HSP90α and blockade of cell cycle progression at elevated temperature. Thus, avian HSF1 and HSF3 redundantly regulate the expression of HSP90α, which is

required for cell cycle transition under stress condition (Morano et al. 1999; Zarzov et al. 1997).

2.8 Future Perspectives

In this chapter, we described mainly the structure and function of four *HSF* genes in vertebrates. The crystal structure and NMR solution structure of the DBD in yeast HSF revealed the precise structure in the DBD. It is proposed that an inert HSF1 monomer is folded for the HR-A/B and HR-C to interact with each other in unstressed condition and is converted to three-bundle, rod-shaped structure in response to heat shock. The structure of full-length HSF1 or domains other than the DBD is not analyzed yet, and these analyses should be done in the future. Among domains of HSFs, amino acid sequences of the DBD and oligomerization domains are highly conserved, and functions of these domains are well known. On the other hand, roles of the regions X and Y and the DHR are little known. There are several conserved sequences termed a to g sites in these regions, whose functions should be revealed.

In mammalian cells, HSF1 is required for the heat shock response, while roles of other HSF family members had hardly been known until recently. It is now known that not only HSF1 but also HSF2, HSF3, and HSF4 regulate proteostasis capacity within a cell to adapt to proteotoxic stresses. However, target genes and activation mechanisms of each HSF are not well known at present. In addition, mechanisms of cooperative and competitive regulation by the HSF family members are still unclear and should be clarified in the future.

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Chapter 3
Regulation of HSF Activation and Repression

Eiichi Takaki and Akira Nakai

Abstract Heat shock response (HSR) is characterized by robust induction of heat shock proteins (HSPs) during heat shock and is regulated mainly at the level of transcription by heat shock factor (HSF). Preexisting inert HSF monomers undergo conformational change to form trimers that bind to DNA and to acquire transcriptional activity during heat shock and other stimuli. These two steps are separated processes and are induced by release from feedback repression by HSPs, direct effects of stimuli, posttranslational modifications, and others. Basal activity of HSF is also regulated in unstressed conditions. In this chapter, we review molecular mechanisms of activation and repression of HSF and describe stimuli that activate HSF by controlling these mechanisms.

Keywords Conformational change • Feedback repression • Posttranslational modification • Small compound • Transcriptional activity • Trimerization

3.1 Introduction

Eukaryotic cells respond to elevated temperatures by a rapid increase in the synthesis of heat shock proteins (HSPs) that facilitate protein folding and non-HSP proteins with diverse functions including protein degradation (Lindquist 1986; Richter et al. 2010). This adaptive response called as the heat shock response (HSR) is regulated mainly at the transcriptional level by heat shock factor (HSF), which is conserved in all eukaryotic species (Wu 1995; Morimoto 1998). HSF preexists mostly as an inert state in unstressed cells and is converted quickly to an active state to induce the heat shock genes including *HSP* genes during heat shock. HSF is also activated at different levels by a variety of environmental and pathophysiological stresses. Thus, regulation of HSF activity plays a pivotal role in controlling proteostasis capacity in a cell (Balch et al. 2008; Morimoto 2011; Wolff et al. 2014; Hipp et al. 2014).

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A single HSF exists in yeast, fly, and worm, while vertebrate cells possess four HSFs (HSF1 to HSF4). Among them, HSF1 is a master regulator of the HSP expression during heat shock in mammalian cells (Akerfelt et al. 2010; Fujimoto and Nakai 2010). In this chapter, we principally describe molecular mechanisms of activation and repression of orthologs of mammalian HSF1 and summarize stimuli that activate them.

3.2 Trimer Formation and the Acquisition of Transcriptional Activity

HSF in budding yeasts constitutively forms a trimer that binds to the HSE (Sorger et al. 1987). Heat shock induces extensive phosphorylation of HSF, which is correlated with the transcriptional activation of *HSP* genes (Sorger and Pelham 1988). It turned out that this hyperphosphorylation serves as a regulatory mechanism to deactivate HSF, rather than being involved in its activation (Høj and Jakobsen 1994). However, these observations indicate that the trimerized HSF should be modified to acquire potent transcriptional activity during heat shock.

Mammalian HSF1 and HSF in fission yeast *Schizosaccharomyces pombe* and *Drosophila* stay mostly as an inactive monomer and are converted to a DNA-binding trimer upon heat shock (Gallo et al. 1991; Clos et al. 1990; Sarge et al. 1991, 1993; Baler et al. 1993). However, the acquisition of the DNA-binding activity is not sufficient for HSF1 to activate *HSP70* gene in some cell lines such as murine erythroleukemia (MEL) and human Y79 retinoblastoma cells (Hensold et al. 1990; Mathur et al. 1994). Furthermore, treatment of human cells with sodium salicylate, an anti-inflammatory agent, induces the DNA-binding activity of HSF1 and its occupancy on HSP70 promoter in vivo. Nevertheless, sodium salicylate does not induce the transcription of *HSP70* gene (Jurivich et al. 1992, 1995). It also induces the DNA binding of *Drosophila* HSF and puff formation in the polytene chromosomes, but does not induce the transcription of *HSP70* gene (Winegarden et al. 1996). These observations indicate that the trimer formation of HSF1 and its acquisition of transcriptional activity are separated processes (Fig. 3.1).

Mammalian HSF1, like yeast HSF, is hyperphosphorylated upon heat shock. Human HSF1 isolated from heat-shocked cells is more extensively phosphorylated than HSF1 activated in vitro, suggesting that the hyperphosphorylation is associated with transcriptional activity of HSF1 (Larson et al. 1988). However, it is dispensable for the acquisition of the transcriptional activity (Newton et al. 1996; Budzyński et al. 2015). Rather, specific residues in HSF1 are covalently modified by thiol oxidation, sumoylation, and acetylation as well as phosphorylation (Björk and Sistonen 2010). Therefore, HSF1 activation including the acquisition of transcriptional activity should be regulated multistep modifications as described below (see Sect. 3.5).

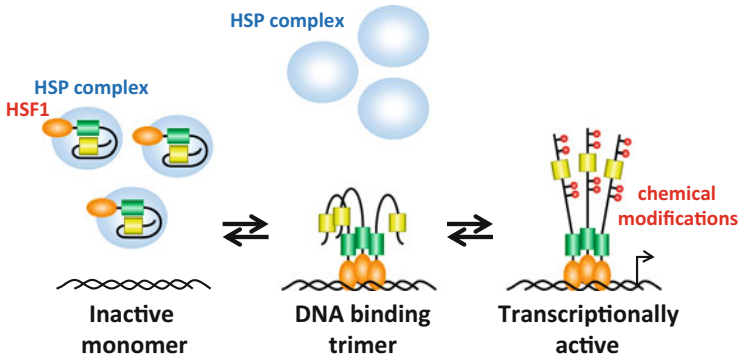

Fig. 3.1 HSF1 activation involves two distinct steps. Metazoan HSF1 stays as an inactive monomer by binding to chaperone machineries (*HSP complex*). It is converted to a DNA-binding trimer upon heat shock and then acquires transcriptional activity by unmasking the activation domain. DNA-binding domain (*orange circle*) and hydrophobic heptad repeats, HR-A/B (*green box*) and HR-C (*yellow box*), are shown (see Chap. 2). Chemical modifications are indicated by flags (*red*)

3.3 Release from Feedback Repression by HSPs

HSR or the accumulation of HSPs is quantitatively related to the degree of heat stress such as heating temperature and duration. How do cells sense these changes in the severity of temperature upshift? The concept that the free pool of HSP70 and other chaperones serves as a cellular sensor or a thermometer that regulates the HSR has been proposed for a long time (Craig and Gross 1991). Lindquist group originally showed that HSP70 expression was proportional to the degree of stress in *Drosophila* cells, and the elevated expression of HSP70 continued when the accumulation of functional HSP70 is blocked (DiDomenico et al. 1982). The bacterium *E. coli* harboring DnaK (HSP70 homolog) mutation failed to turn off the HSR (Tilly et al. 1983). Furthermore, yeast *S. cerevisiae* expressed a high level of HSP90 or activity of the heat shock element (HSE)-driven reporter at normal growth temperature when two *HSP70* genes were mutated (Craig and Jacobsen 1984; Boorstein and Craig 1990). These observations suggest that HSP70 acts as a negative regulator of the HSR.

In *E. coli*, *HSP* genes are under the control of the σ^{32} transcription factor, whose level and activity increase during heat shock (Straus et al. 1989; Tilly et al. 1989). DnaK functions as molecular chaperone in cooperation with DnaJ (HSP40 homolog) and GrpE (HSP110 homolog, a nucleotide exchange factor). As was expected, strains carrying mutations in DnaJ and GrpE as well as DnaK enhanced the synthesis of HSPs at normal growth temperature and failed to shut off the HSR, in part by increased synthesis and stabilization of σ^{32} (Straus et al. 1990). Furthermore, the overexpression of DnaK and DnaJ reduced not only the level of σ^{32} but

also its activity in response to heat shock (Tomoyasu et al. 1998). Thus, the amount of functional DnaK (HSP70) chaperone machinery provides tight control of the level and activity of the σ^{32} transcription factor. The GroEL/S chaperonin also controls the σ^{32} and HSR (Guisbert et al. 2004, 2008).

In mammalian cells, the activity of HSF1 and transcription level of *HSP70* gene are linked to the accumulation of proteins denatured by heat shock. When human HeLa cells grown at 37 °C were exposed to continued heat shocked at 42 °C, HSF1 was modestly activated and attenuated soon. In contrast, the high level of HSF1 activation continued when cells were heat shocked at 43 °C (Abravaya et al. 1991). Furthermore, the threshold temperature for HSF1 activation was decreased when cells were treated with an inhibitor of the synthesis of nascent polypeptides, which consist of major nonfolded proteins present in cells (Baler et al. 1992). Taken together with the fact that HSF1 interacted with HSP70, a product of its target gene, it was suggested that HSP70 acts as an autoregulatory factor of the HSR (Abravaya et al. 1992; Baler et al. 1992).

To understand molecular mechanisms of the autoregulation by HSP70, profiles of HSF1 activation and deactivation were monitored in cells overexpressing HSP70. The acquisition of the DNA-binding activity of HSF1 during heat shock was reduced in human T-cell leukemia cell line by the overexpression of HSP70 (Mosser et al. 1993), whereas it was not affected in rat fibroblasts at all (Rabindran et al. 1994; Kim et al. 1995). Rather, the shutdown of the DNA-binding activity during recovery period was accelerated in common in these cells. Morimoto group analyzed the mechanism in more detail and revealed that HSP70 and HSP40 (HDJ1) interact with the C-terminal activation domain of HSF1 and negatively regulate its transcriptional activity in vivo during attenuation of the HSR (Shi et al. 1998). HSP90 also interacts with HSF1 (Nadeau et al. 1993; Nair et al. 1996), suggesting its inhibitory role in the HSR. Using in vitro HSF1 activation system, Voellmy group found that the treatment of cells with geldanamycin, an inhibitor of HSP90, induced the DNA-binding activity of HSF1 in vitro and demonstrated that HSP90 inhibited the acquisition of the DNA-binding activity in vitro, but HSP70 did not (Zou et al. 1998a). It is proposed later that the HSP90 chaperone machinery including p23 and FKBP52 binds to the regulatory domain of HSF1 and negatively regulates both the monomer-to-trimer transition of HSF1 and its transcriptional activity (Ali et al. 1998; Duina et al. 1998; Bharadwaj et al. 1999; Guo et al. 2001). Analysis in *Drosophila* cells further shows that the synergistic interaction of HSP70 and HSP90 chaperone machineries modulates HSF activity by feedback repression (Marchler and Wu 2001) (Fig. 3.2). Moreover, TRiC/CCT chaperonin complex also interacts and represses the activity of HSF1 (Neef et al. 2014). Taken together, HSF1 is activated by the release from feedback repression by chaperone machineries during heat shock, and the activated HSF1 is subsequently repressed by the increased free pool of chaperones during recovery period.

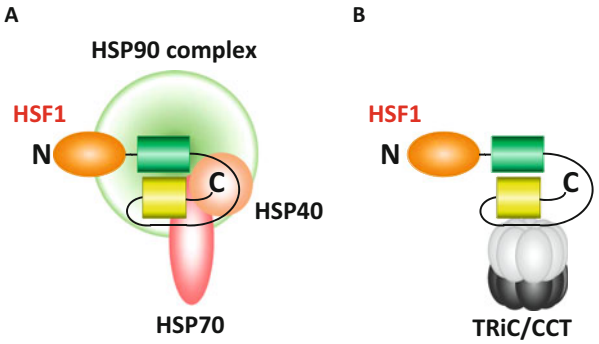

Fig. 3.2 HSF1 is maintained as an inactivate state by chaperone machineries. (**a**) HSP90 chaperone machinery including p23 and FKBP52 binds to the regulatory domain of HSF1, while HSP70 chaperone machinery containing HSP40 interacts with the C-terminal activation domain. These chaperone machineries may cooperatively inhibit the trimerization of HSF1 and suppress its transcriptional activity. (**b**) Chaperonin TRiC/CCT also binds to HSF1 and represses its activity

3.4 HSF Directly Senses Heat and Stimuli

After the development of a cell-free system that exhibits heat-induced activation of human HSF1 in vitro (Larson et al. 1988), signaling pathways that induce the DNA-binding activity have been extensively studied. The DNA-binding activity of human HSF1 in unstressed HeLa cytoplasmic extract was induced in vitro not only by heat shock but also by low pH (pH 6.0), Nonidet P-40, and urea, which affected protein conformation (Mosser et al. 1990). The in vitro HSF1 activation by these reagents was inhibited by glycerol, which stabilized protein structure. Furthermore, the treatment of *Drosophila* SL2 cytoplasmic extract with polyclonal antiserum against *Drosophila* HSF also induced the HSF DNA-binding activity in vitro (Zimarino et al. 1990). These observations suggest that HSF1 or HSF can be activated directly by undergoing a conformational change, without a covalent modification of protein.

Does purified HSF undergo a conformational change in response to heat? Kingston group purified in vitro heat-activated human HSF1 in the HeLa cytoplasmic extract by using an HSE oligonucleotide affinity column and deactivated it by denaturation using guanidine and subsequent renaturation (Larson et al. 1995). They showed that the DNA-binding activity of purified HSF1 was induced by heat shock, and the acquisition of its DNA-binding activity is accompanied with a monomer-to-trimer transition of oligomeric structure, like that in heat-shocked HeLa cells. Mouse HSF1 synthesized in *E. coli* was purified, and its DNA-binding activity was also induced in vitro by heat shock (Goodson and Sarge 1995; Farkas et al. 1998). These observations demonstrate that HSF1 can directly sense temperature upshift. Wu group carefully analyzed kinetics of the dissociation of

Drosophila HSF trimer (Zhong et al. 1998). They infected a baculovirus overexpressing *Drosophila* HSF into insect Sf9 cells and purified it (bac-HSF). A high concentration of bac-HSF solution contained predominantly trimers, which dissociated to monomers when the solution was diluted. The population of trimers reversed upon reconcentration. Furthermore, the dissociation of bac-HSF trimers was inhibited by heat, H_2O_2, and low pH, but not by 2,4-dinitrophenol, ethanol, arsenite, indomethacin, and salicylate, which induce HSF trimerization in intact cells (Zhong et al. 1998, 1999). Thus, some inducers of the HSR act directly to HSF, while others do indirectly.

3.5 Posttranslational Modifications

3.5.1 Phosphorylation

When cells are heat shocked, an apparent molecular weight of mammalian HSF1 on SDS-polyacrylamide gel dramatically increases due to multisite phosphorylation, which is called as hyperphosphorylation (Sorger et al. 1987; Larson et al. 1988; Sarge et al. 1993; Baler et al. 1993). HSF1 becomes hyperphosphorylated by heat shock, heavy metals, and amino acid analogs, but not by anti-inflammatory drugs that induce trimerization but not transcriptional activity (Cotto et al. 1996). Thus, the acquisition of transcriptional activity of HSF1 is generally correlated with its hyperphosphorylation. However, mutation analyses of multi-phosphorylation sites in HSF1 show that the hyperphosphorylation is not necessary for HSF1 to acquire full transcriptional activity during heat shock (Newton et al. 1996; Budzyński et al. 2015). It would be possible that the hyperphosphorylation facilitates dissociation of active HSF1 trimers during recovery period (Xia and Voellmy 1997).

Is there a specific phosphorylation site that promotes HSF1 transcriptional activity? Sistonen group identified that Ser230 was constitutively and stress-inducibly phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) (Holmberg et al. 2001) (Fig. 3.3). Phosphorylation of Ser230 and CaMKII enhanced HSF1 transcriptional activity in response heat shock. Voellmy group carried out an alanine scan of all serines, threonines, and tyrosines in human HSF1 using a reporter assay and also identified phosphorylation sites of exogenously expressed HSF1 in HeLa cells (Guettouche et al. 2005). They found that phosphorylation of only Ser326 contributed to HSF1 activation during heat shock. Phosphorylation on Ser326 increased rapidly during heat shock. It promoted the transcriptional activity of HSF1, but did not affect the DNA-binding activity. It turned out that Ser326 is phosphorylated by ERK1/2, and its phosphorylation promotes carcinogenesis (Dai et al. 2007, 2012). HSF1 activity may also be enhanced by polo-like kinase 1 (PLK1) and protein kinase A (PKA) during heat shock through phosphorylation of Ser216/419 and Ser320, respectively (Kim et al. 2005; Lee et al. 2008; Murshid et al. 2010).

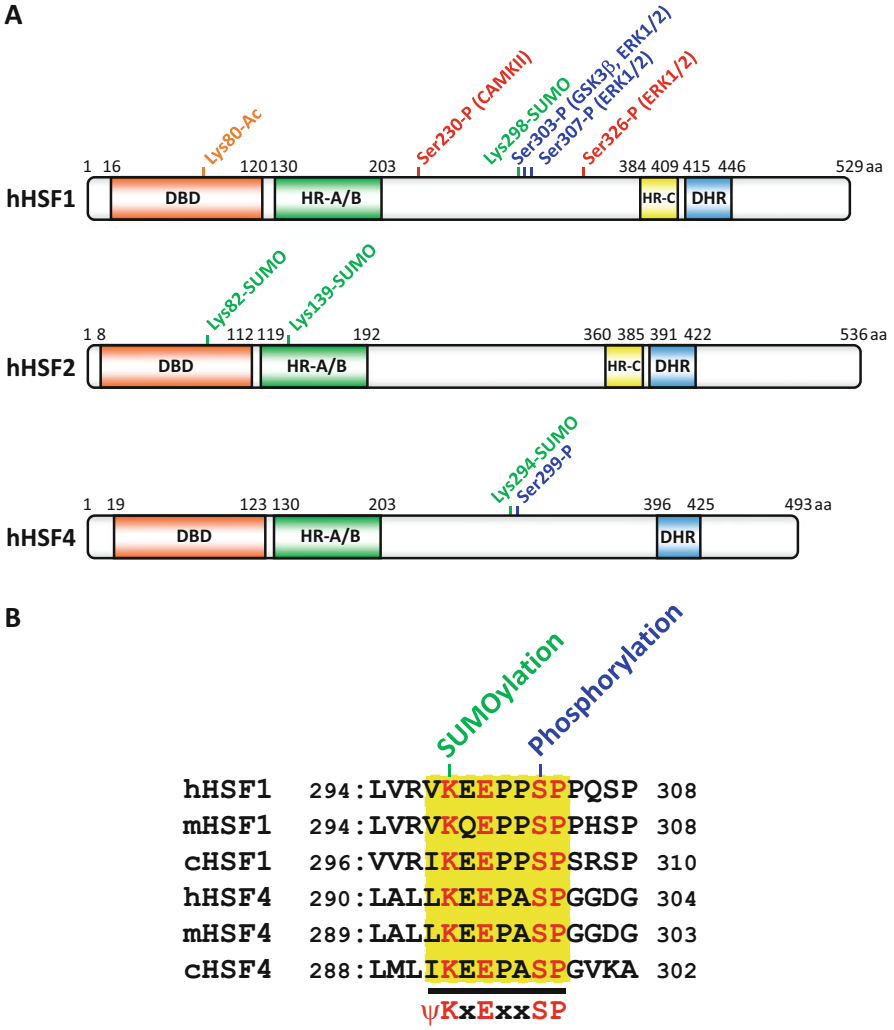

Fig. 3.3 Posttranslational modification of HSF family members. (**a**) Major phosphorylation sites that activate HSF1 are shown in *red*, and those repress it are in *blue*. Protein kinases that phosphorylate each amino acid are indicated in parentheses. Acetylation sites (*orange*) and sumoylation sites (*green*) are also shown. (**b**) Phosphorylation-dependent sumoylation motif (PDSM) in HSF1 and HSF4. Amino acid sequences containing the PDSM motif (*yellow box*) in human, mouse, and chicken HSF1 and HSF4 are aligned, and a consensus sequence is indicated (ψ is acceptable for all hydrophobic amino acids). Numbers indicate position of amino acids

In unstressed condition, the regulatory domain represses activity of the C-terminal transcriptional activation domain of human HSF1 (a.a. 221–310) (Green et al. 1995). Ser303 and Ser307 in the regulatory domain of human HSF1 are constitutively phosphorylated, and phosphorylation of these sites is required for

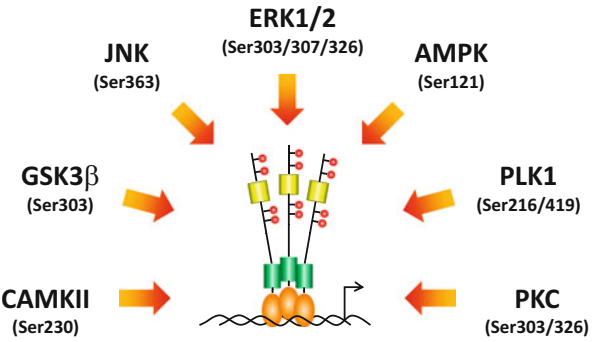

Fig. 3.4 HSF1 is phosphorylated by various kinases. Protein kinases that phosphorylate HSF1 and phosphorylation sites of each kinase are indicated (see text)

the repression of HSF1 transcriptional activity at control temperature (Knauf et al. 1996; Chu et al. 1996; Kline and Morimoto 1997). Ser307 is first phosphorylated by ERK1/2, and this modification is required for phosphorylation of Ser303 by glycogen synthase kinase 3 (GSK3) (Knauf et al. 1996; Chu et al. 1996). Ser303/307 not only regulates the transcriptional activity but also stability of HSF1. Phosphorylated Ser303/307 is recognized by an FBXW7α ubiquitin ligase, which results in degradation of HSF1 (Kourtis et al. 2015). Regulation of Ser303/307 phosphorylation could modulate HSF1 activity in response to growth control signals.

Effects of protein kinases on HSF1 activity in unstressed condition are complex. GSK3 represses not only transcriptional activity of HSF1 but also the trimer formation by unknown mechanisms (Xavier et al. 2000). ERK1/2 represses the activity of HSF1 through phosphorylation of Ser307, but activates it through that of Ser326 as described above. Protein kinase C (PKC) and c-Jun N-terminal kinase (JNK) repress HSF1 activity through Ser363 phosphorylation (Chu et al. 1998; Dai et al. 2000), and AMP-activated protein kinase (AMPK) does so through Ser121 phosphorylation (Dai et al. 2015) (Fig. 3.4).

3.5.2 Sumoylation

Sarge group first found that HSF2, another member of the HSF family, interacted with the SUMO-conjugating (E2) enzyme Ubc9 (Goodson et al. 2001). They showed that HSF2 was constitutively modified by SUMO-1 at Lys82 in the DNA-binding domain, whereas HSF1 underwent heat shock-inducible SUMO-1 modification at Lys298 in the regulatory domain (Hong et al. 2001) (Fig. 3.3). Sistonen group further studied roles of sumoylation in HSF1 function and showed that phosphorylation of Ser303 was prerequisite for the sumoylation of Lys298 (Hietakangas et al. 2003). Phosphorylation of Ser303 in HSF1 is markedly induced during heat shock, which allows Lys298 to be inducibly modified by sumoylation. A motif combining a SUMO consensus site to an adjacent proline-directed

phosphorylation site is generally conserved in other factors including HSF4, GATA1, and MEF2 (Hietakangas et al. 2006) (Fig. 3.3). Sumoylation of Lys298 of HSF1 and Lys294 of HSF4 represses the transcriptional activity of these factors. Effects of sumoylation on the DNA-binding activity are unclear (Goodson et al. 2001; Hong et al. 2001; Anckar et al. 2006), but structural analysis at least demonstrates that SUMO attachment at Lys82 of HSF2 negatively modulates the formation of protein-DNA complex (Tateishi et al. 2009).

3.5.3 *Acetylation*

Stress resistance and metabolic regulation are coupled to protein homeostasis, and key regulatory factors for these mechanisms including HSF1 and a NAD^+-dependent lysine deacetylase SIRT1 are involved in lifespan extension in *C. elegans* (Hsu et al. 2003; Morley and Morimoto 2004). Therefore, Morimoto group examined the regulation of HSF1 by SIRT1 and found that HSF1 was inducible acetylated at Lys80 by p300 during heat shock, whereas it was deacetylated by SIRT1 (Westerheide et al. 2009) (Fig. 3.3). The HSF1-DNA complex dissociates by the acetylation of Lys80. Thus, SIRT1 inhibits the attenuation of the HSR, and p300 promotes it (Fig. 3.5). SIRT1 modulators also regulate HSF1 activity (Raynes

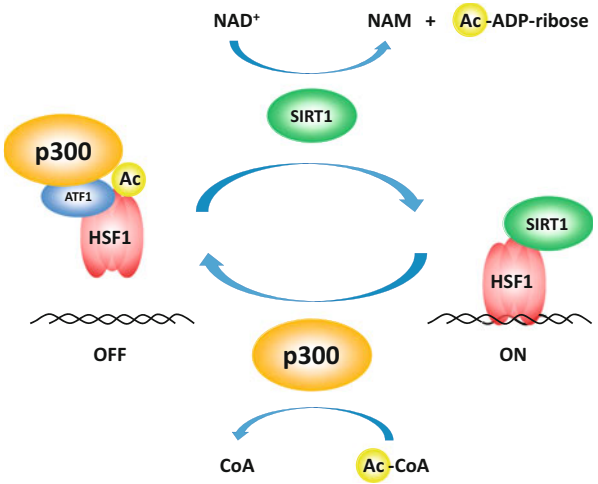

Fig. 3.5 Acetylation and deacetylation of HSF1. HSF1 is largely deacetylated by SIRT1 in unstressed condition. SIRT1 cleaves NAD^+ and produces nicotinamide (*NAM*). Simultaneously, the acetyl group is transferred from HSF1 to the ADP-ribose moiety of NAD^+ to generate O-acetyl-ADP ribose. In response to heat shock, HSF1 binds to the DNA and recruits p300 in a manner that is dependent on ATF1. p300 acetylates HSF1 by transferring an acetyl group from acetyl CoA. Acetylated HSF1 dissociates from the DNA

et al. 2013). In some condition, acetylation by p300 may also control stability of HSF1 through proteasomal degradation (Raychaudhuri et al. 2014).

In general, chromatin-modifying enzymes such as lysine acetyltransferases are enriched in active genes and are correlated with gene expression in the whole genome (Wang et al. 2009). Previous studies suggested that p300, one of histone acetyltransferases, might promote heat-inducible HSP70 expression in *Xenopus* oocytes and mammalian cells (Li et al. 1998; Xu et al. 2008). Does p300 enhance heat shock-inducible expression of HSP70 or inhibit it by deactivating HSF1? Nakai group found that the HSF1-ATF1 complex promoted the recruitment of p300 to the HSP70 promoter during heat shock (Takii et al. 2015). Inhibition of p300 accumulation by disconnecting the interaction delayed the shutdown of HSF1 DNA-binding activity during recovery period, but did not affect histone acetylation on HSP70 promoter in MEF cells. Depletion of a *Drosophila* ortholog of p300 also inhibited only the shutdown of the HSR during recovery period (Ghosh et al. 2011). These observations indicate that p300 negatively regulates the HSR by inhibiting HSF1 activity through acetylation.

3.5.4 Thiol Oxidation

Human and mouse HSF1 possesses five cysteine residues, and redox-dependent regulation of HSF1 activity through these residues has been analyzed. Liu group first showed that trimerization of human HSF1 in the cytoplasmic extracts from HeLa cells during in vitro heat shock was inhibited by diamide, a reagent that promotes disulfide bond formation (Manalo and Liu 2001). Mutation of Cys36 and Cys103 in the DNA-binding domain did not affect sensitivity to diamide, while HSF1 having mutated Cys153 in the HR-A/B domain or Cys373/378 just upstream of the HR-C domain was insensitive to diamide (Manalo et al. 2002). They proposed from these in vitro studies that disulfide bond formation between Cys153 and Cys373 or Cys378 in HSF1 inhibited the trimerization during in vitro heat shock. Thiele group showed another mechanism of redox regulation. They showed that a purified mouse HSF1 underwent a monomer-to-trimer transition by heat and hydrogen peroxide (Ahn and Thiele 2003), like *Drosophila* HSF (Zhong et al. 1998). Mutation of Cys35 and Cys105 (Cys36 and Cys103 in human HSF1, respectively) in the DNA-binding domain inhibited the trimerization and acquisition of DNA-binding activity in vitro by heat shock and hydrogen peroxide. Furthermore, these HSF1 mutants were defective in heat-inducible trimerization and activation of *HSP* genes in vivo in cells (Ahn and Thiele 2003). The heat-induced bonding between Cys36 and Cys103 in human HSF1 may form an intermolecular disulfide bond and is required for trimerization (Lu et al. 2008).

3.6 Other Regulations

Many studies have been conducted to identify proteins interacting HSF1 and regulate its activity. Many of these proteins are involved in regulation of chromatin and HSF1 transcription complexes (see Chap. 4) and in feedback repression and chemical modifications as described above (see Sects. 3.3 and 3.5). We here explain roles of some other factors that regulate the trimerization and DNA-binding activity of HSF1.

CHIP (C-terminus of HSP70-interacting protein) is one of the co-chaperones for HSP70 chaperone machinery and also has ubiquitin ligase activity (Murata et al. 2003). It turned out that overexpression of CHIP, but not other co-chaperones, uniquely induced the HSF1 DNA-binding activity and expression of HSP70 (Dai et al. 2003). Furthermore, CHIP was required for maximal HSP70 induction in cells and tissues. CHIP seems to affect complex formation of HSF1 with chaperone machineries.

HSF1 activity is repressed by DAF-2 or an insulin/IGF-1-like signaling (ILS), which is one of the major regulatory pathways for longevity in *C. elegans* (Hsu et al. 2003; Morley and Morimoto 2004). DDL-1 and DDL-2 are also involved in longevity pathways and form a complex that interacts with and stabilizes HSF1 monomers (Chiang et al. 2012). Increased ILS signaling promotes the formation of this complex, whereas reduction of this signaling results in disruption of the complex and increase in the trimerization of HSF1.

Remarkably, it is proposed that a noncoding RNA regulates the trimerization and DNA-binding activity of HSF1. Nudler group identified a translation elongation factor eEF1A as one of HSF1-interacting proteins (Shamovsky et al. 2006). eEF1A induced the HSF1 DNA-binding activity by recruiting noncoding RNA consisting of ~600 nucleotides, termed as heat shock RNA-1 (HSR1). Both eEF1A and HSR1 were required for induction of the HSF1 DNA-binding activity in vitro and in vivo and for the expression of HSP70. eEF1A also promoted the HSR in part by enhancing the transcriptional activity of HSF1 and by binding to and stabilizing HSP70 mRNA (Vera et al. 2014).

3.7 HSF Activation by Diverse Stresses

3.7.1 Environmental Stimuli

HSR was originally detected as heat-induced puffs in *Drosophila*, and an identical set of puffs could be induced by other agents (Ritossa 1962). Inducers of the heat-induced puffs are inhibitors of oxidative phosphorylation and electron transport (azide, dinitrophenol, rotenone, valinomycin); an inducer of reactive oxygen species (ROS) (menadione), anoxia, and a thiol-reactive reagent (arsenite); and an inhibitor of the synthesis of inflammatory mediators (salicylate) (Ashburner and

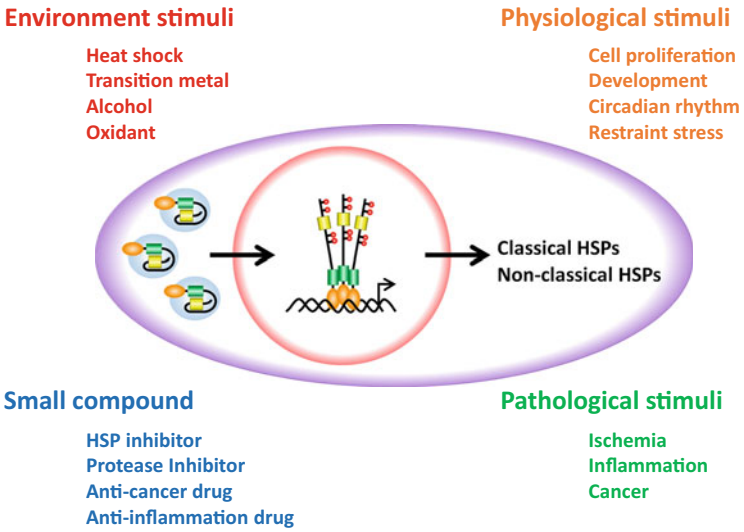

Fig. 3.6 HSF1 can be activated by diverse stimuli. Activity of HSF1 can be induced by environmental, physiological, and pathological stimuli or by the treatment with small compounds, which results in enhanced synthesis of HSPs

Bonner 1979). However, the synthesis of HSPs is not always induced in *Drosophila* and other species by these induces including oxidants and inhibitors of respiration. In mammalian and avian cells, the synthesis of HSPs is strongly induced by environmental stimuli such as heat shock, transition metals (copper, cadmium, zinc, and mercury), arsenite, and ethanol (Levinson et al. 1980; Johnston et al. 1980; Li 1983), which activate HSF1 (Fig. 3.6).

3.7.2 Physiological Stimuli

Activity of HSF1 is regulated during development. In *Drosophila*, the expression of HSPs is induced during development (Zimmerman et al. 1983) and is tightly correlated with the nuclear localization of HSF (Wang and Lindquist 1998). Regulation of HSF activity is complex in mammals because members of HSF family are involved in development (Abane and Mezger 2010) (see Chaps. 6, 7 and 8). Correlation between HSF1 activation and the induction of HSP expression during organogenesis have been shown. DNA-binding activity of HSF1 and the expression of HSPs were markedly induced in the pubertal olfactory epithelium, and HSF1 deficiency resulted in decreased expression of HSPs and impaired olfactory neurogenesis (Takaki et al. 2006). In response to the immunization of mice with sheep red blood cells, B cells proliferate in germinal center in the spleen. Simultaneously, HSF1 was activated and elevated the expression of HSPs (Inouye

et al. 2004). HSF1 deficiency impaired the proliferation of B cells in the germinal center. Mechanisms of HSF1 activation during early development and organogenesis are not known.

HSF1 is one of circadian transcription factors. Activated HSF1 induces the expression of HSPs at the onset of dark phase in mice, when they start to be behaviorally active (Reinke et al. 2008) (see Chap. 10). HSF1 is also activated in vivo by neurohormonal stimuli. Restraint stress or immobilization stress induced the HSP70 expression in the rat adrenal by activating HSF1 (Blake et al. 1991; Fawcett et al. 1994). Hypophysectomy prevented HSF1 activation, and administration of adrenocorticotropic hormone (ACTH) to hypophysectomized rats induced it. Thus, activity of HSF1 is under hormonal control in vivo.

3.7.3 Pathological Stimuli

The expression of HSPs in a tissue is markedly elevated under pathological conditions such as ischemia, trauma, and inflammation. It was shown that HSF1 in the cerebral neocortex was activated in vivo by focal cerebral ischemia, which was produced by occluding the middle cerebral and common carotid arteries in rats (Higashi et al. 1995). A high DNA-binding activity of HSF1 appeared soon after the ischemic treatment and then gradually decreased. HSF1 activation by ischemia and reperfusion was further monitored in isolated rat hearts (Nishizawa et al. 1996). Rat hearts were isolated and perfused with a buffer by the Langendorff method. In the ischemia/reperfusion experiments, isolated hearts were subjected to global ischemia by clamping the aortic cannula and then reperfused. HSF1 was slightly activated at 10 min and quickly attenuated, and reperfusion activated HSF1 again. Furthermore, repetitive ischemia/reperfusion induced a robust activation of HSF1, while its effect was inhibited in the presence of scavengers of reactive oxygen species (ROSs) (Nishizawa et al. 1999). Thus, ROSs may play an important role in the activation of HSF1 in organs by the ischemia/reperfusion injury.

Inflammation is caused by physical, chemical, infectious, and some immunological agents and is associated with increased production of various kinds of mediators (Polla et al. 1998) (see Chap. 9). Among them, proinflammatory cytokines including TNF-α, IL-1α, and IL-6 activated HSF1 in synovial fibroblast-like cells (Schett et al. 1998). HSF1 activation by TNF-α may be in part due to the TNF-α-mediated induction of ROSs (Goossens et al. 1995). HSF1 is also activated by cyclopentenone prostaglandins (PGs) including PGA_1, PGA_2, and PGJ_2, which possess anti-inflammatory, antitumor, and antiviral activities (Holbrook et al. 1992; Amici et al. 1992). These PGs exert biological effects in part through its reaction with cysteine residues of many cellular proteins (Straus and Glass 2001), which could then activate HSF1 (Santagata et al. 2012). Furthermore, a prostaglandin precursor, arachidonic acid, also activates HSF1 and induces the expression of HSPs (Jurivich et al. 1994).

HSF1 facilitates malignant transformation and cancer cell survival (Dai et al. 2007; Min et al. 2007). Protein level of HSF1 is elevated in some cancer cells (Hoang et al. 2000), and high levels of HSF1 is associated with poor prognosis in various cancers originating from the breast, colon, lung, prostate, and pancreas (Santagata et al. 2011; Mendillo et al. 2012). The upregulation of HSF1 in cancer cells is in part due to activation of mitogen-activated protein kinase (MAPK) signaling, which phosphorylates HSF1-Ser326 (Dai et al. 2012; Chuma et al. 2014) (see Chap. 13).

HSF1 inhibits progression of aging and age-related protein misfolding disease models (Hsu et al. 2003; Morley and Morimoto 2004; Fujimoto et al. 2005; Hayashida et al. 2010) (see Chap. 11). However, it is still unclear whether HSF1 is activated by the accumulation of misfolded proteins in the brain of protein misfolding diseases including polyglutamine diseases. HSF1 was not activated by aggregation-prone, polyglutamine-expanded fragments even in cells selected for the highest expression levels (Bersuker et al. 2013).

3.7.4 Small Compounds

HSF1 is activated by a variety of small compounds. Because HSF1 is usually repressed by chaperone machineries, compounds that inhibit chaperone activity may release HSF1 from the feedback repression. In fact, HSF1 is robustly activated when cells are treated with geldanamycin, a benzoquinone ansamycin antibiotic, that inhibits HSP90 function by binding to its ADP/ATP-binding pocket (Zou et al. 1998a, b). Geldanamycin and its derivatives including 17-allylamino-17-demethoxygeldanamycin (17-AAG) could be candidates of therapeutic drug for neurodegenerative disease and cancer (see Chap. 14). Geranylgeranylacetone (GGA), an acyclic polyisoprenoid, is known as an antiulcer drug and also induces the HSR (Hirakawa et al. 1996), in part by binding to HSP70 and disrupting the HSF1-HSP70 interaction (Otaka et al. 2007). Furthermore, HSF1 is activated by proteasome inhibitors including lactacystin and MG132 and amino acid analogs, azetidine, and canavanine (proline and arginine analogs, respectively), by inducing the accumulation of misfolded proteins in cells (Kelley and Schlesinger 1978; Kawazoe et al. 1998; Mathew et al. 1998; Pirkkala et al. 2000). Moreover, a lot of thiol-reactive compounds including natural product celastrol, a quinone methide triterpene, also activate HSF1 probably through modification of cysteines in cellular target proteins (Westerheide et al. 2004; Trott et al. 2008; Santagata et al. 2012). Bimoclomol, a nontoxic hydroxylamine derivative, is a co-inducer of HSPs that elevates levels of HSPs under stress conditions (Vígh et al. 1997; Kieran et al. 2004), in part by binding to HSF1 complex directly (Hargitai et al. 2003). Furthermore, HSF1 is activated by anticancer drugs including 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (an alkylating drug), vincristine (a microtubule-damaging drug), and bleomycin (a DNA-damaging drug) by unknown mechanisms (Kroes et al. 1991; Kim et al. 1999). BCNU strongly induces

the HSR, while the latter two drugs induce only the expression of mitochondrial HSPs. Moreover, anti-inflammatory drugs, sodium salicylate and indomethacin, induce the HSF1 DNA-binding activity without upregulation of the HSP expression (Jurivich et al. 1992; Lee et al. 1995).

3.8 Future Perspectives

In response to proteotoxic stresses including heat shock and proteasome inhibition, the activation of HSF1 is triggered by the rapid elevation of misfolded proteins within cells, which leads the release from feedback repression of HSF1 by HSPs. Heat shock also promotes the monomer-to-trimer transition of HSF1 directly. Furthermore, the activation and shutdown of HSF1 activity are associated with posttranslational modifications including phosphorylation and acetylation. Because these posttranslational modifications are complex, impact of each modification on the regulation of HSF1 activity in response to a specific proteotoxic stress is not well understood. Furthermore, it is still unclear whether is there any other factor than HSPs that directly regulates HSF1 activation during proteotoxic stress.

It is proposed that each cell possesses a unique proteostasis capacity or a buffering capacity against protein misfolding, which is determined by the balance of protein synthesis, folding, and degradation (Gidalevitz et al. 2010). Not only the status of protein folding and degradation but also that of protein synthesis regulates HSF1 activity (Santagata et al. 2013), indicating a tight link between the proteostasis capacity and HSF1 activity even in unstressed conditions. The basal HSF1 activity is required for maintenance of the proteostasis capacity in unstressed conditions and delays physiological aging and the progression of a model of misfolding diseases (Hsu et al. 2003; Morley and Morimoto 2004; Hayashida et al. 2010). Thus, regulation of the basal HSF1 activity may modulate aging and age-related protein misfolding diseases. It should be clarified how HSF1 activity is strictly regulated by metabolic signaling pathways under physiological conditions in future.

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Chapter 4
Transcriptional Regulation by HSF

Mitsuaki Fujimoto

Abstract The induction and inhibition of gene expression occur constantly in cells. Stress-inducible genes are equipped with a mechanism that allows them to be instantaneously induced; RNA polymerase II (Pol II) stops at a position several tens of bases ahead of transcription initiation point in order to prepare for the induction of expression following stress. Of the known stress-inducible genes, the heat shock protein *HSP70* gene has been actively studied using *Drosophila melanogaster* as a model organism. In the normal state, GAGA factor binds to the HSP70 promoter region in order to open the surrounding chromatin structure to prepare the condition to which HSF, Pol II, general transcription factors and coactivators can readily access. The GAGA factor is absent in higher animals, and mammalian HSF1 instead plays its role. Heat stress releases Pol II pausing in both *Drosophila* and higher animals, and HSP70 is rapidly induced, but through different molecular mechanisms in these animals. HSF1 induces not only the classical but also nonclassical HSP groups involved in various processes in the normal state and in response to heat shock. In addition to turning on transcription, HSF1 turns off transcription in conjugation with corepressors. Through these regulations, HSF1 plays roles in balancing the quality and quantity of proteins within a cell.

Keywords HSF1 • HSP70 • RNA polymerase II pausing • GAGA factor

4.1 Introduction

When cells are exposed to high temperatures, they induce the expression of heat shock proteins, which are responsible for adjusting the capacity of protein homeostasis or proteostasis (Lindquist 1986; Balch et al. 2008). This is called as the heat shock response (HSR) and is mainly regulated at the transcriptional level by heat shock factor (HSF) in yeast and *Drosophila* (Wu 1995). To date, HSF1, HSF2, HSF3, and HSF4 have been identified in higher animals, and HSF1 is a master

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regulator of HSP expression (Morimoto 1998; Akerfelt et al. 2010; Fujimoto and Nakai 2010). HSF1 is typically present in cells as an inactive monomer and is converted to a DNA-binding trimer, acquiring the potential to activate transcription upon heat stress. The molecular mechanisms underlying the induction of *HSP70* gene transcription have been analyzed in detail in *Drosophila melanogaster* (Lis 2007; Adelman and Lis 2012; Petesch and Lis 2012). Analyses in higher animals have recently progressed, with differences being identified in the molecular mechanisms responsible for transcriptional regulation by HSF1 from that in *Drosophila*. Thus, we herein described the mechanism underlying general stress-inducible gene expression and the molecular mechanisms of constitutive and heat shock-induced HSP70 expression, as well as the genes targeted by HSF.

4.2 Inducible Gene Expression in General

To rapidly activate gene expression in response to stimuli, RNA polymerase and regulators have to be recruited to the promoter region to initiate transcription (Weake and Workman 2010). In bacteria, RNA polymerase is a multi-subunit complex, and one subunit, σ factor, binds directly to the promoter region. In eukaryotic cells, three types of RNA polymerases (I, II, and III) have been detected in the nucleus (Roeder and Rutter 1969), and Pol II is mainly involved in the transcription of protein-encoding genes. Eukaryotic Pol II itself cannot bind to the promoter region directly. One or several transcription factors initially bind to the specific DNA sequence present in the promoter region and form a complex with coactivators such as histone acetyltransferases and chromatin remodeling complexes (Reyes et al. 1997). This complex facilitates the recruitment of the transcription preinitiation complex (PIC), consisting of basal transcription factors (GTFs) and Pol II, to the promoter region (Fig. 4.1a). In order to stabilize the interaction between these two complexes, a mediator complex bridges them (Kim et al. 1994). TFIIH, a component of GTFs, unwinds the DNA double helix using its helicase activity and also phosphorylates serine at position 5 (Ser5) in the C-terminal domain (CTD) of the Pol II subunit (Kim et al. 2000; Komarnitsky et al. 2000). Pol II dissociates from the GTFs and starts to synthesize RNA. On the stress-inducible *HSP70* gene, Pol II is stalled after moving to a position +20 to +60 from the transcription start site (TSS) (Lee et al. 1992). This phenomenon is termed promoter-proximal pausing and is regulated by negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) (Wada et al. 1998) (Fig. 4.1b). Pol II pausing is released, and the transcription elongation reaction is induced by positive transcription elongation factor b (P-TEFb), which phosphorylates Ser2 of the Pol II CTD (Yamaguchi et al. 1999) (Fig. 4.1c). Pol II pausing had been previously considered to be a mechanism responsible for the rapid induction of inducible genes after stimuli. However, a recent ChIP-seq analysis reveals that Pol II pausing occurs in a lot of active genes in many cells, including embryonic stem (ES) cells (Sun et al. 2010; Min et al. 2011).

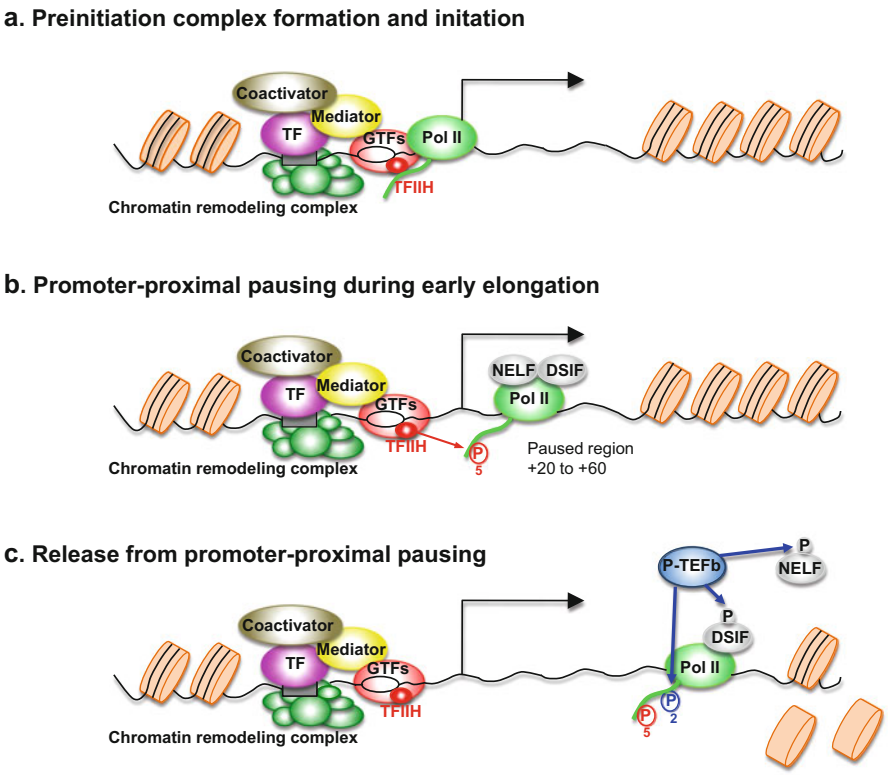

Fig. 4.1 Regulation of transcription initiation and elongation. (**a**) Assembly of PIC and transcriptional initiation. (**b**) RNA polymerase II is stalled at promoter-proximal region. (**c**) Paused Pol II is released. *Pol II* RNA polymerase II, *TF* Transcription factor, *GTFs* General transcription factors, *P-TEFb* Positive transcription elongation factor b, *DSIF* DRB sensitivity-inducing factor, *NELF* Negative elongation factor

4.3 Regulation of Constitutive Gene Expression by HSF1

The accumulation of HSF in developmental genomic loci under normal state has been detected in *Drosophila* (Westwood et al. 1991), and HSF is required for normal development (Jedlicka et al. 1997; Wang and Lindquist 1998). In avian cells, the DNA-binding activity of both HSF1 and HSF3 is heat-inducible, and HSF1 and HSF3 redundantly upregulate the expression of *HSP90α* gene in unstressed chicken B lymphocyte DT40 cells (Nakai and Ishikawa 2001). These results indicate that HSF family members regulate constitutive expression of target genes.

In mammalian cells, HSF1 is mostly present as an inactive monomer in unstressed conditions, but several percent of HSF1 is present as a trimer that binds to DNA (Mosser et al. 1988; Inouye et al. 2007). In fact, the constitutive expression of HSP25, αB-crystallin, and HSP70 in the heart was reduced in HSF1-

null mice (Yan et al. 2002). These mice also exhibit enlargement of the cerebral ventricles and impairment of the sensory organs such as the cochlea and olfactory epithelium (Sugahara et al. 2003; Takaki et al. 2006). Furthermore, HSF2 is involved in neurogenesis and gametogenesis (Kallio et al. 2002; Wang et al. 2003), and HSF4 is required for maintenance of the lens in humans and mice (Bu et al. 2002; Fujimoto et al. 2004). Thus, HSF1 and other family members play significant roles under unstressed conditions.

4.3.1 HSP70 *Gene in* Drosophila

Among the classical heat shock genes, the transcriptional mechanism of *HSP70* gene has been extensively studied. In unstressed *Drosophila* cells, GAGA factor (GAF) constitutively binds to the HSP70 promoter and opens the chromatic structure (Tsukiyama et al. 1994; Shopland et al. 1995) (Fig. 4.2a). As a result, a promoter-proximal region within several hundred bases from the transcription start site (TSS) of the *HSP70* gene does not form nucleosomes, while RNA polymerase II (Pol II) is stalled at a position +20 to +60 downstream of the TSS. ChIP-seq analysis shows that HSF binds in vivo to the promoters of HSP22, HSP23, HSP26, and HSP83, but HSF binding to the HSP70 promoter is too low to be detected (Guertin and Lis 2010). These observations suggest that *Drosophila* HSF influences constitutive gene expression.

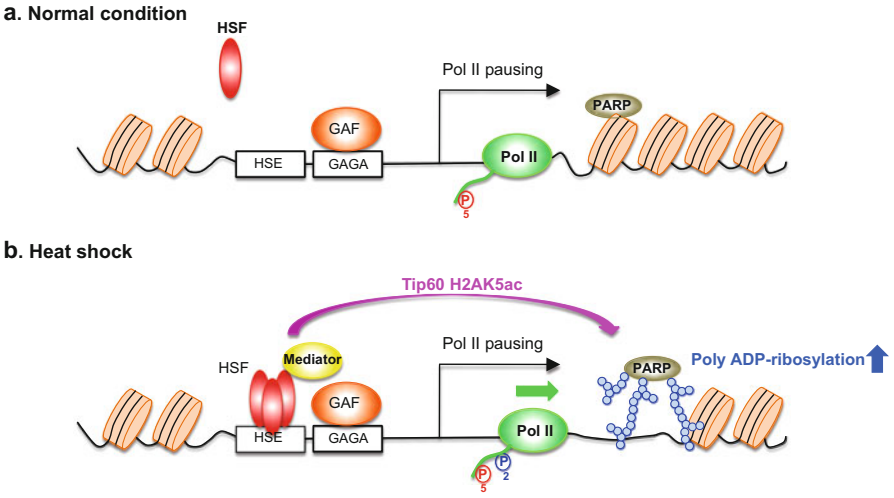

Fig. 4.2 Transcriptional regulation of *HSP70* gene in *Drosophila*. (**a**) Normal condition. (**b**) Heat shock condition. For explanation, see the text

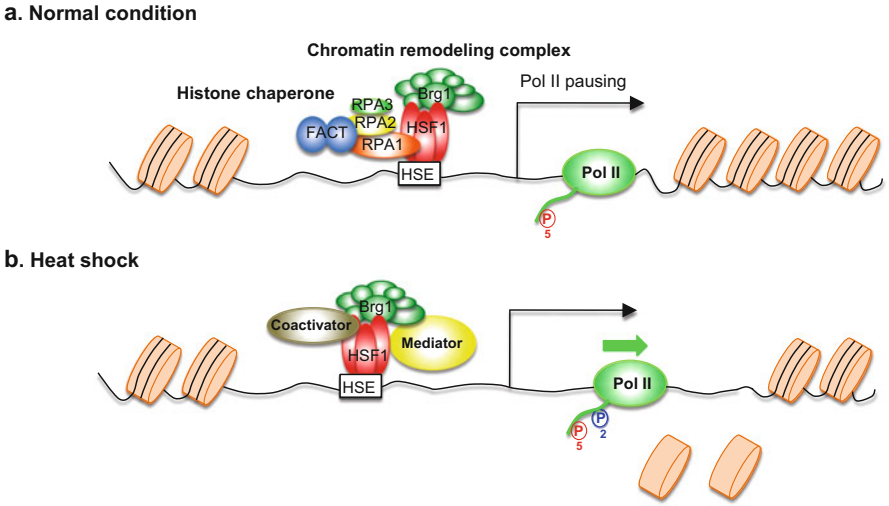

Fig. 4.3 Transcriptional regulation of *HSP70* gene in mammals. (**a**) Normal condition. (**b**) Heat shock condition. For explanation, see the text

4.3.2 HSP70 *Gene in Mammals*

The GAF is absent in higher animals. Instead, HSF1 binds to the HSP70 promoter in normal conditions. However, previous studies show that HSF1 is unable to bind stably to nucleosomal DNA by itself (Becker et al. 1991; Taylor et al. 1991). How does HSF1 access to the nucleosomal DNA? It is revealed that replication protein A (RPA), which is involved in the replication and repair of DNA, is recruited to HSF1. This HSF1-RPA complex binds stably to the HSP70 promoter in vivo by recruiting a histone chaperone and facilitates chromatin transcription (FACT) and a chromatin remodeling complex containing Brahma-related gene 1 (BRG1), resulting in opening of the chromatin structure and facilitating the loading of Pol II (Fujimoto et al. 2012) (Fig. 4.3a). Since HSF4 also interacts with RPA1, it may have a similar function to that of HSF1.

Histone variant mH2A1.1 and poly(ADP-ribose) polymerase 1 (PARP1) are also recruited to the HSP70 promoter in unstressed condition and are involved in induction of the HSP70 expression (Quararhni et al. 2006). It should be determined whether these factors are associated with constitutive expression of HSF1 target genes.

4.4 Regulation of Heat Shock-Induced Gene Expression by HSF

HSF1, which is mostly present as a monomer in unstressed conditions, is released from negative regulation by HSP70 and HSP90 and forms a trimer that binds to the HSE in the HSP promoters upon heat shock (Shi et al. 1998; Zuo et al. 1998). As a result, HSF1 binding robustly increased in the promoters, which is followed by efficient recruitment of the preinitiation complex containing Pol II and coactivators such as SWI/SNF chromatin remodeling complex. The latter removes the nucleosome structure of target genes and promotes elongation reactions (Corey et al. 2003; Brown et al. 1998). Because the removal of nucleosomes in the genes markedly influences the efficiency of transcription by Pol II, mechanisms of opening the chromatin structure by HSF have been extensively studied in *Drosophila* (Core and Lis 2008).

4.4.1 HSP70 *Gene in* Drosophila

In the *HSP70* gene, the initial removal of nucleosomes occurs only 30 s after heat shock (Petesch and Lis 2008). Activated HSF is rapidly recruited to the HSE sequence and promotes the acetylation of histone H2K5 by a Tip60 histone acetyltransferase (Petesch and Lis 2012) (Fig. 4.2b). This modification activates poly(ADP-ribose) polymerase (PARP), which was known to be necessary for heat shock-induced puffing and the induction of HSP70 in *Drosophila* (Tulin and Spradling 2003). PARP is associated with the 5′ end of the *HSP70* gene in unstressed condition (Fig. 4.2a). Activation of PARP causes the redistribution of PARP and the accumulation of poly(ADP-ribose) throughout the *HSP70* loci (Petesch and Lis 2012) (Fig. 4.2b), resulting in opening of the chromatin structure and transcription (Beneke 2012). The HSE-bound HSF1 further promotes the recycling of Pol II through continuous stable binding (Yao et al. 2006).

HSF also promotes the transcription by recruiting coactivators such as a component of mediator complex (Park et al. 2001), a histone methyltransferase trithorax, and a histone acetyltransferase CREB-binding protein (CBP) (Smith et al. 2004). In addition, factors involved in the transcription elongation reaction including Spt6 and FACT are recruited in the HSP70 promoter (Kaplan et al. 2000; Andrulis et al. 2000; Saunders et al. 2003; Ardehali et al. 2009).

4.4.2 HSP70 *Gene in Mammals*

A little amount of HSF1 is present at the HSP70 promoter in vivo in unstressed mammalian cells (Fujimoto et al. 2012), and HSF1 occupancy robustly increases

upon heat shock (Vihervaara et al. 2013; Takii et al. 2015). HSF1 then recruits a SWI/SNF chromatin remodeling complex containing BRG1 through its C-terminal activation domain (Corey et al. 2003), a mediator complex containing a subunit MED26 (Takahashi et al. 2011), a coactivator activating signal cointegrator 2 (ASC-2) (Hong et al. 2004), and a histone methyltransferase mixed-lineage leukemia 1 (MLL1) (Chen et al. 2014) to promote the induction of HSP70 expression (Fig. 4.3b). Another transcription factor, activating transcription factor 1 (ATF1), facilitates the recruitment of BRG1 (Takii et al. 2015). A lysine acetyltransferase p300 is also recruited to the promoter in cooperation with ATF1 and stress-responsive activator of p300 (STRAP) but plays a role in the shutdown of HSF1 activity during recovery phase (Xu et al. 2008; Takii et al. 2015). Because histones in the promoter are highly acetylated during heat shock, some lysine acetyltransferase may be involved in the HSP70 expression (Zelin and Freeman 2015). Screening of genes involved in the transcriptional regulation by HSF1 shows that many chromatin modification factors and nuclear proteasomes are involved in the heat shock response (Raychaudhuri et al. 2014). These findings further suggest that the HSF1 transcription complex is regulated by a lot of factors, which may rapidly assemble to the HSP70 promoter upon heat shock.

4.5 Shutdown of HSF Activity

When cells are released from stress, HSF1-induced transcription is shut down. HSP70 and HSP40 reach excessive levels and bind to the transcription activation domain of HSF1, thereby suppressing its activity (Abravaya et al. 1992; Baler et al. 1992) (Fig. 4.4). In addition, a corepressor CoREST is also recruited to the promoter through an interaction with HSP70 and inhibits HSF1-induced transcription (Gómez et al. 2008). HSF1 has also been shown to recruit the lysine acetyltransferase p300 to the HSP70 promoter and be acetylated. HSF1 acetylation releases the HSF1 trimer from DNA and accelerates the attenuation of the HSR (Westerheide et al. 2009). ATF1 phosphorylation was found to be important for this heat stress-induced recruitment of p300/CBP (Takii et al. 2015). HSP90 interacts with HSF1 and promotes the conversion of DNA-binding trimers to monomers (Zuo et al. 1998). TRiC/CCT chaperone complex also binds to HSF1 and suppresses the expression of HSF1 target genes (Neef et al. 2014). Accordingly, HSF1-induced transcription is attenuated through two processes: reductions in the transcription activation potential and DNA-binding activity of HSF1.

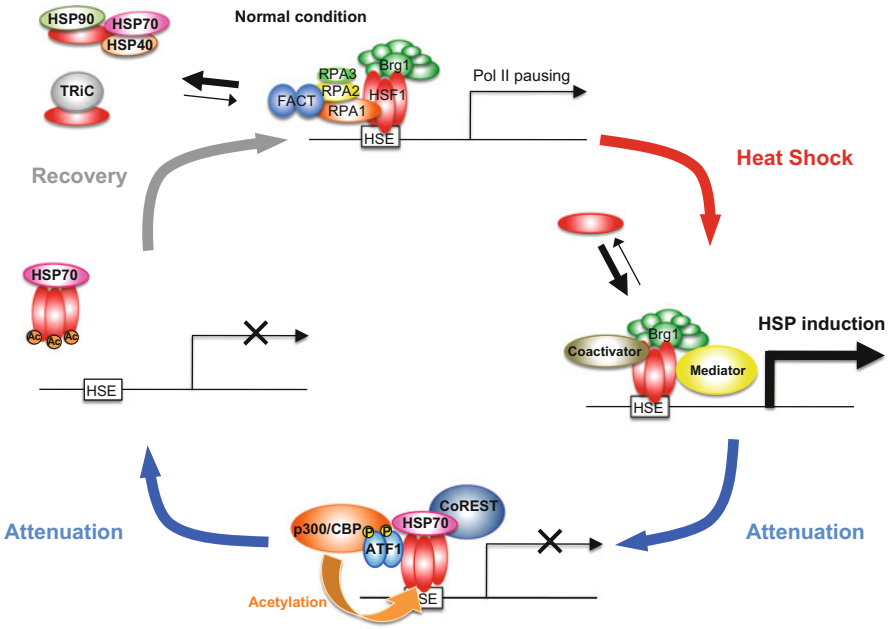

Fig. 4.4 HSF1 activation and attenuation. Monomeric HSF1 interacts with HSP90, HSP70, and HSP40 under unstressed conditions. HSF1 is dissociated from HSP90 and HSP70 upon heat shock, forms a trimer, and binds to the heat shock elements (HSEs). Coactivators including BRG1 and mediators assemble via HSF1 in the promoter region. HSP70, HSP40, and CoREST suppress HSF1 transcriptional activity during attenuation phase, and p300/CBP releases HSF1 from DNA through its acetylation

4.6 Suppression of Gene Expression by HSF

The expression of HSF1 targets including heat shock gene is induced under heat stress conditions, while the transcription of many other HSF1 targets is inhibited. HSF1 at least in part mediates a genome-wide and massive histone deacetylation by recruiting the histone deacetylases, HDAC1 and HDAC2 (Fritah et al. 2009). More generally, the transcript of Alu sequence, which is comprised of a repetitive sequence, is markedly induced by stresses such as heat shock (Liu et al. 1995). This Alu RNA binds to Pol II in the preinitiation complex present on the promoter and inhibits the transcription (Allen et al. 2004; Espinoza et al. 2004; Yakovchuk et al. 2009, 2011).

4.7 Genes Regulated by HSF

HSF was originally discovered as a regulator of HSP expression. Thereafter, DNA microarray and ChIP-seq analyses demonstrated that the HSF family members regulate the expression of not only a small set of *HSP* genes but also several hundreds of *non-HSP* genes in response to heat shock. In yeast, HSF binds to approximately 3 % of the entire genome, with expression being induced in more than half of genes in the bound regions (Hahn et al. 2004; Hu et al. 2007). In this section, we describe target genes of the HSF family members in detail by focusing mainly on the analyses in mouse and human cells.

4.7.1 HSF1

A DNA macroarray analysis was performed using wild-type mouse embryonic fibroblasts (MEF) and HSF1-null MEF cells. This analysis revealed that HSF1 induced and suppressed the expression of many genes other than *HSPs* (Trinklein et al. 2004). Changes in constitutive gene expression were further investigated in HSF1-knockdown MEF cells (our analysis; GEO accession number GSE38412) (Fujimoto et al. 2012). A gene ontology analysis showed that HSF1 promoted the expression of genes involved in transcription, signal transmission, and development. In contrast, HSF1 inhibited the expression of genes involved in immune response, metabolism, and apoptosis (Fig. 4.5a). Under heat shock condition, the induction of genes involved in protein folding including *HSP* genes (response to stress) was observed (Fig. 4.5b). Genes involved in cell proliferation and apoptosis were also induced in this condition, while genes related to the cell cycle and DNA damage were inhibited (Fig. 4.5b). HSF1 was necessary for the induction of HSP genes, but is not required for all of heat-inducible genes. Experiments using HeLa cells also demonstrated that HSF1 caused the induction of *HSP* and *non-HSP* genes during heat shock. HSF1 knockdown resulted in the marked reduction of the expression of genes in categories related to protein folding, anti-apoptosis, RNA splicing, and ubiquitination (Todd et al. 2006). In contrast, the overexpression of actively mutated HSF1 in HeLa cells induced *non-HSP* genes involved in proteostasis, such as *NFATc2* and *CRYAB* (Hayashida et al. 2010). These findings show that HSF1 induces the expression of *HSP* genes in response to heat shock in all cell types, whereas it regulates different sets of *non-HSP* gene in each cell type.

The DNA microarray analysis does not clarify whether HSF1 directly regulates its target genes. To uncover its direct targets, genome-wide HSF1-binding regions have recently been analyzed in *Drosophila* and human cells using the ChIP-seq analysis (Table 4.1). In *Drosophila*, HSF binds to the promoter regions of *HSP* genes after heat shock and is needed to open the chromatin structure around the binding region (Guertin and Lis 2010). Furthermore, HSF is also recruited to the promoters of *non-HSP* genes, including *TOM34*, *TAF7*, and *GSTD*, after heat shock

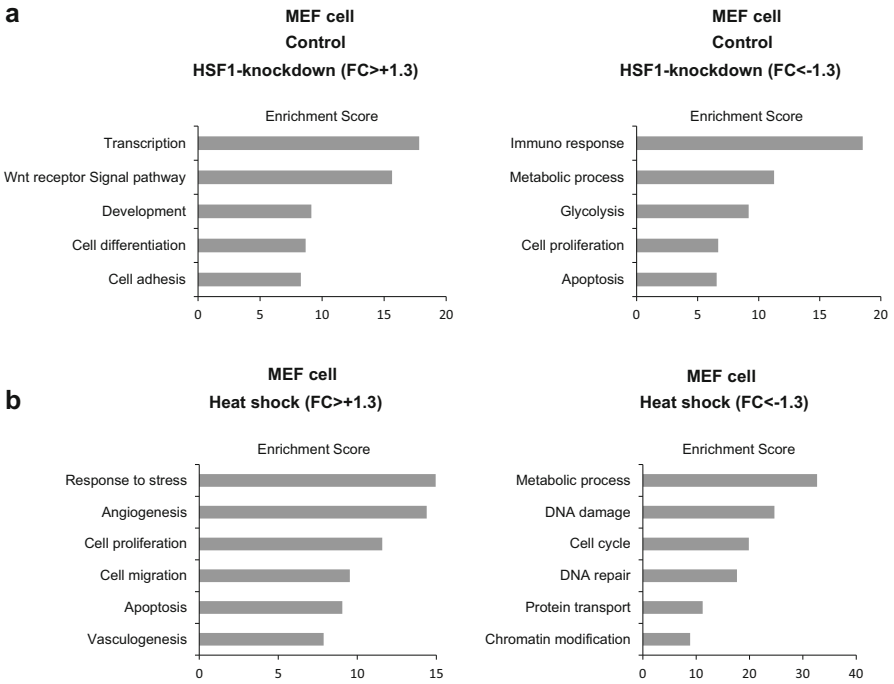

Fig. 4.5 Ontology analysis of the regulated genes under unstressed and heat shock conditions. (**a**) Gene ontology enrichment analysis of unstressed condition in scramble RNA-treated and HSF1-knockdown MEF cells. A DNA microarray analysis was performed using MEF cells, which are infected for 72 h with Ad-sh-mHSF1-KD or Ad-sh-SCR. Fold changes >1.3 or <-1.3 are shown. Gene Expression Omnibus (GEO) accession number is GSE38412 (Fujimoto et al. 2012). (**b**) Gene ontology enrichment analysis of heat-inducible genes in MEF cells. MEF cells were treated with heat shock at 42 °C for 1 h and arrowed to recover at 37 °C for 6 h. Fold changes >1.3 or <-1.3 are shown. GEO accession number is GSE54060

(Gonsalves et al. 2011). These binding regions show 40 % homology with the chromosomal distribution of HSF identified by Westwood in 1991 (Westwood et al. 1991).

In MEF cells, the HSF1-binding region was identified at 316 sites under unstressed condition and 2,291 sites under heat shock condition (Fig. 4.6a) (Takii et al. 2015). HSF1 is weakly recruited to the promoters of *HSP* and co-chaperone *BAG3* genes under unstressed conditions, but the recruitment is rapidly enhanced by heat shock (Fig. 4.6b). A similar effect is observed in the promoter regions of *non-HSP* genes, such as the *NAA30* and *SLC5A3* genes (Fig. 4.6c). It is worth noticing that HSF1 binds to some genes including *IL-6* at a constant level under both unstressed and heat shock conditions (Fig. 4.6d). The ChIP-seq analysis has also been performed on various cancer cells. Genes bound by HSF1 are related to tumorigenesis, cell cycle, apoptosis, energy metabolism, and immune processes (Mendillo et al. 2012). Comparison of the DNA microarray and ChIP-seq analyses

Table 4.1 ChIP-seq analysis of HSF1 and HSF2 target genes

	Species	Assay	Condition	Target gene	Gene ontology	References
HSF1	*Drosophila*	ChIP-seq	Heat shock	HSP26, HSP23, HSP27, HSP68, etc.	–	Guertin and Lis (2010)
	Drosophila	ChIP-seq	Heat shock	HSP83, DNAJ, TOM34, TAF7, etc.	Response to stress	Gonsalves et al. (2011)
					Transferase activity	
					Regulation of transcription	
	Mouse (MEF)	ChIP-seq	Heat shock	HSPA1A, DNAJB1, BAG3, NAA30, SLC5A3, etc.	–	Takii et al. (2015)
	Human (cancer cell)	ChIP-seq	Heat shock	HSPA1A, HSPE1, HSPH1, RPL22, CDC6, IL7R, STAT6, etc.	Protein folding and stress response	Mendillo et al. (2012)
					Translation	
					Cell cycle and signaling	
					Immune process	
	Human (K562)	ChIP-seq	Heat shock	HSPA1A, HSPB1, HSPH1, HSPD1, HSPE1, etc.	–	Vihervaara et al. (2013)
HSF2	Human (K562)	ChIP-seq	Heat shock	HSPA1A, HSPB1, HSPH1, HSPD1, HSPE, MLL, etc.	–	Vihervaara et al. (2013)

indicates that genes involved in immune response and apoptosis are negatively regulated by HSF1. Thus, HSF1 binds to the promoters of *HSP* and *non-HSP* genes and up- or downregulates its target genes depending on cell types.

4.7.2 HSF2

HSF2-null mice suffer from brain abnormalities as well as meiotic and gametogenesis defects in both genders (Kallio et al. 2002). Detailed analysis of the brain abnormality showed that migration of neurons in the superficial layers is impaired, in part due to the reduced expression of p35, an activator of cyclin-dependent kinase 5 (Cdk5). HSF2 binds directly to the p35 promoter directly and regulates the expression of p35, which is essential for neuronal migration (Chang et al. 2006).

Consistent with the fact that both HSF1 and HSF2 recognize the similar consensus HSE sequence in vitro, these two factors cooperatively regulate the

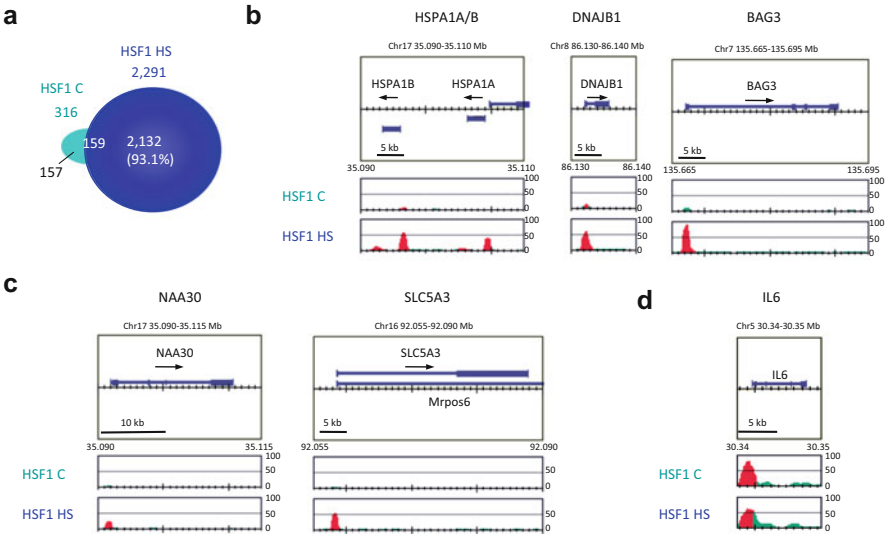

Fig. 4.6 HSF1 occupies the promoters of regulated genes under unstressed and heat shock condition. (**a**) Venn diagram of HSF1 ChIP-seq binding peaks in unstressed (HSF1 C) and heat-shocked (HSF1 HS) MEF cells. Numbers of binding peaks are indicated. (**b**–**d**) ChIP-seq binding profiles for *HSP* (chaperone) genes (*HSPA1A/HSPA1B*, *DNAJB1*), a co-chaperone gene (*BAG3*), *non-HSP* genes (*NAA30*, *SLC5A3*), and *IL6* gene under unstressed and heat shock conditions

expression of HSPs and αB-crystallin by binding directly to the promoters of these genes (Ostling et al. 2007; Shinkawa et al. 2011). To identify direct target genes of HSF1 and HSF2, ChIP-seq analysis was performed using cycling and mitotic K562 cells. HSF2 target genes include *HSPs*, *polyubiquitin*, and many other targets such as a trithorax homologue *MLL* (Vihervaara et al. 2013).

4.7.3 HSF4

HSF4 is highly expressed in the lens of mice, while it is ubiquitously expressed in many other tissues at low levels (Tanabe et al. 1999; Fujimoto et al. 2004). Consistently, *HSF4* mutation is associated with human hereditary cataract (Bu et al. 2002). Analysis of HSF4-null mice showed that the lens fiber cells contain inclusion body-like structure, whereas lens epithelial cells exhibit increased proliferation and premature differentiation (Fujimoto et al. 2004). These abnormalities may be caused in part by dysregulation of HSF4 target genes such as *γ*- and *αB-crystallins* and *FGFs*. HSF4 regulates gene expression in cooperation with a SWI/SNF chromatin remodeling complex containing BRG1. In order to identify the genes regulated by HSF4 and BRG1, a DNA microarray analysis was performed using the lenses of dominant-negative BRG1 transgenic mice and HSF4-null mice. Both HSF4 and BRG1 were required for the expression of *HSPB1*, *CRYAB*,

TFB1M, and *DNASE2b* (He et al. 2010). Furthermore, a two-dimensional electrophoresis was performed to identify differences in the proteins expressed in the lens of wild-type and HSF4-null mice. HSF4 inhibits the expression of enzymes (ALDOC, KPYM, and AL3A1) and an intermediate filament protein vimentin and induces that of crystallins in the lens. HSF4 binds at least to the vimentin promoter in vivo and may directly regulate its expression (Mou et al. 2010). HSF4 target genes in other tissues are not clear yet.

4.8 Future Perspectives

The HSR is a robust and prompt transcriptional response to environmental stresses. Therefore, the induction of HSPs has been considered to be a model system to elucidate the mechanisms of inducible gene expression. Of these genes, *HSP70* gene has been investigated in detail in *Drosophila*. Although the mechanisms underlying transcriptional regulation by HSF have been uncovered in *Drosophila*, some processes remain unclear in higher animals, and the coactivators that accumulate to the HSF1 transcriptional complex during heat shock have not yet been identified. The difficulty associated with the identification of these factors may be due to dynamic changes in HSF1 binding to the coactivators during heat shock. Thus, these factors warrant further study.

Many researchers have identified HSF1 target genes under control and heat shock conditions. However, differences in HSF1-mediated gene expression have been observed among cell lines. Even the genes that are commonly regulated by HSF1 in all cell lines remain unknown, apart from *HSPs*. HSF1 regulates the expression of many genes, which maintain proteostasis capacity in cells and are also involved in cell growth and malignant transformation. Advances in the analyses of HSF1-binding regions and the epigenomes near these regions may clarify the influence of HSF1 on the chromatin structure and its modification.

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Part II
Physiological Roles of HSF

Chapter 5
HSF1 Regulation in Aging and Its Role in Longevity

Netta Shemesh and Anat Ben-Zvi

Abstract Protein folding and clearance machineries are required for the maintenance and function of the proteome. Quality control systems and activation of stress signaling pathways have, therefore, profound consequences on the long-term health of the cell and, by extension, on lifespan. Aging is associated with loss of cellular function, increased vulnerability to stress, and enhanced susceptibility to disease. Over the course of a lifespan, proteome stability is substantially impacted by mutations, by processing errors, and by the acute effects of environmental stresses. Recently, the function of cellular protein quality control networks, as well as stress signaling pathways, was shown to be differentially regulated over the course of life, leading to reduced proteostasis capacity and decreased stress response activation during adulthood. Proteostatic collapse can be partially mitigated by overexpression of stress response transcription factors, such as HSF1, or by enhancing the activity of quality control systems, which can have significant beneficial effects on lifespan extension and suppression of age-related misfolding diseases. However, the link between proteostasis and lifespan can also be uncoupled, for example, by cell-nonautonomous stress signaling. Here, we will examine how proteostasis changes with age. We will then focus on HSF1 and review its roles in lifespan regulation, as well as how HSF1 function is modulated with age. Finally, we will examine the cell-nonautonomous regulation of HSF1, specifically during aging.

Keywords Aging • Chaperone • Dietary restriction • Germline stem cells signaling • Insulin/IGF-1 signaling • Heat shock • HSF1 • Longevity • Sirtuins • Stress

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5.1 Introduction

Cellular quality control networks are comprised of molecular chaperones, degradation machineries, and cytoprotective stress responses, such as the heat shock response (HSR) and the unfolded protein response (UPR) (Akerfelt et al. 2010; Haynes et al. 2013; Hetz 2012). The HSR enables the cell to adjust the expression of chaperones and other cytoprotective genes under proteotoxic conditions, thereby ensuring stress survival, recovery, and adaptation (Akerfelt et al. 2010). At the molecular level, this involves transcriptional regulation of heat shock (HS) genes in a manner proportional to the intensity, duration, and type of stress (Abravaya et al. 1991a, 2010; Gasch et al. 2000). HSF1, as the "master regulator" of the HSR, functions both as a stress sensor and transcriptional regulator of downstream stress response genes that are essential for protecting and remodeling the cell (Akerfelt et al. 2010).

The HSR has been considered a universal molecular response to various stress stimuli. Nonetheless, there are several examples in which the HSR is poorly or incompletely activated, such as the limited response seen in early development, as well as in different tissues of aged animals and in late-onset neurodegenerative diseases, such as Huntington's disease and Alzheimer's disease (Ben-Zvi et al. 2009; Bienz 1984; Heydari et al. 1993; Labbadia et al. 2011; Prahlad et al. 2008; Sprang and Brown 1987; Taylor and Dillin 2011). These observations suggest that the HSR can be spatially and temporally regulated during the lifetime of a multicellular organism.

In the past decade, lifespan-enhancing pathways were shown to be potent modifiers of proteostasis and to suppress protein aggregation and toxicity. Moreover, HSF1 has been shown to be an important factor influencing lifespan (Kenyon 2010b; Labbadia and Morimoto 2014; Tissenbaum 2012). Recent studies mainly conducted in *Caenorhabditis elegans* have uncovered several cell-nonautonomous pathways that modulate cellular quality control systems during the lifespan of the organism, in particular, HSF1. These pathways include cell-nonautonomous regulation of the heat shock response by neurons and germline stem cells, as well as transcellular signaling of proteostatic deficiencies in which the expression of misfolded proteins in one tissue can induce a systemic HSR (Prahlad and Morimoto 2009; Shai et al. 2014; van Oosten-Hawle and Morimoto 2014). Several of these pathways are also linked to lifespan, suggesting that proteostasis networks may show differential sensitivity at different stages over the lifespan of an organism. Here, we review changes in proteostatic function with age, focusing on cell-nonautonomous regulation of HSF1 during aging (mainly in *C. elegans*) to offer insight into how multicellular organisms adjust stress responses during key life stages and how HSF1 function, in turn, can impact lifespan.

5.2 The Loss of Proteostasis Is a Hallmark of Aging

Aging is often associated with the time-dependent loss of cellular function, increased susceptibility to environmental and physiological insults, and increased vulnerability to disease (Gems 2015; Partridge 2014; Riera and Dillin 2015). One of the hallmarks of aging is age-dependent loss of proteostasis. In agreement, aggregation and toxicity of aggregation-prone proteins associated with disease, such as huntingtin, is enhanced with age, while different protein quality control machineries show decreased function (Labbadia and Morimoto 2015a; Shai et al. 2014; Taylor and Dillin 2011). Proteostasis loss and age-dependent aggregation can be recapitulated in different model systems in a time frame related to their aging. In fact, a subnetwork of chaperones was shown to be required in both human brain aging, as well as in brain samples, and *C. elegans* models of age-associated diseases (Brehme et al. 2014). Thus, quality control systems and, specifically, the decline in proteostasis capacity are suggested to play important roles in aging and age-associated diseases (Brehme et al. 2014; Labbadia and Morimoto 2015a).

5.2.1 When Does Proteostasis Collapse?

The failure of proteostasis networks to maintain the proteome of aged animals is commonly explained by the slow accumulation of damage over time. Protein damage and misfolding in aged individuals is, therefore, suggested to result from a constant difference between the error rate and the efficiency of cellular quality control network in repairing or removing misfolded proteins over the course of an organism's life, leading to a gradual accumulation of damaged proteins over time. An alternative explanation for the failure of proteostatic networks in old individuals is that the function of cellular proteostatic networks decline with age (Taylor and Dillin 2011). For example, translation fidelity may decline with age, resulting in an increased load of damaged proteins as the individual ages (Kirstein-Miles et al. 2013). Likewise, the expression and function of cellular quality control networks may be differentially regulated over the lifespan of the organism (Bar-Lavan et al. 2012). Such changes can remodel cellular folding capacity and stress tolerance, thus increasing the risk for age-associated pathology. What distinguishes between these mechanisms is the rate of damage accumulation during adulthood. While the first scenario suggests a passive accumulation of damage over time that can be modulated by the efficacy of the proteostatic network, the second suggests that quality control function is differentially regulated over the lifespan of the organism.

5.2.2 Age-Dependent Changes in HSR Activation

Recent studies mostly conducted with *C. elegans* demonstrated that cellular quality control networks are modified as animals undergo transition to a reproductively mature state. Specifically, rapid changes in the regulation and activation of stress responses were identified. When thermoresistance and induction of heat shock genes were monitored over time in adult *C. elegans*, both declined sharply 12 h following transition to adulthood (Shemesh et al. 2013). Thus, within hours of reaching adulthood, most animals lost the ability to mount an effective HSR and survive the insult. This finding suggests the existence of very strong negative regulation of the HSR upon transition to adulthood, inhibiting HSF1 function. When other stress responses were examined, such as activation of UPR in the endoplasmic reticulum (UPR^{ER}), UPR in the mitochondria (UPR^{mt}), and oxidative stress in response to different perturbations, an early decline in the mounting of a stress response was also observed (Ben-Zvi et al. 2009; Labbadia and Morimoto 2015b; Taylor and Dillin 2013). Likewise, survival from acute oxidative stress and activation of an NRF2-dependent oxidative stress response in adult *Drosophila melanogaster* was strongly dampened, as compared to young animals (Rahman et al. 2013). Altered temporal regulation was also apparent for *C. elegans* c-Jun N-terminal kinase (JNK) signaling. While the JNK homolog KGB-1 enhances DAF-16 nuclear localization and transcriptional regulation during *C. elegans* development, this function was reversed upon transition to adulthood (Twumasi-Boateng et al. 2012). Changes in the activation of JNK signaling were also observed in adult *Drosophila* and old mice, although expression modulation was not monitored early in adulthood in either case (Girardot et al. 2006; Hsieh et al. 2003; Simonsen et al. 2008; Tsakiri et al. 2013). These data suggest that stress-induced transcriptional activation is strongly dampened early in adulthood and exhibits switch-like behavior associated with reproduction onset (Fig. 5.1).

It is important to note that stress response activation can also be remodeled at other stages over the lifespan of an organism. For example, activation of UPR^{mt} can be transmitted to other tissues, albeit only during development (Durieux et al. 2011). Likewise, heat shock response can be modulated during *C. elegans* development by including 5-fluoro-2′-deoxyuridine in the growth medium (Feldman et al. 2014). These observations strongly support the hypothesis that quality control networks can be remodeled during development and with age and suggest that many different pathways modulate cellular responses to stress.

5.2.3 Age-Dependent Changes in Proteostatic Network Composition

Changes in proteostatic function over time are associated with changes in expression of genes encoding quality control machinery components, such as ribosomal

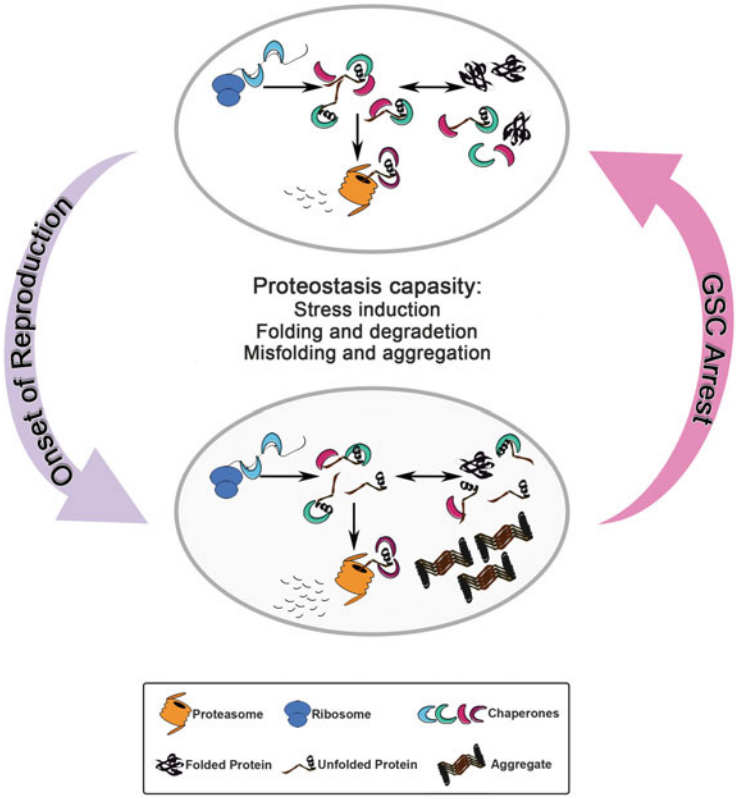

Fig. 5.1 Proteostasis is remodeled upon transition to adulthood. Changes in stress response activation, expression and function of chaperones, the ubiquitin-proteasome system, and autophagy modulate cellular proteostasis capacity, resulting in accumulation of misfolded and aggregated proteins during adulthood. These changes coincide with the onset of oocyte biomass production and can be modulated by GSC arrest

proteins, chaperones, and proteasome- and autophagy-associated proteins (Taylor and Dillin 2011). Relying on a quantitative mass spectrometry proteomics-based approach designed to follow changes in protein expression during *C. elegans* adulthood, it was uncovered that ribosomal abundance strongly declined with age and that ribosome stoichiometry was disrupted, while the abundance of specific small HS proteins (sHSP), proteasome subunits, and some E3 ligases increased with age. These changes were even observed early in adulthood (Walther et al. 2015). In agreement, *C. elegans* epidermal growth factor (EGF)-mediated signaling was shown to upregulate the expression of genes associated with the ubiquitin-proteasome system and downregulate the expression of some chaperones upon transition to reproductive adulthood (Liu et al. 2011). Additional studies observed changes in the expression of genes encoding proteostasis-mediating components during aging. For example, in adult *Drosophila*, changes in the activation of JNK

signaling and in the levels of proteasome subunits were observed (Girardot et al. 2006; Simonsen et al. 2008; Tsakiri et al. 2013). Likewise, expression levels of autophagy-associated genes declined with age in adult *Drosophila* and rats (Kiffin et al. 2007; Simonsen et al. 2008). Thus, the composition of the proteostatic network is also remodeled in early adulthood (Fig. 5.1).

5.2.4 Age-Dependent Changes in Protein Folding and Aggregation

If proteostatic networks are remodeled during adulthood, then one would expect proteostatic capacity to decline within a specific window of time during adulthood. Changes in the activity of quality control networks would impact their interactions with the proteome and, therefore, change rates of protein misfolding. One such example is the onset of aggregation and toxicity in a *C. elegans* model of Huntington's disease. In this model, animals expressing 35 repeats of polyglutamine (polyQ) start to accumulate aggregates following the onset of reproduction (Morley et al. 2002). For animals cultivated at 20 °C, this occurs ~4 days postembryo, while for animals cultivated at 25 °C, this transpires 2.5 days postembryo (Morley et al. 2002; Shemesh et al. 2013). Thus, aggregation-prone proteins quickly became insoluble upon transition to adulthood.

The functions and folding of metastable temperature-sensitive (ts) mutant proteins are also disrupted early in adulthood. Temperature-sensitive mutant proteins assayed for changes in their folding capacity, such as ras, the acetylcholine receptor, and perlecan (UNC-52), all showed a rapid loss of function early in adulthood (days 2–6 of adulthood) (Ben-Zvi et al. 2009; Shemesh et al. 2013). In *C. elegans* muscle, expression of a ts version of myosin gave rise to age-dependent mislocalization of myosin and resulted in uncoordinated movement. Here, myosin misfolding was detected as early as day 3 of adulthood (Ben-Zvi et al. 2009). Likewise, neurons and coelomocytes of animals expressing a ts version of DYN-1 showed age-dependent dysfunction and DYN-1 mislocalization as early as day 2 of adulthood (Ben-Zvi et al. 2009). In such animals, the misfolding of metastable proteins observed early in adulthood was thus general but affected different proteins in different tissues.

Age-dependent protein misfolding associated with functional decline was also apparent for wild-type proteins, although their aggregation started, for the most part, later in adulthood (Ben-Zvi et al. 2009; Haithcock et al. 2005; Shemesh et al. 2013). When wild-type myosin was monitored over time, it showed age-dependent mislocalization and misfolding, similar to what was seen with metastable myosin, albeit later in adulthood than the nonnative protein (Ben-Zvi et al. 2009; Shemesh et al. 2013). An unbiased examination of age-dependent aggregation during *C. elegans* adulthood supported this finding. Using mass spectrometry to systematically examine age-dependent changes in protein solubility,

David et al. identified several hundred proteins that became insoluble over time. Some wild-type proteins aggregated as early as day 3 of adulthood (David et al. 2010). Many of these proteins were also identified in another independent study (Reis-Rodrigues et al. 2012). A recent study using quantitative mass spectrometry to analyze changes in the proteome during *C. elegans* adulthood showed that many proteins underwent more than twofold changes in abundance, linked to widespread aggregation (Walther et al. 2015). Thus, upon transition to reproductive adulthood, there is an onset of age-dependent decline in cellular proteostasis, coinciding with a decline in HSR activation (Fig. 5.1).

The rate of protein clearance is also modulated early in *C. elegans* adulthood. Monitoring the clearance of GFP tagged with an uncleavable ubiquitin tag as a degradation reporter (Ub^{G76V}-GFP) demonstrated that there is a sharp increase in protein degradation upon transition to adulthood. While Ub^{G76V}-GFP was completely degraded during development, it started to accumulate when animals became reproductive adults (Liu et al. 2011). At later stages, protein degradation was shown to decrease in worms, flies, and rats (Hamer et al. 2010; Keller et al. 2000; Liu et al. 2011; Tonoki et al. 2009). Changes in autophagy early in adulthood were not monitored, although reduced autophagic activity was observed in adult *Drosophila* and rats (Kiffin et al. 2007; Simonsen et al. 2008). Given that the two systems are linked for the removal of aggregated proteins (Cha-Molstad et al. 2015), these observations raises interesting questions about how such interactions are modulated with age to change protein clearance.

5.2.5 *Modulating HSF1 Levels Can Affect Proteostatic Collapse*

If the levels and functions of proteostasis-related genes are limiting in adulthood, could modifying the levels of quality control transcription factors, such as HSF1, modulate proteostasis? When HSF1 levels were diminished using RNAi, the loss of function of metastable proteins, such as paramyosin and dynamin, and of aggregation-prone proteins, such as polyQ and Aβ, was exacerbated, resulting in an earlier decline in proteostasis. In contrast, overexpression of HSF1 proved to be protective for metastable and aggregation-prone proteins (Ben-Zvi et al. 2009; Cohen et al. 2006; Hsu et al. 2003; Morley and Morimoto 2004). Modulating HSF1 levels also affected the aggregation of wild-type proteins (Walther et al. 2015). The switch-like behavior of the HSR and the fact that over-expression of HSF1 can rescue proteostasis in adulthood (Ben-Zvi et al. 2009; Labbadia and Morimoto 2015b; Shemesh et al. 2013) suggest that HSF1 and other stress transcription factors are likely differentially regulated over the lifespan of the organism.

5.3 HSF1 Is a Lifespan Regulator

The involvement of HSF1 in setting the function of proteostasis in adulthood suggests that HSF1 can impact aging and lifespan. In agreement, HSF1 was shown to modulate age-associated phenotypes and shorten lifespan in *C. elegans* (Garigan et al. 2002). HSF1 knockdown induced tissue integrity decline of both somatic and germline stem cells, and wild-type animals treated with HSF1 RNAi were short-lived (Garigan et al. 2002). Conversely, the lifespan of animals overexpressing HSF1 was extended by 22–40 % (Hsu et al. 2003; Morley and Morimoto 2004). Thus, HSF1 is a lifespan regulator and it can promote longevity.

5.3.1 HSF1 Is Required for Insulin/IGF-1 Signaling (IIS) Pathway Activity

Downregulation of the insulin/IGF-1 signaling (IIS) pathway promotes longevity and suppresses protein aggregation and toxicity. Occupation of the IIS receptor (DAF-2 in *C. elegans*) initiates a conserved cascade of kinases that results in the phosphorylation and subsequent inactivation of the FOXO transcription factor (DAF-16 in *C. elegans*). In different model organisms, activation of the FOXO transcription factor, as occurs upon reduced IIS receptor function or levels, resulted in an increase in lifespan (Kenyon 2010b). In *C. elegans*, DAF-16 is required for DAF-2-mediated extended lifespan and improved proteostasis (Cohen et al. 2006, 2009; Hsu et al. 2003; Morley et al. 2002), as is HSF1. RNAi knockdown of HSF1 in a DAF-2 mutant background shortened the lifespan of the animal and resulted in a loss of the expected proteostatic improvement (Ben-Zvi et al. 2009; Cohen et al. 2006, 2009; Hsu et al. 2003; Morley et al. 2002).

5.3.2 HSF1 Is Required for Germline Stem Cell (GSC) Signaling

Endocrine signaling from germline stem cells (GSCs) and the somatic gonad links reproduction to aging. Removal of GSCs by laser ablation or induction of GSC arrest through mutations extended the lifespan of *C. elegans* (Arantes-Oliveira et al. 2002; Hsin and Kenyon 1999) and other model animals, such as the nematode *Pristionchus pacificus* (Hsin and Kenyon 1999; Rae et al. 2012) and *Drosophila* (Flatt et al. 2008). Transplantation of young ovaries into old mice also promoted longevity (Cargill et al. 2003; Mason et al. 2009). These studies established a role for the reproductive system, and specifically signals from the somatic gonad and GSCs, in lifespan (Kenyon 2010a). Because removal of the entire gonad did not extend lifespan, it would appear that longevity is not a simple consequence of

sterility but rather depends on communication between the reproductive system and somatic tissues that modulate different signaling pathways, including those involved in metabolism and quality control, to extend lifespan (Antebi 2012). GSC-dependent extension of lifespan requires the activation of many different downstream signaling pathways, one of which includes HSF1 (Antebi 2012; Libina et al. 2003).

5.3.3 *HSF1 Is Required in Part for Dietary Restriction (DR)*

Dietary restriction (DR) is another manipulation that extends life in organisms spanning from yeast to mammals. In fact, DR was the first manipulation described that increased lifespan (McCay et al. 1989). There are many different protocols to induce DR, including limiting food intake (Greer and Brunet 2009). For example, mutant *C. elegans* with a defective *eat-2* pharyngeal pump-encoding gene showed reduced food intake and thus serve as a genetic model of DR (Avery 1993). While different DR regimes all extend lifespan, they activate different nutrient-sensing pathways, including those involving TOR, AMP kinase, and sirtuins (Kenyon 2010b). Only some of these pathways, however, require HSF1 for longevity and reduction of proteotoxicity (Greer and Brunet 2009; Mouchiroud et al. 2011; Sutphin and Kaeberlein 2008; Vora et al. 2013; Zhang et al. 2009). Thus, HSF1 is required for lifespan extension in several different models of longevity that are linked but not always coupled to improved proteostasis.

5.3.4 *HSF1 Is Differentially Regulated in Development and in Adulthood*

Similar to the observed changes in proteostatic composition and function in adulthood, HSF1 regulation by the IIS pathway is also modulated upon transition to adulthood. Using conditional RNAi, Cohen et al. showed that HSF1 function in IIS-dependent reduction of aggregates toxicity was mostly required during larval development. Knocking-down HSF1 levels against a background of reduced IIS signaling (as achieved upon knockdown of the *daf-2* insulin-like receptor-encoding gene) on the first day of adulthood did not reduce IIS-mediated protective effects. In contrast, downregulation of DAF-16 was essential only in adulthood (Cohen et al. 2010). Moreover, HSF1 knockdown during development also abolished IIS-mediated extension of lifespan, while HSF1 downregulation during early or late adulthood had only a mild or no effect on lifespan, respectively (Volovik et al. 2012). Thus, HSF1 function in proteostasis and lifespan when part of the IIS pathway shows switch-like behavior upon transition to adulthood (Fig. 5.1).

5.4 Cell-Autonomous and Cell-Nonautonomous Regulation of HSF1

HSF1 regulation has been studied in depth in both in vitro and in single-cell systems (see Chaps. 1, 2, 3 and 4). In short, upon accumulation of misfolded and damaged protein, the equilibrium of molecular chaperones is altered such that HSF1 monomers are released from repressive complexes that include molecular chaperones (Abravaya et al. 1992; Shi et al. 1998; Zou et al. 1998). Once free, HSF1 acquires DNA-binding activity through trimerization in a process that is also regulated by extensive posttranslational modifications that include phosphorylation (Guettouche et al. 2005; Holmberg et al. 2001; Kline and Morimoto 1997; Knauf et al. 1996; Sorger and Pelham 1988), sumoylation (Anckar et al. 2006; Hietakangas et al. 2003), and acetylation (Westerheide et al. 2009) that can enhance or suppress HSF1 DNA-binding activity (Akerfelt et al. 2010). In the nucleus, HSF1 binds to promoters of target loci (heat shock elements (HSEs)) found in promoter regions of stress-inducible genes, which are maintained in a state that is permissive for HSF1 binding and transcription regulation (Fujimoto et al. 2012; Guertin and Lis 2013; Shopland et al. 1995; Zelin et al. 2012).

In nonstressed metazoan cells, HSF1 is predominantly found in a monomeric non-DNA-binding state, repressed by transient interactions with chaperones including Hsp90, Hsp70, and Hsp40 (Abravaya et al. 1992; Shi et al. 1998; Zou et al. 1998). When the stress signal is dampened, or when damaged proteins are no longer expressed, the HSR is attenuated. Attenuation is associated with the release of HSF1 trimers from DNA and subsequent conversion of the trimers to HSF1 monomers (Anckar and Sistonen 2007; Morimoto and Santoro 1998; Wu 1995). HSR attenuates, in part, due to the accumulation of chaperones that bind to HSF1 and acetylation of HSF1 in the DNA-binding domain (Abravaya et al. 1991b, 1992; Shi et al. 1998; Yao et al. 2006). The combination of these posttranslational modifications and chaperone interactions thus subjects HSF1 to multiple levels of control and feedback loops so as to precisely regulate chaperone levels in the cell. Regulation of the HSR, therefore, occurs through HSF1-dependent and HSF1-independent mechanisms (Fig. 5.2a). However, the mode of regulation whereby HSR is linked to chaperone levels in the cell cannot explain the poor or incomplete activation of the HSR during development and aging. Such variability in HSR induction suggests the existence of additional, cell-nonautonomous modes of HSR regulation in multicellular organisms. Accordingly, we discuss below five novel pathways that are suggested to regulate HSF1 activation, efficacy, or attenuation in a cell-nonautonomous manner (Fig. 5.2b).

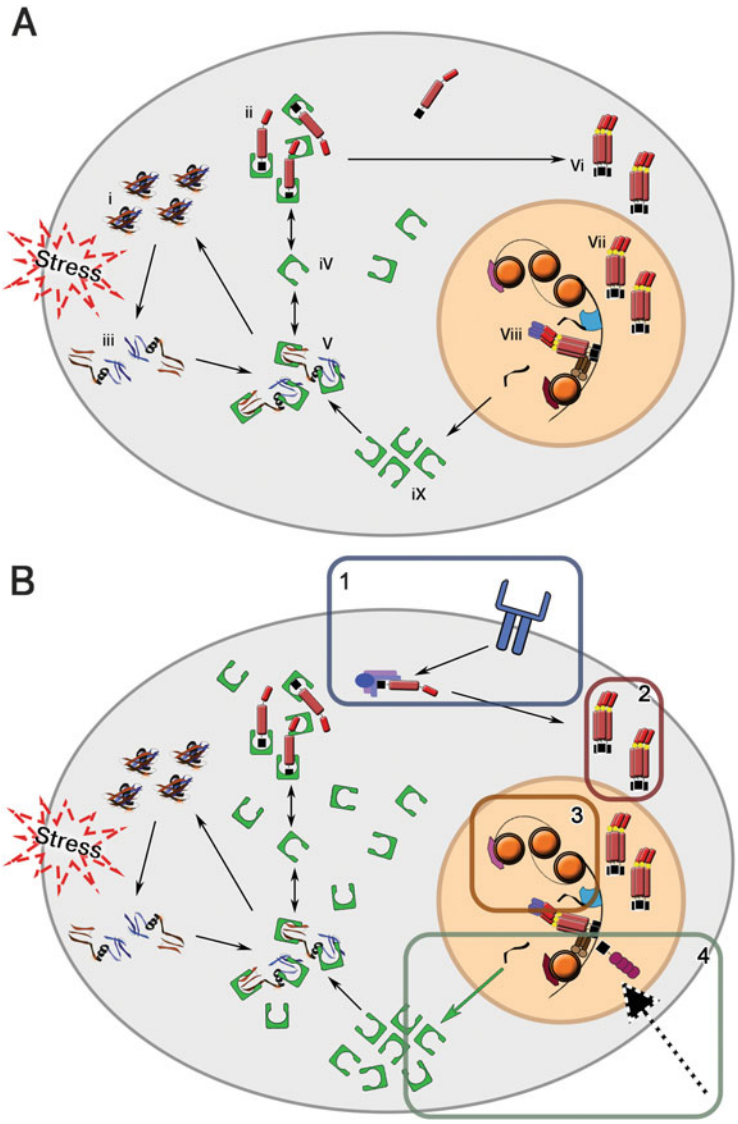

Fig. 5.2 Cell-autonomous and cell-nonautonomous regulation of HSF1. (**a**) Cell-autonomous HSR activation is a stepwise process. (*i–ii*) Stress-induced appearance of misfolded and damaged proteins, resulting in (*iii–v*) an equilibrium shift of chaperones from HSF1 to unfolded species, (*vi*) leading to formation of HSF1 trimers, which (*vii*) translocate to the nucleus, (*viii*) bind to HS gene promoters, and induce transcription of HS genes, leading to (*ix*) elevated levels of chaperones that bind to damaged proteins until the stress signal is alleviated. (**b**) Cell-nonautonomous signals can integrate into the cell-autonomous response. (*1*) HSF1 monomers are sequestered by the DHIC complex (*1*), an event that is regulated by IIS signaling. (*2*) HSF1 function is modulated by posttranslational modifications, such as acetylation that can be activated by cell-nonautonomous signals. (*3*) HSF1 binding can be modified by chromatin remodeling, while (*4*) HSF1 activation can be impacted by other transcription factors that modulate HSP levels and thus their repressive effects on HSF1 trimerization

5.4.1 Neuronal Regulation of HSF1

The first example of cell-nonautonomous regulation of HSF1 was identified as a novel cross talk between an animal's ability to perceive ambient temperature and the ability of the cell to mount a HSR (Prahlad et al. 2008; Prahlad and Morimoto 2011; Tatum et al. 2015). In *C. elegans*, two neurons termed AFD neurons are required to sense temperature and regulate temperature-dependent behaviors, such as feeding and reproduction (Lee and Kenyon 2009; Mori et al. 2007). For example, AFD neurons can modulate foraging behavior according to the history of food availability at a given temperature (Mori et al. 2007). Mutations in *gcy-8*, encoding AFD-specific receptor-type guanylyl cyclases, can disrupt thermotactic behavior (Inada et al. 2006). Work by Prahlad et al. demonstrated that the ability of *gcy-8* mutant animals exposed to an acute HS treatment to mount an effective HSR was blocked in different somatic cells. This resulted in low expression of HS genes and reduced thermo-survival (Prahlad et al. 2008). In agreement, excitation of AFD neurons in the absence of stress using optogenetic tools was sufficient to activate HSF1, resulting in reorganization of HSF1 into nuclear puncta in germ cells (Tatum et al. 2015). In contrast, *gcy-8* mutant animals were able to mount a HSF1-dependent heavy-metal stress response, revealing specificity to a given sensory input (Prahlad et al. 2008). While the ability to respond to acute HS was inhibited, the ability of *gcy-8* mutant animals to upregulate chaperones and maintain proteostasis was enhanced in different somatic cells under chronic stress conditions, such as upon expression of aggregation-prone proteins. Activation of the AFD neurons by a single optogenetic event was sufficient to retard protein aggregation, suggesting that activation of HSR resulted in improved proteostasis (Tatum et al. 2015). Neuronal regulation of the HSR is mediated by the serotonergic neurons ADF and NSM as activated by signals from AFD neurons. Temperature increase resulted in serotonin release in an AFD-dependent manner, leading to preemptive activation of the HSR (Tatum et al. 2015). Thus, in *C. elegans*, the HSR and proteostatic maintenance of somatic cells depend on sensory neuronal function that modulates the organismal response to stress and can distinguish between acute and chronic stresses (Prahlad et al. 2008; Prahlad and Morimoto 2011; Tatum et al. 2015). Somatic tissues can also send feedback information on the cultivation temperature to the AFD neurons. This response requires HSF1 in both neuronal and nonneuronal cells (Sugi et al. 2011). It remains unknown how these signals are integrated into the cellular response to stress or, specifically, how these signals modulate HSF1. Given that *C. elegans* exposed to high concentrations of the dauer pheromone that signal crowdedness present reduced HSR activation, it would appear that other signals may also feed into the neuronal pathway, either directly or via a different route (Prahlad et al. 2008). Thus, signals are also likely to be sent to the AFD neurons, integrating environmental signals into the decision of whether or not to activate the HSR in response to temperature shift (Prahlad et al. 2008; Prahlad and Morimoto 2011; Sugi et al. 2011; Tatum et al. 2015).

5.4.2 IIS-Mediated Regulation of HSF1

IIS-dependent suppression of protein aggregation, as well as lifespan extension, required HSF1 activity, suggesting that cell-nonautonomous regulation of HSF1 plays a role in both proteostasis and lifespan modulation (Alavez et al. 2011; Ben-Zvi et al. 2009; Cohen et al. 2006, 2009; Gidalevitz et al. 2011; Hsu et al. 2003; Morley et al. 2002; Taylor and Dillin 2011). Indeed, Chiang et al. showed that HSF1 is not only held in repressive complexes by chaperones preventing its activation (Abravaya et al. 1992; Shi et al. 1998; Zou et al. 1998) (Fig. 5.1a ii–v) but that HSB-1 and DDL-1/2 also form repressive complexes with HSF1 (Chiang et al. 2012). Formation of these inhibitory complexes, termed DDL-1/2 HSF1 inhibitory complexes (DHIC), is regulated by phosphorylation of DDL-1. DDL-1 phosphorylation, in turn, is regulated by IIS signaling (Chiang et al. 2012). Thus, HSF1 activation can be regulated by its active sequestration into a repressive complex regulated by cell-nonautonomous signals. This mode of regulation may be shared by other regulators, either via phosphorylation of DDL-1 or by assembly of other repressive complexes that affect HSF1 activation in a specific tissue (Fig. 5.2b).

5.4.3 SIRT1-Mediated Regulation of HSF1

Sirtuins are NAD^+-dependent deacetylases that are implicated in lifespan regulation, although the mechanism of such action is not clear (Kenyon 2010b). Given that NAD+/NADH levels are determined by energy homeostasis, sirtuin function is linked to nutrient availability. SIRT1 was shown to deacetylate HSF1 and enhance DNA binding, suggesting that diet can modulate HSF1 release from DNA by removing the acetylation from the K80 acetylation site in the DNA-binding domain of HSF1. This deacetylation promotes the occupancy of HSF1 at the HS promoter sites, enhances HSR activation, and increases thermotolerance (Westerheide et al. 2009). In support of this idea, DR was shown to induce HS gene expression in a SIRT1- (*sir-2.1* in *C. elegans*) dependent manner (Raynes et al. 2012). This suggests that signals that modulate SIRT1 function can affect HSF1. Likewise, other posttranslational modifications of HSF1 could, potentially, be regulated by cell-nonautonomous signals (Fig. 5.2b).

5.4.4 Germline Stem Cell-Mediated Regulation of HSF1

The coincidence of proteostatic collapse and the onset of reproduction, whereby the activation of different stress responses is strongly inhibited after egg laying begins, supports a link between proteostatic remodeling and reproduction. Several studies

have examined the effects of inhibiting reproduction, and specifically GSC arrest, on proteostasis in *C. elegans*. The functions of several stress transcription factors, such as HSF1 and DAF-16, were modulated by GSC ablation or arrest (Arantes-Oliveira et al. 2002; Berman and Kenyon 2006; Ghazi et al. 2009; Hansen et al. 2005; Hsin and Kenyon 1999). Moreover, germline-less mutant animals were shown to have enhanced expression of proteostasis machinery genes, such as *lgg-1*, *bec-1*, and *unc-51* required for autophagy (Lapierre et al. 2011) and the *rpn-6.1* subunit of the proteasome (Vilchez et al. 2012). Whole genome microarray analysis of *P. pacificus* in germline-ablated animals identified ~3000 differentially expressed genes, including ribosomal and translation-, proteasome-, and protein folding- and refolding-associated genes (Rae et al. 2012). Moreover, the expression of stress-inducible genes, such as heat shock genes, in response to different assaults was modulated in germline-less animals (Ghazi et al. 2009; Shemesh et al. 2013). Thus, the proteostatic network, including the translational, chaperone, autophagic, and proteasomal machineries, is remodeled by GSC proliferation (Ghazi et al. 2009; Lapierre et al. 2011; Shemesh et al. 2013; Vilchez et al. 2012). The expression of proteostatic components is dependent on different downstream signaling pathways (Lapierre et al. 2011; Shemesh et al. 2013; Vilchez et al. 2012), suggesting that GSC signaling activates different regulatory programs to modulate somatic functions. This change in proteostatic composition is strongly associated with altered proteostatic function. Germline-less animals showed increased autophagosome numbers (Lapierre et al. 2011), increased levels of chymotrypsin-like proteasome activity, and decreased levels of highly polyubiquitinated proteins (Vilchez et al. 2012). GSC proliferation also modulated protein misfolding and aggregation in somatic cells. Germline-less animals displayed reduced aggregation, as well as reduced toxicity of polyQ proteins (Shemesh et al. 2013). Likewise, the functions of metastable and wild-type proteins that are compromised with age, such as UNC-52(ts) and myosin (Ben-Zvi et al. 2009), were rescued in germline-less animals (Shemesh et al. 2013). Finally, stress survival and the activation of stress responses, such as HS, were also rescued by GSC arrest (Alper et al. 2010; Arantes-Oliveira et al. 2002; Ermolaeva et al. 2013; Libina et al. 2003; Shemesh et al. 2013; Stiernagle 2006; TeKippe and Aballay 2010). Thus, inhibition of GSC proliferation reversed the decline in somatic protein quality control upon transition to adulthood.

Labbadia and Morimoto directly examined how HSF1 function is remodeled upon transition to adulthood and in GSC-arrested animals. They examined different regulation nodes in the HSF1 activation cycle, such as HSF 1 levels, nuclear localization, and DNA binding (Fig. 5.2a). Using chromatin immunoprecipitation coupled to qPCR to address specific HS genes, they found that HSF1 and RNA polymerase II association with HS gene promoters following HS was reduced between the first and second days of adulthood (Labbadia and Morimoto 2015b). At the same time, proteostasis is remodeled (Labbadia and Morimoto 2015b; Shemesh et al. 2013). HSF1 transcriptional repression was associated with reduced chromatin accessibility and specifically, with an increased histone methylation repressive marker, H3K27me3, on HS, as well as other stress genes. Moreover, changes in the expression levels of the H3K27me3 demethylase *jmjd-3.1* were

correlated with the onset of proteostasis collapse (Labbadia and Morimoto 2015b; Shemesh et al. 2013). Thus, the promoter regions of HSF1 target genes are more repressed on the second day of adulthood. In contrast, the levels of *jmjd-3.1* and H3K27me3 markers on the promoter of HS genes in GSC-arrested animals remained low (Labbadia and Morimoto 2015b), in agreement with increased HS gene activation and HS survival collapse (Labbadia and Morimoto 2015b; Shemesh et al. 2013). Taken together, these data strongly suggest that HS activation is actively repressed upon transition to adulthood by cell-nonautonomous signals from the reproductive system (Fig. 5.2b). These signals globally change the chromatin accessibility of HSF1 and likely of other transcription factors, resulting in remodeling of several different signaling pathways upon transition to adulthood by GSC inhibition, with these signaling pathways drastically remodeling somatic proteostasis. This switch-like mechanism links reproduction to maintenance of the soma. However, the nature of the signals sent from the reproductive system to the soma and how they modulate *jmjd-3.1* levels remain unknown.

5.4.5 Tissue-to-Tissue HS Signaling

As noted above, HSF1 is regulated by chaperones, such as Hsp90 and Hsp70 that form a repressive complex with HSF1 and inhibit its trimerization (Abravaya et al. 1992; Shi et al. 1998; Zou et al. 1998). However, chaperone levels are also regulated cell-nonautonomously by transcellular chaperone signaling that is regulated by the FOXA transcription factor PHA-4 in *C. elegans* (van Oosten-Hawle et al. 2013). Enhanced expression of Hsp90 in one tissue was sufficient to elevate the levels of Hsp90 in other tissues and thus block the induction of thermoprotective HSR in the distal tissues. Conversely, knocking down the expression of Hsp90 in a single tissue was sufficient to induce HS genes in distal tissues and protect the animals from stress. Such transcellular chaperone signaling was dependent on PHA-4 regulation of chaperone expression (van Oosten-Hawle et al. 2013). Thus, PHA-4 and possibly other transcription factors that modulate chaperone levels can impact the cell's ability to mount an effective stress response (Fig 5.2b).

5.5 Perspectives

The findings that proteostasis is actively remodeled upon transition to adulthood and that HSF1 plays a role in proteostatic maintenance and lifespan open many questions. For example, how are regulation of proteostasis and lifespan linked? How does the decline in stress activation impact lifespan? While we do not yet know the answers to these questions, it is interesting to note that downregulation of any of the GSC-dependent signaling pathways is required for increased lifespan (Antebi 2012), even though these pathways differentially modulated proteostatic

functions and stress response regulation (Lapierre et al. 2011; Shemesh et al. 2013; Vilchez et al. 2012). This suggests that only the combined actions of many protective pathways is sufficient for lifespan enhancement, while proteostatic function can be modulated by partial activation of these pathways. This is in agreement with chemical and genetic manipulation of lifespan-extending pathways in which lifespan, proteostasis, and stress resistance were mechanistically dissociated (El-Ami et al. 2014; Tissenbaum 2012; Van Raamsdonk and Hekimi 2012; Yu and Driscoll 2011).

Recent studies have identified several signaling pathways that can regulate HSF1, as well as defining points in HSF1 regulation that can be modulated by cell-nonautonomous signals. Still, many parts of this puzzle are missing. For example, how does neuronal regulation modulate HSF1 function? How are the different cell-nonautonomous signals integrated at the level of the cell, as well as at the level of the organism, to mount an effective HSR? Finally, focusing on lifespan modulation and treatment of age-dependent diseases, the most important question asks whether the age-dependent decline in stress activation and proteostasis can be reversed.

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Chapter 6
HSF Modulates Neural Development Under Normal and Stress Conditions

Seiji Ishii and Kazue Hashimoto-Torii

Abstract Over the last decade, laboratories working with knockout mice have contributed data substantiating that heat shock factors 1 and 2 (HSF1, HSF2) play critical roles in the normal development of the central nervous system. More recent studies have determined that these factors also play critical, but altered, roles during pathological brain development elicited by prenatal exposure to environmental stress. Those researches have, in fact, provided new insights into the roles of heat shock factors at the molecular level in both normal and pathological brain development, strengthening the view that the malresponse of HSFs to environmental stress is predisposed or highly influenced by genetic mutations associated with the incidence of neuropsychiatric disorders. In this chapter, we summarize the roles of HSFs in both normal and pathological brain development with a primary focus on the cerebral cortex and discuss potential mechanisms governing the multifaceted roles of HSFs under both normal and pathological conditions.

Keywords HSF1 • HSF2 • Neurogenesis • Cortical development • Prenatal stress

6.1 Introduction

Throughout life, humans are inevitably exposed to many types of stress, principally psychological and environmental. The earliest stress we encounter in life occurs in the womb where prenatal exposure to diverse agents such as alcohol or illegal drugs may cause anatomical and functional anomalies in the developing brain. Even

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subtle disturbances in the cerebral cortex may impair cognitive and memory functions, thereby increasing susceptibility to neuropsychiatric disorders such as autism, attention deficit/hyperactivity disorder (ADHD), and schizophrenia. Accordingly, increased attention is being paid to understanding the underlying epigenetic programs governing neuropsychiatric deficits or disorders. Here, we focus on recent papers that illuminate the roles of HSFs in prenatal environmental stress-induced pathological cortical development and identify the risks posed for the genesis of neuropsychiatric disorders.

6.1.1 Normal Cortical Development and Its Disturbance by Environmental Stress

The initial step of corticogenesis is proliferation of the neural progenitor cells in the germinal zone, known as the ventricular zone (VZ) (Fig. 6.1a). The neural progenitor cells in the cerebral cortex consist of several different cell types defined by their morphological, physiological, and molecular properties (Gleeson and Walsh 2000; Kriegstein and Noctor 2004; Kriegstein et al. 2006; Ayala et al. 2007; Barnes and Polleux 2009; Rakic 2009; Rakic et al. 2009). Among them, radial glial cells have unique characteristics that enable them to produce the layered structure or early scaffold of the cerebral cortex. The radial glial cell extends an elongated shaft that spans the entire thickness of the fetal cerebral wall (Rakic 1988), and neurons differentiated from neural progenitor cells appose to this transient scaffolding and migrate toward the cortical plate (CP) during the neurogenic phase (Fig. 6.1a). In the following gliogenic phase, radial glial cells give rise to oligodendrocytes that are required for myelination of neuronal axons and astrocytes that support neurons physically and metabolically. The radial glial cells are then transformed into adult neural stem cells in the ependymal layer (EL in Fig. 6.1a) (Kriegstein and Alvarez-Buylla 2009).

A number of independent histological analyses using postmortem tissues with a history of exposure to prenatal environmental insult, such as alcohol, have documented various anomalies in the cerebral cortex, including heterotopias, microcephaly, hydrocephaly, and agenesis of the corpus callosum (Clarren and Smith 1978; Roebuck et al. 1998; Muralidharan et al. 2013). In addition, animal exposure models have reproduced many of the morphological and behavioral phenotypes that are seen in human subjects (Thompson et al. 2009). Disturbances in the multiple events involved in corticogenesis, including proliferation, differentiation, apoptosis, and cortical cell migration (Fig. 6.1b), are considered the principle causes underlying these anomalies.

Collectively, corticogenesis consists of numerous extremely intricate multifaceted steps that must be controlled precisely and are therefore vulnerable to impairment by exposure to even minimal levels of environmental stress.

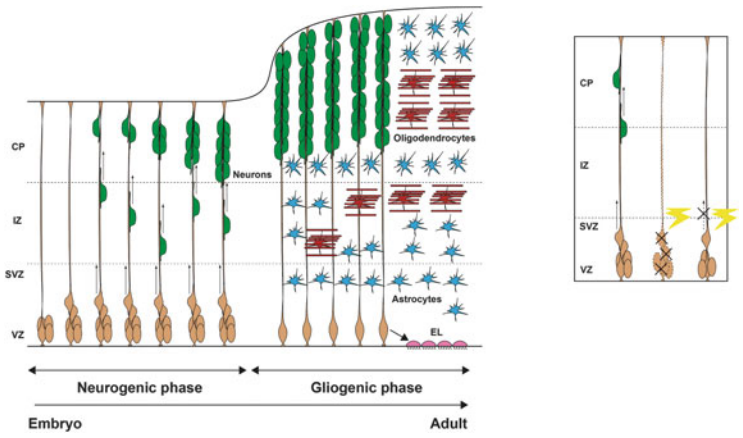

Fig. 6.1 Prenatal exposure to environmental stress increases the cell death and cell cycling arrest and delays neuronal migration in the developing cerebral cortex. (**a**) Cortical neurons are generated from the neural progenitor cells located in the ventricular zone (*VZ*)/subventricular zone (*SVZ*) of the cortex. Then, the produced neurons migrate through the intermediate zone (*IZ*) to the cortical plate (*CP*). Following the neurogenic phase, the oligodendrocytes and astrocytes are produced from the same progenitor cells. (**b**) The cortical malformations caused by prenatal environmental stress are as follows: cells are at increased risk of cell death or cell cycling arrest (*left*), and other cells show impaired migration (*right*)

6.2 Roles of HSFs in Normal Cortical Development

In the course of neural development, both HSF1 and HSF2 are ubiquitously expressed in the developing brain until birth (El Fatimy et al. 2014), and phenotypes of the loss of functions are also prominent in the brains. In the following subsections, we review the data and discuss the potential mechanisms governing how these two genes regulate normal brain development.

6.2.1 HSF1 Controls Normal Neurogenesis, Gliogenesis, and Behavior

Xiao et al. (1999) found that the brains of *Hsf1* knockout mice were generally smaller than their wild-type littermates, becoming discernible only after the first postnatal week. Adding to the list of neuropathological consequences arising from deletion of *Hsf1*, Santos and Saraiva found that brains of *Hsf1* knockout mice were hydrocephalic upon the onset of birth (Santos and Saraiva 2004). The knockout mice showed reduced anxiety and sociability, but increased depressive behavior and aggression (Uchida et al. 2011). By restoring *Hsf1* specifically into the hippocampus, anxiety and depression-like behaviors were partially reversed along with mitigation of impaired synaptic connections. Further, histological analyses of the

brains showed a reduction of spine density in the granule cells (a type of neurons) of the hippocampus. Furthermore, chromatin immunoprecipitation assays demonstrated that the expression of genes encoding polysialytransferases is directly controlled by Hsf1 in the hippocampus (Uchida et al. 2011). Given that the polysialylated-neural cell adhesion molecule (PSA-NCAM) is required for synapse formation (Dityatev et al. 2004) and that the expression level of PSA-NCAM is lowered in the hippocampus of *Hsf1* knockout mice (Uchida et al. 2011), the transcriptional controls of polysialytransferases by HSF1 might be a mechanism governing the behaviors that are altered in *Hsf1* knockout mice. Uchida et al. provided additional evidence of brain pathology showing reduced proliferation of adult neural stem cells and delayed maturation of de novo neurons in the dentate gyrus, the neurogenic site of the hippocampus in young adult mice (Uchida et al. 2011). Although the data nicely demonstrate evidence for a multifaceted role of HSF1 in adult neurogenesis, the underlying mechanisms remain largely undetermined. However, *Hsf1* knockout mice exhibit impaired cilia (Takaki et al. 2007), and thus together with accumulating evidence that ciliopathy is linked to impaired adult neurogenesis (Breunig et al. 2008; Han et al. 2008; Amador-Arjona et al. 2011; Tong et al. 2014), yet another possible mechanism on how HSF1 regulates the adult neurogenesis and behavior might be through the regulation of the primary ciliary structure and functions (please refer to Chap. 7 for the function of HSF1 in cilia formation).

Impaired gliogenesis observed in *Hsf1* knockout mice also suggests a critical role for HSF1 in glial specification and development (Homma et al. 2007). In these mice, the number of glial fibrillary acidic protein (GFAP)-positive astrocytes was increased in the ependymal layers surrounding the lateral ventricles. In contrast, the number of myelin basic protein (MBP) expressing oligodendrocytes was significantly reduced in the corpus callosum and the hippocampus (Homma et al. 2007). Given that radial glial cells, the neural stem cells in the cerebral cortex, give rise to both oligodendrocytes and astrocytes sequentially during development, HSF1 may control gliogenesis in the mouse brain just as it controls proliferation of adult stem cells and differentiation of de novo neurons.

Taken together, this entire body of work strongly supports the critical roles of HSF1 in the brain by both directly and indirectly influencing multiple facets of brain development and, consequently, behavior.

6.2.2 *HSF2 Is Required for Proper Neuronal Migration in the Cerebral Cortex*

Consistent phenotypes were observed in *Hsf2* knockout mice made in different laboratories, including hydrocephalus and higher rates of fetal lethality (Kallio et al. 2002; Wang et al. 2003), similar to rates observed in *Hsf1* knockout mice. A unique phenotype, however, was observed in the cerebral cortex of the *Hsf2*

knockout mice in which Hsf2 was found to be strongly expressed in the VZ. While the effects on proliferation/differentiation of the neural progenitor cells appeared to be minimal, rather prominent effects were found in the descendent neurons that migrate out from the VZ. As a potential mechanism, Mezger's group (Chang et al. 2006) demonstrated direct transcriptional control of Hsf2 on the *p35* gene that is involved in migration control through the phosphorylation of CDK5 (Chae et al. 1997). Another gene, *p39*, the product of which forms a protein complex with p35 (Ko et al. 2001) that is required for neuronal migration, also shows reduced expression in *Hsf2* knockout mice. However, this appears to occur via an indirect Hsf2 control mechanism as the direct binding of Hsf2 on the promoter region of *p39* was not observed.

Furthermore, disturbances were also found in the morphology of the radial glial cells which form the scaffold for neuronal migration and in the number of Cajal-Retzius cells that secrete signals required for neuronal migration (Rice and Curran 2001; Tissir and Goffinet 2003) in *Hsf2* knockout mice, suggesting indirect effects of *Hsf2* depletion on neuronal migration. Genome-wide expression profiling of *Hsf2* knockout mice performed by Mivechi's group (Wang et al. 2003) reported reduced expression of several genes that are potentially involved in the migration of cortical neurons as well as genes involved in corticogenesis. The list of genes exhibiting reduced expression included the *T-box brain gene 1* (*Tbr1*), proximal promoter regions of which include sequences that both HSF1 and HSF2 can bind to. Since the knockout mice showed impaired neuronal migration in the cerebral cortex and also exhibited reduced numbers of Cajal-Retzius cells (Hevner et al. 2001), it is feasible to consider that *Hsf2* may control neuronal migration via transcriptional control of the *Tbr1* gene in Cajal-Retzius cells.

6.3 Roles of HSFs in Cortical Development Under Conditions of Prenatal Exposure to Adverse Conditions

Recent studies demonstrated that both HSF1 and HSF2 play important roles in pathological cortical development provoked by prenatal environmental stress. In the following subsections, we review and discuss the findings obtained in these studies.

6.3.1 HSF1 Protects the Embryonic Cortex from Various Types of Environmental Stress

Although subjects exposed prenatally to various types of environmental challenge share increased susceptibility to late-onset neurological dysfunction, factors that determine the degree of susceptibility among individuals, however, remain obscure.

Hashimoto-Torii et al. (2014) showed that HSF1 serves as a guardian against damage caused by prenatal exposure to various environmental challenges such as alcohol, methylmercury, and maternal seizure (Fig. 6.1b). By chromatin immunoprecipitation, mice exposed in utero to these three types of challenge exhibited increased Hsf1 binding to heat shock element (HSE) in the *Hsp70* gene of the embryonic cerebral cortex. To examine the role of activated Hsf1 under conditions of exposure to such prenatal challenges, *Hsf1* knockout embryos were exposed to subthreshold levels of exposure at the peak of corticogenesis. While subthreshold exposure alone induced neither structural nor behavioral abnormalities in the cerebral cortex, *Hsf1* deficiency plus subthreshold exposure to these challenges increased the frequency of leptomeningeal heterotopias and reduced the size of the cortex (Fig. 6.2). The offspring also exhibited increased seizure susceptibility after birth (Fig. 6.2). These structural abnormalities were attributed to impaired survival and cell cycling of neural progenitor cells due to autonomous cell defects (Fig. 6.3) and secondary effects produced by compromised meninges. These findings uncovered the role of Hsf1 in conferring tolerance to prenatal environmental perturbation in the mouse cerebral cortex, thereby securing a lower incidence of cortical dysplasia than might have occurred in the absence of functional Hsf1.

With regard to humans, there are a number of reports identifying a number of prenatal environmental risk factors thought to potentially cause or contribute to schizophrenia (SZ) (Sullivan 2005). In addition, there are multiple reports demonstrating abnormalities in HSF1-HSP70 signaling (Schwarz et al. 1999; Kim et al. 2001; Pae et al. 2005). To test the possibility that HSF1 activation is altered in humans exposed to harsh prenatal environmental conditions, induced pluripotent stem cells (iPSCs) derived from patients diagnosed with SZ (Brennand et al. 2011) were employed as a model. The same three types of environmental challenges mentioned above were applied in vitro to these cultured human iPSC-derived neural progenitor cells (NPCs) at subthreshold levels for 3 h (Fig. 6.4). The copy number of *HSP70* and *GAPDH* was measured in individual cells, and the results revealed that all cell lines, including control NPCs, showed a robust increase in *HSP70* expression in response to these challenges (Fig. 6.4). Notably, although the mean of both *HSP70* and *GAPDH* expression levels showed no differences between control and SZ NPCs, cell-to-cell variability among the SZ NPCs was found to be significantly larger in *HSP70* expression, but not in the *GAPDH* (Fig. 6.4). The observation of abnormal levels of HSF1 variability among single cells within a subpopulation of SZ NPCs demonstrated different degrees of cell susceptibility upon exposure to adverse conditions. These results support the suggestion that variable responses of HSF1-HSP signaling among a population of SZ neural progenitor cells exposed to environmental stress is predetermined by genetic predisposition and may increase the risk for the onset of schizophrenia and possibly other neuropsychiatric diseases (Hashimoto-Torii et al. 2014; Brennand et al. 2014).

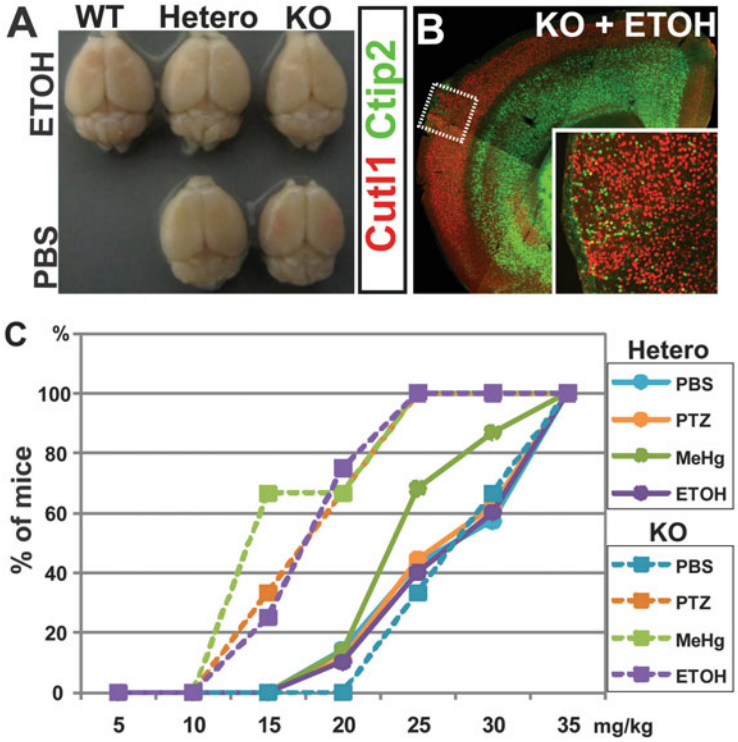

Fig. 6.2 Chances of cortical malformation and risk of epilepsy are increased by combining prenatal challenges and *Hsf1* loss of function. (**a**) An image of whole brains of postnatal day 25 mice with indicated *Hsf1* genotypes and substrate exposure, showing a slightly smaller cerebral cortex in the *Hsf1* KO mouse prenatally exposed to ETOH. (**b**) Immunohistochemistry for Cutl1 (marker for upper layer) and Ctip2 (lower layer) in a cortical section of a P25 *Hsf1* KO mouse prenatally exposed to ETOH, showing the formation of heterotopias (inset). (**c**) *Hsf1* KO mice with a prenatal history of challenge exposure show increased susceptibility to PTZ-induced seizures at the juvenile stage. Dose-response cumulative curves for induction of tonic-clonic seizures demonstrate greater PTZ (convulsant) sensitivity of *Hsf1* KO mice prenatally exposed to challenges (ETOH, MeHg, or PTZ) compared to heterozygous littermates and KO mice exposed to control treatment (This figure is adopted from Hashimoto-Torii et al. (2014))

6.3.2 *HSF1-HSF2 Heterotrimer Participates in the Control of Pathological Cortical Development*

Contrary to fact that heat shock inactivates HSF2 (Mathew et al. 2001), El Fatimy et al. (2014) found that alcohol (ethanol) exposure maintains Hsf2 activity concomitantly with HSF1 de novo activation both in vivo and in vitro brain. Both HSF2 and HSF1 were shown to occupy the promoters of *Hsp70* and *Hsp90*, respectively, in embryonic cortices exposed to alcohol. Furthermore, posttranslational modifications of Hsf1 upon exposure to alcohol were also different from those observed upon heat shock. Reduced acetylation, absence of hyperphosphorylation, and

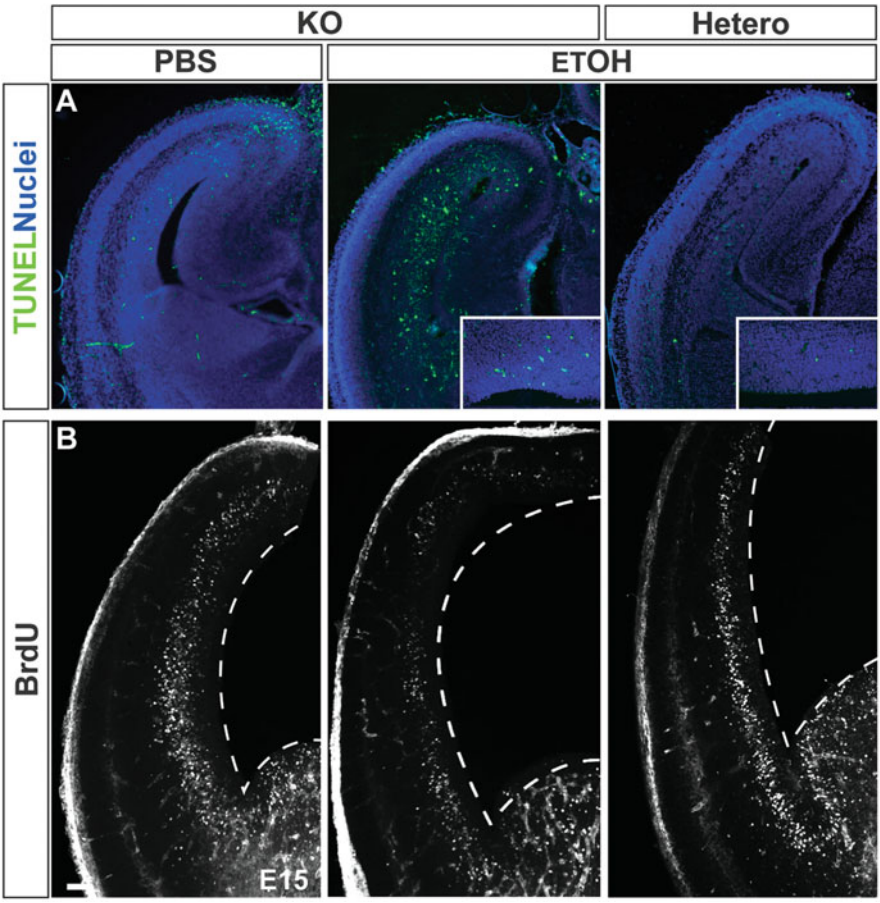

Fig. 6.3 Increase of apoptotic cell death and decrease of cell cycling in the cortex of *Hsf1* KO embryos exposed to challenges. (**a**) TUNEL staining at E16 in cortical slices from embryos with indicated genotypes and prenatal exposure. An increase in apoptosis in the cortex of *Hsf1* KO embryos exposed to ETOH is evident. (**b**) Pulse labeling with BrdU for 30 min at embryonic day 15 in the cortex of PBS- or ETOH-exposed embryos with indicated genotypes. The number of $BrdU^+$ progenitors is decreased in the *Hsf1* KO cortex exposed to ETOH, indicating the decreased cell proliferation/cell cycling (All panels are from Hashimoto-Torii et al. (2014))

delayed sumoylation of Hsf1 occurred in response to alcohol, but not in response to heat shock (El Fatimy et al. 2014).

In addition to such differences between the responses of HSFs to heat shock and alcohol, El Fatimy et al. found similar subcellular distributions of Hsf1 and Hsf2 in response to alcohol exposure, but not to heat shock (El Fatimy et al. 2014). This result suggested that HSF1 and HSF2 may form heterotrimer complexes upon exposure to alcohol in contrast to the predominant formation of HSF1 homotrimer complexes upon exposure to heat shock. Given that depletion of *Hsf2* cannot

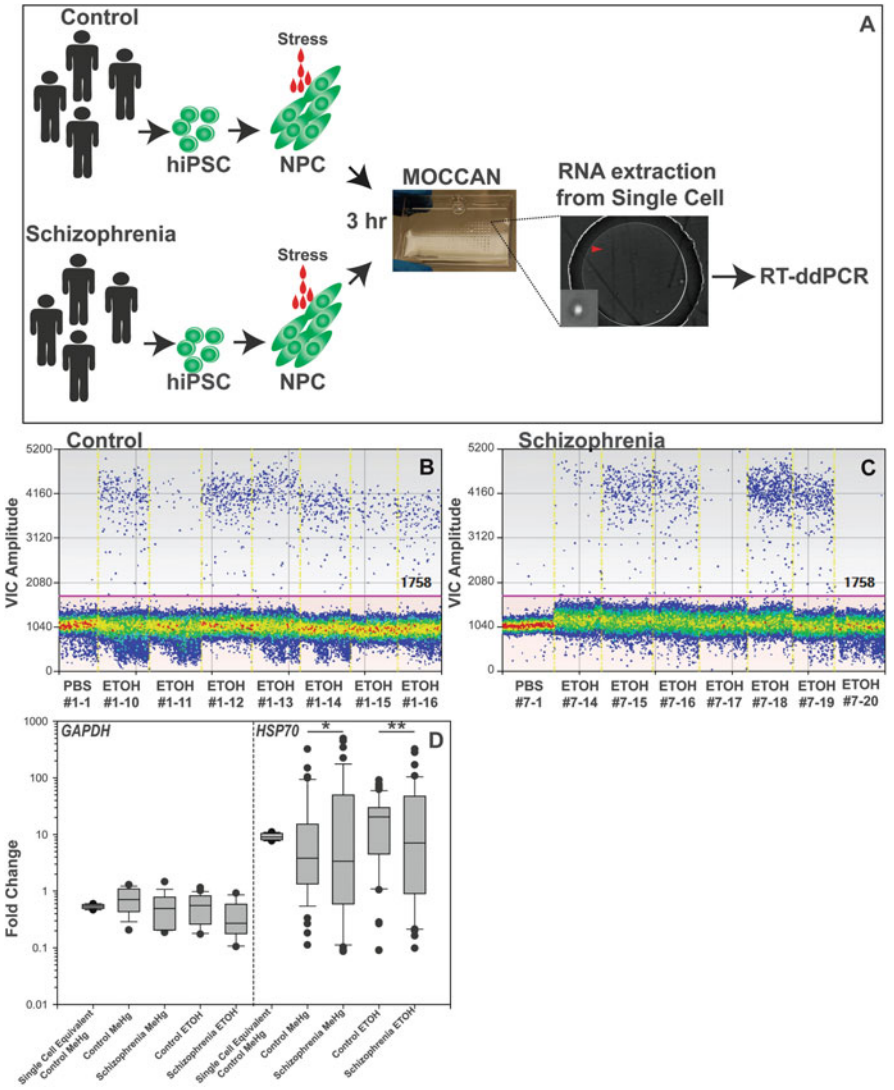

Fig. 6.4 Cell-to-cell variability of *HSP70* mRNA levels in response to environmental challenges is increased in schizophrenia neural progenitor cells. (**a**) Human iPS cells were differentiated to neural progenitor cells (*NPCs*), and the NPCs were exposed to stress for 3 h. Then sorted single cells were subject to single-cell RT-droplet digital PCR of *Hsp70*. (**b**) (**c**) Representative results of single-cell ddPCR of human *HSP70* (VIC label) in control (**b**) and schizophrenia (**c**). The clone number of each cell and substrates applied are shown under the graph. The *red line* shows cutoff of positive and negative droplets. (**d**) Graph shows fold change of *GAPDH* and *HSP70* expression compared with PBS exposure. Significantly increased variability was observed in schizophrenic cells as compared with the control ($^{*}p < 0.0001$, $^{**}p = 0.002$ by Levene's test). Significant differences in the comparison of means were not observed in all sets of comparisons ($p > 0.05$ by Welch's *t*-test). The single-cell equivalents were made by using 1/10 of a pool of ten lysed cells for the template of reverse transcription (All panels are from Hashimoto-Torii et al. (2014))

facilitate Hsf1 binding on the HSE under conditions of exposure to alcohol, Hsf2 is still required for binding of Hsf1 on HSE, thus suggesting the likelihood that HSF1 and HSF2 heterotrimer complexes form to facilitate HSF1 binding to HSE.

In the alcohol-exposed embryonic cortex, El Fatimy et al. (2014) also observed occupancy of HSF1 and HSF2 on the HSEs of genes that control neuronal migration, including *doublecortin (Dcx)*, *doublecortin-like kinase 1 (Dclk1)*, and *p35* (Ayala et al. 2007). Since the binding of Hsf2 homotrimer is required for the expression of these genes during normal neuronal migration in the embryonic cortex (El Fatimy et al. 2014; Chang et al. 2006), the reduction of their transcription upon exposure to alcohol may be caused by the loss of functional HSF2 homotrimer or by dominant occupation of HSF1-HSF2 heterotrimer that does not, by itself, activate transcription of these genes, thereby inhibiting neuronal migration (Fig. 6.5). However, given that the loss of *Hsf2* rescued the impaired radial neuronal migration elicited by alcohol exposure, the HSF1-HSF2 heterotrimer complex seems to play an instructive role in the inhibition of neuronal migration by alcohol exposure (Fig. 6.5). Consistent with the alcohol-reduced expression levels of genes (*Dclk1*, *Dcx*, *p35*, *Chl1*, *Myo10*, *MapT*, and *Mark2*) that pertain to functions required for neuronal migration, it is noteworthy that the impaired migration was reversed in the *Hsf2* knockout embryo (El Fatimy et al. 2014).

6.3.3 *HSF1 May Control Neurogenesis Through the Control of Synaptic Functions and the GABAergic System*

Using microarrays, Harrison and colleagues performed transcriptome analyses on mouse cortical neurons to identify genes that are commonly increased under conditions of exposure to alcohol or heat (Pignataro et al. 2007). Among nine genes identified (*glycoprotein m6a* (*Gpm6a*), *microtubule-associated protein 1B* (*Mtap1b*), *neurogranin* (*Nrgn*), *ELMO domain-containing 1*, *spectrin β2 transcript variant 1* (*Spnb2*), *glypican 5* (*Gpc5*), *SEC23A*, *synaptotagmin 1* (*Syt1*), and *cadherin 13* (*Cdh13*)), *Spnb2*, *Nrgn*, *Cdh13*, *Gpm6a*, and *Syt1* are known to be involved in synaptic functions (Pignataro et al. 2007). By demonstrating that shRNA-mediated *Hsf1* knockdown reduced the expression level of *Syt1n*, Varodayan et al. (2011) proved that the increase of *Syt1* mRNA upon exposure to environmental stress is directly mediated by Hsf1. They also revealed that the expression of another gene encoding the core synaptic vesicle fusion protein, vesicle-associated membrane protein 2 (Vamp2), is increased by exposure to both alcohol and heat shock (Varodayan et al. 2011). Since *Vamp2* is predicted to include HSF binding sites in the second intron (Varodayan et al. 2011), the increase of *Vamp2* mRNA by alcohol may also be mediated by activated HSF1 (Varodayan and Harrison 2013). Altogether these findings suggest that environmental stress-induced disturbance of synaptic functions may be mediated, at least in part, by the transcriptional activity of activated HSF1.

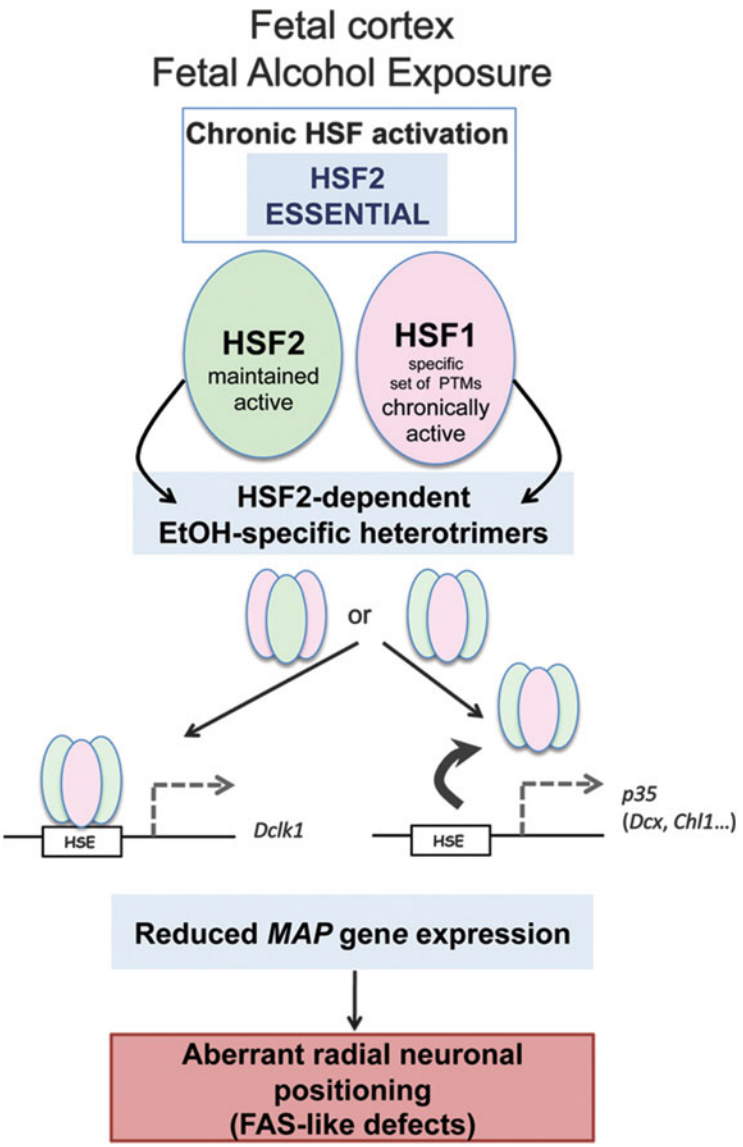

Fig. 6.5 Models for the functions of HSF1-HSF2 heterotrimers in migration defects upon fetal alcohol exposure. Fetal alcohol exposure may lead to persistent HSF1-HSF2 heterotrimers that bind to HSF2 target genes involved in neuronal migration (which are bound by HSF2 homotrimers under normal conditions) and disturb their expressions (*left*). Alternatively, the formation of heterotrimers may simply prevent bindings of HSF2 homotrimers to the HSE (*arrow*) (Figure was modified from El Fatimy et al. (2014))

Environmental insults such as alcohol exposure also increase the release of GABA (*gamma*-aminobutyric acid) and the frequency of inhibitory currents that have the potential to disturb overall brain development and function (Varodayan and Harrison 2013). In addition to all of the previous findings, Harrison's group also obtained evidence that support the possibility that HSF1 is involved in environmental stress-induced dysfunction of the GABAergic system (Pignataro et al. 2007). In the case of both heat shock and alcohol exposure, they observed increased expression of *Gabra4* mRNA that encodes the alpha 4 subunit protein of the GABA receptor in mouse embryonic cortical neurons (Pignataro et al. 2007). Then, using the luciferase assay, they demonstrated that Hsf1 controls *Gabra4* expression by binding to HSE in the promoter, thus proving that the increase of *Gabra4* mRNA by alcohol is mediated by Hsf1 (Pignataro et al. 2007).

These results reported by Harrison et al. add additional support for the critical involvement of HSF1 in environmental stress-elicited brain dysfunction, and they raise the possibility that a reduction in or inhibition of HSF1 functionality might alleviate the pathological consequences emanating from prenatal exposure to harmful agents or conditions.

Interestingly, GABA is also released in the neurogenic domains where neuronal progenitor cells reside, and it plays important roles in the proliferation/differentiation of the neural progenitor cells in both embryonic and adult cortex (LoTurco et al. 1995; Antonopoulos et al. 1997; Haydar et al. 2000; Liu et al. 2005). Therefore, the possibility also exists that the environmental stress-induced disturbance in proliferation/differentiation of neural progenitor cells (Miller and Nowakowski 1991; (Miller 1996) may also be mediated by HSF1-controlled GABAergic signaling.

6.4 Perspective

We are now on the verge of establishing with scientific certainty the importance of HSFs in mediating environmental stress-provoked pathological brain development in animal models. Although both detrimental and beneficial roles of HSFs were documented in the various studies of pathological brain development, it is yet to be determined if excess activation of HSF1 reduces or increases the risk of developing neural pathology and whether observations such as cell-to-cell variability in HSF1 responses to environmental stress in NPCs are random or directly connected to genetic predisposition.

The body of data reviewed in this chapter represents the cornerstone of evidence leading to the formulation of more fundamental questions that need to be addressed. However, further progress for comprehensive understanding of the molecular basis of HSF responses against prenatal environmental stress is inevitably important and may well lead to the discovery of prophylactic and/or therapeutic interventions of the neurodevelopmental diseases.

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Chapter 7
HSF Maintains Sensory Organs

Kazuma Sugahara, Ke Tan, Akira Nakai, and Hiroshi Yamashita

Abstract The heat shock factor (HSF) family consists of four members in mammals and controls proteostasis capacity by regulating the heat shock response (HSR). Members of this family are involved in development of some tissues such as the brain and reproductive organs. In addition, they are involved in development of the lens and olfactory epithelium, which are originated from the sensory placodes. Analysis of these sensory organs reveals that HSF activity reaches to a peak and becomes indispensable for development and maintenance within a few days or weeks after birth. The inner ear is another sensory organ derived from the sensory placodes, and its adaptation to acoustic trauma is highly dependent on HSF1. In this chapter, we summarize roles of HSF family members in the sensory organs and discuss about molecular mechanisms of development and maintenance by the HSF family members.

Keywords Cytokine • Heat shock protein • Inner ear • Lens • Olfactory epithelium • Placode • Sensory organ • Transcription

7.1 Introduction

Heat shock response is characterized by induction of a set of heat shock proteins (HSPs) and is a fundamental adaptive response that maintains proteostasis (Parsell and Lindquist 1993; Balch et al. 2008). This response is regulated mainly at the level of transcription by heat shock factor, which binds to a heat shock element (HSE) that is composed of at least three inverted repeats of the consensus sequence nGAAn (Wu 1995). Among vertebrate HSF family members (HSF1, HSF2, HSF3, and HSF4), HSF1 is required for the induction of *HSP* genes in response to heat

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shock in mammalian cells (Akerfelt et al. 2010). Heat shock triggers conversion of an inert HSF1 monomer to an active trimer that binds to HSEs in the HSP promoters with a high affinity and rapidly activates transcription of these genes. The HSF1-mediated induction of HSPs is required for acquisition of thermotolerance (Fujimoto and Nakai 2010) and protection of cells from various pathophysiological conditions such as aging and neurodegenerative diseases (Morimoto 2011). All members of the HSF family also play critical roles in developmental processes such as gametogenesis (Christians et al. 2000; Nakai et al. 2000; Kallio et al. 2002; Izu et al. 2004; Wang et al. 2004; Metchat et al. 2009) and neurogenesis (Wang et al. 2003; Santos and Saraiva 2004; Chang et al. 2006; Homma et al. 2007; Uchida et al. 2011), in immune response (Inouye et al. 2004, 2007; Zheng and Li 2004; Ichiyanagi et al. 2010), and in maintenance of the ciliated tissues (Takaki et al. 2007). Furthermore, they are required for maintenance of the sensory organs, parts of which are derived cranial placodes.

Cranial placodes are defined as transient embryonic thickenings of the cranial ectoderm that contribute to the peripheral nervous system of the head (Bhattacharyya and Bronner-Fraser 2004; Schlosser 2006). The cranial placodes include the sensory placodes that will give rise to the nasal epithelium, the lens, and the inner ear and the neurogenic placodes from which elements of cranial ganglia are derived (Fig. 7.1). Among them, lens and olfactory precursors arise from a common territory surrounding the anterior neural plate at early developmental stages (Bhattacharyya et al. 2004). A transcription factor PAX6 is expressed in both the olfactory and lens placodes and is required for eye and nasal development

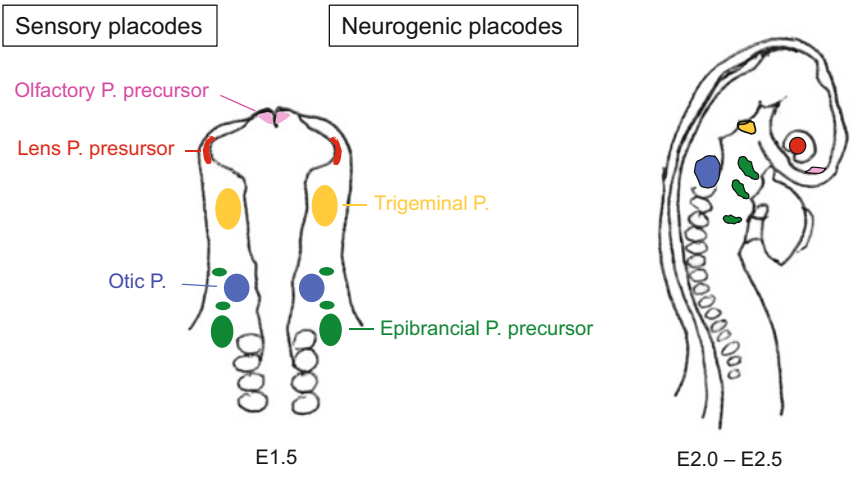

Fig. 7.1 Spatial organization of the cranial sensory and neurogenic placodes. Placodes and placode precursors at embryonic day 1.5 (E1.5) and day 2.0–2.5 (E2.0–E2.5) of chicken embryos are shown (Bhattacharyya and Bronner-Fraser 2004). The olfactory placode precursor (*pink*) is present at the rostral tip of the embryo, and the lens placode precursor (*red*) overlies the lateral portions of the diencephalon. The trigeminal placode (*yellow*) forms in the ectoderm adjacent to the midbrain. The otic placode (*blue*) is surrounded by the epibranchial placode precursor (*green*)

(Grindley et al. 1995; Quinn et al. 1996; Xu et al. 1997). It was demonstrated that HSF1 and HSF4 were required for maintenance of the olfactory epithelium and the lens, respectively. Mutations of *HSF4* gene were associated with human hereditary cataract. Furthermore, HSF1 played a critical role in adaptation of the inner ear against acoustic trauma. In this review, we summarize functions and mechanisms of HSFs in the sensory organs and discuss therapeutic opportunities for treatment of diseases in these organs.

7.2 HSF4 Is Required for Maintenance of the Lens

7.2.1 Mutation of HSF4 *in Human Hereditary Cataract*

HSF4 was identified as a third member of the HSF family (Nakai 1999). HSF1 and HSF2 proteins were highly expressed in most cells and tissues, whereas the expression level of HSF4 protein was relatively low (Nakai et al. 1997; Tanabe et al. 1999). Predicted amino acid sequence of HSF4 uniquely lacked an evolutionally conserved HR-C domain in the HSF family that inhibited trimerization of HSFs, suggesting that HSF4 should be constitutively a trimer that can bind to HSE. These observations implied that HSF4 had physiological roles under unstressed conditions at least in some tissues.

The first report suggesting a physiological role of HSF4 came from the finding that mutations of *HSF4* gene are associated with dominant inherited cataracts in humans (Bu et al. 2002). Thereafter, many patients with hereditary cataract have been shown to be associated with the *HSF4* mutations. Interestingly, autosomal dominant cataract coincides with missense mutations in the DNA-binding domain (Ala20Asp, Lys23Asp, Arg74His, Ile87Val, Arg111Cys, Leu115Pro, Arg120Cys) (Bu et al. 2002; Ke et al. 2006; Lv et al. 2014; Liu et al. 2015), whereas recessive cataract does with missense, deletion, or splice site mutations in the oligomerization domain (HR-A/B) (Arg175Pro, Del595-599) or in the downstream of heptad repeat (DHR) (Arg405X, A1327G) (Smaoui et al. 2004; Forshew et al. 2005; Sajjad et al. 2008) (Fig. 7.2). Patients with the *HSF4* mutations have no other symptom than cataract, suggesting that HSF4 could be dispensable in other tissues. The *HSF4* mutations are also identified in dogs and mice with hereditary cataracts (Mellersh et al. 2006; Talamas et al. 2006).

Age-related cataract is caused by interaction between gene and environmental factors. It was revealed that genetic factors were most important among several risk factors for this disease (Hammond et al. 2000). Analysis of 150 age-related cataract patients identified five mutations of *HSF4* gene, suggesting that *HSF4* mutation also accounts for a small fraction of age-related cataract (Shi et al. 2008). These mutations included missense mutations in the DNA-binding domain (Gln62Arg, Arg117His) and substitutions of nucleotides in the introns.

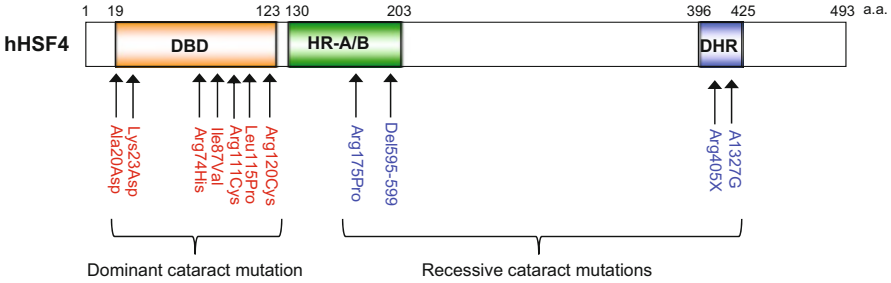

Fig. 7.2 Mutations of *HSF4* gene in patients with hereditary cataract. The schematic indicates the structure of HSF4 protein and the positions of mutations, which are associated with autosomal dominant cataracts (*red*) and autosomal recessive cataracts (*blue*). Substitution of alanine (*Ala*) at amino acid 20 with aspartic acid (*Asp*) is indicated as Ala20Asp. Del595–599 indicates a frameshift deletion of nucleic acids from 595 to 599 that produces a truncated protein (Forshew et al. 2005). Arg405X indicates a substitution of arginine (Arg) at amino acid 405 with a stop codon (X) (Sajjad et al. 2008). A1327G indicates a substitution of A to G at the fourth nucleotide (at nucleotide 1327) in the 5′ splice site of intron 12 (Smaoui et al. 2004). The number of amino acids of each domain in HSF4 is shown. *DBD* DNA-binding domain, *HR* hydrophobic heptad repeat, *DHR* downstream of HR-C

Functional analysis of the HSF4 mutants revealed that the DNA-binding activity of these mutants in patients with autosomal dominant and recessive hereditary cataracts was reduced, constant, or even increased (Enoki et al. 2010; Merath et al. 2013). However, transcriptional activity was severely impaired in most of these HSF4 mutants. In contrast, the DNA-binding activity and transcriptional activity were hardly affected in the HSF4 mutants associated with age-related cataract.

7.2.2 *Inactivation of* HSF4 *Causes Cataract in Mice*

The lens is characterized by its high degree of transparency. Development of the lens occurs by differentiation of epithelial cells into elongated fiber cells that accumulate in concentric layers and lose their nuclei and other organelles (McAvoy et al. 1999). The concentration of protein in the lens fiber cells is extremely high, as much as 450 mg/ml of protein in the center of the lens (Fagerholm et al. 1981). As proteins in the center of the lens cannot turn over and must remain stable and soluble throughout the life of the organism, it is remarkably important to stabilize proteins (Zigler 1994; Bhat 2003). To stabilize proteins in that extreme condition, crystallins constitute nearly 90 % of the soluble protein in the lens fiber cells. The ubiquitous vertebrate crystallins consist of two unrelated families of proteins, the α-crystallins and the β, γ-crystallins (Wistow and Piatigorsky 1988). The former belongs to a small heat shock protein/α-crystallin superfamily. The latter belongs to

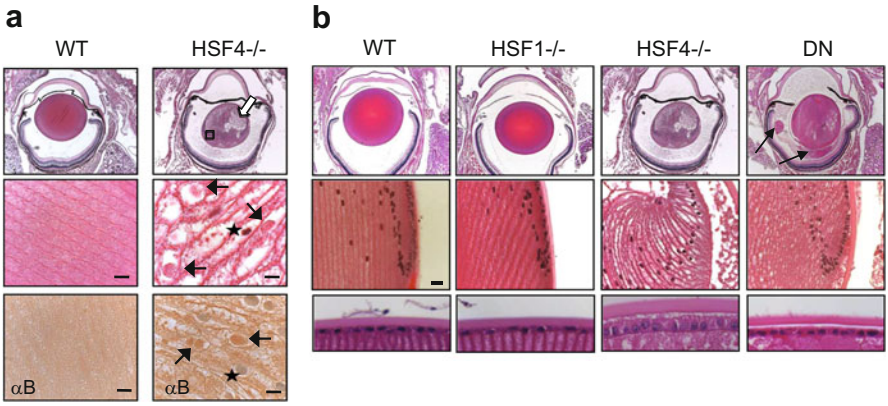

Fig. 7.3 Abnormalities in the lenses of HSF4-null mice. (**a**) The lens fiber cells contain inclusion-like structures in HSF4-null mice (ICR background). Histological examination of the lens sections of the 6-week-old wild-type (WT) and HSF4-null (HSF4−/−) mice stained with HE (*upper*, *middle*) and immunostaining of αB-crystallin were performed (*lower*). An *open arrow* indicates the lesion where structure of the fiber cells was not recognized. The legion indicated by a *square* is enlarged, and cytoplasmic inclusion-like structures are indicated by *arrows*. A *star* indicates the nucleus. Bars, 10 mm. (**b**) Histological examination of the lens sections of 6-week-old wild-type (WT), HSF1-null (HSF1−/−), HSF4-null (HSF4−/−), and double-null (DN) mice. Sections were stained with HE (*upper*). The bow regions (*middle*) and the epithelial cells (*lower*) are magnified (Fujimoto et al. 2004)

a β, γ-crystallin superfamily that may have roles in protein stabilization under dehydrated conditions (Wistow 1990).

It was shown that the level of HSF4 protein in the mouse lens was extremely high compared with those in other tissues, and HSF4 consisted of a major HSE-binding activity in the lens (Somasundaram and Bhat 2000; Fujimoto et al. 2004). Three laboratories independently demonstrated that *HSF4* inactivation in mice caused cataract at early postnatal days (Fujimoto et al. 2004; Min et al. 2004; Shi et al. 2009). Histological examination showed abnormal fiber cells, which contained nuclei and vacuole-like cavity. Nakai group further noticed inclusion-like structures stained heavily with eosin in the fiber cells in mice with ICR background, implicating that the expression of genes involving protein stability was affected by HSF4 deficiency (Fig. 7.3) (Fujimoto et al. 2004). In fact, the expression of heat shock proteins and crystallins was altered. Remarkably, HSP25 was not induced during development of the HSF4-null mouse lens, and the expression of γ-crystallins such as γF- and γS-crystallins decreased (Fujimoto et al. 2004; Min et al. 2004; Shi et al. 2009). These observations suggest that *HSF4* has evolved to adapt to the unique dehydrated environment of the lens in mammals.

7.2.3 *HSF4 Regulates Differentiation into Fiber Cells*

Loss of HSF4 function also resulted in increased proliferation and premature differentiation of the lens epithelial cells (Fig. 7.3) (Fujimoto et al. 2004). These processes are regulated by various cytokines such as fibroblast growth factors (FGFs) (McAvoy and Chamberlain 1989; Robinson et al. 1995; Lovicu and Overbeek 1998). It was revealed that HSF4 inhibited the expression of FGF-1, FGF-4, and FGF-7 in ICR mice and that at least the *FGF-7* gene was a direct target of HSF4. Remarkably, HSF1 inversely activated the similar set of FGFs in the lens that was repressed by HSF4 (Fujimoto et al. 2004). Therefore, abnormal levels of FGFs in the HSF4-null lens reversed to normal levels in the double-null lens, and proliferation and differentiation of the epithelial cells were almost normal in the double-null lens. These results demonstrate that HSF1 and HSF4 competitively regulate expression of FGFs that are essential for cell growth and differentiation (Fig. 7.4). Although the bow region of the lens, where the lens epithelial cells differentiated into fiber cells, was morphologically normal in two other HSF4-null mouse models (Min et al. 2004; Shi et al. 2009), the expression of FGFs could be moderately impaired in these models with C57BL/6 background, like that in an HSF1-null ICR mouse model (Fujimoto et al. 2004). FGFs regulate growth and differentiation of various cell types (Burgess and Maciag 1989); therefore, the regulation of FGF expression by HSF family members might be an important regulatory pathway for development in general.

Abnormal differentiation of the lens fiber cells is generally associated with impaired denucleation. Comprehensive gene expression analyses in the lenses of genetically modified mice revealed that deficiency of HSF4, as well as that of BRG1 or PAX6, reduced the expression of DNase 2β (He et al. 2010), which is

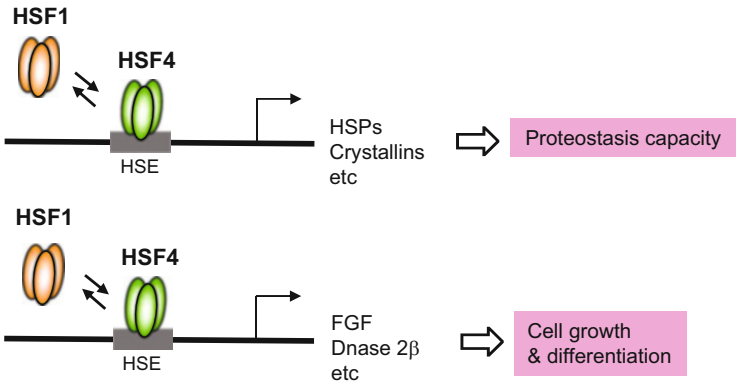

Fig. 7.4 HSF4 and HSF1 control proteostasis capacity, cell growth, and differentiation. HSF4 and HSF1 control proteostasis capacity in the lens fiber cells, by competitively or complementarily regulating the expression of HSPs and crystallins. Furthermore, they are involved in cell growth and differentiation of the lens epithelial cells by regulation the expression of cytokines such as FGFs, DNase 2β, and others. The heat shock response element (*HSE*) is located in the promoters

required for the degradation of nuclear DNA in the lens (Nishimoto et al. 2003). HSF4 also upregulated the expression of DNase 2β directly in the zebrafish lens, and its knockdown reduced the expression of DNase 2β and led to a denucleation defect (Swan et al. 2012; Cui et al. 2013). Furthermore, HSF4 directly repressed the expression of an intermediate filament vimentin, whose overexpression resulted in an impaired denucleation (Mou et al. 2010). Moreover, HSF4 regulated the expression of RAD51 in the lens, which played a role in DNA damage repair and might be involved in cataract formation (Cui et al. 2012). These observations suggest that HSF4 deficiency induces cataracts through multiple pathways.

7.2.4 HSF4 Is Associated with Changes in Chromatin Modification

The lens is composed of only two cell types, and HSF4 starts to be expressed at early stage of lens development in both cell types (Fujimoto et al. 2004; Min et al. 2004). Therefore, it is suitable for comprehensive identification of HSF4-target genes and their functional analysis. Interestingly, levels of HSF1 and HSF2 in the lens were relatively high at embryonic days, but they decreased quickly after birth (Fujimoto et al. 2008). Analysis of the relative location of the HSF4-binding regions revealed that 53 % mapped to the intron and exons and 40 % to the distal parts (>10 kb) of the genes (Fujimoto et al. 2008). Remarkably, only 5 % of HSF4-binding regions mapped to 10 kb promoter-proximal regions. This result is in marked contrast to the localization of canonical HSEs on the 5′-proximal promoter of classical heat shock genes. Furthermore, 70 % of the HSF4-binding regions were occupied by HSF1 and/or HSF2. These observations suggest that HSF4-binding regions are distributed on whole genome and are co-occupied by members of the HSF family.

It is a matter of great interest that HSF binding to genomic regions in stressed and unstressed conditions is linked to gene regulation. Genome-wide analysis showed that HSF1 binding to the promoter does not necessarily induce gene expression in yeast (Hahn et al. 2004; Hu et al. 2007) and in humans (Trinklein et al. 2004; Page et al. 2006). Remarkably, HSF4 binding to genomic regions was closely associated with reduced histone H3K9 methylation, irrespective of the relative location of the HSF4-binding regions, although it was not always correlated with developmental expression of genes on or near the HSF4-binding regions. Furthermore, HSF4 was required for the induction of some *non-HSP* genes during heat shock. These observations suggest that HSF4-mediated changes in chromatin modification are necessary for gene activation in both stressed and unstressed conditions (Fujimoto et al. 2008). HSF4, like HSF1, may access nucleosomal DNA in cooperation with replication protein A (RPA) and a histone chaperone FACT before stimuli (Fujimoto et al. 2012).

7.3 HSF1 Is Required for Olfactory Neurogenesis

7.3.1 Abnormal Nasal Cavity in HSF1-Null Mice

Analysis of sensory organs other than the eye in HSF1- and HSF4-null mice showed normal morphology in the ear, skin, and taste buds in both types of adult mice (Takaki et al. 2006). In contrast, structure of the nasal cavity was abnormal in adult (6 weeks old) HSF1-null mice with ICR and C57BL/6 backgrounds (Takaki et al. 2006). Mice developed chronic sinusitis with accumulation of mucus, which contained mostly dead epithelial cells but not leukocytes. The nasal cavity area increased, which was associated with marked decreases in the turbinate areas and calcified bone (Fig. 7.5). Furthermore, atrophy of the olfactory epithelium containing increased apoptotic cells was observed. These results suggest that two separate processes, the olfactory neurogenesis and clearance of mucus, are impaired in the HSF1-null nasal cavity. Consistent with these abnormalities, olfaction was disturbed in HSF1-null mice.

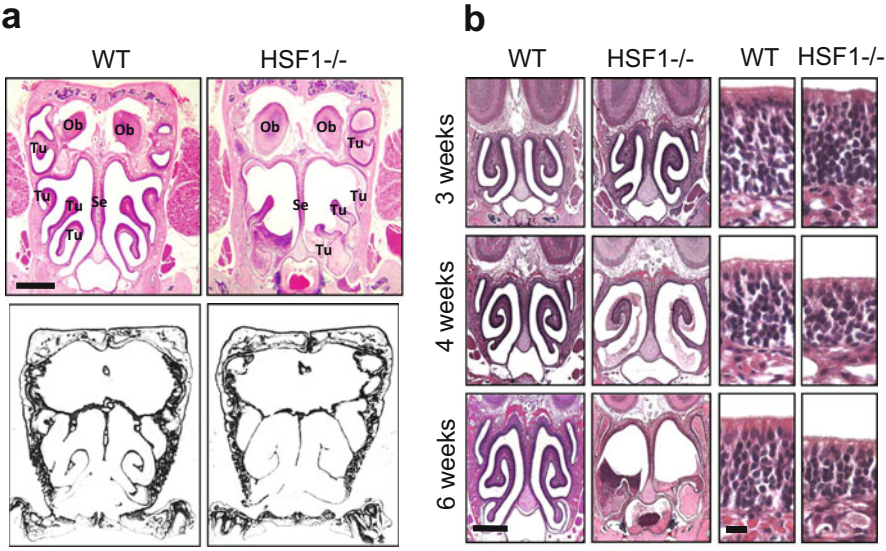

Fig. 7.5 Abnormalities in the nasal cavity of HSF1-null mice. (**a**) Histological examination of the paranasal sections of 6-week-old wild-type (+/+) and HSF1-null (−/−) mice. HE staining was performed (*upper*). *Ob* olfactory bulb, *Se* nasal septum, *Tu* turbinates. Bar, 1 cm. Calcified bone was stained with the Tripp-Mackay method (*lower*). (**b**) HE staining of paranasal sections in 3-, 4-, and 6-week-old wild-type (+/+) and HSF1-null (−/−) mice was performed. The olfactory epithelium is magnified. *Large bar*, 1 mm; *small bar*, 10 μm (Takaki et al. 2006)

7.3.2 Maintenance of Olfactory Neurogenesis by HSF1

HSF1 is not required for development of the olfactory epithelium until 3 weeks old but is indispensable for maintenance of the olfactory epithelium in mice over 4 weeks old (Fig. 7.5) (Takaki et al. 2006). Simultaneously, the DNA-binding activity of HSF1 is induced in the olfactory epithelium in postnatal 4-week-old mice, which is associated with increased levels of major HSPs. In the absence of HSF1, the heat shock proteins are not induced. Requirement of HSF1 in pubertal mice is unique when it is compared with the requirement of transcription factors Mash1 and Ngn1 that control early olfactory neurogenesis (Guillemot et al. 1993; Cau et al. 2002).

Proliferation and differentiation of the olfactory sensory neurons are regulated by many cytokines (Farbman 1994; Mackay-Sima and Chuahb 2000; Schwob 2002), including FGFs, whose expression is regulated by HSF1 and HSF4 in the lens (Fujimoto et al. 2004). Expressions of most genes such as *FGF* genes were constant in the HSF1-null olfactory epithelium (Takaki et al. 2006). In marked contrast, the level of the LIF (leukemia inhibitory factor) expression continued to be high during development of the olfactory epithelium. LIF is essential for normal development of the olfactory sensory neurons (Metcalf 2003). Continuous overexpression of LIF may greatly affect intracellular signalings in the olfactory sensory neuron, in which proteostasis is disturbed due to the reduced expression of HSPs. The LIF expression was inhibited by HSF1 during olfactory development, whereas it was induced by overexpression of HSF4. Thus, HSF1 and HSF4 have opposing effects on the LIF expression (Takaki et al. 2006).

7.3.3 Maintenance of Ciliary Beating by HSF1

Morphological examination showed that mucus was accumulated in the nasal cavity and the ventricles including the aqueduct and fourth ventricle were enlarged in adult HSF1-null mice (Takaki et al. 2007). The latter indicated a communicating hydrocephalus and was observed even in 3-week-old HSF1-null mice but not in 2-week-old mice. It was revealed that not only the mucociliary clearance in the nasal cavity but also the ependymal flow in the lateral ventricles was severely impaired in HSF1-null mice, suggesting ciliary dysfunction in both the respiratory epithelium and ependymal cells (Takaki et al. 2007).

The differential interference contrast image showed beating cilia located on the surface of respiratory cells. In wild-type mice, the whipping movement of the cilia was observed and ciliary beat frequency (CBF) was 13.27 ± 1.53 Hz (Takaki et al. 2007). In marked contrast, the beating amplitude was significantly low, and CBF reduced to 8.70 ± 1.72 Hz in HSF1-null mice. Similarly, CBF in the ventricles of wild-type mice was 18.65 ± 2.54 Hz, whereas that of HSF1-null mice was 8.60 ± 1.82 Hz. Interestingly, the whipping movement of the cilia was markedly

impaired even in the tracheae and oviducts and CBF was decreased. These results indicate that HSF1 deficiency causes severe impairment of ciliary beating in many organs. Examination of the ultrastructure of cilia by electron microscopy showed that about 10 % of the cilia possessed abnormal central microtubules and microtubule doublets such as deletion or transposition, suggesting that assembly or organization of microtubules might be impaired.

Microtubules are formed by protofilaments of α/β-tubulin heterodimers. Among five β-tubulin isotypes, β_i- and β_{iv}-tubulin are found in all axoneme structures (Renthal et al. 1993; Jensen-Smith et al. 2003) and are required for ciliary function and assembly (Vent et al. 2005). It was revealed that level of β_{iv}-tubulin in the cilia as well as that of ciliary α-tubulin specifically decreased in the HSF1-null respiratory epithelium. Furthermore, HSP90 expression disappeared especially in the HSF1-null cilia, while it was highly expressed in wild-type cilia. HSP90 maintains ciliary beating by stabilizing tubulin proteins and facilitating microtubule polymerization (Takaki et al. 2007).

7.4 HSF1 Protects the Sensory Hair Cells from Damage

The inner ear serves auditory and vestibular functions by responding to sound, gravity, and movement. Almost all cell types of the inner ear including sensory hair cells derive from the otic placode, and the sensory hair cells are hardly regenerated in adult animals (Whitfield 2015). The expression of HSP70 in the cochlea of the inner ear is induced not only by whole-body or local hyperthermia (Dechesne et al. 1992; Akizuki et al. 1995) but also by noise overstimulation (Lim et al. 1993). It plays a protective role against damage of the cochlea induced by noise overstimulation and ototoxic drugs including the anticancer agent cisplatin and aminoglycoside antibiotics (Yoshida et al. 1999; Oh et al. 2000; Sugahara et al. 2003; Taleb et al. 2009). Roles of HSF1 in adaptation to these damages have been analyzed. The morphology of the cochlea and the hearing examined by the auditory brainstem response (ABR) were normal in adult HSF1-null mice (Fig. 7.6) (Sugahara et al. 2003). However, the noise-induced loss of the sensory hair cells was much more serious in HSF1-null mice than in wild-type mice. Furthermore, the recovery of hearing from noise overstimulation was more reduced in HSF1-null mice (Fairfield et al. 2005; Gong et al. 2012). Thus, HSF1 protects the sensory hair cells against acoustic trauma.

Geranylgeranylacetone (GGA), which is an oral antiulcer drug, is one of the inducers of the HSR (Hirakawa et al. 1996). It was revealed that the expression of HSP70 was strongly induced in the sensory hair cells and spinal ganglion cells by the administration of GGA (Mikuriya et al. 2005). Pretreatment with GGA suppressed the noise-induced decline of the ABR threshold and the loss of sensory hair cells (Mikuriya et al. 2005). Furthermore, it inhibited the attenuation of progressive hearing loss in a mouse model of age-related hearing loss (Mikuriya et al. 2008). These observations suggest that the administration of inducers of the

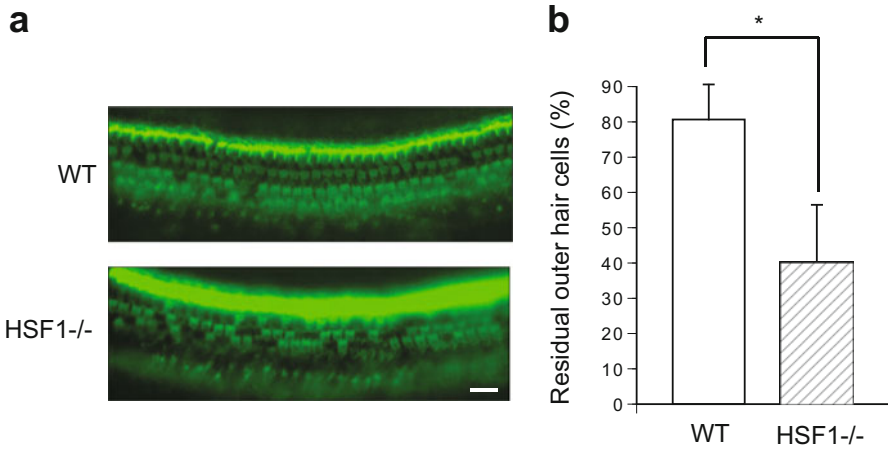

Fig. 7.6 HSF1-null sensory hair cells are highly sensitive to noise overstimulation. (**a**) Surface preparation of the cochlear in wild-type (WT) and HSF1-null (HSF1−/−) mice after the noise overstimulation. The mice were exposed to intense noise for 1 h. After a 6-h interval, the mice were exposed to the same intense noise again for 10 h. (**b**) Percentages of the residual outer hair cells in the cochlear second turn are shown. Means of three independent experiments are shown. *Stars* indicate $p < 0.05$ (Sugahara et al. 2003)

HSR is a promising therapeutic approach for damage-induced or aging-related hearing loss.

7.5 Future Perspectives

The HSF family members play essential roles in early development of the brain and reproductive organs (Kallio et al. 2002; Wang et al. 2003, 2004). HSFs can regulate the expression of some development-related genes, but we did not understand well the difference between the role of HSFs and that of master regulators (transcription factors) of development (Abane and Mezger 2010) (see Chaps. 6 and 8). In contrast, roles of HSFs during development of sensory organs are unique in terms of timing of HSF activation. Activity of HSFs reaches to a peak during postnatal developmental processes, which is required for normal development and maintenance of the lens and olfactory epithelium. As the sensory organs are continuously exposed to external stimuli, the developmental activation of HSFs should be programmed in the genome. It is necessary to uncover mechanisms by which HSF activity is regulated during postnatal periods and effects of activated HSFs on chromatin structure and gene transcription in the future.

Although development of another sensory placode, the otic placode, is normal in young HSF1-null adult mice (Sugahara et al. 2003; Fairfield et al. 2005), age-related hearing loss is suppressed by HSF1 activation (Mikuriya et al. 2008).

Therefore, HSF1 may also play a role in the maintenance of the aged-inner ear function. This point as well as the role of HSF1 in mouse life span should be clarified in the future.

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Chapter 8
HSF Is Required for Gametogenesis

Koji Shiraishi

Abstract Gametogenesis, spermatogenesis and oogenesis, is a unique process that includes meiosis, a step that produces haploid cells. Heat shock factors (HSFs), especially HSF1 and HSF2, play important roles during this process in cell type- and stage-specific manners; however, their functions in spermatogenesis and oogenesis are different because the cellular kinetics of spermatogenesis and oogenesis are completely different. Basically, HSF1 acts in cytoprotection and cell survival, playing an important role in quality control mechanisms by eliminating injured or abnormal cells during spermatogenesis, thereby protecting the organism from abnormal development in the next generation. The role of HSF2 in spermatogenesis is controversial, but it may be involved in the spermatogenic process. The disruption of HSF1 and HSF2 in oocytes led to multiple defects during meiotic maturation, causing female infertility and reduced oogenesis, respectively. The involvement of HSF1 and HSF2 has also been reported in pathological conditions, such as cryptorchidism, varicocele, and genotoxic stress response, where heat stress and oxidative stress are the major factors that inhibit spermatogenesis. The investigation of HSFs in gametogenesis per se may provide important information for understanding the physiology/pathophysiology of spermatogenesis and oogenesis and for establishing new therapeutic approaches for male and female infertility.

Keywords Heat shock factor • Heat shock proteins • Spermatogenesis • Oogenesis

8.1 Introduction

Responses against heat and other harmful cell stresses are regulated mainly by heat shock transcription factors (HSFs) (Wu 1995). By inducing the transcription of the molecular chaperones called heat shock proteins (HSPs), HSFs protect the cell from the deleterious consequences of protein-damaging stress. In vertebrates transcription of HSPs controlled mainly by four HSFs: HSF1, HSF2, HSF3, and HSF4 (Morimoto 1998; Nakai et al. 1995; Wu 1995). HSF3 is not expressed in human

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cells (Fujimoto et al. 2010). Basically HSF1 acts as a cell survival factor against various stresses and protects against cell death by upregulating a specific heat shock gene (Nakai and Ishikawa 2001; Inouye et al. 2003). HSFs play important roles during the process of gametogenesis, i.e., spermatogenesis and oogenesis, which includes meiosis, a step that produces haploid cells. Especially in the testis, which has the most rapid cell cycle in the whole body, the functions of HSFs in spermatogenesis are quite unique. The expression of HSF4 is restricted to the brain and lung and is required for ocular lens development and fiber cell differentiation (Nakai et al. 1997; Fujimoto et al. 2004). This chapter reviews the contribution of HSF1 and HSF2 in spermatogenesis and oogenesis based on findings mainly from mice and mentions the involvement of the HSFs in spermatogenesis during clinical situations.

8.2 Normal Spermatogenesis

Spermatogenesis is a developmental step that occurs in the seminiferous tubules in the testis, involving dynamic changes from spermatogonia to mature spermatozoa through mitosis, meiosis, and differentiation (Fig. 8.1). The final step of spermatogenesis, spermiogenesis, features unique chromatin remodeling as somatic histones

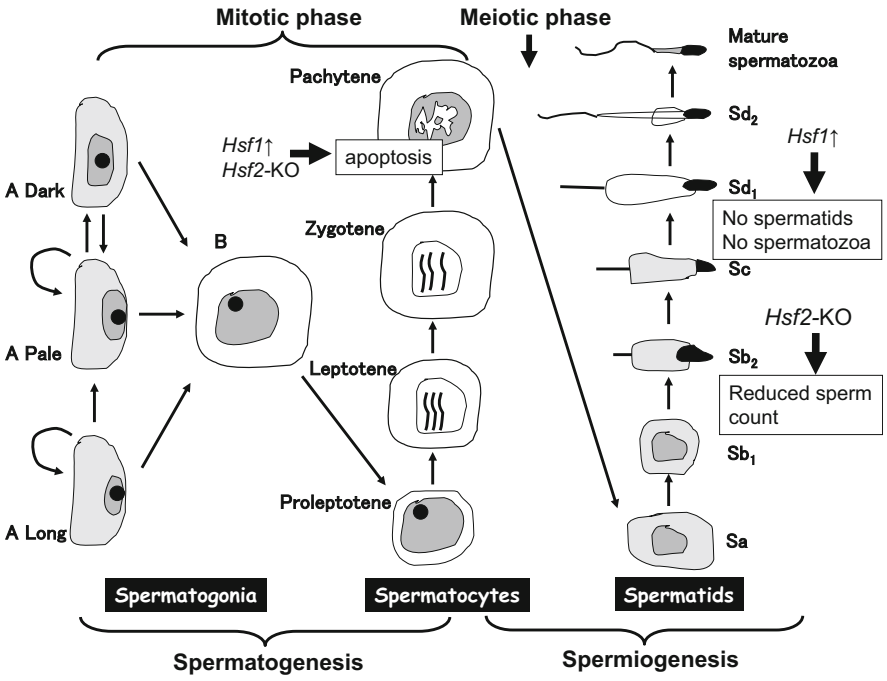

Fig. 8.1 A schema of mammalian spermatogenesis and the contribution of HSF1 and HSF2

and is replaced by DNA packing proteins, such as transition proteins and protamines. Germ cells continuously proliferate to supply a large amount of spermatozoa, whereas up to 75 % of potential spermatozoa are estimated to undergo apoptotic cell death in the seminiferous tubules of adult animals (Print and Loveland 2000). During the process of spermatogenesis, dramatic changes in the patterns of gene expression and chromatin structure are observed with each germ cell type and in a stage-specific manner.

8.2.1 Roles of HSF1 in Normal Spermatogenesis

HSF1 is a highly conserved transcription factor that coordinates stress-induced transcription through binding of heat shock response elements (HSEs) to the promoters of the HSP molecular chaperone genes (He et al. 2003). Basically, HSF1 promotes cell survival against various types of stress by inducing HSPs and other proteins. In the mouse testis, HSF1 is abundantly expressed from pachytene spermatocytes to round spermatids but cannot be detected in spermatogonial stem cells or elongating spermatids (Akerfelt et al. 2010). In the testis, where various types of cells are contained, there are also a variety of HSF1 expression levels in each cell type and the function in each cell type differs. In the normal human testis, distinct stage-dependent expression of HSF1 immunoreactivity was observed in primary spermatocytes (Fig. 8.2a) and postmeiotic spermatids (Fig. 8.2b) (our preliminary data).

Different from oogenesis and the other somatic cells, spermatogenesis is a unique process in which the cell kinetics are extremely high compared with the other organs. A large amount of low-quality spermatozoa are produced and released into the seminal tract, and simultaneously, immature spermatogenic cells undergo apoptosis and phagocytosis by Sertoli cells (Shiratsuchi et al. 1997). Primary spermatocytes are diploid cells, which are characterized by their nuclear appearance including the following: preleptotene, leptotene, zygotene, pachytene, or diplotene stages (Fig. 8.1). Recent studies have demonstrated the existence of a "pachytene checkpoint" in late meiotic prophase; when triggered, it leads to the elimination of defective germ cells by apoptosis (Roeder and Bailis 2000). Involvement of HSF1 is summarized in Table 8.1. Using transgenic mice, Nakai et al. showed that the active form of HSF1 increased the number of apoptotic pachytene spermatocytes and blocked spermatogenesis at this step, leading to spermatogenic dysfunction (Nakai et al. 2000). Further studies using HSF1 overexpression have shown massive degeneration of the seminiferous epithelium and the general absence of round and elongated spermatids (Izu et al. 2004; Hayashida et al. 2006). In other words, HSF1 paradoxically promotes apoptotic cell death of male germ cells rather than cytoprotection (Widlak et al. 2003; Nakai et al. 2000). On the other hand, *Hsf1*-KO male mice were fertile but produced approximately 21 % less sperm than wild-type mice (Salmand et al. 2008; Akerfelt et al. 2010). Taking these observations together, HSF1 could be considered to be

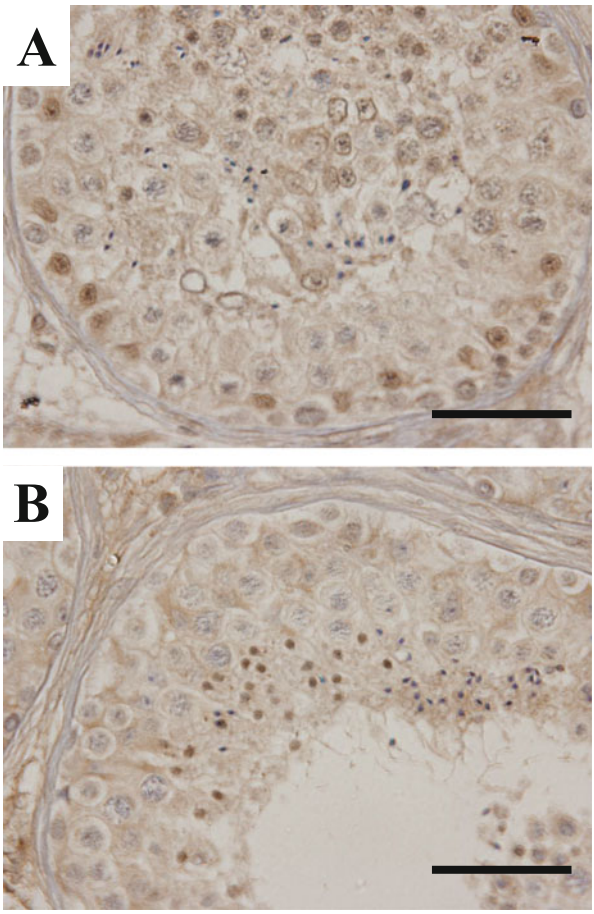

Fig. 8.2 Representative immunostaining for HSF1 in the human testis with normal spermatogenesis (×400, bar: 10 um). Stage-dependent expression of HSF1 was observed in primary spermatocytes (**a**) and postmeiotic spermatids (**b**). These microphotos were obtained from the same testis. Primary antibody was courtesy from Professor Nakai

dispensable for mouse spermatogenesis. Heat-induced apoptosis of primary spermatocytes is inhibited in mice lacking HSF1 (Izu et al. 2004). Furthermore, the inhibition of germ cell apoptosis by caspase inhibitors has also shown to have no merit for long-term spermatogenic function using a rat testicular ischemia-reperfusion model (Shiraishi et al. 2000). Although there is no information regarding the quality of spermatozoa produced from *Hsf1*-KO mice, HSF1 plays an important role in checking quality control mechanisms by eliminating injured or abnormal cells in spermatogenesis, thereby protecting the organism from abnormal development in the next generation.

Previous studies have shown that HSP27, HSP40, HSP60, HSP70, HSP90, and HSP105 are important for spermatogenesis; specifically, HSP70.1, HSP70.2, and HSP90 have been well studied (Sarge 1995; Dix et al. 1996, 1997). The expression levels of major heat shock genes, Hsp110, Hsp90α, Hsp90β, Hsp70.1, Hsp70, Hsp60, Hsp40, Hsp27, and testis-specific Hsp70.2, were not altered or even downregulated in the testes in *Hsf1*-KO mice compared with wild-type mice (Izu

Table 8.1 Reproductive phenotypes of *Hsf1* and *Hsf2* overexpression and knockout mouse

	Male phenotype	Female phenotype
Hsf1 transgenic	Apoptosis of pachytene spermatocyte no meiosis Protection against heat-induced spermatogonia death spermatocyte death	
Hsf1 knockout	No male infertility Lack of genotoxic proliferation block in spermatogonia Lack of genotoxic cell death in meiotic spermatocyte Lack of protection against heat-induced spermatogonia and spermatocyte death	Delay of germinal vesicle breakdown Meiosis arrest Abnormal cytokinesis Apoptosis of prophase oocyte Impaired granule exocytosis and pronuclei formation
Hsf2 transgenic	Not established	Not established
Hsf2 knockout	Reduced testicular size, sperm count, and male infertility Pachytene spermatocyte apoptosis Arrest of meiosis Complete spermatogenic defect in *Hsf1*/*Hsf2* double KO	No female infertility Reduced ovarian weight and female infertility Ovulation defect

et al. 2004). HSP70.2 is synthesized during the meiotic phase of spermatogenesis and is abundant in pachytene spermatocytes, and it is required for Cdc2 to form a heterodimer with cyclin B1, suggesting that it is a chaperone necessary for the progression of meiosis in the germ cells of male mice. Prior to the onset of massive apoptosis caused by the expression of active HSF1 in spermatocytes, a marked decrease in Hsp70.2 mRNA and protein levels in spermatocytes occurs (Widlak et al. 2007). The activation of HSF1 in spermatocytes does not activate these HSPs but leads to a massive activation of the caspase-3 pathway, a common pathway toward apoptosis (Widlak et al. 2003; Vydra et al. 2006), indicating that there are multiple pathways for HSF1-dependent apoptosis in primary spermatocytes. Possible HSF1-dependent factors involved in negative regulation of Hsp70.2 and other testis-specific genes remain to be identified. The level of HSP60 and HSP105 was rather increased in mice overexpressing *Hsf1*, which promotes the apoptosis of germ cells (Vydra et al. 2006).

On the other hand, the original function of HSF1, protection of cells from stress and escape from cell death, is observed in immature germ cells, mainly in spermatogonia (Izu et al. 2004; Akerfelt et al. 2010). Dark A spermatogonia divide to maintain their own numbers, whereas others differentiate into pale type A, then type B, which are the immediate precursors of the spermatocytes. Thus, the dark spermatogonia form a reservoir of resting cells, which divide into the primary spermatocytes (Fig. 8.1). Unlike the primary spermatocytes, diminishing these groups of cells causes permanent loss of germ cell supply, and these cells should be protected against a variety of stresses to maintain spermatogenic function. Therefore, how does HSF1 decide whether to prevent or promote apoptosis?

Hayashida et al. demonstrated a novel survival pathway, which requires a balance between HSP and TDAG51 (T-cell death-associated gene 51)-mediated pathways (Hayashida et al. 2006). TDAG51 is a member of the PHL (pleckstrin homology-like) domain family and acts upstream of the Fas-mediated cell death pathway. There are interplays between proapoptotic TDAG51 and antiapoptotic HSPs, both of which are direct target genes of HSF1. The unique balance between HSPs and TDAG51, in favor of HSPs in spermatogonia and in favor of TDAG51 in spermatocytes, would trigger HSF1-dependent cell survival and apoptosis, respectively.

Spermatids do not undergo further division but gradually mature into spermatozoa by changing their shape and are often described as "round" or "oval" spermatids and may also be called "early" or "late" spermatids. This step is called spermiogenesis (Fig. 8.1). Akerfelt et al. have shown that HSF1 is transiently expressed in meiotic spermatocytes and haploid round spermatids in the mouse testis and is localized to the sex chromatin both prior to and after the meiotic divisions (Akerfelt et al. 2010). HSF1 regulates the postmeiotic expression of X- and Y-chromosome genes that are required for correct packing of the DNA in the sperm (Akerfelt et al. 2010; Toure et al. 2004). These observations demonstrate the role of HSF1 in final spermatid differentiation as well as the mitotic and meiotic processes in spermatogonia and primary spermatocytes.

Testosterone, which binds and activates the androgen receptor (AR), suppressed HSF1 expression in Sertoli cells by promoting binding of the AR to the *Hsf1* promoter. As anticipated, HSF1 was upregulated in Sertoli cell androgen receptor-KO mouse testes compared with wild-type mice (Zhang et al. 2012), indicating that androgens may regulate HSF1 expression. Furthermore, HSF1 partially mediates the effects of androgens on the expression of HSPs in Sertoli cells through AR action (Yang et al. 2014). Germ cells localize to seminiferous tubules, which are isolated from the systemic circulation by the blood-testis barrier, indicating that somatic cells (i.e., Leydig, Sertoli, and endothelial cells) are easily affected by a variety of stresses. Understanding the role of HSF1 in these cells and their endocrinological regulation is therefore important for understanding spermatogenesis.

8.2.2 Roles of HSF2 in Normal Spermatogenesis

In addition to HSF1, HSF2 belongs to the family of HSFs and is most abundantly expressed in the testis, as well as other organs (Fiorenza et al. 1995). HSF2 plays important roles in regulating normal spermatogenesis process in mice (Goodson et al. 1995; Kallio et al. 2002; He et al. 2003; Wang et al. 2003; 2004; Akerfelt et al. 2008) with cell- and stage-specific expression profiles (Sarge et al. 1994; Alastalo et al. 1998; Björk et al. 2010). In rat, the expression of the alternatively spliced HSF2-alpha and HSF2-beta isoforms is developmentally regulated in a stage-specific manner (Alastalo et al. 1998). HSF2 mRNA first appears in the testis between day 14 and 21 of postnatal development and is found mainly in the nuclei

of early pachytene spermatocytes (stages I–IV) and round spermatids (stages V–VII) in the rat (Sarge et al. 1994; Alastalo et al. 1998).

The phenotype of *Hsf2*-KO in spermatogenesis is controversial. *Hsf2*-KO mice exhibit reduced testis and epididymis sizes, vacuolization of the seminiferous tubules, and lower sperm counts (Kallio et al. 2002; Wang et al. 2003). Kallio et al. reported that spermatocytes are eliminated via stage-specific apoptosis at the pachytene spermatocyte stage. They proposed that this apoptosis could be caused by an elevated frequency of synaptonemal complex abnormalities, indicated by the formation of loop-like structures or the appearance of separated centromeres, which can activate the pachytene checkpoint and trigger apoptosis (Roeder and Bailis 2000; de Rooij and de Boer 2003) in *Hsf2*-KO spermatocytes (Kallio et al. 2002). On the other hand, McMillan et al. found that *HSF2*-KO mice showed normal spermatogenesis (McMillan et al. 2002). This discrepancy may be caused by the technical methods for the generation of the knockout mice or the genetic background of the mice (Kallio et al. 2002; McMillan et al. 2002). The inactivation of both *Hsf1* and *Hsf2* results in a more severe phenotype, manifested by arrested spermatogenesis and complete sterility (Wang et al. 2004). It has also been proposed that HSF1 requires cooperation with HSF2 in development (Sandqvist et al. 2009) and that the synergistic action of both HSFs is crucial for spermatogenesis (Wang et al. 2003). On the other hand, the level of HSF2 protein in the testis of *Hsf1*-KO mice was the same as that in wild-type mice, indicating that there is no compensatory expression between HSF1 and HSF2 (Izu et al. 2004).

HSF2 may function to regulate the expression of *HSP* genes during germ cell differentiation; several studies have identified target genes for HSF2, whereas the target genes in spermatogenesis are largely undetermined and the vast majority of potential targets are not heat shock genes. Basically no correlation between the expression of HSF2 and classical HSF targets (e.g., HSPs) has been found (Sarge et al. 1994; Alastalo et al. 1998; Kallio et al. 2002; Wang et al. 2003). HSF2 has been shown to bind to the promoter of the *hsp70.2* gene, which is essential for spermatogenesis, in a cell type- and germinal stage-dependent manner (Sarge et al. 1994), whereas it is not a target of HSF2 (Kallio et al. 2002; Wang et al. 2003; McMillan et al. 2002; Akerfelt et al. 2008). Two splice variants, HSF2α and HSF2β, have been identified and HSF2α is predominantly expressed in the mouse testis (Goodson et al. 1995). Transfection experiments have revealed that the HSF2α isoform has 2.6-fold more transcriptional activity than the HSF2β isoform (Goodson et al. 1995). This alternative splicing of HSF2 is regulated during testis postnatal development, with a change in the expression level from predominantly HSF2β at day 7 to primarily HSF2-a in the adult testis. Different functions have been considered for these splice variants of HSF2 in spermatogenic cells.

It has been shown that HSF2 binds the promoters of a large set of targets and regulates the transcription of sex chromosome multicopy gene promoters in postmeiotic cells (Akerfelt et al. 2008). On the Y chromosome, the possible target genes for HSF2, as identified by ChIP-on-chip analysis, were the following: spermatogenesis-associated glutamate (E)-rich protein 4a (*Speer4a*), *Hspa8* (formerly *Hsc70*), ferritin mitochondrial (*Ftmt*), spermiogenesis-specific transcript on

the Y (Ssty2), Scyp3-like Y-linked (Sly), and Scyp3-like X-linked (*Slx*) (Akerfelt et al. 2008). In the postmeiotic spermatids (i.e., round spermatids), where *Ssty2* is thought to regulate chromatin remodeling, HSF2 is involved in chromatin remodeling during spermatogenesis through direct regulation of *Ssty2* and/or *Sly* (Toure et al. 2004; Akerfelt et al. 2008), which exist in multiple copies throughout the *MSYq* (male-specific Y-chromosome long arm) region. The presence of a Cor1 domain, which helps chromatin binding, in the Sly and Slx proteins and the fact that there is a high occurrence of sperm head abnormalities in some *MSYq* deletions (Ellis et al. 2005) indicate that *MSYq* may cause chromatin remodeling impairment during sperm head condensation. Taken together, the human data suggest that deletions in the MSYq region are the most common genetic cause for spermatogenic failures, resulting in oligo- and azoospermia (Krausz 2005); the role of HSF2 in regulating these genes is crucial for spermatogenesis. More directly, Mou et al. have identified nine synonymous mutations and five missense mutations in *Hsf2*, and the R502H mutation affected the transcriptional regulatory function of HSF2 as measured by the expression level of the target gene *HspA2* in patients with idiopathic azoospermia (Mou et al. 2013). Testis-specific HSPA2 is a member of the HSP70 family (Naaby-Hansen and Herr 2010). This molecular chaperone is present in spermatocytes during meiosis, participating as an element of the synaptonemal complex, and during the maturation stage of spermiogenesis (Georgopoulos and Welch 1993). These observations have obviously demonstrated that HSF2 plays a crucial role in spermatogenesis, in both model organisms and humans.

8.2.3 Another HSF in Spermatogenesis

A heat shock-like factor, HSFY, which shares partial homology with classical HSFs, was discovered on the human Y chromosome (Shinka et al. 2004; Tessari et al. 2004). HSFY contains a HSF-type A DNA-binding domain in the middle portion of the protein that is similar to other *Hsf* genes (Shinka et al. 2004; Tessari et al. 2004). In humans, the expression is observed to be mainly in the testis (Tessari et al. 2004), especially in Sertoli cells and spermatogenic cells (Shinka et al. 2004). The functions were presumed to be different in each cell type. However, the putative DNA-binding domain shows only 30 % homology to that of classical HSFs such as *Hsf1* and *Hsf2* (Shinka et al. 2004), and its exact function in spermatogenesis remains unknown (Vinci et al. 2005; Kinoshita et al. 2006). Therefore, HSFY is postulated to have functions different from those of the classical HSFs.

The HSFY gene is localized in the AZFb region of the Y chromosome, and its deletion causes azoospermia (Ferlin et al. 2003). On the other hand, high HSFY expression was observed in men with a normal spermiogram compared with infertile men (Ferlin et al. 2010), indicating that its expression is a good sign for

spermatogenesis. Furthermore, HSFY expression is altered in infertile patients with hypospermatogenesis (Sato et al. 2006).

8.3 Normal Oogenesis

Completely different from the testis, where huge amounts of sperms are produced every second, the cell kinetics of gametogenesis in the ovary are very low and only one oocyte matures in every month in humans. Mammalian oocytes are highly specialized cells engaged in complex meiotic cell division, characterized by several "stops and starts" prior to the formation of the zygote for fertilization (Mehlmann 2005) (Fig. 8.3). During development, oogonia enter meiosis at 13.5 embryonic days, and oocytes remain blocked at prophase I until they are fully grown. Then, in response to hormonal signals or after experimental isolation from their ovarian follicles, oocytes resume meiosis, enter meiotic maturation, and progress to metaphase II (MII), at which stage they can be fertilized. Basically, oocyte maturation ends with the first meiotic division. Meiosis is characterized by a tightly regulated chromosome chromatid separation, resulting in the extrusion of the first polar body, which contains less than 20 % of the oocyte volume. Although information regarding the role of HSFs in oogenesis is very limited compared with spermatogenesis, it was originally reported that HSF1 was highly expressed in mouse oocytes, whereas the expression of HSF2 was very faint (Christians et al. 1997). Accumulating

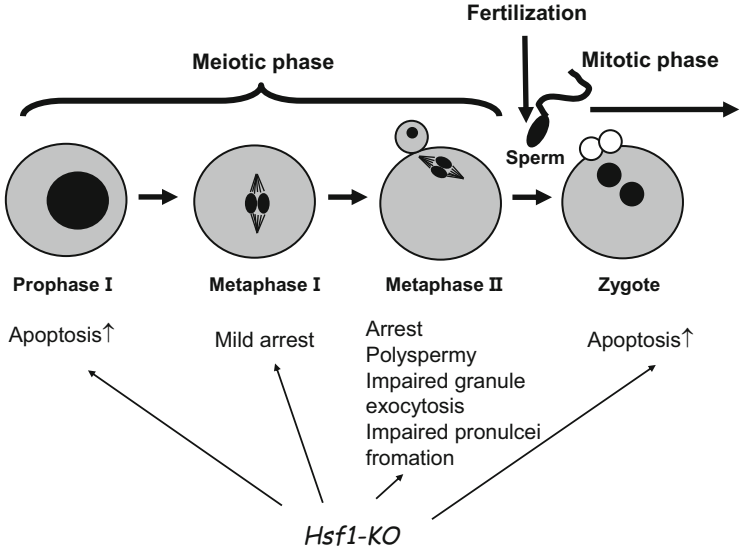

Fig. 8.3 A schema of mammalian oogenesis and the contribution of HSF1

evidence reviewed in this section has shown that HSF1 and 2 definitely play pivotal roles in oogenesis.

8.3.1 Roles of HSF1 in Normal Oogenesis

The involvement of HSF in oogenesis was first reported in a *Drosophila* study (Jedlicka et al. 1997) demonstrating that HSF is essential for oogenesis and HSF's contributions were complex because its role in oogenesis was only partially mediated by the regulation of HSP genes.

It has been reported that *Hsf1*-KO female infertility is linked to the inability of *Hsf1*-KO oocytes to produce viable embryos after natural mating (Christians et al. 2000). *Hsf1*-KO mice have normal ovaries and reproductive tracts, indicating that folliculogenesis and oogenesis were not influenced by the absence of HSF1. However, embryos without HSF1 were unable to develop properly beyond the zygotic stage. HSF1 acts as a transcription factor for meiotic genes during both embryonic and adult oocyte meioses. The resumption of meiosis, characterized by germinal vesicle breakdown (GVBD), was delayed in *Hsf1*-KO oocytes (Metchat et al. 2009). *Hsf1*-KO oocytes exhibited multiple defects during meiotic maturation, a marked block at the metaphase I (MI) stage, and alterations in meiotic asymmetrical cytokinesis, resulting to less than 16 % of mutant oocytes reaching the normal MII stage (Metchat et al. 2009). Subsequently, most of the MII oocytes were unable to cleave to the two-cell stage after fertilization, possibly due to mitochondrial damage and altered redox homeostasis (Bierkamp et al. 2010).

Involvement of HSP90, the major HSP expressed in fully grown oocytes, has been well documented. HSP90 is the major HSF1-dependent chaperone and is markedly downregulated by the absence of HSF1 (Metchat et al. 2009). Both *Hsp90*-KO and the HSP90 inhibitor, 17-(allylamino)-17-demethoxygelda-namycin (17AAG), caused a similar phenotype: impairment of meiosis progression. This finding suggests that Hsp90 deficiency contributes to the developmental defects seen in *Hsf1*-KO oocytes (Metchat et al. 2009). HSF1 directly regulates the transcription of Hsp90α, and the lack of Hsp90α leads to the degradation of kinases such as PLK1 and CDK1. In addition, the mitogen-activated protein kinase pathway was shown to regulate the asymmetry of the first meiotic division and MAPK activity was reduced in *Hsf1*-KO oocytes (Metchat et al. 2009). In addition to the downregulation of many HSPs in oocytes, other HSF1 target genes may contribute to the pleiotropic meiotic phenotype (Metchat et al. 2009): Pre-ovulated *Hsf1*-KO oocytes (1) have cytoskeletal deformities (i.e., Golgi apparatus, cortical actin cytoskeleton, cytoplasmic aggregates) (Christians et al. 2000); (2) have mitochondrial dysfunction, leading to the increased production of reactive oxygen species; and (3) are susceptible to oxidative stress (Bierkamp et al. 2010).

8.3.2 Roles of HSF2 in Normal Oogenesis

Female *Hsf2*-KO mice exhibited multiple fertility defects (Kallio et al. 2002): (1) reduced litter size, (2) the production of abnormal eggs (70 % of fertilized oocytes appear to be abnormal and unable to proceed to the two-cell stage), (3) reduction in ovarian follicle number and the presence of hemorrhagic cystic follicles, and (4) abnormal responses to gonadotropin due to abnormally elevated (50–60 times) levels of luteinizing hormone receptor mRNA (Kallio et al. 2002), which can be rescued by superovulation treatment. Taken together, these findings suggest that HSF2 plays a part in the oogenic process and defects in its function quantitatively alter oogenesis.

Because HSF1 has a role in testis Sertoli cells (Yang et al. 2014), there should also be a role in ovarian somatic cells (i.e., granulose cells and theca cells) to directly or indirectly regulate oogenesis. However, no information has been found to support roles for HSF 1 and 2 in the ovarian somatic cells. Furthermore, no placental defects were identified in the *Hsf2*- KO models, which could have explained embryonic lethality (Kallio et al. 2002).

8.4 HSF in Stressed Spermatogenesis and Its Clinical Significance

8.4.1 Role of HSFs on Heat-Induced Spermatogenic Cell Death

The testes in most mammals are maintained 2–7 °C below body temperature (in humans: 2–3 °C) by their location outside the body cavity in the scrotum and by a countercurrent heat-exchange process that cools the blood entering the testis (Kandell and Swerdloff 1988). Spermatogenesis is disrupted readily by slight increases in temperature or by other environmental stresses, implying that HSFs and HSPs play important roles in spermatogenic cells. In particular, primary spermatocytes are the most sensitive germ cells to heat stress (Yin et al. 1997). On the other hand, information regarding the effects of heat stress on oogenesis is very limited because the ovary is localized in the abdominal cavity and the cell kinetics are much lower than for spermatogenesis. The importance of precise testicular thermoregulation has been shown by the fact that even slight elevations in scrotal temperature, which are common in clinical practice, such as cryptorchidism, varicocele, sauna use, wearing of tight clothing, and even usage of laptop computers, are associated with male infertility. Studies on the effects of temperature on the testis have revealed that all testicular cell types, especially germ cells, are affected readily by heat stress (Shiraishi et al. 2012). Among a variety of cellular functions, HSF plays an important role in protecting the cells or causing apoptotic cell death.

It was found that HSF1 in pachytene spermatocytes was activated at 35 °C, whereas HSF1 from the somatic testis cells was activated at 42 °C (Sarge 1995), indicating that the mode of HSF1 activation is cell type specific. The stage-specific susceptibility to heat stress is supposed to be caused by the function of supporting Sertoli cells (Lue et al. 1999). Few pachytene spermatocytes at stage V died after heat stress in wild-type mice, whereas numerous pachytene spermatocytes at stages XI–XII died. In contrast, few pachytene spermatocytes at stages XI–XII died in HSF1-KO mice (Izu et al. 2004). These results demonstrate that HSF1 promotes heat shock-induced cell death of pachytene spermatocytes. In this section, literature reports describing the expression and role of HSF in pathophysiological conditions are reviewed.

8.4.2 Cryptorchidism

Although an experimental cryptorchidism model is often used to investigate the effects of in vivo heat shock stress on spermatogenesis, cryptorchidism per se is a congenital developmental disorder and the underlying pathophysiology causing deteriorated spermatogenesis still remains controversial. Heat stress in cryptorchidism is not a primary cause but an accompanying detrimental effect due to the retention of the testis in the abdominal cavity or inguinal tract. In other words, an experimental cryptorchidism model is a mild and long-term heat stress model for spermatogenesis. To examine the effects of short-term high-temperature exposure, anesthetized animals were simply submerged in a water bath at 43 °C for several minutes and then allowed to recover for up to several hours.

Shrinkage in the size of the testes, thinning of the germinal epithelium, and loss of germ cells (particularly spermatocytes and spermatids) has been reported in the initial stages of cryptorchidism. Apoptosis, rather than necrosis, has been considered the predominant mechanism of germ cell death in cryptorchidism (Kocak et al. 2002). *Hsf1*-KO and transgenic mice have shown than HSF1 is involved in cell death in primary spermatocytes without HSP induction in an experimental cryptorchid model (Izu et al. 2004; Hayashida et al. 2006). In fact, the constitutive expression of Hsp70.1 did not protect the seminiferous epithelium against cryptorchidism-induced cell death (Vydra et al. 2006). In contrast, it was shown by TUNEL staining that a substantial fraction of the apoptotic cells were observed in the outermost layer of the tubules in *Hsf1*-KO mice, indicating that cell death of undifferentiated germ cells exposed to heat shock is inhibited by HSF1 (Izu et al. 2004). Thus, HSF1 has two opposite roles in male germ cells. HSF1-mediated death signals may play major roles in pachytene spermatocytes, and the HSF1-mediated survival signals may dominate the death signals in undifferentiated germ cells (spermatogonia). Injured germ cells are selectively eliminated by an apoptotic mechanism that may be regulated by the p53 pathway (Yin et al. 1998, 2002). In response to cellular stress, p53 induces cell cycle arrest to allow the opportunity for DNA repair to occur before replication or mitosis. Alternatively, it induces

apoptotic cell death to eliminate irreparably damaged cells (Sionov and Haupt 1999). The choice between growth arrest and apoptosis is determined by many factors, including the cell type. Similar to p53, HSF1 is a major factor that senses proteotoxic stress and transduces it to the expression of proapoptotic and antiapoptotic genes, depending on the cell types. Hayashida et al. have determined that a target gene of HSF1 is Phlda1 (PHL domain, family A, member 1), also known as TDAG51, which subsequently activates apoptosis. They showed that apoptosis of the germ cells was inhibited by *Tdag51*-KO in heat stress (Hayashida et al. 2006). This suggests that the inhibition of spermatogenesis during cryptorchidism is partly attributable to the activation of the HSF1/Phlda1 pathway. Taken together, these observations suggest that HSF1 determines the choice between apoptosis and cell survival in response to heat stress by activating TDAG51 or several HSPs, respectively.

8.4.3 Varicocele

Varicocele is an abnormal enlargement of the pampiniform venous plexus, which is the most frequent cause of male infertility. Reflux of toxic metabolites of adrenal and/or renal origin, deficiency of the hypothalamic gonadal axis, and venous stasis leading to testicular hypoxia are among the main factors associated with varicocele. The most widely accepted mechanism underlying varicocele-dependent infertility is increased testicular temperature. This is because varicocele disrupts the countercurrent heat-exchange mechanism, leading to impaired testicular thermoregulation and increased testicular oxidative stress (Shiraishi et al. 2012). Taken together, the involvement of these factors and the alterations of several HSPs in men with varicocele suggest that HSFs play pivotal roles in the pathophysiology of varicocele.

mRNA expression levels of HSF1 and HSF2 were increased in oligospermic men with varicocele (Ferlin et al. 2010). Taking together the observation that HSF2 binds to the promoters for HsoA11 (Wilkerson et al. 2008), Hspb2, Hsp90aa1, and Fos in mitotic cells (Wilkerson et al. 2007), the expression of HSPA2 could be regulated by HSF2. HSPA2 was highly expressed in human testis and appeared to have an essential role during the meiotic phase of spermatogenesis (Son et al. 1999). The disruption of *Hspa2* resulted in meiosis failure, germ cell apoptosis, and male infertility (Dix et al. 1996, 1997; Govin et al. 2006). Therefore, the transcriptional regulatory function of HSF2 may be considered to be essential to assure the appropriate expression of HSPA2. Afiyani et al. observed significantly higher expression of HSPA2, which mainly localizes to postmeiotic cells such as round spermatid and sperm, in testicular tissue from men with varicocele (Afiyani et al. 2014). This HSP was downregulated in the ejaculated sperm of men with oligozoospermia and increased at 6 months after varicocelectomy (Yesilli et al. 2005). In varicocele patients, it has also been shown that the removal of varicocele by surgery improves HSPA2 expression (Yesilli et al. 2005; Afiyani

et al. 2014). Round spermatids and sperm in the varicocele group had higher HSPA2 levels compared with the control group. These results agreed with the report of increased HSP and HSF expression in sperm from oligozoospermic and varicocele individuals (Ferlin et al. 2010). HSP 32, also known as heme oxygenase-1 (HO-1), is one member of this family with enzymatic activity and is induced by heat stress, heavy metals, inflammatory mediators, and oxidized low-density lipoproteins; it has been shown to be increased in Leydig cells in patients with varicocele (Shiraishi and Naito 2005). In addition, the upregulation of HSP32 in Sertoli cells inhibits caspase-3 activity and alleviates heat-induced impairments in mouse testis (Li et al. 2013). HSF1 negatively regulates HO-1 gene expression in human Hep3B hepatoma cells in response to stimulation with 15-deoxy-delta12,14-prostaglandin J2 and arsenite (Chou et al. 2005); however, direct evidence regarding the role of HSF in Leydig and Sertoli cells is lacking.

Apparently, varicocelectomy improves the semen parameters of sperm concentration and motility. Even in patients whose semen parameters do not improve, the results of intracytoplasmic sperm injection therapy, a commonly used infertility treatment, can be improved after varicocelectomy (Shiraishi et al. 2012), indicating that some sperm factors may be improved. As HSF1 plays roles in the regulation of sperm DNA packing (Akerfelt et al. 2010), further investigation into HSF's role in sperm function is required.

8.4.4 Genotoxic Stress

Doxorubicin, an anticancer agent widely used against various types of neoplasms, belongs to the anthracycline antibiotic family, and it induces DNA damage by inhibiting topoisomerase II and also intercalating DNA. Doxorubicin is one of the famous agents that cause permanent azoospermia, if a majority of spermatogonia undergo cell death by its administration (Shiraishi and Matsuyama 2014).

At 2 days after a single intraperitoneal injection of doxorubicin (5 mg/kg), the proliferation of *Hsf1* wild type but not *Hsf1*-KO spermatogonia and preleptotene primary spermatocyte were inhibited, followed by a decreased number of meiotic cells in *Hsf1* wild type but not *Hsf1*-KO testes (Salmand et al. 2008). Proliferating cells were blocked or lost very rapidly as a result of an increase in cell death signals and phagocytic activity of Sertoli cells (Shiratsuchi et al. 1997). Nevertheless, spermatogenesis showed better signs of recovery in *Hsf1* wild type but not *Hsf1*-KO. These data indicate that acute response to genotoxic stress in the testis involves HSF1-dependent mechanisms that induce apoptotic cell death (Salmand et al. 2008), which is similar to the heat stress response orchestrated by HSF1 (Nakai et al. 2000; Izu et al. 2004). Most likely, these similar observations for HSF1 will be seen after the administration of other anticancer agents, and these responses should be further investigated.

8.5 Summary and Future Perspectives

Previous research, especially the generation of gene-manipulated animals, has greatly contributed to our understanding of the roles for HSF1 and HSF2 in gametogenesis. Obviously, HSF1 and HSF2 play important roles in both spermatogenesis and oogenesis. HSF1 checks the quality control of gametogenesis, whereas there is still controversy regarding the role of HSF2 in spermatogenesis. The generation of HSF2 transgenic mice will elucidate the function in spermatogenesis. Unfortunately, the simplest methodology, in vitro cell culture, is not useful for germ cells because male and female germ cell culture is very difficult in vitro.

Gametogenesis is strictly regulated by the gonadotropins: luteinizing and follicle-stimulating hormone. However, information regarding the effects of these hormones on the actions of HSFs is still lacking. The target cells in the testis and ovary, the Leydig, Sertoli, theca, and granulosa cells, are often cultured in vitro, and the role of HSFs in these endocrinological cells is readily investigated. This approach may contribute to the development of endocrinological stimulation in both spermatogenesis and oogenesis. Because HSFs are involved in a variety of signaling pathways in normal and stressed gametogenesis, the investigation of HSFs in gametogenesis per se may provide information for understanding the physiology/pathophysiology of spermatogenesis and oogenesis and for establishing new therapeutic approaches to combat male and female infertility.

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Chapter 9
HSF Regulates Immune and Inflammatory Response

Sachiye Inouye

Abstract Both heat shock response and immune response are fundamental and indispensable systems for biological defense mechanisms. A key part of the heat shock response is the upregulation of heat shock proteins (HSPs), which are virtually found in all living organisms, from bacteria to humans. The induced expression of HSPs during heat shock is generally controlled by a single transcription factor; in mammals this regulation is performed by heat shock factor 1 (HSF1). The primary function of HSPs is to serve as molecular chaperones, involved in the folding and unfolding of other proteins. Recent works indicate that HSPs play important roles in the immune response, including antigen presentation, as well as activation of macrophages, lymphocytes, and dendritic cells. In addition, HSF1 regulates the expression of inflammatory cytokines directly or indirectly. In this chapter, the coordinate regulation of heat shock response and immune and inflammatory response and the involvement of these responses in inflammatory diseases are summarized.

Keywords Heat shock response • Immune response • Febrile response • Inflammation • HSF • Fever • Arthritis • Inflammatory bowel disease • Cytokine

9.1 Introduction

Cells have developed a number of defense mechanisms to protect themselves from physical (temperature, sound, UV, etc.), chemical (oxygen, heavy metals, toxin, medicine, etc.), and biological (infection, inflammation, and etc.) stresses (Fig. 9.1). Among cellular defense mechanisms, heat shock response and immune response are universal and significant stress responses in all mammalians. The heat shock response is essential for survival in a stressful environment such as elevated temperatures, oxidative stress, heavy metals, toxins, and infections. The mammalian HSF family consists of four members: HSF1, HSF2, HSF3, and HSF4. Among them, HSF1 is the master regulator of heat shock response in vertebrates. The heat

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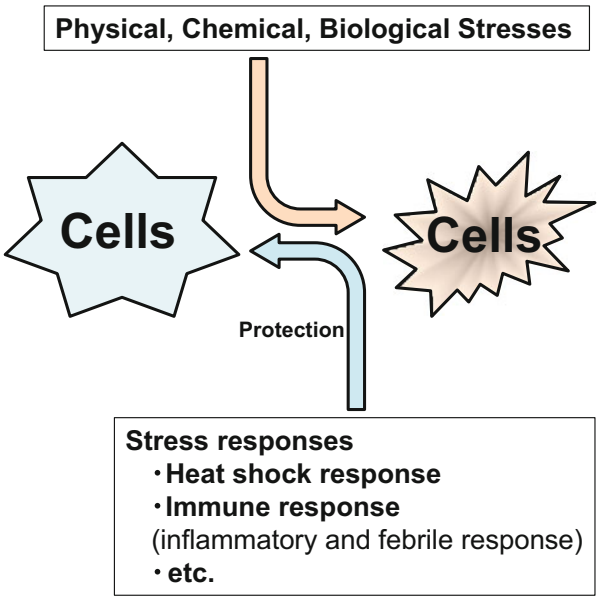

Fig. 9.1 Cellular defense mechanisms. Physical, chemical, and biological stresses evoke damage. Cells protect themselves from stress responses, such as heat shock response and immune response, including inflammatory and febrile response

shock response is initiated by the activation of the heat shock transcription factor 1 (HSF1) (Fujimoto and Nakai 2010; Morimoto 2011; Pirkkala et al. 2001; Westerheide et al. 2012; Akerfelt et al. 2010). HSF1 exists as an inactive monomer in complex with Hsp40, Hsp70, and Hsp90. Upon stress, HSF1 is released from the chaperone complex and trimerizes. Then, HSF1 is translocated into the nucleus where it is hyper-phosphorylated and binds to DNA containing heat shock elements (HSE), activating transcription of HSP genes. Intracellular HSPs, including HSP90, HSP70, and HSP40, have pivotal roles in facilitating protein folding. Extracellular HSPs, including HSP90, HSP70, and gp96, are peptide carriers, inducers of cytokines, and stimulants for immune cells (Schmitt et al. 2007). The immune response is a biological system that protects against antigens (Fig. 9.2). Antigens are engulfed via phagocytosis by antigen-presenting cells (APCs) which process antigens in phagolysosomes and present them on their major histocompatibility complex (MHC) molecules. T-cell receptor (TCR) on helper T cell recognizes these molecules. The activated T-cells release cytokines and other signals that stimulate B cells, macrophages, and killer T cells. In turn, B cells produce specific antibodies which trap antigens, and macrophages engulf pathogen including antigens. Finally killer T cells kill pathogen including antibodies (Beck and Habicht 1996). In this chapter, I focus on the molecular mechanisms underlying the involvement of the heat shock response in the immune and inflammatory responses.

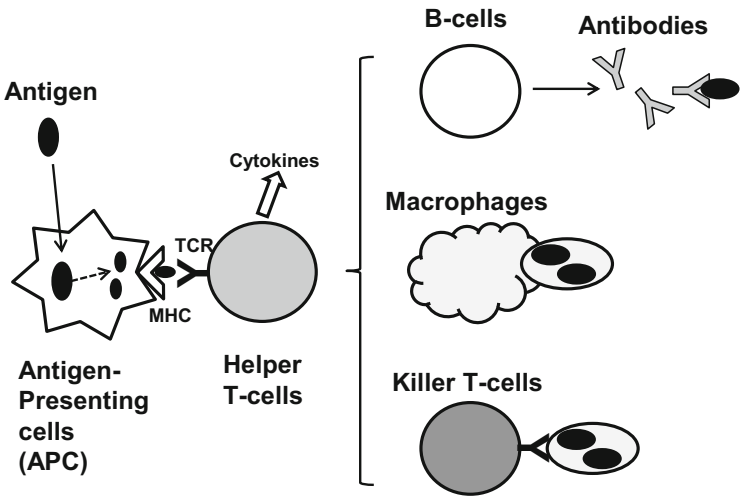

Fig. 9.2 Immune response. Antigen-presenting cells (APCs) engulf antigens by a phagocyte, process antigens in phagolysosome, and present antigen on their MHC class II molecules. Helper T cells recognize these molecules by TCR (T-cell receptor), and then cells are activated. The activated T cells release cytokines and other signals that stimulate B cells, macrophages, and killer T cells. Activated B cells produce antibodies, which trap antibodies. Activated macrophages engulf pathogen including antibodies. Activated killer T cells kill pathogen including antibodies

9.2 Contribution of Immune System in Inflammatory and Febrile Responses

The immune system is a network of cells and tissues that work together to protect the body from harmful stimuli, such as pathogens and damaged cells. In many species, the immune system can be classified into two categories: innate and adaptive immune response (Beck and Habicht 1996). Both immune responses have the ability to distinguish between self- and non-self-molecules. The innate immune response (nonspecific immune system) provides immediate defense against foreign pathogens by recruiting immune cells to sites of infections and removing the pathogens. The adaptive immune response (specific immune system) is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogen growth. The innate immune response is also important in the activation of the adaptive immune response (Alder et al. 2005). In most cases, the immune system keeps the body healthy and prevents infections; however, when this system is impaired, it may lead to an infection and disease.

9.2.1 *Inflammatory Response*

Inflammation is one of the first responses of the immune response that is intended to eliminate the initial cause of cell injury and to initiate the healing process (Kawai and Akira 2006; Ferrero-Miliani et al. 2007). Acute inflammation is short lived, lasting only a few days. If it lasts longer, it is referred to as chronic inflammation. Chronic inflammation may last weeks, months, or even beyond (Ferrero-Miliani et al. 2007).

The classical signs of acute inflammations are pain, heat, redness, and swelling. Acute inflammation is the initial response of the body to harmful stimuli and is produced by eicosanoids and cytokines, which are released by leukocytes or infected cells. Prostaglandins, included in eicosanoids, produce fever and the dilation of blood vessels. Among cytokines, interleukins are involved in the communication between leukocytes, chemokines promote chemotaxis, and interferons have antiviral functions (Serhan and Savill 2005). Leukocytes, including phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells, are moved from blood into the injured tissues. These cells eliminate pathogens, either by attacking or by engulfing microorganisms (Mohamed-Ali et al. 2001). Progressive destruction of tissue in the absence of inflammation would compromise the survival of the organism. On the other hand, chronic inflammation might lead to a variety of host diseases, such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer.

9.2.2 *Febrile Response*

The febrile response is a complex physiological reaction to infection or injury involving a cytokine-mediated rise in body temperature, production of acute phase reactants, and activation of endocrinology and immune systems (Dalal and Zhukovsky 2006; Mackowiak et al. 1997). Fever is defined as the elevation of the core body temperature above normal range around 37 °C. Fever is a common symptom of many conditions such as infectious disease, immunological diseases, tissue destruction, cancers, and metabolic disorders, as summarized to the febrile response (Fig. 9.3). A number of exogenous and endogenous substances, including microorganism, toxins, and tumors, can evoke fever. These pyrogens induce the production of proinflammatory cytokines, such as interleukins 1β (IL-1β) and 6 (IL-6), interferon α (INF-α), and tumor necrosis factor α (TNF-α) by activated leukocytes. These cytokine factors are released into general circulation, where they migrate to the circumventricular organs of the brain, due to easier absorption caused by the blood–brain barrier's reduced filtration action. The cytokine factors, then, bind with endothelial receptors on vessel walls or interact with local microglial cells. When these cytokine factors bind, the arachidonic acid pathway is activated. Thus, these pyrogenic cytokines in central nervous system (CNS) stimulate release

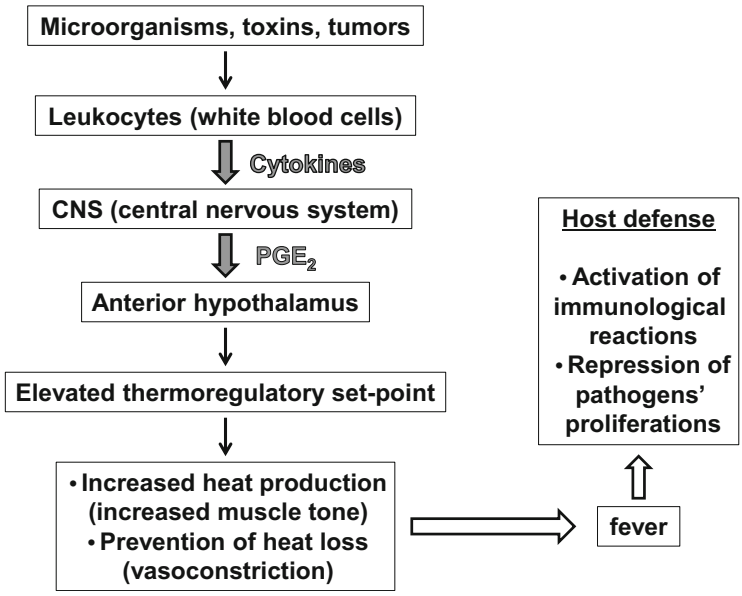

Fig. 9.3 Febrile response. The febrile response is a complex physiological reaction. A number of exogenous and endogenous substances, including microorganism, toxins, and tumors, can evoke fever. These pyrogens induce the production of proinflammatory cytokines, such as interleukins 1β (IL-1β) and 6 (IL-6), interferon α (INF-α), and tumor necrosis factor α (TNF-α) from leukocytes. These pyrogenic cytokines enter central nervous system (CNS) and stimulate release of prostaglandins, such as PGE2. PGE2 resets the hypothalamic thermal set point. Core temperature rises to reach the new set point, by increasing heat production (increased muscle tone) and preventing heat loss (vasoconstriction). Fever can be one of host defense function, such as activation of immunological reactions and repression of pathogens' proliferations

of prostaglandins, such as prostaglandin E2 (PGE2), which reset the hypothalamic thermal set point. Core temperature rises to reach the new set point, by increasing heat production (increased muscle tone) and preventing heat loss (vasoconstriction). Under normal conditions, heat is generated internally during metabolic processes, whereas under inflammatory conditions, heat is produced by increased skeletal muscle activity, such as shivering. Heat loss occurs predominantly from the skin via evaporation; thus the vasoconstriction occurs to prevent it (Watkins et al. 1995). Fever can be one of the host defense functions, such as activation of immunological reactions and repression of proliferation of pathogens (see Sect. 9.3). A fever with an extreme elevation of body temperature, higher than 41.5 °C, is called hyperpyrexia or hyperthermia (McGugan 2001). Such a high temperature is considered a medical emergency, as it may indicate a serious condition. Immediate cooling has been found to improve survival.

9.3 Beneficial Effects of Fever on Pathological Conditions

Fever is often considered to be an important host defense mechanism, since it enhances leukocyte phagocytosis, increases T-cell proliferation, and decreases endotoxin effects and proliferation of pathogens (Dalal and Zhukovsky 2006). Thus, a lack of fever may contribute to lower resistance to infection, delayed recovery, and a higher mortality (Fischler and Reinhart 1997). There are at least four beneficial effects of fever: inducing cytoprotective heat shock proteins (HSPs) in host cells, inducing expression of pathogen-derived HSPs, directly killing or inhibiting growth of pathogens, and modifying and orchestrating host defenses (see Fig. 9.3) (Hasday and Singh 2000; Hasday et al. 2000; Jiang et al. 2000). Two of them directly involve the heat shock response, and one of them relates to the function of heat shock factor 1 (HSF1). HSF1 not only activates HSP expression but also regulates expression of cytokines and early response genes.

An increased body core temperature can activate the heat shock response, during which a set of stress inducible HSPs is expressed. HSPs are highly conserved proteins from microbes through mammals and are grouped in distinct families according to their molecular weight. They are preferentially induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiations, ischemia–reperfusion injury, microorganism infections, nutrient deprivation, and chemicals (Lindquist 1986). HSPs are molecular chaperones essential to maintaining cellular functions by preventing misfolding and aggregation of nascent polypeptides, facilitating protein folding, and targeting denatured proteins for degradation (Hartl and Hayer-Hartl 2002). Thus, fever might confer protection from infection by inducing HSP expression in host cells. These HSPs, induced by thermal stress, preserve essential cellular components.

In addition to HSPs, heat shock also activates cytoprotective genes, Cu/Zn superoxide dismutase (SOD) (Yoo et al. 1999), and heme oxygenase 1 (HO-1) (Ewing and Maines 1991). Superoxide is produced as a by-product of oxygen metabolism and causes many types of cell damage commonly known as oxidative stress. SOD (EC 1.15.1.1) enzymes catalyze the dismutation of the toxic superoxide (O_2^-) radical into two less damaging species: either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Hydrogen peroxide is degraded by other enzymes such as catalase, which decomposes hydrogen peroxide to water and oxygen. Thus, SOD is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Borgstahl et al. 1996). HO-1 (EC 1.14.99.3) is an inducible enzyme in response to stress such as oxidative stress, hypoxia, heavy metals, and cytokines. It catalyzes the degradation of heme, producing biliverdin, iron, and carbon monoxide. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Since free heme is one of the potent prooxidants and its degradation products are antioxidants, HO-1 is also a cytoprotective enzyme against oxidative stress (Ryter et al. 2006).

The second way in which fever may protect the host is to stimulate the expression of pathogen-derived HSPs, which are potent activators of host innate immune

responses. Bacterial HSPs, such as Hsp70, GroEL, and Hsp60, activate macrophages to express and secrete IL-1α, IL-1β, TNFα, IL-6, and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Retzlaff et al. 1994). In addition, members of the Hsp60 class serve as immunodominant targets of T lymphocytes as well as stimulating antibodies during infections (Haregewoin et al. 1989). Fever might be directly cytotoxic or cytostatic for microbial pathogens (Jiang et al. 2000) as bacterial HSPs are also induced by heat shock, to protect themselves. In *Escherichia coli*, RNA polymerase complexes with sigma32 activate transcription of HSPs (Yura 1996), and some pathogens have strict temperature preferences; thus prolonged fever may inhibit their proliferations and kill them.

Fever and elevated expression of HSPs have been shown to have both pro- and anti-inflammatory effects on immune functions, including activation of macrophages (Lee and Repasky 2012). These opposing effects might be explained by the inflammation stages. In the initiation phase, fever enhances the induction of HSPs in macrophages, and the induced HSPs can be released from cells to extracellular environment. In turn, these HSPs enhance the production of LPS-induced proinflammatory cytokines, TNF-α, IL-6, and NO. In resolution phase, fever induces HSF1 activation, which directly or indirectly suppresses proinflammatory cytokines, TNF-α, IL-6, and IL-1β (see Sect. 9.4). Bacterial endotoxin lipopolysaccharide (LPS)- and heat-induced HSPs bind to TNF receptor-associated factor (TRAF6) or to inhibitor κB kinase (IKK) and inhibit activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), leading to repression of proinflammatory cytokines (Lee and Repasky 2012). Additional beneficial effects of fever, in modifying and orchestrating host defenses, are described in Sect. 9.5.

Prolonged fever is associated with an increase in metabolic rate and oxygen consumption, dehydration, and nutritional demands, which may be a problem for critical illness (Styrt and Sugarman 1990). Antipyretic medicines, such as acetaminophen, aspirin, or nonsteroidal anti-inflammatory drugs (NSAIDs), act by lowering the elevated body temperature (Isaacs et al. 1990). The synthesis of PGE2 is mediated by phospholipase A2 (PLA2), cyclooxygenase-2 (COX-2), and prostaglandin E2 synthase. NSAIDs are potent COX-2 inhibitors and acetaminophen is a weak COX-2 inhibitor. Corticosteroids are also effective antipyretics, since they reduce PGE2 synthesis by inhibiting the activity of PLA2, and they also act by blocking the transcription of pyrogenic cytokines mRNA (Isaacs et al. 1990).

9.4 HSF Inhibits Expression of the Inflammatory and Pyrogenic Cytokines

As mentioned above, HSF1 is a master transcription factor of mammalian heat shock response, inducing the expression of HSPs. These HSPs protect cells from stressful influence, in part by functioning as chaperones on denatured proteins (Trinklein et al. 2004; Alder et al. 2005; Morimoto et al. 1992). However, recent

evidences suggest that other genes are also regulated by heat-activated HSF1, including cytokines (Christians et al. 2002; Nagarsekar et al. 2005; Singh and Aballay 2006; Hahn et al. 2004).

9.4.1 Inflammatory Cytokines

Cytokines are low molecular weight polypeptides (~5–20 kDa) that are pleiotropic regulators of host responding to infection, immune responses, and inflammation. Originally they were called lymphokines and monokines, because of their cellular sources. They are important in disease, specifically in host responses to infection, immune responses, inflammation, trauma, sepsis, and cancer. Cytokines are produced by a broad range of cells, including macrophages, B lymphocytes, T lymphocytes, mast cells, endothelial cells, fibroblasts, and vascular cells. Cytokines act through receptors and are especially important in the immune system, as they modulate the balance between humoral and cell-based immune responses (Grignani and Maiolo 2000). Cytokines regulate the maturation, growth, and responsiveness of particular cell populations, and they enhance or inhibit the action of other cytokines in complex ways. Cytokines have been classified as interleukins, lymphokines, monokines, interferons (IFNs), colony-stimulating factors (CSFs), and chemokines, based on their function and producing cells.

Interleukins are a group of cytokines that were initially expressed by white blood cells (leukocytes) and their targets are principally leukocytes. They promote the development and differentiation of T and B lymphocytes and hematopoietic cells. The function of the immune system depends largely on interleukins (Brocker et al. 2010).

Lymphokines are a subset of cytokines that are produced by lymphocytes, typically produced by T cells. They have many roles, including the attraction of macrophages and other lymphocytes to an infected site and their subsequent activation of immune cells to prepare them to mount an immune response. Lymphokines also activate B cells to produce antibodies (Miyajima et al. 1988).

Monokines are a subset of cytokines produced primarily by monocytes and macrophages. IL-1, TNF, IFNs, and CSF are included in monokines (Curfs et al. 1997).

IFNs are the large class of cytokines, and they are named for their ability to "interfere" with viral replication by protecting cells from virus infection. They also have other functions: activate immune cells, such as natural killer cells (NK cells) and macrophages, and increase host defenses by upregulating antigen presentation by virtue of increased expression of MHC antigens. They are typically divided into three classes: type I, type II, and type III IFNs. The type I IFNs present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, which bind to a specific cell surface receptor complex known as the IFN-α/β receptor. The type II IFN in humans is IFN-γ, induced by cytokines such as IL-12, and binds to IFNGR. The expression of type II interferon is restricted to immune cells such as T cells and NK cells. Type III

IFNs including IFN-λ were discovered recently and mediate antiviral protection through a distinct class II cytokine receptor complex such as IFN-α/β receptor alpha chain (IFNLAR1) and IL-10 receptor 2 (IL-10R2) (Vilcek 2003).

CSFs are soluble glycoproteins that bind to receptor proteins on the surfaces of hemopoietic stem cells. The name "colony-stimulating factors" comes from the experiments by which they were discovered. When a single cell starts proliferating, all cells derived from it clustered around it and formed a colony. The substance to stimulate formation of colonies of macrophages was called macrophage colony-stimulating factor (M-CSF). For granulocytes it was called granulocyte colony-stimulating factor (G-CSF), and for both granulocytes and macrophage, it was called granulocyte–macrophage colony-stimulating factor (GM-CSF) (Cohen et al. 1999).

Chemokines are a family of small cytokines (8–10 kDa) secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells; they are *chemo*tactic cyto*kines*. Some chemokines are considered proinflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection. Chemokines have been classified into four subfamilies: CXC, CC, CX3C, and XC, based on the location of the four conserved cysteine residues. All of these proteins exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors (Melik-Parsadaniantz and Rostene 2008).

Another classification of cytokines based on the immune system is type 1 cytokines (IFN-γ, TNF-α, etc.) that enhance cellular immune responses and type2 cytokines (TGF-β, IL-4, IL-10, IL-3, etc.) that promote antibody responses. Some cytokines promote inflammation and worsen the disease (proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α), whereas others serve to reduce inflammation and promote healing (anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IL-35) (Dinarello 2000). Proinflammatory cytokines produce fever, inflammation, tissue destruction, and, in some cases, shock and death. Blocking IL-1β or TN-Fα has been highly successful in patients with rheumatoid arthritis and inflammatory bowel disease (Strober and Fuss 2011). Anti-inflammatory cytokines act mainly by inhibiting of the production of proinflammatory cytokines or by counteracting many biological effects of proinflammatory mediators in different ways.

9.4.2 Pyrogenic Cytokines

Among proinflammatory cytokines, those induce fever in experimental animals are called pyrogenic cytokines, including IL-1β, IL-6, TNF-α, IFN-α, IFN-γ, IL-8, and macrophage inflammatory protein (MIP)-1a (Price et al. 1997). They are produced by activated immune cells and cause the increase in the thermoregulatory set point in the hypothalamus (see Sect. 9.3 and Fig. 9.3). HSF1 represses at least three

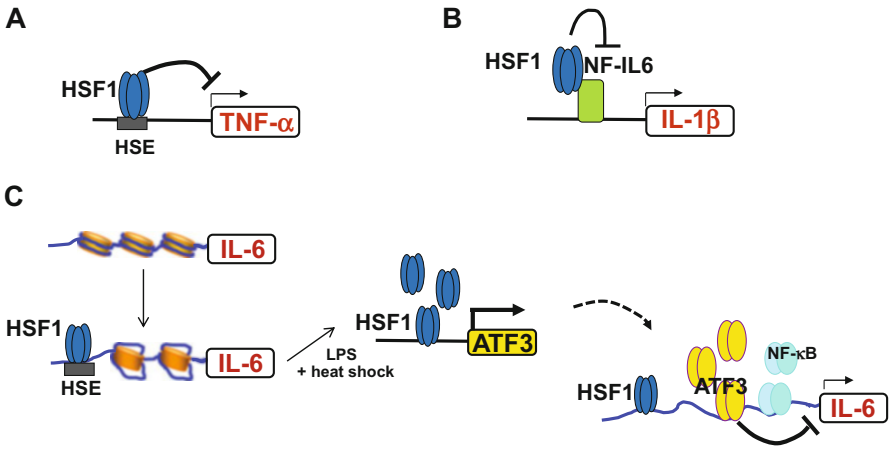

Fig. 9.4 Inhibition of pyrogenic cytokine expression by HSF1. (**a**) HSF1 binds to HSE in the 5′-untranslated region of TNF-α gene and represses the expression directly, during cells exposed to febrile range temperatures. (**b**) HSF1 represses the lipopolysaccharide (LPS)-induced transcription of IL-1β promoter through interaction with the nuclear factor of interleukin 6 (NF-IL6), which is an essential activator in IL-1β transcription. (**c**) HSF1 binds to HSE in the promoter region of IL-6 and opens its chromatin structure. HSF1 activated by heat shock induces the expression of activating transcription factor 3 (ATF3), a negative regulator of IL-6 gene

pyrogenic cytokines, such as TNF-α, IL1β, and IL-6, directly or indirectly (Fig. 9.4).

9.4.3 TNF-α

A cytotoxic factor, having an antitumoral activity, is produced by lymphocytes and macrophages and called tumor necrosis factor (TNF). TNF-α is primarily produced as a 212-amino acid-long transmembrane protein arranged in stable homotrimers. TNF-α elicits a broad spectrum of cellular responses, including cell proliferation, differentiation, inflammation, and apoptosis. Binding of TNF-α to receptors, TNFR1 and TNFR2, results in a complex cascade of signaling steps (Idriss and Naismith 2000). Two transcription factors, AP-1 complex and NF-kB, are activated by this signaling and induce genes mediating proliferative and inflammatory responses (Palanki 2002). The primary role of TNF-α is an early pivotal mediator of the regulation of immune cells. TNF-α is able to induce fever (endogenous pyrogen), apoptotic cell death, and inflammation and to inhibit tumor genesis and viral replication. The function of TNF-α is mediated by binding to TNF receptor type 1 (TNFR1) expressed on the surface membrane of cells of the immune system, where only the membrane-bound homotrimer responds. Although TNF-α is essential for optimal host defense that is essential for survival in infection, persistent high TNF-α expression induces multiorgan failure and death. Hence, the pleiotropic

nature has led to stringent and redundant regulatory mechanisms of expression. Dysregulation of TNF-α production has been implicated in a variety of human diseases, including Alzheimer's disease, cancer, major depression, and inflammatory bowel disease (IBD). It has been reported that febrile range hyperthermia suppresses TNF-α expression in murine peritoneal macrophages, Kupffer cells, Raw 264.7 macrophage cell line, human monocyte-derived macrophages, and THP1 monocyte cell line (Ensor et al. 1995). Macrophages exposed to febrile range temperature (38.5–40 °C) markedly attenuated TNF-α expression induced by LPS. This inhibitory effect is mediated by HSF1 which is activated by febrile range temperature. The minimal regulatory sequence bound by HSF1 is located at the +85-nucleotide (nt) proximal promoter to the −138-nt'-untranslated region (Singh et al. 2000, 2002). The direct HSF1 binding to the HSE-like sequences around the TNF-α gene promoter region has been reported in vitro and in vivo. In addition, HSF1 knockout mice exaggerated TNF-α expression following a challenge with LPS compared with HSF1-sufficient littermates (Xiao et al. 1999). These data indicate that febrile range temperature reduces LPS-induced TNF-α expression through the direct interaction of HSF1 with the TNF-α gene promoter (Fig. 9.4a). HSF1 might repress TNF-α transcription by blocking RNA polymerase processivity. However, another molecular mechanism has been reported, suggesting that the febrile range temperature reduced LPS-induced recruitment of NF-κB p65 to the TNF-α promoter (Cooper et al. 2010). A mild thermal stress that induced HSP70 expression in rat liver prevented the LPS-induced increase in TNF-α level. Its repression was associated with inhibited IκBα degradation and NF-κB p65 nuclear translocation (Dokladny et al. 2010).

9.4.4 IL-1β

Human IL-1β consists of 269 amino acids and is produced by activated macrophages and monocytes in response to a variety of stimuli. The discovery of IL-1β began with studies on the pathogenesis of fever (March et al. 1985). IL-1β plays a fundamental role in protecting animals against infectious agents. IL-1β stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. IL-1 proteins are involved in the inflammatory response, being identified as endogenous pyrogens, and are reported to stimulate the release of prostaglandin and collagenase from synovial cells (Van Damme et al. 1985). The expression and function of IL-1β is regulated at a number of different levels. These include modulation of transcription, mRNA stabilization, posttranslational proteolytic processing of pro-IL-1β, and inhibition of IL-1β receptor binding by a naturally occurring IL-1β antagonist. IL-1β is produced as an inactive precursor and is converted to the active mature protein through proteolytic cleavage by caspase-1 (Rider et al. 2011). IL-1α and IL-1β bind to the same receptor molecule, which is called type I IL-1 receptor (IL-1R). After the formation of receptor complex which is assembled by IL-1α or IL-1β, IL-1RI, and IL-1RAcP,

a complex intracellular signal transduction occurred. These signaling pathways lead to activation of many transcription factors, such as NF-κB, AP-1, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK), which induce the expression of canonical IL-1 target genes (such as IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), COX-2, IκBα, mitogen-activated protein kinase phosphatases1 (MKP-1)) (Weber et al. 2010). The regulation of IL-1β gene transcription is dependent on the activity of the myeloid-specific transcription factor Spi-1/PU.1, which binds specifically to multiple elements in proximal IL-1β promoter and activates transcription. The nuclear factor of interleukin 6 (NF-IL6, also known as CCAAT/enhancer-binding protein (C/EBPbeta)) is the other regulator involved in activation of IL-1β transcription by LPS and is capable of synergistically cooperating with Spi-1, resulting in strong activation of the IL-1β core promoter. NF-IL6 is a bZIP transcription factor of the C/EBP family that is constitutively expressed in resting monocytes and immediately activated by agents such as LPS, PMA, and IL-6. NF-IL6 has been shown to activate IL-1β transcription by binding to the promoter at two different sites (Cahill et al. 1996; Xie et al. 2002a). Considering that IL-1β was highly toxic to animals, produced immediately in response to a wide variety of proinflammatory agents, and affecting the function of a wide variety of targets, negative feedback regulation mechanism should be very important in infectious diseases from both the pathophysiological and pharmacological points of view (Kinoshita et al. 2005). Under heat shock stress condition, HSF1 becomes activated and represses the LPS-induced transcription of IL-1β through direct interaction with NF-IL6, an essential regulator of IL-1β transcription (Fig. 9.4b). This inhibition appears to be the result of competition between HSF1 and Spi.1 to bind to NF-IL6. The interaction between HSF1 and NF-IL6 involves the trimerization and regulatory domains of HSF1 and the bZIP region of NF-IL6 (Xie et al. 2002a). The macrophage colony-stimulating factor (M-CSF) receptor, product of the c-fms gene, is also transcriptionally activated by NF-IL6 and repressed by HSF1 (Xie et al. 2002b).

9.4.5 IL-6

Human IL-6 consists of 184 amino acids with two potential N-glycosylation sites. IL-6 is an interleukin that generally acts as a proinflammatory cytokine. IL-6's role as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF-α and IL-1 and activation of IL-1RA and IL-10. IL-6 is produced by various types of lymphocytes, T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, and tumor cells. IL-6 is originally identified as T-cell-derived lymphokine that induces final maturation of B cells into antibody-producing cells (Kishimoto 1989; Hirano 1992; Akira et al. 1993; Keller et al. 1996). IL-6 signals through a cell surface type I cytokine receptor complex consisting of the ligand-binding IL-6Rα chain (CD126) and the signal-transducing component gp130 (CD130) (Keller et al. 1996). These complexes bring together the intracellular

regions of gp130 to initiate a signal transduction cascade through certain transcription factors, Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). IL-6 acts on various target cells and has pleiotropic actions, as it regulates immune responses, hematopoiesis, and acute phase reactions, indicating its central role in host defense mechanism. IL-6 is the major regulator of the acute phase response in human hepatocytes, inducing C-reactive protein and serum amyloid A synthesis and acting directly on B cells to induce immunoglobulin M, G, and A production. Also, IL-6 is an important factor in primary antigen-receptor-dependent T-cell activation and subsequent proliferation of activated T cells, while inducing not only proliferation but also the differentiation of cytotoxic T cells (CTL) and also enhancing the proliferation of multipotential hematopoietic progenitors. In addition to the function of IL-6 as a growth and differentiation factor in the immune system, IL-6 also stimulates proliferation of various cells. The abnormal expression of IL-6 is directly related to the pathogenesis of several diseases, such as diabetes, atherosclerosis, multiple myeloma, prostate cancer, and rheumatoid arthritis. Therefore, the expression of IL-6 should be strictly regulated. IL-6 is an important mediator of fever as well as the acute phase response (Heinrich et al. 1990). It is capable of crossing the blood–brain barrier and initiating synthesis of PGE2 in the hypothalamus, thereby changing the body's temperature set point (Banks et al. 1994). In muscle and fatty tissue, IL-6 stimulates energy mobilization that leads to an increase in body temperature.

It has been shown that HSF1 is involved in the expression of IL-6, one of a major cytokine for B-cell maturation. Impaired IgG production was observed in HSF1-deficient mice (Inouye et al. 2004). HSF1 binds to the HSE-like sequence (−684 to −659-nt) in mouse IL-6 gene promoter that is highly conserved in the human gene. The reduced expression of CCL5, a CC chemokine that promotes antigen-specific IgG production, was also observed in HSF1-deficient mice. When cells were treated with LPS, IL-6 expression was induced mainly by NF-kB and then suppressed by ATF3, a member of the ATF/CREB family of transcription factors (Gilchrist et al. 2006). In addition, many transcription factors such as NF-IL6, AP1, IFN regulatory factor 1 (IRF-1), and activating transcription factor 2 (ATF2) enhance IL-6 expression (Franchimont et al. 1999; Sanceau et al. 1995). Upon macrophage activation, ATF3 acts as a negative regulator of IL-6 and IL-12β. Because ATF3 itself is induced by LPS, it seems to regulate Toll-like receptor (TLR)-stimulated inflammatory response as part of a negative feedback loop (Gilchrist et al. 2006). Even in the absence of stress, HSF1 partially open the chromatin structure of the IL-6 promoter, by recruiting histone acetylase and nucleosome remodeling complexes to the IL-6 gene. This activity is also important for recruitment of a repressor, ATF3, and an activator, NF-kB, to the promoter, when cells are stimulated with LPS (Inouye et al. 2007). HSF1 activated by heat shock induced the expression of ATF3, a negative regulator of IL-6, and ATF3 was necessary for heat-mediated suppression of IL-6 (Fig. 9.4). It indicates a fever-mediated feedback loop consisting of HSF1 and ATF3 (Takii et al. 2010). A comprehensive analysis of inflammatory gene expression revealed that heat pretreatment suppressed the LPS-induced expression of most genes, demonstrating a novel inhibitory pathway

for inflammatory cytokines such as IL-6, ICAM1, NOS2, CCL5, TRL2, CX3CL1, CXCL16, TNF-α, IL-1β, CCL9, CCL3, etc. (Takii et al. 2010). Among them, the expressions of IL-6, ICAM1, NOS2, CCL5, TRL2, CX3CL1, and CXCL16 are ATF3 dependent, while those of TNF-α, IL-1β, CCL9, and CCL3 are ATF3 independent.

9.4.6 Feedback Regulation of Cytokines by HSF1

LPS from bacteria stimulates fever and endotoxic shock through production of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, by activated monocytes and macrophages, whereas HSF1 represses these expressions as described above. Excessive activation of monocytes/macrophages and lymphocytes often leads to the activation of the acute phase response through production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, which are potentially lethal to the host. Therefore, the negative feedback regulation of cytokine genes by HSF1 may play a crucial regulatory anti-inflammatory role in monocyte/macrophage function, protecting the host during infection and fever (Kluger et al. 1996). Heat shock suppresses LPS-induced expression of many genes involved in the immune system, as shown in mouse embryonic fibroblast and macrophages (Takii et al. 2010). Feedback regulation of pyrogenic cytokines by heat-activated HSF1 is summarized in Fig. 9.5. Pyrogenic cytokines such as IL-6, IL-1β, and TNF-α are produced in response to infections and disease and elicit the febrile response

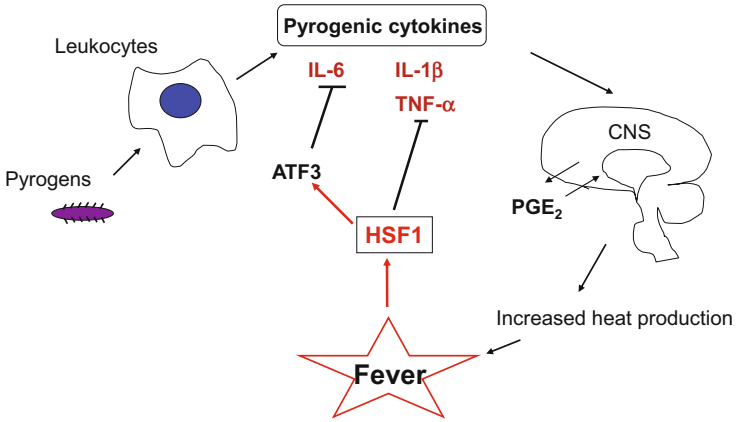

Fig. 9.5 Feedback regulation of pyrogenic cytokines by heat-activated HSF1. Pyrogens, including microorganisms, toxins, and tumor cells, are engulfed by leukocytes. Leukocytes release pyrogenic cytokines, including IL-6, IL-1β, and TNF-α. These cytokines enter CNS (central nervous system) and stimulate release of PGE2 from anterior hypothalamus. Core temperature rises to reach a new set point, by increasing heat production and preventing heat loss. Increased body temperature activates HSF1. The heat-activated HSF1 negatively regulates the expression of IL-6, IL-1β, and TNF-α

including a cytokine-mediated increase in body temperature. Increased body temperature activates HSF1, and in turn, heat-activated HSF1inhibits the expression of TNF-α by binding directly to promoter region; IL-1β by physically interacting with NF-IL6, an activator for the IL-1β gene; and IL-6 by inducing ATF3, a repressor of IL-6 gene (Fig. 9.4). Hence, fever plays beneficial roles in the clinical prognosis of disease; however, hyperthermia could be harmful for the body as described in Fig. 9.3. Thus, the fever-mediated feedback loop is necessary for preventing excessive body reactions during various pathophysiological conditions.

9.5 Modulation of T-Cell, B-Cell, and Dendritic Cell Function

9.5.1 T-Cell, B-Cell, and Dendritic Cells

T cells are a type of lymphocytes that play a central role in cell-mediated immunity and are at the core of adaptive immunity. The "T" stands for "thymus", where they mature and differentiate into various types of mature T cells and become active in the immune system. T cells that are potentially activated against own body tissues are normally killed during this maturation process. There are several different types of mature T cells, which have distinct functions (Gatenby et al. 1984). Helper T cells assist in the maturation of B cells into plasma cells and memory B cells and activation of cytotoxic T cells and macrophages. Cytotoxic T cells (CTL, CD8(+) T cells) destroy virus-infected cells and tumor cells and are also implicated in transplant rejection. Memory T cells are a subset of antigen-specific T cells that persist in long term after an infection has resolved. Regulatory T cells (Treg cells, suppressor T cells) play a major role in shutting down T-cell-mediated immunity toward the end of an immune reaction and in suppressing autoreactive T cells. Natural killer T cells (NKT cells) are also able to recognize and eliminate some tumor cells and cells infected with viruses.

B cells (B lymphocytes) play a role in the humoral immunity of the adaptive immune system. The maturation of B cells takes place in birds in an organ called the bursa of Fabricius, while B cells in mammals mature largely in the bone marrow. Many B cells mature into what are called plasma cells that produce antibodies necessary to fight off infections, while other B cells mature into memory B cells. B cells also release cytokines, which are used for signaling immune regulatory functions.

Dendritic cells (DCs) are a type of APC that performs an important role in the adaptive immune system. The main function of DCs is to process antigens and present them on the surface of T cells. They act as messengers between the innate and the adaptive immune systems. DCs are constantly in communication with other cells by direct cell–cell contact based on the interaction of cell surface proteins.

DCs also contribute to the function of B cells, producing cytokines and other factors that promote B-cell activation and differentiation.

9.5.2 *Roles of HSPs in the Immune Cell Function*

Various stimuli, including inflammation, heat shock, and fever, can induce the expression of HSPs. As mentioned before, the primary function of HSPs is to serve as molecular chaperones essential for maintaining cellular functions, by preventing misfolding and aggregation of nascent polypeptides and assisting protein folding (Hartl and Hayer-Hartl 2002; Sakahira et al. 2002). Heat shock can also induce the expression of immunologically important molecules such as nonclassical MHC antigen and Iκ-Bα or repress the expression of IL-6, IL-1β, and TNF-α (Srivastava 2002a, b). HSPs have been reported to play important roles in antigen presentation and cross-presentation, activation of macrophages and lymphocytes, and activation and maturation of DCs (Li et al. 2002; Calderwood et al. 2007; Pockley et al. 2008; Tsan and Gao 2009). The various roles of HSPs in the modulation of the immune response are summarized in Figs. 9.6 and 9.7. In the innate immune system (Fig. 9.6a), HSPs produced by damaged cells are recognized by CD40 or TLR-4 and activate the transcription factor NF-κB, which plays a key role in regulating the immune response to infection, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κB is considered to be responsible for cytokine production and cell survival (O'Donnell et al. 2006; Perkins 2007). Upon activation of NF-κB, cytokines (TNF-α, IL-1β, IL-12, GM-CSF) and chemokines (MCP-1, CCL5, CCL3) are produced by leukocytes (Fig. 9.6a). In the adaptive immune response (Fig. 9.6b), HSP-chaperoned peptides are recognized by CD91 on APCs, i.e., macrophages and DCs. These peptides are engulfed by phagocytosis, released from endosome, and represented by MHC class II molecules. The TCR on the CD4(+) T lymphocytes recognize the presented peptides (antigen presentation), leading to the activation of CD4(+) T cells; then signals are transferred to helper T cells. Some HSP-chaperoned peptides are degraded by proteasome. The resulting peptides enter the endoplasmic reticulum, represented by MHC class I molecules (cross-presentation), and stimulate CD8(+) T cells. Thus, HSPs are a class of host-derived inflammatory mediators that perform the dual function of both chaperoning MHC class I-restricted epitopes to the cross-presentation pathway of DCs and inducing the activation/maturation of these DCs to allow priming of cognate CD8(+) T-cell effector responses. Activated CD8(+) T cells induce the proliferation and maturation of cytotoxic T cells (Fig. 9.6b) (Li et al. 2002; Srivastava 2008; Basu et al. 2001).

In addition to the HSP function of immune activation, their effects on immunosuppression are also reported (Hauet-Broere et al. 2006; Multhoff 2006; Kim et al. 2006). The observation that eukaryotic and prokaryotic HSPs have high sequence homology prompted that HSPs could act as potentially dangerous autoantigens. There are two sources of extracellular HSPs, pathogen- and host-derived HSPs

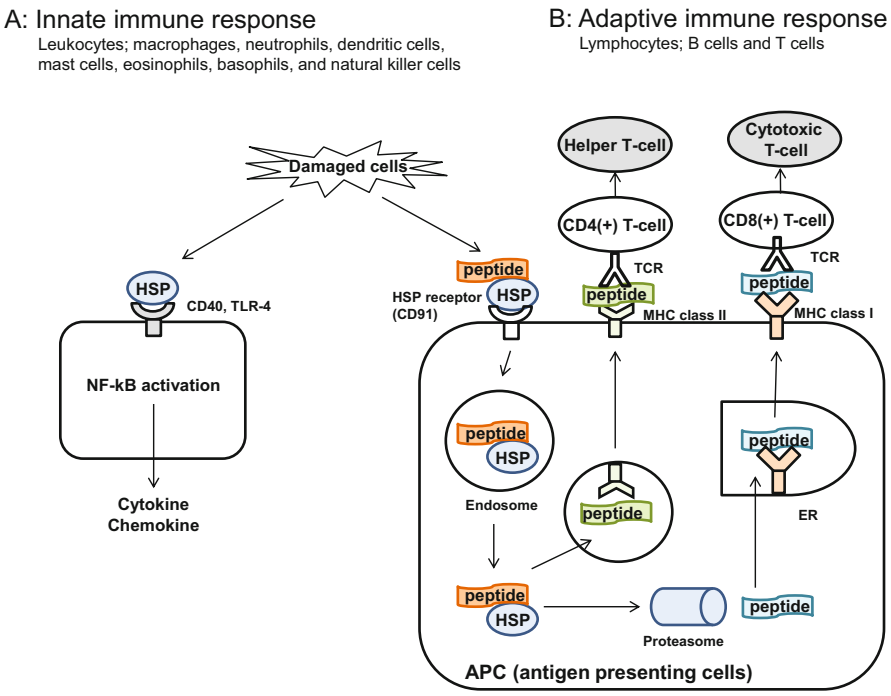

Fig. 9.6 Role of heat shock protein (HSP) in immune response. (**a**) In the innate immune response, HSP produced from damaged cells is recognized as CD40 or Toll-like receptor 4 (TRL-4), and NF-kB activation occurred. Cytokines (TNF-α, IL-1β, IL-12, GM-CSF) and chemokines (MCP-1, CCL5, CCL3) are released from leukocytes. (**b**) In the adaptive immune response, HSP-chaperoned peptide is recognized by CD91 and engulfed by phagocytosis. The peptide released from endosome is represented by MHC class II molecule and stimulates CD4(+) T lymphocytes. HSP-chaperoned peptide is degraded by proteasome. The resulting peptide is entered into endoplasmic reticulum, represented by MHC class I molecule, and stimulates CD8(+) T cells

(Joly et al. 2010). Extracellular HSPs induce the maturation of DCs and present peptide molecules to APCs; those serve as a link between the innate and adaptive immune response. Although the immune response is a remarkably effective host defense system against pathogens, its failures fall into autoimmune disorders (Miller 1993). Elevated HSP levels and anti-HSP antibodies are present in autoimmune inflammatory responses such as arthritis, multiple sclerosis, and diabetes. However, HSPs exhibit regulatory activities in controlling and preventing autoimmunity in addition to exerting immune activation and mediating protection against diseases. Immunosuppression effects of extracellular HSPs were also observed (Fig. 9.7), as HSPs can bind to receptors and be endocytosed, leading to immunosuppression. In DC cells, HSP70 induces IL-6 and IL-10 production, but suppresses expression of CD40 on DCs, resulting in decreased expression of CD86, MHC II, and TNF-α. In myeloid-derived suppressor cells (MDSC), the anti-inflammatory cytokine IL-10 production is

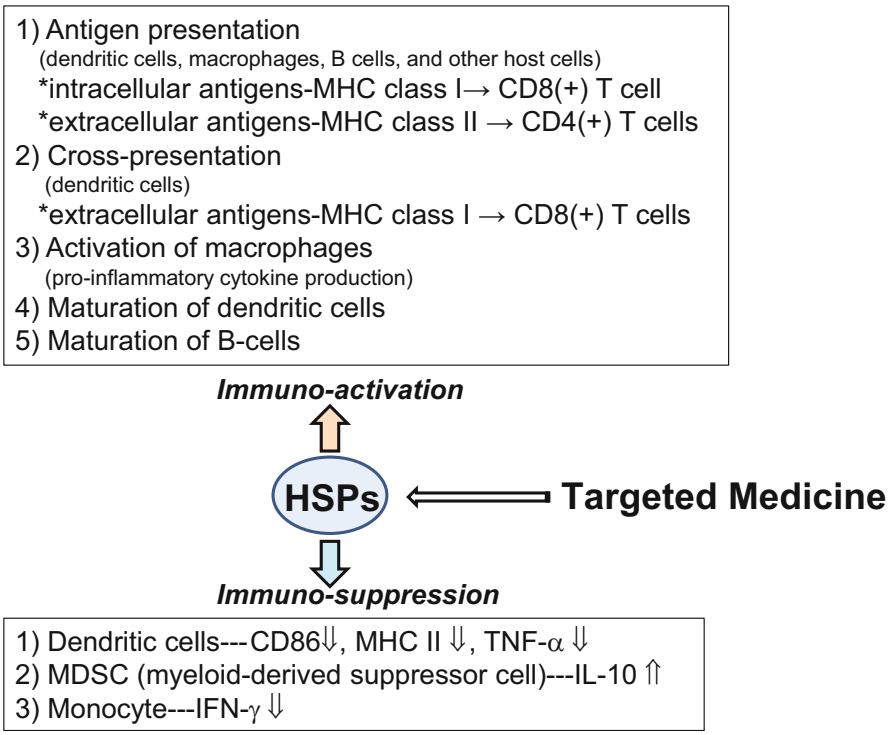

Fig. 9.7 HSPs as regulators of the immune response. HSPs are involved in the immuno-activation and the immunosuppression. Thus, some molecule-targeted medicines are created against HSPs, which decrease the immuno-activation effects of HSPs and enhance immunosuppression effects of HSPs

induced, which in monocytes, the production of IFN-γ is inhibited (Borges et al. 2012). The self-HSP cross-reactive peptides induced protection against arthritis via the induction of self-reactive regulatory T cells (Anderton et al. 1995; van Eden et al. 1995), in associating with reduced frequency of IL-10-producing CD4(+) T cells and enhancement of IFN-γ-producing CD8(+) T cells (Jarnicki et al. 2008; Toomey et al. 2008). An HSP-driven model of autoimmunity led to the unexpected development of regulatory T-cell and MDSC (myeloid-derived suppressor cells) populations. These populations are possible negative regulators of inflammation and reduce the expression of IL-10 (Thaxton et al. 2014). Exosomes from protozoa containing HSP100 modulated human monocyte cytokine responses to IFN-γ in a bimodal fashion by promoting IL-10 production and inhibiting that of TNF-α. Moreover, these exosomes were inhibitory with respect to cytokine responses (IL-12p70, TNF-α, and IL-10) by human monocyte-derived DCs. Thus, packaging of proteins into exosomes is dependent, in part, on pathogen-derived HSP100 (Silverman et al. 2010). In addition, it is evident that the heat shock response has a complex role in the function of monocytes, because extracellular HSPs, such as HSP60 and

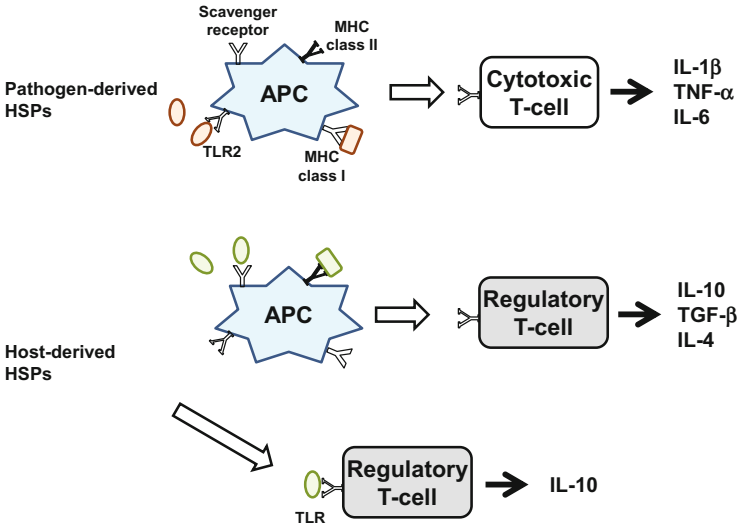

Fig. 9.8 Summary of the function of extracellular HSPs. Pathogen-derived HSPs are loaded on MHC class I of APCs and thus cross-presented to CD8(+) cytotoxic T cells to initiate production of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6. Host-derived HSPs are loaded on the MHC class II of APCs, inducing the functional phenotype of regulatory T cells and leading to the production of anti-inflammatory cytokines, such as IL-10, IL-4, and TGF-β. Host-derived HSPs might directly interact with regulatory T cells, eventually inducing an anti-inflammatory cytokine, IL-10

HSP70, can activate cytokine production, whereas intracellular HSF1 and HSP70 act as repressors. Extracellular HSPs bind to pattern recognition receptors and stimulate inflammation, whereas intracellular components of the heat shock response target the promoters of proinflammatory genes and inhibit septic shock (Ferm et al. 1992). Thus, HSPs are potential targets of some medicines for inflammations (Fig. 9.7).

The regulation of extracellular HSPs on cell-mediated immune responses is summarized in Fig. 9.8. Pathogen-derived HSPs are loaded on MHC class I of APCs by default to achieve cross-presentation, thus inducing cytotoxic T-cell responses, eventually eliciting proinflammatory cytokine production, such as IL-1β, TNF-α, and IL-6. Host-derived HSPs are uploaded in the MHC class II of APCs, inducing the functional phenotype of regulatory T cells and leading to the production of anti-inflammatory cytokines, such as IL-10, IL-4, and TGF-β. Host-derived HSPs might directly interact with regulatory T cells, eventually inducing the anti-inflammatory cytokine IL-10 (Liu et al. 2014).

9.6 Inflammatory Diseases (Arthritis, Inflammatory Bowel Disease, etc.)

The immune system is designed to protect the body from any invading disease-causing microbial agents such as bacteria and viruses (Moudgil and Sercarz 2005; Amador-Patarroyo et al. 2012). This immunity against foreign pathogens is mediated primarily through T lymphocytes and antibodies, and there are complex regulatory mechanisms of other immune effector components. However, immune system disorders result in immunodeficiencies, autoimmune diseases, and hypersensitivities (Fig. 9.9). Under certain constellations of genetic predisposition, environmental triggers, and deregulated immune system, the immune system can turn against the host and cause damage to the body's own cells and tissues (autoimmune diseases) (Fig. 9.9). Diseases like rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), systemic lupus erythematosus (SLE), Sjögren's syndrome, and

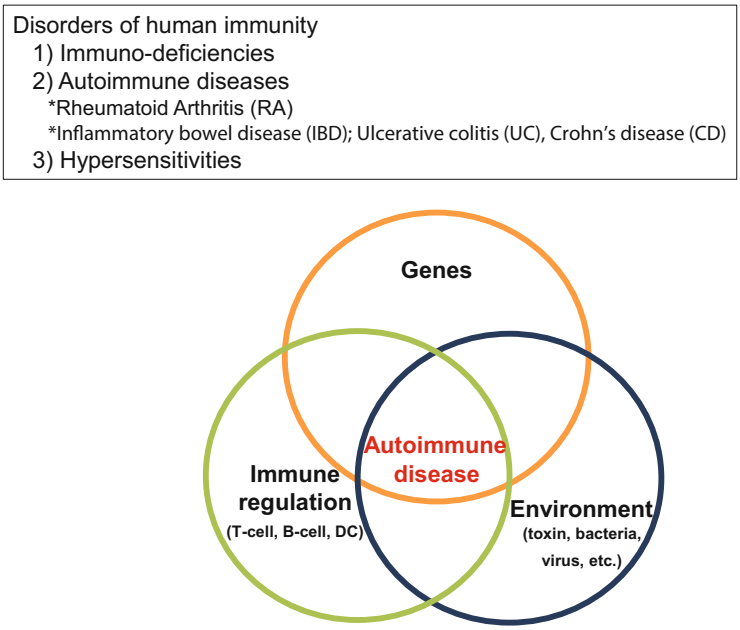

Fig. 9.9 Requirements for the development of an autoimmune disease. Failures of host defense cause immunodeficiencies, autoimmune diseases, and hypersensitivities. Immunodeficiencies occur when the components of the immune system are inactive. Autoimmunity and an overactive immune system occur when the immune system fails to properly distinguish between self and non-self and attacks tissues and organs, for example, rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn's disease (CD). Hypersensitivity is an immune response that also damages one's own body, such as allergy, cytotoxic hypersensitivity, immune complexes, and infectious diseases. Genetic factors, environmental factors, and immune regulations are involved in the development of autoimmune diseases

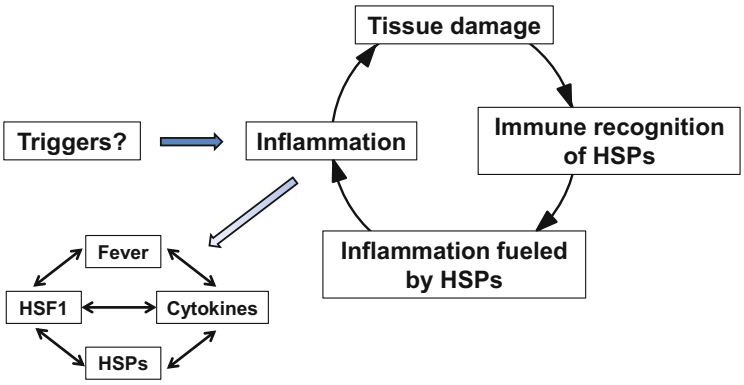

Fig. 9.10 Schematic illustration of autoimmune inflammation modulated by HSPs and HSF1. HSPs are involved in chronic inflammation cycle as described in the text. Fever, cytokines, HSPs, and HSF1 comprise complex reciprocal interactions

autoimmune thyroiditis (AITD) represent the autoimmune diseases (Ermann and Fathman 2001). Such diseases are classified into two types, diseases restricted to certain organs (organ-specific autoimmune disease) and diseases involving a particular tissue in different places (systemic autoimmune disease). For example, rheumatoid arthritis (RA) is a systemic autoimmune disease, whereas inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are organ-specific autoimmune diseases.

Inflammation is part of the body's immune response. Initially, it is beneficial when the immune system properly distinguishes between self and non-self. However, prolonged inflammation, known as chronic inflammation, leads to a progressive destruction of tissues, resulting in the release of HSPs from cells. Since HSPs are ubiquitous, highly conserved proteins and possess strong immunogenicity, they are thought to contribute to modulation of autoimmune inflammation. Thus, chronic inflammation can cause further inflammation, becoming self-perpetuating (Fig. 9.10). During this self-perpetuating loop, fever, HSF1, HSPs, and cytokines comprise reciprocal interactions.

9.6.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common, chronic autoimmune disease characterized by excessive immune responses resulting in inflammation of the joints (Akerfelt et al. 2010). It may lead to deformed and painful joints, such as hands, feet, cervical spine, shoulder, and knee, which can lead to loss of function (Taylor et al. 2004). The joint capsules are swollen, warm, painful, and stiff. Fibrous tissue is developed in synovia, and bone and cartilage have focal erosions, with thinning and destruction (Fig. 9.11) (Feldmann et al. 1996). The cause of RA is not fully understood and

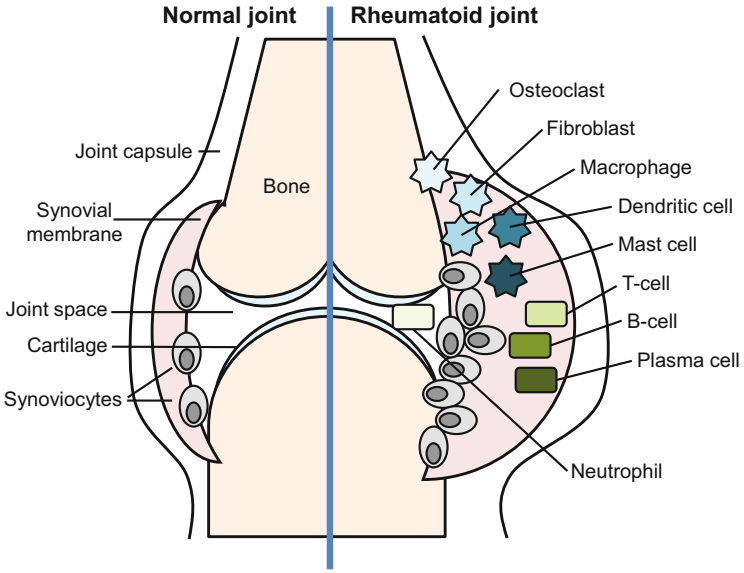

Fig. 9.11 Rheumatoid joint indicating major cell types and sites of joint destruction. For explanation, see text

it is thought to reflect interplayed between genetic factors and an infectious agent or another immune-activating agent. Important candidate gene is a region of class II human leukocyte antigen (HLA) locus. Proinflammatory cytokines derived from macrophages and fibroblast are abundant in the rheumatoid synovia, including IL-1, TNF-α, GM-CSF, IL-6, and numerous chemokines. Once the abnormal immune response has been established, plasma cells derived from B cells produce rheumatoid factors (RF) and antibodies to citrullinated peptides of IgG and IgM classes. RF is the autoantibody against the Fc portion of IgG, where RF and IgG join to form immune complexes that contribute to the disease process (Hermann et al. 1986).

In addition to anti-inflammatory agents such as NSAIDs, COX-2 inhibitors, and glucocorticoids, biological agents have been used for the treatment of RA. Reducing the biological activities of proinflammatory cytokines, such as TNF, IL-1, and IL-6, is accomplished by highly specific strategies, involving neutralizing antibodies, soluble receptors, and receptor antagonist (Dinarello 2000). Stress-induced extracellular BiP, binding immunoglobulin protein or heat shock 70 kDa protein 5 (HSPA5), has potential for long-lasting drug-free therapy in RA (Panayi and Corrigall 2014). A bacterial extract containing HSP60 and HSP70 is used for oral administration in the treatment of disease models for RA (Wendling and Farine 1998).

9.6.2 *Inflammatory Bowel Disease*

Inflammatory bowel disease (IBD) is defined as a chronic intestinal inflammation. IBDs are a group of autoimmune diseases that are characterized by inflammation of both the small and large intestines, in which the body's own immune system attacks elements of the digestive system (Baumgart and Sandborn 2007, 2012; Baumgart and Carding 2007; Goyette et al. 2007). IBD encompasses two major forms known as Crohn's disease (CD) or ulcerative colitis (UC). One of the differences between CD and UC is the colitis regions: CD affects any part of the gastrointestinal tract; UD affects mainly the large intestine. While UC can generally be cured by surgical removal of the entire large intestine, CD cannot be cured by surgery, which is used just to remove the damaged parts of the intestine and reconnecting the healthy parts. Patients affected by IBDs experience symptoms, including abdominal pain, vomiting, diarrhea, bloody stools, and weight loss. Fever, less than 39 °C, may also be present. There are no direct known causes for IBD as yet, because IBDs are a complex disease which arises as a result of the interaction of environmental and genetic factors (Fakhoury et al. 2014; Maynard et al. 2012). The dietary factors, including the increased consumption of milk protein, animal protein, polyunsaturated fatty acids, and alcoholic beverages, increase the risk for IBD, and consumption of tobacco influences the development of the disease. In genetic variations, for instance, a mutation of the NOD2 (nucleotide-binding oligomerization domain protein 2) gene is associated with an increase susceptibility to IBD. Other genetic factors, OCTN1 (organic cation transporter 1) and OCTN2 (organic cation transporter 2), are also suggested to be linked to UC. ATG16L1 (autophagy 16-like 1), a component of a large protein complex essential for autophagy, as well as IL23R (IL-23 receptor), may increase risk of CD (Siakavellas and Bamias 2012).

Intestinal barrier is an important defense mechanism against bacterial infections (Fig. 9.12). It forms a barrier between the lumen of gastrointestinal tract and the internal organs. The physical barriers such as tightly adhering intestinal epithelial cells, secretory IgA (sIgA) produced by IgA(+) plasma cells, and mucin secreted by goblet cells prevent bacterial infections (Maynard et al. 2012). IBD is characterized by defect in the integrity of the epithelial barrier, resulting in translocation of pathogens and other external toxins. Exposure of the internal immune system to external luminal contents significantly increases the production of proinflammatory cytokines and favors a differentiation of T cell to helper T cells (Th1 and Th2 cells). In the case of IBD, the balance between Th1 and Th2 cells is disordered (Arseneau et al. 2007; Zenewicz et al. 2009; Sanjabi et al. 2009; Strober and Fuss 2011).

Anti-inflammatory medicines, such as 5-aminosalicylic acid, and immunomodulators, such as methotrexate, regulate the immune system by triggering a Th2-mediated response and reducing Th1-mediated inflammation. Classically, IBD was thought to be primarily mediated by Th1 cells in CD or Th2 cells in UC; however, recent works indicate that Th17 cells and their related cytokines are crucial for both diseases (Galvez 2014). Th17 cells producing IL-17 promote autoimmunity and inflammation, and anti-TNF-α antibodies are frequently used

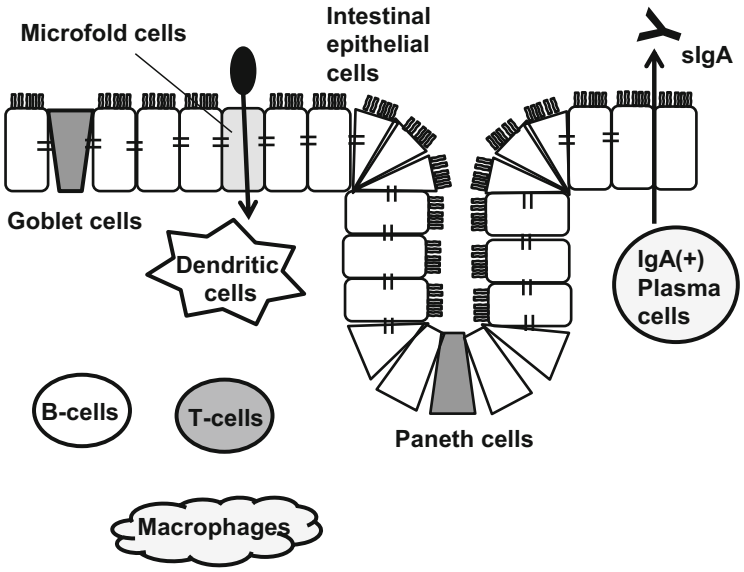

Fig. 9.12 Structure and function of intestinal epithelium. Intestinal epithelial cells (IEPs) are integrated into a single cell layer connected by tight junctions, which make a barrier between apical and basal regions. The IgA(+) plasma cells produce sIgA into the lumen, which limit microbial interaction with epithelium. The epithelial innate and adaptive immune systems are stimulated in response to pathogen invasion and mucosal injury

to treat IBD (van Deventer 1999; Lee and Fedorak 2010; van Montfrans et al. 1998).

9.7 Future Perspectives

HSF1 was originally identified as a specific transcriptional regulator of the heat-induced expression of HSPs, molecular chaperones. However, recent genome-wide studies have revealed that HSF1 is involved in diverse cellular functions in stressed and non-stressed conditions, such as metabolism, aging, and pathogenesis. It has been demonstrated that HSF1 activation at fever range temperature is critical for T-cell, B-cell, and hematopoietic stem cell proliferation (Gandhapudi et al. 2013). As described in Sect. 9.4, HSF1 is involved in suppression of expression of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and cell adhesion molecules (CAMs), such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In addition, heat shock modulates the expression of other immunologically important molecules, such as chemokines (Nagarsekar et al. 2005; Takii et al. 2010). Blocking IL-1 and TNF has been successful in patients with RA and IBD (Taylor et al. 2004). One of the key

inflammatory mediators is NF-κB, a nuclear transcription factor. Incorrect regulation of NF-κB has been linked to cancer, inflammatory, and autoimmune diseases, septic shock, viral infection, and improper immune development. Heat shock reduces the activity of IKK (IκB kinase), thereby reducing the phosphorylation of I-kB (inhibitor of κB) and preventing activation of NF-κB. Thus, heat-induced inhibition of IKK leads to decrease in NF-κB activation and then to suppress expression of cytokines and chemokines (Ryan and Levy 2003). Although a number of essential aspects of HSF1 remain to be elucidated, HSF1 function is reflected in pathogenesis, such as diseases and cancer, where an imbalanced HSF1 activity facilitates disease onset.

As molecular chaperones, HSPs that have specialized functions in protein folding are now well established to play important roles in antigen presentation and cross-presentation, activation, and maturation of macrophages, lymphocytes, and dendritic cells (Figs. 9.6, 9.7, and 9.8) (Tsan and Gao 2009). Since eukaryotic and prokaryotic HSPs have high sequence homology, HSPs have a dual function: immuno-activation and immunosuppression. The elevated HSP levels and anti-HSP antibodies are present in autoimmune inflammatory diseases, including RA and IBD.

The HSPs play a significant role on intestinal mucosal injury as internal cytoprotective and cellular repair factors. In fact, HSP70 play a role in protecting against IBD-related colitis (Tanaka et al. 2007; Tanaka and Mizushima 2009). These protective roles against colitis involve not only molecular chaperones (Otaka et al. 2006) but also suppression of expression of proinflammatory cytokines and cell adhesion molecules (CAMs). The disruption of intestinal barrier function in IBD produces pathogen- and host-derived HSPs in intestinal mucosa. These HSPs affect immune responses and pathogenesis of autoimmune diseases including IBD (Otaka et al. 2006; Fust et al. 2012; Prohaszka and Fust 2004; Dotan and Rachmilewitz 2005; Rokutan 2000).

In experimental disease models, HSP administration can prevent or arrest inflammatory diseases. In initial clinical trials in patients with chronic diseases, HSP peptides have been shown to improve inflammatory conditions (Borges et al. 2012). The therapeutic approaches via exogenous administration of HSPs, enhancing antigen-specific regulatory T-cell (Treg) function, will benefit from current therapies (Akerfelt et al. 2010; Borges et al. 2012; van Herwijnen et al. 2012). Thus, HSPs offer a great potential as a therapeutic target given the diverse array of human diseases including autoimmunity (Multhoff 2006).

Elucidation of the diverse functions of HSF1 and HSPs in the immune and inflammatory response provides new strategies for therapy. However, care should be taken in choosing the right targets, because HSPs and HSF1 possess both pro- and anti-inflammatory effects as described.

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Chapter 10
Heat Shock Factors Modulate Circadian Rhythms

Tsuyoshi Hirota and Yoshitaka Fukada

Abstract Temperature changes have a variety of effects on physiological processes including the circadian clock that generates diurnal rhythms of sleep/wake behavior, hormone release, metabolism, and so on. Even in homeothermic mammals, body temperature fluctuates in a circadian manner that transmits the time information to peripheral tissues and cells. The body temperature rhythms cause cyclic activation of the transcription factor HSF1 and its targets such as *HSP* genes and a clock gene *Per2*, resulting in adjustment of the circadian oscillation in peripheral cells for synchronization. Loss of function of HSF1 therefore leads to reduced synchronization of the clock against temperature changes. HSF1 inhibition also slows down the speed of the clock oscillation and impairs the mechanism that maintains the oscillation speed constant under varying temperature. In the chick pineal gland, a photosensitive clock tissue, HSF and *HSP* genes are activated by light pulse at a specific time of the day, suggesting a role of the HSF pathway in light-dependent synchronization of the circadian clock. Together, HSF has substantial roles in modulating the circadian clock function in response to environmental changes.

Keywords Circadian clock • Clock genes • Temperature • Light • Mammals • Chick pineal gland • HSF

10.1 Introduction

We wake up in the morning and sleep at night. Almost all organisms on the earth show daily rhythms of physiology and behavior under the control of an endogenous biological time-keeping mechanism called circadian clock (Mohawk et al. 2012).

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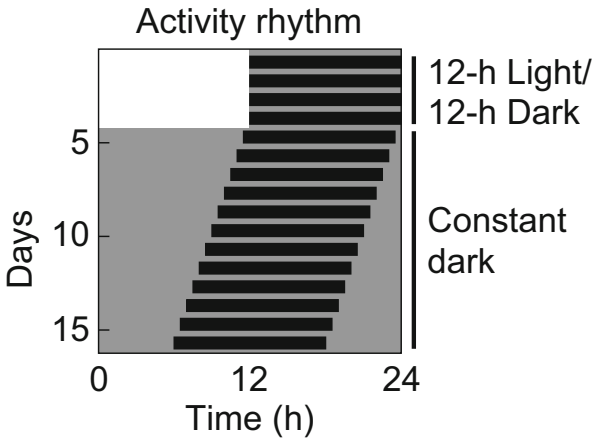

Fig. 10.1 Behavioral circadian rhythm and its entrainment to a light-dark cycle. In the schematic diagram, 1 line represents 1 day (24 h) in which active time is indicated by a *solid bar*. Mouse is a nocturnal animal and hence active at night under a daily cycle of 12-h light (*white area*) and 12-h dark (*gray area*) from day 1 to day 4. When the mouse is transferred to a constant dark condition from day 5, its active time starts to shift day by day with the free-running period of slightly shorter than 24 h (23.5 h in this case). This behavioral rhythm demonstrates the presence of the endogenous circadian clock

The circadian clock oscillates with a period of ~24 h even in the absence of any external time cues. Because the intrinsic period of the circadian clock deviates from exact 24 h that is a rotation period of the light-dark cycle of the earth, the organisms use periodic environmental signals such as light and temperature to adjust the phase of the clock for synchronization, a process called entrainment (Fig. 10.1). In mammals, light information is perceived in the retina and transmitted to the suprachiasmatic nucleus (SCN) of the hypothalamus, a center of the body clock controlling the behavioral rhythms (Welsh et al. 2010). The SCN coordinates peripheral clocks located in most tissues in a hierarchical manner through neuronal, hormonal, and metabolic signals (Fig. 10.2). Although the body temperature of homeothermic animals is kept within a narrow range irrespective of the ambient temperature, it shows daily fluctuations under the control of the circadian clock (1–4 °C, varying among species) and acts as an entrainment signal of the peripheral clocks (Saini et al. 2011). In this chapter, we describe temperature-dependent regulation of the mammalian circadian clock and a role of HSF in this process. We also discuss possible roles of HSF in the light response of the circadian clock in the chick pineal gland, a unique photosensitive clock tissue.

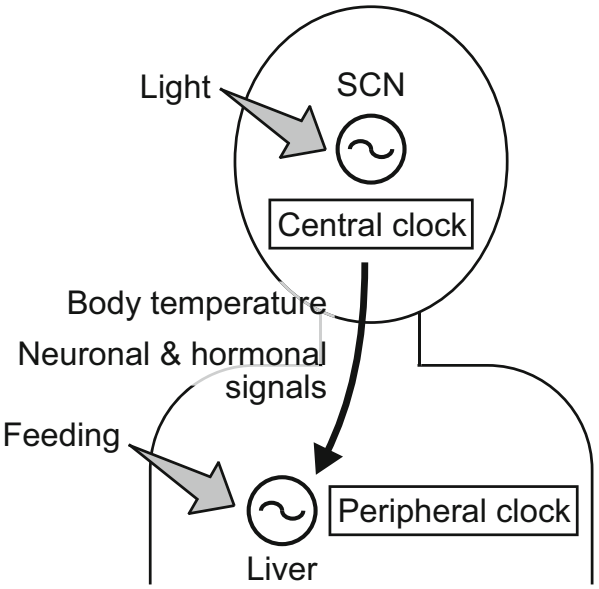

Fig. 10.2 Hierarchical organization of the mammalian circadian clock. The central clock resides in the hypothalamic SCN and orchestrates organismal circadian rhythms including behavior, body temperature, neuronal activity, hormone release, and feeding time that control peripheral clocks in many tissues such as the liver. Light is a dominant time cue for the central clock, while the peripheral clocks can be entrained to feeding time independent of the central clock

10.2 Molecular Mechanism of the Circadian Clock in Mammals

Genetic and molecular biological studies in the past two decades identified more than a dozen of "clock genes" that are required for normal oscillation of the circadian clock with ~24-h period in mammals (Takahashi et al. 2008; Mohawk et al. 2012). The clock genes form transcription- and translation-based feedback loops that cycle once a day (Fig. 10.3). A heterodimer of bHLH-PAS transcription factors CLOCK and BMAL1 activates transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes by binding to E box *cis*-regulatory elements in their promoter region. Translated PER (PER1 and PER2) and CRY (CRY1 and CRY2) that are PAS domain-containing proteins and photolyase homologs, respectively, form a complex to enter the nucleus and repress CLOCK-BMAL1-dependent transcriptional activation. Degradation of PER and CRY through ubiquitin-proteasome pathway releases CLOCK-BMAL1 from repression, resulting in restart of the cycle. This "core loop" couples with "sub-loops" consisting of other transcription factors such as DBP, E4BP4, ROR, and REV-ERB to generate rhythmic expression of a variety of output genes regulating physiological processes. As examples of the clock output, CLOCK-BMAL1 regulates rhythmic expression of *Nampt* gene encoding rate-limiting enzyme of NAD^+ biosynthesis to drive circadian change in NAD^+ levels (Nakahata et al. 2009; Ramsey et al. 2009), and REB-ERBα controls *Ucp1* gene encoding uncoupling protein 1 in brown adipose tissue to generate body temperature rhythms (Gerhart-Hines et al. 2013).

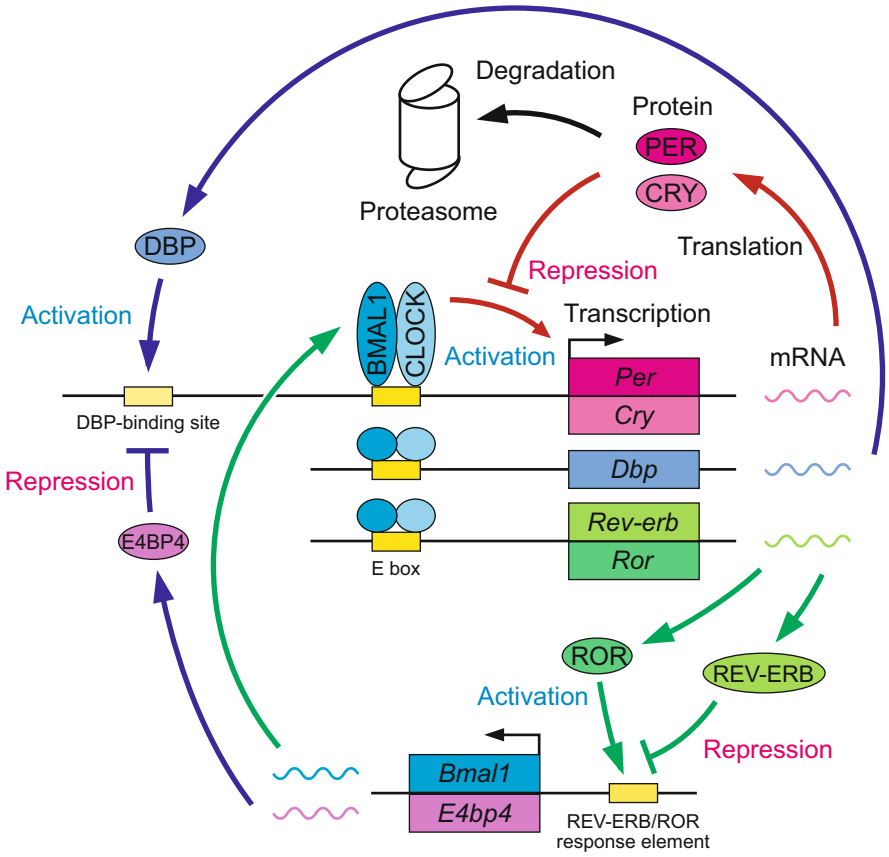

Fig. 10.3 Feedback loops of the mammalian circadian clock. In the core loop (*red arrows*), BMAL1-CLOCK transcription factor complex activates expression of *Per* and *Cry* genes. Translated PER and CRY proteins in turn repress their own transcription to form negative feedback loop. Degradation of PER and CRY through the proteasomal pathway reinitiates transcription of *Per* and *Cry* genes. The core loop is tightly connected with sub-loops composed of nuclear hormone receptors ROR and REV-ERB (*green arrows*) and bZip transcription factors DBP and E4BP4 (*blue arrows*)

In order to facilitate understanding of this review, here we explain several technical terms of the circadian clock research. The circadian rhythms can be characterized by a combination of three parameters: period, phase, and amplitude (Fig. 10.4). The period is the time required for the clock to oscillate one cycle under a free-running condition without any external signals, and it is close but not equal to exact 24 h. The phase is the angle of the rhythm relative to a reference rhythm such as the external day–night cycle. Phase shift is an important property of the circadian clock to synchronize with the environmental 24-h rhythm. The amplitude is the difference between the peak (or trough) and the mean value of the rhythm that

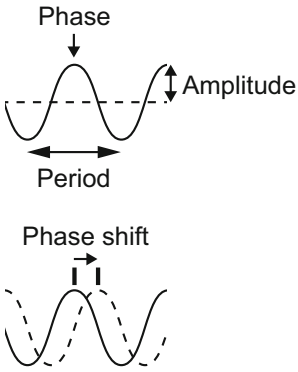

Fig. 10.4 Parameters of the circadian rhythm. See the text for details

represents the strength of fluctuation. A decrease of the amplitude with progressing cycles is called damping.

The oscillatory mechanism of the circadian clock resides in each cell of the entire body and generates long-lasting rhythms at the single cell level (Leise et al. 2012). In the central SCN clock, the cellular clocks communicate with each other by using neural signals such as neurotransmitters and electric activities to form a tightly connected network that produces robust circadian rhythms at the tissue level (Welsh et al. 2010). In contrast, peripheral clocks in most tissues and cultured cell lines do not have such a strong coupling and show rapid damping as a population because of desynchronization among individual cells (Nagoshi et al. 2004; Welsh et al. 2004). It was found that serum shock induces (or resets) rhythmic gene expression in cultured cell lines (Balsalobre et al. 1998), and this discovery led to identification of a variety of resetting stimuli such as a synthetic glucocorticoid dexamethasone, an adenylate cyclase activator forskolin, a PKC activator phorbol 12-myristate 13-acetate, growth factors FGF and TGF-β, glucose, and pH (Akashi and Nishida 2000; Balsalobre et al. 2000a, b; Yagita and Okamura 2000; Hirota et al. 2002; Kon et al. 2008). The cell lines therefore have been used as a model system to study the resetting mechanism of the peripheral clocks.

10.3 Effect of Temperature on Circadian Clock Function

One of the hallmarks of the circadian clock is "temperature compensation." Most biochemical reactions depend on temperature, and their reaction rate varies two to threefold with a temperature change of 10 °C ($Q_{10} = 2–3$). In contrast, the period of the circadian clock oscillation is much less affected by temperature. Q_{10} is 0.99 in cultured rat SCN (Ruby et al. 1999), 0.85–0.88 in rat-1 fibroblasts (Izumo et al. 2003), and 0.88 in NIH3T3 fibroblasts (Tsuchiya et al. 2003), indicating that the oscillation speed of the mammalian circadian clock is affected only slightly by the change of the temperature (few-hour period change in response to 10 °C difference).

While the period length is temperature compensated, the clock system responds to temperature changes to adjust its phase. In cultured fibroblasts, imposing a square temperature cycle (a repeat of 12 h at 33 °C and 12 h at 37 °C) or a simulated cycle of mouse body temperature (35.5–38.5 °C) results in rhythmic expression of the clock genes (Brown et al. 2002; Saini et al. 2012), reflecting synchronization of individual cellular clocks. Although efficiency of the synchronization becomes reduced when the amplitude of the temperature cycle decreases, a difference as small as 1 °C (36.5–37.5 °C) is sufficient to entrain the rhythms (Saini et al. 2012). Peripheral clocks of mouse pituitary glands and lungs in culture are also entrained to a square temperature cycle (a repeat of 12 h at 36 °C and 12 h at 38.5 °C), whereas the central clock of the mouse SCN is unaffected (Buhr et al. 2010). These results suggest that physiological body temperature cycles act as a time cue for the peripheral clocks but not for the SCN clock.

In addition to temperature cycles, a single down- or upshift change of temperature (37–33 °C, 33–37 °C, or 37–42 °C) can induce rhythmic expression of clock genes in fibroblasts (Brown et al. 2002; Tsuchiya et al. 2003), suggesting an acute resetting of individual cellular clock in response to such a temperature shift. Indeed, a brief pulse of high temperature (38.5 °C for 1 or 6 h from 36 °C) strongly phase-shifts or resets preexisting rhythms of mouse pituitary glands and lungs in culture (Buhr et al. 2010). In contrast, the central clock exhibits much smaller responses to temperature pulse in the rat SCN (Ruby et al. 1999) or is mostly unaffected in the mouse SCN (Buhr et al. 2010). Interestingly, the mouse SCN becomes to show peripheral clock-like properties of strong resetting when the intercellular SCN communication is reduced by treatment with a voltage-gated Na^+ channel blocker tetrodotoxin or an L-type calcium channel blocker nimodipine, as well as by physical separation of the dorsomedial and ventrolateral SCN regions (Buhr et al. 2010). Therefore, the strong coupling among the SCN neurons appears to confer resistance against temperature changes. This property is reminiscent of the coupling-mediated robustness against mutation of the clock genes (Liu et al. 2007).

Evaluating the effect of temperature on the circadian clock in vivo is complicated because altered ambient temperature affects the patterns of not only the body temperature but also the feeding that is a major time cue for the peripheral clock (Damiola et al. 2000; Stokkan et al. 2001). Nonetheless, exposing mice to an ambient temperature cycle (24 °C during the day and 37 °C during the night) synchronizes the phase of the clock gene expression in the liver without affecting the central clock in the SCN (Brown et al. 2002). Furthermore, a 2-h treatment of mice with 41 °C water bath causes phase shift by several hours in the kidney, liver, and submandibular gland as revealed by in vivo imaging of the clock gene expression (Ohnishi et al. 2014).

10.4 Regulation of Circadian Gene Expression by HSF1

Connection between HSF and the circadian rhythm in mammals has emerged from two unbiased screenings both done by the Schibler laboratory at the University of Geneva to understand the mechanism underlying rhythmic gene expression.

Transcriptome profiling analyses revealed thousands of genes exhibiting rhythmic expression patterns in each tissue (Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002; Yoshitane et al. 2014; Zhang et al. 2014). However, it was not known how much contribution to the gene expression rhythms comes from the local clock in the tissue and from systemic signals such as hormones and body temperature. To address this question, Kornmann and colleagues developed a transgenic mouse strain to stop the circadian clock specifically in the liver in a conditional manner (Kornmann et al. 2007). The transgene consists of a *Rev-erbα* coding sequence under the control of tetracycline-responsive elements and a gene encoding tetracycline-dependent transactivator under the control of a liver-specific promoter. In the absence of doxycycline, a tetracycline analog, mRNA and protein expression of *Rev-erbα* continuously elevates only in the liver and suppresses transcription of the core clock gene *Bmal1*, resulting in liver-specific shutdown of the clock oscillation. By using this animal, hepatic circadian gene expression was analyzed with genome-wide transcriptome profiling. A set of 351 genes showed circadian expression in the liver with functional clock, and more than 90 % of them became arrhythmic when the liver clock was ablated, indicating a dominant role of the local clock in regulating circadian gene expression. In contrast, a set of 31 genes exhibited rhythmic expression even in the absence of the functional clock. These genes contain a variety of heat shock protein (*HSP*) genes with the expression peaking at a time of highest body temperature, suggesting a role of temperature cycles and HSF in mediating systemic signals to impose extra-hepatic rhythms. Interestingly, the core clock gene *Per2* showed circadian rhythms in the clock-deficient liver (Kornmann et al. 2007). Expression of *Per2* is induced by a heat shock (from 37 °C to 40 °C) in the liver explants (Kornmann et al. 2007), and the *Per2* promoter has functional HSEs, heat shock elements (Tamaru et al. 2011), further supporting the potential role of HSF in the rhythmic gene expression.

In contrast, Reinke and colleagues developed a new technique, differential display of DNA-binding proteins (DDDP), in order to identify transcription factors that exhibit rhythmic DNA-binding activity in vitro (Reinke et al. 2008). They constructed a random DNA library and conducted an EMSA screening of 400 probes with liver nuclear extracts prepared at six different time points across a day. Known circadian transcription factors DBP, REV-ERBα, and CLOCK-BMAL1 were found to show cyclic patterns of DNA-binding activities, indicating the suitability of the assay. The screen further identified three rhythmic probes containing HSE. Among the HSF family members, HSF1 is responsible for the DNA-binding rhythms as revealed by supershifting with anti-HSF1 antibody and EMSA with liver nuclear extracts of *Hsf1*-deficient mice. Consistent with the major role of posttranslational regulation in controlling HSF1 activity (Fujimoto and

Nakai 2010), the nuclear HSF1 level and degree of its phosphorylation exhibit circadian rhythms while *Hsf1* mRNA and total HSF1 protein levels are constant throughout the day. A ChIP experiment indicated circadian rhythms of HSF1 binding to the promoter region of *HSP* genes in the liver in vivo with a phase compatible with the target genes. In cultured fibroblasts, a simulated cycle of the mouse body temperature (35–39 °C) drives circadian expression of a luciferase reporter harboring four copies of HSE, suggesting rhythmic activation of HSF1 by physiological temperature changes (Reinke et al. 2008).

Collectively, HSF1 may function as a key transcription factor that imposes the body temperature cycles to the local transcriptional rhythms of target genes.

10.5 Role of HSF1 in Temperature-Dependent Regulation of the Circadian Clock

Recent studies using a small molecule inhibitor and gene knockout/knockdown demonstrate a central role of HSF1 in mediating the effect of temperature on the circadian clock function.

KNK437, a benzylidene lactam compound, was shown to block heat shock-dependent induction of *HSP* genes and to inhibit interaction of HSF1 with HSE (Yokota et al. 2000; Ohnishi et al. 2004). Buhr and colleagues applied KNK437 to investigate the role of heat shock response pathways in the clock entrainment (Buhr et al. 2010). Interestingly, a 1-h pulse treatment with the compound causes a strong phase shift that is similar to a 1-h cold pulse (from 36 °C to 33.5 °C) and is different from a 1-h warm pulse (from 36 °C to 38.5 °C) in cultured mouse pituitary glands and lungs. Therefore, inhibition of the heat shock response pathway mimics a condition with reduced temperature. Consistently, KNK437 treatment completely blocks the phase-shifting effect of the warm pulse given at the same timing. A flavonoid compound quercetin, another heat shock response inhibitor that acts through HSF1 (Hosokawa et al. 1992), also attenuates the phase shift induced by the warm pulse (Buhr et al. 2010). The role of HSF1 in the temperature resetting is supported by the study of Tamaru and colleagues who demonstrated that the induction of the circadian rhythms by heat shock (43 °C for 30 min) in embryonic fibroblasts from wild-type mice is abolished in the cells from *Hsf1*-deficient mice (Tamaru et al. 2011). Furthermore, Saini and colleagues revealed that the entrainment of circadian gene expression to physiological temperature cycles (35.5–38.5 °C) is delayed by *Hsf1* knockdown in NIH3T3 fibroblasts and severely attenuated in primary fibroblasts of *Hsf1*-deficient mice (Saini et al. 2012). In contrast, *Hsf2* knockdown has almost no effect on the kinetics of the temperature entrainment. Collectively, these results indicate the principal role of HSF1 in the regulation of circadian phases of peripheral clocks in response to temperature change.

In contrast to the peripheral clocks, the phase of the central clock in the SCN is resistant to the pulse treatment with KNK437. Interestingly, however, chronic treatment with KNK437 has a pronounced period lengthening effect in cultured SCN as well as pituitary glands and lungs in a dose-dependent manner (Buhr et al. 2010). Consistently, *Hsf1*-deficient mice show a long free-running period of the behavioral rhythms (Reinke et al. 2008), and their embryonic fibroblasts also exhibit a long period of the gene expression rhythms (Tamaru et al. 2011). Based on the chronic effect, Buhr and colleagues further revealed that KNK437 treatment impairs temperature compensation of the circadian period (Buhr et al. 2010). The period length under control condition shows only a ~1-h difference between the culture temperatures of 30 °C and 38 °C, but the KNK437 treatment expands the period difference to ~7 h in both the SCN and the pituitary. Therefore, the HSF1 pathway also contributes to the mechanism maintaining the period length constant at various temperatures.

10.6 Cross Talk Between HSF and Light Response in the Chick Pineal Gland

In addition to the role of HSF in temperature-dependent regulation of the mammalian circadian clock, a study using the chick pineal gland identified an unexpected link between HSF and the light input pathway of the clock. Light is one of the most prominent signals mediating synchronization of the endogenous clock. Similar to temperature, the light-dark cycle entrains the clock, and a light pulse shifts its phase. Importantly, the phase-shifting effect of light differs depending on the timing when the pulse is provided. A light pulse causes delay and advance of the phase at the early and late night, respectively, while it has no effect during the day in constant dark condition. To approach the molecular mechanism underlying this time-of-day-dependent response of the clock to light, Hatori and colleagues conducted genome-wide expression analysis of light-responsive genes (Hatori et al. 2011). The chick pineal gland provides a unique model system because it contains both the central clock and the light input pathway for entrainment (Takahashi et al. 1989), while in mammals, these functions are separated into the SCN and the retina. Comparison of the gene expression in the chick pineal gland with and without 1-h light pulse at three different time points (early night, late night, and daytime) identified more than 100 light-inducible transcripts (Hatori et al. 2011). Most of the transcripts show stronger induction at one time point than the others, indicating circadian rhythms of these light responses. Of note, many of the genes strongly induced at late night are related to protein folding and known to respond to heat shock or endoplasmic reticulum (ER) stress. Consistent with the gene expression profiles, their upstream regulators are light-activated: HSF1 and HSF2 but not HSF3 accumulate in the nucleus, and expression level of spliced *Xbp1* mRNA increases to produce the activated form of XBP1 transcription factor, the

master regulator of ER stress response (Mori 2009), in the pineal gland after the light pulse (Hatori et al. 2011). While heat shock regulates nuclear accumulation of HSF1 and HSF3 (Fujimoto and Nakai 2010), the light pulse stimulates that of HSF1 and HSF2, suggesting a unique mechanism independent of temperature change to transmit the light information to the clock at late night. Furthermore, the light pulse at early night was discovered to induce posttranslational activation of SREBP transcription factor to stimulate expression of *E4bp4* gene (Hatori et al. 2011). E4BP4 is a transcription factor that represses *Per2* expression (Doi et al. 2001, 2004), connecting light-dependent activation of SREBP at early night to the phase delay of the core clock mechanism. These results together provide a starting point to understand the molecular mechanism of time-of-day-dependent regulation of the circadian phase, in which HSF1, HSF2, XBP1, and SREBP may play key roles.

10.7 Future Perspectives

It is straightforward that high temperature activates HSF to induce expression of the clock gene *Per2* for resetting of the circadian clock. *Per2* induction also plays a key role in light-dependent phase shift of the mammalian clock (Albrecht et al. 2007). Therefore, the light-dependent activation of HSF (Hatori et al. 2011) is likely to induce *Per2* expression to reset the clock in the chick pineal gland. Future studies need to reveal the role of HSF in this process as well as the mechanism how HSF1 and HSF2 are activated by light in a time-of-day-dependent manner. Such studies will lead to identification of a unique regulatory mechanism of HSFs that mediate environmental light information to the pineal circadian clock controlling melatonin release.

In addition to *HSP* genes, temperature cycles drive rhythmic expression of *Cirp* gene encoding cold-inducible RNA-binding protein (Kornmann et al. 2007; Morf et al. 2012). CIRP protein interacts with *Clock* mRNA, and knockdown of *Cirp* causes reduction of cytosolic *Clock* mRNA level and CLOCK protein expression, resulting in a strong decrease of the amplitude of clock gene expression rhythms and faster entrainment to temperature cycles (Morf et al. 2012). Therefore, HSF and CIRP seem to have opposite effect on the temperature entrainment of the clock, and their interplay needs to be addressed to understand how the peripheral clock synchronizes with temperature cycles.

The temperature compensation of the circadian period has been a long-standing question among the researchers of the circadian clock, and its molecular mechanism is still unknown. Phosphorylation of the clock proteins is temperature-compensated and/or plays a key role in the temperature compensation in a variety of organisms such as cyanobacteria, fungi, plants, and mammals (Tosini and Menaker 1998; Nakajima et al. 2005; Isojima et al. 2009; Mehra et al. 2009; Portoles and Mas 2010). It is necessary to reveal the mechanism underlying reduced temperature compensation by HSF inhibitor KNK437 (Buhr et al. 2010) and to explore its potential link with phosphorylation of clock proteins. In order to further identify

essential components of the temperature compensation, it would be a promising strategy to apply recent advances of cell-based RNAi and small molecule screens of circadian clock modifiers (Hirota et al. 2008, 2010, 2012; Hirota and Kay 2009, 2015; Isojima et al. 2009; Maier et al. 2009; Zhang et al. 2009; Lee et al. 2011; Chen et al. 2012, 2013).

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Part III
Diseases Associated with HSF Function

Chapter 11
HSF Inhibits the Progression of Age-Related Neurodegenerative Diseases

Naohide Kondo, Masahisa Katsuno, Yuichi Riku, and Gen Sobue

Abstract Age-related neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and polyglutamine diseases, are refractory conditions that appear during the prime of life. The heat shock factor (HSF)–heat shock protein (HSP) pathway, which controls and facilitates physiological protein homeostasis, may hold the key to understanding the neurodegenerative process. In this chapter, we describe the clinical and pathological features of representative age-related neurodegenerative diseases and the pathomechanisms underlying those disorders. Furthermore, we discuss the role of the HSF–HSP pathway in the aging and degeneration of neurons, the roles of HSPs in model organisms of neurodegenerative diseases, and therapeutic candidates for activating HSF and HSPs. Finally, we discuss the relationship between prion diseases and neurodegenerative conditions with a special focus on the HSF–HSP pathway.

Keywords Neurodegenerative diseases • Protein homeostasis • Proteostasis • Aggregation • Heat shock factor (HSF) • Heat shock protein (HSP) • Propagation • Prion

11.1 Introduction

Most neurodegenerative diseases are characterized by an age-dependent onset and progressive neuronal cell death in specific lesions of the central nervous system. The mechanisms underlying these features are unknown, and no effective therapies for these devastating disorders currently exist (Taylor et al. 2002; Petrucelli and Dawson 2004; Katsuno et al. 2012a). Some age-related neurodegenerative diseases are single-gene hereditary disorders, whereas the etiology of sporadic diseases may involve multiple genetic and environmental factors. Huntington's disease (HD), spinal bulbar and muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and several forms of spinocerebellar ataxia (SCA) are a group of hereditary neurodegenerative disorders caused by the expansion of a CAG repeat,

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Table 11.1 Clinical and pathological features of neurodegenerative diseases

Age-related neurodegenerative diseases	Aggregation-prone protein	Pathological aggregation	Major susceptible lesion of the aggregation	Main clinical symptoms
Alzheimer's disease (AD)	Amyloid-β Tau	Senile plaque Neurofibrillary tangle	Cerebral cortex neuron (extracellular and cytoplasm)	Dementia
Parkinson's disease (PD)	α-synuclein	Lewy body	Dopaminergic neuron and glia (cytoplasm)	Tremor, bradykinesia, rigidity, and postural instability
Amyotrophic lateral sclerosis (ALS)	TDP-43 FUS	Skein like inclusion	Cerebral and spinal motor neuron (cytoplasm)	Muscular atrophy and weakness
Huntington's disease (HD)	Huntingtin (HTT)	Nuclear inclusion body	Caudate and putamen neuron (nuclear)	Chorea and dementia
Spinal and bulbar muscular atrophy (SBMA)	Androgen receptor (AR)	Nuclear inclusion body	Spinal and brainstem motor neuron (nuclear)	Muscle atrophy and weakness
Spinocerebellar ataxia (SCA) 1, 2, 3, 6, and 17	Ataxin-1, ataxin-2, ataxin-3, α1A calcium channel subunit, and TATA-binding protein	Nuclear inclusion body	Cerebellar neuron (nuclear and cytoplasm)	Ataxia

which encodes polyglutamine, in specific genes (Orr and Zoghbi 2007). By contrast, although Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are inheritable diseases in a subset of the population, the vast majority of cases appear sporadically (Table 11.1). The causative proteins in the familial forms of these diseases, such as amyloid-beta (Aβ) in AD, α-synuclein in PD, and TDP-43 in ALS, are also likely implicated in the pathogenesis of the sporadic forms of the diseases. Thus, both hereditary and sporadic forms of neurodegenerative diseases appear to share critical molecular mechanisms. Here, we describe the role of HSF–HSP pathway in age-related neurodegenerative diseases and therapeutic strategies for these devastating disorders using HSF–HSP-activating compounds.

11.2 Features of Neurodegenerative Diseases

11.2.1 Age-Related Neurodegenerative Diseases

Polyglutamine Diseases

HD, SBMA, DRPLA, and six forms of SCA (SCAs 1, 2, 3, 6, 7, and 17) are categorized as polyglutamine diseases. The expansion of a CAG triplet repeat in the gene that encodes the polyglutamine tract instigates neuronal damage via a toxic-gain-of-function (La Spada and Taylor 2010). In these disorders, pathogenic proteins accumulate in the nuclei of neurons within specific lesions with aging. The disruption of normal biological systems due to the accumulation of these polyglutamine-expanded proteins leads to cell death. Polyglutamine diseases share several clinical and genetic features. For example, the CAG repeat size correlates with the age of onset of neurological deficits in each disease. CAG repeats also exhibit both somatic and germline instability and have a strong tendency to expand further in successive generations; this leads to the phenomenon of anticipation, in which subsequent generations experience an earlier disease onset and more severe symptoms (Katsuno et al. 2014).

Huntington's disease is an inherited autosomal dominant disorder caused by a CAG trinucleotide repeat in the first exon of the *huntingtin* (*HTT*) gene (Snell et al. 1993). This disease involves progressive neurodegeneration chiefly in the cerebral cortex and basal ganglia. HD has two prominent features: choreoathetosis and dementia. The onset of symptoms generally occurs while patients are in their 30s and 40s; however, the pathological condition has an earlier onset (Ross and Tabrizi 2011). An earlier onset, which is called anticipation and is usually associated with a larger CAG repeat size, often results in quite different symptoms, such as slow movement, rigidity, seizures, and a faster disease progression, particularly when the disease is inherited from the father. A recent study on anticipation in HD reported that the longest expanded allele was the sole determinant of the age at onset of motor symptoms; the normal allele CAG length, the interaction between the expanded and normal allele, and the presence of a second expanded allele had no influence on the age at onset (Lee et al. 2012). Most cases of HD exhibit gyral atrophy in the frontal and temporal lobes, and gross atrophy of the head of the caudate nucleus and the putamen is prominent in advanced HD. In patients with HD, the abnormal huntingtin protein accumulates preferentially in the neurons of the striatum and parts of the cortex.

SBMA, or Kennedy disease, is a rare inherited recessive X-linked neuromuscular disease caused by the expansion of a CAG repeat in the first exon of the *androgen receptor* (*AR*) gene (Kennedy et al. 1968; La Spada et al. 1991). SBMA affects only men due to a testosterone-dependent mechanism of the pathology. Patients with SBMA have progressive muscle weakness in the tongue, arms, and legs. Severe cramps, tongue atrophy, and contraction fasciculation are the distinguishing symptoms of SBMA, although symptoms of androgen insensitivity

such as gynecomastia, erectile dysfunction, and low sperm count may be present in certain cases (Sobue et al. 1989). SBMA causes the loss of lower motor neurons, including those in the brainstem that supply the bulbar muscles (Li et al. 1998). The unique pathogenesis of this disease is marked by the ligand-dependent accumulation of the abnormal AR in the nucleus of the spinal anterior horn neurons as well as nonneuronal cells including scrotal skin epithelial cells (Katsuno et al. 2002; Adachi et al. 2005).

SCAs are a group of heterogeneous neurodegenerative diseases that affect the cerebellum, brainstem, and spinal cord and commonly induce a slowly progressive incoordination in speech as well as movement in the arms, legs, and eyes. The inheritance pattern depends on the disease and may be autosomal dominant, autosomal recessive, or X-linked (Matilla-Duenas et al. 2012; Seidel et al. 2012). Among the autosomal dominant SCAs, DRPLA and SCAs 1, 2, 3, 6, 7, and 17 are polyglutamine diseases caused by the aberrant expansion of a CAG repeat in defective genes (Durr 2010; Orr 2012).

Alzheimer's Disease

AD, which is the most common neurodegenerative disease and accounts for 60–70 % of dementia cases (Burns and Iliffe 2009), may afflict more than 20 million people worldwide. Increasing age is the greatest risk factor for AD; after the age of 65, the risk of developing AD doubles approximately every 5 years. In addition to genetic factors (e.g., apolipoprotein E), poor diet, lack of exercise, and smoking and alcohol use are associated with a higher risk of developing AD. Microscopic amyloid plaques and neurofibrillary tangles have been detected in the postmortem brain of patients with AD (Arriagada et al. 1992). These deposits are likely associated with neuronal loss, synaptic dysfunction, and severe atrophy of the cerebral cortex. Amyloid plaques, which are cellular material found outside the neuron, consist of insoluble deposits of Aβ42, whereas neurofibrillary tangles in the neurons are composed of hyperphosphorylated tau. Aβ, the key protein of AD, is a small portion of amyloid precursor protein (APP) that is normally bound to the membranes of neurons. The Aβ protein is formed from the cleavage of APP by β- and γ-secretases. Although APP is cleaved by α-secretase, this cleavage does not produce Aβ. One pivotal hypothesis states that the cleavage of APP by these enzymes gives rise to Aβ residues of different lengths (Sisodia and St George-Hyslop 2002). At the first step, APP is cleaved by either α- or β-secretase. The products of this initial cleavage then are cleaved by γ-secretase to produce small fragments. Although the products that result from the sequential cleavage by α- and γ-secretases are not toxic to neurons, the sequential cleavage by the β- and γ-secretases produces the 40-amino acid peptide, Aβ40, and the longer 42-amino acid form, Aβ42; the latter is thought to be toxic to neurons in the AD brain (Terry 1996). Furthermore, the ratio of Aβ42 to Aβ40 is critical to neuronal toxicity in the pathogenesis of AD. This view is strongly supported by the fact that all known causative genes in the hereditary form of AD, including *APP* and *presenilin* 1 and

2, encode proteins that are involved in the Aβ generation pathway. Although the majority of the molecular-targeted therapies for AD have been developed on the basis of the amyloid cascade hypothesis, the effects of such treatment have not been confirmed in clinical trials despite clear evidence of neuroprotection in animal models. Several studies have indicated that the inhibition of Aβ aggregation, which appears to be initiated a few decades prior to the clinical onset of AD, is not sufficient for mitigating the disease progression at the symptomatic stage (Hampel et al. 2010). However, these unfavorable results from anti-amyloid therapies may also stem from an amyloid-independent pathogenesis.

Parkinson's Disease

PD, which is the second most common neurodegenerative disease after AD, is characterized by a progressive movement disorder. According to the PD foundation, more than seven million people worldwide are afflicted with PD. Although less is known about the mechanisms underlying the idiopathic form of PD compared with AD, more treatment options are available, including medication and surgery to control the symptoms of PD. The pathognomonic findings in PD include tremor of the hands, arms, legs, jaw, and face; bradykinesia or slowness of movement; rigidity or stiffness of the limbs and trunk; and postural instability with impaired balance and coordination. The most remarkable histopathological finding in PD is the loss of dopaminergic neurons, which are pigmented cells found in the substantia nigra, nuclei of the locus coeruleus, and dorsal motor nucleus of the vagus (Obeso et al. 2008; Lewitt 2008). Microscopic analysis reveals the characteristic presence of pigmented nuclei that contain eosinophilic cytoplasmic inclusions surrounded by a faint halo, which are called Lewy bodies. The accumulation of α-synuclein in Lewy bodies is a distinctive mechanism of PD (Jellinger 2014). Although the pathogenesis of idiopathic PD is not well known, mutations in specific genes, including *SNCA*, *PRKN*, *LRRK2*, *PINK1*, *DJ-1,* and *ATP13A2*, have been reported in several familial forms of PD (Lesage and Brice 2009). Given that some of these molecules are implicated in the quality control of mitochondria and the ubiquitin–proteasome system, the disruption of protein homeostasis appears to be a major pathological factor in PD (Grenier et al. 2013).

Amyotrophic Lateral Sclerosis (ALS)

ALS is an adult-onset motor neuron disease caused by motor neuron cell death in the brain, brainstem, and spinal cord (Rowland and Shneide 2001). The clinical features of ALS are weakness in the limbs and face as well as difficulties with speech, swallowing, and breathing. Signs of cell death in both the upper motor neurons (overactive tendon reflexes, Hoffman signs, clonus, and Babinski signs) and lower motor neurons (muscle atrophy, weakness, and fasciculation) are found in the advanced stage of this condition. The characteristic histopathological finding

of ALS is the loss of both upper and lower motor neurons in the cerebral motor cortex, spinal anterior horn, and lower portion of the brainstem. Large α motor neurons tend to be affected before small motor neurons during the progression of the disease. In addition, gliosis and the proliferation of microglial cells are detected in the microscopic analysis, and surviving nerve cells are usually small, shrunken, and filled with lipofuscin. Furthermore, motor neurons often have cytoplasmic inclusions that are present in both the neurons and glia. Most of these inclusions are ubiquitinated and composed of TDP-43 or FUS, both of which are DNA-/RNA-binding proteins. Approximately 10 % of patients with ALS have the familial form of the disorder caused by specific mutations (Iguchi et al. 2013) inherited mainly in an autosomal dominant pattern. Some familial ALS is related to a hexanucleotide expansion in the *C9orf72* gene, and surprisingly, several ostensibly sporadic cases exhibit mutations in this gene (Majounie et al. 2012). A mutation in *superoxide dismutase 1* (*SOD1*) is another major cause of autosomal dominant familial ALS. Moreover, the intermediate CAG repeat expansions in *ATXN-2*, the causative gene of SCA2, are also associated with susceptibility to sporadic ALS (Elden et al. 2010). It also has been reported that mutations in *TDP-43* or *FUS* are associated with approximately 5 % of familial and 2 % of sporadic ALS cases. Therefore, dysregulation of RNA metabolism may underlie the pathogenesis of ALS (Wojciechowska and Krzyzosiak 2011; Cleary and Ranum 2013), and both the familial and sporadic forms of this disease could share common pathomechanisms (Andersen and Al-Chalabi 2011). It has been difficult to develop therapies for ALS due to the complex pathogenesis. Although a number of compounds have been shown to slow disease progression in mutant *SOD1* mouse models, only riluzole, a glutamate-release inhibitor, has demonstrated effectiveness in clinical trials (Bensimon et al. 1994).

11.2.2 Common Mechanisms of Neurodegenerative Disease

Conformational Changes and Protein Aggregation

Abnormal protein aggregation is detected in most neurodegenerative diseases regardless of the mode of inheritance (Fig. 11.1). The aggregation of abnormal proteins such as amyloid, tau, synuclein, TDP-43, androgen receptor, and huntingtin, which may have undergone posttranslational modification, leads to disruption of the normal protein homeostasis machinery (i.e., proteostasis). This disruption further accelerates the aberrant protein accumulation. Each disease is categorized on the type of protein aggregate, e.g., tauopathy, synucleinopathy, and TDP-43 proteinopathy (Spillantini et al. 1997; Nonaka et al. 2005; Arai et al. 2006; Neumann et al. 2006). Proteostasis destruction is described in more detail below.

Another hallmark of age-related neurodegenerative diseases is that disease-causing erratic protein accumulation occurs in specific regions of the central nervous system and involves anatomically and physiologically related systems of

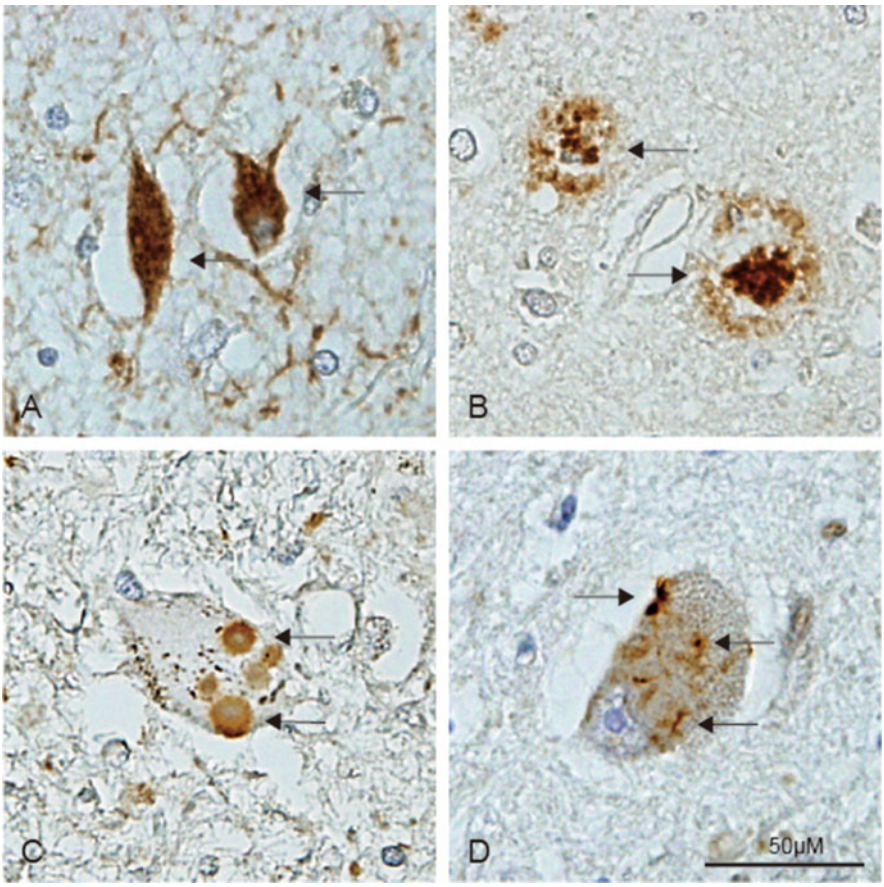

Fig. 11.1 Morphological abnormalities of age-related neurodegenerative diseases. Photomicrograph of neurofibrillary tangles with tau in Alzheimer's disease (**a**), senile plaque with amyloid-β in Alzheimer's disease (**b**), Lewy body with α-synuclein in Parkinson's disease (**c**), and skein like inclusion with TDP-43 in amyotrophic lateral sclerosis (**d**)

neurons. In the ALS patient, pathological changes are limited to the motor neurons of the cerebral cortex, brainstem, and spinal cord. In patients with some types of SCA, abnormal protein aggregation chiefly affects the Purkinje cells of the cerebellum. Although the mechanism underlying the selective distribution of lesions is not well known (Rowland and Shneider 2001; Braak et al. 2013; Margulis and Finkbeiner 2014), it is of interest that the affected lesions of each neurodegenerative disease are anatomically and physiologically related. This observation provides a theoretical basis for the protein propagation theory (Jucker and Walker 2013), which proposes that abnormal proteins propagate from cell to cell and result in the progression of neurodegeneration.

Several hypotheses for the cause of age-related neurodegenerative diseases have been proposed, including proteostasis disruption, transcriptional dysregulation,

axonal transport disruption, synaptic dysfunction, mitochondrial dysfunction, and excitotoxicity. Age-related neurodegenerative diseases are also called protein-misfolding diseases because these diseases are characterized by accumulation of misfolded proteins in the neurons and/or nonneuronal cells. Dysfunction and neuronal death in neurodegenerative diseases are commonly caused by the accumulation of toxic aggregate species, which are diffusible oligomeric forms of misfolded proteins (Kayed et al. 2003; Chiti and Dobson 2006). The structure of these oligomeric proteins is disrupted, which exposes hydrophobic amino acid residues on unpaired peptide β-strands with sticky surfaces that form abnormal interactions with other proteins embedded within the cellular membrane. These toxic oligomers are considered to interfere with the normal cellular machinery and instigate cell death cascades. Although the misfolded proteins also form histopathologically visible aggregates, various studies have indicated that these inclusion bodies are protective rather than toxic to the cells, which is contrary to expectations. This neuroprotection may be due to the sequestration of the sticky surfaces of the toxic oligomers, which reduces the interaction between aberrant proteins and normal proteins (Miller et al. 2011; Hipp et al. 2014). This result is clearly demonstrated in cell-based studies of HD, in which cells bearing inclusion bodies are more resistant to the toxicity of the polyglutamine-expanded huntingtin protein compared with those with diffuse accumulation of the pathogenic proteins (Arrasate et al. 2004). There also is increasing evidence that soluble Aβ and tau oligomers trigger synaptic dysfunction in the early stages of AD (Spires-Jones and Hyman 2014).

Proteostasis Disruption

In addition to the increased propensity for abnormal proteins to aggregate, there is increasing evidence that several mechanisms underlying the disruption of the protective or robust system in the cells conspire to induce detrimental conditions and consequently lead to neurodegeneration. The leading hypothesis is that the disruption of proteostasis underlies age-related neurodegenerative diseases, and therapeutic strategies have been developed to target this mechanism (Hartl et al. 2011). Several studies have suggested that the accumulation of misfolded proteins attenuates robust proteostasis in the neuronal cells and eventually leads to neurodegenerative diseases. There are several lines of evidence that implicate the impairment of proteostasis in protein aggregation-related diseases (Bence et al. 2001; Gidalevitz et al. 2006). The genetic disruption of the ubiquitin–proteasome system in murine neurons has been shown to induce neurodegeneration that mimics AD and ALS (Irmler et al. 2012; Tashiro et al. 2012). In addition, the suppression of neuronal autophagy results in massive neuronal loss in the cerebral and cerebellar cortices (Komatsu et al. 2006). It also has been shown that the cellular ability of proteostasis is blunted by aging, which provides a theoretical basis for the age dependency of neurodegeneration (Vilchez et al. 2014; Yang et al. 2014).

Transcriptional Dysregulation

Transcriptional dysregulation has been implicated in the early pathogenesis of neurodegenerative diseases. Microarray analyses have demonstrated the profound alteration of transcriptomes in a variety of neurodegenerative disorders (Dietz and Casaccia 2010). In polyglutamine diseases, transcriptional factors such as specificity protein 1 (SP1), TATA-box-binding protein-associated factor II (TAFII130), and cAMP response element-binding protein (CREB) interact with the polyglutamine-expanded proteins and are sequestered by the abnormal protein aggregates, which eventually leads to transcriptional dysfunction (Nucifora et al. 2001; Buckley et al. 2010). Furthermore, it has been reported that aberrant histone acetylation also induces transcriptional dysregulation in polyglutamine diseases and ALS, which provides a clue to the development of therapies (Minamiyama et al. 2004; Ryu et al. 2005).

Axonal Transport Disruption of Axonal Transport

The impairment of axonal transport, in which axonal cargo such as neurofilament accumulates in affected neurons, is one of the earliest molecular events in the pathogenesis of ALS (De Vos et al. 2008). Mutations in the gene encoding dynactin 1, a motor protein that regulates retrograde axonal transport, result in lower motor degeneration, and the mRNA level of dynactin 1 is decreased in SBMA and ALS (Puls et al. 2003; Jiang et al. 2005; Katsuno et al. 2006). Another study has shown that pathogenic AR interrupts kinesin-1 microtubule-binding activity and consequently disrupts anterograde axonal transport via c-Jun N-terminal kinase (JNK) activation (Morfini et al. 2006). Pathogenic huntingtin also blocks fast axonal transport via the stimulation of JNK3 and phosphorylating kinesin (Morfini et al. 2009). Deficits in the axonal transport of mitochondria are also reported in cellular and animal models of AD and PD (Liu et al. 2012; Krstic and Knuesel 2013).

Synaptic Dysfunction

Synaptic dysfunction has been implicated in the pathogenesis of AD, especially in the early stage of the disease. Many studies indicate that Aβ plays a physiological role in normal synaptic function. For example, in hippocampal slices, the activity of β-secretase is enhanced by synaptic activity, and the resulting Aβ peptides depress excitatory transmission via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors (Kamenetz et al. 2003). Moreover, there is a substantial correlation between synaptic activity and concentration of Aβ in the interstitial fluid of an APP transgenic mouse model (Cirrito et al. 2005). It has been reported that Aβ plays an important role in

controlling synaptic plasticity. Another study revealed that Aβ toxicity employs the NMDA receptor and p25/cyclin-dependent kinase (Cdk) 5 pathway in the pathogenesis of synaptic plasticity (Seo et al. 2014). LRRK2 and α-synuclein, the causative proteins of familial PD, also regulate synaptic integrity; the dysfunction of these proteins appears to underlie the pathogenesis of PD (Vekrellis et al. 2011).

Mitochondrial Dysfunction

Mitochondrial dysfunction and oxidative stress have been implicated explicitly in the molecular mechanisms underlying normal aging and various disease conditions, including neurodegenerative disorders. The pathogenic role of mitochondrial deficits in neurodegeneration has been studied extensively in PD (Beal 2003). The Parkinsonism-inducing toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is known to induce mitochondrial perturbation. More importantly, some of the causative proteins of familial PD play an essential role in the cellular regulation of mitochondrial levels and functions, and a recent study reported that these proteins interact in the pathological process, which indicates a strong relationship between mitochondrial dysfunction and PD pathogenesis (Exner et al. 2012; Winklhofer and Haass 2010; Okatsu et al. 2012). Polyglutamine-expanded proteins also affect mitochondria via a direct interaction and the transcriptional dysregulation of genes such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a transcriptional coactivator that regulates mitochondrial biogenesis and function (Cui et al. 2006; Ranganathan et al. 2009). Indeed, the expression level of PGC-1α is downregulated in the skeletal muscle in both a familial ALS mouse model and a sporadic ALS patient and in the spinal cord and skeletal muscle in an SBMA mouse model (Thau et al. 2012; Iida et al. 2015). The disruption of mitochondrial calcium metabolism and mitochondrial DNA damage has been suggested as the machinery of mitochondrial turbulence in HD (Wang et al. 2013; Ayala-Pena 2013). Moreover, mitochondrial dysfunction is also reported in the early pathology of AD, although the cause and effect of this phenomenon are still under debate (Caldeira et al. 2013).

Excitotoxicity

Excessive stimulation by neurotransmitters such as glutamate is another molecular mechanism underlying the pathogenesis of neurodegenerative diseases (Trippier et al. 2013). Abundant amounts of Q/R site-unedited GluR2, a subunit of the AMPA receptor, in the motor neurons of sporadic ALS cases support the excitotoxicity theory (Kawahara et al. 2004). The activation of NMDA receptors (NMDARs) gives rise to synaptic dysfunction in early AD, which provides a theoretical basis for therapies that inhibit NDMAR-mediated signaling, such as memantine (Zadori et al. 2014). In addition, in mouse models of HD, the uptake of glutamate by

astrocytes is decreased, which is partially due to the downregulation of glutamate transporter 1 (Hassel et al. 2008).

11.3 HSF Suppresses the Progression of Polyglutamine Diseases, Alzheimer's Disease, and Parkinson's Disease

11.3.1 The Role of the HSF–HSP Pathway in Protein-Misfolding Diseases

The cornerstone of life is protein dynamics, and protein dynamics are also important in neurodegenerative disorders. Protein homeostasis is deeply associated with neurodegenerative disorders, including AD, PD, polyglutamine diseases, and ALS. Heat shock factor 1 (HSF-1) is a key transcriptional factor of stress-dependent gene expression and protein homeostasis. Heat shock proteins (HSPs), which are regulated downstream of HSF-1 regulation, play an important role in the neurodegenerative process by refolding and solubilizing pathogenic proteins (Kim et al. 2013). The refolding of pathogenic proteins by HSPs facilitates the proteasomal degradation of these toxic molecules. HSP-induced autophagy further accelerates the clearance of aberrant proteins (Bauer et al. 2010). HSPs also are known to inhibit the apoptotic pathway, which is construed as a major pathomechanism of neuronal cell death in various diseases (Kennedy et al. 2014). Despite these neuroprotective properties of HSPs, their localization and expression are affected in neurodegenerative diseases. HSPs are shown to co-localize with aggregates of the toxic polyglutamine-expanded proteins in various polyglutamine diseases (Wyttenbach 2004; Muchowski and Wacker 2005). HSPs similarly co-localize with α-synuclein in PD (Uryu et al. 2006). The decreased expression of HSPs is demonstrated in affected brain regions of AD and HD (Dou et al. 2003; Hay et al. 2004). Several studies have reported that the expression levels of HSP70 are strongly associated with the vulnerability of cells to the toxic insults of polyglutamine-expanded proteins (Tagawa et al. 2007). This view is exemplified in an animal study showing that the heterozygous knockout of *HSF-1* in SBMA model mice leads to the extended distribution of pathogenic AR accumulation in neuronal and nonneuronal tissues as well as exacerbated neuromuscular phenotype (Kondo et al. 2013). These observations suggest a tight link between HSPs and neurodegeneration. Furthermore, considerable research indicates that HSPs, including HSP70, influence the pathogenesis of protein-misfolding diseases at several stages of disease progression (Fig. 11.2). Although clinical applications of these strategies treat and reverse age-related neurodegenerative diseases have not been successfully developed, in vitro and in vivo experiments have determined that the HSF–HSP pathway ameliorates these devastating conditions.

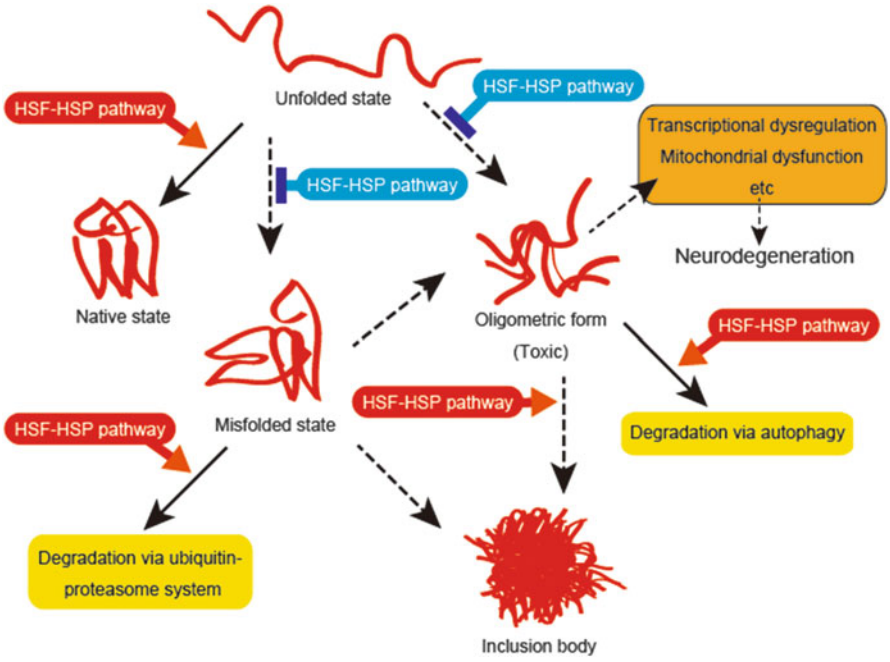

Fig. 11.2 Schematic illustration of protein homeostasis network and the roles of HSF–HSP pathway during the neurodegenerative process of diseases. *Arrows* indicate physiological course of protein autoregulation. *Arrows* with *dashed line* indicate the neurodegenerative process of age-related neuronal disorders. *Red* and *blue* items are activating and inhibiting roles of HSF–HSP pathway, respectively

11.3.2 HSF Is a Key Player in Protein-Misfolding Diseases and Aging

Aberrant protein accumulation in cells is a common characteristic of normal aging and neurodegenerative disease (Kenyon 2010; Kourtis and Tavernarakis 2011). There is increasing evidence that the expression level of stress-dependent chaperone proteins is controlled by HSF-1 and Forkhead box O (FOXO), also known as DAF-16 in *C. elegans* (Demontis and Perrimon 2010; Hartl et al. 2011). FOXO is downstream of insulin/IGF-1 signaling and a regulator of the expression of several genes that are associated with aging and longevity (Ogg et al. 1997; Giannakou et al. 2004). HSF-1 and FOXO together activate the expression of small HSPs, resulting in longevity (Hsu et al. 2003). Namely, insulin/IGF-1 signaling suppresses HSF-1 activity due to the inhibition of the phosphorylation of the HSF-1 regulators, DDL-1 and DDL-2 (Chiang et al. 2012). Moreover, HSF-1 and FOXO are the target of sirtuin (SIRT), which is also known as nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-1, a master regulator of aging and longevity (Sauve et al. 2006). In addition, dietary restriction as a strategy for longevity is effective

through the SIRT1-HSF1/FOXO pathways (Guarente 2013). Thus, HSF-1 is not only a regulator of HSPs but also a key player in aging and age-related machinery.

11.3.3 HSF Suppresses the Progression of Alzheimer's Disease, Parkinson's Disease, Polyglutamine Diseases, and Amyotrophic Lateral Sclerosis

Among the HSFs, HSF-1 is a potent inducer of molecular chaperones, including HSPs (Fujimoto and Nakai 2010). The genetic elimination of HSF-1 has been shown to induce neurodegeneration, which suggests a critical role of this transcriptional factor in the maintenance of neuronal function and survival (Santos and Saraiva 2004; Homma et al. 2007). There is abundant evidence from in vitro and in vivo models that the HSF–HSP pathway ameliorates several age-related disorders, including AD, PD, polyglutamine disease, and ALS. HSF-1 mainly acts against neurodegeneration through HSP70 induction, although it also has been reported that HSF-1 is capable of protecting neurons via an HSP-independent pathway (Verma et al. 2014). Moreover, the pharmacological activation of the HSF–HSP machinery has been shown to consistently provide positive effects on neurodegenerative diseases. We have summarized the existing studies on the effects of HSP70 overexpression in mouse models of neurodegenerative disorders in Table 11.2.

Table 11.2 The effect of HSP70 in age-related neurodegenerative diseases model mice

Age-related neurodegenerative disease	Model mouse	Effect of HSP70 overexpression	References
Alzheimer' disease (AD)	Mutant APP transgenic mouse	Improve phenotype and pathology	Hoshino et al. (2011)
Parkinson's disease (PD)	MPTP-induced PD mouse	Improve pathology	Dong et al. (2005)
Amyotrophic lateral sclerosis (ALS)	G93A mutant SOD1 mouse	Improve phenotype and pathology	Gifondorwa et al. (2007)
Huntington's disease (HD)	Transgenic mouse (R6/2)	Improve phenotype	Hansson et al. (2003)
Spinal and bulbar muscular atrophy (SBMA)	Mutant AR transgenic mouse	Improve phenotype and pathology	Adachi et al. (2003)
Spinocerebellar ataxia (SCA)	SCA1 model mouse SCA7 model mouse	Improve phenotype and pathology Not effective	Cummings et al. (2001); Helmlinger et al. 2004

AD

Numerous studies have shown that the HSF–HSP pathway has a protective effect against the toxicity of tau and Aβ accumulation in an AD model organism. For example, Hsp70/40 and Hsp90 block Aβ self-assembly in cultured cells (Evans et al. 2006). HSP70 overexpression ameliorates both pathological and functional phenotypes in AD mice that express the mutant form of APP by upregulating the expression of Aβ-degrading enzymes and TGF-β (Hoshino et al. 2011). The co-chaperone carboxyl terminus of HSP70-interacting protein (CHIP) and HSP70 also facilitates tau ubiquitination, degradation, and aggregation (Petrucelli et al. 2004; Dickey et al. 2007).

PD

The HSF–HSP pathway also plays a protective role in the pathogenesis of PD. -Dominant-positive HSF-1 decreases the level and cytotoxicity of α-synuclein (Liangliang et al. 2010). The overexpression of HSP70 prevents dopaminergic neuronal loss induced by α-synuclein in *Drosophila* (Auluck et al. 2002). CHIP facilitates α-synuclein degradation via proteasomal and lysosomal pathways (Shin et al. 2005). The adeno-associated viral overexpression of HSP70 mitigates damage to the mouse dopaminergic system in the MPTP-induced PD-like phenotype in mice (Dong et al. 2005).

Polyglutamine Diseases

Both HSF-1 and HSPs have been shown to suppress the aggregation of polyglutamine-expanded proteins (Fujimoto et al. 2005; Neef et al. 2011). The overexpression of HSF-1 induces HSP70 and mitigates neuronal damage in a mouse model of SBMA, whereas the deletion of HSF-1 exacerbates neurodegeneration in mouse models of HD and SBMA (Hayashida et al. 2010; Kondo et al. 2013). The overexpression of HSP70 ameliorates neurodegeneration in mouse models of SBMA and SCA1 (Cummings et al. 2001; Bailey et al. 2002; Adachi et al. 2003). Intracerebral injection of the adeno-associated virus expressing HSP40 suppresses pathogenic huntingtin aggregation in the striatum of the R6/2 mouse model of HD (Popiel et al. 2012). Pathogenic AR-induced motor neuron degeneration also is attenuated by the overexpression of CHIP, whereas the genetic reduction of this co-chaperone exacerbates neurodegeneration in a mouse model of SCA3 (Adachi et al. 2007; Williams et al. 2009). Nevertheless, the effect of HSP70 enhancement is not sufficient to suppress neurodegeneration in mouse models of HD and SCA7 (Hansson et al. 2003; Helmlinger et al. 2004). Further studies are necessary to understand the molecular basis for the discrepancy regarding the

biological effects of the HSF–HSP pathway in the pathogenesis of different polyglutamine diseases.

ALS

The intraperitoneal injection of recombinant human HSP70 increases the lifespan of the mutant SOD1 mouse model of familial ALS. However, the homeostatic overexpression of HSP70 in this model does not improve the phenotype, presumably due to insufficient expression levels of HSP70 (Gifondorwa et al. 2007). Additionally, it has been confirmed that the aggregation of TDP-43, a histopathological hallmark of sporadic ALS, is regulated by HSF-1 in *C. elegans* (Zhang et al. 2011). A recent study has indicated that SIRT1 overexpression ameliorates neurodegeneration in mutant SOD1 mice with a mild phenotype via activation of the HSF–HSP70 pathway (Watanabe et al. 2014). Hence, these upstream of the HSF–HSP pathway have lately attracted considerable attention from the therapeutic point of view.

11.3.4 Pharmacological Induction of the HSF–HSP Pathway as a Therapeutic Strategy for Neurodegenerative Disease

From a clinical perspective, the pharmacological activation of the HSF–HSP pathway is a promising strategy to treat and reverse the damage from age-related neurodegenerative diseases. There are many inducers and co-inducers of HSPs that have been reported in studies using neurodegenerative disease model organisms. For example, HSP90 inhibitors and HSF-1 activators have been identified as promising therapeutic compounds in the treatment of neurodegeneration (Waza et al. 2005; Katsuno et al. 2005; Neef et al. 2010). However, ideal therapeutic compounds for human patients must be both safe and effective. Given these requirements, there is adequate proof from animal models that HSP90 inhibitors such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and celastrol and HSF-1 activators such as geranylgeranylacetone (GGA) and arimoclomol are safe and effective in the treatment of late-onset neurodegenerative diseases (Waza et al. 2005; Paris et al. 2010; Katsuno et al. 2005; Kieran et al. 2004). Additionally, HSF1A, riluzole, nonsteroidal anti-inflammatory drugs (NSAIDs), and gedunin have been proposed as candidates for HSP–HSF pathway activation. HSF1A is a novel pharmacological activator of HSF-1 that has been detected by yeast screening (Neef et al. 2010). Riluzole, an approved drug for the treatment of ALS, also induces HSP expression via HSF-1 upregulation (Yang et al. 2008a). Furthermore, NSAIDs have some effect on HSF-1 induction through an unknown mechanism

(Jurivich et al. 1992; Lee et al. 1995), and recent studies have identified gedunin as another type of Hsp90 inhibitor (Brandt et al. 2008).

17-AAG

The HSP90 inhibitor 17-AAG has the potent ability to induce the expression of HSPs and thus is a promising therapeutic agent for several neurodegenerative disorders, including polyglutamine diseases (Nagai et al. 2010). Pathogenic AR-mediated neuronal toxicity in a mouse model of SBMA is ameliorated by 17-AAG via the degradation of androgen receptors by HSPs and proteasomes (Waza et al. 2005). In *Drosophila* models of SCA3 and HD, 17-AAG abrogated the neurodegenerative process by inducing HSPs (Fujikake et al. 2008). This compound also is known to activate autophagy in addition to chaperones in a cellular model of SBMA (Rusmini et al. 2011). In a mouse model of AD, Hsp90 inhibition by 17-AAG restores synaptic function via not only HSP induction but also the upregulation of presynaptic and postsynaptic proteins in neurons, such as synapsin I, synaptophysin, and PSD95 (Chen et al. 2014). The compound 17-AAG also inhibits the aggregation of TDP-43 and α-synuclein, the key pathological features of sporadic ALS and PD, respectively (Jinwal et al. 2012; Chang et al. 2013; Riedel et al. 2010).

Celastrol

Celastrol is another HSP90 inhibitor that has been shown to improve disease condition in animal models of AD, PD, HD, and ALS via HSP induction (Salminen et al. 2010). For instance, celastrol reduced Aβ pathology by preventing NFκB activation and inhibiting BACE-1 expression in an AD model mouse (Paris et al. 2010). In addition, celastrol has been shown to attenuate the loss of dopaminergic neurons in the MPTP-induced mouse model of PD and improved the pathology in an HD model rat (Cleren et al. 2005). In addition, treatment with this agent significantly ameliorates the phenotype in an ALS mouse model (Kiaei et al. 2005). Although the mechanism is unknown, the stimulation of HSF-1 activity by celastrol has been observed in various experiments (Hieronymus et al. 2006; Yang et al. 2006). It has been hypothesized that these plural pathways play a synergistic therapeutic role in the neurodegenerative process of age-related central nervous system impairments.

GGA

GGA is an acyclic isoprenoid compound with a retinoid skeleton that induces HSP synthesis in various tissues, including the central nervous system. GGA stimulates the induction of HSPs via the activation of HSF-1 and ameliorates the phenotypes

of polyglutamine-induced neurodegeneration in an SBMA model mouse (Katsuno et al. 2005). Moreover, the oral administration of GGA mitigates AD-related phenotypes and pathological changes, such as cognitive impairment, Aβ accumulation, and synaptic loss (Hoshino et al. 2013). This compound has also been shown to be safe in humans, given that the drug has been widely used as an antiulcer agent.

Arimoclomol

Arimoclomol, a strong co-inducer of HSPs, is a promising drug for the treatment of ALS. Recent research has revealed that arimoclomol mitigates motor neuron degeneration and mortality in ALS model mice (Kieran et al. 2004; Kalmar et al. 2008). Furthermore, this compound improves muscle power and motor neuron survival in a mouse model of SBMA (Malik et al. 2013). Most studies have shown that arimoclomol is effective even after the visible onset of neurological deficits in mice, which provides a considerable advantage as a clinical treatment option in humans.

Paeoniflorin

Paeoniflorin is the principal active ingredient extracted from the roots of the Paeonia plant, which possesses various biological and biomodulating activities including improvements in memory, antioxidant activity, antiepileptic activity, anti-stroke properties, and anti-neuroinflammation. Paeoniflorin is shown to ameliorate motor neuron degeneration in a mouse model of SBMA by activating both the molecular chaperone-ubiquitin proteasome system and autophagy (Tohnai et al. 2013).

11.4 Propagation Modulated by the HSF–HSP Pathway

Several studies in disorders similar to age-related neurodegenerative diseases have been conducted to identify the mechanisms underlying neurodegeneration. One important finding that has emerged from this research is the discovery of the prion, which is a self-propagating misfolded protein. Prion diseases are any of various chronic neurodegenerative disorders caused by prions, including Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cattle (Collinge 2001; Flechsig and Weissmann 2004; Jucker and Walker 2013). Proteinaceous infectious particles can serve as self-propagating seeds for the instigation and progression of prion diseases (Prusiner 2001). In prion diseases, the normal form of the prion protein (PrPC) is converted into a misfolded isoform protein (PrPSc) that has the feature of self-propagation.

PrPSc infection to PrPCs leads to the conformational conversion of PrPC and engenders an aberrant form of PrPSc (Caughey 2003).

Recently, the disease-causing misfolded proteins of neurodegenerative diseases were shown to propagate from cell to cell, which provides a molecular basis for the regional expansion of neurodegeneration (Jucker and Walker 2013). Aggregates of misfolded proteins function as seeds that can continuously recruit endogenous proteins and form larger aggregates over time. For example, Lewy bodies were shown to form in transplanted fetal mesencephalic dopaminergic neurons in the brains of patients with PD (Li et al. 2008). More direct evidence has been shown in animal studies, which have demonstrated the expansion of lesions along neural networks resulting from the intracerebral injection of insoluble α-synuclein (Luk et al. 2012; Masuda-Suzukake et al. 2014). A similar lesion expansion has been induced by the intracerebral injection of tau aggregation in wild-type mice (Clavaguera et al. 2013). Although in vivo evidence is lacking, the prion-like transmission of TDP-43 and SOD1 has been suggested in cellular experiments (Furukawa et al. 2011). These observations strongly suggest that the propagation of misfolded proteins is an important pathomechanism underlying the expansion of neurodegeneration through neural networks (Kanouchi et al. 2012). The HSF1–HSP pathway plays a crucial role in propagation of prions and misfolded proteins that cause age-related neurodegenerative diseases. For prion diseases, molecular chaperones such as HSP104 inhibit the propagation of yeast prions partially by suppressing the transformation of large prion aggregates into polymers, which function more efficiently as seeds for propagation (Song et al. 2005). With respect to PD, HSP70 is shown to reduce the release of α-synuclein oligomers into the extracellular space and mitigate related toxicity (Danzer et al. 2011).

11.5 Future Perspectives

Compounds that activate the heat shock factor (HSF)–heat shock protein (HSP) pathway could abrogate neuronal damage in age-related neurodegenerative diseases. Preclinical studies using animal models of neurodegenerative disorders show the therapeutic effects of HSP inducers, including 17-AAG, celastrol, GGA, arimoclomol, and paeoniflorin. Translation of these results in human patients, however, faces several hurdles (Katsuno et al. 2012b). To successfully translate these results, several issues need to be resolved, including the adequacy of animal models; the reproducibility of preclinical studies; the dosage, timing, and duration of intervention; and the drug delivery.

11.5.1 Adequacy of Animal Models

Ideal animal models of neurodegenerative diseases should recapitulate human conditions. A number of models carrying the gene mutations responsible for familial forms of neurodegenerative diseases have been created and used for the analysis of pathophysiology, based on the assumption that sporadic and familial forms of neurodegenerative disorders share similar clinical and pathological features (Turner and Talbot 2008; Mackenzie et al. 2010). Rodent models chiefly contribute to the understanding of the pathomechanism, while smaller model organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and zebra fish, have been utilized to screen therapeutic compounds, due to their short life cycles (Gotz and Ittner 2008; Teschendorf and Link 2009). Although many disease-modifying compounds showed positive results in animal studies, few drugs have shown efficacy in clinical trials (Katsuno et al. 2012a). These unsuccessful clinical trial results imply that the results of preclinical animal experiments using familial models may not be applicable to sporadic neurodegenerative disorders. Nevertheless, there are no established animal models of sporadic neurodegenerative disease, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), despite sporadic cases representing the majority of patients. Moreover, the use of animal models is complicated by non-cell autonomous causes of disease. Both mutant genes in neurons and environmental conditions, such as neighboring glial cells, can determine disease pathogenesis (Yamanaka et al. 2008; Ilieva et al. 2009). Drug screening and preclinical studies using induced pluripotent stem cells from neurodegenerative disease patients may resolve this issue (Bellin et al. 2012; Ross and Akimov 2014). Nonhuman primate models (Yang et al. 2008b; Nagahara et al. 2009) are also promising strategies to increase the possibility of applying therapeutic compounds in human patients.

11.5.2 Reproducibility of Preclinical Studies

Animal studies should be repeated to assure reproducibility. The failure of promising animal studies to translate is partially attributed to the inadequate validity of preclinical studies (Jucker 2010). Independent preclinical animal studies are necessary to ensure blinded treatment allocation and outcome evaluations. Public or private animal institutes, which assess new drug candidates for age-related neurodegenerative diseases, may be useful to perform independent preclinical animal studies. A checklist to design animal study quality control arguments may help predict the ability to translate preclinical studies to human patients (Ludolph et al. 2010).

11.5.3 Dosage, Timing, and Duration of Intervention

The amount of drug used in an animal model is not always applicable in human patients. HSP-activating drugs may cause adverse events while achieving therapeutic effects. Given that side effects are a common cause of failure in clinical trials, deliberate dose determination is necessary to apply activators of HSF-1–HSP. In humans, intervention is primarily initiated after disease onset. Therefore, it is of particular importance to test compounds in animal models at the asymptomatic stage (Ludolph et al. 2010). In contrast, clinical trials using prodromal subjects with neurodegenerative disease have gained attention, since various studies strongly indicate that biological changes precede clinical onset by more than a decade in familial forms of neurodegenerative disease (Bateman et al. 2012; Ross et al. 2014). To conduct such studies, appropriate biomarkers need to be utilized to assess the effects of these therapies in subjects without overt neurological symptoms. In this regard, it is of particular interest that creatine appears to attenuate brain atrophy in asymptomatic mutation carriers of Huntington's disease (Rosas et al. 2014). Given the potential side effects with long-term use of neuroprotective agents, appropriate duration of therapy, in addition to the right timing, must be determined.

11.5.4 Drug Delivery

The delivery of many potential therapeutic and diagnostic compounds to specific areas of the central nervous system is restricted by barriers, including the blood-brain barrier (BBB) and the blood–cerebrospinal fluid (CSF) barrier. Recent studies have shown numerous roles for these barriers, including neurodevelopment, the control of cerebral blood flow, and neurodegenerative disease pathology. Thus, from a clinical perspective, drugs should easily pass these barriers without affecting their physiological function (Zlokovic 2008; Hanson and Frey 2008). Development of drug delivery systems, including viral vectors, is another key to the successful translation of basic studies into the clinic (Miyazaki et al. 2012).

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Chapter 12
HSF and Heart Diseases

Jie Yuan and Yunzeng Zou

Abstract In the heart, HSF1 has a wide range of expression in many kinds of cells, including cardiac myocytes, fibroblasts, and endothelial cells, which governs the activation of heat shock proteins and plays a protective role against different pathological stimuli. HSF1 is activated by phosphorylation and transferred into the nucleus. HSPs also regulate HSF1 activity. HSF1 activity is required to maintain redox state and attenuate oxidative damage in the heart under normal physiological conditions. HSF1 protects against ischemia/reperfusion injury and myocardial infarction by inhibiting oxidative stress and cardiomyocyte apoptosis. HSF1 ameliorated death of cardiomyocytes and cardiac fibrosis and thereby prevented cardiac dysfunction as well as hypertrophy induced by chronic pressure overload. HSF1 promotes cardiac angiogenesis during chronic pressure overload, leading to the maintenance of cardiac adaptation. In atherosclerosis, however, HSF1 is activated and highly expressed in atherosclerotic lesions and that proinflammatory cytokine stimulation and disturbed mechanical stress to the vessel are primarily responsible for HSF1 activation in smooth muscle cells. In the failing hearts, HSF1 is increased, but nuclear translocation of the HSF1 is markedly reduced in the viable myocardium upon the pathological stresses. Thus, cardiac protective HSP induction is impaired in the failing heart.

Keywords HSF1 • Activation • Myocardial infarction • Ischemia/reperfusion injury • Cardiac pathologic hypertrophy • Atherosclerosis • Heart failure

12.1 Introduction

The heart is a muscular organ in humans and other animals, which functions as a pump in the circulatory system to provide a continuous circulation of blood throughout the body. By means of this circulation, the heart can provide sufficient oxygenated blood containing nutrients and metabolites, meet metabolic needs, and preserve a constant internal milieu. Its two essential characteristics are contractility

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and rhythmicity. In the regulation of these, the nervous system and neurohumoral effects modulate relationships between venous returns, outflow resistance, frequency of contraction, and inotropic state. There are also intrinsic cardiac autoregulatory mechanisms.

Ischemic heart disease (IHD) accounts for the most of the global heart disease burden, which occurs when the heart is not sufficiently supplied with blood causing damage to the heart muscle. Common IHDs develop when coronary blood supply to myocardium is reduced, either in terms of absolute flow rate (low-flow or no-flow ischemia) or relative to increased tissue demand (demand ischemia). Atherosclerosis, myocardial infarction (MI), ischemia, and reperfusion are major causes for IHD. In addition, pathological cardiac hypertrophy is accompanied with a variable degree of myocardial ischemia. Obviously, IHDs are actually a result of imbalance between myocardial blood supply and oxygen. Long-standing IHDs will lead to heart failure. In fact, heart failure is a complex disorder that leads to disturbance of the normal pumping of blood to the peripheral organs to meet the metabolic demands of the body, which is the final outcome of various cardiovascular diseases such as hypertension, ischemic heart disease, myocarditis, valvular insufficiency, or cardiomyopathy.

12.2 Protein Metabolism and Signaling in Heart Cells

12.2.1 HSF1 and HSF2 in the Heart

At present, four different HSFs (HSF1, HSF2, HSF3, and HSF4) have been identified in vertebrates. HSF1, HSF2, and HSF4 are expressed in the human tissues (Nakai et al. 1997). HSF1 is primarily involved in the stress response and has been found to be a "multifaceted factor" involving in various pathogenesis. In the heart, HSF1 has a wide range of expression in many kinds of cells, including cardiac myocytes, fibroblasts, and endothelial cells, which governs the activation of heat shock proteins (HSPs). HSPs act to ensure the proper protein folding, as well as to prevent protein misfolding and assist in protein refolding to the correct state. Expression of HSPs is mainly regulated by HSF1 at the transcriptional level. In cardiomyocytes, knockout of HSF1 has no effect on basal levels of HSP72 and HSP27, but results in loss of the stress-induced increase in these proteins. In fibroblasts, knockout of HSF1 is associated with loss of both basal expression and stress-mediated induction of HSP72 and HSP27 (Xiao et al. 1999).

Unlike HSF1, HSF2 is not activated by classical stress stimuli, but plays a role in embryogenesis. HSF2 is present in two isoforms (HSF2-α and HSF2-β) (Fiorenza et al. 1995). Both HSF1 and HSF2 are strongly expressed in the developing heart. HSF2 DNA-binding activity is transiently induced after cardiac looping when chambers maturate and valves are formed (Eriksson et al. 2000). HSF2 expression and activation have no temporal or spatial correlation with heat shock gene expression, which indicates that HSF2 activation is associated with specific stages of heart

formation but is not involved in the regulation of inducible heat shock gene expression (Eriksson et al. 2000). The HSF2-α form is transcriptionally more active than the HSF2-β form (Snoeckx et al. 2001). Interestingly, HSF2 operates additionally to HSF1. When both heat shock factors are activated by hemin treatment and heat shock, respectively, transcription of the hsp70 gene is more potent than after activation of HSF1 alone (Tani et al. 1997).

12.2.2 Control of HSF1 Activation

In the nonstressed state, HSF1 exists as a latent monomer in the cytoplasm, with repressed DNA-binding and transcriptional activity (Fig. 12.1). Upon activation, HSF1 undergoes rapid and reversible relocalization within seconds into specific subnuclear structures, termed stress granules (Jolly et al. 1999), which include a monomer-to-trimer transition, nuclear accumulation, and transcriptional activation. First, serine residue 230 of HSF1 in the cytosol is phosphorylated, and then the transcription factor is transferred into the nucleus, where HSF1 trimers bind with high affinity to the heat shock element (HSE) that consist of multiple contiguous invert repeats of the pentamer sequence nGAAn located in the promoter region of target genes (Holmberg et al. 2001; Westerheide and Morimoto 2005). Upon recovery from stress such as heat shock, HSF1 rapidly dissipates from these stress granules to a diffuse nucleoplasmic distribution, typical of unstressed cells (Jolly et al. 1999). There are several phosphorylation sites in HSF1 (Fig. 12.1). Inducible serine 230 phosphorylation positively contributes to the transcriptional competence of HSF1, which is necessary to exert primarily the function of the HSF1 after heat shock stress (Holmberg et al. 2002). However, constitutive residues (serine 303, serine307, or serine363) repress the transcriptional activation of HSF1 (Kline and Morimoto 1997). In stress granules, HSF1 undergoes posttranslational modification by covalent conjugation of a small ubiquitin-like modifier 1 (SUMO-1) protein to lysine 298 preceded by phosphorylation of serine 303 (Chi and Karliner 2004). Negative regulators of HSF1-driven transcription also include the mitogen-activated protein kinase ERK and c-Jun NH2-terminal kinase (Dai et al. 2000). These kinases recognize their substrates via a small domain (D domain) in which phosphorylation of serine 363 appears to be the major target leading to reduced transcriptional activity (Dai et al. 2000). Additionally, phosphorylation on Ser303 in HSF1 by glycogen synthase kinase 3β (GSK3β) reportedly has a negative regulatory effect on HSF1 activation (Chu et al. 1996; Xavier et al. 2000). Substitution of this serine residue with alanine, such as in the HSF1 S303A mutant, results in increased transcriptional activity (Lepore et al. 2001). HSF1 knockout is associated with a chronic increase in tumor necrosis factor-α (TNF-α) levels and increased susceptibility to endotoxin (Xiao et al. 1999). The promoter for TNF-α contains an HSF1 binding site that represses transcription, and thus, loss of this repressor results in sustained expression of TNF-α (Singh et al. 2002). However, TNF-α transiently inhibits activation of HSF1 via TNF-R1, and activation of phosphatase HSF1-heat shock element DNA binding is not sufficient to elicit

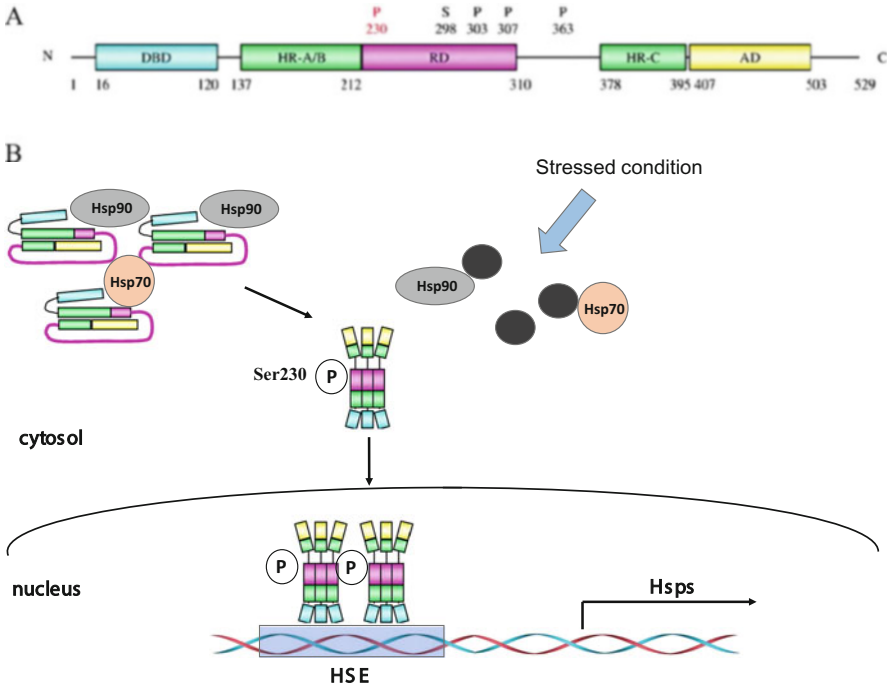

Fig. 12.1 Schematic representation to show the potential regulatory mechanism of HSF1. (**A**) Structure of HSF1. DBD indicates DNA-binding domain; HR, hydrophobic repeat; RD, regulatory domain; AD, transcriptional activation domain; P, phosphorylated site (the activating site is indicated in *red*); S, sumoylated site. (**B**) Under nonstressful conditions, HSF1 exists as a monomer whose transcriptional activity is repressed with HSP70 and HSP90 chaperones that interact with the activation domain of HSF1 and thereby block its activation potential. Under stressed condition, HSP70 and HSP90 switch to interactions with denatured proteins, releasing HSF1 and activation domain. Phosphorylation of the activating site (Ser230) is enhanced, thereby promoting the transcriptional activity of the trimerized HSF1. (Toko et al. 2008)

maximal transcription of the HSP genes, so it is necessary for HSF1 to be modified by phosphorylation and sumoylation to increase its transcriptional activity (Knowlton 2006). Therefore, phosphorylation is key to both activation and inhibition of HSF1, and dephosphorylation mediated by TNF-α can lead to inactivation.

HSPs also regulate HSF1 activity. Under nonstressful conditions, the proteins composed of HSP90, HSP40, heat shock cognate protein (HSC70), and carboxy-terminus of HSC70-interacting protein (CHIP) form a complex with HSF1 (Neef et al. 2011). The formation of this protein complex including HSF1 prevents the translocation of HSF1 from the cytosol to the nucleus and thus suppresses the activation of HSF1. Upon exposure to stress, HSF1 dissociates from the HSP90 protein complex and is translocated into the nucleus, where it binds to the heat shock element in the promoter region of various molecular chaperone genes for their expression (Guo et al. 2001).

12.2.3 Regulatory Role of HSF1 in the Heart

In cardiomyocytes, HSF1 can induce transcription of synapse-associated protein 97 (SAP97) through a SIRT1 (a deacetylase and longevity factor)-dependent interaction with HSEs (Ting et al. 2011). As a linker protein, SAP97 creates the scaffold complex that anchors several channels in the plasma membrane (Kim and Sheng 1996; Gardoni et al. 2007). Therefore, the increase in SAP97 through activation of HSF1 results in stabilization of voltage-dependent K^+ channels ($K_v1.5$ channels) and activation of both I_{Kur} and I_{to} in the cardiomyocytes and modification of responses to adrenergic signaling in the heart, influencing the shape and duration of action potentials in both atrial and ventricular myocytes (Ting et al. 2011).

In vascular smooth muscle cells, reduction of HSF1 amplifies the inflammatory response to angiotensin II (Chen and Currie 2006). HSF1 is primarily known as the transcription factor for the FasL, which has heat shock elements (HSE) in its promoter and is upregulated by HSF1 (Cooper et al. 2010). Activation of both NFκB and AP-1 increases with reduction in HSF1. HSF1 blocks the activation and recruitment of SP1 and AP-1 (Chen et al. 2004; Cooper et al. 2010). HSF1 has also been reported to block transcription of IL-1β by binding its transcription factor and to block expression of c*fos* and c*fms* (Xiao et al. 1999; Xie et al. 2002, 2003), which may explain the increased activation of AP-1 with loss of HSF1.

HSF1 activity is required to maintain redox state and attenuate oxidative damage in the heart under normal physiological conditions. Constitutive expression of HSP chaperones requires HSF1 activity. HSF1-dependent function is linked directly to a major antioxidative pathway through effects on the activity of glucose-6-phosphate dehydrogenase (G6PD). HSF1 deficiency reduces cardiac expression of Hsp25, αB-crystallin, and Hsp70, but not Hsp60 and Hsp90. Consistent with the downregulation of Hsp25, for example, a significantly lower glutathione (GSH)/glutathione disulfide (GSSG) ratio was associated with the decreased activity, but not protein content of G6PD. That such HSF1-dependent requirements are directly and functionally linked to maintain redox homeostasis and antioxidative defenses at normal states (Yan et al. 2002).

12.2.4 Heat Shock Paradox

Induction of the heat shock response with increased expression of the heat shock proteins is well established as a protective response (Currie and Karmazyn 1990). However, when an inflammatory stimulus precedes heat shock, there is an unexpected increase in injury, known as the heat shock paradox (Buchman et al. 1993; Wizorek et al. 2004). Adult cardiac myocytes would exhibit the heat shock paradox. HSF1 was a critical element for injury, and HSP60 is increased as part of the heat shock paradox despite the overall protective effects of the expression of heat shock

proteins (HSPs). Using TNF-α as the inflammatory stimulus followed by heat shock causes more apoptotic injury than any of the other treatments, although HS prior to TNF is protective. TNF/HS can cause a marked increase in NFκB activity. Overall, the heat shock paradox reflects a disassembly of the balance between HSPs and NFκB, viability and apoptosis, and health and inflammation (Kobba et al. 2011).

12.3 Ischemic Heart Disease and HSF

Ischemic heart disease (IHD) or myocardial ischemia is one of the leading causes of mortality all over the world. Myocardial ischemia develops when coronary blood supply to myocardium is reduced. HSF1, as a transcription factor that modulates the cytoprotective response, plays a significantly protective role in cardiac ischemic injury, including apoptosis, fibrosis, and angiogenesis. There is a complex interaction among HSF1, heat shock proteins, NFκB, and TNF-α. This interaction may produce fine-tuning of the inflammatory response. AP-1 activation is also modulated by HSF1.

12.3.1 HSF1 and Myocardial Infarction

HSF1, the transcription factor for heat shock proteins, is expressed in hearts, which has been found to play protective roles in ischemia/reperfusion injury and myocardial infarction. Hearts of the transgenic mice overexpressing a constitutively active form of HSF1 or inducible Hsp70 were more resistant to ischemia/reperfusion injury (Marber et al. 1995; Zou et al. 2003), as indicated by faster recovery of ST-segment elevation in ECG, smaller infarct size, and less apoptosis of cardiomyocytes. In the heart with acute myocardial infarction (MI), HSP72 is rapidly expressed to afford tolerance against myocardial injury under ischemic conditions (Hutter et al. 1994). Therefore, an increase in HSP72 after MI is beneficial for reducing the myocardial damage generated during the development of heart failure. HSP72 expression is predominantly regulated by HSF1.

The main therapeutic intervention after myocardial infarction is to reestablish the coronary blood flow supply. However, restoration of flow is accompanied by detrimental manifestation known as reperfusion injury (Verma et al. 2002). Normally, the reperfusion injury is triggered by a large burst of oxidant molecules accompanying with the inflammatory process. Intracellular reactive oxygen species (ROS), which is accumulated in the oxidative stress, induces myocardial damages. However, oxidative stress also initiates a counterregulatory pathway through the activation of cytoprotective mediators. Oxidative injury leads to activation of HSF1, a transcription factor that modulates the cytoprotective response through gene expression of HSPs (Knowlton and Sun 2001), in terms of translocation to the nucleus and accumulation of HSP70 and HSP90 expression in the ischemic-

reperfused heart (Nishizawa et al. 1999; Costa et al. 2009). Specifically, HSP70 is rapidly induced in response to ischemia and directly protects against myocardial damage, improves metabolic recovery, and reduces infarct size in hearts of transgenic mice (Marber et al. 1995; Suzuki et al. 1997; Lepore et al. 2001; Okubo et al. 2001).

HSF1 activation during ischemia may be induced by multiple cellular stress responses. A decrease in the concentration of high-energy phosphate compounds may be sufficient to activate HSF1 (Benjamin et al. 1992). Intracellular acidosis may also serve as an additional stimulus. Alterations in redox state have been documented to activate cardiac HSF1 DNA binding (Paroo et al. 2002) and activate HSF1 acutely during ischemia/reperfusion (Nishizawa et al. 1999). The δ isoform of CaMKII ($Ca2^{+}$/calmodulin-dependent protein kinase II), predominantly expressed in the heart, serves as antiapoptotic effect of cardiomyocyte triggered by oxidant stress, I/R injury, hypoxia, and Ang II stimulation via phosphorylation of HSF1 and subsequent induction of HSP72 (Peng et al. 2010). Granulocyte colony-stimulating factor (G-CSF) can induce cardioprotection against ischemia/reperfusion (I/R) through enhanced transcriptional activity of HSF1 and increase the association of HSF1 with signal transducer and activator of transcription 3 (Stat3), which contributed to the antiapoptotic effects on cardiomyocytes (Ma et al. 2012). In cardiomyocytes, protein kinase C (PKC) isoforms are involved in cardioprotection (Liu et al. 1999; Fryer et al. 2001). PKC-α not only increases the expression of HSP70 but also protects against simulated ischemia/reperfusion. An increase in PKC-α expression results in an increase in HSP70 gene transcription, but this transcriptional activation is HSF1 independent (Coaxum et al. 2007). Peroxisome proliferator-activated receptor-γ ligands, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), exert cardioprotective effects and attenuate myocardial reperfusion injury by enhancing DNA binding of HSF1 and upregulating the expression of the cardioprotective HSP70 (Zingarelli et al. 2007). It appears that a common consequence of stimuli that activate HSF1 is an increase in the concentrations of unfolded proteins within the cell, which may provide a common stimulus for induction of HSP gene expression (Williams and Benjamin 2000).

Negative regulation of HSF1 may be due to several mechanisms during unstressed conditions. In reperfusion injury, poly(ADP-ribose) polymerase (PARP), a chromatin-associated nuclear enzyme that is activated by stranded DNA nicks and breaks in damaged cells (Lautier et al. 1993), is a repressing factor of HSF1 activation and HSP70 expression (Zingarelli et al. 2004). Another negative regulator of HSF1-mediated transcription is glycogen synthase kinase 3β (GSK3β), which also impairs HSF1 DNA binding (Xavier et al. 2000).

Ischemic and reperfusion activate cardiac myocyte apoptosis, which is an important feature in the progression of ischemic heart disease. HSF1 has critical antiapoptotic features not only in tumors but also in heart cells. HSF1 protects cardiomyocyte from apoptosis under oxidative stress via activation of Akt/protein kinase B, downregulation of intracellular ROS generation, and inhibition of inactivation of Jun N-terminal kinase and caspase 3 (Zou et al. 2003). Apoptosis signal-regulating kinase-1 (ASK1) can affect the inhibitory effects of HSF1 on ROS

generation, JNK activity, and cardiomyocyte injury (Zhang et al. 2011). High-mobility group box 1 (HMGB1) is secreted by active inflammatory cells or impaired tissue cells under stress conditions (Rauvala and Rouhiainen 2010). Once released, extracellular HMGB1 participates in cardiac ischemia/reperfusion responses (Andrassy et al. 2008). HSF1 can effectively inhibit H_2O_2-induced cardiomyocyte death via negatively regulating HMGB1 expression at the early stage of oxidative stress and prevention of HMGB1 translocation at the late stage, which was associated with HSP27 and HSP90 upregulated by HSF1 overexpression (Yu et al. 2012). Ischemia/reperfusion (I/R) injury will stimulate extracellular and endogenous tumor necrosis factor-α (TNF-α) level and induce cardiomyocyte damages (Al-Shudiefat et al. 2013). HSF1 attenuated TNF-α-induced cardiomyocyte death via suppression of NFκB pathway, which not only inhibits activation of RelA at the early stage but also prevents its translocation from the cytoplasm to the nucleus afterward (Wu et al. 2013).

An increased oxidative stress caused by HSF1 deficiency has a detrimental effect on critical molecular targets such as adenine nucleotide translocator 1 (ANT1) and defines such functional consequences at the level of mitochondrial permeability transition pore (MPTP) opening in vivo (Yan et al. 2002). Moreover, Mehlen et al. have found that HSP27 protects cells from death through its conserved ability to raise the pool of reduced glutathione (GSH), which decreases the intracellular ROS level, and HSP25 plays a crucial role in the heart for cellular redox homeostasis (Mehlen et al. 1996). Therefore, HSF1 may downregulate ROS by inducing small HSPs such as HSP27 and HSP25.

12.3.2 HSF1 and Cardiac Pathologic Hypertrophy

Cardiac hypertrophy is one of the key causes of heart failure. The Framingham Heart Study revealed that cardiac hypertrophy is an independent risk factor for heart failure, arrhythmia, myocardial infarction, and sudden death (Levy et al. 1990; Haider et al. 1998). Cardiac hypertrophy is an adaptive response to increased wall stress. At the beginning, cardiac hypertrophy has beneficial effects to maintain cardiac output by reducing wall stress, which is called "adaptive hypertrophy" (Ruwhof and van der Laarse 2000); however, long-term stresses induce systolic dysfunction, leading to "maladaptive hypertrophy" or "pathologic hypertrophy" in the chronic phase, resulting in heart failure (Katz 1990). On the other hand, regular exercise can also induce cardiac hypertrophy without causing systolic or diastolic dysfunction, which is called "physiologic hypertrophy" (Pluim et al. 2000). Maladaptive hypertrophy is actually a result of imbalance between myocardial blood supply and oxygen demand, accompanying with cardiac fibrosis and cellular apoptosis.

HSF1 was first identified as a critical transcription factor that regulates cardiac hypertrophy in 2006. In the high-throughput DNA chip analysis, this study found *HSP* genes (*Hsp70* and *Hsp27*) were markedly upregulated in physiologic cardiac

hypertrophy. Accordingly, HSF1, which regulates HSP gene expression, is activated only in the heart of physiologic hypertrophy but not in chronic pressure overload-induced cardiac hypertrophy (Sakamoto et al. 2006). Constitutive activation of HSF1 in the heart (Nakai et al. 2000) significantly ameliorated death of cardiomyocytes and cardiac fibrosis and thereby prevented cardiac dysfunction as well as hypertrophy induced by chronic pressure overload (which is thought to induce pathologic cardiac hypertrophy). Conversely, when heterozygous HSF1$^{\pm}$ mice (Inouye et al. 2004) were forced to exercise (which is thought to induce physiologic cardiac hypertrophy), significant systolic dysfunction occurred. Likewise, cardiac function was significantly impaired from the early phase of pressure overload, when HSF1 activation was inhibited. Therefore, HSF1 is a key molecule for preservation of systolic function during the development of cardiac hypertrophy under both pathologic and physiologic conditions, which plays a critical role in the transition between adaptive and maladaptive hypertrophy.

Accumulation and aggregation of unfolded proteins are associated with an increase of protein synthesis in hypertrophied hearts and induce cardiomyocyte death that eventually leads to systolic dysfunction (Okada et al. 2005). Thus, the protective effects of HSF1 may be attributable to the functions of HSPs in protein folding and degradation (Toko et al. 2008). In addition to such well-known functions, accumulating evidence indicates that different HSPs directly act on the cell death machinery and inhibit the signaling pathway for cell death at various points (Sreedhar and Csermely 2004). For example, Hsp27 binds to cytochrome c and prevents it from binding to Apaf-1 (Bruey et al. 2000), whereas Hsp70 prevents Apaf-1 from recruiting procaspase-9 (Beere et al. 2000), thereby inhibiting apoptotic cell death. It is conceivable that sustained activation of HSF1 prevents the onset of cardiac dysfunction in hypertrophic hearts through the mechanisms involving a direct action of HSPs on the cell death machinery as well as their functions in protein degradation (Toko et al. 2008).

The production of autocrine/paracrine factors such as angiotensin II and endothelin 1 is increased by pathologic stimuli and plays a critical role in inducing pathologic cardiac hypertrophy. These factors bind to G protein-coupled receptors, leading to dissociation of the Gαq subunit and activation of downstream signaling molecules, which include negative regulators of HSF1 such as ERK and JNK. Accordingly, this signaling pathway may induce pathologic cardiac hypertrophy partly via the inactivation of HSF1 (Toko et al. 2008), although there is a conflicting report that angiotensin II does not influence the activity of HSF1 (Nishizawa et al. 2002).

For cardiac angiogenesis crucially involved in the adaptive mechanism of cardiac hypertrophy, HSF1 promotes cardiac angiogenesis through suppression of p53 and subsequent upregulation of HIF-1 in endothelial cells during chronic pressure overload, leading to the maintenance of cardiac adaptation (Zou et al. 2011).

12.3.3 HSF1 and Atherosclerosis

Atherosclerosis is an inflammatory- and immune-mediated disease, in which HSPs were repeatedly implicated to be crucial (Xu 2002). Several families of HSPs have been demonstrated to be expressed at high levels in atherosclerotic lesions (Berberian et al. 1990; Kleindienst et al. 1993; Rocnik et al. 2000; Kanwar et al. 2001). These highly expressed HSPs in lesions could promote disease progression via evoking proinflammatory and autoimmune responses (Xu 2002). Accordingly, there is an increased HSF1 activity in atherosclerotic lesions in vivo. In atherosclerotic lesions, HSF1 but not HSF2 is mainly localized in the nuclei, and the molecular weight of HSF1 from lesional extracts was larger than that of normal vessels, indicating that HSF1 in lesions was phosphorylated and activated (Metzler et al. 2003). Usually, HSF1 proteins in cultured cells do not markedly change in response to stress, e.g., heat shock or mechanical stress. However, protein levels of HSF1 in atherosclerotic lesions were much higher compared to normal vessels, suggesting different mechanisms of HSF1 activation and regulation of HSP expression in vivo from in vitro cultured cells (Metzler et al. 2003). Therefore, in vivo microenvironment of the vessel wall is of importance in regulation of HSF1 production and activation.

It is believed that Low-Density Lipoprotein (LDL) and oxidized LDL are important in the development of atherosclerosis. Oxidized LDL possesses several proatherogenic properties, including interactions with several receptors, leading to the engorgement of cells with lipids, inhibition of endothelium-dependent vascular relaxation, cytotoxicity to proliferating cells, and stimulation of chemoattractant secretion (Witztum and Steinberg 2001). In vitro, Triglyceride-Rich Lipoprotein (TRLP), oxidized TRLP, LDL, and oxidized LDL do not activate HSF1. However, oxidized LDL and oxidized TRLP may exert their role in HSF1 activation in vivo via stimulating cells producing a panel of cytokines (Niemann-Jonsson et al. 2000). TNF-α, which is present in high concentrations in atherosclerotic lesions (Hansson et al. 2002), can activate HSF1 in smooth muscle cells (SMCs), supporting the role of cytokines in HSF1 activation in atherosclerotic lesions.

Mechanical stretch is a crucial factor in the pathogenesis of atherosclerosis. Using an in vitro mechanical stress model, it has been found that we provide mechanical forces that can evoke rapid activation of HSF1 in SMCs, followed by elevated HSP70 protein levels (Metzler et al. 2003). Interestingly, SMC lines stably expressing dominant negative Rac (Rac N17) abolished HSP protein production and HSF1 activation induced by mechanical forces, whereas a significant reduction of HSF1 activities was seen in Ras N17-transfected cell lines (Xu et al. 2000). Therefore, mechanical stretch-induced HSF1 activation was regulated by Rac/Ras GTP-binding proteins (Xu et al. 2000) that may be primarily responsible for HSF1 activation seen in atherosclerotic lesions.

In summary, risk factors for atherosclerosis, such as biomechanical stress and cytokines induced by hypercholesterolemia, directly stimulate cells of the arterial wall to express high levels of HSF1, which leads to increased expression of HSPs.

Pathologically, overstimulation by the risk factors results in cell death, which releases intracellular HSPs into intercellular spaces to form soluble HSPs that lead to proinflammatory and autoimmune responses (Metzler et al. 2003).

12.4 Heart Failure and HSF

Heart failure is the final common endpoint of various heart diseases, leading to cardiovascular death. Heart failure is also considered as a chronic state of inflammation and stress on the heart tissue. It is generally accepted that heat shock (HS) provides myocardial protection of cardiac mechanical function and metabolism via the enhanced HSF1 activation and synthesis of several HSPs (Yellon et al. 1992).

In the failing hearts, both HSF1 and HSF2, the transcription factors controlling HSP expression, are increased. But nuclear translocation of the HSF1 was markedly reduced in the viable myocardium upon the pathological stresses. HSF1 in the cytosolic fraction and the HSP90 chaperone complex containing HSF1, a repressor of HSF1, were increased, whereas that of HSF1 in the nuclear fraction was reduced. So an increase in the multichaperone complex, especially the HSF1-HSP90 interaction, is associated with attenuation of HSF1 translocation into the nucleus (Marunouchi et al. 2013a). On the other hand, reduced levels of phosphorylated GSK3β (inactive) lead to an increase in the amount of the active GSK3β enzyme for the phosphorylation of HSF1 Ser303, and the activation of HSF1 can be impaired via the increased phosphorylation of the Ser303. Accordingly, cardiac protective HSP72 induction is impaired in the failing heart (Marunouchi et al. 2013b).

HSP72 is a ubiquitous protective protein that is well established as cardioprotective in heart failure. Although HSF1 is increased in the failing heart, HSF72 is not increased accordingly, which shows the activation of HSF1 is suppressed. Because there is no increased phosphorylation of serine 230 or serine 303/307 in HSF1, which is thought to regulate its activity, and electrophoretic mobility shift assay (EMSA) showed no increase in HSF-binding activity with heart failure (Wang et al. 2010). In contrast, HSP60 levels are upregulated in the failing heart. HSP60 on the surface of cardiac myocytes from failing hearts was associated with apoptosis, which may be deleterious (Lin et al. 2007). The increase in HSP60 expression in heart failure is potentially driven by NFκB activation not by activation of HSF1 from the inflammatory state of heart failure (Wang et al. 2010).

There are several different phosphorylation sites for HSF1 activation in response stress. There was no difference in phosphorylation of serine 303/307 between the control and congestive heart failure (CHF). Phosphorylation at serine 230 can increase activation of HSF1, but in failing hearts, there is no significant change in phosphorylation at this site (Holmberg et al. 2001). Other phosphorylation sites are likely involved in the regulation of HSF1 activation, which remains verification (Guettouche et al. 2005).

It is conceivable that sustained activation of HSF1 prevents the onset of cardiac dysfunction in hypertrophic hearts through the mechanisms involving a direct action of HSPs on the cell death machinery as well as their functions in protein degradation.

12.5 Future Perspectives

The past several years have seen a lot of studies of the protective role of HSF in cardiovascular disease during the response to various stressors. These studies could lead to a new strategy for prevention and treatment of cardiovascular diseases such as hypertension, ischemic heart disease, and atherosclerosis. However, the mechanisms underlying the activation of HSF, including its regulation by phosphorylation, sumoylation, and acetylation, remain fully understood. Because there have been many reports that induction of HSF1 and HSPs has a beneficial effect in animal models of cardiovascular disease, activation of HSF1 and HSPs could be a novel therapeutic strategy for various cardiovascular diseases. In the failing heart, the induction of HSP72 was decreased in spite of an increase in HSF1 because of the HSF1-HSP90 interaction associated with attenuation of HSF1 translocation into the nucleus. This suggests the complex regulatory roles of HSF1 and other HSPs in the protection of the heart upon the stress. However, the signal transduction pathways leading to full activation of the various *HSP* genes in the human heart and blood vessels are still incompletely understood. Knowledge of these pathways could lead to the development of well-directed synthetic drugs activating *HSP* genes for patients with cardiovascular disease and provide novel therapeutic strategies.

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Chapter 13
HSF Supports Cancer

Makoto Chuma

Abstract Heat-shock factors (HSFs) are a family of transcription factors whose name is derived from the discovery of their activation following heat shock. Of these, HSF1 is the most well studied and is the master transcriptional regulator of cellular responses to not only heat but also a wide variety of other stressors. During the progression of many types of cancer, HSF1 becomes co-opted by mechanisms that are evidently triggered in the emerging tumor cell. Concerted activation of HSF1 participates in many of the traits (initiation, promotion, proliferation, invasion, metabolism, anti-apoptosis, and inhibition of replicative senescence) that permit the malignant phenotype. Indeed, overexpression of HSF1 has been found in many organ tumor tissues and is associated with poor prognosis. In this chapter we describe (1) the biology of HSF1-associated cancers; (2) the role of HSF1 in cancer initiation, promotion, and progression; (3) cancer-related pathways regulated by HSF1; and (4) HSF1 and its targets as prognostic factors for cancer patients.

Keywords Cancer • Proteostasis • Oncogene • Senescence • Proliferation • Invasion • Metastasis • Metabolism • Prognosis

13.1 Introduction

The mammalian heat-shock factor (HSF) family consists of four members (HSF1, HSF2, HSF3, and HSF4), with HSF1 being the main regulator of the heat-shock response, which is involved in protecting cells and organisms from heat, ischemia, inflammation, oxidative stress, and other noxious conditions (Pirkkala et al. 2001; Sorger 1991). Under various forms of physiological stress, HSF1 drives the production of heat-shock proteins (HSPs) such as HSP27, HSP70, and HSP90, which act as protein chaperones (Guertin and Lis 2010). The function of HSF1 is not limited to increasing the expression of chaperones; HSF1 also modulates the expression of hundreds of non-chaperone genes that are critical for survival under

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an array of potentially lethal stressors (Mendillo et al. 2012; Page et al. 2006). As a result, HSF1 influences fundamental cellular processes such as cell-cycle control, protein translation, glucose metabolism, and proliferation (Dai et al. 2007; Hayashida et al. 2006; Jacobs and Marnett 2007; Vydra et al. 2006). In human tumors, constitutive expression of HSP27, HSP70, and HSP90 at high levels predicts poor prognosis and resistance to therapy (Neckers and Workman 2012; Khalil et al. 2011). These effects are often attributable to HSF1-dependent mechanisms (Cai and Zhu 2003). Thus, as a master regulator of cellular processes, the roles of HSF1 in carcinogenesis and tumor progression are now emerging. Several recent investigations using mouse models have suggested that HSF1 is involved in carcinogenesis (Dai et al. 2007; Min et al. 2007). In clinical samples, HSF1 is often constitutively expressed at high levels in a variety of tumors. In this chapter we describe (1) the biology of HSF1-associated cancers; (2) the role of HSF1 in cancer initiation, promotion, and progression; (3) cancer-related pathways regulated by HSF1; and (4) HSF1 and its targets as prognostic factors of cancer patients.

13.2 Biology of HSF1-Associated Cancers

13.2.1 Cancer Cells Are Generally Exposed to Stressful Environments That Promote HSF1 Activity

In humans, tumorigenesis is a multistep process that includes genome instabilities (mutation and chromosomal deletion) and epigenetic changes (abnormal histone acetylation and DNA methylation). During tumorigenesis, mutated nonnative proteins are synthesized and deregulated and abnormal signal transduction activity is observed. Moreover, abnormal conditions observed within the tumor microenvironment include hypoxia, acidity, lack of nutrients, ATP depletion, and low glucose levels. Therefore, cancer cells are generally exposed to more stresses compared to normal cells (Hanahan and Weinberg 2011), and these stresses promote HSF1 activity.

HSF1 is the most well-studied HSF and has been found at elevated levels in tumors with high metastatic potential and is associated with poor prognosis. Nevertheless, HSF1 operates as a major multifaceted enhancer of tumorigenesis through the induction of classical heat-shock genes, as well as “nonclassical” targets (Fujimoto and Nakai 2010). In this chapter, we focus on describing the different physiological roles of HSF1 in tumorigenesis.

13.2.2 Oncogene Versus Non-oncogene Addiction

Cancer cells harbor vast numbers of genetic and epigenetic alterations, whereas a select few arise nonrandomly and drive the cancer phenotype. Among this category are activating mutations in oncogenes. Because many cancers require increased activity of these oncogenes for tumor initiation and maintenance, this dependence has been coined "oncogene addiction" (Weinstein 2002).

Despite the focus on oncogenes as targets of cancer therapeutics, there are solid genetic arguments, based on experimental evidence, for a larger class of non-oncogene drug targets. Many, if not all, tumor-promoting proteins can be rate limiting to tumor-promoting pathways and represent potential drug targets. Solimini (Solimini et al. 2007) termed this phenomenon "non-oncogene addiction" in reference to the increased dependence of cancer cells on the normal cellular functions of certain genes, which themselves are not classical oncogenes.

Cancer cells are known to exhibit high levels of reactive oxygen species, spontaneous DNA damage, and aneuploidy, each of which represents a form of cellular stress. These cellular stresses might burden the chaperone and proteasome pathways, thus compromising folding of essential cellular proteins and leading to the elevated levels of heat-shock proteins observed in many tumors (Whitesell and Lindquist 2005). Thus, cancer cells are highly dependent upon these general stress responses, which exemplify non-oncogene addiction.

13.2.3 HSF1 Is Implicated in the Hallmarks of Cancer

It has been suggested that there are key traits, described as "the hallmarks of cancer," required for the emergence of a complete malignant phenotype (Hanahan and Weinberg 2011). HSF1 has been implicated in causing these hallmarks (Ciocca et al. 2013), specifically we describe five key traits: (1) maintenance of sustained proliferative signaling, (2) inhibition of replicative senescence, (3) activation of invasion and metastasis, (4) reprogramming of energy metabolism, and (5) resisting cell death (anti-apoptosis). In this chapter, we first describe the role of HSF1 in cancer initiation/promotion and subsequently describe the cancer-related pathways (pertaining to the five key traits) regulated by HSF1.

13.3 HSF Supports Cancer Initiation, Promotion, and Progression

13.3.1 HSF1 Plays a Role in Enabling the Initiation and Maintenance of Cancer

HSF1 by itself does not act as a classical oncogene or tumor suppressor. Neither induced overexpression nor knockout directly drives transformation. However, recent studies have reported that HSF1 supports the malignant phenotype by initiating and promoting oncogenic signal transduction pathways, proliferation, survival, protein synthesis, and glucose metabolism (Dai et al. 2012a; Whitesell and Lindquist 2009; Vydra et al. 2014). HSF1 is dispensable for viability and growth of non-transformed human cells, as well as in whole animals (Dai et al. 2007; Hunt et al. 2004; Meng et al. 2011). The specific dependence of cancer cells on HSF1 has been termed "non-oncogene addiction" (Solimini et al. 2007). HSF1 has itself been implicated in the promotion of tumorigenesis. Dai et al. (2007) found that HSF1 plays a major role in enabling the initiation and maintenance of cancer in several mouse tumor models. Knockdown of HSF1 inhibited cell viability in several cancer cell lines, whereas it had no effect on normal human fibroblasts. They concluded that HSF1 function helps maintain the growth and survival of human cancer cells.

HSF1 is also required for epidermal growth factor receptor-2 (HER2)-induced transformation of MCF-10A cells (Meng et al. 2010) and cooperates with HER2 to promote mammary tumorigenesis (Xi et al. 2012). In addition, HSF1 promotes lymphomas in p53-deficient mice (Min et al. 2007). These findings suggest that HSF1 plays a role in enabling the initiation and maintenance of cancer.

13.3.2 HSF1 Affects Oncogene-Induced Senescence

Cellular senescence was originally described as a limit to the number of divisions that a normal cell can undergo. Senescence can be triggered by telomere shortening or stressful treatments and is associated with activation of p53 and accumulation of the cell-cycle inhibitors p21 and/or p16 (Ben-Porath and Weinberg 2005). Although tumor cells are immortal and divide indefinitely, they often retain functional senescence programs and can become senescent in response to various DNA-damaging drugs and radiation (Chang et al. 2002), which is especially relevant to solid tumors of epithelial origin. Importantly, cell senescence can be triggered upon activation of oncogenes, e.g., Ras or Raf (Bihani et al. 2004; Lowe et al. 2004), which has been observed in various systems. Therefore, a novel concept has emerged whereby oncogene-induced senescence (OIS) represents the major block on the path toward neoplastic transformation, and cells in emerging tumors must acquire adaptations/mutations that allow the bypass of senescence

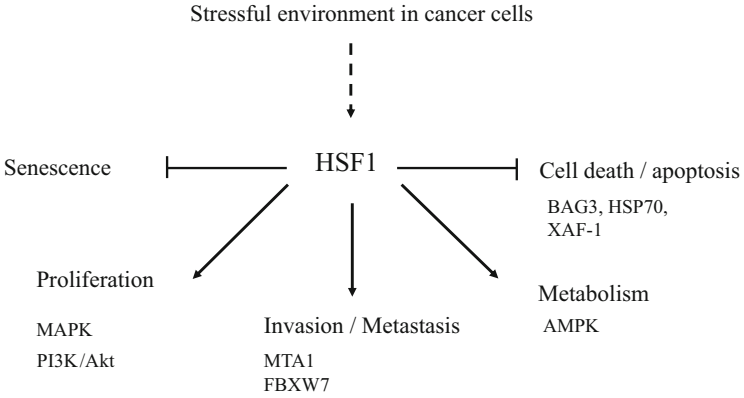

Fig. 13.1 Schematic model of the role of HSF1 in cancer. Stressful environments in cancer cells promote HSF1 activity/overexpression. The HSF1 transcriptome regulates multiple traits associated with malignant transformation, tumorigenesis (proliferation, inhibition of replicative senescence, invasion and metastasis, energy metabolism, and resistance to cell death), and related molecule pathways. *BAG3* Bcl-2-associated athanogene domain 3, *HSP70* heat-shock protein 70, *XAF-1* XIAP-associated factor 1, *ERK* extracellular signal-regulated kinase, *MAPK* mitogen-activated protein kinase, *PI3K* phosphatidylinositol 3-kinase, *AMPK* AMP-activated protein kinase, *MTA1* metastasis-associated factor 1, *FBXW7* F-box/WD40 repeat-containing protein 7

programs (Braig and Schmitt 2006). Gabai et al. (2009) have found that HSF1 plays an important role in the evasion of OIS (Gabai et al. 2009; Meng et al. 2010, 2011), which is critical in the early stages of neoplastic transformation. HSF1 is involved in prevention of the OIS caused by HER2, phosphoinositide 3-kinase catalytic subunit (PIK3CA), and Ras oncogenes (Fig. 13.1).

13.3.3 Progression

HSF1 supports cancer progression by promoting proliferation, invasion, and metastasis. These processes are described as “cancer-related pathways” in the next chapter.

13.4 Cancer-Related Pathways Regulated by HSF1

13.4.1 HSF1-Bound Genes Are Involved in Many Facets of Tumorigenesis

HSF1 binds genes that are involved in regulating the cell cycle, apoptosis, energy metabolism, and other processes. Mendillo et al. (2012) used chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-Seq) to identify genes bound by HSF1. These authors defined a distinct genome-wide transcriptional program coordinated by HSF1 in malignancy. They established an "HSF1-cancer signature" of 456 genes that were bound by HSF1 near their transcription start sites and found that this HSF1 cancer program is active in several types of tumors and is strongly associated with metastasis and mortality (Mendillo et al. 2012).

13.4.2 Signaling Pathways of Cancer Alter HSF1 Activity

Dysregulation of signaling pathways in cancer could also drive posttranslational modifications to HSF1. Some of these will likely be shared with heat-shocked cells, while others will likely be unique to cancer. Indeed, it seems extremely likely that different mechanisms of activation will operate in different cancers. Several pathways activated in cancer have been reported to alter HSF1 activity, including the epidermal growth factor receptor (EGFR)/HER2 axis (Zhao et al. 2009), the RAS/mitogen-activated protein kinase (MAPK) (Stanhill et al. 2006), and the insulin growth factor system (Chiang et al. 2012). Additional modes of cancer-specific regulation might include epigenetic states common to cancer and proliferating cells and transcriptional coregulators.

13.4.3 Maintenance of Sustained Proliferative Signaling

HSF1 supports cancer cell proliferation via many signaling pathways. The MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways govern fundamental physiological processes, such as proliferation and survival (Baines and Molkentin 2005; Mullonkal and Toledo-Pereyra 2007). The extracellular signal-regulated kinase (ERK)/MAPK and PI3K/Akt signaling pathways are frequently constitutively activated in many human cancers and are associated with carcinogenesis (Rho et al. 2011). Growth and survival of many cancer cells critically depend on aberrant signaling by the ERK/MAPK and PI3K/Akt pathways, which are also involved in intensive cross talk (Aksamitiene et al. 2012). Several investigations have reported on the role of HSF1 in supporting MAPK signaling (Dai

et al. 2012b; O'Callaghan-Sunol and Sherman 2006; Chuma et al. 2014). Yang demonstrated that HSF1 promotes breast cancer cell proliferation by regulating the ERK/MAPK and PI3K/Akt signaling pathway (Yang et al. 2014). Tang showed MEK phosphorylate HSF1 (Ser326) and MEK guard proteome stability and inhibit tumor-suppressive amyloidogenesis via HSF1 (Tang et al. 2015) (Fig. 13.1).

One of the mechanisms by which Ras proteins influence cell growth is through the regulation of intracellular levels of reactive oxygen species (ROS) molecules that affect a variety of cellular processes including proliferation. Recently it was shown that repression of the sestrin 3 (SESN3) gene can play a critical role in Ras-induced ROS upregulation. Zamkova et al. (2013) found that Ras-induced repression of SESN3 expression and ROS upregulation is mediated via alterations in HSF1 transcriptional activity, thereby affecting proliferation.

13.4.4 Activation of Invasion and Metastasis Pathways

Cancer metastasis is a multistep process that involves cell detachment from the primary tumor, entry into the vascular or lymphatic system, dispersal through the circulation, and proliferation after extravasation in target organs (Poste and Fidler 1980). Many studies have examined the importance and mechanisms of invasion, one of the first steps in metastasis. Loss of cell–cell contact caused by self-regulation of E-cadherin (Hirohashi 1998) and increased cell motility or migration (Genda et al. 1999; Takamura et al. 2001) is a critical step in this process. There is a growing evidence that actin polymerization and the dynamic reorganization of the actin cytoskeleton play important roles in the regulation of cell migration (Chen et al. 2009). During the invasion process, tumor cells become detached from the substratum and each other and dispersed through the circulation where they are liberated from the signals associated with cell growth and cell survival generated by cell–substratum interaction (Meredith et al. 1993; Frisch and Francis 1994; Frisch and Ruoslahti 1997). Some cancer cells can survive without these signals, a process which is termed anchorage-independent growth (AIG).

O'Callaghan-Sunol and Sherman (2006) showed that immortalized mouse embryonic fibroblast cells (MEF) derived from HSF1−/− mice were deficient in both basal and EGF-induced migration. Fang et al. (2012) demonstrated the effects of HSF1 on hepatocellular carcinoma (HCC) cell migration and invasion using in vitro migration and invasion assays. Furthermore, induced expression of HSF1 can stimulate the reorganization of actin, leading to the formation of stress fiber-like structures in HCC cells, which can be ablated with HSF1 knockdown. These findings indicate that HSF1 promotes HCC cell migration and invasion, possibly by regulating actin reorganization.

Expression of active HSF1 promotes AIG in vitro, as well as metastases in animal models. Furthermore, active HSF1 was found to enhance cell motility, reduce the adherence of cells to a fibronectin-coated surface or a wound healing assay (Nakamura et al. 2014), and affect the actin cytoskeleton. Concerning the

molecular mechanism of HSF1 in cancer invasion, Toma-Jonik et al. (2015) showed that HSF1-induced downregulation of the focal adhesion protein vinculin increased motility and reduced cellular adherence. Khaleque et al. (2008) showed that HSF1 could be involved in promoting cancer cell invasion and metastasis through its ability to associate with metastasis-associated factor 1 (MTA1) in mammary carcinoma cells activated by the transforming cytokine heregulin. MTA1 is a gene corepressor and may function to decrease expression of genes that inhibit invasion and metastasis (Mazumdar et al. 2001) (Fig. 13.1).

Protein stability controlled by the ubiquitin proteasome pathway is emerging in human cancer. FBXW7 (F-box/WD40 repeat-containing protein 7), a substrate-targeting subunit of the SCF (Skp1–Cul1–F-box) ubiquitin ligase complex (Skaar et al. 2013), targets several key regulators of proliferation, growth, and apoptosis for proteasomal degradation (Davis et al. 2014; Inuzuka et al. 2011). FBXW7 is mutated in a significant portion of diverse human cancers (Akhoondi et al. 2007). Kourtis et al. show that FBXW7 controls the stability of nuclear HSF1 and modulates the attenuation phase of the heat-shock response (Kourtis et al. 2015). Moreover, FBXW7 deficiency enhances the metastaticability of melanoma through HSF1 stabilization and alteration of the HSF1 malignant transcriptional program. Together, they concluded that a tumor suppressor, FBXW7, regulates heat-shock response and cancer cell stress response and metastatic potential through modification of HSF1.

These results suggest that HSF1 may be involved in the regulation of cancer metastasis.

13.4.5 HSF1 Regulates Anabolic Metabolism

Cancer cells undergo profound changes in metabolism involving a switch from oxidative phosphorylation to glycolysis, as first observed in the classical studies of Warburg (1956). This leads to elevated glucose consumption and an increased rate of metabolism in malignant cells (Gullino 1966). A role for HSF1 in this metabolic switch was suggested by the findings that glucose uptake in cancer cells is decreased in HSF1 knockout mice and that HSF1 is required for the expression of the glycolytic intermediate lactate dehydrogenase in cells expressing the oncogene HER2 (Dai et al. 2007; Zhao et al. 2009). HSF1 activation stimulates hepatocyte lipid biosynthesis and perpetuates chronic hepatic metabolic disease induced by carcinogens through the promotion of premalignant cell growth in a mouse model (Jin et al. 2011). Furthermore, inactivation of HSF1 impairs cancer progression, mitigating adverse effects of carcinogens on hepatic metabolism by enhancing insulin sensitivity and sensitizing activation of AMP-activated protein kinase (AMPK), an important regulator of energy homeostasis and inhibitor of lipid synthesis (Fig. 13.1). These studies imply that HSF1 has a central role in HCC development, as it modulates proteostasis and metabolic pathways by regulating access to two critical elements, glucose and lipids.

13.4.6 HSF1 Plays a Role in Apoptosis Pathways

HSF1 plays a role in regulating apoptosis in several cancer cell lines, including multiple myeloma (Heimberger et al. 2013), gastrointestinal cancer (Wang et al. 2006), colorectal cancer (Wang et al. 2006; Kim et al. 2013; Benderska et al. 2014), HCC (Chuma et al. 2014), and pancreatobiliary cancer cells (Dudeja et al. 2011). Silencing HSF1 is thought to reduce anti-apoptotic proteins and/or induce apoptosis-related protein. Dudeja et al. (2011) showed that downregulation of HSF1 expression in pancreatobiliary tumors induces annexin V, TdT-mediated dUTP nick end labeling (TUNEL) positivity, and caspase-3 activation, which is indicative of activation of a caspase-dependent apoptotic pathway.

HSF1 induces HSP70 (heat-shock protein 70) and BAG3 (Bcl-2-associated athanogene domain 3) protein. HSP70 and its co-chaperone BAG3 have been reported to protect cells from apoptosis by stabilizing anti-apoptotic Bcl-2 family proteins. Silencing of HSF1 suppresses HSP70 and BAG3, as well as significantly decreasing the amounts of BCL-2 and BCL-xL protein, thereby inducing apoptotic cell death (Jacobs and Marnett 2009; Kim et al. 2013; Chuma et al. 2014). Another report found that HSF1-mediated BAG3 expression attenuates apoptosis in 4-hydroxynonenal-treated colon cancer cells via stabilization of anti-apoptotic Bcl-2 proteins (Jacobs and Marnett 2009) (Fig. 13.1).

XAF1 (XIAP-associated factor 1) is an inhibitor of apoptosis interacting proteins that antagonizes the cytoprotective role of XIAP. The expression of XAF1 and HSF1 was negatively correlated in gastrointestinal cancer cell lines. Upregulation of HSF1 suppresses XAF1 expression, implicating the synergistic effects of HSP and inhibitors of apoptosis in cytoprotection under conditions of cellular stress (Wang et al. 2006).

Endogenous stress in cancer cells sustains the elevated expression levels of HSF1 and subsequently suppresses apoptosis-related proteins, implicating the synergistic effects of anti-apoptotic proteins.

On the other hand, there are reports that activation or overexpression of HSF1 leads to enhanced induction of apoptosis. Benderska et al. (2014) reported that transient overexpression of HSF1 protein accelerates apoptosis. Death-associated protein kinase (DAPK) is a serine-threonine kinase that functions in tumor suppression. The DAPK-HSF1 interaction is a positive feedback mechanism that stimulates tumor necrosis factor (TNF)-induced apoptosis in colorectal cancer cells. They showed that transient overexpression of HSF1 protein led to an increase in DAPK mRNA levels and consequently to increased apoptosis. Further studies are needed to clarify the involvement of HSF1 in apoptosis.

13.5 HSF and Its Targets as Prognostic Factors in Cancer Patients

The role of altered HSF1 expression in various cancers appears to be tissue specific (Table 13.1).

HSF1 is elevated in a number of cells and tissues from a variety of cancers, including breast carcinoma (Santagata et al. 2011; Mendillo et al. 2012; Gabai et al. 2012), endometrial carcinoma (Engerud et al. 2014), pancreatic cancer (Dudeja et al. 2011), prostate carcinoma (Hoang et al. 2000), oral squamous cell carcinoma (OSCC) (Ishiwata et al. 2012), and HCC (Fang et al. 2012; Zhang et al. 2013; Chuma et al. 2014; Li et al. 2014). Studies of breast carcinoma, endometrial carcinoma, and HCC have shown the upregulation of HSF1 in cancers and its association with increased aggressiveness and lower survival rates. High expression of HSF1 mRNA in human breast cancer was correlated with Elston grade, metastasis, and poor prognosis (Gabai et al. 2012). HSF1 protein expression has been investigated in large samples. Santagata et al. (2011) showed that HSF1 expression in breast cancer samples (1,841 participants) was associated with high histologic grade, larger tumor size, and nodal involvement, and that increased levels of HSF1 are closely correlated with poor prognosis in estrogen receptor (ER)-positive breast carcinomas. On the other hand, at the time of diagnosis, the majority of breast cancer patients have ER-positive tumors and early-stage disease (ER-positive/lymph node-negative tumors). These patients will experience a recurrence and might benefit from more aggressive treatment, but it is currently very

Table 13.1 HSF1 expression in human cancers

Cancer type	Authors	Clinicopathological finding	Methods
Breast cancer	Santagata[a]	Clinical stage, differentiation	IHC
		Tumor size, lymph node positive	
		ER negative, HER2 positive	
	Mendillo[a]	ER positive/lymph node negative	IHC
	Gabai[a]	Elston grade	RT-PCR
Hepatocellular carcinoma	Fang[a]	No. of tumor nodules, venous invasion	IHC
		Edmondson grade, capsular formation	
	Zhang[a]	No correlation	IHC
	Chuma[a]	Differentiation, no. of tumor nodules	IHC
		Tumor size, TNM stage	
	Li[b]	Differentiation TNM stage	
Endometrial carcinoma	Engerud[a]	Histologic type, grade	IHC
		Metastatic node, ploidy	
Oral squamous cell carcinoma	Ishiwata[c]	Histopathologic type, tumor size	IHC

IHC immunohistochemistry, *RT-PCR* reverse transcription polymerase chain reaction, *ER* estrogen receptor, *HER2* epidermal growth factor receptor-2

HSF1 expression is associated with poor prognosis[a] or not associated with prognosis[b]

[c]Correlation between HSF1 expression and survival was not described

difficult to identify them in advance. Mendillo et al. (2012) found that HSF1 expression was significantly associated with metastatic recurrence in women initially diagnosed with ER-positive/lymph node-negative tumors and showed association with high HSF1 protein expression and poor prognosis. In endometrial carcinoma, high expression of HSF1 protein is significantly associated with aggressive disease (histological type, high-grade metastatic nodes, and aneuploidy) and poor survival (Engerud et al. 2014). In OSCC, higher nuclear HSF1 expression was closely related to tumor size and histopathologic types (Ishiwata et al. 2012). However, the correlation between HSF1 expression and prognosis in OSCC was not described in this study. HSF1 expression in HCC has been associated with aggressive disease, tumor number, tumor size, poor differentiation, venous invasion, Edmondson grade, etc. Four studies demonstrated that high expression of HSF1 protein is significantly associated with poor survival (Fang et al. 2012; Zhang et al. 2013; Chuma et al. 2014; Li et al. 2014). These reports indicated that HSF1 is a prognostic factor for cancer patients, and targeting HSF1 represents a potentially attractive treatment strategy for several cancers.

13.6 Future Perspectives

HSF1 thus acts as multifaceted enhancer of tumorigenesis by regulating diverse functions that include initiation, promotion, proliferation, invasion, metabolism, apoptosis, and replicative senescence. Furthermore, overexpression of HSF1 in clinical samples is associated with aggressive tumor characteristics and poor prognosis. Therefore, HSF1 is an attractive potential target for cancer therapy. However, several factors must be considered when targeting HSF1 for therapeutics. Firstly, inhibition of HSF1 might, in parallel, accelerate neurodegenerative processes and aging. Indeed, HSF1 displays a protective effect against neurodegenerative diseases, partly through the induction of chaperones, such as HSP27, that inhibit protein aggregation (Lu et al. 2002). Therefore, in the development of new drugs that target HSF1, it would appear important that such compounds are not capable of crossing the blood-brain barrier, so that neurodegenerative risks are minimized. Secondly, it remains to be convincingly demonstrated whether HSF1 is a useful molecular marker for predicting which cancer types and patients in particular will most benefit from an individualized anticancer strategy. Thirdly, the challenge is to design the most appropriate approach to pharmacologically perturbing HSF1 in order to promote cancer cell death while minimizing adverse effects on normal cells. Future studies are needed to understand the mechanisms whereby the HSF1 transcriptional system is activated in cancer, which may permit rational approaches to therapies based on this target.

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Chapter 14
HSF1 Activation by Small Chemical Compounds for the Treatment of Neurodegenerative Diseases

Nobuhiro Fujikake, Toshihide Takeuchi, and Yoshitaka Nagai

Abstract Heat shock transcription factor 1 (HSF1) is a transcription factor that is activated upon the exposure of cells to various types of proteotoxic stress, such as heat shock stress and oxidative stress, which induces the expression of various molecular chaperones. HSF1-induced molecular chaperones, including heat shock protein 40 (Hsp40) and Hsp70, suppress protein misfolding through binding to structurally unstable proteins and thereby protect cells from proteotoxic stress. Therefore, activation of HSF1 is considered as a therapeutic approach against a group of neurodegenerative diseases that are caused by protein misfolding, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and the polyglutamine diseases. In fact, many compounds that activate HSF1 have been tested for their potential as therapeutic agents against neurodegenerative diseases. In this chapter, we introduce various HSF1-activating compounds, their mechanisms of activation of HSF1, and their therapeutic effects against neurodegenerative diseases.

Keywords Heat shock transcription factor 1 • Molecular chaperones • Neurodegenerative diseases • Polyglutamine diseases • Amyotrophic lateral sclerosis • Parkinson's disease

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14.1 Introduction

Heat shock transcription factor 1 (HSF1) is a transcription factor that protects cells from various types of proteotoxic stress, including heat shock stress and oxidative stress, by inducing the expression of various types of molecular chaperones. Under unstressed conditions, HSF1 is inactivated by its interaction with molecular chaperones, including heat shock protein 40 (Hsp40), Hsp70, and TCP-1 ring complex (TRiC)/chaperonin in the cytoplasm (Zou et al. 1998; Neef et al. 2014; Shi et al. 1998). Upon exposure to stress, HSF1 is quickly released from the chaperone complex, translocates into the nucleus, and binds to the heat shock element (HSE) in the promoter region of various molecular chaperone genes to induce their expression (Sarge et al. 1993; Baler et al. 1993). During this activation process, HSF1 forms a homotrimer and is posttranslationally modified, such as by phosphorylation and sumoylation (Chu et al. 1996; Hietakangas et al. 2003). After acute transcriptional activation, HSF1 is acetylated by the histone acetyltransferase p300/CBP, which attenuates the binding of HSF1 to HSEs, leading to a reduction in its transcriptional activity (Westerheide et al. 2009) (Fig. 14.1). HSF1-induced

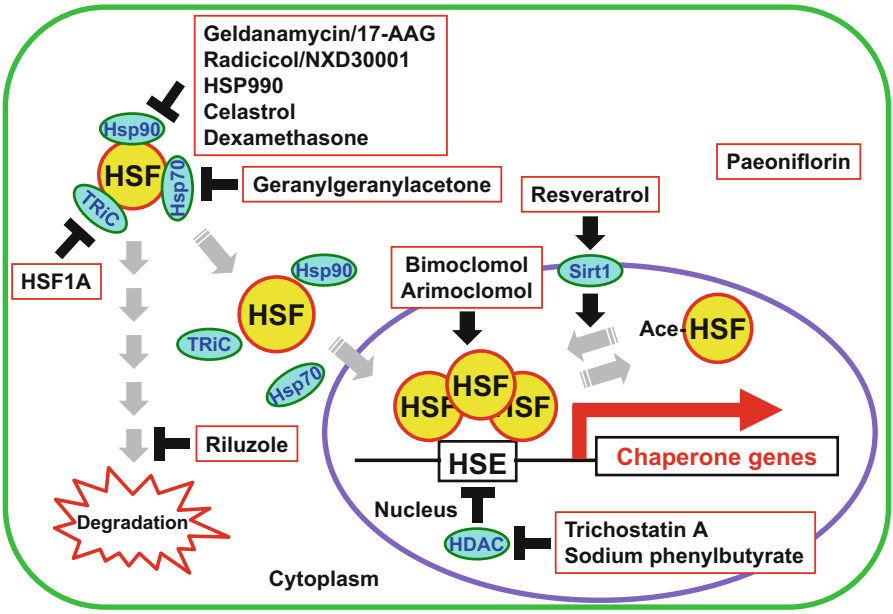
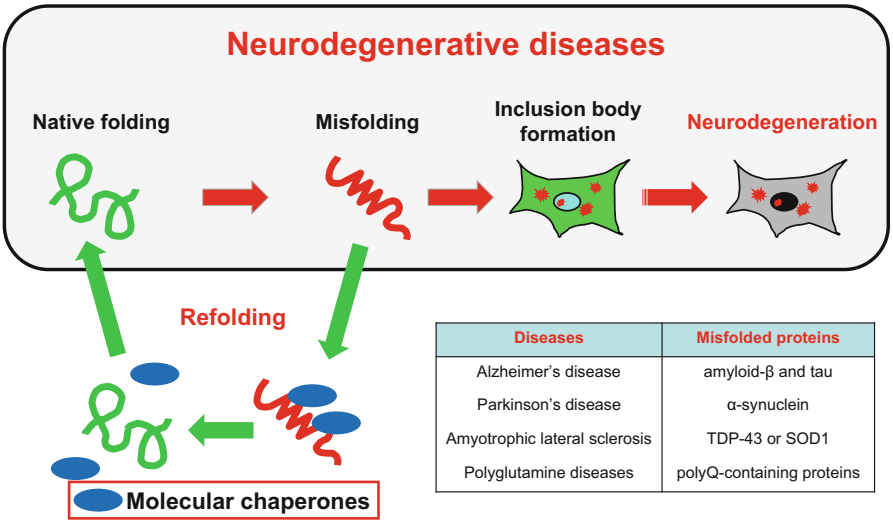


Fig. 14.1 Mechanisms of HSF1 activation by small compounds. Upon exposure to stress, HSF1 is quickly released from the chaperone complex, translocates into the nucleus, forms a homotrimer, and binds to the heat shock element (HSE) in the promoter region of various molecular chaperone genes. After its acute transcriptional activation, HSF1 is acetylated to attenuate its transcriptional activity. Various HSF1-activating compounds targeting various steps of HSF1 activation are shown in *red boxes*. Although paeoniflorin was reported to activate HSF1, the mechanisms remain unknown

molecular chaperones, such as Hsp40, Hsp70, Hsp90, Hsp110, TRiC, and small Hsps, suppress protein misfolding through binding to structurally unstable proteins and assist the refolding of misfolded proteins.

Protein misfolding has been considered to be involved in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and the polyglutamine (polyQ) diseases. The aggregates of misfolded proteins that accumulate as inclusions in the central nervous system are a common pathological hallmark of various neurodegenerative diseases, whereas the main component protein of the inclusions is different in each disease (amyloid-β and tau in Alzheimer's disease, α-synuclein in Parkinson's disease, TAR DNA-binding protein 43 (TDP-43) or superoxide dismutase 1 (SOD1) in ALS, and polyQ-containing proteins in the polyQ diseases). In addition, many genetic mutations that are responsible for the inherited forms of neurodegenerative diseases confer a propensity for misfolding on the disease-causing proteins, resulting in aggregation of these proteins. These facts strongly indicate that protein misfolding is a common pathogenesis of various neurodegenerative diseases (Taylor et al. 2002). Therefore, the suppression of protein misfolding by molecular chaperones is considered to be a common therapeutic approach for the currently untreatable neurodegenerative diseases (Fig. 14.2).



Diseases	Misfolded proteins
Alzheimer's disease	amyloid-β and tau
Parkinson's disease	α-synuclein
Amyotrophic lateral sclerosis	TDP-43 or SOD1
Polyglutamine diseases	polyQ-containing proteins

Fig. 14.2 Protein misfolding in the pathomechanisms of neurodegenerative diseases and its suppression by molecular chaperones. Protein misfolding has been considered as a common pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and the polyglutamine diseases. Molecular chaperones assist the refolding of misfolded proteins, and hence the suppression of protein misfolding by molecular chaperones is considered to be a common therapeutic approach for the currently untreatable neurodegenerative diseases

In fact, the overexpression of molecular chaperones has been demonstrated to lead to therapeutic effects in cell culture and animal models of various neurodegenerative diseases (Auluck et al. 2002; Bruening et al. 1999; Cummings et al. 1998, 2001; Klucken et al. 2004; Shimura et al. 2004). Importantly, the simultaneous expression of several molecular chaperones was shown to result in stronger therapeutic effects than the expression of a single type of chaperone, in various models of neurodegenerative diseases (Chan et al. 2000; Ishihara et al. 2003), indicating that additional and synergistic therapeutic effects are expected through the induction of multiple molecular chaperones. Therefore, the expression of multiple molecular chaperones by HSF1 activation is expected to result in the greatest benefits against neurodegenerative diseases. The first trial to investigate the effects of HSF1 activation against neurodegenerative diseases was reported in 2001. Zimarino and colleagues showed that the genetic overexpression of a constitutively active HSF1 mutant reduces polyQ aggregation through the induction of Hsp70 in a cell culture model (Rimoldi et al. 2001). A constitutively active HSF1 mutant was also shown to ameliorate the disease phenotypes of polyQ disease model mice through the induction of multiple molecular chaperones (Fujimoto et al. 2005). These facts suggest that the genetic overexpression of exogenous HSF1 would ameliorate the symptoms of patients with neurodegenerative diseases, through the induction of multiple molecular chaperones, although the expression of exogenous genes in the human brain is currently very limited owing to safety concerns and the lack of efficient methods for gene delivery.

On the other hand, it had remained unclear as to whether endogenous HSF1 is activated in the brains of patients with protein misfolding neurodegenerative diseases. Interestingly, Bates and colleagues found that the expression levels of molecular chaperones unexpectedly decreased with disease progression in polyQ disease model mice (Hay et al. 2004), suggesting that endogenous HSF1 is gradually inactivated in the brains of these mice. Subsequently, Nukina and colleagues revealed that endogenous HSF1 is inactivated in the brains of polyQ disease model mice (Yamanaka et al. 2008), consistent with the inactivation of HSF1 under chronic heat shock stress (Kline and Morimoto 1997). These facts strongly suggest that endogenous HSF1 is inactivated in the brains of patients with protein misfolding diseases, which are under chronic proteotoxic stress, and hence the activation of endogenous HSF1 is expected to exert therapeutic effects for patients with neurodegenerative diseases.

In this chapter, we focus on small compounds that activate endogenous HSF1 and lead to the induction of molecular chaperones (Fig. 14.1), which are promising candidates as a common therapy against neurodegenerative diseases.

14.2 HSF1 Activation by Hsp90 Inhibitors

The molecular chaperone Hsp90 binds to many proteins, including steroid hormone receptors, protein kinases, and transcription factors, to stabilize them under unstressed conditions. Hsp90 also binds to the regulatory domain of HSF1 to keep HSF1 as an inactive monomer in the cytoplasm, and hence the pharmacological inhibition of Hsp90 is able to activate HSF1.

Geldanamycin, a benzoquinone ansamycin antitumor antibiotic, has been shown to specifically bind to the N-terminal ATP-binding pocket of Hsp90 and inhibit its activity, resulting in the activation of HSF1, leading to the induction of molecular chaperones (Dehner et al. 2003; Whitesell et al. 1994; Zou et al. 1998; Roe et al. 1999). In 2001, Wanker and colleagues reported for the first time the therapeutic effect of the pharmacological induction of molecular chaperones in a neurodegenerative disease model (Sittler et al. 2001). They showed that treatment with geldanamycin suppresses aggregation of the polyQ protein through the induction of multiple molecular chaperones in a cell culture model. A decrease in the number of polyQ aggregates by geldanamycin treatment was also subsequently reported in brain slice cultures derived from polyQ disease model mice (Hay et al. 2004). We also demonstrated that the oral administration of geldanamycin and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a less toxic derivative of geldanamycin, suppresses not only the aggregation of polyQ proteins but also neurodegeneration in polyQ disease model flies, through HSF1-mediated induction of multiple molecular chaperones (Fujikake et al. 2008), indicating that geldanamycin and its derivative are effective in vivo. In addition, Bonini and colleagues showed the therapeutic effects of geldanamycin on the progressive loss of dopaminergic neurons in Parkinson's disease model flies (Auluck and Bonini 2002). Furthermore, the cytotoxicity of mutant SOD1 in a cell culture model of ALS was shown to be reduced by geldanamycin treatment (Batulan et al. 2006). These facts suggest that geldanamycin and its derivatives have the potential to be developed as common therapeutic agents against various neurodegenerative diseases.

Radicicol, an antifungal macrolactone antibiotic, was also shown to activate HSF1 similarly to geldanamycin by inhibiting Hsp90 through its direct binding to the N-terminal domain of Hsp90 (Schulte et al. 1998; Roe et al. 1999; Bagatell et al. 2000). Radicicol was shown to decrease the number of polyQ aggregates in brain slice cultures derived from polyQ disease model mice (Hay et al. 2004). We also demonstrated that radicicol suppresses neurodegeneration as well as the aggregation of polyQ proteins in flies (Fujikake et al. 2008). Therefore, the potential of radicicol to be developed as a therapeutic agent is similar to that of geldanamycin.

HSP990 is a compound that was developed by Novartis as an inhibitor of the ATPase activity of Hsp90, by binding to the N-terminal ATP-binding pocket of Hsp90 (Machajewski et al. 2007). Bates and colleagues demonstrated that oral administration of HSP990 transiently improves the motor performance and reduces

the aggregates of polyQ disease model mice at early stages of disease, through the induction of molecular chaperones via the activation of HSF1 (Labbadia et al. 2011). However, the induction of molecular chaperones as well as the therapeutic effects of HSP990 gradually diminished with disease progression, although HSF1 was dissociated from Hsp90, phosphorylated, and localized in the nucleus of neurons. They demonstrated that the binding of HSF1 to HSE is significantly decreased in the brains of polyQ disease model mice and that the chromatin structure and acetylation of histones are altered with disease progression, suggesting that the accessibility of HSF1 to the HSE sequence on the genome is decreased by chronic proteotoxic stress in neurodegenerative diseases. These data suggest that oral administration of HSP990 is effective against neurodegenerative diseases at least early stages of the diseases.

14.3 HSF1 Activation by an Hsp70 Inhibitor

The molecular chaperone Hsp70 has a major role in assisting the proper folding of newly synthesized polypeptides and in preventing the misfolding of mature proteins (Hendrick and Hartl 1995). Upon exposure to stress, Hsp70 expression is quickly induced by activated HSF1, resulting in the binding of Hsp70 to structurally unstable proteins, to prevent the misfolding of these proteins. Hsp70 also binds to the transactivation domain of HSF1 to disassemble the active HSF1 trimer into inactive monomers and to stabilize the monomers (Abravaya et al. 1992; Mosser et al. 1993; Shi et al. 1998).

In 1996, geranylgeranylacetone (GGA), an antiulcer drug, was demonstrated to induce molecular chaperones via the activation of HSF1 (Hirakawa et al. 1996). About 10 years later, GGA was shown to bind to and inhibit Hsp70, leading to the dissociation of HSF1 from Hsp70, resulting in the activation of HSF1 (Otaka et al. 2007). Sobue and colleagues showed that the oral administration of GGA induces the expression of multiple molecular chaperones in the spinal cord of polyQ disease model mice, which suppresses the accumulation of polyQ proteins, resulting in the improvement of motor performance (Katsuno et al. 2005). GGA is considered as a strong candidate agent for patients with neurodegenerative diseases, since it has already been used as an antiulcer drug for humans.

14.4 HSF1 Activation by a TRiC Inhibitor

TRiC is a eukaryotic chaperonin composed of eight paralogous subunits (Tcp1 and Cct2-8) (Lopez et al. 2015). Although TRiC was considered to specifically assist the folding of cytoskeletal proteins, such as α-tubulin and actin, it was later estimated to interact with 6–7 % of cytosolic proteins, probably to assist their

folding (Yam et al. 2008). TRiC directly binds to HSF1 and suppresses its activation, similarly to Hsp90 and Hsp70.

HSF1A, a benzyl pyrazole derivative, was recently identified as an HSF1-activating compound by a humanized yeast screen and was confirmed to also activate HSF1 in human cells (Neef et al. 2010, 2011). HSF1A has been shown to interact with at least four TRiC subunits (Tcp1, Cct2, Cct5, and Cct8) and to mildly inhibit its chaperone activity, leading to the activation of HSF1 (Neef et al. 2014). Treatment with HSF1A was shown to reduce the aggregates and cytotoxicity of polyQ proteins in a cell culture model and to attenuate a disease phenotype in polyQ diseases model flies (Neef et al. 2010). These facts indicate that TRiC is a new target to develop HSF1-mediated therapies against neurodegenerative diseases.

14.5 Prolongation of HSF1 Activity

Silent information regulator 2 (Sir2), an NAD-dependent protein deacetylase in budding yeast, was found to be a determinant of their lifespan (Kaeberlein et al. 1999). The overexpression of Sir2 slowed the aging process in yeast, whereas Sir2 mutants had a shortened lifespan. Subsequently, an increase in lifespan by the overexpression of Sir2 orthologs has been demonstrated in worms and flies (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Furthermore, the overexpression of sirtuin 1 (Sirt1), a mammalian ortholog of Sir2, results in healthy aging in mice, such as a reduction in DNA damage and fewer spontaneous carcinomas and sarcomas (Herranz et al. 2010). Morimoto and colleagues demonstrated that the DNA-binding domain of HSF1 is acetylated after its acute transcriptional activation, to reduce its transcriptional activity (Westerheide et al. 2009). Importantly, they further showed that Sirt1 deacetylates the acetylated HSF1, leading to its prolonged transcriptional activity, which may be involved in the mechanisms of longevity and healthy aging by Sirt1.

By screening small compound libraries for modulators of Sirt1 activity, resveratrol, a polyphenol found in red wine, was identified as an activator of Sirt1 (Howitz et al. 2003). Resveratrol treatment extended the lifespan of budding yeast in a Sirt1-dependent manner and conferred resistance to ionizing radiation in human cultured cells, accompanied with the deacetylation of p53, which is a known target of Sirt1. Treatment of ALS model mice with resveratrol by chronic intraperitoneal injection was reported to successfully extend their lifespan, decrease motor neuron death, and delay the onset of muscle weakness (Han et al. 2012). Although the effect of resveratrol on protein aggregation was not investigated, the authors demonstrated a decrease in acetylated HSF1 accompanied by the induction of Hsp27 and Hsp70 in the spinal cord of the resveratrol-treated ALS model mice, suggesting that the therapeutic effects of resveratrol are mediated by prolonged HSF1 activity.

14.6 Enhancement of HSF1 Activity

Bimoclomol, a derivative of hydroxylamine, has been reported to enhance the induction of molecular chaperones in cultured cells upon heat shock stress, whereas it does not induce molecular chaperones under unstressed conditions (Vigh et al. 1997). Although the specific mechanisms by which bimoclomol enhances the induction of molecular chaperones are not fully understood, Csermely and colleagues reported that bimoclomol directly binds to HSF1, resulting in significant prolongation of the binding of HSF1 to HSE (Hargitai et al. 2003). On the other hand, Vigh and colleagues reported that bimoclomol interacts with acidic lipids and modifies the fluidity of the plasma membrane, similarly to the effect of heat shock stress on the membrane, leading to an enhancement of HSF1 activity (Torok et al. 2003).

Arimoclomol, an orally active derivative of bimoclomol, has been shown to induce molecular chaperones in the pancreas of rats with pancreatitis, upon its intragastric administration (Rakonczay et al. 2002). Arimoclomol has also been shown to induce multiple molecular chaperones via the activation of HSF1 in the spinal cord upon intraperitoneal injection, resulting in the alleviation of disease symptoms of ALS model mice (Kieran et al. 2004). Furthermore, the lifespan of ALS model mice was extended by arimoclomol treatment, when treatment was started at the time of symptom onset (Kieran et al. 2004), indicating that this compound is expected to exert therapeutic effects in the early stages of ALS. In a phase II clinical trial, arimoclomol was shown to be safe and well tolerated in patients with ALS and confirmed to be delivered to the cerebrospinal fluid upon oral administration (Cudkowicz et al. 2008). Currently, a phase II/III clinical trial of arimoclomol to ALS patients is ongoing (ClinicalTrials.gov Identifier: NCT00706147).

14.7 Inhibition of HSF1 Degradation

Riluzole, a compound that acts against glutamate toxicity, is the clinically approved agent for the treatment of ALS. Although the therapeutic effects of riluzole were believed to be from its protective effects against glutamate toxicity (Bensimon et al. 1994), the effect of riluzole on HSF1 has recently been clarified. Treatment of cultured cells with riluzole was reported to accelerate the induction of Hsp70 upon heat shock stress by increasing the amount of HSF1, whereas riluzole treatment under unstressed conditions only increased the amount of steady-state HSF1 and did not induce the activation of HSF1 (Yang et al. 2008; Liu et al. 2011). Since the expression of HSF1 mRNA was not altered, riluzole is thought to inhibit degradation of the HSF1 protein. These results suggest that the increase in the amount of HSF1 may be involved in the therapeutic effects of riluzole against ALS.

14.8 Medicinal Plants

Root extracts from the *Celastraceae* family are used for the treatment of fever, chills, joint pain, inflammation, edema, rheumatoid arthritis, and bacterial infection. Celastrol, which is isolated from the root extract of the *Celastraceae* family, was identified as an inducer of molecular chaperones through HSF1 activation (Westerheide et al. 2004). Although the mechanisms by which celastrol activates HSF1 are not fully understood, celastrol is thought to inhibit the binding of Hsp90 to ATP without affecting the N-terminal ATP-binding pocket of Hsp90 (Zhang et al. 2009; Hieronymus et al. 2006). Treatment of a cell culture model of the polyQ diseases with celastrol was reported to induce Hsp70 in an HSF1-dependent manner and to suppress polyQ aggregation, leading to a reduction in cell death (Zhang and Sarge 2007). However, since celastrol was reported to have multiple targets, such as Cdc37, IKKβ, and the proteasome, and, furthermore, since it directly suppresses the aggregation of purified polyQ proteins in vitro, other mechanisms may also contribute to its therapeutic effects (Yang et al. 2006; Lee et al. 2006; Sreeramulu et al. 2009; Zhang and Sarge 2007; Wang et al. 2005).

Paeonia lactiflora is a medicinal plant used for nourishing blood, alleviating pain, reducing irritability, and treating liver disease and cancer. Paeoniflorin, one of the main compounds extracted from *Paeonia lactiflora*, was reported to induce multiple molecular chaperones via the activation of HSF1, although the mechanisms remain unknown (Yan et al. 2004). Furthermore, treatment with paeoniflorin was reported to induce molecular chaperones in a polyQ disease cell culture model, resulting in the suppression of polyQ protein aggregation (Chang et al. 2013). In addition, intraperitoneal injection of paeoniflorin to polyQ disease model mice was shown to induce the expression of not only molecular chaperones but also nuclear factor-YA (NF-YA), a transcriptional factor (Tohnai et al. 2014). NF-YA increases the expression levels of transcription factor EB (TFEB) and carboxyl-terminus of Hsc70-interacting proteins (CHIP), which are proteins involved in the lysosome and proteasome protein degradation system, respectively (Sardiello et al. 2009; Stankiewicz et al. 2010), leading to the degradation of polyQ proteins, resulting in the amelioration of motor performance and extension of lifespan of polyQ disease model mice. These results suggest that paeoniflorin targets not only HSF1 but also other molecules, demonstrating its additional therapeutic effects against neurodegenerative diseases.

14.9 Dexamethasone

Dexamethasone, a synthetic corticosteroid used as an antiinflammatory agent, was shown to induce Hsp70 but not Hsp27 and Hsp60 via the activation of HSF1 in rat cardiac myocytes (Sun et al. 2000). Jana and colleagues found that the expression levels of HSF1 mRNA and protein are decreased in the eyes and brains of polyQ

disease model flies and mice, respectively (Maheshwari et al. 2014). They tested the effects of dexamethasone on these models and found that dexamethasone treatment increases the expression level of HSF1 to a similar level to that in wild-type mice. Furthermore, dexamethasone not only increased the expression levels of HSF1 but also induced HSF1 activation in the brains of a mouse model of the polyQ diseases, leading to a decrease in the number of aggregates and an improvement in motor performance through the induction of Hsp70. Although the mechanisms by which dexamethasone increases the expression level of HSF1 and induces its activation are unclear, they showed that dexamethasone reduces the expression level of Hsp90, which probably contributes to the activation of HSF1.

14.10 Inhibition of Histone Deacetylase

Heat shock stress induces not only the activation of HSF1 but also the acetylation of histones, which alters the chromatin structure in the promoter region of molecular chaperone genes. The acetylation of core histones weakens the interaction of histones with DNA, leading to the open chromatin structure that confers high accessibility for transcription factors to the target sequence on the genome. In fact, hyperacetylation of histones by treatment with histone deacetylase (HDAC) inhibitors has been reported to increase the accessibility of HSF1 to the HSE sequence and to induce the expression of molecular chaperones (Chen et al. 2002; Zhao et al. 2005; Marinova et al. 2011; Ovakim and Heikkila 2003). Therefore, HDAC inhibitors can be regarded as activators of HSF1-mediated induction of molecular chaperones *in trans*. Treatment with HDAC inhibitors, such as trichostatin A and sodium phenylbutyrate, has indeed been shown to exert therapeutic effects against various models of neurodegenerative diseases, including Alzheimer's disease, Parkinson's diseases, ALS, and the polyQ diseases (Ricobaraza et al. 2009; Kontopoulos et al. 2006; Ryu et al. 2005; Steffan et al. 2001; Minamiyama et al. 2004), although the contribution of HSF1 to these effects was not investigated. These facts suggest that induction of molecular chaperones via HSF1 may contribute to the therapeutic effects of HDAC inhibitors against neurodegenerative disease models.

14.11 Future Perspectives

Here we introduced various studies investigating the therapeutic effects of various small compounds that induce the activation of HSF1, for the treatment of various neurodegenerative diseases involving protein misfolding. To date, several HSF1-activating compounds were demonstrated as potential therapeutic agents using mouse models of various neurodegenerative diseases. However, we note here that in almost all studies showing the therapeutic effects of HSF1 activators in mouse

models, treatments were started before disease onset. Bates and colleagues demonstrated an important issue, namely, that the induction of molecular chaperones is gradually diminished with disease progression in the brains of neurodegenerative disease model mice, through alterations in chromatin structure, which decreases the accessibility of activated HSF1 to the HSE sequence (Labbadia et al. 2011). Considering that the majority of patients with neurodegenerative diseases are diagnosed after disease onset, the chromatin structure of the HSE sequence in patients' brains may already be altered, which would interfere with activated HSF1 accessing the HSE, and hence this issue should be solved toward developing a therapy for human patients.

One plausible strategy is the rearrangement of the altered chromatin structure, allowing it to be accessed by HSF1. Huang and colleagues clearly showed that HDAC inhibitor treatment rearranges the chromatin structure, increasing the accessibility of HSF1 to the HSE sequence (Chen et al. 2002). Hence, a combination of HSF1-activating compounds and HDAC inhibitors is expected to show synergistic effects against the neurodegenerative diseases. In fact, Marsh and colleagues showed that combinatorial treatment with an Hsp90 inhibitor (geldanamycin) and an HDAC inhibitor (suberoylanilide hydroxamic acid) results in much greater suppression of neurodegeneration than treatment with each compound alone in polyQ disease model flies, although the induction of molecular chaperones was not examined (Agrawal et al. 2005). This robust suppression may be caused by the efficient induction of molecular chaperones via the activation of HSF1 both *in cis* and *in trans*.

Another strategy is based on the novel concept of exosome-mediated intercellular chaperone transmission, which contributes to the maintenance of protein homeostasis at the organismal level. We recently reported that molecular chaperones, including Hsp40 and Hsp70, are secreted from cells via exosomes, transmitted to other cells, and suppress polyQ aggregation in other cultured cells (Takeuchi et al. 2015). Furthermore, the overexpression of molecular chaperones in nonneuronal tissues, such as the muscle and fat body non-cell, autonomously suppresses neurodegeneration in the eyes of polyQ disease model flies, probably through intercellular chaperone transmission. Therefore, our study indicates that even if molecular chaperones cannot be induced in the brain through the activation of HSF1, their induction in peripheral tissues or supplying chaperone-containing exosomes from the periphery is expected to suppress protein misfolding in patients' brains in a non-cell autonomous manner.

In summary, the pharmacological activation of HSF1 is a promising therapeutic approach against various protein misfolding neurodegenerative diseases. Toward developing HSF1-mediated therapies against neurodegenerative diseases, additional studies toward understanding the mechanisms of activation of HSF1 under chronic proteotoxic stress should be performed in the future.

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