Heat Shock Proteins 17
Series Editors: Alexzander A. A. Asea · Stuart K. Calderwood

Alexzander A. A. Asea
Punit Kaur *Editors*

Heat Shock Proteins in Signaling Pathways

Springer

Heat Shock Proteins

Volume 17

Series editors
Alexzander A. A. Asea
Professor, Department of Medicine and Director
Precision Therapeutics Proteogenomics Diagnostic Center
Eleanor N. Dana Cancer Center
University of Toledo College of Medicine and Life Sciences
Toledo, USA

Stuart K. Calderwood
Professor and Director, Division of Molecular and Cellular Radiation Oncology
Department of Radiation Oncology
Beth Israel Deaconess Medical Center and Harvard Medical School
Boston, USA

Heat Shock Proteins: key mediators of Health and Disease. Heat shock proteins (HSP) are essential molecules conserved through cellular evolution required for cells to survive the stresses encountered in the environment and in the tissues of the developing and aging organism. These proteins play the essential roles in stress of preventing the initiation of programmed cell death and repairing damage to the proteome permitting resumption of normal metabolism. Loss of the HSP is lethal either in the short-term in cases of acute stress or in the long-term when exposure to stress is chronic. Cells appear to walk a fine line in terms of HSP expression. If expression falls below a certain level, cells become sensitive to oxidative damage that influences aging and protein aggregation disease. If HSP levels rise above the normal range, inflammatory and oncogenic changes occur. It is becoming clear that HSP are emerging as remarkably versatile mediators of health and disease. The aim of this series of volumes is to examine how HSP regulation and expression become altered in pathological states and how this may be remedied by pharmacological and other interventions.

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Alexzander A. A. Asea
Professor, Department of Medicine and
Director, Precision Therapeutics
Proteogenomics Diagnostic Center
Eleanor N. Dana Cancer Center
University of Toledo College of Medicine
and Life Sciences
Toledo, OH, USA

Punit Kaur
Department of Experimental Radiation
Oncology
MD Anderson Cancer Center
Houston, TX, USA

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Preface

Signaling pathway is a comprehensive mechanism by which all cellular organisms communicate internally and externally with their microenvironment. This is a highly complex and exact process. Errors in signaling pathways and in the processing of cellular information are known to be responsible for the majority of diseases including cancer and inflammatory and neurological disorders. Knowledge gained from the better understanding of signaling pathways will help in elucidating disease processes and will assist in the development and design of novel targeted treatment therapies to combat human diseases and disorders. Heat shock proteins (HSP) are uniquely involved in a number of critical signaling pathways.

The book ***Heat Shock Proteins in Signaling Pathways*** provides the most comprehensive review on contemporary knowledge on the role of HSP in signaling pathways relevant to a number of diseases. Using an integrative approach, the contributors provide a synopsis of novel mechanisms, signal transduction pathways. To enhance the ease of reading and comprehension, this book has been subdivided into various sections: Section I reviews current progress on our understanding of inflammatory signaling pathways, Section II focuses on oncology signaling pathways, and Section III emphasizes neurological signaling pathways.

Key basic and clinical research laboratories from major universities, academic medical hospitals, and biotechnology and pharmaceutical laboratories around the world have contributed chapters that review present research activity and importantly project the field into the future. The book is a must-read for graduate students, medical students, basic science researchers, and postdoctoral scholars in the fields of Translational Medicine, Clinical Research, Human Physiology, Biotechnology, and Cell and Molecular Medicine and also for pharmaceutical scientists and researchers involved in drug discovery.

Toledo, OH, USA — Alexzander A. A. Asea
Houston, TX, USA — Punit Kaur

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About the Editors

Prof. Dr. Alexzander A. A. Asea is a highly innovative and accomplished world renowned clinical and basic research scientist and visionary executive leader who has exceptional experience spearheading clinical and basic science research, training, education, and commercialization initiatives within top ranked academic biomedical institutes. Prof. Dr. Asea's initial findings studying the effects of Hsp72 on human monocytes lead to the proposal of a novel paradigm that Hsp72, previously known to be an intracellular molecular chaperones, can be found in the extracellular milieu where it has regulatory effects on immuno-competent cells - a term now called chaperokine. Prof. Asea has authored over 255 scientific publications including peer-reviewed articles, reviews, books, book chapters, editorials, and news headliners in a wide range of biomedical-related disciplines. Prof. Asea is the series editor of the widely successful book series *Heat Shock Proteins* (Springer Nature Publishing) and is an editorial board member of numerous scientific peer-reviewed journals. Currently, Prof. Dr. Asea is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Dr. Punit Kaur is an expert in onco-proteogenomics, with extensive training and experience in quantitative mass spectrometry imaging, protein chemistry and biomarker discovery. Her main research focus is on the use of heat-induced nanotechnology in combination with radiotherapy and chemotherapy in the cancer stem cell therapy. She has published more than 40 scientific articles, book chapters, and reviews, and currently serves as editorial board member for the *European Journal of Cancer Prevention* and the *Journal of Proteomics and Bioinformatics*. She is an editor of eight books in the highly successful *Heat Shock Proteins* book series by Springer Nature Publishers. Currently, she is a Visiting Scientist Professor at the University of Texas MD Anderson Cancer Center in Houston, USA.

Part I
Inflammatory Signaling Pathways

Chapter 1
Thiol-Based Redox Signaling: Impacts on Molecular Chaperones and Cellular Proteostasis

Amy E. Ford and Kevin A. Morano

Abstract Signaling through protein cysteine residues to regulate diverse biological processes is widely conserved from bacterial to human cells. Differential cysteine reactivity enables cells to sense and respond to perturbations in the cellular redox environment, which may impact protein structure and activity. This chapter will focus on how redox signaling regulates components of the protein quality control network to mitigate proteotoxic stress caused by redox active compounds. While specifics of redox-based activation of the endoplasmic reticulum unfolded protein response and the cytoplasmic heat shock and oxidative stress responses differ, the presence of regulatory proteins containing reactive cysteines is a common feature. Moreover, several protein chaperones are reversibly regulated via cysteine switches that govern their ability to protect or refold damaged polypeptides. These responses are biologically indispensable, given the propensity of dysregulated cells to produce endogenous reactive oxygen species and the prevalence of thiol-reactive xenobiotics in the external environment.

Keywords Chaperone · Oxidative stress · Proteostasis · Reactive oxygen species · Redox · Signaling

A. E. Ford
Department of Microbiology and Molecular Genetics, University of Texas McGovern Medical School at Houston, Houston, TX, USA

MD Anderson UT Health Graduate School of Biomedical Sciences, Houston, TX, USA

K. A. Morano (✉)
Department of Microbiology and Molecular Genetics, University of Texas McGovern Medical School at Houston, Houston, TX, USA
e-mail: kevin.a.morano@uth.tmc.edu

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Abbreviations

CRD	cysteine-rich domain
Cys	cysteine
ER	endoplasmic reticulum
HMW	high molecular weight
HS	heat shock
HSP	heat shock protein
HSR	heat shock response
LMW	low molecular weight
NBD	nucleotide binding domain
NEF	nucleotide exchange factor
OS	oxidative stress
OSR	oxidative stress response
PDI	protein disulfide isomerase
PQC	protein quality control
Prx	peroxiredoxin
ROS	reactive oxygen species
SOH	sulfenic acid
TF	transcription factor
Ub	ubiquitin
UPR	unfolded protein response

1.1 Introduction

Cysteine (Cys) is one of the least abundant amino acids, but serves critical and unique roles in protein structure and chemistry due to its irreplaceable functionality as the only amino acid with a readily ionizable thiol group (Marino and Gladyshev 2010). Thiol reactivity depends on its accessibility and protonation state (pK_a), the latter of which is influenced by local protein microenvironment properties such as pH, secondary structure, and hydrogen bonding (Kortemme and Creighton 1995; Ferrer-Sueta et al. 2011). Although methionine also contains a sulfur atom, the thioether is in a relatively less reactive form and is typically not involved in biologically relevant reactions. Cys residues are most often buried within the interior of the protein structure; however, they can also be found exposed to the solvent (Poole 2015). Additionally, cysteines are typically clustered into two or more groups, characteristic of metal binding and redox centers. These chemical and functional properties allow for rapid and reversible redox regulation of protein activity, frequently but not exclusively through the formation of intramolecular disulfide bonds, to sense and control diverse cellular states and processes.

Reactive oxygen species (ROS) produced as a byproduct of aerobic metabolism, oxidative protein folding, and exposure to oxidants and highly toxic xenobiotics

have the potential to modify reactive thiols (Marnett et al. 2003; Tu and Weissman 2004; West et al. 2012). Oxidants such as hydrogen peroxide and diamide can react with protein thiols to form both reversible and irreversible thiolations (Winterbourn and Hampton 2008; Paulsen and Carroll 2010). Following initial formation of sulfenic acid (SOH), the modified thiol can either be further oxidized into sulfinic (SOOH) or sulfonic (SOOOH) acid or form a disulfide bond with a nearby free thiol (e.g. intramolecularly with a proximal Cys residue or with glutathione). These modifications play biological roles in sensing and regulation of activity of redox enzymes and transcriptional programs. The highly toxic heavy metal cadmium and metalloid-anion arsenite can target proteins in multiple ways – covalent binding of free thiols, metal ion displacement, and catalyzing oxidation (Tamás et al. 2014). In addition to oxidants and heavy metals, Cys residues are susceptible to modification by organic electrophiles, which form thiol adducts and may induce intermolecular cross-links between proteins (Zhang et al. 1995; Sánchez-Gómez et al. 2010). While xenobiotics are not involved in normal, steady state redox regulation, exposure to these agents can mimic endogenous modifications and induce similar downstream signaling.

Protein homeostasis ("proteostasis") is essential for cellular function, and is defined as the status of the protein complement of a cell as determined by protein synthesis, assembly and degradation/turnover. Molecular chaperones assist proteins in their proper folding and prevent non-native conformations that lead to misfolding and aggregation (reviewed by Verghese et al. 2012). Proteins that cannot be folded properly or any non-native confirmations that arise are shuttled to specific protein aggregation sites and/or degradation pathways. These functions are performed by a variety of different chaperone classes and machines that make up the protein quality control (PQC) network. Members of the highly conserved Hsp70 class of chaperones are located in all major subcellular compartments and function in many aspects of proteostasis including native folding, transport, disaggregation, and degradation. Hsp70 performs these functions with the assistance of co-chaperones such as J-domain-containing Hsp40 proteins and nucleotide exchange factors (NEF), including the Hsp110, HspBP1 and Bag protein families (Bracher and Verghese 2015). Unlike Hsp70, the conserved Hsp90 system of chaperones interacts with specific "client" proteins, including kinases, receptors, and transcription factors, to aid in protein maturation and assembly of macromolecular complexes (Röhl et al. 2013). Cells also utilize small heat shock proteins that form multimers to aid in disaggregation (Verghese et al. 2012).

Cys modification by thiol-reactive compounds (described above) has the potential to alter protein structure and affect protein stability and solubility. Using in vitro folding assays, live cell imaging, and proteomic approaches, thiol stress has been found to induce protein aggregation (Sharma et al. 2008; Jacobson et al. 2012, 2017; Weids et al. 2016). Accumulation of protein aggregates resulting from exposure to these compounds can be toxic to cells as demonstrated by dose-dependent loss of cell viability (West et al. 2011). Protein aggregation is linked to diverse human diseases including diabetes, cancers, and neurodegenerative disorders such as Alzheimer's, Parkinson's and Lou Gehrig's diseases (Valastyan and Lindquist 2014;

Hipp et al. 2014). In addition to protein aggregation, oxidative stress (OS) and metal dyshomeostasis have been implicated, suggesting that disruption of redox balance and, therefore, redox regulation, as a contributing factor to these diseases.

Multiple studies have investigated the in vivo redox state of thiol-containing proteins during steady state conditions, peroxide stress, and changes to redox status due to aging or genetic mutations (Le Moan et al. 2006; Brandes et al. 2011, 2016). A common theme amongst these studies is the diversity of cellular processes that depend on redox-active thiol-containing proteins – redox systems, energy metabolism, translation, and, notably, protein folding. In this chapter, we will discuss the interplay between the PQC network and redox signaling with respect to changes in the protein folding environment.

1.1.1 Regulation of Stress Responses

The ability to respond and adapt to environmental changes through transcriptional reprogramming is essential for survival and proliferation. Bacterial responses to stress are numerous due to the diversity of niches and are regulated by specific or over-lapping stresses (Chalancon and Madan Babu 2011; Helmann 2011). Transcriptional activators, repressors and alternative sigma factors block or recruit RNA polymerase and additional co-regulators to regulate gene expression. Activity of these proteins is often controlled through anti-sigma factors that act as stress sensors and interact with the transcriptional regulator to sequester or facilitate its degradation (Hughes et al. 1998; Zhou et al. 2001; Arsène and Tomoyasu 2000). The major chaperone Hsp70 system composed of DnaK/DnaJ/GrpE (*E. coli*) and the Hsp60 chaperonin (GroEL/ES in *E. coli*) machines protect nascent polypeptides from insults to the folding environment and assist in refolding or degradation of damaged proteins. The two chaperone systems have been implicated in stress response sensing and regulation, most notably the Hsp70 system that regulates stability of the bacterial stress factor σ^{32} (Arsène and Tomoyasu 2000).

A distinguishing feature of eukaryotes is the presence of membrane-bound organelles that allow for the compartmentalization of distinct protein folding environments that differ in redox status: a reducing environment predominates in the cytosol and nucleus, and an oxidizing one is characteristic of the ER and mitochondrial inner membrane space, as well as the extracellular milieu. Changes in the redox balance within these compartments are sensed via protein thiol modifications which lead to activation of transcriptional responses (see Fig. 1.1). Within the ER, the response to redox imbalance is well characterized and is known as the unfolded protein response (UPR). In the cytosol, cells activate a specific transcriptional program to oxidative stress called the oxidative stress response (OSR). On the other hand, the response to misfolded proteins, classically termed the heat shock response (HSR), is primarily modulated by the transcription factor (TF) Hsf1; however, the mechanism of Hsf1 activation by oxidation of the reducing environment is unclear. Within the last 10 years, studies have investigated the connection between OS and

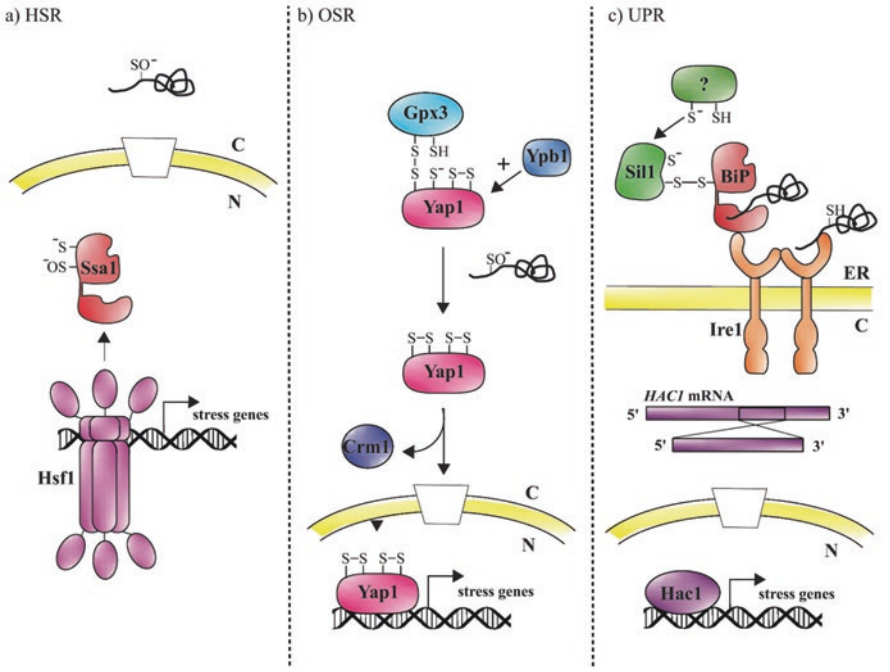

Fig. 1.1 Redox regulation of stress responses in the eukaryotic model budding yeast. (**a**) Proposed mechanism of redox regulation of the Heat Shock Response (HSR) following oxidative or thiol-reactive stress. Modification of the Hsp70 Ssa1 cysteines in the nucleotide-binding domain (NBD) decreases Ssa1 interaction with Hsf1 activating domains leading to de-repression of the HSR and protection of redox sensitive substrates. (**b**) Yap1-mediated redox regulation of the Oxidative Stress Response (OSR). Following oxidative or thiol-reactive stress, Yap1 undergoes oxidative re-arrangement through interactions with Gpx3 and Ypb1 allowing translocation into the nucleus and transcription of stress-protective genes. (**c**) Redox regulation of the ER Unfolded Protein Response (UPR). Redox sensing through the Hsp70 BiP and its co-chaperone Sil1 as well as unfolded peptides triggers the UPR through Ire1-mediated activation of Hac1

protein folding in the cytosol in the budding yeast *Saccharomyces cerevisiae*. Specifically, transcriptional profiling experiments demonstrated that thiol-reactive compounds induce both the HSR and OSR, implying evolutionary pressure to mobilize the PQC network in response to both environmental insults (Trott et al. 2008; Wang et al. 2012). Although these compartmentalized responses control distinct transcriptional programs, the different stress response pathways (UPR, HSR) occasionally converge to co-regulate expression of the same stress genes, indicating crosstalk between different cellular stresses and the responses they elicit (Liu and Thiele 1996; Sugiyama et al. 2000; Solis et al. 2016; Morano et al. 2012). This is further shown in how reductive stress in one compartment such as the ER can alter redox state of the cytosol (Delic et al. 2012). This section will discuss how chaperones play a role in regulation of each of these stress responses by either direct interaction with TFs or upstream effectors.

1.1.1.1 Heat Shock Response

Bacterial regulation of the HSR is mediated through negative autoregulation of the transcriptional activator σ^{32} (reviewed by Arsene et al. 2000). Under normal growth conditions, direct binding of the DnaK chaperone system facilitates σ^{32} rapid degradation via the FtsH mediated protease. Cellular stress such as HS induces dissociation of this complex and subsequent activation of stress defense genes. Glutathione modification of DnaK following dual HS-OS results in loss of chaperone activity, suggesting a potential mechanism of dissociation and subsequent σ^{32} activation (Winter et al. 2005). This notion is further supported by the finding that glutathione-modified DnaK is impaired in peptide binding and its interaction with σ^{32} (Zhang et al. 2016).

In yeast, the HSR activator Hsf1 exists as a stable trimer constitutively bound to high affinity HSEs located upstream of heat shock induced genes (Hahn et al. 2004). The prevailing model for Hsf1 activation stipulated that protein misfolding due to heat shock is sensed by chaperones such as Hsp70 being titrated away from Hsf1, allowing productive recruitment of Mediator and RNA polymerase complexes, thereby resulting in transcription of heat shock genes. However, definitive experimental support for this mechanism was lacking. Recent work has shed considerable light on the precise mechanism of how the Hsp70 yeast homolog Ssa1 regulates the HSR, with the discovery of reversible binding to sites identified in the N- and C-terminal transcriptional activation domains of Hsf1 (Peffer and Morano, unpublished results, Zheng et al. 2016). These findings are consistent with an earlier study that established a role for Ssa1 in regulation of Hsf1 in response to thiol stress. Two Cys residues (C264, C303) in the Ssa1 ATPase domain were found to be required for activation of the HSR in response to multiple thiol-reactive compounds ranging from H_2O_2 to organic electrophiles (Wang et al. 2012) (see Fig. 1.1a). Interestingly, the cysteines are not required for HSR activation in response to HS, suggesting distinct mechanisms regulate the HSR in response to different types of stress. The precise role the cysteines play in modulating the Ssa1-Hsf1 regulatory circuit remains to be established.

How the models of HSR redox sensing through Hsp70 in bacteria and yeast might apply to mammalian biology is unclear. For example, Hsf1 activation and repression domains are not precisely conserved from yeast to humans. Although all Hsf1 homologs possess a C-terminal activation domain that appears to include a putative Hsp70 binding site similar to that identified in yeast, human and other metazoan HSF1 homologs lack an N-terminal activation domain. Furthermore, human and murine HSF1 contain two cysteines in the DNA binding domain that form a reversible disulfide bond upon exposure to HS and OS (Ahn and Thiele 2003). Disulfide bond formation between C35 and C105 activates Hsf1 trimerization, nuclear localization, and target gene expression, indicating a direct sensing mechanism. This redox regulation of nuclear import is similar to what has been shown for the OSR TF Yap1 (described below). However, yeast Hsf1 lacks any cysteines, necessitating a partner protein(s) capable of sensing OS, possibly Ssa1. In additional contrast to yeast, mammalian Hsf1 exists primarily as an Hsp90-repressed

monomer in the cytoplasm that trimerizes and localizes to the nucleus to activate stress genes (Bharadwaj et al. 1999; Abravaya et al. 1992; Grunwald et al. 2014). Following oxidative stress produced by xenobiotics, Hsp90 is cleaved resulting in loss of chaperone activity indicating redox inactivation of Hsp90 and a potential role in Hsf1 regulation in response to OS (Shen et al. 2008; Beck et al. 2009). Collectively, evidence supports thiol-mediated loss of chaperone function as a likely mechanism of HSR redox regulation.

1.1.1.2 Oxidative Stress Response

In the yeast cytosol, OS is sensed primarily through the Yap1 TF (see Fig. 1.1b) (Harshman et al. 1988; Kuge and Jones 1994). Yap1 contains six Cys residues located in both N- and C-terminal cysteine-rich domains (CRD). Under normal growth conditions, these residues exist in a reduced state which allows binding of the *trans*-regulator Crm1 to the C-terminal CRD and nuclear export (Kuge et al. 1998; Yan et al. 1998). In oxidizing conditions, Yap1 undergoes oxidative protein folding with the help of glutathione peroxidase Gpx3 and Ypb1, the Yap1 binding protein. Gpx3 induces disulfide bond formation of Cys pairs between the CRD domains through a transient mixed disulfide mechanism (Delauney et al. 2002), while Ybp1 is thought to enhance oxidative folding efficiency. This alternative conformation blocks Crm1 binding and allows nuclear import (Delauney et al. 2002; Veal et al. 2003; Wood et al. 2004). Yap1 can then activate the OSR by inducing transcription of antioxidant defense genes.

1.1.1.3 Unfolded Protein Response

As proteins destined for secretion are synthesized and translocated across the ER membrane, the oxidative environment along with chaperones enable the formation of disulfide bonds necessary for proper protein folding. A switch to either a reducing or hyperoxidative environment prevents proper disulfide bond formation resulting in protein misfolding and activation of the UPR (Braakman et al. 1992; Haynes et al. 2004; Merksamer et al. 2008; Hetz et al. 2011). In yeast, protein misfolding is sensed through the ER resident Hsp70 chaperone BiP via binding of exposed hydrophobic patches of unfolded polypeptides (Wei and Hendershot 1995; Knittler and Haas 1992). Substrate-bound BiP and/or misfolded substrates directly interact with the ER lumenal domain of the transmembrane endonuclease Ire1 inducing dimerization and cytosolic intron splicing of the UPR TF *HAC1* precursor mRNA (Ng et al. 1992; Gardner and Walter 2011; Mori et al. 2000). Splicing of *HAC1* mRNA allows translation and downstream expression of ER stress genes such chaperones, disulfide isomerases, and those involved in ER-associated degradation (Rüegsegger et al. 2001; Travers et al. 2000). In mammals, ER stress is sensed through the Ire1 mechanism as well as other pathways (Haze et al. 1999; Yoshida et al. 2001; Harding et al. 2003) Each of these pathways utilizes BiP to some degree to regulate UPR activation and attenuation.

Oxidative protein folding produces peroxides that contribute to the oxidative environment and have the potential to tip the redox balance to unfavorable folding conditions (Tu and Weissman 2004). Recently, studies have elucidated the role of BiP and its co-chaperone Sil1 in the response to OS using budding yeast. Following exposure to OS, hyperoxidation of the ER lumen triggers the UPR via BiP/Sil1 redox sensing (see Fig. 1.1c). Oxidation of BiP leads to inactivation of its ATPase activity similar to the mechanism described for bacterial Hsp70 DnaK (Winter et al. 2005). However, unlike DnaK, loss of ATPase function in BiP leads to enhancement of the holdase activity and prevention of protein aggregation (Xu et al. 2016). Sil1 acts as a reductant to reduce BiP following return to optimal conditions (Seigenthaler et al. 2017). Together they help to integrate redox balance with proteostasis in the ER. BiP/Sil1 redox sensing may enable fine tuning of UPR regulation.

1.1.2 Regulation of Cytosolic Hsp70

The Hsp70 chaperone functions in all aspects of a protein's life span including proper native protein folding, aggregation prevention, and protein degradation. The protein structure and "cradle to grave" function is highly conserved from bacteria to humans (Verghese et al. 2012). The Hsp70 structure contains an N-terminal nucleotide binding domain (NBD) in which co-chaperones interact to enhance ATP hydrolysis and nucleotide exchange, and a C-terminal substrate binding domain (SBD) that cooperates allosterically with the NBD to bind and release peptides.

The bacterial Hsp70 DnaK functions with the co-chaperones DnaJ and GrpE forming a protein folding machine that protects substrates from aggregation during cellular stress such as HS (Szabo et al. 1994; Arsene et al. 2000). However, during OS or dual OS-HS, DnaK loses this protective property (see Fig. 1.2a) (Winter et al. 2005; Zhang et al. 2016). OS results in a drop in ATP levels due to oxidation of metabolic enzymes such as Gap1 that are involved in glycolytic and mitochondrial ADP phosphorylation pathways (Hyslop et al. 1988). When depleted of ATP, ATP-free DnaK is unstable and undergoes domain unfolding exposing its single cysteine to the oxidative environment. Oxidation-induced glutathionylation of C15 leads to inactivation of DnaK, which can be reversed upon return to normal, reducing conditions (Winter et al. 2005).

Hsp70 redox regulation in yeast is poorly understood. As mentioned in the previous section, Cys 264 and Cys 303 in the yeast Hsp70 homolog Ssa1 can be modified in vitro and in vivo by thiol-reactive compounds, specifically the formation of covalent adducts with organic electrophiles (see Fig. 1.2b). The two redox-active residues are located within the NBD and Cys 303 is analogous to Cys 306 in mammalian Hsp72, suggesting that thiol modification may lead to loss of ATPase activity in the yeast isoform as it does for Hsp72 (see below). Although the precise mechanisms of thiol-based regulation of Hsp70 may differ between prokaryotes and eukaryotes, it is striking that this chaperone exhibits conserved redox regulation across such a striking evolutionary distance.

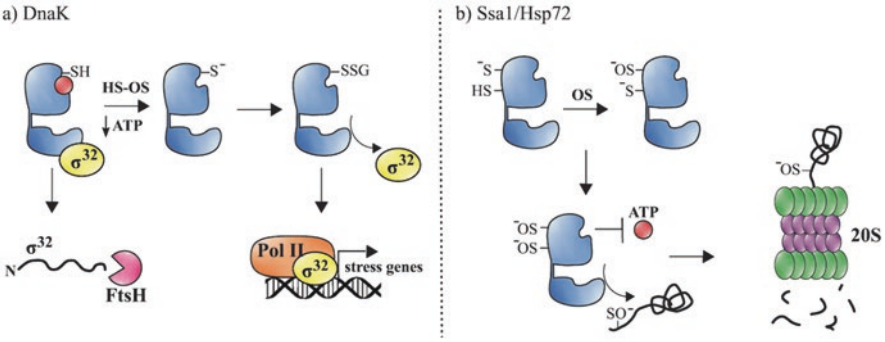

Fig. 1.2 Redox regulation of the cytosolic Hsp70 chaperone. (**a**) Modification of the bacterial DnaK following dual heat shock and oxidative stress (HS-OS). In optimal conditions, DnaK is active as a holdase and facilitates degradation of the σ^{32} transcriptional activator by the protease FtsH. Following dual HS-OS, ATP levels drop resulting in instability of nucleotide-binding domain (NBD). This leads to cysteine oxidation and inactivation of DnaK. Inactive DnaK releases σ^{32} and allows activation of stress genes. (**b**) Potential mechanism of yeast Ssa1 and mammalian Hsp72 cysteine modification during oxidative or thiol-reactive stress (OS). Following redox stress, modification of cysteine(s) leads to conformational changes in the NBD, which may prevent ATP-binding and chaperone "holdase" function leading to degradation of damaged proteins

Multiple isoforms of Hsp70 are expressed in mammalian cells including the constitutively expressed Hsc70 and stress-inducible Hsp72 (Hageman et al. 2011). Through chemical screening, the thiol oxidant methylene blue was identified to inhibit activity of only the inducible isoform Hsp72 (Kelner and Alexander 1985; Wang et al. 2010; Miyata et al. 2012). Inhibition was mediated through oxidation of C306 resulting in conformational changes in the NBD that may block ATP binding (see Fig. 1.2c). Additionally, peroxide was found to inactivate Hsp72 by a similar mechanism. Hsp72 has been implicated in modulation of stability of the Alzheimer's disease-linked protein tau, and inactivation of Hsp72 led to decreased levels of tau (Petrucelli et al. 2004). This suggests that redox modulation of Hsp72 may result in loss of stability for specific clients that require the chaperone at one or more points during their lifetime.

1.1.3 Redox Sensing in the ER

The ER is a specialized compartment for oxidative protein folding of secretory, membrane, and organelle-targeted proteins, many of which require glycosylation and formation of disulfide bonds between Cys residues for proper folding; thus, necessitating an oxidative environment. Consequently, redox characteristics of the ER have been extensively studied using both yeast and mammalian systems. Folding of proteins is aided by oxidoreductases that catalyze and isomerize disulfide bonds using molecular oxygen and the glutathione pool. This leads to accumulation of

glutathione disulfide and peroxide within the ER lumen, which is potentially detrimental at high levels (Tu and Weissman 2004). Therefore, eukaryotic cells have evolved mechanisms to sense and respond to changes in ER redox status (discussed above). Additionally, the resident ER Hsp70 folding system of chaperones (BiP and co-factors) functions along with the oxidoreductases to assist in protein maturation. Here, we focus on how these two types of folding chaperones switch roles during ER stress to promote proteostasis.

1.1.3.1 PDI, the Classic Redox Chaperone

Protein disulfide isomerase, or PDI, is located within the ER lumen and assists in proper protein folding by catalyzing disulfide bond formation, reduction and isomerization of protein thiols (Givol et al. 1964; Laboissière et al. 1995; Freedman et al. 1994). Following translocation across the ER membrane, oxidized PDI will catalyze formation of disulfide bonds between reduced cysteines to promote protein folding and stability. Reduced PDI can also isomerize non-native disulfides that arise and recognizes polypeptide regions that are misfolded due to improper disulfide linkages via a chaperone-like substrate recognition domain. PDI is itself converted between oxidized and reduced states by the oxidoreductase Ero1, which uses molecular oxygen to form the catalytic disulfide, and the glutathione pool, respectively (Frand and Kaiser 1998; Tu et al. 2000; Molteni et al. 2004). PDI has low peptide binding specificity and was shown to prevent protein aggregation of a non-disulfide bond containing protein indicating its chaperone function is independent of its disulfide isomerase activity (Wang and Tsou 1993; Cai et al. 1994; Gillece et al. 1999). In support of this ability, PDI interacts with ER peptides through an acidic amino acid-rich region at the C-terminus and not its redox catalytic sites (Noiva et al. 1993; Klappa et al. 1995). Following ER stress, PDI enzymatic activity is diminished coinciding with oxidization of Ero1, suggesting the inability of Ero1 to regenerate PDI to a reduced state (Nardai et al. 2005). Additionally, PDI expression is induced at the transcriptional level, likely promoting protein oxidation and preventing protein aggregation (Delic et al. 2002). PDI may functionally switch from an oxidoreductase to a molecular chaperone under ER stress conditions that result in modification of its Cys residues. This scenario is similar to what has been shown for other redox-regulated chaperones (see below) (Jang et al. 2004; Zhou et al. 2006).

1.1.3.2 BiP, the Resident ER Hsp70

The Hsp70 family chaperone BiP/GRP78 is the most abundant and best characterized ER-resident chaperone. Like other Hsp70s, BiP binds hydrophobic peptide stretches and facilitates protein folding through the binding and hydrolysis of ATP, which is aided by nucleotide exchange factors and Hsp40 co-chaperones (Knittler

and Haas 1992; Wei and Hendershot 1995; Meunier et al. 2002). Recently, BiP has been shown to be a sensor for redox changes in the ER lumen. The Sevier group found that the conserved cysteine residue C63 in the ATP binding pocket can be oxidized into a SOH and further glutathionylated, leading to decreased ATPase activity (Wang et al. 2014; Xu et al. 2016; Wang and Sevier 2016). As demonstrated using in vitro assays, oxidized BiP retains the capacity to prevent protein aggregation. Passive substrate holdase activity may even be enhanced in the absence of ATP-driven substrate binding and release. Oxidation of BiP may additionally reduce translocation of polypeptides into the ER lumen until redox balance is restored, limiting synthetic flux to promote ER proteostasis.

1.1.3.3 Sil1 Is a BiP Reductase

As an Hsp70-family chaperone, BiP utilizes an ATP hydrolysis cycle to efficiently assist in proper protein folding. Within the yeast ER, the NEFs Sil1 and Lhs1 exchange ADP for ATP to promote BiP ATPase activity, and thereby accelerate the folding cycle. As previously discussed, the redox status of BiP may transmit the redox state of the ER lumen to trigger a response to OS (Wang and Sevier 2016). Redox status of C63 within the yeast BiP Kar2 was found to be reversible and governed by Sil1 (Seigenthaler et al. 2017). Within its N-terminal domain, Sil1 contains a pair of cysteines (C42 and C57) that resemble a thioredoxin oxidoreductase motif characteristic of proteins such as PDI and the peroxiredoxin Tsa1. Seigenthaler et al. found that both cysteines can act as either the attacking nucleophile or resolving cysteine to reduce oxidized BiP. Sil1 specifically interacts with BiP to form a mixed disulfide-bonded intermediate, reducing the protein thiols to restore BiP ATPase activity. Loss of Sil1-BiP interaction prevents recycling of BiP and attenuation of the cellular response to OS shown by increased resistance to the thiol oxidant diamide. This redox regulated pair therefore allowed cells to rapidly sense, respond, and recover from changes in the ER redox state relevant to proteostasis.

1.1.4 Atypical Heat Shock Proteins

Several unrelated and atypical HSP respond to OS by acquiring or activating chaperone activity normally quiescent in the absence of redox stress. Unlike small HSP that form large oligomeric structures and aid in protein disaggregation via passive chaperone “holdase” activity, these chaperones frequently act as dimers to prevent protein aggregation (Jakob et al. 1993). Furthermore, regulation of chaperone activity is governed via distinct mechanistic routes with the common theme of generating robust protein holdase capacity in the presence of thiol-reactive stress.

1.1.4.1 Bacterial Hsp33

The bacterial chaperone Hsp33 is a novel family of ATP-independent chaperones conserved among prokaryotes (Jakob et al. 1999). Hsp33 is regulated by the redox potential of the cellular environment to protect proteins from stress-induced misfolding and aggregation. In optimal, reducing conditions, Hsp33 is maintained as an inactive monomer through C-terminal stabilization of a cluster of four Cys residues by zinc coordination – C232, C234, C265, and C268 (Graumann et al. 2001; Graf et al. 2004). In this conformation, both the substrate binding and dimerization domains are masked. Exposure to OS induces dissociation of zinc from the Cys cluster resulting in a partially unfolded conformation that enables accessibility of the cysteines and substrate binding domain. Subsequent disulfide bond formation between the two Cys pairs induces further conformational changes and dimerization (Barbirz et al. 2000; Graf et al. 2004). These active, oxidized dimers act as passive chaperones with substrate holdase ability (Graumann et al. 2001). Following return to reducing conditions, the oxidized dimer will reduce, release substrate, and disassociate with the combined aid of the thioredoxin and DnaK folding systems (Hoffmann et al. 2004). Deletion of Hsp33 results in an increase in sensitivity to OS and severe dual OS-HS. Conversely, overexpression enhances survival when cells are exposed to severe stress (Jakob et al. 1999; Winter et al. 2005). Furthermore, redox activation of Hsp33, perhaps, functionally replaces OS-inactivated DnaK as described in a previous section.

1.1.4.2 The DJ-1 Family of HSP

The DJ-1/ThiJ/Pfp1 superfamily of HSP is present in bacteria, yeast, and humans (Lee et al. 2003; Wilson et al. 2004; Skoneczna et al. 2007). All contain a conserved nucleophile elbow Cys-containing motif and protease-like catalytic triad, suggestive of peptidase activity; however, structural differences indicate they may have distinct functions. Here we specifically focus on Hsp31 and its role in oxidative stress protection.

The bacterial Hsp31 (bHsp31), was first identified in *E. coli* to be transcriptionally induced by HS via the general stress response regulator σ^S (Richmond et al. 1999; Mujacic and Baneyx 2006). Studies have found bHsp31 functions dually as a glyoxylase and molecular chaperone to prevent protein aggregation in cooperation with the DnaK/DnaJ/GrpE protein folding system (Malki et al. 2003; Mujacic et al. 2004; Subedi et al. 2011). In optimal growth conditions, Hsp31 eliminates toxic metabolic products such as methylglyoxal and acetic acid. Upon exposure to severe HS, bHsp31 undergoes heat-induced conformational changes to expose the conserved hydrophobic patch as well as the catalytic site allowing enhanced peptide binding (Quigley et al. 2004; Sastry et al. 2004). In yeast, deletion of Hsp31 (yHsp31) renders cells sensitive to thiol-reactive stresses including peroxide and cadmium, due to the intracellular accumulation of ROS (Skoneczna et al. 2007). yHsp31 expression is induced by Yap1 indicating a role in protection against

OS. More recent studies have shown yHsp31 to act as a homodimer to eliminate toxic ROS and prevent protein aggregation (Bankapalli et al. 2015; Tsai et al. 2015). How the two homologs functionally switch and whether this activity is redox regulated similarly to human Hsp31 (described below) is yet to be determined. The domain unfolding mechanism of inducing chaperone activity is similar to what has been shown for Hsp33 (described above). Furthermore, heat-induced unfolding exposes the Cys-containing catalytic triad, which may be susceptible to modification in dual HS-OS resulting in deactivation of glyoxylase activity and activation of chaperone activity.

The human Hsp31 homolog DJ-1 has been implicated in oxidative stress resistance as DJ-1 deficient human cells exhibit increased sensitivity to peroxide ultimately leading to cell death (Martinat et al. 2004). In contrast to bacteria and yeast, regulation of its chaperone activity is better understood and more developed. Like the bacterial Hsp33 (described above), DJ-1 chaperone activity is regulated by the redox environment as shown by in vitro "holdase" assays in the presence of the reducing agent DTT and oxidizing peroxide (Shendelman et al. 2004). DJ-1 contains three Cys residues that are all susceptible to oxidation: C106, C53, and C46 (Zhou et al. 2006). Oxidized DJ-1 has been shown to inhibit α-synuclein fibril formation and aggregation, a hallmark of Parkinson's disease, both in vitro and mammalian cell culture, albeit with conflicting results on whether oxidation of C106 or C53 plays the primary role in mediating chaperone activation (Shendelman et al. 2004; Zhou et al. 2006). Overall, oxidation of DJ-1 activates chaperone activity to block protein aggregation and allow cell survival in response to changes in redox status or a disease state.

1.1.4.3 Peroxiredoxins

The yeast thioredoxin-dependent peroxidases, or peroxiredoxin (Prx), are part of the Trx-fold superfamily of antioxidant proteins, which include thioredoxin, PDI, and Dsb proteins (Schröder and Ponting 1998; Jang et al. 2004). These proteins contain a dithiol CxxC motif that functions in protein folding, and enzymatic detoxification of ROS and xenobiotics. Prxs are classified into two groups based on Cys conservation and mechanism of multimerization: 1-Cys and 2-Cys. Specifically, the yeast 2-Cys Prx, Tsa1, forms homomultimers in sizes ranging from low molecular weight (LMW) dimers to >1000 kDa high molecular weight (HMW) structures and is abundant in the cytosol. The multimeric state or size is associated with a specific function: peroxidase or molecular chaperone. As a homodimer or LMW tetramer, Tsa1 functions as a peroxidase to serve as an antioxidant and reduce protein thiols in coordination with thioredoxin. During hyperoxidative conditions, Tsa1 undergoes a thiol-dependent functional switch. Oxidation of the peroxidase active site Cys47 results in inactivation of peroxidase activity and multimerization. These HMW structures are associated with chaperone activity and have been shown to prevent protein aggregation. These chaperones typically form multimeric complexes and are thought to intercalate within protein aggregates as they are found in

isolated insoluble protein fractions (de Jong et al. 1993; Wallace et al. 2015). Furthermore, it has been shown that Tsa1 will localize to sites of aggregation in vivo in response to both HS and OS conditions (Weids and Grant 2014). Although similar to small HSP such as Hsp42 that normally form multimeric complexes and function in protein disaggregation regardless of redox status, Tsa1 is unique in that both multimerization and function are redox regulated.

1.1.5 Regulation of the Ubiquitin-Proteasome System

The eukaryotic proteasome is a specialized protein degradation machine that has both ubiquitin (Ub) dependent and independent conformations (reviewed by Bhattacharyya et al. 2014). Both conformations function as part of the protein quality control network to maintain proteostasis by degrading normal and aberrant proteins. The Ub-dependent 26S proteasome contains the evolutionarily conserved 20S proteolytic core and a 19S regulatory subunit that utilizes ATP and the Ub-machinery to degrade protein substrates. In contrast, the Ub-independent 20S proteasome lacks both the 19S regulatory subunit and requirement for ATP. The core particle consists of two outer alpha rings that regulate proteasome activity and two inner beta rings that utilize caspase-like, trypsin-like, and chymotrypsin-like activity to degrade substrates (Coux, et al. 1996: Groll et al. 1997). The regulatory particle acts an ATPase that recognizes, unfolds and translocates ubiquitinated substrates into the core for degradation.

During OS, there is a preferential switch to primarily utilize Ub-independent proteasomal degradation of damaged proteins (Pajares et al. 2015). Evidence suggests this is due to the increased sensitivity of the 19S regulatory subunit and Ub machinery to OS-induced modifications, such as glutathionylation, as compared to the 20S core (Jahngen-Hodge et al. 1997; Reinheckel et al. 1998). The majority of modifications lead to decreased activity. For example, the regulatory subunits Rpn1 and Rpn2, which allow translocation of Ub-substrates into the 20S core, undergo glutathionylation following exposure to OS resulting in inhibition of 26S proteasome function (Zmijewski et al. 2009). On the other hand, a study has shown that glutathionylation of C76 and C221 of the 20S α5 subunit increased proteasomal activity by triggering and maintaining the core in an open conformation (Silva et al. 2012). Again, this suggests that the Ub-independent machine is preferentially activated for degradation of oxidized proteins whereas the Ub-dependent iteration is inactivated. Furthermore, in human cells, the majority of oxidized proteins are not ubiquitinated with the exception of the molecular chaperones Hsp70, Hsp90, and Hsp60, which is a late consequence of OS; while the purpose of chaperone ubiquitination is unclear, it may help maintain appropriate chaperone levels and facilitate recovery from OS (Kästle et al. 2012).

1.2 Conclusions

The intersection of redox regulation and protein homeostasis is a burgeoning area of investigation that has enabled identification of novel redox sensors and atypical chaperones. These and other known redox PQC components are regulated through cysteine modifications that alter protein activity to control stress responses and promote protein stability and cell viability. Notably, many aspects of integrated redox and protein quality control biology are conserved from bacteria to humans. It is becoming increasingly clear how perturbations in the redox environment are sensed in each compartment. A key goal of future research is to better understand connections between sensing mechanisms and cellular responses. Additionally, while crosstalk at the gene expression level is apparent, how this is achieved locally is unclear. Because many human diseases are now recognized to be fundamentally linked to defects in proteostasis and dysregulation of ROS metabolism, it is expected that insights into how these two phenomena are linked will be of significant future therapeutic benefit.

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Chapter 2
Heat Shock Proteins (HSP) in Stress-Related Inflammatory Diseases

Burcu Hasdemir, Dina Shakran, Sreenivasan Paruthiyil, and Aditi Bhargava

Abstract When first observed nearly 56 years ago, the existence of heat shock proteins was thought to be an anomaly. Since then, heat shock proteins have been shown to play an important role in protecting and preserving protein structure and function in response to various stressors. An estimated 57 million people in the US alone suffer from stress-related disorders. Twice as many women as men suffer from these conditions, which range from autoimmune disorders, anxiety and depression to gastrointestinal and reproductive disorders. In 1936, Hans Selye defined stress as "the non-specific response of the body to any demand of change". Thus, stress is a broad term encompassing psychological, physical, and cellular stress in response to exposure to traumatic experiences, temperature changes, environmental factors, chemicals, and toxins. Exposure to stress can result in inflammatory responses, activation of mitogen-activated protein kinase (MAPK) signaling, and alterations in expression of various heat shock proteins (HSP). The roles of HSP70 and HSP90 remain best characterized in the normal functioning of cells and during diseased state. In this chapter, we will review the role of various heat shock proteins in stress-related inflammatory pathophysiologies of the gastrointestinal tract and the female reproductive system.

Keywords CRF_2 · Endometriosis · IBD · MAPK · Pancreatitis · Preeclampsia · Pregnancy · Rodent models · Sex differences

B. Hasdemir · D. Shakran · S. Paruthiyil
Osher Center for Integrative Medicine, University of California, San Francisco, CA, USA

A. Bhargava (✉)
Osher Center for Integrative Medicine, University of California, San Francisco, CA, USA

Department of ObGyn, University of California, San Francisco, CA, USA
e-mail: Aditi.Bhargava@ucsf.edu

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Abbreviations

Akt	AKT serine/threonine kinase 1
CD	Crohn's disease
CFTR	cystic fibrosis transmembrane conductance regulator
CRF	corticotropin-releasing factor
DSS	dextran sodium sulfate
ER	endoplasmic reticulum
ER-α	estrogen receptor alpha
ER-β	estrogen receptor beta
GRP78	glucose regulated protein 78
HSP	heat shock proteins
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IRE1	inositol requiring enzyme 1
MCP	monocyte chemotactic protein
NF-κB	nuclear factor-kappa B
PERK	PRK-like protein kinase
PRSS1	protease serine 1
PSTI	pancreatic secretory trypsin inhibitor
SPINK1	serine protease inhibitor Kazal-type 1
TLR	toll-like receptor
TNBS	trinitrobenzenesulfonic acid
UC	ulcerative colitis
UPR	unfolded protein response
VCAM-1	vascular cell adhesion molecule 1

2.1 Introduction

Heat ***s***hock ***p***roteins (HSP) serve as molecular chaperones by protecting signaling proteins from being prematurely activated and by keeping proteins properly folded under conditions of various cellular stressors. HSP are one of most versatile class of proteins capable of responding to a myriad of cellular stressors that range from change in temperature, hypoxia, nutritional deficiency, exposure to toxins, physical and psychological stress. While working on types of nuclei acids synthesized in the puffs of polytene chromosomes from the salivary gland of fruit flies in the early 1960s, Ferruccio Ritossa found himself looking at a novel and very rapid puff pattern in response to shift in temperature. Apparently, some co-worker had unintentionally changed the temperature of the incubator being used (Ritossa 1962). Not only did Ritossa have difficulty publishing his findings, the existence of heat shock proteins was thought to be an anomaly. Subsequent researchers including Susan Lindquist were questioned about studying proteins of no physiological importance

(Trivedi 2012). A body of work from the Lindquist Lab established the importance of HSP90 in a plethora of cellular functions, including the role HSP90 in cancer and prion disease (DebBurman et al. 1997; Whitesell and Lindquist 2005). Since their serendipitous discovery, heat shock proteins have been shown to play an important role in regulation of a number of cellular signaling pathways, both under normal physiological and pathophysiological conditions.

Under normal physiological conditions or in the absence of any superimposed stress, constitutively expressed HSP serve housekeeping functions. For example, HSP10 forms a complex with HSP60, which is responsible for accelerating folding, reactivating denatured proteins, and decreasing protein aggregates (Bukau and Horwich 1998). HSP are responsible for trafficking nascent proteins between intracellular compartments and prevent untimely activation of newly transcribed proteins. Additionally, HSP are responsible for tagging highly misfolded proteins or protein complexes for degradation through proteolytic pathways such as proteasome. Interactions of the HSP70 and HSP90 chaperone complexes have distinct functional consequences. The HSP70 complex prevents nascent proteins from untimely activation or interactions, thereby preventing misfolding and aggregation. The HSP90 complex on the other hand, helps maintain the native state of the proteins and keeps them poised for action.

In presence of stressful conditions, HSP are rapidly induced as initially observed by Ritossa (Caggese et al. 1979). A variety of stressful stimuli such as thermal stress, ischemia, hypoxia, nutritional deficiency, oxidants, subsets of interferon and cytokines (inflammation), radiation, industrial xenobiotics such as heavy metals and carbon monoxide, induce HSP synthesis. Infections by bacteria, viruses, parasites, and fungi also result in synthesis of HSP. Other than the extensively studied intracellular HSP, extracellular HSP of uncertain origin are also found. Extracellular HSP70 released from damaged and stressed necrotic cells can elicit innate and adaptive proinflammatory immune responses (Pockley 2003). HSP can turn into immunodominant molecules. Antibodies against endogenous HSP (autoantibodies) as well as antibodies against the HSP of pathogens can serve as biomarkers for diagnosis of various diseases (Wu and Tanguay 2006). In this chapter, we will review the role of heat shock proteins in stress-related pathophysiologies of the peripheral organs that include the gastrointestinal tract and pregnancy-related complications.

2.1.1 HSP and Signaling Pathways in Inflammatory Gastrointestinal Diseases

Heat shock proteins (HSP) interact directly or indirectly with multiple components of mitogen activated protein kinase (MAPK) signaling pathways to alter cellular function during inflammatory stress. MAPKs such as p38-MAPK, JNK-MAPK, the phosphatidylinositol-3 kinase (PI3K)/Akt, and the nuclear factor-kappa B (NF-κB) signaling pathways are all influenced by HSP (Abraham and Cho 2009;

Hasdemir et al. 2016). Through their chaperone activity HSP90 and HSP70 tightly regulate NF-κB signaling—a critical pathway in mediating inflammatory responses (Sevin et al. 2015).

2.1.1.1 HSP and Signaling in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory condition and is classified into two subtypes: Ulcerative colitis (UC) and Crohn's disease (CD). UC involves inflammation of the rectum and colon in an uninterrupted pattern, whereas CD can affect any region of the intestine, often discontinuously, although its presentation occurs primarily in the ileum and colon. In UC, the inflammation is usually confined to the mucosal layer of colonic tissue, whereas in CD, it is transmural, involving all the tissue layers of the gastrointestinal lining. The etiology of IBD is not completely understood, but it is thought that genetic factors, the abnormal immune response of the intestinal immune system to environmental factors, and the dysfunction of the intestinal mucosal barrier against enteric bacteria have a pivotal role in the pathogenesis of the disease (Abraham and Cho 2009). Some studies suggest that psychological stress, depression, and anxiety prolong the clinical course of IBD in terms of symptom severity and relapses (Mikocka-Walus et al. 2007; Vlachos et al. 2014). However, the mechanisms by which stressors contribute to IBD flares are not completely understood. We recently showed that components of the stress response system that include neuropeptide hormones corticotropin-releasing factor (CRF), urocortins, and their G protein-coupled receptors are dysregulated in pediatric patients with CD (Hasdemir et al. 2016). Psychological stress has been shown to induce systemic and mucosal proinflammatory responses in IBD, particularly in UC patients (Mawdsley et al. 2006).

Microbial factors clearly seem to play a role in the initiation and maintenance of the disease. Although their efficacy in ameliorating IBD symptoms is equivocal, ways to manipulate the intestinal flora beneficially, through probiotics or fecal microbial transplants for example, have been considered as therapeutic options (Jonkers and Stockbrugger 2003; Suskind et al. 2015). Changes in diet, use of medication, treatment with immunomodulators, and biologics such as anti-TNF-α antibodies, corticosteroids, aminosalicylates (5-ASA), and antibiotics are all used to manage IBD symptoms. Eventually, approximately 23–45% of UC patients and ~75% of CD patients will require surgery as the available treatments are no longer effective.

In IBD patients, the intestinal immune system fails to adequately regulate itself. Dysbiosis of the gut-associated bacteria in IBD patients also contributes to abnormal immune signaling pathways. Dysregulation of signaling pathways including toll-like receptor and NF-κB disrupts the intestinal barrier resulting in uninhibitedly release of effector T cells, key immune cells mediating inflammation in Crohn's disease (Wei and Feng 2010). Elevated levels of Hsp60 and Hsp10 have been found in the colon mucosa in IBD compared to normal controls (Rodolico et al. 2010). Possible explanations include both a non-disease specific reaction to mucosal stress

and an anti-pathogenic/cytoprotection mechanism as well as the opposite: a pathogenic effect typical of autoimmune diseases where Hsp60 and Hsp10 became auto-antigens perhaps due to structural alterations they suffered (Rodolico et al. 2010). Mucosal expression of the constitutive HSP70 (HSP70c) and inducible (HSP70i) forms has been reported in biopsies from patients with IBD (Ludwig et al. 1999). Compared to non-IBD individuals, expression of HSP70 was significantly increased in patients with ulcerative colitis and to a lesser degree in patients with Crohn's disease (Ludwig et al. 1999). HSP70c and HSP70i exhibited strong epithelial staining in both disease subtypes. In biopsies of patients with active UC, HSP70c was downregulated in inflamed epithelium (Vlachos et al. 2014). The different pattern of HSP70 expression in Crohn's disease compared to ulcerative colitis points to a distinct protective and immunological function (Ludwig et al. 1999). Mucosal and submucosal mononuclear cells showed increased HSP70c expression in Crohn's disease patients and, to a lesser degree, in ulcerative colitis patients. In patients with active cryptitis, neutrophils expressed inducible HSP70i (Vlachos et al. 2014). Regression analysis showed a strong positive relationship between HSP70i expression in the neutrophils and depression and anxiety scores in UC patients (Vlachos et al. 2014). Thus, HSP appear to regulate signaling in both mucosal and immune cells of the gut.

2.1.1.2 HSP in Murine Models of IBD

In mice with dextran sodium sulfate (DSS)-induced colitis and in regulatory T-cells upon exposure to heat shock or inflammatory stress, HSP90 levels were increased. HSP90 inhibitor 17-allylaminogeldanamycin showed efficacy in reduction of colonic inflammation in DSS-induced and adoptive T-cell transfer models of colitis (Collins et al. 2013). In another murine model of colitis that used trinitrobenzenesulfonic acid (TNBS), HSP90 levels were decreased in colons of mice (Hasdemir et al. 2016). In DSS-induced colitis, T-cells and TNF-α do not play a significant role, whereas both T-cells and TNF-α are involved in the TNBS-induced colitis. This may explain the opposite effect on colonic HSP90 levels in these two models of colitis. While both studies used C57BL/6 mice, the study by Collins et al. used male mice housed in specific pathogen free conditions, whereas the study by Hasdemir et al. used both male and female mice housed in standard housing. Gut microbiome plays an important role in modulating inflammation and signaling in both animal models of colitis and in people with IBD. Thus, modulation of HSP90 is complex. Hasdemir et al. further showed that in wild-type mice with no inflammation, female mice have less HSP90 levels in colons compared with their male counterparts. This sex difference in baseline expression of HSP90 was abolished in mice with haploinsufficiency of stress receptor CRF_2. During colitis, male and female mice had comparable levels of HSP90. On the other hand, baseline phospho-HSP27 levels did not differ between the sexes and did not change with colitis induction in wild-type male and female mice. However, in mice haploinsufficient for CRF_2 receptor, phospho-HSP27 levels decreased during colitis (Hasdemir et al. 2016).

HSP90 and HSP27 are known to regulate p38 MAPK signaling (Larsen et al. 1997; Ota et al. 2010). Levels of phospho-p38 (p-p38) MAPK tended to increase in the colons of male and female wild-type C57BL/6 mice compared with control mice with no colitis (Hasdemir et al. 2016). In the same study, male and female CRF_2 haploinsufficient mice, showed decreased levels of phospho-p38 MAPK failed to increase in response to TNBS-induced colitis. Data analysis revealed that colonic HSP90 and phospho-p38 MAPK levels showed an inverse correlation in wild-type male mice, whereas no correlation was seen in female wild-type mice Fig. 2.1. HSP are known to modulate expression of pro-inflammatory cytokines, including that of TNF-α. Consistent with this idea, we found an inverse correlation between HSP90 and TNF-α levels during colitis in both male and female wild-type mice (Fig. 2.2). Previously, we have shown that TNF-α levels increased and HSP90 levels decreased in the colons of mice with colitis, but a correlation was not established (Hasdemir et al. 2016). Very few studies have examined sex-specific expression of HSP. Thus, stressful stimuli, context, and sex-specific dichotomy may be important in designing therapeutics that target HSP and MAPK pathways in gastrointestinal diseases.

The significance of altered HSP levels and presence of more severe ER ultrastructure damage in CRF_2 deficient mice during inflammatory stress (Hasdemir et al. 2016; Kubat et al. 2013) can be explained by a recent observation we made (Hasdemir et al. 2017). We found HSP70 in the CRF receptor complex as determined using immunoprecipitation and mass spectrometry studies in HEK293 cells. CRF_2 receptors show both cell surface and intracellular localization and mediate stresscoping cellular function. When intracellular, CRF_2 maybe associated with the ER membrane (Fuenzalida et al. 2014). Taken together, these data suggested to us that intracellular CRF_2 mediates stresscoping actions by incorporating HSP70 in its supercomplex and help preserve nascent proteins that are crucial for bring cell function back to homeostasis from being misfolded or aggregated.

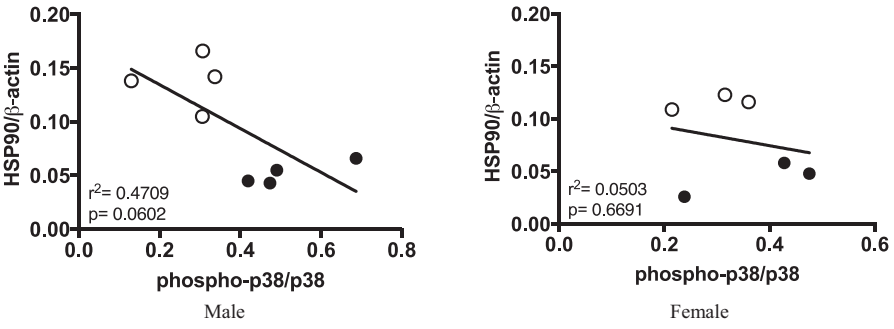

Fig. 2.1 Relationship between phospho-p38 and HSP90 levels in the colons of mice. Regression analysis revealed an inverse relationship between colonic phospho-p38 levels and HSP90 levels in male mice, whereas no relationship was seen in female mice. Phospho-p38 levels increased and HSP90 levels decreased during TNBS-colitis. ○: Saline-treated control mice, ●: TNBS-treated mice

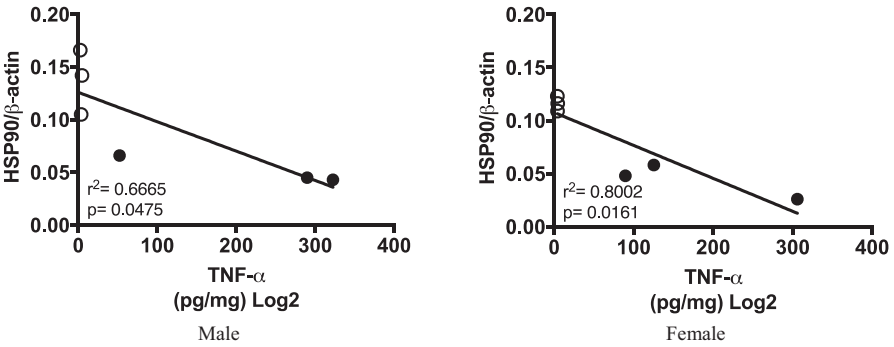

Fig. 2.2 Exposure to inflammatory stress increased TNF-α levels and decreased HSP90 levels in the colons of both male and female mice. Regression analysis revealed a negative relationship between colonic TNF-α and HSP90 levels. ○: Saline-treated control mice, ●: TNBS-treated mice

The PI3K/Akt signal transduction pathway is closely associated with the regulation and release of cytokines such as the cytokine TNF-α, which play an important role in the abnormal immune response and development and progression of ulcerative colitis. In a mouse model of colitis, treatment with a PI3K inhibitor, wortmannin, reduced the levels of p-Akt and TNF-α in the colon and significantly alleviated the inflammation (Huang et al. 2011). In cultured cells, inhibition of PI3K with pharmacological agents reduced HSP70 and HSP90 levels in an Akt-dependent manner (Zhou et al. 2004). Thus, there is a bidirectional regulation between HSP and signaling pathways, such as MAPK and PI3K/Akt in the gut.

2.1.1.3 HSP Modulation in Rodent Models of Physical and Psychological Stress

Physical stressors induce HSP in colonic tissue in vivo. Restraining C57BL/6 mice for 2 h effectively induced HSP70 in colonic epithelia (Matsuo et al. 2009). Glucocorticoid levels increase during restraint stress via activation of the hypothalamic-pituitary-adrenal axis (Bhatnagar and Dallman 1998). Antagonism of glucocorticoid receptors or treatment of mice with antibiotics abolished stress-induced upregulation of HSP70 (Matsuo et al. 2009). HSP target the cell surface toll-like receptors (TLRs) (Schaefer 2014). Stress-induced glucocorticoids compromised the integrity of the epithelial tight junctions, thereby allowing LPS from the commensal flora to access the lamina propria and activate TLR4. TLR4 activation in turn was required for HSP70 induction in this study (Matsuo et al. 2009).

We find that in BALB/c strain of male mice exposed to a regimen of chronic stressor showed an increase in splenic T-cells and fecal corticosterone levels (Gurfein et al. 2017). In the colons of mice exposed to chronic stressors described in that study, HSP90 levels showed an inverse relationship with fecal corticosterone levels (Fig. 2.3). Our finding that HSP90 levels decreased as stress biomarker

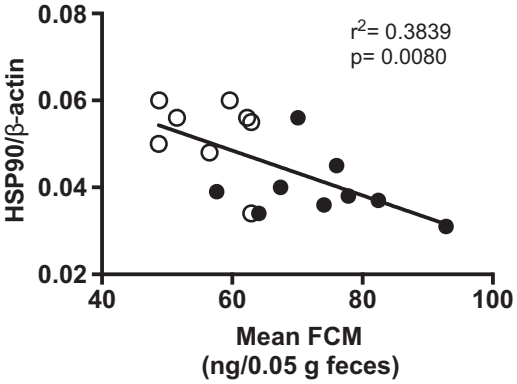

Fig. 2.3 Exposure to stress increased mean fecal corticosterone metabolites (FCM) and decreased colonic HSP90 levels in male mice. Regression analysis revealed a negative relationship between mean FCM concentration and colonic HSP90 expression (r2 = 0.3839, p = 0.0080). ○: Control mice housed in enriched environment, ●: Stressed mice

corticosterone levels increased, is in agreement with our observations that HSP90 levels decreased in experimental colitis (Hasdemir et al. 2016). Thus, depending on the type of stress or insult, HSP90 levels are modulated in a distinct manner. This paradoxical role of HSP90 is consistent with the observations made by Lindquist, who proposed that HSP90 functions as a capacitor and HSP90 isoforms play a dual role in cell physiology (Rutherford and Lindquist 1998).

2.1.2 HSP Signalling in Exocrine Pancreactic Disease

Pancreatitis is a progressive inflammatory disease affecting the exocrine pancreas (Giri et al. 2017; Lankisch et al. 2015; Sah et al. 2014). Based on the severity and clinical presentation of the disease, pancreatitis is classified into acute and chronic. Pancreatitis is idiopathic in origin, with risk factors such as gallstones, endoscopic retrograde cholangiopancreatography, smoking, and alcohol. Although acute pancreatitis appears to be equally prevalent in both sexes, chronic pancreatitis is more common in men (Yadav and Lowenfels 2013; Yadav et al. 2014). Chronic pancreatitis is a progressive and irreversible inflammatory condition resulting in fibrotic pancreas, blocked ducts, and both exocrine and endocrine insufficiency (Kloppel and Maillet 1995). Nonalcoholic chronic pancreatitis is usually idiopathic and is also associated with abnormal epithelial ion transport and/or mutations in several genes including cystic fibrosis gene (CFTR), trypsinogen (PRSS1), and trypsin inhibitors (PSTI or SPINK1). Presence of mutations in both CFTR and SPINK1 increases chronic pancreatitis risk dramatically (Noone et al. 2001). Elevated serum amylase and lipase levels along with medical history and physical exam are used to diagnose pancreatitis.

2.1.2.1 HSP, Unfolded Protein Response, and MAPK Signaling in Rodent Models of Pancreatitis

While a number of animal models of acute pancreatitis have been developed, few models exist for chronic pancreatitis (Gorelick and Lerch 2017). Administration of secretagogue such as caerulein (analog of cholecystokinin) and nutrient overload with arginine are two most commonly used models of acute pancreatitis in mice and rats. Treatment with these agents result in elevated trypsinogen, serum amylase levels, and Ca^{2+} overload, zymogen activation, endoplasmic reticulum and mitochondrial dysfunction, and cytokine release in acinar cells. Elevated intracellular Ca^{2+} levels are accompanied by activation of the endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in rodent pancreatitis models.

The heat shock protein HSPA5 (GRP78 or BiP) is part of the general chaperone response machinery activated as part of the UPR and ER stress signaling (Pfaffenbach and Lee 2011). In unstressed conditions, GPR78 is bound to the three UPR transmembrane sensors, namely: (i) activating transcription factor 6 (ATF6), (ii) PRK-like protein kinase (PERK), and (iii) inositol requiring enzyme 1 (IRE1). GRP78-bound UPR sensors remain inactive, thereby preventing UPR from being activated under homeostasis. By the virtue of their ability to protect proteins from being misfolded and damaged, HSP play a significant role in pancreatic disease (Dudeja et al. 2009). Under conditions of acinar stress, injury, and ER stress, accumulation of unfolded proteins results in sensor proteins being released from GPR78 to activate unfolded protein response. Activation of UPR feeds forward to further increase GRP78 expression levels. In turn, GRP78 binds to misfolded and unfolded proteins to keep them in a functional state and bring cells back to homeostasis (Yang et al. 2000). GRP78 and other members of the HSP70 family recognize and bind to hydrophobic patches on a misfolded or unfolded protein in an ATP-dependent manner and increase the residence time of these proteins in ER and ensure incorrectly folded proteins do not leave the lumen (Kaufman et al. 2002).

In a rat model of acute pancreatitis, GRP78 has been associated with apoptosis of neutrophils in a PI3K/Akt mediated pathway and results in deterioration of inflammation and severe acute pancreatitis (Xu et al. 2015). Several molecular mechanisms that lead to changes in HSP expression acute pancreatitis models have been proposed, including: (i) decrease in cellular contents of ATP, (ii) accumulation of unfolded proteins, and (iii) ischemia due to compromised microcirculation (Plusczyk et al. 1997). Agents that cause pancreatitis such as caerulein, bile acids, palmitoleic acid, and palmitoleic acid ethyl ester decrease levels of ATP in pancreas. Decreased intracellular ATP levels serve as a strong signal for triggering stress response. Thus, based on this body of work, it appears that damage to ER and mitochondria during pancreatitis, comprises the ability of HSP to perform their chaperone function.

Sex differences in UPR and endoplasmic reticulum stress signaling were reported in a murine model of caerulein-induced pancreatitis (Kubat et al. 2013). C57BL/6 wild-type mice with caerulein-induced pancreatitis showed higher histological damage than female wild mice, but milder endoplasmic reticulum damage as

determined by electron microscopy ultrastructure (Kubat et al. 2013). In mice lacking the stress receptor CRF_2, caerulein-induced endoplasmic reticulum ultrastructure damage was much more pronounced compared with wild-type male littermates. Female mice showed milder histological damage than male mice. Interestingly, in this model of acute pancreatitis, pERK1/2 pathway appeared to be activated only in female mice (Kubat et al. 2013). However, studies have yet to address the sex specific role of HSP in pancreatitis.

Expression of HSP27 and HSP70 is increased rodent models of pancreatitis (Dudeja et al. 2009; Ethridge et al. 2000; Tashiro et al. 2001). Both HSP27 and HSP70 exert anti-apoptotic function (Garrido et al. 2006). HSP27 was activated as a result of cellular stress and activated Akt/PKB pathway (Konishi et al. 1997). Prior induction of HSP70 expression by a variety of stimulants appeared to protect acinar cell damage induced by caerulein or arginine in rats (Bhagat et al. 2008; Frossard et al. 2002; Giri et al. 2017). These studies showed that HSP70 provided protection during inflammatory stress, irrespective of the insult that resulted in its induction.

2.1.2.2 HSP and Signaling in People with Chronic Pancreatitis

Chronic pancreatitis describes a broad range of fibro inflammatory disorders of the exocrine pancreas. Chronic pancreatitis and the accompanying inflammation is a risk for pancreatic cancer (Maisonneuve and Lowenfels 2002). HSP27 levels were elevated in serum of chronic pancreatitis and pancreatic cancer patients compared to healthy controls (Liao et al. 2009). HSP70 overexpression was demonstrated in pancreatic tissues of people with chronic pancreatitis (Gress et al. 1994). In humans, three members of HSP70 have been reported (Milner and Campbell 1990). HSP70-2 gene mutant allele was present in significantly higher frequency in patients with acute pancreatitis and pancreatic carcinoma compared to control population (Srivastava et al. 2012). Frequency of mutant allele was not associated with disease severity. While association between HSP90 and TNF-α expression have been observed in other gastrointestinal inflammatory diseases (Fig. 2.2), it is unclear whether TNF-α and TNF-β present on the chromosome 6 in close proximity to HSP70-2, serve as genetic modifiers of HSP70 expression in pancreatitis (Srivastava et al. 2012). As pancreatic biopsy tissues from people with acute pancreatitis are rarely obtained, mechanistic studies in human acute pancreatic tissue are lacking.

2.1.3 HSP Signalling in Inflammatory Obstetric and Gynecological Disorders

2.1.3.1 HSP in Normal Pregnancy

The expression of heat shock proteins is well characterized in pregnancy, but the potential sources of HSP in pregnancy is not yet fully established. Role of HSP70 is best studied in pregnancy and related pathophysiologies. HSP70 was present in the

serum of healthy non-pregnant and pregnant individuals (Molvarec et al. 2010). Two forms of HSP70 are present in pregnancy: extracellular HSP70 and intracellular HSP70 (Basu et al. 2000; Multhoff and Hightower 1996; Soltys and Gupta 1997). Extracellular HSP70 acts as an intercellular stress-signaling molecule and represents an ancestral danger signal of cellular stress or damage that elicits the innate and adaptive proinflammatory immune response (Pockley 2003). Extracellular HSP70 acts through binding to various surface receptors on antigen presenting cells, stimulating expression of their proinflammatory cytokines, chemokines, nitric oxide production, and co-stimulatory molecule expression (Asea 2005; Asea et al. 2000, 2002; Basu et al. 2000, 2001). Extracellular HSP70 induces the maturation, migration and activation of dendritic cells and stimulates the cytolytic activity of natural killer cells and upregulating γ/δ T-cells (Asea 2005; Basu et al. 2000; Millar et al. 2003; Wang et al. 2002). These immune cells are part of the cell-mediated (cytotoxic) immune response typically suppressed in normal pregnancy (Wegmann et al. 1993). To increase the chances of a successful pregnancy, monocytes and granulocytes from the maternal innate immune system sequester HSP70, which decreases their concentration in the bloodstream and maintains immunologic tolerance to the fetus. Failure of the maternal immune system to remove HSP from the peripheral circulation results in several pregnancy complications, including rejection of the fetus (Delneste et al. 2002; Sacks et al. 1999; Theriault et al. 2005). Thus, HSP70 works as a rheostat to fine tune the maternal immune system responses to the developing fetus.

2.1.3.2 HSP in Preeclampsia

Preeclampsia is a multi-system pregnancy-specific disorder that affects maternal vascular function and fetal growth (Ekambaram 2011). Specific characterizations of the disease include systolic blood pressure greater than 140 mmHg and a diastolic blood pressure greater than 90 mmgHg and proteinuria, among other factors (American College of Obstetricians and Gynecologists and Pregnancy 2013). The key characteristic of preeclampsia is endothelial dysfunction (Molvarec et al. 2011), which results in symptoms such as visual disturbances, persistent headaches, and epigastric pain (Gardner 2005; Practice 2002). There are two subtypes of preeclampsia: early-onset preeclampsia and late-onset preeclampsia (Staff et al. 2013). Early-onset preeclampsia occurs less than 33 gestational weeks into the pregnancy and late-onset preeclampsia occurs at or after 34 gestational weeks, with the latter being more prevalent. Preeclampsia is one of the leading cases of maternal and perinatal morbidity and mortality globally, accounting for 50–60,000 deaths annually worldwide (American College of Obstetricians and Gynecologists and Pregnancy 2013). Etiology and pathogenesis of preeclampsia is not fully understood and the only known treatment is to deliver the fetus and placental removal.

Endothelial cell dysfunction is a key symptom and contributes to the maternal inflammatory response system in preeclampsia (Ekambaram 2011). HSP70 levels were increased during preeclampsia and showed a positive correlation with C-reactive protein, an acute phase reactant and a marker for inflammation as well as

with malondialdehyde, a marker for oxidative stress (Molvarec et al. 2010). Oxidative stress in the placental environment leads to hypertension by reducing vasodilating agents, which may cause endothelial cell dysfunction (Mutlu-Turkoglu et al. 1999). The dysfunctional endothelial cells can be activated by internalized HSP70 (Molvarec et al. 2011), which produce adhesion molecules that mediate the adherence of inflammatory cells, resulting in the production of cytokines such as IL-6 or IL-1 (Ekambaram 2011). Increased levels of HSP70 help in protein refolding and maintaining multi-protein complexes under oxidative stress to bring cellular function back to homeostatic state (Ogi et al. 1999; Padmini and Lavanya 2011). Increased risk of apoptosis and necrosis was seen in preeclampsia (Walsh and Wang 1993; Wiktor et al. 2000). HSP70 blocked apoptosis by inhibiting the release of pro-apoptotic factors from the mitochondria (Mosser et al. 2000). However, over production of HSP70 inhibited cell growth or induced cell death as a protective measure (Ekambaram 2011).

2.1.3.3 HSP in Endometriosis

Endometriosis affects women during their reproductive period, causing symptoms such as pelvic pain, and often result in infertility. The disease is characterized by the presence of endometrial glands and stroma outside of the uterine cavity, primarily in the pelvic compartment. Retrograde menstruation is widely accepted as a major cause of the disease. Endometrial fragments driven through the fallopian tubes reach the peritoneal cavity, implant, grow and invade onto pelvic structures (Vercellini et al. 2014). The disease is considered hormone-dependent with estrogen and progesterone playing a key role in the pathogenesis of endometriosis. Estrogens fuel ectopic endometrium growth, and altered estrogen signaling pathways have been associated with the disease (Bulun 2009; Bulun et al. 2012). The overexpression of the estrogen receptor contributes to the resistance to selective actions of progesterone in these cells (Burney et al. 2007). Due to progesterone resistance in ectopic endometrial growth, genes essential to embryo implantation (Wei et al. 2009), are dysregulated in the endometrium of affected women and lead to infertility (Burney et al. 2007).

While the pathophysiology of endometriosis is not fully known, the immune system plays a role in the growth and persistence of endometriosis (Khan et al. 2009). The endometrium of patients with endometriosis showed alternations in cellular and humoral immunity as a result of the tissue spreading outside the uterus. These abnormalities include an increased number of peritoneal macrophages, γ/δ T-cells and B cells (Ota et al. 1996a, b; Witz et al. 1994). Increased LPS concentration in peritoneal fluid of women with endometriosis compared to women who did not have the disease was reported (Khan et al. 2009). LPS was shown to stimulate macrophage to release cytokines and growth factors such as VEGF, IL-6 and TNF-α. Thus, LPS can cause pelvic inflammation in an autocrine or paracrine mechanism (Khan et al. 2005, 2009).

Like in preeclampsia, endometriosis is also associated with increased systemic oxidative stress; however, the mechanisms by which endometriosis could trigger oxidative stress is still poorly understood (Kajihara et al. 2011). Increased circulating levels of HSP70 correlated with increased LPS concentration. It is thought that HSP70-bound LPS in turn bound to the surface receptors, including TLR2 and TLR4 and stimulated the production of proinflammatory cytokines and chemokines (Asea 2005; Asea et al. 2000, 2002; Basu et al. 2000, 2001). The proinflammatory cytokines induced pelvic inflammation and TLR4-mediated growth of endometriosis (Kajihara et al. 2011; Khan et al. 2008).

HSP60 was detected in the peritoneal fluids from women with endometriosis and may have originated from two sources. The first source was ectopic endometrium itself, where presence of HSP was first shown, and the second source was locally activated macrophages (Koga et al. 1989; Tabibzadeh et al. 1996). HSP60 released at the site of implanted endometrium lead to chronic macrophage and T-cell activation thereby impeding fertility (Kligman et al. 1996). T-lymphocytes were activated by surface-expressed HSP60 and released interferon γ, which further stimulated macrophages and released proinflammatory cytokines IL-1 and TNF-α (Ferm et al. 1992; Koga et al. 1989). HSP60 released from ectopic endometrium may be associated with immune activation and endometriosis-associated infertility (Kligman et al. 1996). Besides HSP70 and HSP60, HSP27 plays a role in endometriosis. HSP27 levels were also increased in the serum of women with endometriosis (Ota et al. 1997). HSP27 is thought to be a marker for macrophages- or T-cells-mediated immune responses in endometriosis (Ota et al. 1996a, b; Witz et al. 1994).

2.2 Conclusions

HSP are versatile proteins involved in the regulation of a myriad of cellular processes. While the physiological role of HSP is to keep proteins from being prematurely activated, forming aggregates, and/or properly folded, HSP can exert both protective and damaging effects under conditions of pathophysiology. In inflammatory gastrointestinal diseases where stress is a major contributing factor, presence of HSP has been described. In the gut, HSP70, HSP90, HSP27 all appear to regulate MAPK, JNK, ERK, and PI3K/Akt signaling and cytokine expression. Very few studies have examined whether HSP regulate signaling differentially between the sexes. HSP90 appears to inversely correlate with phospho-p38 expression in colons of male mice with inflammation, whereas it is inversely correlated with TNF-α in both sexes. In pancreatic pathophysiology, the role of HSPA5 or GRP78 is best characterized. HSPA5 plays a significant role in oxidative and ER stress pathways activated in the acinar cells during pancreatitis. CRF_2, a GPCR that mediates stress-coping functions in cells might do so by bringing HSP70 in close proximity to nascent proteins whose functions are crucial in bringing cells back to homeostasis. HSP70 in turn would then prevent misfolding and aggregation of proteins in the CRF_2 supercomplex under conditions of stress and inflammation. In healthy

pregnancy, HSP70 is removed from the circulation by maternal innate immune response. In inflammatory conditions associated with the female reproductive system, such as preeclampsia and endometriosis, the role of HSP70 is best characterized and modulates signaling of both the adaptive and innate immune systems. Role of other HSP is less known in peripheral organs; while role of HSP in mediating sex-specific signaling under normal physiological conditions or pathophysiological conditions is lacking. HSP are being investigated as therapeutic targets in many diseases; thus, their role in modulating function and signaling in the body needs to be better characterized to minimize side effects from HSP-directed treatments.

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Chapter 3
Heat Shock Response and Metabolism in Skeletal Muscle

Yuki Tamura

Abstract Skeletal muscle comprises approximately 40% of the total body mass in humans. It plays important roles in locomotion, metabolism and endocrine signaling. We and others have previously described the biological responses/adaptations of skeletal muscle to heat stress, the contributions of heat shock proteins to the cellular processes underlying the muscle response to heat stress, and the therapeutic potential of manipulating heat stress and heat shock proteins in skeletal muscle. In this chapter, I briefly summarize current understanding of the heat stress-induced regulation of protein, glucose and mitochondrial metabolism in skeletal muscle. Furthermore, I overview future perspectives on studies of the heat shock response in skeletal muscle biology.

Keywords Atrophy · Glucose · Heat shock proteins · Hypertrophy · Mitochondria · Skeletal muscle

Abbreviations

AMPK	AMP-activated protein kinase
BGP-15	O-[3-piperidino-2-hydroxy-1-propyl]-nicotinic amidoxime
CaMKII	Ca2+/calmodulin-dependent protein kinase II
DEPTORDEP	domain-containing mTOR-interacting protein
FOXO	forkhead box O
GLUT	glucose transporter
HSE	heat shock element

Y. Tamura (✉)
Graduate School of Health and Sport Science, Nippon Sport Science University, Tokyo, Japan

Faculty of Sport Science, Nippon Sport Science University, Tokyo, Japan

Center for Coaching Excellence, Nippon Sport Science University, Tokyo, Japan

Research Institute for Sport Science, Nippon Sport Science University, Tokyo, Japan
e-mail: y-tamura@nittai.ac.jp

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HSF1	heat shock factor 1
HSP	heat shock protein
IGF-1	insulin-like growth factor 1
JNK	c-Jun N-terminal kinases
LC3	microtubule-associated proteins 1A/1B light chain 3
MAPK	mitogen-activated protein kinase
MFN2	mitofusion 2
MLST8	MTOR associated protein
mTOR	mechanistic target of rapamycin
NRF	nuclear respiratory factor
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PP2A	protein phosphatase 2
PPAR	peroxisome proliferator-activated receptor
PRAS	proline-rich Akt substrate
RAPTOR	regulatory-associated protein
ROS	reactive oxygen species
UPR	unfolded protein response
VDAC	voltage-dependent anion channels

3.1 Introduction

In humans, skeletal muscle comprises approximately 40% of total body mass. It clearly plays important roles in the activities of daily life and in exercise/sport. Furthermore, it is the largest tissue regulating systemic metabolism. For example, 70–80% of circulating glucose is taken up by and metabolized in skeletal muscle (DeFronzo et al. 1981). In addition, skeletal muscle can convert kynurenine (a liver-derived molecule associated with psychological depression) into kynurenic acid, improving stress-induced depression (Agudelo et al. 2014). More generally, skeletal muscle-initiated organ-crosstalk is a research field that has expanded recently (Schnyder and Handschin 2015). For instance, muscle-derived hormone-like peptides (myokines) such as IL6, irisin and meteorin-like trigger adipose thermogenic remodeling and promote hippocampus memory formation (Schnyder and Handschin 2015). Skeletal muscle is highly plastic in response to local and systemic conditions such as the mechanical and nutritional environment. Exercise/training improves skeletal muscle mass and functions. In contrast, skeletal muscle health is impaired by disuse, injury, aging and disease. Taken together, emerging evidence strongly indicates that the maintenance or improvement of skeletal muscle mass and function could be a good therapeutic approach for improving quality of life. We and others have previously described (1) biological responses/adaptations to heat stress, (2) the contributions of heat shock proteins (HSP) to the underlying cellular processes, and (3) the therapeutic potential of targeting heat stress and HSP in skeletal muscle. In this section, I briefly summarize current understanding of the heat stress-induced regulation of protein, glucose and mitochondrial metabolism in skeletal muscle.

3.1.1 Heat Shock Response and Heat Shock Proteins in Skeletal Muscle

The heat shock response and induction of HSP are believed to be essential biological processes for the maintenance of protein homeostasis (proteostasis) (Schlesinger 1990). The most well-accepted, basic role of HSP is their function as molecular chaperones. HSP helps de novo protein folding, the refolding of denaturized proteins, protein trafficking, and proteolysis. Stress-inducible HSP expression is driven via the activation of heat shock responsive transcriptional factor 1 (HSF1). Under basal conditions, HSF1 exists as monomer and in a repressed state for heat shock element (HSE) binding and transcriptional activity. In contrast, under stress conditions including heat shock, proteotoxicity or physiological stress (for example, mechanical stress, osmolality shock, energy imbalance), HSF1 forms a trimer, binds to HSE and promotes the transcription of target genes (especially HSP). Induction of the heat shock response and HSP in skeletal muscle can be triggered by exercise and drugs (for example, BGP-15, celastrol) as well as heat stress (Henstridge et al. 2014; Ma et al. 2015). Both a single bout of endurance exercise and chronic endurance training increase several types of HSP (for example, HSP72, HSP60, HSP10, HSP25) (Morton et al. 2009). Interestingly, HSP induced by a single bout of exercise return to pre-exercise level within 2–4 days (Oishi and Ogata 2012). However, elevated HSP induced by chronic endurance training does not return to pre-training levels for at least 28 days (Oishi and Ogata 2012). This suggests that the protein stability or half-life of HSP can be modulated by repeated bouts of exercise. However, the regulation of HSP protein stability is not well understood.

3.1.2 Heat Shock Response and Myofibril Remodeling

Fundamentally, skeletal muscle mass is determined by the net balance between protein synthesis and protein breakdown. Mechanistic (mammalian) target of rapamycin complex 1 (mTORC1) has been extensively studied as a key phosphokinase that controls protein synthesis. mTORC1 is a protein complex composed of mTOR, RAPTOR, MLST 8, PRAS 40 and DEPTOR. mTORC1 is activated by amino acids (especially leucine), mechanical loads, insulin and growth factors (for example, insulin-like growth factor-1 [IGF-1]). When mTORC1 is activated, it both inhibits the translation initiation inhibitor, and activates ribosomes. In recent years, ribosomal biogenesis has also attracted attention as a factor controlling skeletal muscle protein synthesis. For further details on protein synthesis in skeletal muscle, please refer to a review by Yoon (2017).

On the other hand, protein degradation mechanisms can be divided into (1) the ubiquitin–proteasome system, (2) the autophagy–lysosome system, and (3) the apoptosis system. The ubiquitin–proteasome system is a mechanism to selectively degrade proteins labeled with poly-ubiquitin chains. MuRF1 and Atrogin 1 have

been identified as the E3 ubiquitin ligases of myofibers in skeletal muscle. The autophagy–lysosomal system is a mechanism for the non-selective proteolysis of proteins following their delivery by autophagosomes to lysosomes. Interestingly, autophagy acts as double-edged sword in the regulation of skeletal muscle mass—skeletal muscle atrophy is induced by both excessive and insufficient autophagy. Although autophagy was originally discovered as a non-selective proteolysis mechanism, some types of selective autophagy are now known (for example, mitochondria-selective, "mitophagy"; described below). Apoptosis is a type of programmed cell death. Following the release of cytochrome c from the mitochondria into the cytoplasm, several steps of caspase signal transduction are activated culminating in caspase-3 cleaving genomic DNA. In recent years, the importance of mitochondria-independent apoptosis (that is, endoplasmic reticulum stress-initiated cell death) in skeletal muscle mass regulation has increasingly been recognized. These protein degradation systems can act cooperatively or independently, depending on the cellular environment. The expression of genes encoding proteolytic enzymes is controlled by the FoxO transcription factor family. For further details on proteolysis mechanisms, please refer to a review by Bonaldo and Sandri (2013).

The heat shock response and HSP affect skeletal muscle mass in the basal state and in overloading/unloading conditions. In studies using cultured cells, it has been shown that myotube diameter is decreased by HSP 72 knockdown (Gwag et al. 2015). An analysis of skeletal muscle from HSF1-deficient mice showed that the proportion of slow muscle fibers was reduced (Ohno et al. 2015). Consistent with this observation, continuous heat stress promotes the differentiation of myoblasts to slow-fiber dominant myotubes (Yamaguchi et al. 2013). There is a consensus that heat stress suppresses disuse atrophy in skeletal muscle, based on various experimental approaches. Interestingly, attenuation of skeletal muscle atrophy is also observed following relatively mild heat stress that is insufficient for HSP induction (Naito et al. 2012). This suggests that HSP72 is sufficient but not necessary for the heat stress-induced attenuation of muscle atrophy. Yoshihara and coworkers reported that transient heat stress activates the Akt/mTORC1 pathway in rat skeletal muscle. Interestingly, activation of the Akt/mTORC1 pathway by heat stress is temperature dependent (that is, higher temperature results in more activation) (Yoshihara et al. 2013). Studies on cultured cells have reported that the inhibition of mTORC1 activation by heat stress results in insufficient activation of HSF1 and HSP induction (Chou et al. 2012). Therefore, the primary significance of mTORC1 activation by heat stress is likely to be cell protection.

In recent years, studies on cultured cells have been employed to clarify mechanisms underlying the beneficial effects of heat stress on muscle atrophy. Tsuchida and coworkers recently established an in vitro experimental model of the heat stress-induced attenuation of muscle atrophy (glucocorticoid-induced myotube atrophy and heat stress) (Tsuchida et al. 2017). They found that its molecular basis was the suppression of decreased mTORC1 pathway activity by glucocorticoids and the reduced expression of enzymes related to FoxO and the ubiquitin proteasome system. On the other hand, studies on rat muscles in vivo have shown that heat stress suppresses the increase in apoptosis induced by hindlimb suspension (Yoshihara

et al. 2015). Interestingly, suppression of the ubiquitin–proteasome system by heat stress was observed only in the soleus muscle (slow muscle fiber-dominant) (Yoshihara et al. 2015). This suggests that the molecular mechanism of muscle atrophy suppression by heat stress may be dependent on muscle fiber type. We have also shown that heat stress partially suppresses age-related muscle atrophy (Tamura et al. 2017). However, since this effect was characterized mainly on the basis of muscle weight, it will be necessary to support our conclusion with physiological measurements such as muscular strength and endurance.

In addition to perspectives from rehabilitation, heat stress can be an effective strategy to promote exercise/training adaptations. It is well-accepted that resistance exercise is the most effective option for inducing skeletal muscle hypertrophy and preventing disuse atrophy. It is now known that heat stress both during and after resistance exercise potentiates the activation of Akt/mTORC1 signaling (Kakigi et al. 2011). Although techniques to enhance the effect of resistance exercise have focused mainly on nutritional approaches, these results suggest that heat stress therapy could be another option.

3.1.3 Heat Shock Response and Glucose Metabolism in Skeletal Muscle

Skeletal muscle is the largest tissue which takes up and metabolizes circulating glucose. Therefore, improving the insulin sensitivity, glucose uptake, oxidation and storage capacity of skeletal muscle are promising strategies for preventing and treating type 2 diabetes. Glucose uptake in skeletal muscle can be divided into the insulin-dependent and -independent pathways. When insulin is secreted in response to elevated circulating glucose levels, it binds to the insulin receptor on the cell membrane of skeletal muscle and activates intracellular signal transduction. Subsequently, cytoplasmic glucose transporter 4 (GLUT4) translocates to the cell membrane and takes up circulating glucose. On the other hand, it has been shown that the increases in adenosine monophosphate/triphosphate and calcium concentration associated with muscle contraction activate adenosine monophosphate-activated protein kinase (AMPK). AMPK also induces the translocation of GLUT4 to the cell membrane. Therefore, as a transient cellular response, it is important to sufficiently induce the translocation of GLUT4. Based on these findings, it is considered that exercise training is the best way to reduce circulating glucose levels in healthy and diabetic individuals.

It has been reported that insulin resistance in patients with type 2 diabetes is improved by hot tub therapy (Hooper 1999). Even in studies on experimental animals, it has been shown that insulin resistance due to a high fat diet is also improved by local heat stress to skeletal muscle (Gupte et al. 2009). These authors also found that daily heat stress suppresses continuous activation of the c-Jun N-terminal kinase (JNK) signaling cascade (the JNK pathway is involved in the

development of insulin resistance) (Gupte et al. 2009). They concluded that the attenuation of JNK activation by heat stress is at least in part mediated by HSP72, based on experiments with an HSP72 inhibitor (Gupte et al. 2009). Furthermore, it has been shown that muscle-specific over-expression of HSP72 improves the insulin resistance associated with a high fat diet (Henstridge et al. 2014). Moreover, improvement of insulin resistance is also triggered by administering BGP-15, an inducer of HSP72 expression, supporting data from genetically modified animals. As described above, expression of HSP72 is also induced by endurance exercise and training. It has been shown that, when induction of HSP72 expression is attenuated by endurance exercise in a cold environment, improvements in glucose metabolism are also attenuated (Tsuzuki et al. 2017). To integrate these findings, increasing HSP72 in skeletal muscle appears to be a sufficient intervention for improving insulin resistance. In addition, we found that daily heat stress increases the GLUT4 protein content of skeletal muscle (Tamura et al. 2015). Therefore, it is thought that daily heat stress can up-regulate the de novo glucose uptake capacity of skeletal muscle.

On the other hand, it has been shown that skeletal muscle glucose uptake is also promoted immediately after a single heat stress treatment. Since increased HSP72 was not observed in this context, it appears that an HSP72-independent pathway also contributes to improved glucose metabolism (Koshinaka et al. 2013). This and other studies employed an isolated skeletal muscle model (an ex vivo model) to interrogate the direct role of skeletal muscle in glucose uptake. It has been shown that a single bout of heat stress increases glucose uptake in isolated skeletal muscle (Goto et al. 2015). Interestingly, there is an additive effect of heat stress and insulin on glucose uptake. Goto et al. have shown that AMPK is activated by heat stress, and pharmacological inhibition of AMPK prevents the promotion of glucose uptake by heat stress (Goto et al. 2015). However, the effects of heat stress on AMPK activity are controversial. For example, it has been shown that AMPK inactivation is observed when heat stress is applied to various cultured cells, including skeletal muscle cells (Wang et al. 2010). The molecular mechanism of this effect has been shown to involve an increase in the phospholipid, PP2A (a component of the cell membrane). AMPK inactivation by heat stress has also been shown to be an essential response for the induction of HSP72. We have confirmed that AMPK is inactivated by heat stress in mouse skeletal muscle (Tamura et al. 2014). Overall, the effect of heat stress on AMPK activity is more likely attributable to differences in heat stress conditions, such as temperature and time, rather than to in vitro, ex vivo and in vivo differences such as humoral factors. In addition, as for the physiological significance of inducing HSP expression, there may be different molecular mechanisms depending on the temperature range. AMPK has been shown to be involved in various biological processes such as mitochondrial biogenesis (described below) and is positively involved in autophagic regulation (Sanchez et al. 2012). Therefore, it is important to better understand how the response of AMPK is affected by different experimental conditions. To summarize this section, several lines of evidence indicate that heat stress can be an effective alternative strategy for diabetic patients with low physical fitness.

3.1.4 Heat Shock and Mitochondrial Biogenesis/Turnover in Skeletal Muscle

Mitochondria are organelles responsible for energy production. In particular, improving the content and function of mitochondria in skeletal muscle contributes to improved exercise performance at sub-maximal intensity through sparing glycogen (Fitts et al. 1975). Furthermore, mitochondria trigger and/or mediate various biological processes and signal transduction pathways by buffering intracellular Ca2+ concentrations, producing reactive oxygen spices (ROS), and regulating apoptotic cell death. Recent emerging evidence demonstrates that decreased and/or dysfunctional mitochondria in skeletal muscle cause skeletal muscle atrophy with disuse and aging, and systemic metabolic disease with the dysfunction of other organs affected through neural or circulating factors (Tezze et al. 2017). In this section, we outline our recent findings on heat stress and mitochondrial adaptation in skeletal muscle. For further information on heat stress and mitochondrial adaptation, please also refer to our recent review (Tamura and Hatta 2017). For more general information about the adaptive mechanism and biological significance of mitochondria in skeletal muscle, see recent reviews by Hood and coworkers (Tryon et al. 2014; Carter et al. 2015; Hood et al. 2015, 2016; Kim et al. 2017).

Mitochondrial content is determined by the net balance between mitochondrial biogenesis and breakdown (mitophagy). Mitochondrial biogenesis can be divided into three steps: (1) transcription of mitochondria-related genes, (2) translation of mitochondria-related gene products, and (3) the processing of mitochondrial-related proteins by post-translational modification and transport, folding and assembly. Among these steps, the transcription process has been most extensively studied. It is accepted that PGC-1α plays important roles in the transcription of mitochondria-related genes. PGC-1α acts as a transcriptional booster, cooperating with transcription factors (for example, NRF1/2, p53, PPARs). Mitochondria-related genes are encoded by both nuclear and mitochondrial DNA. In the basal state, PGC-1α is mainly localized in the cytosol. However, when PGC-1α is activated, it translocates into the nucleus and mitochondria, and then promotes the transcription of mitochondria-related genes. PGC-1α activation has been shown to be induced following activation of upstream factors such as AMPK, p38 MAPK, CaMKII and mTORC1. In contrast, the mechanisms involved in the translation and subsequent processing of mitochondria-related gene products are not well understood. Importantly, this limitation is common in mitochondrial biology, and does not apply only to skeletal muscle.

The content of mitochondria is determined not only by mitochondrial biogenesis but also by mitochondrial degradation. The cellular machinery of mitochondrial breakdown (mitochondrial selective autophagy—mitophagy) has been a rapidly growing research field in recent years (Drake and Yan 2017). There are several mitophagic pathways, but here, we briefly discuss Parkin-mediated mitophagy. Dysfunctional and unhealthy mitochondria produce high levels of ROS and impair

cellular processes and integrity. Therefore, it is necessary to remove dysfunctional mitochondria from cells. The mitochondrial E3 ubiquitin ligase, Parkin, translocates from the cytosol to the mitochondrial outer-membrane in response to a decrease in mitochondrial membrane potential and the accumulation of oxidative stress. Parkin promotes the poly-ubiquitination of mitochondrial outer-membrane proteins (for example, VDAC, Mfn2). The poly-ubiquitin chain is then recognized by p62/SQSTM1, which is known as an autophagic substrate and an adapter protein for autophagosomes. LC3-II, a key autophagosome molecule, recognizes p62/SQSTM1 and targets the bound mitochondria for breakdown, transport to lysosomes, and finally, degradation.

Liu and Brooks first demonstrated that heat stress increases mitochondrial content in cultured cells (Liu and Brooks 2012). Furthermore, we have recently reported that heat stress-induced mitochondrial biogenesis is observed in mouse skeletal muscle (Tamura et al. 2014). To explore the significance of our observation in vivo, we have also investigated heat stress interventions as post-exercise therapy. We found that heat stress after exercise has additive effects with endurance training on enhancing mitochondrial biogenesis (Tamura et al. 2014). Interestingly, this additive effect of heat stress depends on the in vivo environment. It has been shown that a high-fat diet increases mitochondrial content in skeletal muscle (Hancock et al. 2008). However, it has also been found that the mitochondrial biogenesis associated with a high-fat diet disappears when mice are subjected to heat stress (Gupte et al. 2009). We have also examined the mechanism by which mitochondrial biogenesis is induced by heat stress. We found that a single bout of heat stress increased PGC-1α protein content in nuclear and mitochondrial fractions (unpublished observations). We have confirmed that heat stress acutely increases the mRNA level of mitochondria-related genes (unpublished observations), consistent with the translocation of PGC-1α. We interpret these observations as heat stress activating the transcriptional step of mitochondrial biogenesis in a PGC-1α-centered manner.

We next examined the mechanism underlying PGC-1α activation by heat stress. We found that p38 MAPK and mTORC1 are activated by heat stress (Tamura et al. 2014). Interestingly, a pioneering study by Liu and Brooks showed that the activation of mitochondrial biogenesis by heat stress is mediated by activation of AMPK (Liu and Brooks 2012). However, as described above, AMPK was inactivated in our study. Therefore, we suggest that various factors contribute to mitochondrial biogenesis induction by heat stress. In addition, mechanisms underlying mitochondrial biogenesis might involve not only the repeated transient activation of PGC-1α, but also a stable increase in PGC-1α content. We have confirmed that the content of PGC-1α mRNA is increased by heat stress (unpublished observations). Interestingly, it has recently been clarified that HSF1 functions as a transcription factor for PGC-1α (Ma et al. 2015). Also, in a study of the liver, it was shown that liver mitochondria are decreased following knockout of HSF1 (Qiao et al. 2017). Therefore, the heat shock response and oxidative energy metabolism are much more closely-linked than conventional understanding would suggest. It has been reported that the over-expression of HSP72 in skeletal muscle increased mitochondrial content. Interestingly, overexpression of HSP72 does not alter the amount of

PGC-1α (Henstridge et al. 2014). Moreover, an HSP72 knockout mouse displays abnormal mitochondria (Drew et al. 2014). Therefore, it will be necessary in the future to carefully study the control of protein transport and folding for mechanistic insights into the contribution of HSP72.

Based on our findings showing that heat stress increases mitochondrial content, we examined whether heat stress also counteracts the mitochondrial loss associated with skeletal muscle disuse. Reduction and dysfunction of mitochondria in skeletal muscle have also been shown to cause muscle atrophy following disuse (Powers et al. 2012). We examined whether heat stress can suppress the decrease of mitochondria caused by experimental muscle disuse (sciatic nerve resection; denervation). As expected, we found that heat stress partially suppressed the decrease in mitochondrial content and skeletal muscle atrophy caused by denervation (Tamura et al. 2015). Although we initially thought that PGC-1α-mediated mitochondrial biogenesis could have contributed to this effect, we did not obtain any results supporting this possibility. On the other hand, since mitophagy is activated by denervation, we decided to study the effect of heat stress on mitophagy. Interestingly, heat stress attenuated the activation of mitophagy induced by denervation. We next tested a molecular mechanism potentially underlying the contribution of heat stress. It was revealed that mitochondrially localized Parkin, the state of mitochondrial poly-ubiquitination, and the amount of p62 bound to mitochondria were all increased by denervation, but these increases were attenuated by heat stress.

However, this result should be interpreted carefully. If the mitochondria to be broken down are not degraded, the attenuation of mitochondrial clearance by heat stress is not always preferable (because unhealthy mitochondria would accumulate). Therefore, we decided to evaluate mitochondrial oxidative stress, because oxidative stress and mitochondrial dysfunction can be both cause and consequence, forming a vicious circle. Although mitophagy is a quality control mechanism at the organelle level, in recent years, the mitochondrial unfolded protein response (UPR^{mt}) has received attention as a quality control mechanism at the molecular level. We have also examined the mitochondrial stress response and the response of mitochondrial proteases from the viewpoint of the UPR^{mt}. As a result, both mitochondrial oxidative stress and mitochondrial proteases were increased by denervation, but it became clear that heat stress improves than rather worsens the UPR^{mt} (Tamura et al. 2015, Tamura et al. unpublished observations). Therefore, we consider that the primary effect of heat stress on denervated mitochondria is to improve mitochondrial health. Subsequently, the need to degrade mitochondria is reduced. In recent years, attention has been paid to the function and dynamics of HSP in mitochondrial health. Over-expression of mitochondrial HSP in skeletal muscle has been shown to suppress mitochondrial dysfunction (Sarangi et al. 2013). It has also been shown that HSP72, which was considered to be a cytoplasmic HSP, translocates to mitochondria in response to mitochondrial stress, interacting with parkin (Drew et al. 2014). These observations suggest that an HSP-centered, dynamic mitochondrial quality control mechanism is becoming more important to our understanding of mitochondrial and muscle biology.

3.2 Conclusions

I believe that more attention should be paid to the physiological roles of the HSF1-dependent heat shock response and HSP other than their function as molecular chaperones. In recent years, roles of the transcription factor, HSF1 other than HSP synthesis have received the most attention (Li et al. 2017; Gomez-Pastor et al. 2017). In addition, the regulation of HSF1 activity has been examined in the past from the perspectives of the content and post-translational modification of HSF1. It is also necessary to pay attention to the epigenetic regulation of HSF1 transcriptional activity, such as changes in the chromatin structure of HSF1 target genes. We have recently performed transcriptome and bioinformatics analyses to discover novel responses to heat stress in skeletal muscle. We detected the activation of pathways other than HSF1–HSP (unpublished observation). Overall, searching for new targets regulated by HSF1 and integrating our understanding of heat shock responses other than HSF1 will be necessary in the future.

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Chapter 4
Temperature Stress and Redox Homeostasis: The Synergistic Network of Redox and Chaperone System in Response to Stress in Plants

Hui-Chen Wu, Florence Vignols, and Tsung-Luo Jinn

Abstract A remarkable number of strategies has been developed by living organisms to mitigate conflict with environmental changes. The global environment rising with ambient temperature has a wide range of effects on plant growth, and therefore activation of various molecular defenses before the appearance of heat damage. Evidence revealed key components of stress that trigger enhanced tolerance, and some determinants for plant tolerance have been identified. The interplay between heat shock proteins (HSP) and redox proteins is supposed to be vital for the survival under extreme stress conditions. Any circumstance in which cellular redox homeostasis is disrupted can lead to the generation of reactive oxygen species (ROS) that are continuously generated in cells as an unavoidable consequence of aerobic life. Integrative network analysis of synthetic genetic interactions, protein-protein interactions, and functional annotations revealed many new functional processes linked to heat stress (HS) and oxidative stress (OS) tolerance, implicated upstream regulators activated by the either HS or OS, and revealed new connections between them. We present different models of acquired stress resistance to interpret the condition-specific involvement of genes. Considering the basic concepts and the recent advances, the following subsections provide an overview of calcium ion (Ca^{2+}) and ROS interplay in abiotic signaling pathways; further we introduce several examples of chaperone and redox proteins that respond the change of cellular redox status under environmental circumstances. Thus, the involvement or

H.-C. Wu (✉)
Department of Biological Sciences and Technology, National University of Tainan, Tainan, Taiwan
e-mail: huichenwu@mail.nutn.edu.tw

F. Vignols
BPMP, CNRS, INRA, Montpellier SupAgro, Univ Montpellier, Montpellier, France

T.-L. Jinn (✉)
Department of Life Science and Institute of Plant Biology, National Taiwan University, Taipei, Taiwan
e-mail: jinnt@ntu.edu.tw

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contribution of redox proteins through the functional switching in conjunction with the HSP that prevent heat- and oxidative-induced protein aggregation in plants.

Keywords Calcium ion · Chaperone · Heat shock proteins · Heat stress · Oxidative stress · Reactive oxygen species · Redox proteins

Abbreviations

ABA	abscisic acid
AOX	alternative oxidase
AsA	ascorbic acid
APX	ascorbate peroxidase
Ca^{2+}	calcium ion
CaM	calmodulin
CML	CaM-like protein
CAT	catalase
Cys	cysteine
GRX	glutaredoxin
GR	glutathione reductase
GSH	glutathione
GST1	glutathione-S-transferase 1
GPX	glutathione peroxidase
HIP	Hsp70-interacting protein
HS	heat stress
HSE	heat shock element
HSG	heat shock granule
HSP	heat shock protein
HSF	heat shock transcription factor
HSR	heat shock response
H_2O_2	hydrogen peroxide
$OH^{\cdot}$	hydroxyl radical
HMW	high molecular weight
LMW	low molecular weight
NTR	NADPH-dependent TrxR
NO	nitric oxide
OS	oxidative stress
PDI	protein disulfide isomerase
PRX	peroxiredoxin
RBOH	respiratory burst oxidase homolog
ROS	reactive oxygen species
$^1O_2$	singlet oxygen
$O_2^{\cdot-}$	superoxide anion
SOD	superoxide dismutase

TRX	thioredoxin
TrxR	thioredoxin reductases
TPR	tetratricopeptide repeat
RNR	ribonucleotide reductase

4.1 Introduction

As sessile organisms, plants are exposed to persistent changes in environmental conditions, and therefore they have developed intricate defense mechanisms to recognize and interpret them appropriately. Global warming is one of the most serious challenges to the crop production due to the negative effects of high temperatures on the plant development and crop production (Bita and Gerats 2013). A rising ambient temperature is one of the most detrimental stresses due to the global climate change that leads to 1.5–5.8 °C higher temperature than the current level by 2100 (Hemantaranjan et al. 2014; Rosenzweig et al. 2001). Heat stress (HS) due to increased temperature is one of the most prominent abiotic stresses affecting plants including crops. Thus, the extremely high temperature is becoming a serious problem as it affects vital physiological processes like photosynthesis, respiration, membrane stability, and resulting in devastating damage to crop production. Therefore, cells need to precisely tune defense mechanisms to maintain cellular homeostasis and, accordingly, they have involved elaborate ways to sense and to respond to stresses.

HS is defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause a broad spectrum of cellular damages, by extensive denaturation and aggregation of intracellular proteins, modifying membrane fluidity and permeability, and subsequently disrupting the balance of metabolic processes that cause the accumulation of toxic products. Consequently, HS is an agricultural problem, leading to a drastic reduction in economic yield in many areas. In nature, HS condition is a chronic and/or recurring phenomenon (Bäurle 2016), and therefore plants have developed diverse strategies to cope with this stress. Among them, the heat shock response (HSR) is a highly conserved stress response mechanism, originally discovered in heated Drosophila salivary-gland tissue by Ritossa (1962), and observed as a new pattern of puffs, a manifestation of increased gene expression on the polytene chromosomes. HSR defines the total sum of cellular high temperature-related defense activities in the cell by the induction of a large set of heat stress proteins (HSP) which are chaperone proteins, to prevent damage and aggregation at the proteome level and protect cellular homeostasis against heat and other stress stimuli (Lindquist and Craig 1988). It has a complex impact on cellular functions, suggesting that many processes are involved in thermotolerance to minimize damage and ensure the protection of cellular homeostasis in plants.

Oxidative stress (OS) is also a major phenomenon for all living cells in an oxygen-based world. A very close relationship appears to exist between the response of HS and OS in plant cells. Particularly, elevated temperatures result in an accelerated generation of ROS, causing an imbalance between the ROS production and the

ability of scavenging systems to detoxify and remove the reactive intermediates. It has been shown that very short heat pulses resulted in an oxidative burst of hydrogen peroxide (H_2O_2) to increase thermotolerance, suggesting a considerable linking between HS and OS response and signaling (Dat et al. 1998; Vallelian-Bindschedler et al. 1998). Furthermore, the induction of stress-related proteins and ROS production constitute the major plant responses to HS (Hemantaranjan et al. 2014; Saidi et al. 2011). Increasing evidence has shown that ROS can induce expression of *HSP* genes to provide a protective function against OS (Driedonks et al. 2015). Thus, more recently, the involvement of HS in inducing oxidative damage has attracted great interest. Several researches have shown that calcium ion (Ca^{2+}) and H_2O_2 as signaling molecules are involved in plant development as well as in response to abiotic stresses. The mutual interplay of these signal molecules functioning together in signal transduction of cross-adaptation responses and in plant normal physiological processes. The HSP plays a positive role in thermotolerance by protecting the conformations and activities of ROS scavenging protein, resulting in a lower ROS production (Kong et al. 2014). However, the mechanism for the crosstalk signaling between the chaperone and redox systems in response to HS and OS is not well understood, and is a topic of great debate in the field. Part of the repertoire of dynamic adjustments involved in chaperone-redox balancing system may be by increasing the cellular level of Ca^{2+} and H_2O_2. They were established as key players and as necessary parts of the intracellular communication system, by constituting the most intracellular signaling molecules and participating in the integration of diverse cellular functions.

The redox status of thiol-containing molecules is important to cellular functions, such as the regulation of the protein synthesis, their folding and activity, preventing the aggregation of the non-native protein and maintaining proteins in functional conformations that are particularly important for cells survival under stress. To protect cells from OS damage and ROS-mediated protein unfolding and aggregation, organisms are equipped with a wide range of antioxidant proteins, and diverse forms of molecular chaperones, which have essential roles in protecting cells from the potential lethal effects of stress. Among them, thiol-containing molecules, such as thioredoxins (TRX) and glutaredoxins (GRX) define redox switches by reversible cysteine (Cys) modifications to control many cell functions, such as the catalysis of oxidative protein folding via protein–protein interactions with potential chaperone controlling protein folding and to reversibly modify the structure and function of proteins.

Distinct HSP families have been shown to be regulated by their activity and/or conformation change by their redox status and members of TRX family. For example, the cytosolic Hsp33, a bacterial redox-regulated chaperone protein, undergoes oxidation upon H_2O_2 uses through a Cys-containing thiol switch to rapidly respond to stress conditions (Jakob et al. 1999). In the absence of stress, Hsp33 requires reduction by the TRX or GRX system prior complete substrate release (Hoffmann et al. 2004). Although on numerous single components involved in redox-regulated chaperone have been identified, how these components with individual behaviors integrate common, interrelated mechanisms of these multiple elements needs to be elucidated in more details.

This review of aims at highlighting some of the major knowledge advances in the complexity of interrelated HS and OS networks in recent years, including their considerable compatibility but also their differences in the control of regulatory pathways in distinct conditions, involving various cell components and activation of specific signaling pathways, by providing both specificity and backup for protection from cellular damage. Furthermore, we attempt to summarize the current knowledge about the interplay between HSP and TRX for HSR and redox regulation, and how thiol-containing proteins synergistically work with chaperone networks. To draw attention to the member of HSP and TRX family involved in protein folding and chaperone activity, and discuss their co-operations and/or interactions with other stress-induced components, which are essential for HSR and cellular redox homeostasis.

4.2 Simultaneous Activation of Heat Shock Response and Oxidative Stress

Heat shock proteins (HSP) assist in protein folding and protect cellular homeostasis against heat, they also play a role in a number of other cellular processes that occur during and after exposure to the OS; thus, the increased HSP synthesis can not only correct protein misfolding but actively prevent protein damage following OS (Kalmar and Greensmith 2009). In addition, the ability of plants to develop heat tolerance following a mild heat pre-treatment, the so-called acquired thermotolerance was shown to be mediated by enhancing cellular mechanisms that prevented oxidative damage under the HS (Larkindale and Huang 2004). NADPH oxidases known as respiratory burst oxidase homolog (RBOH) can enhance the ROS production and maintenance that is crucial for basal thermotolerance (Miller et al. 2008).

Oxidative stress (OS) has been shown to play a major role in HS possibly because many cellular antioxidant proteins are thermally unstable (Davidson et al. 1996). Thus, HS- and OS-responsive genes were identified during the HS will facilitate the understanding of the cross-talk between the HS and OS. The intimate relationship between the HSR and OS is the activation of heat shock transcription factors (HSF) during these processes. Considerable evidence supporting a possible role for the HSF as direct sensors of ROS is found in studies of mammalian cells, Drosophila, plants, and yeast (*Saccharomyces cerevisiae*). The human Hsf1 and Drosophila HSF have been shown to be regulated in a redox-dependent manner to sense H_2O_2 directly and reversibly be assembled into active homotrimer, driven by oxidant-induced disulfide bond formation (Ahn and Thiele 2003). In particular, two Cys residues located within and near the DNA-binding domain (DBD) of Hsf1, were shown to be required for intramolecular disulfide bond formation in response to HS or H_2O_2. In Drosophila, H_2O_2 was found to be involved in the transactivation, nuclear translocation, and DNA binding activity of Hsf1 (Jacquier-Sarlin and Polla 1996). Furthermore, the DNA binding of the Drosophila HSF was shown to be reversibly regulated by H_2O_2 as well

as by high temperature (Zhong et al. 1998). The induction of TRX, which are responsible for restoring the DNA-binding activity of oxidized HSF functions as an endogenous regeneration system for HSF activity during the HS and OS (Jacquier-Sarlin and Polla 1996). Mammalian Hsf1 is directly activated by heat and H_2O_2 in a reversible redox regulated manner through the N-terminal 290 amino acid residues (Ahn and Thiele 2003). Therefore, the redox regulation of Hsf1 requires Cys residues for stress-activation of Hsf1 multimerization, nuclear accumulation, HS-responsive genes activation, and the protection of cells from stress-induced apoptosis (Hahn et al. 2004). The activation of Arabidopsis (*Arabidopsis thaliana*) *HsfA1a* (*AtHsFA1a*) in response to heat and H_2O_2 stress occurs via its trimerization in a redox-dependent manner (Liu et al. 2013). The HSR in Arabidopsis can be largely inhibited by effective removal of H_2O_2 because expression of genes such as those encoding *AtHsfA4a*, and *Ascorbate Peroxidase1* (*Apx1*) are modulated by ROS signals (Davletova et al. 2005). Thus, it is possible that impaired HSR may further enhance ROS and contribute to increased cell death especially when cells are exposed to persistent OS.

4.3 Redox Status Regulates in Various Aspects of Cellular Function

The cellular compartmentalization provides well distinct chambers for a variety of vital biochemical processes in the cell. One of the most obvious features of the different compartments is their redox status, which refers to the reduction potential or reducing capacity in the biological system. In aerobic organisms, a continuous flux of electrons to molecular O_2 requires the simultaneous presence of both oxidized and reduced forms of electron carriers, which modifies a reducing environment, from multiple sites in the photosynthetic and respiratory electron transport chains. At the first level of regulation, redox-triggered responses compensate for redox imbalances and are crucial for avoiding the second phase of redox disturbances that are associated with the excessive amounts generation of ROS. Therefore, it is a critical determinate of cellular function, and any major imbalances can cause cells severe damage or death.

4.3.1 Redox Status

Cellular redox status primarily is determined by the balance between cellular levels of reductants and oxidants. The first example of regulation by redox modification from photosynthetic organisms was several enzymes in the Calvin cycle (Buchanan et al. 1979). Later, oxidative disorders, those observed during various events of abiotic and biotic stress were recognized as triggers for redox regulation by plants. Examples of redox-related regulation are cell cycle control, adjustments of photosynthesis, stress acclimation, pathogen defense, and the initiation of cell death

(Grant and Loake 2000; Mahalingam et al. 2005; Pastori and Foyer 2002). The cellular redox status needs to be detected inaccuracies from redox homeostasis and modulated prior to such imbalances occur. Importantly, the redox status can be as an integrating element of cell parameters, and a transducing factor acting on specific targets. Thus, the cellular redox environment has emerged as the functional redox regulatory network to be finely balanced with the coordinated induction of proteins with antioxidant properties and protective activities as shown in Fig. 4.1.

The mechanism of redox-regulation is based on the post-translational modification of key regulatory proteins that contain essential Cys residues at their catalytic sites. In organelles, various redox-active components are involved in these

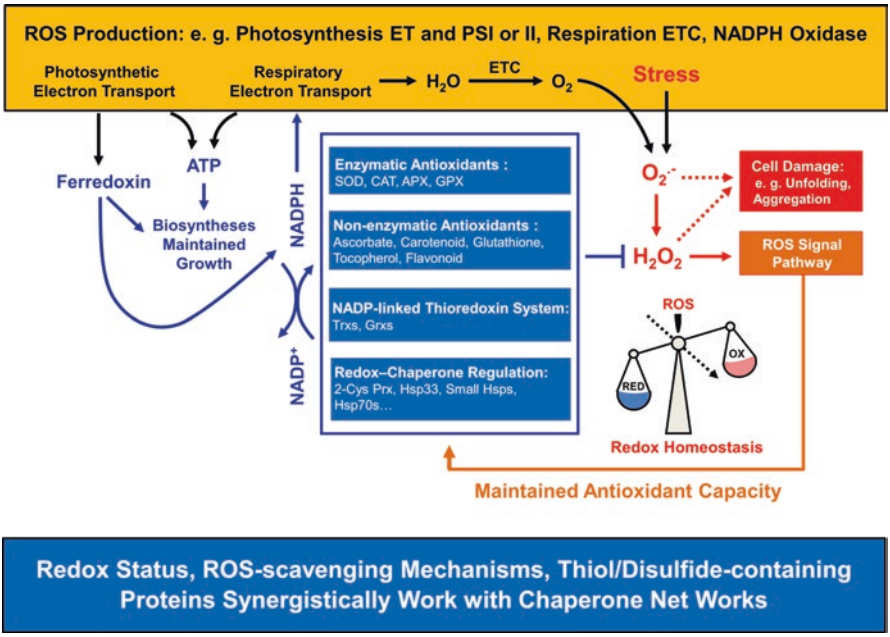

Fig. 4.1 The regulation of redox homeostasis interacts with numerous cellular components to perceive stress and maintain physiological growth. A model of redox homeostasis summarizes that the network of the antioxidant-chaperone interaction driven by oxidant-induced disulfide bond formation is involved in the metabolic regulation and the signal transduction. The extent of ROS is governed by the presence of antioxidant system (reductant–antioxidant–oxidant interactions), which maintains redox homeostasis and cellular components in an active state for metabolism. The production of the superoxide anion ($O_2^{\cdot-}$) and H_2O_2 can be induced under certain conditions which are continuously produced by NADPH oxidases, electron transport components (photosynthetic and respiratory metabolisms) or stressed, leading to increased oxidative charge on the antioxidant system. Excessive production of ROS is avoided by enzymatic or non-enzymatic processing of H_2O_2 or by TRX and GRX systems function as disulfide reductases and molecular chaperones which directly modify the cellular redox state or synergistically work with HSP chaperone networks (see text). Increased availability of ROS acts as a signaling molecule which may relatively effects on proteins, particularly transcription factors, which results in gene expression to mediate the capacity of an antioxidant system

processes, including TRX, GRX, and other thiol/disulfide-containing proteins that maintain the reduced state through a constant input of metabolic energy. Many of these key components are also regulated by interactions with molecular chaperones under various stresses. Redox regulation of molecular chaperones originally identified as the HSP, they facilitate de novo proteins folding under non-stressed conditions, prevent protein aggregations during stressful conditions, and then promote protein refolding following recovery from stress, thereby reducing the pool of proteins with non-native conformation (Deuerling et al. 1999). OS is known to cause carbonylation of amino acids and thermal instability of many proteins and ultimately leads to the unfolding and aggregation of cellular proteins (Berlett and Stadtman 1997). It is therefore not surprising that all these conditions can also induce the expression of HSP to modulate cellular redox homeostasis. It is worthy to mention that TRX can participate at multiple stages in protein folding by different ways and function in chaperone activity that was proved by a wealth of proteins interacting with TRX. Such as *E. coli* Trx1 and the TRX homolog YbbN refolded citrate synthase and α-glucosidase with efficiency comparable to those of chaperones like DnaK and different HSP (Caldas et al. 2006; Kern et al. 2003). Therefore, redox balance system is constitutively expressed to maintain redox homeostasis during non-stress conditions and to integrate the response to the HS and OS.

4.3.2 Reactive Oxygen Species (ROS) and ROS-Scavenging Mechanisms in Cells

Plants have evolved complex regulatory mechanisms for rapid and precise perception of internal and external stimuli that exert adverse effects on plant growth and development at different extents. One of the consequences from both normal metabolism and stress situations is the triggering of intracellular oxidizing molecule levels to generate ROS production, such as H_2O_2, superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$), as well as reactive sulfur and nitrogen species. ROS can be generated endogenously during certain developmental transitions such as seed maturation and as a result of normal, unstressed, photosynthetic and respiratory metabolism. Therefore, the production of ROS in plants is mainly those with high electron transport rates localized in chloroplasts, mitochondria, and peroxisomes as illustrated in Fig. 4.2.

Major ROS producers such as peroxidase and amine oxidase located in cell walls, NADPH oxidase located in the plasma membrane, and intracellular oxidase and peroxidase in chloroplasts, mitochondria, and peroxisomes (Bolwell et al. 2002). Some locations are much less commonly known, such as the nucleus, Golgi apparatus, and ER. It has been shown that the production of ROS from chloroplast during photosynthesis can pass through the retrograde signaling to nucleus (Nott et al. 2006). The ROS signaling networks between the organelles are not yet characterized in detail and have recently raised considerable interest.

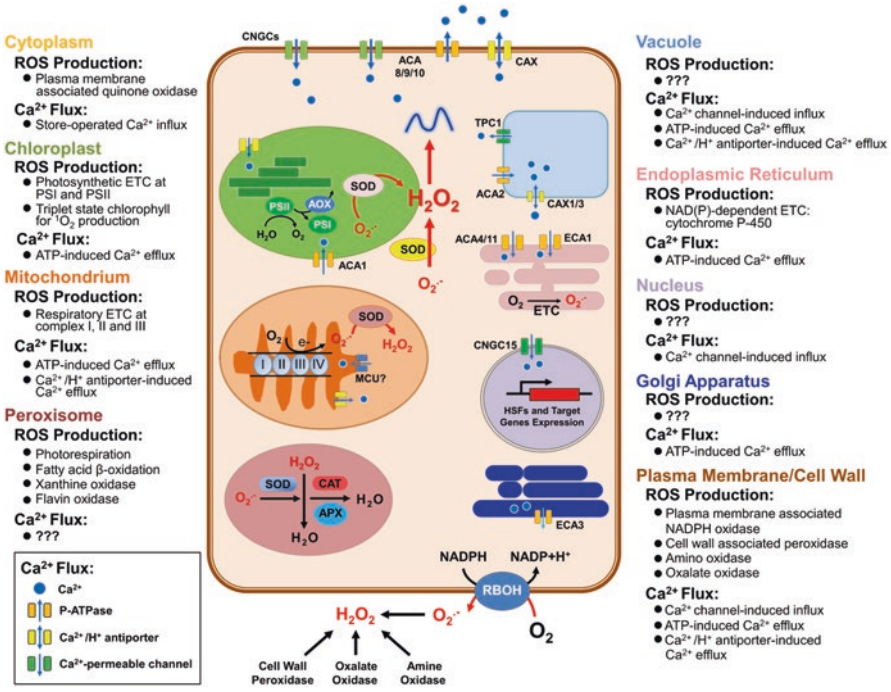

Fig. 4.2 A possible interrelationship between ROS and Ca^{2+} signaling network in plant cells. ROS and Ca^{2+} are crucial second messengers in the intra- and extracellular signaling cascades in the response to variety of stimuli like biotic and abiotic stresses. The mutual interplay between ROS and Ca^{2+} signaling can be considered as bidirectional, the Ca^{2+} signal is essential for increasing the production of the ROS, while ROS can significantly affect Ca^{2+} flux into the cell and intracellular Ca^{2+} stores. The localization of ROS production and scavenging pathways is presented mainly in chloroplast, peroxisome, cytosol and mitochondria. The production of $O_2^{\cdot-}$ in the thylakoid membrane of chloroplast is mainly due to electron transfer from photosystem I to O_2 generated by photosystem II, while chlorophyll and its tetrapyrrole derivatives near both photosystems are main sources of $^1O_2$. Mitochondrial electron transport chain (ETC) contributes to plant ROS production, peroxisomes also produce H_2O_2 through the photorespiratory glycolate oxidase reaction (GOX), fatty acid β-oxidation by acyl-CoA oxidase (ACO), enzymatic reactions of flavin oxidases, and the disproportionation of $O_2^{\cdot-}$ radicals by SOD. Endoplasmic reticulum $O_2^{\cdot-}$ originates from a NAD(P)H-dependent ETC involving a cytochrome p450. In addition, plasma membrane NADPH oxidases and apoplastic enzymes, such as POXs, oxalate- and amine-oxidases, also contribute to ROS generation in plants. In addition to ROS formation, cellular Ca^{2+} transport is the most tightly controlled within all membrane-bound organisms. An increase in $[Ca^{2+}]_{cyt}$ is carried out by Ca^{2+} influx which is mediated by Ca^{2+}-permeable ion channels to the cytosol, either from the apoplast across the plasma membrane, or from intracellular stores such as ER or vacuole. On the contrary, Ca^{2+}-ATPases and the Ca^{2+}/H^+ antiporter systems are responsible for Ca^{2+} extrusion out of the cytosol. *ACAs* the autoinhibited Ca^{2+}-ATPases, *APC* adenine nucleotide/phosphate carrier, *CAXs* Ca^{2+}/H^+ cation antiporters, *CNGC* cyclic nucleotide-gated ion channels, *ECAs* ER-type calcium ATPases, *GLR3.5* glutamate receptor 3.5, *HMA1* heavy metal translocating P-type ATPase, *LETM1* leucine zipper-EF-hand-containing transmembrane protein 1, *MCU* mitochondrial calcium uniporter, *SOD* superoxide dismutase, *TPC1* two-pore voltage-gated channel 1, *PSI* photosystem I, *PS II* photosystem II, *RBOH* respiratory burst oxidase homolog

There is growing evidence that ROS and redox regulation is central to cellular signaling and to both transcriptional and post-transcriptional regulation, across a wide range of organisms (Droge 2002; Foyer and Noctor 2005; Sauer et al. 2001). When concentrations of ROS rise above a certain level, cellular scavengers are no longer sufficient to prevent oxidative damage to membrane lipids, proteins, and DNA; they are believed to be the major contributing factors to stress injuries and to cause rapid cellular damage (Prasad 1996). ROS have been shown to be toxic but also function as signaling molecules, which are involved in many biogenesis; however, being a toxic molecule, it is also capable of injuring cells. The resolution of this conflict remains largely unknown; nonetheless, it is clear that the steady-state level of ROS in cells needs to be tightly regulated. Consequently, the evolution of all aerobic organisms has been dependent on the development of efficient ROS-scavenging mechanisms, thereby maintaining the level of ROS below a toxic threshold.

Highly specific mechanisms have been evolved that form the fundamental oxidant scavenging system by constitutively expressing detoxifying enzymes including the superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), peroxiredoxin (PRX), etc. The balance between SOD and APX or CAT activities in cells is considered to be crucial in determining the steady-state level of $O_2^{\cdot-}$ and H_2O_2 (Bowler et al. 1991). Cellular levels of APX (μM range) and CAT (mM range) showed the different affinities for scavenging H_2O_2, APX might be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during the stress (Mittler 2002). Together with the non-enzymatic antioxidants ascorbic acid (AsA) and glutathione (GSH) provide cells with highly efficient machinery to detoxify $O_2^{\cdot-}$ and H_2O_2. These compounds can be as substrates in enzyme-catalyzed detoxification reactions. Such as the reaction of AsA with H_2O_2 can occur directly or it can be catalyzed by APX. The AsA–GSH cycle are almost found in all cellular compartments, as well as the high affinity of APX for H_2O_2, suggests that this cycle plays a central role in controlling levels of ROS in these compartments, e.g. cytosol, chloroplasts, mitochondria, peroxisomes, and apoplast. In addition, the original oxidation status of cytosolic protein thiols is maintained and rapidly restored by the action of redox-balancing systems. Other evidence indicates that TRX act as regulators of scavenging mechanisms and as components of signaling pathways in the plant antioxidant network. Further efforts are necessary to characterize the targets and molecular functions of ROS, as well as the complex interplay of ROS-generating and -scavenging mechanisms.

4.3.3 Hydrogen Peroxide (H_2O_2) as a Signaling Molecule

One of the consequences of many stresses is a dramatic increase in the cellular level of ROS, which is subsequently converted to H_2O_2. It is formed as a result of SOD action, there are potential sources of apoplastic origin of H_2O_2 including

peroxidases, amine oxidases and oxalate oxidases (Auh and Murphy 1995). Because of its relatively higher stability with long life (cellular half-life ~1 ms, steady-state levels ~10^{-7} M) and highly permeability across membranes, is to be accepted as a second messenger for signaling function in defense responses (Yang and Poovaiah 2002). Sustaining the H_2O_2 concentration at an appropriate level of cells can promote plant growth and reinforce resistance by modulating the transcripts coding for proteins involved in the response to environmental stimuli. In addition to being a toxicant, H_2O_2 has been given much attention and ample evidence has been regarded as a central signaling mediator and a regulator of the expression of some genes in cells. These include genes encoding antioxidant, cell rescue/defense protein, and signaling protein such as kinase, phosphatase, and transcription factor, which are subsequently converted to H_2O_2 (Cheng and Song 2006).

The altered redox status also contributed to the expression of heat-responsive genes including HSF and HSP that are important for plant thermotolerance (Cheng and Song 2006; Desikan et al. 2001; Liu et al. 2015). It has been suggested that H_2O_2 might be directly involved in modification of HSF for activation of trimerization (Miller and Mittler 2006), the exact mechanism of action is still unclear. The HSF such as AtHsfA1a contains one Cys residue located at the N-terminal region of the trimerization domain, may be sensitive to H_2O_2 via Cys redox sensory mechanism (Hübel and Schöffl 1994; Mittler et al. 2011). The Cys residue of AtHsfA8 is responsible for the cytosol-to-nucleus translocation upon H_2O_2 (Giesguth et al. 2015). The steady-state transcript and protein levels of many ROS-scavenging enzymes were found to be elevated by HS (Mittler et al. 2004; Rizhsky et al. 2002). For example, Arabidopsis *APX* genes show enhanced transcript accumulation in response to a short-term HS in an HSF-dependent mechanism (Panchuk et al. 2002). AtHsfA2 plays an important role in linking the signaling of HS and OS by modulating the expression of stress responsive genes such as *HSP* and cytosolic *Apx1* (Li et al. 2005). AtHsfA4a, acts in a ROS-mediated HS response upstream of the redox sensors *Apx1* and zinc finger protein *Zat12* through its binding capacity at the heat shock elements (HSE; 5′-GAAnnTTCnnGAA-3′) in *Apx1* and *Zat12* promoter (Miller and Mittler 2006). Thus, the thiol-based regulation by H_2O_2 is the key component in the acclimation of plant adaption to abiotic stress.

4.3.4 Mutual Interplay Between H_2O_2 and Calcium Ion (Ca^{2+})

Multiple pieces of evidence show the modulation of HS and OS by triggering H_2O_2 and Ca^{2+} signaling. Abiotic and biotic stresses cause an elevation of $[Ca^{2+}]_{cyt}$ to stimulate the H_2O_2 production, which diffuses into cells as a messenger to induce the physiological response (Saxena et al. 2016). Furthermore, changes in intracellular Ca^{2+} and redox homeostasis are unifying consequences of abiotic and biotic stresses (Yang and Poovaiah 2002). A stress-induced change in $[Ca^{2+}]_{cyt}$ might be one of the primary transduction mechanisms whereby gene expression and biochemical events are altered to adapt the environmental stresses (Sanders et al. 2002; van der Luit

et al. 1999). The patterns of Ca^{2+} signatures may differ in the amplitude, duration, frequency, and localization of Ca^{2+} oscillations, and there is evidence that these parameters are used to encode the information required to initiate specific and appropriate responses to a given stimulus (White and Broadley 2003). From Ca^{2+} influx channels and Ca^{2+} efflux, transporters function as modulators of Ca^{2+} in shaping the Ca^{2+} signature in plants. A schematic summary of the stimulus induced specific Ca^{2+} signature containing the influx and efflux pathway through Ca^{2+} channels and transporters is illustrated in Fig. 4.2.

Despite the ubiquitous nature of the HSR, little is known about how the plant senses the elevating temperature to transmit a signal resulting in *HSP* genes induction and acquired thermotolerance. Several key components involved in Ca^{2+} signaling pathways in response to distinct external stimuli have been identified in plants up to now. They include non-protein determinants such as the phytohormone abscisic acid (ABA), ROS, and phospholipids (Xue et al. 2009), as well as a large variety of proteins among which calmodulin (CaM) have been identified as playing crucial role in HS signaling (Gong et al. 1998; Liu et al. 2003, 2005; Wu et al. 2012). CaM is highly conserved and considered to be a multifunctional protein, mostly acting as a general transducer of Ca^{2+}-mediated signal cascades in eukaryotes submitted to various developmental and external stimuli. In plants, a huge number of *CaM* and *CaM-like* (*CML*) genes has been inventoried by genome sequencing programs and phylogenic analyses. The transduction of environmental signals regulating *CaM* genes expression are in part regulated by the elevation $[Ca^{2+}]_{cyt}$ levels (Braam and Davis 1990).

Wheat *CaM1-2* gene expression started to increase after HS at 37 °C for 10 min, then reached its maximum 20 min after HS by northern analysis (Liu et al. 2003). Knocking out or overexpressing specific CaM isoforms in Arabidopsis transgenic plants was shown to significantly reduce or increase thermotolerance, suggesting that *CaM* genes are key components in HS signal transduction (Zhang et al. 2009). HS induces not only Ca^{2+}-dependent CaM in Arabidopsis, but also regulates a CaM-binding protein kinase, which promotes activation of *HSF* and *HSP* genes expression (Liu et al. 2008). HS-triggered a biphasic $[Ca^{2+}]_{cyt}$ elevation together with OsCaM1-1 has also been investigated in rice (*Oryza sativa* L.) seedlings, linking to early transcriptome changes of HS-responsive genes in rice (Wu et al. 2012).

In Fig. 4.2 depicts a possible interrelationship between Ca^{2+} and H_2O_2 signaling network in plant cells, suggesting a mutual codependence of these two factors. The interaction of Ca^{2+} and H_2O_2 signaling can be considered as bi-directional, wherein Ca^{2+} homeostasis is required for ROS formation, while H_2O_2 can directly regulate cellular Ca^{2+} signaling (Görlach et al. 2015). For example, Ca^{2+} signaling is involved in maintaining ROS homeostasis by increasing the expression of ROS related genes while H_2O_2 could activate ROS homeostasis by inducing antioxidant genes, weakening Ca^{2+} signaling response to salt stress in perennial ryegrass (*Lolium perenne*) (Hu et al. 2016). In the leaf of maize (*Zea mays* L.), it has been demonstrated that Ca^{2+} functioning as an upstream of H_2O_2 stress, through the activation of antioxidant enzyme CAT activity to relief the level of H_2O_2 (Jiang and Zhang 2003). In addition,

a crosstalk between Ca^{2+} and H_2O_2 signaling was involved in the ABA-induced antioxidant defense when plants exposed to water stress (Hu et al. 2008). Plasma membrane-located ROS-generating NADPH oxidases also function in the oxidative burst occurring during HS (Suzuki et al. 2011); they can be activated by HS via increased membrane fluidity and/or via an elevation of cytosolic Ca^{2+} controlled by a Ca^{2+} permeable channel, that leads to the increase of ROS (Sun and Guo 2016). Furthermore, Ca^{2+} and H_2O_2 have involved in the expression of heat-responsive genes exhibiting HSE in their promoter region, such as those encoding HSF, HSP, and cytosolic APX.

A link between H_2O_2-triggered a rises in $[Ca^{2+}]_{cyt}$ and antioxidant gene induction, indicating a direct connection between H_2O_2 and Ca^{2+} signaling pathways is enable for cell-to-cell communication, and further for long-distance transmission of signals in plants (Steinhorst and Kudla 2013). In aequorin-expressing tobacco cell cultures (*Nicotiana tabacum* L. cv. Wisconsin-38) and Arabidopsis seedlings, H_2O_2 triggered a biphasic $[Ca^{2+}]_{cyt}$ elevation (Lecourieux et al. 2002; Rentel and Knight 2004). Ca^{2+} signaling in linking H_2O_2-induced expression of *glutathione-S-transferase1* (*GST1*) gene, suggesting that Ca^{2+} signals may be triggered by changes in the balance of redox status, the level of which provides plant cells for appropriate induction of antioxidant gene expression (Rentel and Knight 2004). Ca^{2+} has dual functions in regulating ROS homeostasis, which in turn influences redox regulation in response to environmental signals. As a positive regulation, extracellular signals induce an elevation $[Ca^{2+}]_{cyt}$ resulting in the increased H_2O_2 generation, whereas through Ca^{2+}/CaM complex stimulate the CAT activity leading to degradation of H_2O_2. Thus, Ca^{2+} and CaM play a role in down-regulated H_2O_2 levels by stimulating the activity of CAT in plants (Yang and Poovaiah 2002).

Pretreatment with $CaCl_2$ solution significantly raised $[Ca^{2+}]_{cyt}$ and CaM proteins enhanced intrinsic heat tolerance, and furthermore, the addition of external Ca^{2+} keep relatively higher activities of SOD, CAT, and APX, and lower levels of lipid peroxidation in maize seedlings (Gong et al. 1998). The deprivation of Ca^{2+} strongly inhibited the activity of GR and changed the cellular redox state in *Digitalis thapsi*, reversely external Ca^{2+} could enhancement of GR activity protect the chloroplastic components to against oxidative damage under HS (Paranhos et al. 1999). Ca^{2+}/CaM play a critical role in balancing ROS that caused oxidative damage by HS is exacerbated by pretreatment with CaM inhibitors and Ca^{2+} channel blockers in Arabidopsis (Larkindale and Knight 2002); whereas, these effects of inhibitors can be reversed by exogenous Ca^{2+}, implying that Ca^{2+}/CaM is required for protection against heat-induced oxidative damage. Ca^{2+}/CaM signaling mediated SOD and APX activation to remove effectively excessive formation of ROS, and in the protection against HS-induced oxidative damage in tomato (*Solanum lycopersicum* L.) (Ding et al. 2012). Therefore, Ca^{2+}/CaM signaling is involved in the protection against HS-induced oxidative damage through activation of antioxidant enzymes to eliminate over accumulation of ROS.

4.4 Redox-Mediated Functional Changes Contribute to Stress Responses

4.4.1 Potential Redox-Regulated Chaperones

Molecular chaperones are ubiquitous, highly conserved proteins in all free-living organisms. The term molecular chaperone, coined by Laskey in 1978 (Laskey et al. 1978), was first described an activity associated with nucleoplasmin in *Xenopus* oocytes. They are essential for contributing to cellular homeostasis in cells under both normal and stress conditions. Molecular chaperones have been expanded to respond in cell viability by that aid nascent polypeptide correct folding, disassembly of macromolecular complexes or aggregates, translocation, and degradation in a broad array of normal cellular processes. They also function in the stabilization of proteins and membranes, transit across cellular and organelle membranes, and assist in protein refolding under stress conditions, as well as the re-establishing normal protein conformation that affect biological functions, such as signaling (Young et al. 2004). Moreover, many of them were stress proteins and originally identified as the HSP which expressed in a sudden change in the cellular environment to which the cell is not prepared to respond, such as HS.

There are many types of HSP chaperones, and most are conveniently grouped into families and are conservatively recognized: the Hsp100 (Clp) family, the Hsp90 family, the Hsp70 (DnaK) family, the Hsp60 (GroEL, chaperonin) family, the Hsp40 family, and the small HSP (sHSP) family (Fig. 4.3). Genome sequencing has revealed that many organisms contain multiple members of both the DnaK (Hsp70) family and their co-chaperone partner J-domain protein (JDP), belonging to the DnaJ (Hsp40) family. Aside from these major families, there are other proteins with chaperone functions, such as PDI and calnexin/calreticulin, which assist in protein folding of the endoplasmic reticulum (ER).

Molecular chaperones/HSP are found in the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and ER (Wang et al. 2004). Different classes of molecular chaperones appear to bind to specific non-native substrates and states. Despite expression patterns that differ with respect to the physiological status of the cell, all chaperones function via a similar mechanism: molecular chaperones recognize the exposed hydrophobic regions of non-native proteins and bind specifically to partially folded proteins, preventing aggregation and misfolding. As a group, chaperones operate in a functional network in which some chaperones are "holderases" and others are "folderases" (Lund 2006).

4.4.2 Chaperone Mechanism-Holdases and Foldases

Molecular chaperones have the ability to bind folding intermediates and to prevent their non-specific aggregation (Ellis 1987). The fate of the substrate proteins once bound to the chaperones mostly relies on the individual chaperone mechanism.

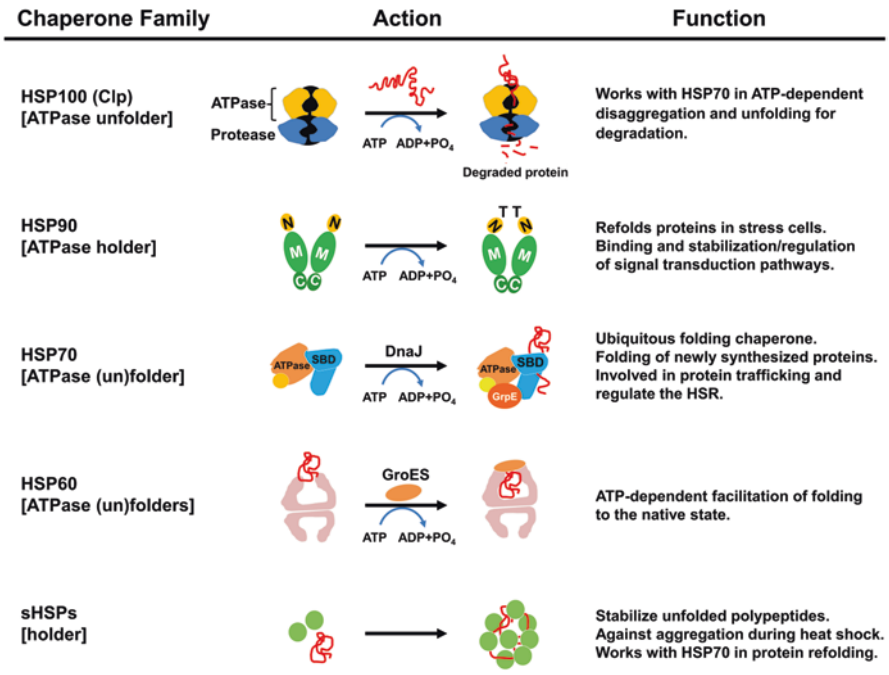

Fig. 4.3 A schematic summary of the major molecular chaperone families. Heat shock proteins (HSP) are classified according to molecular weight, such as Hsp100, Hsp90, Hsp70, Hsp60, and the small HSP (sHSP) families. They can be classified functionally based on their mode of action. Folders (or foldases) generally with an ATP-dependent mechanism to active unfoldes proteins, including Hsp70 and Hsp60 chaperones. Holders (or holdases) are capable of binding the folding intermediates and prevent their aggregation, such as small HSP. In addition to, they can be as disaggregates, primarily act on protein aggregates into small peptide fragments such as the members of AAA^+ ATPase family, the Hsp100 chaperone

Molecular chaperones are often divided into two main mechanistic groups, chaperone foldases (e.g., Hsp70/DnaK and Hsp60) and chaperone holdases (e.g., sHSP and Hsp33, and HdeA). As chaperone "foldases", they actively support the folding of the proteins to their native state. Other chaperones work as "holdases" bind tightly to folding intermediates, efficiently preventing protein aggregation, but do not support the refolding of the protein (Beissinger and Buchner 1998).

In order to change the affinity for the substrates and allow their productive folding, certain chaperone foldases typically utilize ATP-binding and hydrolysis to cycle between high-and low-affinity substrate binding states. In the DnaK (Hsp70) chaperone machinery, chaperone foldases use cycles of ATP binding, and hydrolysis to specifically regulate their affinity for unfolding proteins. This ATP-dependent chaperone folding system supports a de novo folding of proteins under non-stressed conditions, prevent protein aggregation during stress conditions, and promote protein refolding upon recovery from stresses (Deuerling and Bukau 2004). Holdases, on the other hand, are usually ATP-independent chaperones and often work to

deliver substrate proteins to either the proteolytic system or to chaperone foldases (Beissinger and Buchner 1998). Such as DnaJ, Hsp33, and the sHSP appear to bind to unfolded substrate proteins or folding intermediates via hydrophobic interactions, but are usually unable to support their refolding to the native state directly (Hoffmann et al. 2004; Linke and Jakob 2003; Rüdiger et al. 2001). Owing to unable to regulate substrates binding and release by ATP-driven binding and hydrolysis, chaperone holdases have developed an alternative-induced conformational switch from a low to a high-affinity binding state under stresses. Such as Hsp33, using a Cys-containing thiol switch rapidly respond to stress conditions that lead to protein unfolding. Otherwise, some holdases have been shown to transfer their substrate proteins to corresponding chaperone foldases such as the DnaK system, which then provides for the refolding of the respective proteins (Hoffmann et al. 2004).

Both of these novel post-translational regulatory strategies appear one ultimate goal: to significantly increase the substrate binding affinity of the affected chaperones under exactly those stress conditions that require their highest chaperone activity. This ensures that protein folding intermediates remain bound to the chaperones under stress conditions and are released only after the cells return to non-stressed conditions. Under non-permissive folding conditions, the unfolded substrates remain bound to chaperone holdases. Only after return to non-stress conditions are the substrates relayed onto the foldase-system for refolding (Hoffmann et al. 2004). This interplay between chaperone holdases and foldases is tightly regulated, so that unproductive folding effort is prevented.

4.4.3 The Hsp70 Chaperone Machinery

Members of the 70-kDa HSP (Hsp70) family are the most extensively studied group of stress proteins to date. Hsp70 chaperones are considered to be the most highly conserved multigene family in evolution, with 50% identical residues between the *E. coli* homolog DnaK and the eukaryotic Hsp70. They play essential housekeeping functions in preventing aggregation and in assisting refolding of non-native proteins under both normal and stress conditions. Some members of this family may be constitutively expressed as termed Hsc70 (70-kDa heat-shock cognate protein), and are often involved in assisting the folding of de novo synthesized polypeptides and the import/translocation of precursor proteins. Other family members are expressed only when the organisms are challenged by environmental assaults hence their classification as Hsp70s. Up-regulation of the inducible form of Hsp70s has been most closely associated with the development of thermotolerance. They are also involved in quality control processes, such as protein refolding after a stress injury, and control the activity of regulatory proteins in signal transduction pathways. In addition, because the Cys residue is responsive for cellular redox states, yeast Hsp70s have been linked to redox regulation based on its reactive Cys residues (Vignols et al. 2003). The conserved Cys20 in all the Hsp70 chaperones, this residue might be a target of redox regulation of chaperone binding activity (Vignols et al. 2003); while

the true mechanism of redox regulation in Hsp70 chaperone activity remains unsolved in plants.

Structurally, Hsp70 is composed of an amino (N)-terminal approximately 40–45 kDa ATPase domain and a carboxyl (C)-terminal approximately 25–30 kDa peptide-binding domain, both are highly conserved as shown in Fig. 4.4a. The inter-domain linker connecting the ATPase domain to the peptide-binding domain is highly conserved, and plays a critical role in the allosteric regulation of Hsp70s. Sequence variations among Hsp70s occur in the extreme end of N- and C-terminus, where the information for subcellular localization and for intramolecular and intermolecular interactions resides (Lin et al. 2001). A short C-terminal domain of largely unknown function interacts with various partner proteins to modulate the chaperone function (Mayer and Bukau 2005; Young et al. 2004). For example, cytosolic Hsp70s contain a G/P-rich C-terminal region containing an EEVD-motif that mediates their binding to tetratricopeptide repeat (TPR)-domain containing co-chaperones such as the C-terminus-Hsp70-Interacting Protein CHIP (Ballinger et al. 1999).

In the eukaryote, members of the Hsp70s multigene family are localized to distinct subcellular compartments of the cell: cytoplasm, plastids, mitochondria, and ER. The various subcellular localization of Hsp70s implies both functional specificity and phylogenetic divergence (Parsell and Lindquist 1993). Therefore, the specific roles of individual Hsp70 proteins are likely to be determined by their location in different subcellular compartments. For example, the cytosolic Hsc70 prevents protein aggregation, assists de novo protein folding and maintains the organelle precursor proteins in an import-competent stage. The ER Bip (binding immunoglobulin protein), mitochondrial and chloroplastic Hsp70 proteins are involved in precursor protein import and translocation. The specific roles of Hsp70 proteins are also determined by their interaction with specific sets of Hsp70-associated proteins. The interaction of the Hsp70 chaperones has also been reported to be involved in protein import and translocation into chloroplasts and mitochondria (Huang et al. 1999), as well as in the cell-to-cell movement of proteins and viruses through the plasmodesmata (Peremyslov et al. 1999). The cytosolic Hsc70 cooperated with other chaperones (e.g. 14-3-3 proteins), keeping mitochondrial or chloroplastic precursor proteins in an unfolded, yet import competent state. Once precursor proteins were transported by cytosolic Hsc70 through the membranes and then arrives in the matrix or stroma, the mitochondrial or chloroplastic Hsp70 proteins, together with their co-chaperones, interact with the precursor protein and allow cleavage of the leader or signal peptide by the peptidases (May and Soll 2000).

Hsp70s cycling between two stable conformations with different affinities for substrates (Fig. 4.4b): the ADP-bound state represents the high-affinity binding state. DnaK-ADP complexes tightly bind substrate proteins and prepare them for their refolding. In contrast, the ATP-bound state of DnaK represents the low-affinity state that undergoes weak interactions with the unfolded substrate proteins and has a high on/off rate of substrate binding (Mayer and Bukau 2005). Therefore, the modulation of the affinity for peptide substrates is mediated by the repositioning of an α-helical lid over the substrate binding pocket, is governed by the nucleotide

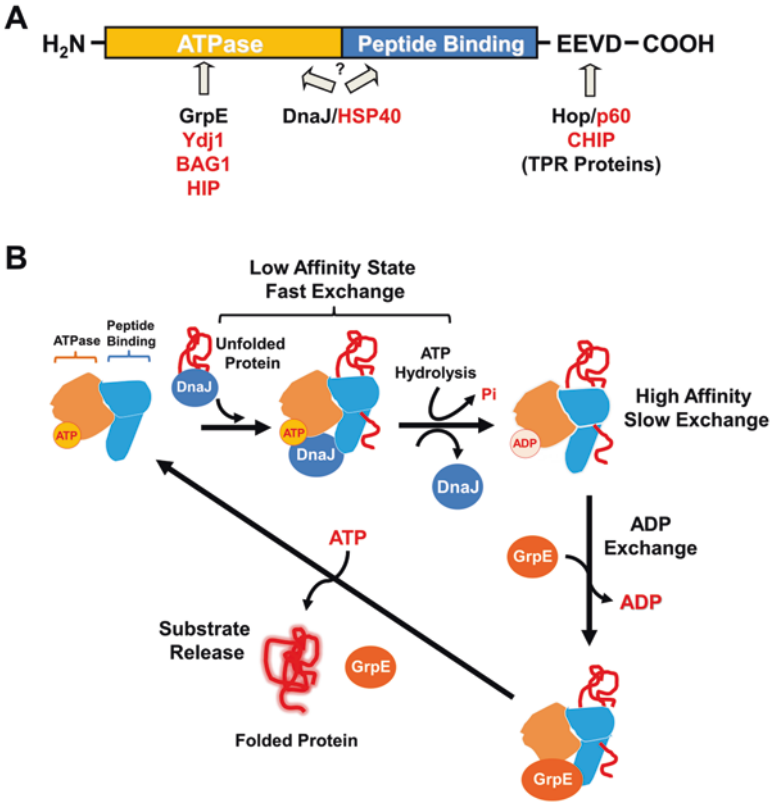

Fig. 4.4 The schematic model of Hsp70 chaperone mechanisms. The representative ATP-dependent cycle of peptide binding and release is best understood for the bacterial Hsp70 homolog DnaK and its cofactors DnaJ and GrpE. (**a**) Structurally, Hsp70 comprises a highly conserved N-terminal ATPase domain and a C-terminal peptide-binding domain. Sequence variation among Hsp70s occurs in the extreme N and C ends, where show the information for subcellular localization and for interactions resides (EEVD is a conserved motif in cytosolic forms). The interaction of prokaryotic (black) and eukaryotic (red) co-chaperones is shown schematically (bottom). No GrpE is found in the cytosol of eukaryotic cells. Instead, Ydj1 (yeast DnaJ homolog) combines the function of DnaK and GrpE in yeast. BAG1 acts as a negative regulator of Hsp70 by completing HIP binding to Hsp70. HIP is an oligomeric complex that binds to the ATPase domain of Hsp70 through its TPR domain. HIP is an activator of folding activity to stabilize the ADP state of Hsp70 and maintain a high affinity for substrate binding. (**b**) DnaK/Hsp70 as "foldase" assists in the folding of the non-native proteins via a continuously ATP-dependent cycle utilizing ATP binding and hydrolysis to switch from a low- to a high-affinity binding state. DnaJ interacts first with an unfolded protein and then targets it to DnaK which binds to unfolded protein with high affinity in its ATP-bound state. Conversely, substrate binding stimulates the hydrolysis of ATP. The nucleotide-exchange factor GrpE promotes the release of ADP from DnaK and substrate dissociates from DnaK upon subsequent ATP binding to DnaK

occupancy and status in the ATPase domain (Mayer and Bukau 2005). Thus, this continuous cycle of ADP/ATP-induced binding and release of substrate proteins is essential for the successful refolding of the folding intermediates to the native state.

The successive Hsp70/DnaK cycles of substrate binding and release are coupled to the intrinsic ATPase activity of Hsp70s, which requires the participation of its co-chaperones such as Hsp40/DnaJ and GrpE. A growing number of other cofactors also found in eukaryotic Hsp70 chaperones (e.g., HIP, auxilin, BAG1) (Alberti et al. 2003; Young et al. 2004). Such as HIP (Hsp70-interacting protein) with the TPR domain, binds to the ATPase domain and increases the chaperone activity of Hsp70s by stabilizing the ADP state, which is the substrate-bound state of Hsp70s (Höhfeld et al. 1995). The TPR domain is composed of a 34-amino-acid motif that was originally identified in yeast (Hirano et al. 1990), and then found in a large number of both prokaryotic and eukaryotic organisms (Lamb et al. 1995). TPR-containing proteins play diverse roles in many cellular processes such as cell cycle regulation, transcriptional repression, protein kinase inhibition, peroxisomal protein transport, and HSR. They mediate specific interactions with partner proteins, either forming active multiprotein complexes or acting as co-chaperones involved in the folding of substrates. The BAG1, by contrast, inhibits the chaperone activity of Hsp70s in part by accelerating nucleotide exchange, which affects the premature release of the unfolded substrate (Höhfeld and Jentsch 1997). HIP and BAG1 bind to Hsp70s at the same site on the ATPase domain and directly compete to influence Hsp70s chaperone activity. The biological role of these co-chaperones in the regulation of Hsp70, however, is still not well understood. The DnaK/DnaJ/GrpE chaperone system also is temperature regulated, switching from a folding to a holding mode during the HS.

4.4.4 HSP70s and Signal Transduction

In addition to the general chaperone functions of Hsp70, it also plays a regulatory role in other stress-associated gene expressions and the differential expression profile under various conditions and stimuli (Lee and Schöffl 1996; Sung et al. 2001). Expression profile analysis of the Arabidopsis *Hsp70* genes demonstrated that members of Hsp70 chaperones are expressed in response to environmental stress conditions such as heat, cold, and drought, as well as to chemical and other stresses (Sung et al. 2001).

Hsp70s together with Hsp90 have been suggested as a negative regulator of HSF mediated transcriptional activation during attenuation of stress response (Morimoto 1998; Ohama et al. 2017). Hsp70s prevent the formation of HSF trimerization and binding of HSF to the HSE, thereby blocking the transcriptional activation of HS-responsive genes by their HSF (Kim et al. 2002). Analysis of transcript profile revealed that *AtHsfA2* and *AtHsfA4a* have a potential role in the induction of defense system in response to the OS (Scarpeci et al. 2008). Even though the mechanism of Hsp70s regulatory under HS conditions is poorly understood in plants, Hsp70s chaperones can modulate the signal transducers such as protein kinase A, protein

kinase C, and protein phosphatase (Sung and Guy 2003). Several models of the regulatory interaction between Hsp70 and HSF have been proposed in tomato, the activation of HsfA1 and HsfB1 are induced by the demand of Hsp70 and Hsp90 due to the increasing amounts of denatured proteins under HS; however, the restoration of Hsp70 and HSP90 results in inactivation of HsfA1 by Hsp70-induced release from DNA under recovery or attenuation (Hahn et al. 2011). In this respect, the Hsp70 chaperones might play a broad role by participating in modulating the expression of many downstream genes in signal transduction pathways both during stress and under normal growth conditions.

4.4.5 Small Heat Shock Proteins (sHSP)

Plants express multiple *sHSP* gene families that appear to have evolved independently after the divergence of plants and animals (Scharf et al. 2001). All *sHSP* gene monomers consist of a conserved α-crystallin domain (ACD) in approximately 90 amino acid C-terminal domain, bordered by variable N- and C-terminal extensions (Vierling 1991; Waters et al. 2008). Plant sHSP with a molecular mass range from 15 to 42 kDa, which are ubiquitous stress proteins, classified into different subfamilies as shown in Fig. 4.5a, according to amino acid sequence similarity and localization to distinct subcellular compartments (Sarkar et al. 2009; Scharf et al. 2001). Nucleocytoplasmic isoforms of the sHSP have been divided in 9–11 sub-classes (so-called CI to CXI subclasses) in the model plants Arabidopsis and rice, respectively (Sarkar et al. 2009; Siddique et al. 2008). In specific situations of HS and recovery phase, some organelles sHSP have been shown to relocate in the cytoplasm in a time- and temperature-dependent fashion which may be essential for acquiring thermotolerance. Additionally, plant genomes also code for several other isoforms with an appropriate organelle-targeting signal predicted or functionally localized in organelles such as mitochondria, plastids, peroxisomes and the ER. It has been shown that mitochondria- and chloroplast-localized sHSP are involved in the protection of proteins during HS and increased plant thermotolerance, respectively (Kim et al. 2012; Sanmiya et al. 2004). The mitochondrial sHSP has been reported to be involved with the protection of mitochondrial proteins during HS and with increased thermotolerance.

Strong evidence supports that the sHSP functions as molecular chaperones that bind to partially folded or denatured substrates and thereby prevent irreversible aggregation or promote correct substrate folding. The highly conserved *α*-crystallin domain might be important for chaperone activity. Mutations within the N-terminal phenylalanine-rich region of the α-crystallin abolished chaperone activity in vitro (Plater et al. 1996). The same class sHSP from different plant species can carry out subunit exchange (van Montfort et al. 2001), but hetero-oligomers of sHSP from different classes are absence (Kirschner et al. 2000; Plater et al. 1996). The interaction of sHSP with their protein substrates seems to involve oligomer dissociation which can be induced by heat and reassembly with denatured proteins into larger complexes.

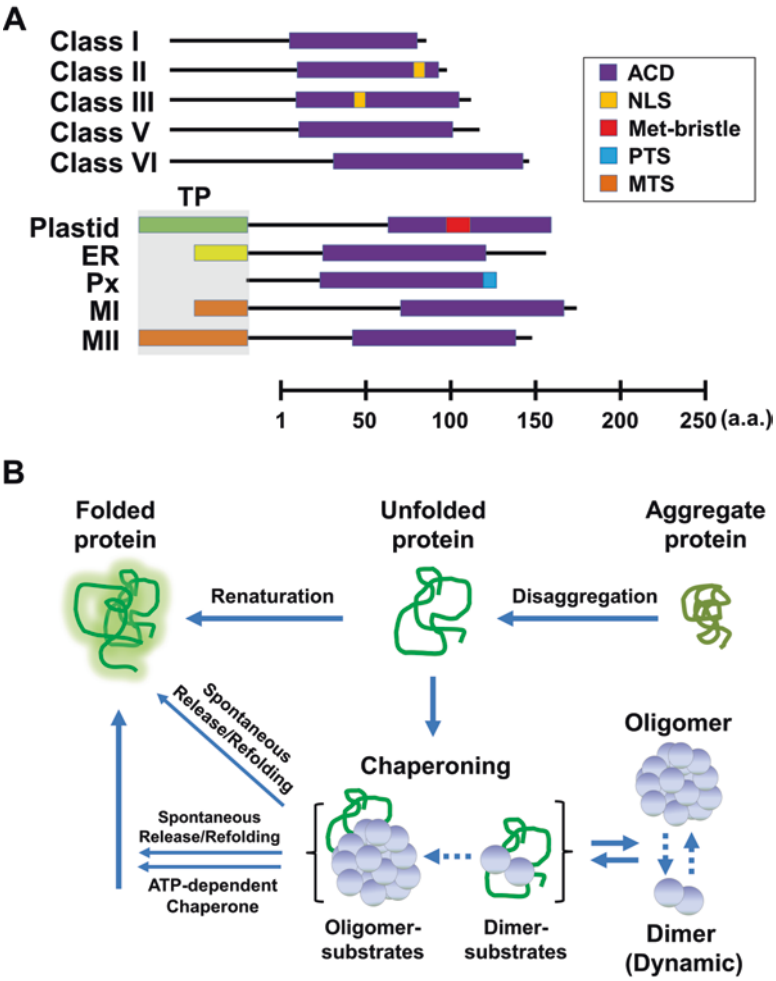

Fig. 4.5 Oligomerization and chaperone activity of small HSP. (**a**) Schematic illustration of plant sHSP subfamilies. The conserved α-crystallin domain (ACD) of sHSP is shown in purple. The transit peptide (TP) of the organellar sHSP, the NLS of CII and CIII sHSP, the methionine rich region (Met-bristle) of plastidic sHSP, peroxisomal targeting signal (PTS; SKL), ER targeting sequence (ETS), chloroplast targeting sequence (CTS), and mitochondrial targeting sequence (MTS) are marked. (Adapted from Sarkar et al. 2009). (**b**) Exposure to stress generates non-native proteins which form the aggregation of proteins. Partially unfolded, non-native proteins may bind large sHSP oligomers and smaller complexes such as dimers, thereby preventing irreversible aggregations. Upon return to favorable conditions, non-native proteins associated with the sHSP are released and refolded spontaneously or with the assistance of ATP-dependent chaperones, including Hsp70 and Hsp60. sHSP exists as oligomers in a functionally important dynamic equilibrium with dimers or other small complexes

Most generally, the sHSP has been proposed to act as chaperones for HS-damaged proteins, their synthesis being induced by a rapid increase of temperature. The members of sHSP subfamilies can incorporate into large oligomeric complexes or HSG, which are composed of 12 subunits approximately molecular weight 200–350 kDa and 40 nm in diameter, during a long-term HS treatment (van Montfort et al. 2001). The HSG is mainly composed of cytosolic sHSP from both classes CI and CII and other factors, such as Hsp40/Hsp70 chaperone machinery, HsfA2, cytoskeleton elements, unfolded proteins, and mRNAs that may participate in the formation of HSG (Port et al. 2004; Scharf et al. 2001; Weber et al. 2008). The formation and dissociation of HSG are important for the sHSP function and losing the capacity of oligomerization causes loss of chaperone activity (Fig. 4.5b). Thus, HSG has been demonstrated to be important for plants to survive under continuous stress conditions at a sub-lethal temperature (Giese and Vierling 2002, 2004; Miroshnichenko et al. 2005). The complexes of denatured proteins–sHSP oligomers can be stored transiently in HSG that can be disintegrated during the recovery period. Experiments performed in vitro with sHSP from diverse organisms have demonstrated that sHSP particularly effective in preventing thermal aggregation of other proteins by an ATP-independent mechanism (Lee et al. 1995).

The current model for the chaperone function of sHSP can bind selectively non-native proteins, prevent their aggregation, and maintain them in a state competent for ATP-dependent refolding by other chaperones. The sHSP can undergo temperature-dependent conformational changes, resulting in either dissociation of the oligomeric complex or increase the affinity for substrate binding to protect cells from heat- and oxidative stress (Jung et al. 2015). In mammalian cells, cytosolic Hsp27 can form smaller and larger formers of oligomers under the HS or OS (Arrigo et al. 1988). Chaperone-inactive of smaller sized Hsp27 tetramers was phosphorylated on Ser15, Ser78, and Ser82; whereas large non-phosphorylated Hsp27 oligomers displayed a chaperone activity (Härndahl et al. 2001). Methionine residue in proteins acts as the major target of ROS attack (Hoshi and Heinemann 2001). Arabidopsis chloroplast Hsp21 contain a conserved methionine rich domain at its N terminus exposed an amphipathic alpha helix, and can be oxidized easily and reversibly by H_2O_2, and reduction in Hsp21 oligomer size with resultant loss of chaperone activity (Härndahl et al. 2001). Rice chloroplast Hsp26 (OsHsp26) plays a key role in the protection of photosystem II (PSII) under both heat and oxidative stresses (Kim et al. 2012). Thus, the chaperone activity of sHSP can prevent aggregation of other proteins is dependent on the cellular redox state during heat or oxidative stress.

4.5 Thioredoxin and Chaperone Regulation

Oxidative stress-induced the activity of thiol-containing proteins helps to restore the cellular redox homeostasis and functions as a feedback regulator to inactivate redox-regulated stress transcription factors (e.g., OxyR and Yap1p) and shut down the

oxidative stress response (Paget and Buttner 2003). Besides the general function of TRX by disulfide oxidoreductases activity, it is important to note that some functions of TRX are independent of their redox activity, for instance *E. coli* TRX as a subunit of the T7 DNA polymerase complex, the redox regulation of apoptosis signaling kinase1 by human TRX (Berndt et al. 2008). Several chaperones have been characterized to be redox regulated by reversible oxidation of thiol groups (Cumming et al. 2004; Jakob et al. 1999). Such as, ERp29 is a ubiquitously expressed rat ER protein conserved in mammalian species (Sargsyan et al. 2002). It presented a TRX-like domain homologous to human PDI, suggesting a role related to the chaperone function of PDI. Such as TRX catalyzes the reduction of protein disulfides in *E. coli* Hsp33 (Hoffmann et al. 2004), yeast Hsp70 Ssb2 (Vignols et al. 2003), and human DnaJ homolog Hdj2 (Choi et al. 2006). A growing body of evidence supports the view that TRX participate in a variety of cellular processes due to the activity of disulfide oxidoreductase and chaperone regulation. The importance of TRX in its activity as a molecular chaperone to promote the folding of proteins separateness of their disulfide oxidoreductases activity both by directly promoting protein folding and by enhancing the refolding activity of other molecular chaperones. Several lines of initial evidence show that cellular redox regulation is necessary for chaperone activity of several proteins both in prokaryotic and eukaryotic systems.

4.5.1 Thioredoxins (TRX) and Glutaredoxins (GRX) as Regulators of Protein Folding and Chaperone Activity

Oxidoreductases of the TRX fold superfamily including TRX and GRX are widely distributed proteins that present in all types of living organisms and function in a broad range of cellular conditions. The first TRX was discovered in *E. coli*, and identified as a hydrogen donor for ribonucleotide reductase (RNR), an essential enzyme for DNA synthesis (Laurent et al. 1964). TRX shows a preference for protein–protein interactions, employing the TRX fold as an universal interaction scaffold to act as chaperones and isomerases of disulfides to generate a native fold (Berndt et al. 2008). Thus, the Cys residue of TRX and GRX is by far the most commonly used amino acid, which play an important role in such processes as DNA synthesis and refolding of proteins, and the regulation of the structure and activity of enzymes, receptors, and transcription factors.

In all organisms, this group of enzymes is central for DNA synthesis both during replication and repair (Nordlund and Reichard 2006). In most of their functions, TRX serves as general protein disulfide oxidoreductases containing a conserved structural motif named the TRX fold with two redox-active Cys separated by a pair of amino acids (Cys-X-X-Cys motif; X: any amino acid). This conserved motif for protein binding has been identified in the TRX superfamily and common to a variety of functional proteins, for instance, thiol-disulfide oxidoreductases, disulfide isomerases, glutathione S-transferases, thiol dependent peroxidases (Choi et al. 1998; Nishida et al. 1998). In all organisms, thioredoxin reductases (TrxR) are the sole

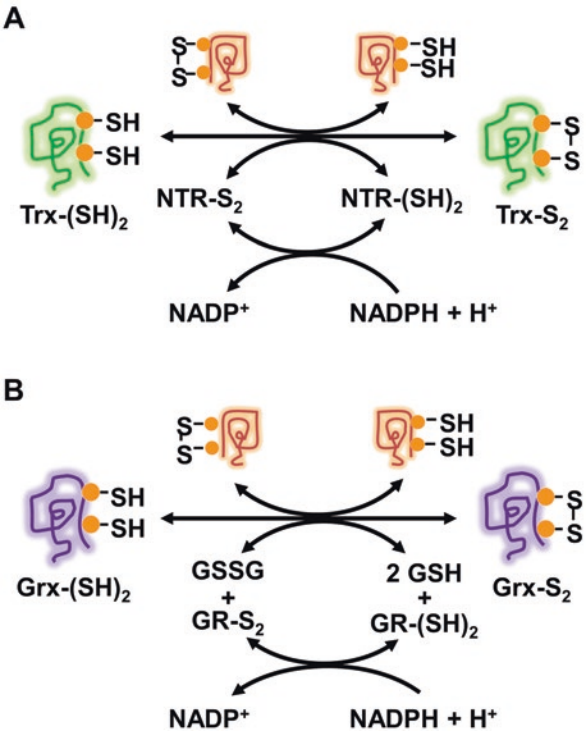

Fig. 4.6 The catalyzed reaction by oxidoreductases of the thioredoxin family. (**a**) The TRX system composes of TRX and TRX reductase (TrxR) using electrons from NADPH, utilizing both Cys residue in their Cys-X-X-Cys active site and reducing the target disulfide to form a covalent-mixed disulfide intermediate. The TRX in turn reduced by TrxR by a similar manner. (**b**) The GRX system consists of GRX, GSH, and NADPH-dependent GSH reductase (GR). The mechanism of protein disulfides reduction by GRX similar to those of the TRX system. GRX reduced by GSH leading to a mixed disulfide which is sequentially reduced by a second GSH molecule. Finally, glutathione disulfide (GSSG) is regenerated by GR at the expense of NADPH

known enzymes to reduce the action of TRX at the expense of NADPH. Thus the TRX system is consisted of TRX, TrxR and NADPH, in which TRX obtains electrons from NADPH as a source of reducing power via the NADPH–dependent TrxR (NTR) (Fig. 4.6a). In the classical scheme, the first Cys of TRX fold probably forms a mixed disulfide intermediate with the target protein such that the lifetime of the intermediate complex between TRX and its target is extremely short. Then the established disulfide bond between the two redox partners is attacked by another Cys residue of TRX fold. Consequently, both the reduced target and the oxidized TRX are released. Thus, TRX either reduces specific disulfide bridges on target proteins, thereby altering their activity, or serves as a substrate reductant in an enzymatic reaction. They act on a growing number of regulatory processes, including enzyme regulation, response to OS, transcription, and translation (Buchanan and Balmer 2005).

Current knowledge supports that plants possess the greatest complement of TRX found in all organisms. Indeed, at least 40 *TRX* genes have been reported in the Arabidopsis whole sequenced genome (Meyer et al. 2008, 2012). Although plant TRX displays a striking diversity not found in other organisms, many of their physiological roles have yet to be determined. This remarkable diversity of plant TRX suggests either functional specialization or a high level of redundancy depending on isoforms. Bacteria, typified by *E. coli*, and yeast present a simple cytosolic TRX system composed of two TRX and an NTR. In mammals, functional TRX has been found in the cytosol and mitochondria, both encoded by single members (Reichheld et al. 2007). Given that plants contain several distinct NTR proteins, there is likely to be a level of specificity in the interaction between NTR and TRX. In chloroplasts and oxygenic photosynthetic prokaryotes such as cyanobacteria, the reduction of TRX is linked not to NADP, but to ferredoxin by the novel Fe–S enzyme, ferredoxin–dependent TrxR (FTR) which reduces chloroplastic thioredoxin *m* and *f* (TXR *m* and TXR *f*) (Arnér and Holmgren 2000).

Glutaredoxins (GRX), one member of TRX superfamily, are small proteins usually around 9–15 kDa, was discovered as a GSH dependent electron donor for RNR in an *E. coli* mutant lacking TRX (Holmgren 1976). They exist in a large number of isoforms in basically all GSH-containing life forms. The GRX system consists of GRX, GSH and NADPH-dependent GSH reductase (GR) as shown in Fig. 4.6b. GRX can be divided into two subgroups based on their active site motifs, including the dithiol GRX (Cys-Pro-Tyr-Cys) and the monothiol GRX (Cys-Gly-Phe-Ser). Dithiol GRX was reduced via non-enzymatically with GSH. GR reduces oxidized form glutathione disulfide (GSSG) with electrons from NADPH in a NADPH dependent manner. Numerous functions have been described for GRX, both as electron donors and regulators of cellular function in response to OS, for instance, in sulfur assimilation (Berndt et al. 2008), dehydroascorbate reduction (Wells et al. 1990), and the regulation of cellular differentiation (Takashima et al. 1999), transcription (Bandyopadhyay et al. 1998), and apoptosis (Chrestensen et al. 2000). Monothiol GRX from *E. coli*, yeast, human, and recently found in plants are involved in Fe-S cluster biosynthesis and the regulation of iron homeostasis (Couturier et al. 2014; Fernandes et al. 2005; Ojeda et al. 2006; Wingert et al. 2005). As chaperone proteins, TRX and GRX are not the necessity of disulfide reductases activity under certain conditions, but it can promote disulfide bond formation and synergistically work with protein disulfide isomerase (PDI) and/or chaperones (Berndt et al. 2008).

4.5.2 Thiol-Based Dependent Chaperone Activity

Hallmarks of the TRX domain consists of a central core of five β-strands enclosed by four α-helices and TRX fold located on the loop connecting the end of a β-strand and at the beginning of a long α-helix (Martin 1995). One part of the area around the active site is hydrophobic and has been suggested to be the main interaction site for

other proteins. The interaction between TRX and substrate proteins has been suggested to involve the TRX fold and several residues, including cis-Pro76 and Gly92, which form a moderately hydrophobic surface around the active site and facilitate interactions with other enzymes (Lennon et al. 2000). It was well known that one identity of chaperones is using hydrophobic substrate interaction sites to bind and segregate these proteins folding intermediates, thereby reducing the protein aggregation and promoting cell survival. TRX covalently linked to the protein of target may act as molecular chaperone to prevent the fused proteins precipitation and aggregation until those achieve a stable folding state (LaVallie et al. 2003). During the binding with TRX, the structure of the substrate is shortened involving a conformational change of the polypeptide. This may well operate in the chaperone activity of TRX with unfolded proteins. For instance, *E. coli* TrxR, formed stable complexes with unfolded proteins, suggesting that it bond and unfolded substrate-proteins, thus facilitating the access of their disulfides to the redox site of TRX (Kern et al. 2003).

4.5.3 Thiol-Based Independent Chaperone Activity

The TRX system in *E. coli*, in addition to its protein disulfide isomerase activity, possesses chaperone-like properties that are involved in protein folding and protein renaturation (Kern et al. 2003). TRX increases the refolding of MglB galactose receptor, a protein without any Cys in *E. coli* (Kern et al. 2003). This suggests that the chaperone properties of TRX were at least partially independent from their active-site Cys. For instance, YbbN (a TRX homolog protein) is characterized as a weak protein disulfide oxidoreductase and a molecular chaperone, which presents a strong homology in its N-terminal part with two TRXs (Trx1 and 2) in *E. coli*, but exists a C-terminal part of an unknown function. However, YbbN does not possess the canonical Cys-X-X-Cys active site of TRX that catalyzes protein disulfide exchange, but instead a Ser-X-X-Cys site (Kthiri et al. 2008).

It has been proposed that YbbN acts as a chaperone rather than as an oxidoreductase to aid in DNA synthesis and HSR (Kthiri et al. 2008; Le et al. 2011). YbbN could interact efficiently with unfolded proteins such as urea-denatured citrate synthase, in contrast with TRX which does not form stable complexes with unfolded proteins. It has been shown that reduced YbbN migrates as a dimer, whereas oxidized YbbN migrates as a mixture of tetramers, hexamers, and higher molecular weight oligomers as a characteristic feature of chaperones (Caldas et al. 2006). Thus, YbbN possesses chaperone properties, promoting protein folding after urea denaturation and forming complexes with unfolded proteins. The YbbN-disrupted strain, further, displayed a sensitivity to thermal stress with decreased expressions of several cytoplasmic proteins, including EF-Tu, DnaK, GroEL, suggesting that the chaperone properties of YbbN are more important than its redox properties. YbbN specifically interacted with DnaK, increasing fourfold the rate of protein renaturation in vitro by the DnaK chaperone machine, suggesting that it cooperates

with DnaK for the optimal expression of several cytoplasmic proteins. Remarkably, neither the redox state of the TRX nor the redox active site important for redox regulation is required for chaperone activity (Kern et al. 2003; McGee et al. 2006).

4.5.4 Prokaryote Hsp33 Activated by Oxidative Stress

The *E. coli* heat shock protein Hsp33 was discovered as redox-regulated chaperone constituting a Cys-rich zinc finger motif, its activity turned on when exposed to oxidizing conditions (Jakob et al. 1999). Hsp33 was originally identified as new HS regulated proteins in *E. coli* (Chuang and Blattner 1993), and as a well-conserved protein with homologs identified in the vast majority of prokaryotic species, where it is located in the reducing environment of the cytoplasm. Hsp33 functions as a highly specialized chaperone holdase, which is specifically activated by ROS and protects cells against the lethal consequences of OS which lead to protein unfolding and aggregation (Winter et al. 2005).

Hsp33 contains four absolutely conserved Cys residues in its C-terminal redox switch domain, arranged in a Cys-X-Cys-X and Cys-X-X-Cys motif and highly affinity coordinate with zinc (II) as shown in Fig. 4.7a. Importantly, this compact zinc binding domain has been found to be substrate bound-site of Hsp33 and to block its interface of dimerization, both of which are located in the N-terminus of Hsp33 (Graf et al. 2004). The Zn-coordinated renders reduced Hsp33 as a monomer, and inactive the chaperone ability of Hsp33 (Graumann et al. 2001). Zn binding is needed to keep Hsp33 inactive under non-stressed conditions and seems equally important for its rapid activation under oxidative stress conditions. After exposure of Zn-coordinated Hsp33 to H_2O_2 or other oxidants such as $OH^{\cdot}$, four conserved Cys residues quickly oxidize to form two intramolecular disulfide bonds and caused the release of Zn(II), to expose a hydrophobic substrate binding site in Hsp33, a prerequisite for efficient substrate binding by molecular chaperones. Oxidized Hsp33 monomers were shown to exert partial chaperone activity but were found to be unable to protect cells against severe OS. As a consequence, oxidized Hsp33 assembles into the dimeric, fully chaperone holdase activity by showing extensive surface-exposed hydrophobic patches, which may explain its high affinity for unfolding substrate proteins and prevents protein aggregation.

Oxidative stress causes an intensely drop cellular ATP levels, which is largely due to the ROS-mediated inactivation of enzymes (e.g., GapDH) for cellular ATP metabolism (Winter et al. 2005). Therefore, when protein unfolding occurs during OS, cells can no longer rely on ATP-dependent foldases but require ATP-independent holdases of Hsp33 to compensate for the functional loss of ATP-dependent foldases and to conquer the stress. Once cells return to non-stressed conditions, the ATP-independence holdase activity of Hsp33 combined with its redox-mediated activation would cause the sudden release of unfolded substrate proteins, while the lack of ATP binding and hydrolysis would prevent HSP33 from participating in the refolding of the substrate proteins. For this reason, the inactivation of Hsp33 is tightly

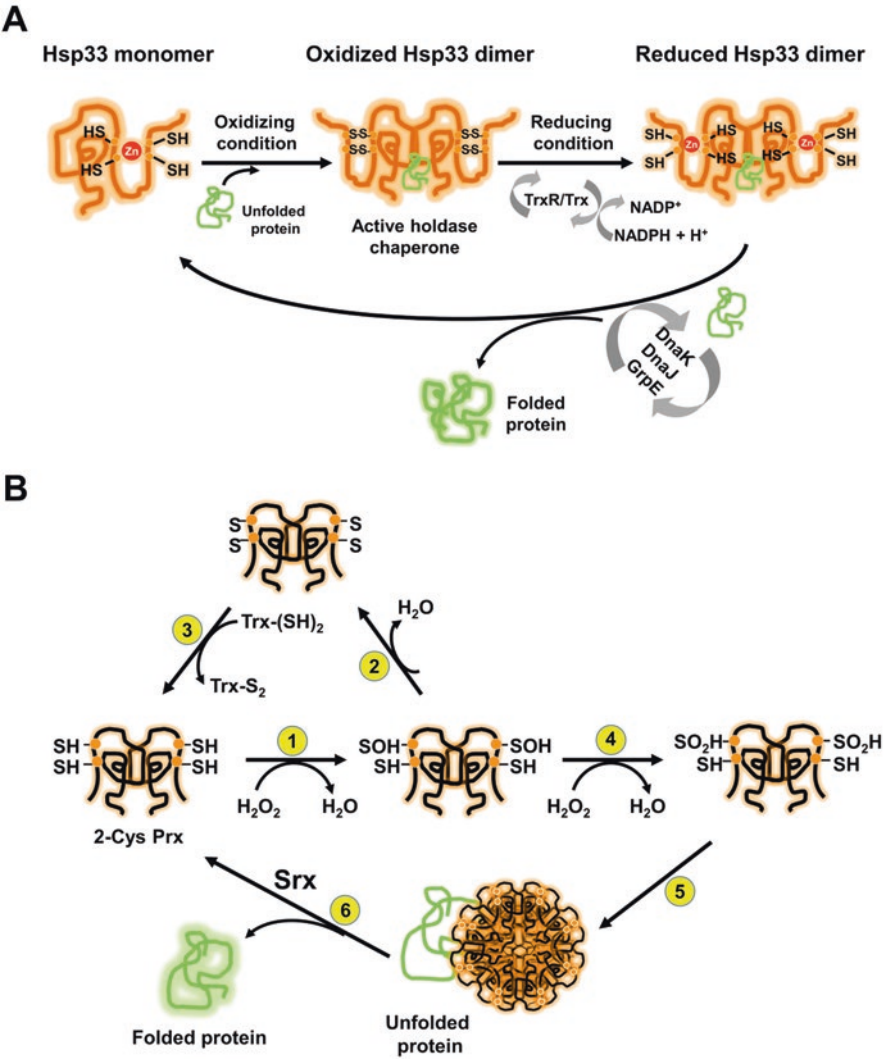

Fig. 4.7 Examples of redox-regulated chaperone pathways. (**a**) The redox-chaperone model of Hsp33. Upon exposure of Hsp33 to oxidative stress, the substrate binding site and the dimerization interface of the C-terminal redox domain was exposed by the disulfide bond switch and releasing zinc ion (Zn), which quickly associate to the fully active Hsp33 dimer and sequentially reduced either by the TRX or GRX system. Upon return to non-stressed conditions, the substrate proteins can be released from reduced Hsp33 dimers and refolded by the DnaK/DnaJ/GrpE machinery. (**b**) Functional chaperone activity of 2-Cys PRX by disulfide switch. Under nonstress conditions, PRX catalyzes the reduction of H_2O_2 to H_2O to form sulfenic acid (-SOH) (*1*). The resolving Cys of the other subunit attacks sulfenic acid, and an intermolecular disulfide bond is formed (*2*). The disulfide bond is reduced by the TRX system (*3*). Under oxidative stress conditions, sulfenic acid reacts with H_2O_2 molecule to lead the sulfinic acid (-SO_2H) formation (*4*). Sulfinic acid inactivates the peroxidase activity of 2-Cys PRX and supports the assembly into a high-molecular weight (HMW) complex with chaperone activity for the prevalence of unfolding protein aggregations in vitro (*5*). Neither the precise structure of the HMW complexes nor the binding site for unfolding proteins has been described. Reduction of overoxidized PRX is catalyzed by sulfiredoxin (SRX) upon return to nonstress conditions and facilitate protein refolding (*6*)

regulated as its activation. With a return to reducing conditions, the activation of Hsp33 dimers is necessary reduced either by the cellular TRX or GRX system (Ilbert et al. 2007). Then the substrate proteins were released from reduced Hsp33 dimers, and can be refolded by the DnaK/DnaJ/GrpE system; meanwhile, reduced Hsp33 dimers dissociate into oxidized monomers and were inactivated. Thus, Hsp33 played an important role in bacterial defenses against OS.

Hsp33 is apparently exclusively activated by simultaneous H_2O_2 and elevated heat, but not by either stress alone (Winter et al. 2005); it is tightly regulated on both transcriptional and posttranslational levels. Whereas OS triggers the post-translational disulfide bond formation in Hsp33, HS increase the Hsp33 concentration and stimulate its dimerization. In *E. coli*, the expression of *Hsp33* gene is controlled by the HSF, σ32, and is strongly induced upon HS treatment (Chuang and Blattner 1993; Nonaka et al. 2006). *Hsp33* is constitutively expressed under non-stressed conditions, however, HS exposure triggers to a massive RNA-level of *Hsp33* increases by as much as 30-fold. This translates into about twofold on the protein level, which increases the cellular HspP33 concentration to about 3 μM (Jakob et al. 1999). It indicated that the transcriptional regulation controls the cellular amount of Hsp33.

4.5.5 Peroxiredoxins (PRX)

PRX has been classified as novel members of the TRX-fold superfamily, play a functional switch from a disulfide reductase to a molecular chaperone. PRX form a highly conserved superfamily of around 20–30 kDa antioxidant enzymes in which redox-active Cys residues participate in the reduction of H_2O_2 (Chae et al. 1994). Based on their catalytic mechanism, PRX was divided into three classes: typical 2-Cys, atypical 2-Cys, and 1-Cys PRX (Wood et al. 2003). All PRX share the same basic catalytic mechanism, in which an active-site is oxidized to a sulfenic acid (-SOH) by the peroxide substrate. The typical 2-Cys PRX is a TRX-dependent peroxidase, in that the oxidoreductase of TRX is required to restore their activity by reducing the disulfide between the catalytic Cys. They have been shown to detoxify H_2O_2, through their catalytic Cys residues, resulting in the formation of disulfide bonds of the proteins. Subsequently, the disulfide bond is reduced with the help of various reducing systems, such as TRX, GRX or cyclophilin as reductants, thereby maintaining the intracellular redox balance and protecting organisms against oxidative stress.

However, TRX peroxidase-independent activities have also been identified for typical 2-Cys PRX in signal transduction and as chaperones (Moon et al. 2005; Veal et al. 2004). A detailed catalytic cycle has been derived for typical 2-Cys PRX, including a model for the redox-regulated oligomeric state proposed to control enzyme activity (Fig. 4.7b). Particularly, it is worth mentioning that PRX serve as a chaperone under oxidized or over-oxidized conditions (Poole et al. 2000). During the H_2O_2-catalyzing process, the peroxidase activity of 2-Cys PRX in eukaryotic cells including yeast, plant and mammalian is completely inactivated by the high concentrations of H_2O_2, producing reversible sulfenic acid (-SOH) intermediate and

the hyperoxidized sulfinic acid form (-SO_2H) of the proteins. Accompanying with the structural changes from low molecular weight (LMW) to high molecular weight (HMW) oligomers, 2-Cys PRX is shown to rapidly and reversibly switch from a peroxidase to a molecular chaperone, which can prevent the misfolding or aggregation of intracellular macromolecules caused by external stresses (Kumsta and Jakob 2009). Neither the precise structure of the HMW complexes nor the binding site for unfolding proteins has been described. Instead of using disulfide bond formation as the redox switch, 2-Cys PRX apparently use a unique "sulfinic acid switch" to convert from a peroxidase under non-stressed conditions to a molecular chaperone under severe oxidative stress conditions (Moon et al. 2005). Sulfinic acid formation inactivates the peroxidase activity and supports the assembly of PRX into chaperone-active HMW complexes, which prevent the aggregation of unfolding proteins in vitro. Disulfide bond formation and over-oxidation seem to be competing for events in the catalytic cycle of 2-Cys PRX. While disulfide bond formation requires the local unfolding of the active site, over-oxidation appears to occur in the folded conformation (Kumsta and Jakob 2009). The yeast PRX and the human Prx2 are temperature-dependent multimers, thereby increasing chaperone activity and interacting with unfolded substrates to suppress thermal degradation of citrate synthase. In contrast, H_2O_2 induced chaperone activity of yeast and human Prx2 requires the active site cysteinyl side chains and the TRX system as a cofactor (Moon et al. 2005).

4.6 Conclusions

The precise molecular connections of the redox components in eukaryotic chaperone systems, such as how TRX families work as molecular chaperones or synergistically coordinate with HSP chaperone to participate in protein folding, to prevent cellular damage and to re-establish cellular homeostasis. Potential target proteins may reveal the interactions between TRX and molecular chaperones. For example, by affinity chromatography using various TRX as bait proteins revealed Arabidopsis Hsp70s and Hsp17 as putative TRX targets (Buchanan and Balmer 2005). Glutathionylated human Hsc70 was characterized as a substrate for Grx1, the synergistic effect of Grx1 and Hsc70-SSG suggests that glutathionylation acts as a signal for cooperative binding between these two proteins to enhance chaperone activity under OS (Hoppe et al. 2004). We propose the possible cross-talk between redox and chaperone network is described in Fig. 4.8. Although most of these studies were carried out in other organisms, for example, prokaryotic Hsp33 proteins, similar cross-talk mechanisms might be operated in plants. However, the exact action of TRX as the regulator of chaperone folding interplaying in cellular redox states in plants remains to be established. The questions to be answered next concern the underlying mechanisms and the identification of TRX molecular targets. Ongoing intensely focus on the functional and structural aspects of HSP and TRX, and cross-talk with other chaperone systems may provide further elucidation of their functions.

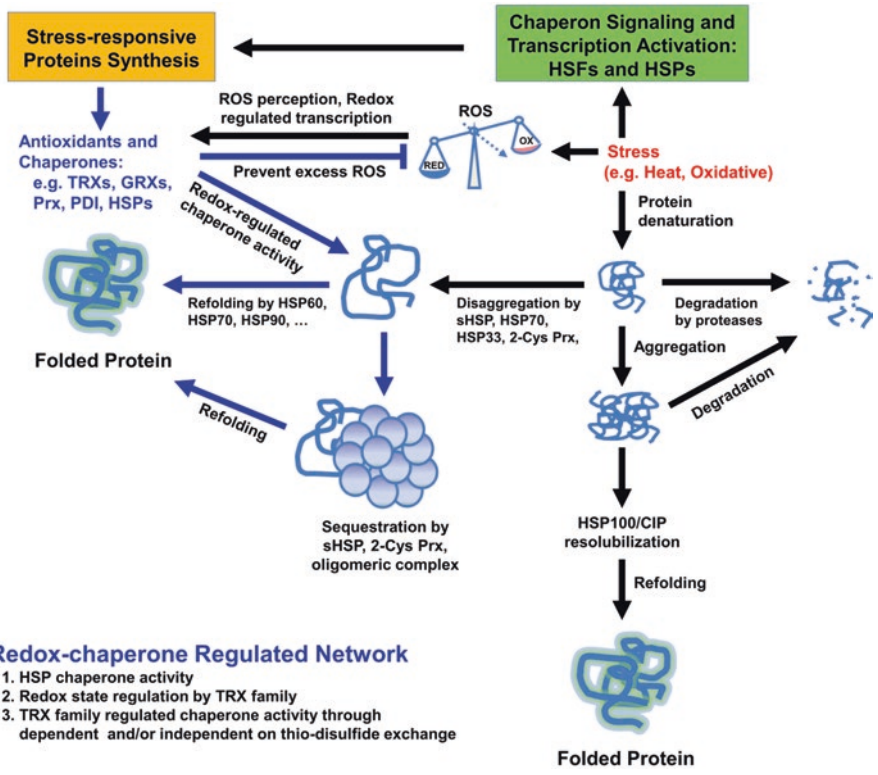

Fig. 4.8 A proposed mechanism of cross-talk between redox and chaperone network. To maintain cellular homeostasis, members of the chaperone families e.g. Hsp70s stabilize protein conformation, prevent aggregation and thereby maintain the non-native protein in a competent state for subsequent refolding, which is achieved by other chaperones (e.g. Hsp60, Hsp70, and Hsp90). When denatured or misfolded proteins form aggregates, they can be re-solubilized by Hsp100/Clp followed by refolding or degraded by a protease. Some chaperones (e.g. Hsp70, Hsp90) that controlled by the HSF accompany the signal transduction and transcription activation that leads to the synthesis of other chaperones and other stress-responsive proteins (e.g. antioxidants). At least two mechanisms are required for cells to regulate the intracellular level of ROS, one is able to fine modulate the low levels of ROS for signaling, and the other is able to detoxify the excess of ROS. ROS such as H_2O_2 can be a specific signaling molecule and interact with Cys residues within antioxidant proteins (e.g. TRX and GRX) which through the reversible formation of inter- or intramolecular disulfides is a prerequisite for the proper folding and function of a protein or protein complex, play complementary and overlapping roles with other chaperones and therefore initiating subsequent cellular responses in preventing dysfunction of proteins from the stress

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Chapter 5
Dynamics of Heat Shock Proteins in Immunity and Aging

Udayakumar Prithika and Krishnaswamy Balamurugan

Abstract Heat Shock Proteins (HSP) are one of the classical molecules that regulate cellular homeostasis. HSP play multifunctional roles that are crucial for folding/unfolding of proteins, cell-cycle control and signaling, and protection of cells against stress/apoptosis. HSP have also been implicated in antigen presentation with the role of chaperoning and transferring antigenic peptides and providing immunity. HSP have been referred as molecular chaperones since they assist in the repair of denatured proteins or promote their degradation after stress or injury. Moreover, HSP are likely to have anti-apoptotic properties and have been reported to be significantly elevated in a plethora of human cancers. The increase in expression levels of HSP has been robustly related with therapeutic resistance and poor survival. The immunological functions and prospective immunological repertoire of HSP put them in critical position that serves as important therapeutic implications for specific drug targets. In this chapter, we have discussed on the existing scientific data about HSP with an effort to highlight the possible future implication of HSP during stress, aging, apoptosis and their status at post-translational and mitochondrial level and the possible drug targets for improving prognosis and treatment of various diseases.

Keywords Aging · *C. elegans* · Heat shock factor -1 · Heat shock proteins · Immunity

Abbreviations

HSF	heat shock factor
HSP	heat shock proteins
PTM	post translational modification
RNAi	ribonucleic acid interference
ROS	reactive oxygen species

U. Prithika · K. Balamurugan (✉)
Department of Biotechnology, Science Campus, Alagappa University, Karaikudi, Tamil Nadu, India

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5.1 Introduction

In the primordial time, it was believed that immunity for the human system was rendered by network of cells, tissues and organs. The elaborate and dynamic communication networks provide ideal immunity against foreign particles (bacteria, virus, fungi and parasites). Although researchers have learned much about the immune system, they continue to study how the body prepares to fight against and destroy invading microbes, infected cells and tumors, making an exception to the healthy tissues. Millions of cells organize as subsets and transfer the information to and fro during an infection. Innate immune response being the first line of defense produces phagocytic cells such as neutrophils, macrophages, cytotoxic natural killer (NK) cells, and granulocytes, while, adaptive immune response delivers antigen specific response through T cell and B cell (Shojadoost et al. 2017; Marcus et al. 2014; Li et al. 2017; Palazon et al. 2017). In the early 1960s pioneering work of Ferrucio Ritossa on heat shock proteins (HSP) found in fruit fly *Drosophila melanogaster* was the first report on HSP (Ritossa 1962). In addition to the various immune cells that come into the action during infection, HSP are known to aid in regulating immune response. HSP express during different kind of stresses such as temperature (heat), hypoxia, nutrient deprivation, irradiation and heavy metals, as well as oxidative stress, infections and exposure to inflammatory cytokines (Lindquist and Craig 1988; Jaattela 1999) (Fig. 5.1). These stress proteins have high cytoprotective effects and acts as molecular chaperones and maintain the protein homeostasis (Lanneau et al. 2008). The HSP families include proteins of different molecular weight 100, 90, 70, 60 and 40 kilodaltons (kDa), as well as the small HSP of 15–30 kDa. The propagation of HSP is conserved through all plant and animal species including humans (Li and srivastava 2004). This has led to the study of HSP in a nematode model system, *Caenorhabditis elegans* which is one of the closely related organism to the human systems. *C. elegans* is the lowest eukaryote and bilateral ancestor of *Danio rerio* and *Drosophila melanogaster.* With a very simple life-cycle, this soil nematode gives a wider platform in biomedical research (Kaletta and Hengartner 2006).

C. elegans has been used as disease model since 1963, which was proposed by South African biologist Syndney Brenner. It is relatively small (~1 mm for an adult worm) making it instrumental to study a variety of biological processes including cell polarity, cell cycle, cell signalling, gene regulation, metabolism, apoptosis, aging and sex determination (Riddle and Albert 1997). Since HSP are implicated in aging phenotypes and control of lifespan across species, the roles of HSP in an invertebrate system like *C. elegans* aids in finding function in novel targets. Indeed, the roles of HSP have been positively found to correlate with aging and immunity of *C. elegans*. This book chapter will discuss on various aspects of HSP such as function and the possible mechanisms that confer immunity against pathogens or response to any stress with respect to the model *C. elegans.*

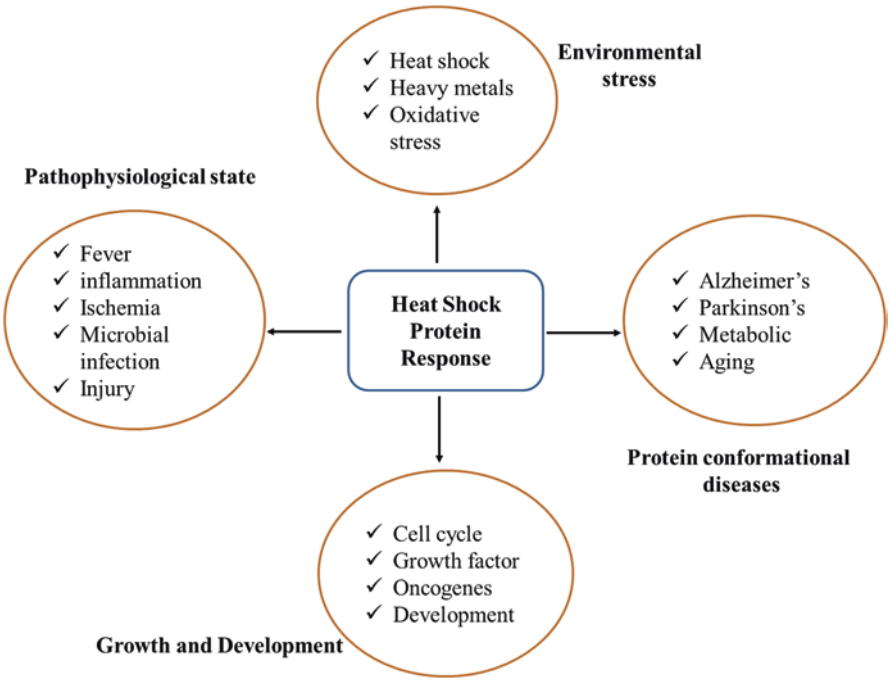

Fig. 5.1 Schematic representation of different types of responses produced by HSP. The role of molecular chaperones in protein folding, translocation, and protection against the deleterious consequences of misfolded proteins; and the role of the heat-shock response and heat-shock proteins in cytoprotection against disease

5.1.1 HSP and Stress Response

Cells respond to rapid changes in their protein biogenesis by exposure to extreme environmental conditions, such as heat, oxidative stress or transition metals, by inducing a highly conserved set of genes (the stress response) (Morimoto 1998). During stress, most HSP act as molecular chaperones that interact with other proteins and prevent aggregation by regulating protein folding, protein translocation and assembly of protein complexes. This plethora of stress signals assist in repair of protein damage (Wu 1995). HSP even facilitate the refolding of malfolded proteins by binding them to the protein complexes. *C. elegans* grow at an optimum temperature of 20–25 °C, any change in the ideal temperature will lead to developmental delay. Increase in temperature above 27 °C will have decreased sustainability, while, decrease in temperature below 16 °C will lead to fecundity (Zevian and Yanowitz 2014). The temperature within the range of 35–37 °C was used for the experiments dealt with *C. elegans* to analyse the HSP and conveniently a lethal dose to restrict the development of new stage (Prithika et al. 2016; Link et al. 1999). Higher level of HSP-16 was found in thermotolerant strains and significant increase in longevity was observed (Walker et al. 2001). Upstream to the HSP is HSF-1, a conserved

transcription that binds to heat shock elements (HSE) that produces a response to the heat stress (Guha Thakurtha et al. 2002). Kumsta et al. (2017) also proposes that overexpression of HSF-1 is enough to recapitulate the effects of HSP.

Oxidative stress also induces wide range of genes, while HSP are one among them. The expression level of *hsp-16.2* was reported to be significantly elevated during oxidative stress (Park et al. 2009; Crombie et al. 2016). Similar to oxidative stress, heavy metal stress also provokes heat shock response especially *hsp-70* with a 60-fold increase in expression level. Transition metals that could elicit heat shock response includes iron (Fe), zinc (Zn), copper (Cu), cobalt (Co), manganese (Mn) and molybdenum (Mo), however, only the higher levels of these would cause a lethal effect on the host (Caito et al. 2012). The toxicity of cadmium (Cd), lead (Pb), chromium (Cr), and arsenic (As) on *C. elegans* was investigated, out of which, cadmium exhibited a higher level of tolerance suggesting that it could be used as sensitive biomarker for environmental monitoring and risk assessment (Roh et al. 2006). A very recent report by Ezemaduka et al. (2017) also validates the role of HSP-17 in the resistance of metal stress condition against cadmium and zinc.

5.2 HSP in Aging and Immunity

Aging in most system is caused by cell senescence or certain age related diseases (Tower 2009). The HSP are increasingly associated with aging phenotypes and control of lifespan across species (Morimoto 2008). Forward genetic screens have helped to identify the individual molecular chaperones such as HSP-70 and sHSP family of proteins has been implicated in extending the lifespan of nematode (Yokayama et al. 2002; Walker and Lithgow 2003). Overexpression of HSF-1 also resulted in subsequent activation of HSP-70 leading to an increase in the lifespan (Morley and Morimoto 2004). While insulin signaling pathway is the classical longevity pathway, stress response produced by HSP have consistently proven to aid the molecular mechanisms that elongate the age of the nematode (Zhou et al. 2011). Pre- treatment of the host with a short term exposure of heat also exhibits positive effects on the healthspan and lifespan of *C. elegans* (Prithika et al., 2016). However the major role is played by HSF-1, wherein the chaperone molecules are expressed only under the control this transcription factor. HSF-1 appears to also play a central role in many pathophysiological functions underlying in protein misfolding diseases such as Alzheimer's and Huntington's (Calderwood and Murshid 2017).

In addition to the function played during stress, HSF also contribute to more complex organismal physiological processes such as development, growth, reproduction, immunity and aging (Morton and Lamitina 2013). A typical HSF-1 contains N-terminal DNA binding and trimerization domains, with a recognizable transactivation domain at the C-terminus (Hajdu-Cronin et al. 2004). Knockdown of *hsf-1*by RNA interference (RNAi) gives rise to aged phenotype, while overexpression of *hsf-1* promotes longevity and delays age-related protein misfolding and proteotoxicity

(Garigan et al. 2002; Hsu et al. 2003; Cohen et al. 2006). Other important roles of HSF-1 and HSP include important functional roles in *C. elegans* innate immunity by inhibiting the pathogen induced protein aggregation (Singh and Aballay 2006; Mohri-Shiomi and Garsin 2008; Jebamercy et al. 2016).

5.3 HSP Targets at the Post-translational Level

Post translational modification (PTM) is an essential mechanism wherein the polypeptide chain undergoes proper folding and modifies the protein for specificity and targeting. Any mis-regulation in this process might lead to severe consequences on cell growth, division and survival (Henze et al. 2016). There are more than 200 different types which majorly include phosphorylation, oxidation, glycosylation, acetylation, deamidation, sumoylation, etc. All these PTMs regulate cellular and protein homeostasis under optimal conditions. PTMs also help in sensing the damaged protein and to coordinate the activities of protective stress response pathways and chaperone networks for adaptation and survival (Balch et al. 2008). Even though, the chaperonic activity restore folding equilibrium, the cell does not possess adequate mechanism to control the conformationally challenged aggregation-prone proteins that are expressed in cancer, metabolic disease, and neurodegenerative disease. This links stress with protein homeostasis and implicates that restoration activities compromises the integrity of the proteome paving the way to influence aging related genes (Morimoto 2008).

HSP are also regulated by PTMs such as phosphorylation, sumoylation and acetylation (Voellmy 2004). Various stresses that cause protein mis-folding will therefore grounds changes in the amount and disposal of HSP that will be transduced to their networks of clients, thereby altering signaling pathways, protein localization, stability, and affecting overall changes in the cell's physiology (Fig. 5.2). AMPylation of HSP-70 covalently modifies core histones and translation elongation factors which helps in protection of host from *Pseudomonas aeruginosa* infection (Truttmann et al. 2016). Similarly, the HSF-1 also undergoes PTMs which is also essential for protection and appears to predominantly regulate disaggregation activity (Cohen et al. 2006). HSF-1 is post translationally modified by phosphorylation activity in the presence of heat shock to make them bind to the HSE (Chiang et al. 2012).

5.4 HSP Role at the Mitochondrial Level

Mitochondria are almost entirely composed of proteins encoded by the nuclear genome and often challenged by toxic by-products of metabolism as well as by pathogen attack (Melo and Ruvkun 2012). Mitochondrial damage induced chaperone genes *hsp-6* and *hsp-60* to go for xenobiotic detoxification and pathogen-response pathway (Liu et al. 2014; Durieux et al. 2011). Report by Kimura also suggests that knockdown of mitochondrial HSP-70 leads to aging phenotype in

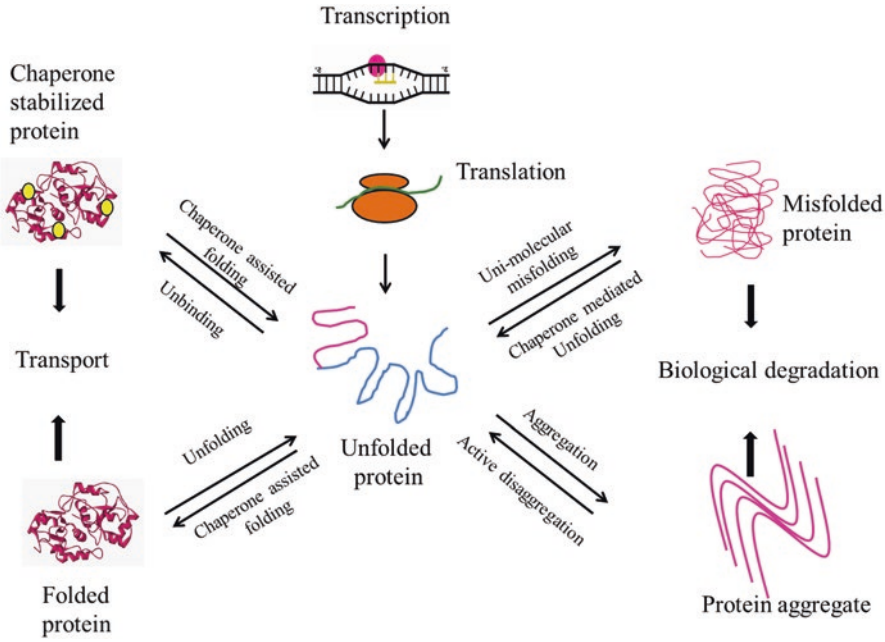

Fig. 5.2 Pictorial representation of the events happening after the translation of proteins. Proteins are synthesized at ribosomes based on the information in mRNA. The proteostasis network integrates chaperone pathways for the folding of newly synthesized proteins, for the remodeling of misfolded states, and for disaggregation with protein degradation that is mediated by the Ubiquitin Proteosome pathway and autophagy system. More than 180 different chaperone components and their regulators orchestrate these processes in mammalian cells

young adult nematodes. Moreover, reduction in the levels of HSP-6 caused the mitochondrial HSP-60 to alleviate therefore leading to mortality of *C. elegans* (Kimura et al., 2007). Similar study by Bennett et al. 2014) suggests that *hsp-6* and *hsp-60* are among the mitochondrial stress response genes that played an important role in longevity by activating the unfolded protein response (UPR). Increase in level of Reactive oxygen Species (ROS) which happens in the mitochondria also induces the *hsp-16* and *hsp-16.2* thereby leading to the extension of post growth span (Munkacsy and Rea (2014). Furthermore, expression of HSP has also been reported to elicit UPR in endoplasmic reticulum (Durai et al. 2014; Jovaisaite et al. 2014).

5.5 HSP Role in Death Receptor Pathway and Apoptosis

Following a cellular damage, HSP level get induced which prevents the cells from programmed cell death by activating either intrinsic or extrinsic death pathways (Beere 2005). The former is associated with mitochondrial outer membrane permeabilization and the latter is categorized by ligation of cell surface death receptors.

These pathways aid in paradoxical activation of pre-apoptotic or anti-apoptotic events which averts the commencement and tenacity of a disease state. One of the main causes of apoptosis in *C. elegans* is the activation of the cysteine protease *ced-3*. The activator protein *ced-4* undergoes oligomerization which mediates the activation of *ced-3* (Yuan and Horvitz 2004). Out of 1090 somatic cells in *C. elegans*, 131 cells go through apoptosis in a lineage-specific and, to a large extent, cell autonomous manner. These events are supported by few caspases that involves cascade of genes. During apoptosis caspases involve in cleavage of the translation initiation factor especially preventing the capping of mRNA at the 5′ end. Alteration in the levels of *hsp-3* and *hsp-4* modulated the cap-dependant translational efficiency and induced apoptosis in *C. elegans* (Morrison et al., 2014). Another report by Qian et al. suggests that *hsp-16* level is elevated and *ced-3* is decreased when there is an over expression of *bmk-1*, which is found to function in chromosome segregation (Qian et al. 2015).

5.6 Conclusions

HSP are functional in maintaining protein homeostasis in a normal cell. They are ubiquitously expressed and found to be abundant in mammalian cells. With wide range of proteins involving in signal transduction, protein assembly, apoptosis and immunity, HSP have been recognized as a potential disease target. A critical understanding of target protein, their subunit-subunit interaction, cytoskeletal elements need to be characterized. Furthermore, the physiological and biological functions of the HSP have to be up to date. This presumably suggests more research has to be done to target these stress chaperones and cell specific targeting depending on the stratification of diseases is mandatory.

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Chapter 6
Heat Shock Proteins, Exercise and Inflammation

Fabiano T. Amorim and Micah N. Zuhl

Abstract The heat shock response (HSR) plays a regulatory role in controlling inflammatory events within a cell. The activation/induction and interplay of heat shock factor 1 (HSF1) and heat shock proteins (HSP) have inhibitory effect on nuclear factor-kappa B (NFκB) inflammatory pathway, c-Jun N-terminal kinases (JNK) regulation, and preventing free radical damage. Exercise training induces the HSR and has the potential to reduce inflammation. The current chapter examines the regulatory impacts of the HSR on inflammation and the role of the HSR in chronic inflammatory disease states, such as skeletal muscle insulin resistance and ischemia/reperfusion injury of the myocardium. In addition, we discuss the inflammatory role of exercise training in activating the HSR, improving insulin signaling, reducing vasculature inflammation, and promoting cardioprotection against ischemia/reperfusion injury of the myocardium.

Keywords Cardioprotection · Cardiovascular disease · Free radical damage · Insulin resistance · Ischemia/reperfusion · Type 2 diabetes mellitus

Abbreviations

AKT	protein kinase B
AMPK	5′ adenosine monophosphate-activated protein kinase
AP-1	activation protein-1
ERK1/2	extracellular signal-regulated protein kinases 1 and 2
GLUT4	glucose transporter type 4
HIIT	high intensity interval training
HOMA	homeostasis model assessment index

F. T. Amorim (✉) · M. N. Zuhl
Exercise Science Program, Department of Health, Exercise, and Sports Sciences, University of New Mexico, Albuquerque, NM, USA
e-mail: amorim@unm.edu

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HSF-1	heat shock factor 1
HSP	heat shock proteins
HSP27	heat shock protein 27
HSP60	heat shock protein 60
HSP70 or HSPA	family of heat shock protein 70 kda
Hsp72 or HSPA1A	heat shock protein 72 kda
HSR	heat shock response
IKK	inhibitor of NF-κB kinase
IL-1β	interleukin-1 β
IMTG	intramuscular triglyceride
IR	insulin resistance
IRS-1	insulin receptor substrate 1
JNK	c-jun amino terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
NFκB	nuclear factor-kappa B
OGTT	oral glucose tolerance test
PBMC	peripheral blood mononuclear cell
RNS	reactive nitrogen species
ROS	reactive oxygen species
T2DM	type 2 diabetes mellitus
TNF-α	tumor necrosis factor-alpha
UCP-3	uncoupling protein 3
β-HAD	β-hydroxyacyl-CoA-dehydrogenase

6.1 Introduction

The heat shock response (HSR) is an intracellular chaperone mediated protein maintenance system involved in successful folding/re-folding of damaged proteins upon exposure to a wide range of stressors (e.g. heat, oxidative stress, bacteria, virus) (Feder and Hofmann 1999). The activation and induction of HSR components, namely the inducible heat shock protein 70 (HSP70 or HSPA) family, confer protection against subsequent exposure to a damaging stimuli; and support adaptations to the stressful environment (i.e. heat tolerance and heat acclimation). Further, overexpression of HSP70 from heat preconditioning or genetic engineering provides protection against lethal cellular insult such as lipopolysaccharide (LPS) exposure. A robust amount of literature exists examining the protective affects of the HSR chaperone system in various tissues (skeletal muscle, heart, brain, liver) under conditions of cellular insult (e.g. exercise, ischemia, heat stress). However, in the early 2000s, it was determined that the HSR may play a regulatory role in controlling inflammatory events within a cell (van Eden et al. 2005; Yoo et al. 2000). In this function, activation of the HSR controls pro-inflammatory signaling, such as cytokine production and release, that may lead to organismal injury or death.

The purpose of this chapter is to examine the regulatory impacts of the HSR on inflammatory pathways. In Sect. 6.1, we discuss the mechanism of how the HSR regulates inflammatory pathways. Section 6.2 highlights the role of the HSR in chronic inflammatory disease states. In addition, throughout the chapter, the role of exercise in activating the HSR is discussed.

6.1.1 Inflammatory Events Activating the HSR

A host of molecular events trigger cellular activation of the HSR. The wide range of signaling factors allows organisms to survive and adapt to an expanse number of environmental challenges. The obvious inducible factor is heat or hyperthermia; however, cold stress, oxidative, bacterial, and viral insults all activate the HSR (Hartl 1996). The HSR serves as a direct protective mechanism within the cell through re-folding of damage proteins to their native state, and prevention of apoptosis (Morimoto et al. 1997; Morimoto and Santoro 1998; Mosser et al. 2000; Yenari et al. 2005). This is demonstrated by greater cell survival in myocardial and brain tissue after ischemic (oxidative damage) insult among transgenic mice overexpressing HSP70. More recent, an anti-inflammatory role of the HSR has been identified, and has been referred to as the heat shock regulatory pathway (Yenari et al. 2005). This is most identifiable in a model of sepsis where invading pathogens promote the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) by leukocytes (neutrophils, monocytes, macrophages) leading to cellular destruction (Bruemmer-Smith et al. 2001). Global induction of HSP70 through heat pretreatment increases recovery from septic shock in animals (Hotchkiss et al. 1993). The regulatory effect of the HSR is mediated through suppression of inflammatory cytokine producing pathways and overall reduction in cellular damage. While the bacterial insult alone may activate heat shock factor 1 (HSF-1) leading to upregulation of multiple heat shock proteins (HSP) and cellular protection, evidence suggests that both HSF-1 and HSP70 have dual, and possibly separate roles, in controlling inflammation (Singh et al. 2004). An example of these binary roles was demonstrated in mouse macrophages where heat induced activation of HSF-1 DNA binding suppressed TNF-α production in the absence of HSP70 induction (Singh et al. 2004). In addition, overexpression of HSP70 in the absence of HSF-1 in human peripheral monocytes decreases TNF-α and interleukin-1 β (IL-1β) levels during LPS exposure (Ding et al. 1998, 2001). It is very difficult to differentiate the regulatory effects of HSF-1 and HSP70 on inflammation. Evidence suggests that HSF-1 may play a role in transcriptional control of inflammatory molecules (i.e. TNF- α, vascular adhesion molecules); while HSP70 may regulate upstream inflammatory pathways [i.e. Nuclear factor-kappa B (NFκB), c-jun amino terminal kinase (JNK)] (Mizushima 2010). This simplified model is diagrammed in Fig. 6.1. However, these conclusions are controversial and dependent upon cell/tissue type, experimental model (e.g. cell culture, rodent, human), and type of stressor (e.g. heat, oxidative, bacterial). Throughout Sect. 6.1 of this chapter we will detail the regulatory control

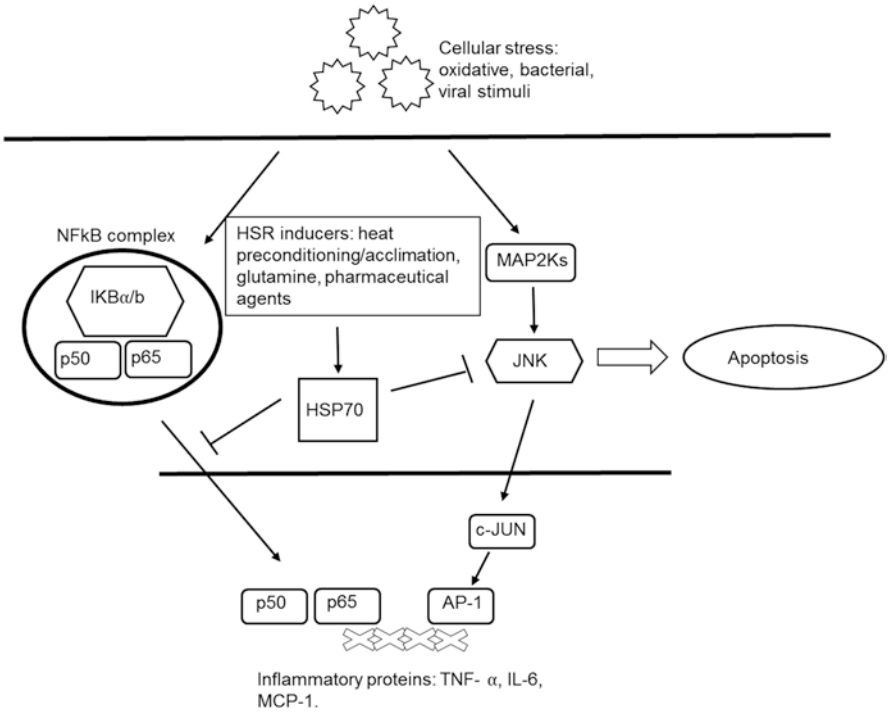

Fig. 6.1 The regulatory role of the HSR on NFκB and JNK pathways. Cellular stress activates both pathways (NFκB and JNK) leading to production of pro-inflammatory cytokines and apoptosis. Induction of the HSR through heat preconditioning and acclimation regulates inflammatory events though inhibition of NFκB and AP-1 transcriptional activity. *NFκB* nuclear factor enhancer of activated B cells, *IκBα/β* nuclear factor inhibitor, *p50* NFκB subunit, *p65* NFκB subunit, *MAP2K* MAP kinase kinases, *JNK* c-Jun N-terminal kinase, *AP-1* activator protein 1, *HSP70* heat shock protein 70, *TNF-α* tumor necrosis factor alpha, *IL-6* interluekin 6, *MCP-1* monocyte chemoattrant protein 1

of the HSR on both the NFκB and JNK inflammatory pathways. We will also briefly comment on the role of the HSR in modulating damage induced by free radicals. In each discussion, we will attempt to differentiate the roles of HSF-1 and HSP70 where appropriate. Lastly, HSR induction through exercise and nutritional supplements will be highlighted.

6.1.2 Regulation of NFκB Inflammatory Pathway

Transcriptional activity of pro-inflammatory cytokines is regulated by the NFκB inflammatory pathway, which is activated under conditions of cellular stress (heat, oxidative, sepsis, exercise) (Gloire et al. 2006; Selkirk et al. 2008). NFκB is

inactively bound in the cytosol to a complex of proteins (IκBα and IκBβ), and upon phosphorylation by inhibitor of NF-κB kinase (IKK), translocates to the nucleus to activate genes of inflammatory proteins (Liu and Malik 2006). The NFκB pathway is tightly controlled and important for innate immunity and cellular protection against bacterial insults; however it has been implicated in chronic low grade inflammatory conditions such as irritable bowel diseases, rheumatoid arthritis, and chronic obstructive pulmonary disease (Bektas et al. 2018; Holgate 2004). Robust activation of the NFκB in massive inflammatory events such as septic shock have resulted in cellular death, where inhibition in this situation improves survival (Li et al. 2009). For these reasons drugs targeting the NFκB pathway have been explored for treatment of various inflammatory states (Miller et al. 2010).

Utilizing various experimental models, researchers have shown that mild heat pretreatment prior to bacterial exposure resulted in suppression of NFκB activation, which correlated with increased HSP70 levels (Guzhova et al. 1997; Schell et al. 2005). This has been demonstrated in human lymphoma cells pretreated at 43 °C for 15 min prior to bacterial insult; brain tissue from rodents injected with LPS after whole body exposure to 41 °C for 20 min; and mouse macrophages immersed in a 42 °C water bath for 1 h before treatment with LPS (Guzhova et al. 1997; Heneka et al. 2003; Shi et al. 2006). In an interesting experiment, Brunt et al. (2017) exposed cultured endothelial cells to oxidative stress after supplementation with serum from heat treated humans who underwent 8-weeks of passive heat therapy by water immersion. NFκB activation and pro-inflammatory cytokine production were markedly reduced. HSP70 cellular inhibitory effects on NFκB may be through HSP70 physical protein binding with the **rel65** subunit of the NFκB complex preventing phosphorylation activity, and activation (Kizelsztein et al. 2009; Sun et al. 2005). In addition, HSP70 may have a regulatory role by preventing IKB degradation and nuclear translocation of NFκB, which appears to be independent of HSF-1 activation (Dokladny et al. 2010).

Acute, high intensity exercise and heat stress have been shown to activate NFκB in peripheral blood mononuclear cells (PBMCs) (Cuevas et al. 2005; Selkirk et al. 2008; Vider et al. 2001). Recently, we have demonstrated that overexpression of HSP70 in human PBMCs through glutamine supplementation suppresses NFκB activation in response to exertional heat stress (Dokladny et al. 2013; Zuhl et al. 2014, 2015). The PBMC overexpression of HSP70 further exhibited an inhibitory effect on interleukin 6 (IL-6) and TNF-α release and mRNA expression in response to a bacterial insult (Dokladny et al. 2010; Sun et al. 2005). In addition, work from our lab has shown that inhibiting PBMC HSP70 response to heat and exercise stress through anti-oxidant supplementation reduces the cytoprotective ability of the cells (Kuennen et al. 2011). In summary, activation of the HSR through heat pretreatment or glutamine supplementation has an inhibitory effect on the NFκB inflammatory pathway. Regulation is mediated through HSP70 control of NFkB by binding to rel65 unit, and preservation of IKBα inhibitory protein.

6.1.3 Regulation of JNK Pathway

The JNK pathway (sometimes referred to as stress activated protein kinase) is part of the mitogen activated protein kinase (MAPK) family and is responsible for inducing pro-apoptotic and inflammatory proteins (Dhanasekaran and Johnson 2007; Dhanasekaran and Reddy 2008). Ultraviolet irradiation, oxidative stress, heat, bacterial, cytokines, and ethanol exposure activate upstream MAP kinase kinases (MAP 2Ks) leading to phosphorylation and translocation of JNK to the nucleus. JNK phosphorylates and transactivates c-JUN ultimately forming activation protein-1 (AP-1), which is involved in transcription of a variety of proteins (Chang and Karin 2001; Dhanasekaran and Reddy 2008; Turjanski et al. 2007). Overexpression alone of JNK in human embryonic kidney cells causes profound cellular cytotoxicity and death (Chen et al. 1996). Drugs targeting the JNK pathway have become an emphasis for pharmaceutical researchers because overexpression of JNK has been linked to chronic inflammatory diseases such as diabetes, obesity, irritable bowel diseases, and atherosclerosis (Kaneto et al. 2004; Karin and Gallagher 2005; Ricci et al. 2004).

The HSR interferes with JNK signaling and improves cell survival upon exposure to damaging stimuli, and appears to be independent, or in the absence of protein damage repair (Gabai et al. 1998). In this model, the HSR inhibits the pro-apoptotic mechanisms of JNK activation under conditions of mild UV damage or TNF-α exposure, which does not induce protein damage (Gabai et al. 1998). HSP70 induced by both heat pre-treatment and transfection experiments drastically reduced apoptosis and inhibited JNK signaling in human lymphoid tumor cells (Gabai et al. 1997). This has been further demonstrated in rodent liver tissue, along with both animal and human skeletal muscle and macrophages (Adachi et al. 2010; Chung et al. 2008; Gupte et al. 2009). In both liver and skeletal muscle tissue, the inhibitory effect of HSP induction on JNK resulted in improved glucose uptake. In macrophages, HSP70 overexpression resulted in reduced pro-inflammatory cytokine release during LPS exposure, which was mediated by JNK inactivation (Wang et al. 2002). It appears that HSP70 has regulatory control by physically binding to JNK thus acting as a direct inhibitor (Park et al. 2001). In addition, HSP70 has been shown to have an indirect regulatory effect on JNK by decreasing release of TNF-α by tissue macrophages, which is a known activator of the JNK pathway (Liang et al. 2009).

Interestingly, in normal functioning skeletal muscle, JNK activation suppresses HSF-1 transcriptional activity and may serve as a controller of the cellular stress response (Park et al. 2001). However, continuous JNK induction as evident in chronic inflammatory states (e.g. insulin resistance), leads to HSR disruption which contributes to the progression of disease (Hooper and Hooper 2009). Mild heat therapy that activates HSP70 also inactivates JNK and improves glucose uptake in a rodent model of insulin resistance (Gupte et al. 2009; Hooper and Hooper 2009). This highlights the complexity of the interaction between the HSR and JNK signaling pathway. HSP70 overexpression protects cells from damaging stimuli by inhibiting JNK, and improves cell survival. However, JNK activation further suppresses HSF-1 function, possibly acting as a control mechanism in normal cellular function. In chronic inflammatory states, JNK overexpression may further inhibit the ability of an organism to fully activate the HSR.

Passive heat treatment mediates JNK levels through induction of HSR proteins, which confers protection against ischemic insult, along with improving glucose uptake in skeletal muscle (Chung et al. 2008; Yang et al. 2003). Short term passive heat acclimation (2 days) in rodents resulted in a threefold increase in HSR gene expression, which coincided with a twofold decrease in JNK genes in myocardial tissue (Horowitz et al. 2004). This was further demonstrated in rodent myocardial tissue after 30-days of passive heat acclimation (Horowitz et al. 2004). In addition, ischemia induced damage to both liver and myocardial tissue is reduced after heat preconditioning, which was mediated by HSP70 inhibition of JNK (Knight and Buxton 1996; Selzner et al. 2003). This demonstrates that inducing HSR through heat treatment may be a serviceable therapy to protect against myocardial injury such as infarction.

Any drug/supplement that upregulates components of the HSR should in theory inhibit the JNK pathway. This has been demonstrated using the pharmacological inhibitor geldanamycin, which prevented cell apoptosis induced by oxidative stress via inactivation of JNK (Choi et al. 2014). However, limited research exists exploring the effect of known nutritional HSR inducers (e.g. glutamine, zinc) on JNK activation in human models; especially under conditions of cellular stress (e.g. heat, bacterial, oxidative). Exercise induced HSR also appears to control JNK activity, but only in a pathological state such as metabolic or myocardial disease (Abubaker et al. 2013). The improvement in glucose uptake in insulin resistant tissue (skeletal muscle and liver) appears to be partially mediated by JNK inactivation, which may be due to HSR upregulation. The anti-inflammatory effects of exercise in chronic inflammatory diseases also appear to be manifested through JNK regulation; but this response is independent of HSR protein changes.

6.1.4 Prevention of Free Radical Damage

Excessive production of free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) have been implicated in a host of pathological states such cardiovascular and neurological disease, along with cancer (Valko et al. 2007). The damaging stimulus induced by free radicals is commonly called oxidative stress and/or nitrosactive stress, and causes DNA, lipid, and protein damages. To remain concise, we will only discuss reactive oxygen species in the following section. Mitochondria are the main site for ROS production which occurs from partially reduced oxygen (superoxide anion) forming in the electron transport chain Complexes I and III (Muller et al. 2004). Additional sites of ROS production are peroxisomes and phagocytes (mainly neutrophils) during respiratory bursts (Decoursey and Ligeti 2005; Valko et al. 2007). Cellular damage can occur by excessive ROS production, a decrease in the activity of antioxidant defense systems, or both; and the balance between the two is the redox state of the cell. While excessive ROS production alone causes profound cellular damage, the change in the redox state has cellular signaling properties that further activate both inflammatory

and apoptosis pathways. For example, ROS have been shown to activate the NFκB pathway, along with AP-1 possibly though JNK activation (Ma et al. 1997; Pande and Ramos 2005; Valko et al. 2006). A major pathophysiology in the condition of heart failure is due to excessive ROS production, which mediates both chronic inflammation (through NFκB activation) and apoptosis (through AP-1 activation) (Moris et al. 2017). Damaging stimuli such as UV radiation, alcohol, smoking, asbestos, and ischemia are activators of the NFκB pathway through ROS production in various tissues (Valko et al. 2007).

Oxidative stress is a known activator of the HSR where mild repetitive ischemic/reperfusion insults of the heart resulted in burst activation of HSF-1 and led to an increase in HSP70 mRNA (Nishizawa et al. 1999). The upregulation of key heat shock proteins upon exposure to ROS is important for cellular adaptation to oxidative stress (Madamanchi et al. 2001). Ischemic/reperfusion preconditioning induction of HSP70 protects against subsequent lethal ischemia in rat hippocampus tissue, and profound cellular damage occurs when HSP70 is inhibited (Sun et al. 2010; Wang et al. 2011). Similar to preconditioning, in HSP70 overexpression experiments, cellular protection against reactive oxygen species is well established (Kalmar and Greensmith 2009; Wang et al. 2011). For these reasons, targeted therapy for HSR activation in ischemic injury disorders such as myocardial infarction and stroke have been explored; but the appropriate timing for administration is not well understood (Kalmar and Greensmith 2009). In summary, reactive oxygen species play an important role in disease pathology due to excessive oxidative stress. The HSR system is activated by ROS, and upregulation to these exposures is critical for cellular adaptation to chronic oxidative stress and confers protection against more severe exposure. In addition, overexpressing HSR proteins may be a preventive strategy against ischemic disease states.

6.2 Exercise Induce HSP and Reduce Inflammation

6.2.1 Insulin Resistance/Diabetes

Insulin resistance (IR) is characterized by a blunted effect of insulin on reducing circulating blood glucose at whole-body, or lower response of certain tissues to the action of this hormone, such as skeletal muscle, liver, and adipose tissue (Kasuga 2006). Skeletal muscle is responsible for about 70–80% of insulin-stimulated postprandial glucose uptake and plays a key role in the maintenance of whole-body insulin sensitivity and control of glycemic homeostasis (DeFronzo et al. 1981; Zierath et al. 2000). Additionally, IR in skeletal muscle is one of the earliest detectable defects preceding hyperglycemia even 10 years before type 2 diabetes mellitus (T2DM) is diagnosed (Di Meo et al. 2017). In the early stages of IR, an excessive release of insulin by the pancreas is needed to the cellular action of this hormone and no signs of impaired glucose disposal is present (no presence of fasting blood hyperglycemia). As it progresses, the pancreas produces extra insulin, but it can no

longer bring down sugar levels and a condition called pre-diabetes type 2 develops (fasting glucose of 100–125 mg/dl, hemoglobin A1C of >5.7–6.4% or glucose concentration after 2 h of oral glucose tolerance test (OGTT) between 140 and 199 mg/dl). In the late stages of IR, a pancreatic β cell dysfunction is present, and T2DM is diagnosed (fasting glucose of >126 mg/dl, hemoglobin A1C >6.5% or glucose concentration after 2 h OGTT >200 mg/dl). The Center of Disease Control estimates that 33.9% of United States adults aged 18 years or older (84.1 million people) had prediabetes in 2015. There is no current data reporting the prevalence of IR in the United States population, but it might be over 50% of the adult population.

A multitude of inflammatory molecules are involved in the disruption of the insulin signaling. Insulin signaling requires a cascade of protein phosphorylation that initiate with autophosphorylation of the insulin receptor tyrosine kinase followed by tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and activation of downstream targets, including protein kinase B (AKT) and glucose transporter type 4 (GLUT4). The NF-κB pathway is directly involved in the pathogenesis of IR by upregulating genes that encode pro-inflammatory molecules such as IL-6, TNF-α and IL-1 β. For example, there is strong evidence that TNF-α is implicated in the etiology of insulin resistance and T2DM, primarily by reducing tyrosine phosphorylation of IRS-1. Uysal et al. (1997) demonstrated that TNF-α is a mediator of IR and that mutation of the gene encoding TNF-α and those encoding the two receptors for TNF-α in mice improves insulin sensitivity in the context of obesity. The authors concluded that TNF-α is an important mediator of insulin resistance in obesity through its effects on several important sites of insulin action. Other inflammatory serine/threonine kinases can also cause inhibitory phosphorylation on insulin-signaling molecules. The JNK, contributes to inflammation and IR possibly by the interplay between JNK and pro-inflammatory cytokines (Hirosumi et al. 2002).

The first evidence that there is an association between IR/T2DM and HSR was provided by Kurucz et al. (2002). The authors reported decreased expression of heat shock protein 72 kda (Hsp72 or HSPA1A) in skeletal muscle from patients with T2DM (tenfold lower than control individuals), and that the level of Hsp-72 mRNA correlated with the rate of insulin-stimulated glucose uptake and lipid turnover and glucose tolerance (Kurucz et al. 2002). Supporting evidences were also provided by Bruce et al. (2003) which also reported a reduction in the basal expression of Hsp72 mRNA in the skeletal muscle of patients with T2DM. The authors further demonstrated a significant correlation between the expression of Hsp72 mRNA and muscle oxidative capacity, as well as a moderate relationship between intramuscular triglyceride (IMTG) accumulation and Hsp72 mRNA (Bruce et al. 2003). Confirmatory data from Chung et al. (2008) latter demonstrated that insulin resistant humans have reduced Hsp72 protein expression and increased markers of inflammation (i.e. JNK phosphorylation) in skeletal muscle. We further reported that insulin sensitive obese individuals have lower HSP70 expression and higher JNK phosphorylation in skeletal muscle compared to non-obese individuals. The presence of IR resulted in a further increase in JNK phosphorylation and lower HSP70 expression (de Matos et al. 2014). Henstridge et al. (2010) also demonstrated in humans that Hsp72 protein expression in skeletal muscle is inversely correlated

with percentage body fat and positively correlated with insulin sensitivity in healthy individuals (Henstridge et al. 2010). Based on these initial studies, the intracellular concentration of Hsp72 (or HSR) is low in the skeletal muscle of individuals with insulin resistance and might be related to the inflammation observed in this condition.

Evidences exist between the potential therapeutic role of HSR in IR conditions. Chung et al. (2008) tested whether activation of Hsp72 through heat therapy (core temperature of 41 °C, once a week, for 16 weeks), transgenic overexpression ($Hsp72^{+/+}$), and pharmacologic means (BGP-15 drug, 15 mg/kg per day in 200 μl of saline for 15 days) either specifically in skeletal muscle or globally in mice would protect against the development of IR in the context of high fat diet and obesity. The authors reported that regardless of the means used to achieve an elevation in Hsp72 protein, protection against diet- or obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and IR was observed (Chung et al. 2008). This protection was tightly associated with the prevention of JNK phosphorylation. In this regard, Gupte et al. (2009) confirmed the findings by Chung et al. (2008) showing that heat therapy or overexpression of Hsp72 restored glucose uptake and improve insulin signaling in skeletal muscle from rats fed a high-fat diet. The authors indicated the underlying mechanism suggesting that Hsp72 and 25 may prevent an increase in JNK phosphorylation and IKK-β activation, possibly through direct interaction (Gupte et al. 2009). Also, heat treatment increased mitochondrial heat shock protein 60 (HSP60) and uncoupling protein 3 (UCP-3) expression and maintained mitochondrial enzyme activity in the presence of a high-fat diet. Moreover, Henstridge et al. (2014) showed that induction of HSP72 using BGP-15 drug or transgenic overexpression ($Hsp72^{+/+}$) in skeletal muscle can protect mice from high fat diet-induced insulin resistance and provided evidences that mechanisms other than blocking inflammation (JNK activation) is involved. It was shown that overexpression of Hsp72 in skeletal muscle enhanced muscle oxidative metabolism, thereby preventing ectopic lipid accumulation, increased mitochondrial number and oxidative metabolism, improved exercise performance and insulin action in mice fed a high-fat diet. The authors concluded that increased muscle Hsp72 promotes mitochondrial biogenesis and enhanced oxidative metabolism, likely via a mechanism involving increased 5′ adenosine monophosphate-activated protein kinase (AMPK) activity and sirtuin activation (Henstridge et al. 2014). Collectively, these findings suggest a potential therapeutic treatment for insulin resistance and identify an essential role for HSP blocking inflammation and/or improving maintaining oxidative metabolism in skeletal muscle in the context of high-fat diet-induced obesity.

Endurance exercise is a well-known non-pharmacological strategy for the prevention or treatment of IR and T2DM. In a meta-analysis conducted by Snowling and Hopkins (2006) using 27 studies evaluating the effect of aerobic, resistance, and combined training (aerobic + resistance) reported that all forms of exercise training result in small to moderate benefits in glucose control (measured by hemoglobin A1C). Also, the effects of exercise are similar to those of dietary, drug, and insulin treatments in type 2 diabetic patients (Snowling and Hopkins 2006). Although the

effect of exercise training on prevention and treatment of IR is well established, the mechanisms are less explored and known. An acute bout of exercise significantly enhances insulin's ability to stimulate glucose uptake in skeletal muscle and counteract insulin resistance. The effects of exercise in the skeletal muscle are mainly related to different cellular stressors including hyperthermia, hypoxia, mechanical and oxidative stress, energy depletion, acidosis, and increased calcium concentration (reviewed by Kregel 2002). In regard to exercise training, cellular adaptations occur to better cope with the acute stress of exercise, such as higher oxidant and oxidative capacity, and thermal tolerance. All of these stressors stimulate the HSR in skeletal muscle and may contribute directly or indirectly to enhance insulin action through a reduction in pro-inflammatory signaling.

Our group investigated the effects of a single session of aerobic exercise on the expression of HSP70, JNK, and IRS-1 serine residue phosphorylation in the skeletal muscle of obese and IR individuals. At rest, obese insulin sensitive individuals (determined by the Homeostasis Model Assessment index – HOMA >2.71) had higher levels of p-JNK and p-IRS-1 serine 612 and reduced HSP70 expression in the skeletal muscle than paired normal weight controls. In obese insulin resistant individuals (HOMA >2.71), we observed a further increase in JNK phosphorylation and decrease in HSP70 expression (de Matos et al. 2014). A significant positive correlation between plasma insulin concentration and JNK phosphorylation in the skeletal muscle was also observed. A single session of exercise reduced skeletal muscle JNK and p-IRS-1serine 612 phosphorylation levels in obese insulin resistant individuals. A main effect of exercise on HSP70 expression was also reported. JNK is an important negative feedback regulator for insulin signaling through inhibitory phosphorylation of IRS-1 in humans and a single exercise session is able to reduce JNK (Lee et al. 2003).

Exercise training adaptations result from the cumulative effect of acute stress of the exercise. The transient changes in mRNA and protein expression after each exercise session may restore skeletal muscle HSR of insulin resistant individuals reducing inflammation and enhancing insulin sensitivity. Atalay et al. (2004) showed that endurance training (8 weeks for 5 days/week) upregulates Hsp72 levels in skeletal muscle of diabetic rats induced by streptozotocin. However, this induction was several folds lower in diabetic animals than in nondiabetic control rats and HSF-1 activation occurred only in the control group (Atalay et al. 2004). Recently, we compared the effect of high intensity interval training (HIIT) on proteins involved in the insulin signaling pathway, MAPKs, and Hsp72 in the skeletal muscle of insulin-resistant and non-insulin-resistant obese individuals (de Matos et al. 2018). HIIT induced a significant reduction in fasting blood insulin concentration and insulin resistance measured by HOMA1-IR only in obese insulin resistant individuals. HIIT also increased phosphorylation of IRS-1 tyrosine 612 and Akt (Ser473), reduced extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation, but did not change JNK1/2, Hsp72 and p38 phosphorylation. Also, HIIT promoted increased expression of β-hydroxyacyl-CoA-dehydrogenase (β-HAD) and cytochrome c oxidase subunit IV (COX-IV), possibly indicating an improvement in oxidative metabolism and, perhaps, this may have contributed to the improvement of insulin signaling. This result suggested that expression of

exercise-induced Hsp72 may not be necessary to obtain the beneficial effects of exercise on insulin resistance. However, Tsuzuki et al. (2017) reported that attenuation of exercise-induced Hsp72 expression in the skeletal muscle partially blunts the improvement in whole-body insulin resistance in type 2 diabetic rats. The authors trained diabetics rats (Otsuka Long-Evans Tokushima Fatty rats) 5 days/week for 10 weeks in two different environmental conditions: temperate (25 °C) or cold environment (4 °C) (Tsuzuki et al. 2017). The insulin levels during an intraperitoneal glucose tolerance test was higher in the cold than in the temperate environment group indicating a lower effect of the exercise training in the IR. Also, Hsp72 expression in the gastrocnemius muscle and liver was significantly lower in the cold than temperate environment group. Therefore, the role of Hsp72 induced by exercise training in reducing inflammation and improving insulin resistance in the skeletal muscle of humans remains unclear.

6.2.2 Cardiovascular Disease

Cardiovascular diseases are a group of disorders of the heart and blood vessels and the leading cause of death worldwide coronary (WHO 2017). An estimated 17.7 million people died from cardiovascular disease in 2015, representing 31% of all global deaths (WHO 2017). Inflammation has been linked, both experimentally and clinically, to cardiovascular disease (Pearson et al. 2003). It has been demonstrated that exercise training reduces cardiovascular risk factors and increase cardioprotection against ischemia followed by reperfusion injury through a direct effect on the myocardium (reviewed by Borges and Lessa 2015). The proposed mechanisms to explain the cardioprotective effects of exercise include induction of intracellular HSP. In the regard to the vasculature, exercise results in vascular stress through the increase in shear stress and mechanical stretch. Fluid shear stress induces the phosphorylation of heat shock protein 27 (Hsp27) in vascular endothelial cells (Li et al. 1996). Exercise pre-conditioning strengthens the endothelial barrier resulting in reduced brain injury by decreasing cerebral permeability and enhancing brain integrity after stroke (Ding et al. 2006). In fact, Silver et al. (2012) reported that a single bout of exercise in rats increased HSP70 mRNA in large intermyofibrillar blood vessels (Silver et al. 2012). Milne et al. (2012) suggested that the protection offered by exercise induce HSP70 against ischemia reperfusion injury may lie in its accumulation in the cardiac vasculature. The authors observed that the accumulation of HSP70 24 h after a single exercise bout (30 m/min for 60 min and 2% incline) or 5 days of training was predominantly located in large blood vessels and, in particular, colocalized with a marker of smooth muscle in rats (Milne et al. 2012). Furthermore, higher core temperatures attained during exercise led to more abundant accumulation in smaller vessels and the endothelium. In the context of vascular inflammation, HSP70 induced by a heat shock has been shown to inhibit TNF-α-induced expression of intercellular adhesion molecule-1 in human endothelial cells (Kohn et al. 2002). A popular prescribed drug class to lower serum cholesterol

concentrations and prevent arteriosclerosis increases HSP70 and nuclear translocation of HSF-1 (Uchiyama et al. 2006). Also, HSF-1 upregulation induces anticoagulation and eNOS expression and decreases endothelin-1 and plasminogen activator inhibitor-1 expression in vascular endothelial cells. Therefore, exercise may inhibit vascular inflammation via activation of HSR (Uchiyama et al. 2007). However, this association needs to be tested.

Disorders characterized by ischemia followed by reperfusion, such as myocardial infarction, stroke, and peripheral vascular disease, results in tissue damage and the accumulation of misfolded proteins. These misfolded proteins are toxic to cardiomyocytes and can cause cardiomyocyte death and heart failure (Pattison et al. 2008). ROS production increases dramatically with ischemia/reperfusion and is associated with tissue damage and accumulation of misfolded proteins. Exercise training (as few as five bouts of exercise on consecutive days) provides cardioprotection against ischemia/reperfusion injury of the myocardium (Powers et al. 2014). The mechanism of the exercise induced cardioprotection resistance against ischemia followed by reperfusion is complex and may involve the HSR. It is suggested that a HSR may protect the myocardium against ischemia/reperfusion injury by increasing myocardial antioxidant capacity, protecting mitochondria and cytosolic proteins against ischemia/reperfusion injury, and preventing apoptosis (Powers et al. 2014). Hsp72 upregulation through thermal preconditioning attenuates inflammation (measured by leukocyte-endothelial migration) induced by ischemia/reperfusion injury (McCormick et al. 2003). Preconditioning attenuated the effects of ischemia/reperfusion, and reduced the number of adherent and migrating leukocytes to control levels, at both the 30- and 60-min postischemia time points. Mice overexpressing HSP70 constitutively in the myocardium demonstrated enhanced recovery of high energy phosphate stores and correction of metabolic acidosis and greater myocardial preservation following brief periods of global ischemia (Radford et al. 1996). Locke et al. (1995) reported that both heat shock and exercise training improved post-ischemic recovery and suggested that Hsp72 was associated with ischemia/reperfusion injury prevention to rat hearts (Locke et al. 1995). The further involvement of HSP70 response to exercise conferred cardioprotection was investigated using a sexual dimorphism model. After exercising, male rats, compared with intact female rats, demonstrated a twofold greater cardiac HSP70 content. Removal of the ovaries, resulted in post-exercise HSP70 levels that were similar to those observed in male rats. The authors reported that the physiological importance of this sexual dimorphism is reflected in the finding that exercise improved post-ischemic cardiac function in male rats and ovariectomized female rats, which exhibited marked induction of HSP70 with exercise, but not in intact female rats (non-ovariectomized), which demonstrated relatively low post-exercise HSP70 expression (Paroo et al. 2002). Although many studies have shown an association between higher cardioprotection and Hsp72 with exercise training, Quindry et al. (2007) demonstrated that elevated cardiac levels of Hsp72 are not essential to achieve exercise-induced cardioprotection against ischemia/reperfusion -induced myocardial infarction or apoptotic cell death following ischemia/reperfusion in the rat. The authors observed that exercise in a cold environment inhibited an increase in body

temperature and prevented the increase in myocardial Hsp72. However, animals trained in the cold environment exhibited cardioprotection against ischemia/reperfusion-induced myocardial infarction and apoptosis similar to animals exercised in the warm environment (Quindry et al. 2007). Although exercise is cardioprotective against ischemia/reperfusion injury, its precise mechanisms offering protection have not been fully defined. The increase in Hsp72 expression promotes cardioprotection against ischemia/reperfusion injury of the myocardium, but it is not essential for exercise-induced cardioprotection.

6.3 Conclusions

The HSR plays a regulatory role in controlling inflammatory events within a cell that may lead to organismal injury or death. Although the exact mechanism still debatable, activation/induction and interplay of HSF1 and HSP have inhibitory effect on NFκB inflammatory pathway, JNK regulation and antioxidant capacity. These effects have potential to alter inflammatory related diseases, such as insulin resistance and cardiovascular diseases. Exercise training induces a HSR, improves insulin signaling, reduces vasculature inflammation, and protects myocardium against ischemia/reperfusion injury. Despite exercise induces a HSR and has anti-inflammatory effect, it cannot be confirmed that a HSR is essential for the anti-inflammatory effect of exercise.

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Chapter 7
Heat Shock Proteins and Alarmins in Autoimmunity

Anil K. Chauhan

Abstract Autoimmunity represents a diverse group of diseases, which demonstrate complex immuno-pathological responses. Heat shock proteins (Hsp) and danger signaling proteins such as HMGB1 and RAGE, grouped as alarmins play a crucial role in autoimmunity. These proteins are present at elevated levels in the patient's plasma. Hsp bind and stabilize large protein complexes such as immune complexes (ICs), which are formed with autoantibodies generated against modified proteins and nucleic acids that are released from apoptotic and dead cells. Alarmins protect nucleic acids from degrading and enhance ICs capability to produce proinflammatory cytokines. Our current understanding of the role of Hsp in disease is largely based on the studies performed in innate immune cells. In autoimmunity, $CD4^+$ T cells are a major contributor of pathology in inflamed tissue. Activation of $CD4^+$ T cells by ICs triggers upregulation of a large set of genes that encode Hsp and also the HMGB1. HMGB1 associates with the low affinity Fc receptor, which trigger the release of proinflammatory cytokines from ICs ligation. This chapter will address our current understanding of the role and interplay of Hsp with other alarmins in autoimmune pathology. Additionally, it will also address the possible role of low affinity Fc receptors in triggering Hsp and alarmins mediated responses in autoimmune pathology.

Keywords Alarmins · Autoimmunity · $CD4^+$ T cells · Heat shock proteins · HMGB1 · Immune cells · Immune complexes

A. K. Chauhan (✉)
Division of Adult and Pediatric Rheumatology, Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, MO, USA
e-mail: anil.k.chauhan@health.slu.edu

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Abbreviations

17-AAG	allyamino-17-dimethoxygeldamycin
APC	antigen presenting cells
BCR	B cell receptor
CpG	cytosine-phosphate- guanine
DAMPs	damage-associated molecular patterns
DC	dendrocyte
ER	endoplasmic reticulum
Grp-78	glucose-regulated protein 78
HMGB1	high mobility group box 1
Hsp	heat shock proteins
HSPB1	heat shock protein family B member 1
ICs	immune complexes
IFNs	interferons
JIA	juvenile idiopathic arthritis
LPS	lipopolysaccharides
MAP	mitogen activated protein kinase
MMP	matrix metalloproteinase
PAMPs	pathogen-associated molecular patterns
pDCs	plasmacytoid dendritic cells
PRRs	pattern recognition receptors
PS	phosphotidyl serine
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
SLE	systemic lupus erythematosus
TCR	T cell receptor
T_E	effector T cells
TLRs	toll-like receptors
TRAF-6	TNF receptor associated

7.1 Introduction

In mild stress innate immune cells produce heat shock proteins (Hsp), which provide protection from subsequent severe stress. Thus, a major role of these proteins is to act as “molecular chaperons” that guide protein folding and prevent protein aggregation. In autoimmunity, both innate and adaptive immune responses are heightened, which is accompanied by formation of large protein complexes with a tendency to precipitate and enhance cell signaling. This necessitates upregulation of the synthesis of chaperon proteins that regulate protein folding. Mis-folded proteins expose the internal buried peptide domains that are prone to chemical modifications and mis-folded proteins also tend to get precipitated. Protein modifications, such as

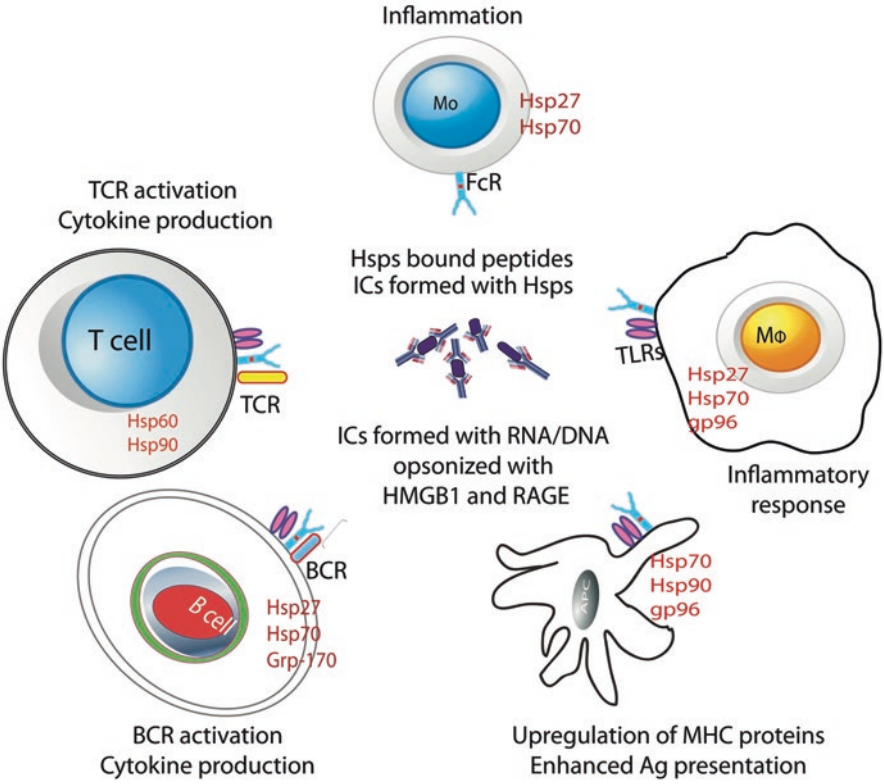

Fig. 7.1 Hsp expressed on immune cells that contribute to autoimmunity. Biochemical modification to Hsp leads to the autoantibody production and ICs formation. Hsp bind to ICs formed with autoantigens generated in autoimmune pathology. ICs opsonize with alarmins such as HMGB1 and RAGE engage immune receptors on the cell surface, enhance signaling and production of proinflammatory cytokines

altered glycation, citrullination, ubiquitination and undesired phosphorylation leads to the development of neo-epitopes that are recognized as foreign and which trigger autoantibody development (Fig. 7.1). Hsp typically reside in the cellular organelles, but they also exist in the extracellular fluid. Enhanced apoptosis is associated with several autoimmune disorders, however it is the necrotic cell death that releases Hsp in the extracellular fluid (Srivastava 2002b). Hsp27 and Hsp70 provide resistance to apoptosis mediated stress (Samali and Orrenius 1998; Xanthoudakis and Nicholson 2000). Many Hsp bind to cell surface glycoproteins that act as scavenger receptors on monocyte derived DCs, myeloid DCs, macrophages, monocytes and B cells (Arnold-Schild et al. 1999; Binder et al. 2000). Various Hsp expression is observed in different immune cell types (Fig. 7.1). These cells contribute to the various immunological responses that are altered and enhanced in autoimmunity. Hsp recognize lipopolysaccharides (LPS) and lipoteichoic acids by pattern recognition. They also bind to membrane phospholipids i.e. phosphatidylserine (PS). Target proteins that

are recognized by Hsp such as pattern recognition receptors (PRRs) play a critical role in the pathogenesis of autoimmune disorders. Toll-like receptors (TLRs) particularly those recognizing nucleic acids, such as nucleic acid binding TLRs (NA-TLRs) recognize ligands in the endosomal compartment and trigger nucleic acid sensing pathways, both in the plasmacytoid dendritic cells (pDCs) and B cells (Blasius and Beutler 2010). TLR2 and TLR4 act as receptors for Hsp. Hsp70 transduce signals via TLR2 and TLR4, which then activate mitogen-activated protein (MAP) kinase cascade and NF-κB signaling pathway (Asea et al. 2002; Vabulas et al. 2002). Toll/IL-1 receptor signaling pathway is activated by Hsp70, which utilizes TNF receptor associated factor (TRAF)-6, a key intracellular signaling pathway protein that drives proinflammatory cytokine production and is a known key contributor of auto-inflammation. Folding of TLR proteins is dependent on dimerization of Hsp, gp96 (also known as glucose-regulated protein-94, Grp-94). All TLR proteins except TLR3 are client proteins for gp96 (Liu et al. 2010). For the host immune system, extracellular Hsp act as pathogen-associated molecular patterns (PAMPs), and are self-adjuvants that promote innate immune responses. In autoimmunity, cells succumb to immunogenic variant of apoptosis, and expose calreticulin on the cell surface. These cells also expose or release Hsp70 and Hsp90, which facilitate the uptake of the dying cells by phagocytes. Further, dying cells release high mobility group binding 1 (HMGB1) protein, which is recognized by DCs through receptor for advanced glycation end-products (RAGE), TLR2 or TLR4. HMGB1 is essential for the cell death to be immunogenic. Surface-expression of calreticulin, Hsp70 and Hsp90 affect the function of DCs that is to present antigen and these proteins also bind to CD91 and TLRs.

Small peptides of 10–30 aa in length with low affinity and specificity associate to Hsp. Antigenic peptides of both viral and bacterial origin associate with cytosolic Hsp70, Hsp90, ER calreticulin and gp96 (Navaratnam et al. 2001; Nieland et al. 1996; Rapp and Kaufmann 2004; Zugel et al. 2001). These interactions lead to productive peptide presentation by MHC class I (MHC I). Peptide loaded MHC I heavy (H) chains associate with β2-macroglobulin and this complex is transported via Golgi to the cell surface, where it is recognized by T cells leading to enhanced development of proinflammatory T_E cells. Hsp70, Hsp90, gp96, and calreticulin transport peptides from proteasomes to MHC I (Srivastava et al. 1994). Interferons (IFNs) are key cytokines in SLE pathology and these cytokines induce Hsp70 and Hsp90, which provide an extraordinary efficiency for antigen cross-presentation, a mechanism that contribute to the autoimmunity. IFN-γ is a cytokine produced by T_H1 cells, and it induces expression of Hsp proteins in addition to MHC I (Anderson et al. 1994). Hsp in particular gp96 contribute to the folding of nascent peptides in ER. Hsp-peptide complexes are capable of priming T cell responses and synergize response with other molecules such as HMGB1 and dsDNA (Gallo and Gallucci 2013). These studies propose diverse roles and mechanisms by which Hsp contribute to the autoimmune pathology.

7.1.1 Hsp in Arthritis

Hsp role in arthritis is controversial, as both protective and inflammatory responses from these proteins in this disease has been observed (Pockley et al. 2008). Hsp influence both innate and adaptive immune responses in rheumatoid arthritis (RA). It has been argued that the context such as cell type and the environment govern Hsp responses where they happen. Serological studies have implicated Hsp40, Hsp60, Hsp70, Grp-78, and Hsp90 in modulating immune responses in RA. In arthritis, inflammatory responses in the joints trigger up-regulated expression of Hsp in the cells that form synovial tissue in the joints. Several studies have shown increased membrane expression of Hsp60 and Hsp70 in the fibroblasts like synovial cells (synovial fibroblasts) isolated from arthritis-affected joints of RA and juvenile idiopathic arthritis (JIA) subjects (Boog et al. 1992; Nguyen et al. 2006; Sedlackova et al. 2009). Levels of soluble Hsp60 are elevated in oligo and polyarticular JIA. ICs isolated from JIA patients contain Hsp40 and mitochondrial peptides along with IgG and IgM rheumatoid factor (RF) (Moore et al. 1995). In oligoarticular JIA, disease remission is associated with proliferative T lymphocyte responses to human Hsp60 (Massa et al. 2007; Prakken et al. 1996). Studies have further shown that Hsp60 successfully suppressed adjuvant arthritis (Van Eden et al. 2007). Elevated levels of Hsp70 are present in both the inflamed synovium and synovial fluid of RA patients. Increase in the extracellular Hsp70 in arthritic joints is profoundly correlative to the disease (Sedlackova et al. 2009). Expression of Hsp70 was also observed on the surface of DCs and in the extracellular compartment in rheumatoid joints (Martin et al. 2003). Both Hsp60 and Hsp70 of mycobacterial origin trigger proliferation of T cells isolated from either SF or peripheral blood of RA and JIA patients (Sedlackova et al. 2006). Citrullination of filaggrin and myelin occurs in RA patient's sera and this modification is a marker for disease activity in RA. In RA patient's, antibodies to hyper-citrullinated Hsp90 are observed (Travers et al. 2016). Another Hsp gp96, a member of Hsp90 is highly expressed in RA synovial tissue and protein levels correlate with the joint lining thickness and inflammation. Furthermore, gp96 is significantly increased in RA SF, demonstrating that this endoplasmic reticulum-localized chaperone is released during chronic inflammation. In addition to signaling through TLR2, gp96 also induces the expression of TLR2. The concentration of gp96 in RA SF correlates with the level of TLR2 expressed on synovial macrophages. These observations suggest that in RA gp96 is released and functions as an endogenous TLR2 ligand, capable of promoting the self-perpetuating activation of synovial macrophages (Huang et al. 2009). These studies establish that several Hsp influence the disease in RA pathology and utilize multiple mechanisms, including the signaling via surface TLR proteins.

A limited number of studies have also suggested the participation of TLR signaling proteins in $CD4^+$ T cell responses (Gelman et al. 2004; Mills 2011). Extracellular Hsp70 binds to the cell surface receptors via TLR2 and TLR4, which than induces IL-10 production (Asea et al. 2002; Borges et al. 2012). Hsp60 has also been shown to enhance regulatory T cells (Tregs) function via TLR2 signaling (Zanin-Zhorov

et al. 2006). IL-10 is an anti-inflammatory cytokines produced by Tregs and it counter balances the inflammatory response triggered by cytokines produced by Th1 and/or Th17 cells (Niu et al. 2012). Both of these cell types participate and contribute to RA disease pathology. Thus these data suggest that by regulating the Treg production both Hsp60 and Hsp70 regulate immune responses in RA.

7.1.2 Hsp in Systemic Lupus Erythematosus

SLE is an autoimmune pathology that is linked to aberrant nucleic acid recognition. Disturbances in several immunological pathways contribute to SLE pathology. ICs formed with nucleic acids and autoantibodies trigger innate immune activation, leading to the plasma B cell development producing autoantibodies and activating complement pathways that lead to apoptosis. Elevated levels of ICs formed with nucleic acids are present in the plasma of SLE patients. ICs are central player in tissue damage, and are often present together with hyper responsive $CD4^+$ T cells, which contribute to the breakdown of B cell tolerance. The defective clearance of ICs is a confounding factor for SLE pathology (Davies et al. 1990). Apoptotic blebs on the cell membrane, in particular on lymphocytes accumulate and release modified proteins and nucleic acids, which trigger humoral responses. This process also contributes to the epitope spreading. In SLE, autoantibodies and T cell clones against Hsp are observed. Both abnormalities in the expression and/or localization of endogenous Hsp contribute to autoimmune response in SLE. High levels of both Hsp72 (inducible form of Hsp70) and Hsp90 are present in SLE patients, which correlate with disease activity (Dhillon et al. 1993, 1994). Hsp90 is largely present in the cytoplasm, but under stress it is released into the extracellular compartment. One of the Hsp90 variant with a unique hydrophobic terminal (HSP90N) localizes to the cell membrane, both in the lymphocytes and monocytes. The membrane bound HSP90N will assist in retaining high density of solubilized signaling proteins on the cell surface, thus enhancing the signaling responses such as those observed in B cell receptor (BCR) and T cell receptor (TCR) complexes during disease activity. As such the formation of immunological synapse is mandatory for TCR signaling, which results in the accumulation of signaling proteins at a single site where the CD3 complex is located (Dustin 2002). This is usually the site where the antigen-presenting cell (APC) makes a contact with T cell during antigen presentation. A role for Hsp in upregulating the costimulatory molecules on APCs are proposed. Hsp70, Hsp90, calreticulin or gp96 enhance the expression of costimulatory proteins, CD80, CD86, CD40 and MHC II (Basu et al. 2000; Srivastava 2002a). Hsp90 co-precipitates with MHC I molecules suggesting a role for Hsp in class I antigen presentation. High-density of protein complexes and membrane rafts accumulate at a single site in $CD4^+$ T cells forming "innate immune synapse" (Chauhan and Moore 2011). These are also the site where ICs bind to FcγRIIIa on activated $CD4^+$ T cells (Chauhan et al. 2015). Also Hsp90 containing ICs are observed in SLE and this protein is present within the immune deposits formed in the kidney of SLE patients

associated with glomerulonephritis (Manderson et al. 2004). In SLE mouse model MRL/lpr elevated expression of Hsp90 is observed. These mice also show age dependent expression of Hsp72 in several tissues. Chronic expression of gp96 on the surface of DCs induces cell activation and SLE-like phenotypes in mice (Han et al. 2010). IgM isotype antibodies against Hsp90 are found in 35% of SLE patients and 26–50% show IgG subclasses (Conroy et al. 1994; Fink 1999; Hightower and Hendershot 1997). High levels of Hsp90 autoantibodies observed in SLE patients correlate with C3 levels suggesting complement consumption (Kenderov et al. 2002). Hsp90 deposition is also observed in the kidney biopsies of patients with lupus nephritis (Kenderov et al. 2002). Extracellular Hsp90 present in the serum of SLE patients correlate and associate with levels of extracellular self-DNA and DNA-ICs. Hsp90 bound to self-DNA triggers the production of proinflammatory cytokines (Okuya et al. 2010). Hsp90 opsonized ICs are more potent in producing IFN-α (Saito et al. 2015). Hsp90 inhibitor 17-AAG, suppresses IFN-α, TNF-α, and IL-6 production post TLR7 and TLR9 activation (Saito et al. 2015). Both TLR7 and TLR9 associate with Hsp90 as observed by co-precipitating experiments (Saito et al. 2015). NA-TLRs drive expression of IFNs that drives SLE pathology (Christensen et al. 2006). Hsp90 empowers the chaperoned ligands to activate pro-inflammatory immune responses. Unmethylated single-stranded DNA containing a cytosine–phosphate–guanine (CpG) motif binds to endosomal TLR9 receptors in pDCs and B cells, which results in IFN-α responses that drive disease pathology (Blasius and Beutler 2010; Shrivastav and Niewold 2013). In human pDCs, Hsp90-CpG-A ODN complexes are more potent to enhance IFN-α production compared to the monomeric CpG-A ODN (Okuya et al. 2010). The cellular localization of TLR9 discriminates between self-DNA and viral-DNA (Barton et al. 2006). The recognition of modified self-nucleic acids by NA-TLRs is critical for BCR activation and autoantibody production (Pelka et al. 2016). The extracellular Hsp90 is able to chaperone CpGs in early endosomes leading to robust IFN responses. Therefore, Hsp are crucial player in the pathogenesis of SLE. Hsp90 associates and deliver TLR7 and TLR9 from ER to early endosome, where they recognize their ligands. In addition, gp96 an ER resident Hsp is shown to be a master immune chaperon for TLR1, 2, 4, 5, 7, and 9 in macrophages (Yang et al. 2007). Macrophages lacking do not respond to the TLR ligands (Yang et al. 2007). Grp-170 is a large Hsp that modulate NF-κB signaling pathways and IFN-β cytokine produced upon TLR3 signaling. Grp-170 binds to immunoglobulins in B cells and compensate for gp96 in the assembly of immunoglobulins (Liu and Li 2008) Forced expression of gp96 on the cell surface in DCs induces lupus like disease in mice and upregulate the myeloid differentiation primary response 88 (MyD88) signaling (Liu et al. 2003). It will be of significant interest to determine whether the Hsp play a role in recruiting endosomal NA-TLRs to the cell surface, where they can bind to altered self-nucleic acid (Chauhan 2017). Thus it is safe to conclude that in SLE, extracellular Hsp contribute to the disease pathology by enhancing the nucleic acid sensing via TLRs, one of the several key mechanisms that contribute to the SLE pathology. In SLE, Hsp are critical in inducing both T and B cell responsiveness, which will result in tolerance breakdown.

7.1.3 Other Alarmins in Autoimmunity

Alarmins mediated signal enhancement in lymphocytes is crucial for the pathogenesis of autoimmune disorders. In addition to Hsp, several additional alarmins have been recognized, which include HMGB1, S100 proteins (notably S100A8/A9 and S100A12), serum amyloid A, purine metabolites (uric acid and ATP), altered matrix proteins and IL-33. In this chapter I will focus on the role of HMGB1 and RAGE, since these proteins opsonize and enhance the IC mediated immune responses. HMGB1 is critical in driving the host inflammatory response to invading pathogens and sterile inflammation in autoimmunity. Elevated levels of HMGB1 are present in the sera of RA and SLE patients, and in the SF of RA patients (Hamada et al. 2008; Lu et al. 2015). Invading pathogens trigger release of HMGB1 from macrophages, DCs and NK cells, which are at the frontline of host defense. However during autoimmunity, HMGB1 is secreted in the extracellular space by molecular mechanisms triggered by downstream cellular signaling events. We have shown that the FcγRIIIa ligation by ICs is one of such singling event that promotes HMGB1 expression and its subcellular redistribution Fig. 7.1. HMGB1 acts as a DNA chaperon that stabilizes nucleosome formation. It is released in the extracellular milieu by necrosis or actively secreted by inflammatory macrophages and monocytes. It has also been observed that the monocytes exposed to apoptotic blebs release HMGB1 (Qin et al. 2006). Extracellular HMGB1 upon binding to RAGE, decrease mTOR activity, which enhances the autophagy (Kang et al. 2010). Nuclear HMGB1 regulates the expression of heat shock protein family B (small) member 1 (HSPB1) and promote mitophagy (Tang et al. 2011). Reactive oxygen species production is triggered upon Fc-receptor signaling, and this could trigger translocation of HMGB1 to the cytosol resulting in the release of beclin by inhibiting Bcl2 (Tang et al. 2010). Beclin plays a critical role both in apoptosis and autophagy by forming the phosphotydylionositol 3-phosphate complex, which mediates vesicle trafficking. The increase in cytosolic HMGB1 also promotes autophagy. Extracellular levels of HMGB1 protein is important in SLE pathology, since HMGB1 binds to ICs and protects nucleic acids present in these complexes from degradation by nuclease such as three prime repair exonuclease 1 (Lee-Kirsch et al. 2007). This enhances the TLR mediated immune response triggered by RNA/DNA-ICs in pDCs and B cells. HMGB1 coordinate cellular responses through RAGE, TLRs, TIM-3, CXCR4 and CD24-Siglecs G/10. HMGB1 binds to TLR4 on synovial fibroblasts of RA patients, present in inflammatory cytokine milieu and signal via TLR4 to mediate tissue damage (Yang et al. 2010). HMGB1 protein functions as a modulator for the immunogenic potential of nucleic acids and other PAMPs and DAMPs. A cross-talk between FcRs expressed on the cell surface and surface TLRs trigger production of proinflammatory cytokines i.e. TNFα, IL-1β, IL6, and IL-23 in human DCs (den Dunnen et al. 2012). We have shown that the human naïve $CD4^+$ T cells upon activation via FcγRIIIa cosignaling upregulate the expression of HMGB1 and TLRs (Chauhan 2017; Chauhan et al. 2016). In addition, this signal also promotes the movement of HMGB1 protein towards the cell surface, where it associates and colocalizes with FcγRIIIa protein Fig. 7.1. MyD88 the downstream signaling protein of TLR signaling pathway is

also up-regulated upon FcγRIIIa cosignaling and associate with FcγRIIIa protein (Chauhan 2017). Also TLR4 binds to MyD88, which then localizes to cell surface (Barton and Kagan 2009; van Egmond et al. 2015). FcγRIIIa cosignaling contributes to the formation of Myddosomes like signaling complex on the surface in human $CD4^+$ T cells Fig. 7.1 (Hamerman et al. 2016). Co-precipitation of these proteins confirmed their association in activated $CD4^+$ T cell lines (Chauhan 2017). A synergistic co-signaling among FcγRIIIa and TLR9 in the human naïve $CD4^+$ T cells upregulates the secretion of proinflammatory cytokines IFN-γ and IL-17A, which contribute to autoimmunity (Chauhan 2017). In addition, Hsp72 during oxidative stress translocate to the nucleus, where it interacts with HMGB1, which prevent oxidative stress (H_2O_2)-induced HMGB1 cytoplasmic translocation and its release. The nuclear Hsp72-HMGB1 interaction may be a universal nuclear stress response to various adverse stimuli (Tang et al. 2007). These studies strongly suggest a role for HMGB1 protein in regulating autoimmune pathology and this protein is a known therapeutic target for various immune pathologies.

Stressed cells markedly enhance RAGE expression, which is implicated in the pathogenesis of a variety of inflammatory diseases. RAGE binds to HMGB1 and regulate the inflammatory responses in autoimmune pathology (Sims et al. 2010). RAGE is a multi-ligand receptor that recognizes non-AGE ligands such as HMGB1, S100, Mac1 and several surface TLRs (Chen and Nunez 2010). RAGE mediates the cytokine activity of HMGB1. Activated platelets trigger NETosis from HMGB1 activation, which result in the engagement of RAGE on neutrophils. NETosis is one of the established mechanism that contributes to the autoimmune pathology (Papayannopoulos 2018). RAGE is expressed by T lymphocytes and it plays a role in the activation and differentiation of these cells. The evidence that soluble RAGE prevented joint inflammation and bone loss in arthritic mice model suggest its involvement in this disease (Hofmann et al. 2002). RAGE expressing cells show matrix metalloproteinase-mediated inflammation and higher binding to S100 protein. The FcγRIIIa cosignaling in human naïve $CD4^+$ T cells upregulates RNA transcripts that encode S100, Fig. 7.2. RAGE also binds to PS in the outer plasma membrane. Lupus mice B6-MRL Fas lpr/j with *Ager* mutation (deletion of RAGE) show delayed apoptosis of T lymphocytes. *Ager* exacerbates autoimmunity, organ injury and lymphoproliferative disorder. RAGE/RAGE ligand interactions are associated with the cell survival and inflammation mediated by the phosphorylation of the extracellular signal-regulated kinase (ERK) and an increase in NF-κB p65 subunit expression (Bierhaus et al. 2001). Cardiovascular pathology observed in SLE patients is linked to RAGE (Nienhuis et al. 2009). In 2005, Means et al. showed that TLR7 and TLR9 recognize DNA/RNA-ICs and trigger upregulation of IFN signature genes and contribute to SLE pathology (Means et al. 2005). Another study that followed soon showed that HMGB1 was an essential component of DNA-ICs that produce IFN-α in both pDCs and B cells. This study also showed that indeed the CpG-A ODN-HMGB1 complexes utilize RAGE to produce IFN-α in pDCs, and other RAGE ligands such as S100 does not show similar affect (Tian et al. 2007). A role for RAGE in the subcellular localization and/or retention of DNA-TLR9 complexes in the endosome has also been shown (Tian et al. 2007). It is now

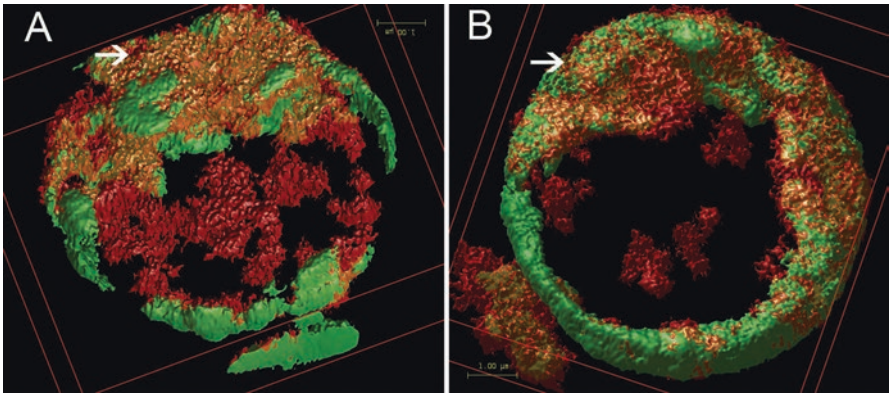

Fig. 7.2 HMGB1 and MyD88 in CD4⁺ T cells upon FcγRIIIa cosignaling moves to cell surface and colocalize with IC staining. (**a**) 3D confocal image shown with double staining of IC binding (Alexa Fluor 488, green) and HMGB1 (Alexa Fluor 594, red). (**b**) 3D confocal image shown with double staining of IC binding (Alexa Fluor 488, green) and MyD88 (Alexa Fluor 594, red)

accepted that the surface expression of TLR9 protein is essential for the recognition of altered self-DNA (Barton et al. 2006; Chauhan 2017). It is thus important to explore the role for Hsp and RAGE in subcellular localization of NA-TLRS and joint signaling events to produce proinflammatory cytokines in various immune cells. Collectively, evidence suggests that alarmins interact with Hsp and nucleic acid containing ICs and contribute to inflammatory responses in autoimmunity. Both of these protein groups are critical contributor for the generation of sterile inflammatory responses.

7.1.4 Hsp in Activated CD4⁺ T Cells

Naïve CD4⁺ T cells express TCR, which upon engaging pep-MHC on APCs trigger cellular proliferation. In addition to this primary signal, a secondary co-stimulatory signal is delivered by CD28. In the absence of CD28 cosignal these cells become anergic and die via apoptosis (Esensten et al. 2016). However, in autoimmunity naïve CD4⁺ T cells differentiate into T_E subsets in the absence of CD28 cosignal. The proinflammatory effector T cell (T_E) subsets generated upon TCR activation produce several proinflammatory cytokines such as IFN-γ, IL-17, IL-21, and TNF-α. Many of these cytokines in particular IFN-γ and IL-21 are efficient B cell help provider. Of particular interest of these T_E subsets are follicular helper T_E cells (Tfh) that form cytoconjugates with B cells in the germinal centers (GCs) and drive the development of plasma B cells that produce autoantibodies. A role for Hsp in adaptive immune response has not yet been fully addressed. We have shown that the FcγRIIIa cosignaling successfully substitute the CD28 requirement for the

generation of T_E cells (Chauhan et al. 2015, 2016; Chauhan and Moore 2011). We have also shown that this cosignal drives the generation of Tfh subset in vitro and $CD4^+$ T cells in vivo bind to ICs, suggesting the expression of FcγRIIIa on these cells (Chauhan et al. 2012). These cells also express B cell CLL/lymphoma 6 (Bcl6), a transcription regulator of Tfh and produce IFN-γ and IL-21. Our RNA-seq data of human naïve $CD4^+$ T cells that were activated via FcγRIIIa cosignaling showed upregulation of a large subset of Hsp RNA transcripts, Fig. 7.2. This increase was observed over the transcript levels present in paired $CD4^+$ T cells from canonical CD28 cosignaling (Chauhan 2017). These data suggest that the FcγRIIIa cosignaling by upregulating the expression of Hsp contribute to stress response. A statistically significant increase in gene transcripts of DNAJP5, DNAJB6, DNAJC2, DNAJC5, DNAJC5B, HSF2, HSFY2, HSP90AB3P, HSPB7, HSPE1, S100A7A, S100A7L2, and S100G was noted (Chauhan 2017). S100A2, S100A5, and S100Z were overexpressed upon CD28 cosignaling (Fig. 7.3). Transcripts that encode proteins, which regulate protein folding, refolding and negative regulation of inclusion body assembly were also upregulated (Chauhan 2017). FcγRIIIa cosignaling upregulated genes that contributes to proteasome assembly and ubiquitination. An alternate mechanism by which Hsp can contribute to T cell activation is by forming ICs with autoantibodies against chemically modified Hsp (Fig. 7.3). Combined these studies points to the role for Hsp in the regulation of $CD4^+$ T cells responses during autoimmunity (Srivastava et al. 1998). Lck is a Src kinase that is a major abundant signaling protein in the TCR complex. CD28 protein primarily utilizes the Lck for downstream signaling (Esensten et al. 2016). CD28 bound Lck phosphorylates phosphoionositide-dependent kinase 1, which activates protein kinase C Φ and mediates signaling events that lead to activation of NF-κB, AP-1, and NF-At transcription factors (Dodson et al. 2009; Esensten et al. 2016). A single study have shown that Hsp90 protects degradation of Lck in activated TCR and regulate ubiquitination of Lck (Giannini and Bijlmakers 2004). FcγRIIIa mediated signaling phosphorylates Lck both in human naïve $CD4^+$ T cells and Jurkat cells (Chauhan and Moore 2011). Thus Hsp90 upregulation likely stabilizes the primary substrate for cosignaling protein CD28 in $CD4^+$ T cells. Also an indirect role for Hsp70 in enhancing the expression of CD28 on T cells has been shown (Kumar et al. 2016). These studies advocate for a role of Hsp proteins in influencing the outcome of $CD4^+$ T cell by altering the threshold of TCR signal during immune responses in autoimmunity.

7.2 Conclusions

A role for Hsp and alarmins in autoimmune pathology is now established. Contribution of the Hsp and alarmins to the inflammatory responses has attracted interest for them to be evaluated as the potential therapeutic target for both autoimmune pathology and cancers. Our current knowledge of Hsp contribution to autoimmune disease pathology is largely based from studying the role of these proteins in

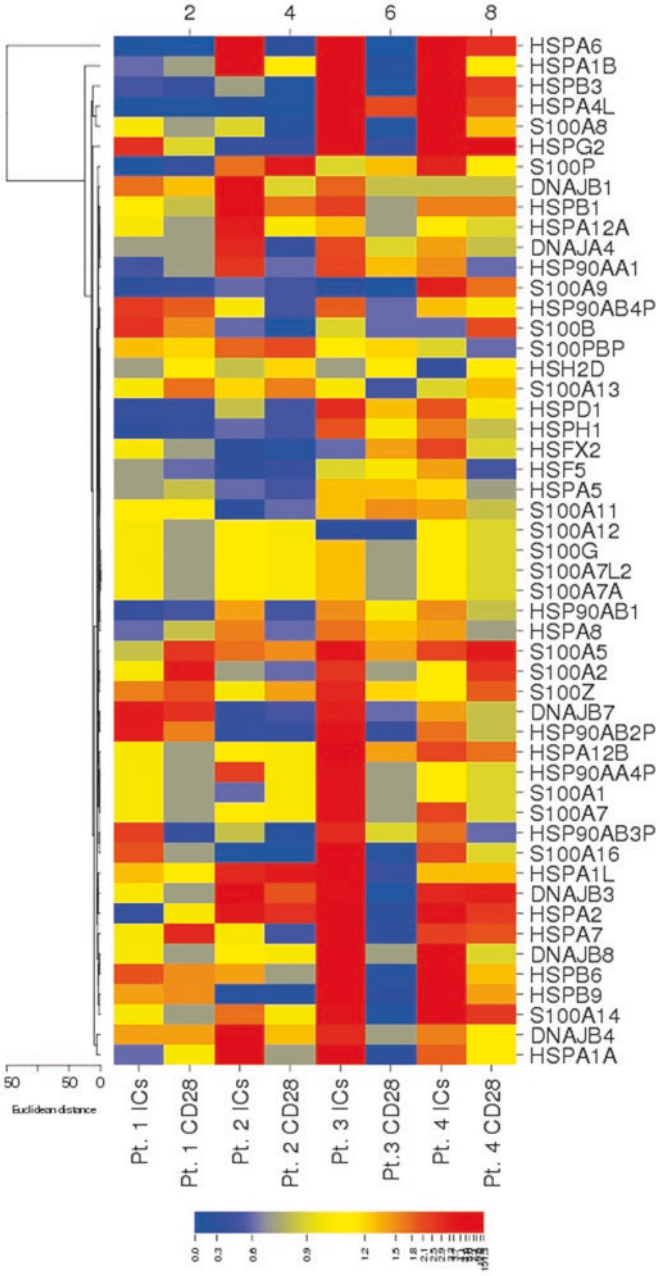

Fig. 7.3 FcγRIIIa cosignaling upregulate the Hsp expression. Relative expression of Hsp RNA transcripts in $CD4^+$ T cells upon FcγRIIIa cosignaling (plate-bound ICs) and CD28 cosignal (plate-bound anti-CD28) in four different subjects. Several transcripts encoding the S100 proteins are overexpressed upon FcγRIIIa cosignaling

innate responses. It remains to be determined how Hsp influence adaptive immune responses and what is the role these adaptive responses in autoimmune pathology. It will also be important to examine if the Hsp mediated responses differ from the traditional responses observed in lymphocytes. Studies from B cells have now conclusively shown that the immune receptors such as BCR and TLRs synergistically upregulate the B cell responses and enhance the production of inflammatory cytokines. It will be of significant interest to examine the role of Hsp in the synergistic responses observed for FcRs and TLR signaling in $CD4^+$ T cell responses. It also remains to be determined whether induced expression of Hsp contributes to the enhanced cell surface-signaling, which is observed during autoimmunity, in particular T and B-lymphocytes. Such studies will enhance our understanding of the role of Hsp mediated events in immune tolerance breakdown. Over past two decades significant advancement in our understanding of the role of Hsp in autoimmune diseases has occurred. However with current advances in T cell based therapies, the role of these proteins in adaptive immunity should be further explored. Hsp may successfully provide effective adjuvant therapy for vaccine development in autoimmunity and cancers.

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Chapter 8
HO-1/HSP32 and Cardiac Stress Signaling

Lakshmikanthan Panneerselvam, Azhwar Raghunath, Kiruthika Sundarraj, and Ekambaram Perumal

Abstract In recent years, increased attention has been gained in elucidating the therapeutic potential of heme oxygenase-1 (HO-1), a rate limiting enzyme involved in the heme metabolism and their pathophysiology of various cardiovascular diseases and heart failure models. HO-1 exhibits multi-faceted role in the systemic regulation of redox, energy homeostasis and boosts the survival and vascular function in various cardiomyopathies. Induction of HO-1 has been known to modulate vascular cell proliferation, inflammation, endothelialization, oxidative damages, apoptosis, fibrosis, ischemic/perfusion injuries, neovascularization and prevent atherosclerotic lesion formation in the pathophysiology of experimental cardiomyopathies. The present chapter summarizes the cardioprotective mechanism of HO-1 and their molecular regulations, potential interaction/cross talk with myocardial cell survival and death signaling cascade. This chapter further reveals the genetic ablation of HO-1, overexpression of transgenic mutants and ascertains the role of HO-1 in cardiac stem cell therapy and xenograft survival in ischemic hearts. Eventually, we examined the pharmacological modulators of HO-1 from the natural and dietary therapeutic polyphenols and their mode of cardioprotection in both in vivo and in vitro models. In this context, the present chapter will append the existing knowledge of HO-1 and their regulation in the cardiac stress signaling cascades.

Keywords Cardiac HO-1/Hsp32 · Cardiac ischemia · Cardiac redox signaling · Cardiac stress · Cardiovascular pharmacological modulators

Authors Lakshmikanthan Panneerselvam, Azhwar Raghunath and Kiruthika Sundarraj have been equally contributed to this chapter.

L. Panneerselvam · A. Raghunath · K. Sundarraj · E. Perumal (✉)
Molecular Toxicology Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India
e-mail: ekas2009@buc.edu.in

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Abbreviations

AP-1	activator protein-1
AP-2	activator protein-2
Bach1	broad-complex, tramtrack and bric-a-brac and cap'n'collar homology 1
CAM	cell adhesion molecule
CaMKKb	Ca^{2+}/calmodulin-dependent protein kinase
CO	carbon monoxide
CREB1	cAMP response element-binding protein 1
CTGF	connective tissue growth factor
DCM	diabetic cardiomyopathy
EBP	emopamil-binding protein
ER	endoplasmic reticulum
HO-1	heme oxygenase-1
Hsp32	heat shock protein 32
ICAM1	intracellular adhesion molecule 1
IL	interleukin
MAPK	mitogen activated protein kinase
NF-κB	nuclear factor-κB
Nrf2	nuclear factor erythroid 2-related factor 2
PKC1	protein kinase C1
SDF	stromal cell-derived factor
SER	smooth endoplasmic reticulum
STATx	signal transducers and activators of transcription x
TNF-α	tumor necrosis factor-α
VCAM1	vascular cell adhesion molecule 1

8.1 Introduction

Heme oxygenases (HO) are one among the prevalent enzymes that are present in both the animal and plant kingdoms (Wilks 2002a). HO is tethered to the endoplasmic reticulum (ER) and catalyzes an important rate-limiting reaction wherein it oxidizes the heme through a series of oxidation and reduction reactions (Montellano 2000). Heme, being the cofactor exists within an array of cellular proteins: cytochromes, hemoglobin, myoglobin, cytochrome P-450, peroxidases, guanyl cyclase, FixL (oxygen sensing protein), and CooA (carbon monoxide and redox state sensing protein) (Wilks 2002b). These heme proteins carry out diverse functions within the cell. The bound heme when becomes a free heme is toxic and hence has to be metabolized by HO to avoid toxicity (Jeney et al. 2002). Though an intrinsically attached to the smooth ER (SER), HO-1 can be found in the caveoli, mitochondria and nucleus. The cleavage of the anchorage C-terminal of HO-1 from the SER make the HO-1 transfer into the nucleus (Lin et al. 2007).

This enzymatic nature of HO in microsomes was first characterized by Tenhunen et al. (1968). In the early 1970s, there was a debate among the groups of scientists in the heme catabolic system. Some argued that HO as the terminal oxidase whereas others said it was cytochrome P450 as there was dissimilitude in the distribution (Tenhunen et al. 1972). This dispute was put to an end as cobalt – the unnatural substrate for HO, induced HO in the liver (Maines and Kappas 1974, 1975) and the study in pig spleen microsome (Yoshida et al. 1974) unaffiliated cytochrome p450 as a terminal oxidase. The presence of two isoforms of HOs: HO-1 and HO-2 was demonstrated (Maines et al. 1986). Of these two forms, HO-1 is inducible whereas HO-2 is expressed constitutively. HO-1 is induced by various non heme stimuli, which includes cytokines, endotoxins, growth factors, heat shock, heavy metals, hormones, hydrogen peroxide (H_2O_2), hypoxia, oxidized low density lipoproteins, shear stress, UV radiation and so on (Agarwal and Nick 2000). This manifold stimuli clearly indicate that HO-1 regulates diverse biological processes.

HO-1 and HO-2 are transcribed and translated from two different genes, the former is generated from one transcript and the latter results from the two transcripts of a single gene due to polyadenylation difference (McCoubrey et al. 1992). The presence of heat shock element in the promoter region of the HO-1 triggered the transcription of HO-1 in response to heat stress in the rat glioma cells (Shibahara et al. 1987). Hence, HO-1 can also serve as a heat shock protein in response to different stress factors. With a molecular mass of 32 kDa, HO-1 is also categorized as heat shock protein 32 (Hsp32). Though HO-1 shares identity with the heat shock elements of other HSP genes, HO-1 does not exhibit chaperone activity and shows no amino acid sequence homology with other HSP.

The primary function displayed by HO-1 is to metabolize heme into three important components: biliverdin, carbon monoxide (CO) and iron (Ryter et al. 2006). Each of these products, exhibits a multitude of functions within the biological systems that includes antiapoptosis, antiinflammation, antioxidant, cytoprotection, immunomodulation, and vasodilation (Otterbein et al. 2003). This versatility of HO-1 makes it an essential enzyme for the regulation of such a wide range of processes. The external stimuli activate c-Jun N-terminal kinase, extracellular signal regulated kinase, and p38 mitogen-activated protein kinase (p38 MAPK) that in turn activates transcription factors ultimately triggers the transcription of HO-1 expression (Kietzmann et al. 2003). Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the HO-1 transcription during oxidative stress by binding to the response elements of the HO-1 gene (Reichard et al. 2007). Broad-complex, tramtrack and bric-a-brac and cap'n'collar homology 1 (Bach1) protein – a heme binding protein inhibits the HO-1 transcription. The other transcription factors and signaling molecules that regulate HO-1 expression are activator protein-1 (AP-1), activator protein-2 (AP-2), activating transcription factor 2 (ATF2), cyclic adenosine monophosphate-responsive element-binding protein (CREB), hepatocyte nuclear factor-1 (HNF1), phosphotidyl inositol 3-kinase/AKT (PI3K/AKT), protein kinase A (PKA), protein kinase C (PKC), signal transducers and activators of transcription x (STATx) and nuclear factor-κB (NF-κB) (Ryter et al. 2006).

8.1.1 Role of HO-1 on Cardiovascular Stress

Oxidative stress in the heart leads to various cardiovascular diseases. The induced expression of HO-1 due to stress offers protection to the heart. The levels of oxidative stress proportionately increased the expression of HO-1 in rat neonatal myocardial primary cultures (Hoshida et al. 1996). Heavy metals, heme compounds, hydrogen peroxide (H_2O_2), sulfhydryls have the potential to induce HO-1 in different tissues (Maines and Kappas 1976). The depletion of cellular glutathione by these agents led to the enhanced HO-1 synthesis. Being a stress protein, HO-1 expression elevated in both the diseased and stressed conditions and hence can serve as a marker of oxidative stress (Abraham et al. 1988). The HO-1 overexpression offered protection against various cardiac dysfunctions such as myocardial ischemia-reperfusion (L'Abbate et al. 2007), chronic heart failure (Collino et al. 2013), cardiac hypertrophy (Brunt et al. 2009), and myocardial ischemia (Issan et al. 2014). Hypoxia induced myocardial ischemia through aggravated oxidative stress in cobalt-protoporphyrin treated streptozotocin-induced diabetic mice. HO-1 rescued the cardiac function by lowering oxidative stress in hypoxia induced myocardial ischemia, elevated pAKT, lowered pGSK3β thus retained the mitochondrial membrane potential (Zhao et al. 2013; Issan et al. 2014). A contradictory role of HO-1 is the induction of oxidative stress in streptozotocin-treated diabetic rats (Farhangkhoee et al. 2003). The pro-oxidant activity of HO-1 may be exhibited by the iron – one of the products of heme catabolism. In Takotsubo cardiomyopathy, the increased expression of HO-1 in cardiac and aortic macrophages offered protection against oxidative stress (Ueyama et al. 2009).

8.1.2 Role of HO-1 in Cardiac Inflammation

HO-1 possesses anti-inflammatory properties. HO-1 inhibits various proinflammatory agents such as tumor necrosis factor-α (TNF-α), interleukin-1b (IL-1b), and IL-6 (Chen et al. 2013; Konrad et al. 2016). Besides the above inflammatory factors, IL-18 activates NF-kB and induces the expression of TNF-α ultimately triggering systemic and acute inflammation. HO-1 prevents both the inflammation by impeding the IL-18 signaling in human cardiac endothelial cell (Zabalgoitia et al. 2008). The IL-10 anti-inflammatory response requires HO-1, whenever the HO-1 activity is abolished by pharmacological inhibitors and IL-10 elicitation of anti-inflammation is reduced (Lee and Chau 2002; Chen et al. 2005). IL-10 is known to induce the expression of HO-1 (Gomez-Hurtado et al. 2011).

Cell adhesion molecules (CAMs) play a major role in inflammation and is associated with many cardiovascular diseases. Oxidative stress is known to activate CAMs such as intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) eventually causing inflammation (Nakashima et al. 1998; Madamanchi et al. 2005). Inflammation causes these ICAM1 and VCAM1 to end in circulation from endothelial and hence both ICAM1 and VCAM1 serve as

potential biomarkers for cardiac inflammation and atherosclerosis (Lu et al. 2010). The elevated levels of HO-1 inhibited the expression of ICAM-1 and VCAM-1 thereby inflammation (Wagener et al. 1997, 1999). The decreased expression of ICAM-1 in arterial injury was observed with an increased expression of HO-1 prior to injury (Rucker et al. 2001).

In endotoxaemic rats, baicalein recuperated the myocardial contractility through the induction of HO-1 thereby suppressing the inflammation (Lee et al. 2011). Hypertension induces inflammatory cytokines, leading to inflammation. Celastrol – a triterpenoid mitigated hypertension via increased expression of HO-1 thus reduced inflammation (Yu et al. 2010). The post-ischemic cardiac inflammation was aggravated due to the deficiency of HO-1 expression in mice. The regional gene therapy using human HO-1 diminished inflammation in the heart of both the murine and porcine after ischemia and reperfusion (Hinkel et al. 2015). In diabetic cardiomyopathy (DCM), the phosphorylation of AKT and 5'-adenosine monophosphate-activated protein kinase (AMPK) was restored to normal levels by the elevated levels of HO-1, thus HO-1 inhibited inflammation and prevented DCM (Zhao et al. 2013). HO-1 recuperates the vascular endothelial function in humans and mice by inhibiting the pro-inflammatory phenotype of monocytes (Wenzel et al. 2015). Niacin – water-soluble vitamin is a potential anti-inflammatory agent (Kuvin et al. 2006). It slows down the progression of atherosclerotic cardiovascular disease by downregulating NF-kB signaling pathway (Meyers et al. 2004). Niacin induces the expression of HO-1 in both the in vitro and in vivo models and offers protection against coronary artery disease (Wu et al. 2012). All these evidence demonstrated HO-1 offers protection against cardiovascular diseases due to inflammation.

8.1.3 Role of HO-1 on Cardiovascular Redox Signaling

The endogenous synthesis of HO-1 – a major redox potential enzyme in the antioxidant defense mechanism is regulated by either glutathione homeostasis or HO-1/Nrf2 signaling axis and thus exhibits multi-faceted role in the pathophysiology of various cardiomyopathies and cardiotoxic studies (Zhao et al. 2015; Liu et al. 2017). Emerging studies insinuate that the redox potential of cardiac HO-1 confers cardioprotection against pro-oxidants, angiotensin II, pressure overload and cardiac hypertrophic models (Cao et al. 2011; Han et al. 2015). Activation of HO-1/MAPK/Nrf2 signaling molecules attenuates H_2O_2 induced cardiomyocytes hypertrophy against the combined treatment of heme precursor 5-Aminolevulinic acid with sodium ferrous citrate (Zhao et al. 2015). The myocardial transactivation of Nrf2/HO-1 axis therapeutically rescued the ventricular arrhythmias through attenuating the NGF-induced sympathetic innervation in lithium chloride treated rats (Lee et al. 2014). In another report, selective β1-adrenergic receptor agonist stimulates the expression of HO1/Nrf2 axis and regulates the cardioprotection through PI3K/p38MAPK signaling against hypoxia/reperfusion – induced neonatal rat cardiomyocytes injury in vitro (Wang et al. 2015a, b). Induction of nitrative stress stimulates

the upregulation of HO-1 via over expression of ARE/Nrf2 complex, which bestows adaptive response in the vascular smooth muscle cell exposed to nitric oxide (Liu et al. 2007).

The implication of HO-1 plays a crucial role in the regulation of hypoxia, vascular smooth muscle cell proliferation and their impairments cause pulmonary hypertension. Yet et al. (1999) reported that hypoxic pulmonary vasculature causes severe ventricular diastolic pressure and increased myocardial oxidative damage and apoptosis were observed in HO-1 knockout mice. In this study, depletion of HO-1 in rat cardiomyocytes possess maladaptive response against pulmonary hypertension. MAPK pathway regulates the stress stimulus, proliferation, differentiation and signals the cell to proceed either prosurvival or apoptotic cell death mechanism. The interaction of JNK/MAPK/AP-1 signling kinase molecules positively correlated to the prosurvival mechanism through the upregulation of HO-1 in H_2O_2 treated H9c2 cells (Aggeli et al. 2006). In the progression of diabetic cardiovascular complications and diseases, induction of HO-1 and Nrf2/JNK/Akt abrogates the formation of advanced glycation end products in endothelial cells (He et al. 2011). The overexpression of HO-1 attenuates proxidant stimulus, heme content, urinary 8-epi-prostaglandin F2 levels and rescue the endothelial cell damage, shedding in the hyperglycemic induced vascular injury in diabetic rats. The findings of this study highlighting the prophylactic approach of the HO system in the regulation of vascular complications in the diabetic heart (Abraham et al. 2004). In addition, upregulation of Nox2 and NF-κB and regression of Nox4, Nrf2 directed HO-1 were associated with the pathogenesis of vascular oxidative stress in seasonal and diabetes induced guinea-pigs and rat models (Gajos-Draus et al. 2017). In contrast, the pro-oxidant activity of HO-1 was due to increased accumulation of redox active irons mediated oxidative damage in the diabetic heart (Farhangkhoee et al. 2003). In the advanced atherosclerotic disease models, potential interaction of AKT and HO-1 exhibits codependence activity via a positive feedback loop of mechanism and attenuates H_2O_2 induced oxidative stress mediated apoptosis in human vascular smooth muscle (Brunt et al. 2006). Similarly, the transcriptional cooperation between protein kinase Cε (PKCε) – cAMP response element-binding protein 1 (CREB1) and Nrf2 regulates the HO-1 activity in the endothelial homeostasis and enhances resistance to inflammatory and apoptotic stimulus (Mylroie et al. 2015).

Guo et al. (2017) reported that alteration or modulation of MAPK1 and its target (HIF1/HO-1) were speculating in the pathophysiology of coronary artery disease (CAD). The genotyping studies reveal that mutations within the MAPK1/HIF1/HO-1 could potentially involve in the development of perimenopausal CAD risk. Furthermore, among the HO-1 interacting proteins, emopamil-binding protein (EBP) is closely associated in the lipid metabolism. The over expression of HO-1 and EBP interaction inhibits carbohydrate metabolism through activation of AKT/Nrf2/mTOR pathways and restores the cardiomyocytes function in both in vivo and in vitro models (Jin et al. 2017a). In another report, HO-1 alleviates cholesterol induced oxidative impairments through activation of Nrf2/Erk2 and inhibition of PI3K/AKT/c-Myc signaling pathway in endothelial cells (Jin et al. 2017b). Cardiac specific HO-1 deficiency has been implicated in the transcriptional regulation of

mitochondrial biogenesis, autophagy/mitophagy and leads to cell death mechanisms. Suliman et al. (2017) reported that depletion of HO-1 impairs the redox/mitophagy sensitive transcriptional cascades (Nrf1, Pink1, Park2) which regulates the mitochondrial quality control mediated vascular remodeling and cell death mechanism.

HO-1/Nrf2 interactions have been shown to regulate myocardial apoptosis in the progression of cardiomyopathies (Zhu et al. 2017). Activation of Nrf2/HO-1 axis lessens the classical mitochondrial apoptotic maker expressions (Bax/Bcl2, caspase cascade and TUNEL labelling) in the myocardial injury of coronary microembolized rats (Liang et al. 2017). It has been reported that arsenic induces vascular remodeling and angiogenesis through HO-1 dependent mechanism. The induction of HO-1 influences the cell migration, tube formation and promotes the HO-1/Nrf2 binding interaction via dissociation of site specific transcriptional repressor Bach1 (Meng et al. 2010). In addition, acute fluoride exposure modulates myocardial HO-1 expression in rats. In this study, the upregulation of HO-1 could serve as a balance between prosurvival and death signal upon fluoride exposure (Panneerselvam et al. 2017). Induction of HO-1 aids prognostic marker of oxidative stress upon xenobiotic insults. Environmental exposure of single intrapharyngeal instillation of single-wall carbon nanotubes induces systemic expression of HO-1 was associated with mitochondrial oxidative damage and inflammation in the aorta of HO-1 reporter transgenic mice (Li et al. 2007).

8.1.4 Role of HO-1 on Cardiac Ischemia and Remodeling

The obstruction or improper blood flow in the coronary arteries in the heart lead to cardiac ischemia. Myocardial ischemia mediated remodeling changes severely impair the cardiac functions in various cardiomyopathies. Numerous reports have shown that activation of HO-1 advances the survival of cardiomyocytes and attenuates the myocardial damages in the experimental ischemic heart models (Csonka et al. 1999; Lakkisto et al. 2002; Jancsó et al. 2007). Interestingly, long term administration of hemin significantly regulates the myocardial HO-1 activity in the infarcted rats and inhibits the oxidants mediated DNA damage, inflammation and apoptosis in chronic heart failure model (Collino et al. 2013). Ono et al. (2004) reported that the variation in the *HO-1* gene promoter activity lessens the affinity between HO-1 and ischemic heart disease in the Japanese. The preemptive and direct gene delivery mediated overexpression of myocardial HO-1 prevents the inflammatory response, fibrosis and upregulates the phosphorylation of survival proteins and ceases the myocardial apoptotic cell death in the I/R rat model (Melo et al. 2002; Pachori et al. 2006). Further, induction of HO-1 regulates the coronary endothelial cell activation, proliferation and promotes angiogenesis (Deramaudt et al. 1998). The synergistic effect of HO-1 has been enthralled in cardioprotective action during cardiac ischemia-reperfusion. The balanced expression of HO-1 and ubiquitin exhibits an adaptive response in the treatment of porcine heart subjected to ischemia and reperfusion mediated cellular deterioration (Sharma et al. 1996).

Induction of HO-1 pathways alleviates hyperthermia induced delayed cardioprotection could be due to regulation of carbon monoxide/cyclic guanylate cyclase signaling pathway in the I/R model (Lu et al. 2002). In addition, coordinated expression of HSP60 and HO-1 was disclosed as a critical prognostic marker in acute myocardial infarction associated pathology in the serum of human subjects (Novo et al. 2011).

Induction of HO-1 has been implicated in the cardiac stem cell therapy and xenograft survival in ischemic hearts (Soares et al. 1998). Genetically engineered mesenchymal stem cells (MSC) with HO-1 vector reduces the inflammatory cytokines, apoptotic cell death and improves the graft MSC survival in the infarcted heart (Tang et al. 2005). Increased circulation of Splenic $Ly6C^{hi}$ monocytes severely impairs the left ventricular function and causes ventricular remodeling in HO-1 deficient mice. In this study, the underlying mechanism associated with the splenic $Ly6C^{hi}$ monocytes recruitment and HO-1 remains obscure (Tomczyk et al. 2017). HO-1 regulates the cardiovascular pathologies in the diabetic cardiomyopathies. The cardiac specific overexpression of HO-1 diminishes the myocardial infarction. HO-1 deficiency caused hyperglycemia mediated oxidative damage and left ventricular dysfunction in diabetic mice (Liu et al. 2005). The cardiac HO-1 expression has been shown to prevent the vasoconstriction by modulating the nitric oxide synthase (NOS) and serum adiponectin levels in the diabetic rats. HO-1/HO-2 signaling pathway prevents the uncoupling of endogenous NOS and oxidative damage through its antioxidant, antiinflammatory and antiatherogenic properties (L'Abbate et al. 2007). Further, HO-1 exhibits antifibrogenic properties through dowregulation of profibrotic connective tissue growth factor (CTGF) and enhances the proliferation and repair via TGF-β1 in the cardiomyocytes of post infarcted rat heart (Lakkisto et al. 2011).

8.1.5 Cross Talk Between Cardiac HO-1 and AMPK Signaling Kinase

AMPK is the master regulator of fatty acid oxidation and glucose metabolism in the energy homeostasis. The dysregulation of AMPK signaling cascade and its target kinases drives the progression of chronic metabolic syndromes. The precise cross talk between HO-1 and AMPK regulates the cellular redox and energy homeostasis in the treatment of cardiomyopathies. Pharmacological activation of xanthohumol enhances the molecular crosstalk between AMPK and Nrf2/HO-1 axis and stimulates the reduction of unfolded protein response mediated endoplasmic reticulum stress in the mouse embryonic fibroblasts (Zimmermann et al. 2015). Inhibition of AMPK and HO-1 promotes the platelet-derived growth factor, vascular smooth muscle cell proliferation and ROS production during cilostazol treatment was exerted on the development of therapeutic cardiovascular measures (Kim et al. 2011). The dysregulation or damage of vascular endothelialization plays an important manifestation in the progress of DCM. Induction of HO-1 is indispensable for the endothelium-selective activation of AMP mediated secretion of stromal

cell-derived factor (SDF) and vascular endothelialization in AMPK transgenic mice with wire-induced carotid denudation (Li et al. 2012). Previous studies have documented that molecular cross talk of both AMPK and HO-1 induces endothelial cell proliferation and confers cardioprotection via inhibiting oxidative stress and apoptosis (Zou and Wu 2008; Liu et al. 2011). Moreover, HO-1 regulates endothelial function directly without the interaction of adiponectin activity in the aorta of obese rats. The cardioprotective mechanism is primarily aided by the activation of AMPK-PI3K/Akt-eNOS signaling pathways (Han et al. 2015). On the other hand, therapeutic intervention of ramipril, an inhibitor of angiotensin converting enzyme activates the Ca^{2+}/calmodulin-dependent protein kinase (CaMKKb), AMPK/Nrf2/HO-1 pathway in high glucose induced endothelial dysfunction in the human aortic endothelial cell and a type 2 diabetic animal model. Dysregulation of CaMKKb or AMPK in the endothelial cells suppress the HO-1 activity and enhances insulin resistance and endothelial dysfunction (Tian et al. 2014). The upregulation of HO-1 exacerbates the phosphorylation of liver kinase b1, AMPK, AKT, GSK3 signaling kinase, adiponectin and restore the microvascular function in DCM rats (Kusmic et al. 2010). Thus, the induction of HO-1, pAMPK and adiponectin potentiates the vascular recruitment of normal stem cells and enhance the interaction between the endothelial progenitor cells in the aortic walls of diabetic rats (Sambuceti et al. 2009). In addition, the systemic overexpression of HO-1 protects cardiomyocytes and impedes the ROS mediated oxidative damage, inflammatory reactions and apoptosis. The phosphorylation of AMPK directly enhances the expression of myocardial autophagic markers in DCM rats (Zhao et al. 2013). However, the molecular insight into the regulation of HO-1 and phosphorylation of AMPK in DCM remains unknown.

8.1.6 Pharmacological Modulators of Cardiovascular HO-1

Numerous epidemiological studies have revealed that pharmacological induction of HO-1 exhibits vascular protection in both clinical and experimental cardiomyopathies (Farhangkhoee et al. 2003; Kusmic et al. 2014). In general, dietary supplementation of natural food compounds has been primarily targeted for the induction of HO-1 signaling axis in the treatment of chronic metabolic diseases (Barbagallo et al. 2013). The direct mechanism by which induction of HO-1 has been reported via transcriptional regulation of either Nrf2/HO axis or HSF1 mediated signaling pathways. Indirect activation of HO-1 requires the anti/prooxidant stimulus and secretion of chemokine or cytokine production in the site of injury. Despite of the natural activator, synthetic compounds such as protoporphyrin IX cobalt chloride and hemin were commonly used for induction of HO-1 in various cardiomyopathies (Eyssen-Hernandez et al. 1996; Kusmic et al. 2014). In this context, we have documented the wide array of both natural and synthetic HO-1 activators and their molecular insights into the treatment of cardiomyopathies were summarized in the Table 8.1.

Table 8.1 Summary of the natural and synthetic HO-1 inducers and their molecular targets in the treatment of cardiomyopathies

Compounds	Experimental models	Molecular targets	Mechanisms	References
2-Methoxycinnamaldehyde	Male Sprague-Dawley rats	↑HO-1 and ↓HMGB1	Reduces myocardial injury by VCAM-1 and HO-1 expression	Hwa et al. (2012)
2-Methoxycinnamaldehyde	Human umbilical vein endothelial cells	↓VCAM-1 and ↓NF-kB	Inhibition of NF-kB by activated TNF-α	Hwa et al. (2012)
Epoxyeicosatrienoic acid	*Mus musculus*	↑HO-1, ↑adiponectin, ↑p-eNOS and ↓Bach1	Increased neovascularization and cardiac function by adiponectin and p-eNOS expression	Cao et al. (2015)
Epoxyeicosatrienoic acid	Human microvascular endothelial cells	↑HO-1 and ↓Bach1	Expression of HO-1 increases neoangiogenesis	Cao et al. (2015)
α-melanocyte-stimulating hormone	Male Sprague-Dawley rats	↑HO-1	Expression of HO-1 due to cytoprotective effects of α-melanocyte stimulating hormone	Vecsernyes et al. (2017)
Caffeic acid phenethyl ester	Rat primary aortic vascular smooth muscle cells	↑HO-1 and ↑phosphorylation of p38 MAPK	Inhibit the cellular proliferation by interfering with G0/1 to S-phase	Roos et al. (2011)
Hydrogen sulfide	Inbred male Balb/c mice	↑HO-1 and ↓iNOS	Over expression of HO-1 and inhibition of iNOS increases cardioprotection in CVB3-incuduced myocarditis in mice	Hua et al. (2013)
Estrogen and Raloxifene	Female Wistar rats	↑HO-1 and ↑HO-2	Estrogen and Raloxifene administration increases the expression of HO-1 and HO-2 attenuate the MPO activity and cardiovascular ischemia damage	Posa et al. (2017)
Malvidin	Male albino Wistar rats	↑HO-1and ↑Nrf2	Malvidin treatrment decreases the levels of cytokines and inhibited the IKK and IkBa phosphorylation	Wei et al. (2017)
Celastrol	Male Sprague-Dawlry rats	↑HO-1, inhibition of ERK, MAPK and AKT	Treatment of celastrol inhibiting the mRNA levels of inflammatory cytokines and increases the expression of HO-1	Yu et al. (2010)

Celastrol	Male Wistar-Kyoto rats	↑HO-1, ↑HSP70, ↓iNOS, ↓TNF-α, ↓NF-kB, ↓caspase3	Induction of HO-1 and HSP70 prevent the circulatory failure	Wang et al. (2015a, b)
Curcumin	Human adult atrial myoblast cells	↑HO-1	Curcumin induced HO-1 expression reduces cold storage induced damage	Abuarqoub et al. (2007)
Curcumin	Bovine aortic endothelial cells	↑HO-1/HSP32	Increased expression of HO-1 mediates cytoprotection against oxidative stress	Motterlini et al. (2000)
Curcumin	Hepatocarcinoma cell line	↑HO-1, ↑Nrf2 and ↑MAPK	Curcumin induces ROS, activate the Nrf2 and MAPkinase molecules and inhibit the phosphatase activity	Mcnally et al. (2007)
Dimethyl sulfoxide	Rat embryonic cardiomyoblast derived cell line	↑HO-1, activation of p38 MAPK and Nrf2	Dimethyl sulfoxide activating the expression of p38 MAPK, Nrf2, HO-1 exerts cardioprotection	Man et al. (2014)
Epigallocatechin	Human aortic endothelial cells	↑HO-1, ↑p38 MAPK and ↑Nrf2	Expression of p38 MAPK induces the activation of Nrf2 resulting in the expression HO-1 and suppressess TNF-α expression	Pullikotil et al. (2012)
Epoxyeicosatrienoic acid	C57BL/L mice	↓NF-kB, ↑HO-1and ↑PPARα/γ	Suppression of NF-kB mediated by induction of PPARα/γ and HO-1 regulates macrophage polarization	Dai et al. (2015)
Aprotinin	Inbred WKY and SHR rats	↑HO-1, ↑cyclin D, ↑p21 and ↓CDK4	Induction of HO-1 suppresses the proliferation of vascular smooth muscle cells	Choi et al. (2009)
Higenamine	Male Sprague-Dawley rats	↑HO-1, ↑BCL-2, ↓cytochrome c, ↓caspase-3 and ↓Bax	HO-1 expression induced by higenamine plays a protective role in myocardial injury	Lee et al. (2006)
L-carnitine	Male C57B1/6j mice	↑p38 MAPK-Nrf2, ↑HO-1, ↑NQO1, ↑caspase3 and ↓Bax	L-camitine treatment inhibits the ROS and apoptosis mediating cardioprotective effect	Fan et al. (2017)

(continued)

Table 8.1 (continued)

Compounds	Experimental models	Molecular targets	Mechanisms	References
α-Lipoic acid	Male Sprague-Dawley rats	↑PI3K/AKT, ↑Nrf2 and ↑HO-1	α-Lipoic acid reduces necrosis, apoptosis and inflammation mediated by PI3K/AKT pathway and upregulation of HO-1	Deng et al. (2013)
Cobalt protoporphyrin	Human embryonic stem cell-derived cardiomyocytes	↑HO-1	Higher expression of HO-1, AKT phosphorylation with apoptosis inhibition reduce hypoxia injury cardiomyocytes	Luo et al. (2014)
Caffeic acid + Pyrrolidine	Sprague-Dawley rats	↑HO-1, ↑AKT and ↑Mn-SOD	Upregulation of HO-1, Mn-SOD, AKT exhibits cardiac function improvement	Ku et al. (2016)
Myricetin	*Mus musculus*	↑HO-1, ↑Nrf2, inhibition of IkB-α/NF-kB/p65 and TGFβ/Smad	Inhibition of IkB-α/NF-kB/p65 and TGFβ/Smad, upregulation of Nrf2, HO-1 possesses higher protective effects on diabetic cardiomyopathy	Liao et al. (2017)
N-acetylcysteine + Allopurinol	Sprague Dawley rats	↑HO-1 and ↑HIF-1 α,	Combination treatment normalizes the levels of HO-1 and HIF-1 α	Mao et al. (2013)
Niacin	Human coronary artery endothelial cells	↑HO-1, activation of Nrf2/p38 MAPK and inhibition of TNF-α	Niacin treatment induces anti-inflammatory property by inducing HO-1 expression	Wu et al. (2012)
Plamatine	Male Sprague-Dawley rats	↓COX-1 and ↓iNOS	Plamatine treatment reduces oxidative stress and inflammation	Kim et al. (2009)
Plamatine	Human aortic endothelial cells	↑HO-1	Plamatine treatment reduces oxidative stress and inflammation	Kim et al. (2009)
Probucol	Rabbit aortic balloon-injury model	↑HO-1	Expression of HO-1 inhibits the smooth muscle cell proliferation and thickening	Deng et al. (2004)
Resveratrol	Male SD rats	↑p-AKT, ↑p-eNOS, ↑Trx-1, ↑HO-1 and ↑VEGF	Resveratrol treatment induces NO and Trx-1 expression responsible for cardioprotective mechanism	Thirunavukkarasu et al. (2007)

Resveratrol	Male BALB/c nude mice	↑HO-1 and ↓p53	Resveratrol induces HO-1 expression plays a protective role in DOX induced cardiomyocyte apoptosis	Gu et al. (2012)
Sulforaphane	Sprague Dawley rats	↑HO-1, ↑Nrf2 and ↑NQO1	Activation of Nrf2 inhibits the fibrosis, inflammation, oxidative stress induced by DOX	Bai et al. (2017)
Tetramethylpyrazine	Sprague Dawley rats	↑HO-1	Tetramethylpyrazine inhibits the neutrophil and oxidative stress via induction of HO-1	Chen et al. (2006)
Sodium tanshinone IIA sulfonate	Sprague Dawley rats	↑HO-1 and ↑NF-kB	Sodium tanshinone IIA sulfonate modulates the expression of HO-1 expression and regulate the antioxidant system involved in cardioprotection	Wei et al. (2014)
Curcumin	H9c2 cardiomyoblasts	↑HO-1, ↓cleaved caspase −3 and ↑PI3K/AKT	PI3K/AKT mediated upregulation of HO-1 inhibits apoptosis	Yang et al. (2017)

8.2 Conclusions

HO-1 is a crucial redox sensitive enzyme which catalyzes the heme catabolism to biliverdin, ferrous iron, and CO. Existing evidence shows that HO-1 is the master regulator in the cell survival and death signaling against various cardiomyopathies. This chapter has documented that HO-1 exerts cardioprotection against ischemic injury mediated ventricular remodeling, endothelial dysfunction, diabetic cardiomyopathies, aortic vessels, neovascularization, oxidative insults, apoptosis and fibrosis. The cytoprotection is mainly due to its antioxidant, anti-inflammatory and antiatherogenic properties. The molecular mechanism behind the interaction/cross talk/activation of HO-1 with other signaling pathways remains elusive. In general, the overexpression of HO system either pharmacological or gene therapy modulates the cardiovascular associated pathologies in both clinical and experimental models. As evidenced from various studies, the induction of HO-1 offers promises in the development of therapeutics against various cardiovascular dysfunctions. However, much more detailed research on HO-1 and cardiac stress signaling pathways are warranted in the near future.

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Chapter 9
Role of Heat Shock Protein 90 in Regulating Downstream Signal Transduction Cascades

Wei Lei, David Duron, Carrie Stine, and John M. Streicher

Abstract Heat shock protein 90 (Hsp90) regulates a broad swathe of proteins critical for normal and pathological cell function. One major class of regulated proteins are signal transduction molecules, such as the Mitogen Activated Protein Kinases (MAPK), G Protein Coupled Receptor (GPCR) regulatory kinases, and similar. Hsp90 regulates these signaling proteins by promoting proper folding and protein stability, however, Hsp90 also regulates signaling activation and association/targeting of mature proteins during the course of acute signal transduction. As these signaling proteins are ubiquitously expressed in most cells and are downstream of numerous different receptor systems, Hsp90 regulation of signaling proteins is strongly and broadly impactful. In this chapter, we will discuss the main themes of signaling protein regulation by Hsp90, and highlight several crucial signaling protein families. We will discuss the impact of Hsp90 on signaling downstream of multiple receptor systems, and subsequent effects on physiology and pathophysiology. We will also suggest means to manipulate these regulatory relationships to improve clinical therapy, and future directions for the field of Hsp90 signaling regulation.

Keywords Hsp90 · Kinase · Signal transduction · MAPK · Small GTPase · Receptor

Abbreviations

AMPK	adenosine monophosphate-activated protein kinase
CDK	cyclin-dependent kinase
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase

Authors Wei Lei, David Duron and Carrie Stine have equally contributed to this chapter.

W. Lei · D. Duron · C. Stine · J. M. Streicher (✉)
Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ, USA
e-mail: JStreicher@email.arizona.edu

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FAK	focal adhesion kinase
GPCR	G protein coupled receptor
GRK	G protein-coupled receptor kinases
GSK-3	glycogen synthase kinase-3
Hsp90	heat shock protein 90
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MOR	Mu opioid receptor
PKA	protein kinase A
PKC	protein kinase C
RTK	receptor tyrosine kinases
VEGFR	vascular endothelial growth factor receptor

9.1 Introduction

Heat shock protein 90 (Hsp90) is one of the central protein regulators of the cell. Through specific and direct client interactions, Hsp90 promotes proper protein folding and maturation late in the protein maturation process [for a general review of Hsp90 function, see Li and Buchner (2013)]. This prominent role could tempt one to think that Hsp90 protein regulation only occurs during early maturation or heat shock, without a role in moment-to-moment cell physiology. However, Hsp90 has been shown to acutely regulate the activation state, mature stability, cellular location, and interaction partners of proteins intimately involved in cell physiology, such as receptors and signaling kinases [see Li and Buchner (2013), and also below]. Through these mechanisms, Hsp90 regulates the translation, interaction partners, location, and activation states of hundreds of client proteins, including receptors (adrenergic, cannabinoid, etc.), channels (CFTR, hERG, etc.), transcription factors (FOX, PPAR, etc.), signaling kinases (MAPKs, Protein Kinase C [PKC], etc.), pathological proteins like tau protein, and many more [see Table 9.1 and Echeverria

Table 9.1 Selected classes of signaling proteins regulated by Hsp90. Selected families and groups of signaling proteins known to interact with Hsp90 are shown here

Selected families of signaling proteins regulated by Hsp90					
ACVR	**Cyclin**	ERBB	**ERK MAPK**	PDK	SGK
Akt	**CDK**	**GRK**	**JNK MAPK**	PIM	Src-related
ALK	DDR	**GSK3**	**p38 MAPK**	**PKC**	SRPK
AMPK	DAPK	**GTPases**	Atyp MAPK	PRK	STK
ASK1	DYRK	**IKK**	mTOR	PTK	TAK1
c-Abl	eEF-2	JAK	NTRK	RIP	TrkA/B
CAMK	EPH	**MAPK-Ks**	p90RSK	RPS6K	TSSK

Families and groups covered in this chapter are marked in **bold**. (Taken primarily from Echeverria et al. 2011). Also see the site of Dr. Didier Picard from the University of Geneva (www.picard.ch)

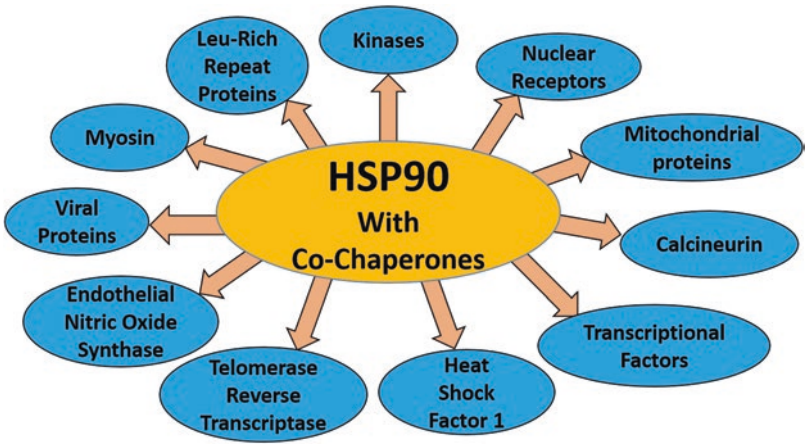

Fig. 9.1 Summary of protein types shown to interact with Hsp90. Here are summarized some of the diverse protein types shown to interact and complex with Hsp90 and its co-chaperones. Through this rich diversity of interaction, Hsp90 impacts many different types of cell and organism physiology, from acute signal transduction regulation to cytoskeletal formation to viral infection

et al. (2011)]. Of note, in this chapter we will focus on the cytosolic Hsp90 isoforms, Hsp90α and Hsp90β. The mitochondrial (TRAP1) and endoplasmic reticulum (Grp94) isoforms will not be discussed. We will also highlight research from multiple eukaryotic species, including yeast, all of which possess conserved Hsp90 proteins. A broad overview of Hsp90 interaction with different protein types is diagrammed in Fig. 9.1.

9.2 Hsp90 Regulation of Signaling Kinases

Most receptor systems transduce signal transduction in whole or in part through the activation of a network of mid-level signaling kinases. These kinases in turn phosphorylate downstream targets to elicit changes in cell physiology, such as activation of ion channels or transcription factors. Many receptors can transduce specific effects through signaling kinases they share in common, so downstream signaling kinases are heavily regulated by chaperone proteins (including Hsp90, 14-3-3, etc.), scaffold proteins (AKAPs, axin, etc.), and similar to control the location and interaction partners of activated kinases.

Hsp90 is one such central regulator of signaling kinases. After translation, the Hsp90 co-chaperone Cdc37 specifically directs nascent kinases to the Hsp90 complex for final folding and maturation (Li and Buchner 2013; Odunuga et al. 2004). However, as discussed above, Hsp90 regulation of kinases extends well beyond protein folding and maturation. Hsp90 in concert with Cdc37 or with other proteins regulates the activation state of mature kinases (Li and Buchner 2013), regulates

kinase location in the cell before and after activation [e.g. cytosolic vs. nuclear, Mahony et al. (1998)], regulates autophosphorylation of kinases (Ota et al. 2010), and similar. Through these mechanisms, Hsp90 differentially regulates signal transduction downstream of numerous or even all receptor systems through regulation of these signaling kinases, with a concomitant broad impact on cell physiology and pathophysiology.

A broad (and incomplete) overview of the families of signaling kinases regulated by Hsp90 is shown in Table 9.1. An incredible diversity of proteins are clients, including the MAPK family, small GTPases (Rho, Ras, etc.), PKC, the IκK kinases, and many more. In this chapter, we will highlight a selection of the major families of signaling kinases regulated by Hsp90. We will explore the various mechanisms of regulation found in each family, and how those mechanisms impact signaling by those kinases. We will also explore the effects of this regulation on receptor signaling, cell physiology, and disease pathophysiology. Lastly, we will explore some of the potential means to exploit these identified mechanisms to improve clinical therapy.

9.2.1 MAPK Family

The MAPKs, especially Extracellular Signal-Regulated Kinase (ERK) MAPK, are crucial in the regulation of cell survival and proliferation. For this reason, they have been heavily studied for their interaction with Hsp90, as Hsp90 inhibitors are a major strategy in developing anti-cancer therapies (Mo et al. 2016; Nagaraju et al. 2016; Park et al. 2017; Sidera and Patsavoudi 2014). MAPKs are downstream of many receptor systems, and carry out many cellular functions besides cell survival and proliferation. They are organized in a signaling hierarchy, with upstream MAPK Kinases (MAP 4K, MAP 3K, MAP 2K) sequentially activating downstream MAPK kinases, with final activation of one of the three main MAPKs (p38, ERK, c-Jun N-Terminal Kinase [JNK]) (i.e. Ras-Raf-MEK-ERK). For a broad review of the organization of MAPK signaling, see Peti and Page (2013). There are also non-canonical MAPKs that are regulated by Hsp90, such as ERK5 and MAPK4, 6, 7, and 15, but they will not be covered in this chapter.

9.2.1.1 MAPK Kinases

There are many identified MAPK Kinases, with some members having overlapping specificity for downstream MAPK activation. Despite this complexity, MAPK Kinase interaction with Hsp90 has been very poorly studied, *with the exception of Ras and Raf in the ERK MAPK cascade*. One recent study found that the MAP 3Ks MLK3 and MLK4β are stabilized by Hsp90, and that Hsp90 inhibition led to decreased protein levels of these MAP 3Ks with subsequent changes to JNK and p38 activity (Blessing et al. 2017; Zhang et al. 2004). Similarly, Hsp90-Cdc37 was

shown to stabilize the MAP 2K Wis1, and promote stress-induced signaling through this kinase (Tatebe and Shiozaki 2003). In a different mechanism than protein stability, Hsp90 was also shown to promote acute phosphorylation and activation of ERK MAPK via the MAP 2K MEK2 (but not MEK1) in mouse brain (Setalo et al. 2002).

9.2.1.2 Ras and Raf Kinases

Ras and Raf are upstream MAPK Kinases of ERK MAPK, and are crucial in the regulation of cell survival and proliferation. They are also frequently mutated in different cancer types. As Hsp90 inhibitors are being heavily investigated as cancer therapies, they are frequently used in the literature *in the context of* Ras or Raf mutant cells, *without testing the direct interaction of Hsp90 with Ras or Raf*. In light of this, care must be taken when interpreting the literature [see the following for examples: Bar et al. (2017), Haarberg et al. (2013), and Park et al. (2017)].

However, Hsp90 has been shown to have a significant role in directly regulating the activity of both Ras and Raf. One theme that has emerged is that continuous association of Raf with Hsp90 in signaling complexes is necessary for acute Raf signaling activity. The mechanism is not yet clear but this represents a direct regulation of acute Raf signaling by Hsp90 (i.e. not just by protein stability) (Cissel and Beaven 2000; Diedrich et al. 2017; Grammatikakis et al. 1999; Mitra et al. 2016; Zhao et al. 2012). Hsp90-Raf complexes thus promote Raf and ERK MAPK signaling, and have been shown to have an important role in promoting cell survival and proliferation. This interaction explains in part the efficacy of Hsp90 inhibitors in blocking cancer cell proliferation.

Interestingly, Ras has been shown to have an important role in regulating Hsp90-Raf complex formation. Ras signaling promotes Hsp90-Raf formation, which in turn promotes association of the Hsp90-Raf complex with Ras in the membrane compartment. This association promotes the activation of Raf in the Ras-Raf-MEK-ERK cascade (Cissel and Beaven 2000; Diedrich et al. 2017; Mitra et al. 2016; Schulte et al. 1995). In contrast to these more specific regulatory roles, Hsp90 has also been shown to stabilize Raf protein expression, leading to loss of expression and signaling with Hsp90 inhibitor treatment (Dou et al. 2005; Stancato et al. 1997). Lastly, Hsp90 has been shown to *repress* Ras-Protein Kinase A (PKA) signaling in yeast, demonstrating the importance of context in determining Hsp90 regulation of signaling (Shapiro et al. 2009).

9.2.1.3 ERK MAPK

As Ras and Raf are both MAPK Kinases for ERK, Hsp90 can indirectly promote ERK MAPK signaling as discussed above. However, direct studies have shown that ERK regulation by Hsp90 can be strongly context-dependent; increased in some tissues/contexts and decreased in others. One example is downstream of the opioid receptors. Hsp90 has only been studied in a handful of papers relating to opioid

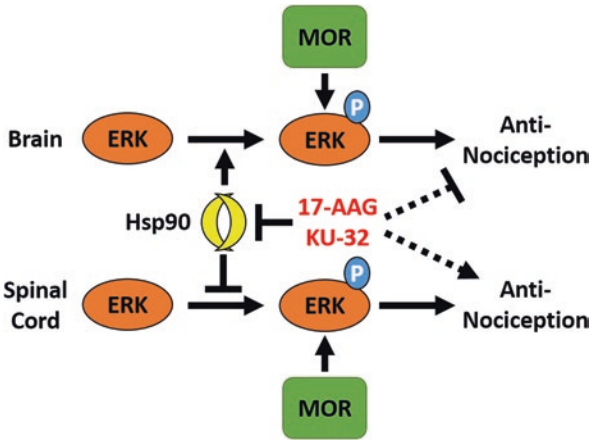

Fig. 9.2 Summary of the differential role of Hsp90 in regulating ERK MAPK in the brain vs. spinal cord. This figure demonstrates how both in the brain and spinal cord ERK MAPK phosphorylation driven by the mu opioid receptor (MOR) promotes opioid anti-nociception in pain states. Hsp90 differentially regulates this process by promoting ERK phosphorylation in the brain and repressing it in the spinal cord. Treatment with Hsp90 inhibitors (17-AAG, KU-32) reverses these processes, resulting in reduced ERK activation in the brain and increased ERK activation in the spinal cord. This has the physiological impact of decreasing opioid anti-nociception when applied to the brain and increasing it when applied to the spinal cord (dashed lines). (Data taken from Lei et al. (2017) and unpublished data from the Streicher Lab)

signaling; Hsp90 inhibition in an in vitro model blocked chronic opioid cAMP superactivation (Koshimizu et al. 2010), while Hsp90 inhibitor treatment in an in vivo model of opioid dependence and withdrawal strongly reduced withdrawal behavior (Abul-Husn et al. 2011).

In our own work, we found that Hsp90 strongly *promotes* ERK MAPK signaling in the brain, resulting in strong or total decreases in morphine analgesia in some pain states with inhibitor treatment and concomitant loss of ERK activation (Lei et al. 2017). In contrast, we found that Hsp90 strongly *blocks* ERK MAPK signaling in the spinal cord, meaning that Hsp90 inhibitor treatment strongly increased ERK signaling in response to opioids. This led to strong increases in morphine analgesia in some pain states (unpublished data, Fig. 9.2). These results demonstrate how differential Hsp90 regulation of ERK in different contexts can have sharply divergent physiological consequences.

This theme continues with other studies of Hsp90 and ERK in the literature. Hsp90 was shown to complex with and promote ERK signaling in the brain (Setalo et al. 2002), and promote ERK signaling in lymphoma cells (Georgakis et al. 2006). Hsp90 was similarly shown to promote ERK signaling in other contexts, in that Hsp90 inhibitor treatment reduced basal or evoked ERK activation (Rice et al. 2008; Wang et al. 2014; Yun et al. 2011). In contrast, Hsp90 inhibition promoted ERK signaling in cervical cancer cells (Lin et al. 2015). Lastly, Hsp90 was shown to complex ERK MAPK with the phosphatase PP5, which regulates both ERK and Raf activity (Mazalouskas et al. 2014).

9.2.1.4 p38 MAPK

In contrast to the context-dependent role of Hsp90 in ERK regulation, the regulation of p38 MAPK by Hsp90 appears to be far more consistent. In nearly all papers that study the question, Hsp90 has been shown to repress p38 MAPK phosphorylation and activity, in that treatment with Hsp90 inhibitors leads to p38 activation. This in turn causes p38-mediated changes in cell physiology, including the promotion of CYP2E1 toxicity in HepG2 cells (Dey and Cederbaum 2007), the promotion of erythroid differentiation (Morceau et al. 2008), the desensitization of Epidermal Growth Factor receptors (Adachi et al. 2010), switching from autophagy to apoptosis (Jiang et al. 2014), and the promotion of Interleukin-6 cytokine production (Fujita et al. 2017). Repression of p38 MAPK signaling by Hsp90 is thus highly physiologically relevant.

An elegant study by the lab of Yibin Wang may have found the mechanism to this negative regulation. In a proteomic study from heart, they found that Hsp90-Cdc37 complexed constitutively with p38 (Ota et al. 2010). This association did not prevent canonical activation by upstream MAPK Kinases, however, it did prevent TAB1/TAK1 mediated phosphorylation [TAK1 also regulated by Hsp90, see Shi et al. (2009)]. Through the use of p38 inhibitors, the authors found that TAB1/TAK1 promotes p38 *autophosphorylation*, which Hsp90 specifically acts to prevent. This may explain the more consistent role of Hsp90 with p38, as intrinsic autophosphorylation would be less likely to differ by context than other types of activation, and suggests a consistent role for Hsp90 in preventing autophosphorylation without altering canonical activation.

In contrast to the consistent results cited above, there have been a few studies where Hsp90 inhibitor treatment decreased p38 phosphorylation (Shi et al. 2009; Yun et al. 2011). Generally speaking these studies showed an impairment in the upstream activators of p38 MAPK, leading to the loss of p38 activation. While this mechanism does not argue against Hsp90 blocking p38 autophosphorylation, it's unclear why a loss of upstream activation overpowered the loss of autophosphorylation regulation in these studies.

9.2.1.5 JNK MAPK

Hsp90 regulation of JNK MAPK has not been as strongly studied as for p38 and ERK. Nevertheless, a common theme of Hsp90 promotion of JNK signaling has emerged from the literature. We could find no evidence that Hsp90 inhibitor treatment caused JNK activation. In contrast, we found studies showing that Hsp90 inhibition led to decreases in JNK signaling in inflammatory stimulation (Rice et al. 2008), stroke (Wen et al. 2008), apoptosis in leukemia cells (Nieto-Miguel et al. 2008), Interleukin-1β treatment (Shi et al. 2009), inflammatory macrophage activation (Yun et al. 2011), and pancreatic cancer cells (Nagaraju et al. 2016). The mechanism of this signaling promotion occurs at least in part through the formation of Hsp90-JNK protein complexes (Nieto-Miguel et al. 2008) and through the

stabilization of JNK protein (Chen et al. 2014). As discussed above, Hsp90 can also promote JNK signaling through promoting the stability or signaling of the JNK MAPK Kinases MLK3 and MLK4 (Blessing et al. 2017; Wen et al. 2008; Zhang et al. 2004). The most common physiological consequence from Hsp90 regulation of JNK signaling in the studies cited above was the promotion of inflammatory signaling, in which JNK has a well-established role.

9.2.2 GTPase and Related Signaling

GTPases are an influential class of signaling molecules. They are roughly divided into heterotrimeric G proteins (α, β, γ) which directly transduce signaling downstream of G Protein Coupled Receptors (GPCR) and small GTPases (Ras, Rac, Rho, etc.) which are mid-level signaling effectors for multiple receptor families, including GPCRs and Receptor Tyrosine Kinases (RTK). These two superfamilies are responsible for an incredible diversity of impactful signaling. GPCRs are the largest superfamily of receptors with at least 800 members (olfactory account for about half), and represent the largest target for clinical drugs, with about ~40% of all clinical drugs targeting GPCRs. Small GTPases are highly impactful in both GPCR and RTK (and other) signaling, making their physiological impact even larger. Ras discussed above is a small GTPase, which was heavily studied in the Hsp90 field for its role in regulating proliferation and survival, which is highly relevant to the role of Hsp90 in cancer. This broad role in signaling and physiology makes the regulation of GTPases by Hsp90 potentially highly impactful. See Gurevich and Gurevich (2017) for a review of the molecular mechanisms of heterotrimeric G protein signaling through GPCRs; see Mott and Owen (2015) for a review of the molecular mechanisms of small GTPase function.

9.2.2.1 Heterotrimeric G Proteins

A relatively small number of studies have examined Hsp90 regulation of the heterotrimeric G proteins. Considering their crucial role downstream of all GPCRs, future work should focus on this question. Intriguingly, Hsp90 was shown to specifically direct mature $G\alpha_{12}$ (but not $G\alpha_{13}$) to lipid rafts and the mitochondria, and is necessary for $G\alpha_{12}$ signaling, suggesting a role in directing specific signaling evoked by GPCRs that couple to this G protein. This regulation had a direct impact on tight junction formation in MDCK cells (Andreeva et al. 2008; Montgomery et al. 2014; Sabath et al. 2008; Vaiskunaite et al. 2001; Waheed and Jones 2002). In a similar specific signaling role, Hsp90 was shown to direct the interaction of $G\alpha_{i2}$ with the cannabinoid type-2 receptor, which may have a broad impact on cannabinoid regulation in the CNS (He et al. 2007). Hsp90 was also shown to form a specific protein complex with $G\alpha_S$, through which Hsp90 regulated GDP/GTP exchange of this protein. This again could have a very large impact on the function of many $G\alpha_S$ coupled

GPCRs (Gibbs et al. 2009). Less specifically, Hsp90 also maintains the expression of $G\alpha_o$, which will generally promote signaling through this protein (Busconi et al. 2000). Lastly, Hsp90 was shown to associate with the Gβ/γ complex, but no function has yet been found for this association (Inanobe et al. 1994).

9.2.2.2 Small GTPases

The crucial small GTPase Ras is discussed above, and will not be covered here. Hsp90 has been shown to associate with and regulate many small GTPases besides Ras. For one, Hsp90 has been shown to regulate multiple members of the Rab family. Hsp90 was shown to complex with Rab and the regulatory protein alpha-GDI. Stimulation of the cell resulted in Hsp90-dependent complex dissociation, permitting alpha-GDI to regulate GDP/GTP exchange of Rab, demonstrating an acute signal-dependent role for Hsp90 in regulating Rab activity (Chen and Balch 2006; Chen et al. 2005; Raffaniello et al. 2009; Sakisaka et al. 2002). Hsp90 also specifically complexes with Rab11 and Rab5 to regulate vesicle internalization and sorting (Allonby et al. 2014; Bozza et al. 2014; Liu et al. 2009). Similarly, Hsp90 regulates membrane association by Rab3 to impact vesicle internalization by that protein (Chen et al. 2013b). Interestingly, the relationship is reciprocal, in that Rab27 has been shown to be crucial in the processing and secretion of cleaved forms of Hsp90, which have been shown to promote cancer progression (Hendrix et al. 2010).

In the Rho family, Hsp90 specifically couples RhoA to the Vascular Endothelial Growth Factor Receptor (VEGFR), and is necessary for the VEGFR to transactivate Focal Adhesion Kinase (FAK) via RhoA (Le Boeuf et al. 2004). This relationship should be highly impactful in vasculogenesis in response to VEGF. Hsp90 was also shown to *repress* Rho activity/signaling in the context of cytoskeletal remodeling, in that Hsp90 inhibition led to Rho activation and actin filament formation (Amiri et al. 2007). Hsp90 may thus be important in preventing irregular cytoskeletal remodeling, and is perhaps related to cell motility. Supporting this hypothesis, Hsp90 was found to block cancer migration by promoting RhoC expression (Willmer et al. 2013). Hsp90 regulates the Rho GDP exchange regulatory protein Vav3, and in a similar manner to alpha-GDI discussed above, indirectly regulates a broad swathe of Rho activity (Wu et al. 2013). Hsp90 also regulates complex assembly and activity of the irregular Rho GTPase DBC2, and as DBC2 is a tumor-suppressor, this relationship impacts tumorigenesis and metastasis (Manjarrez et al. 2014). Lastly, Hsp90 potentiates Src-mediated RhoA activation in endothelial cells in response to lipopolysaccharide, suggesting a role in promoting macrophage extravasation and edema in response to inflammation (Joshi et al. 2014).

Hsp90 also regulates the Rac GTPases, mostly in the context of inflammation and immunity. Hsp90 was shown to complex with Rac1, and promote Rac1 activity which was crucial in the innate immunity response (Thao et al. 2007). Hsp90-Rac1 complex formation was similarly shown to activate NADPH oxidase and reactive oxygen species production in *H. pylori*-infected gastric epithelial cells, and could be

a target to mitigate cell damage in *H. pylori* infection (Cha et al. 2010). Rac1 was also shown to be crucial in the formation of Hsp90-promoted PP5-ERK MAPK complexes, discussed above in the section on ERK MAPK (Mazalouskas et al. 2014)

9.2.2.3 G Protein-Coupled Receptor Kinases (GRK)

Hsp90 also interacts with regulatory signaling components closely associated with GTPase signaling above. One such crucial interaction are the GRKs. GRKs phosphorylate activated GPCRs, and are responsible for desensitization and internalization of the receptors via recruitment of βarrestin. This has made the GRKs a target of considerable interest, as this process is implicated in drug tolerance, dependence, side effects, and other negative effects of drug therapy [see Violin et al. (2014) for a representative discussion in the opioid/pain field]. Hsp90 has been shown to stabilize and promote maturation of GRK2, 3, 5, and 6, imparting a broad potential role to Hsp90 (Luo and Benovic 2003; Penela 2016; Wu et al. 2012). This interaction for GRK3 was shown to be acutely regulated by calcium in neuroblastoma cells, suggesting a potential acute regulatory role in activated neurons (Salim and Eikenburg 2007). Beyond protein maturation and stability, Hsp90 was also shown to target GRK2 to the mitochondria in the heart after ischemic stress in response to ERK MAPK. This mitochondrial targeting of GRK2 was shown to contribute to the mitochondrial permeability transition and to cell death; these results suggest that Hsp90 could be a target for intervention in ischemia-reperfusion injury after myocardial infarction (Chen et al. 2013a)

9.2.3 Protein Kinase C (PKC)

PKC is a central node in the signaling network of the cell, downstream of most receptors and signaling pathways, with a large number of target effectors and thus physiological roles. PKC is most responsive to calcium and ligand signaling, as most PKC isoforms are activated by calcium, diacylglycerol, or both. Despite this centrality, only a small number of studies have examined *specific* Hsp90-PKC interaction, suggesting PKC as a major target for future investigation.

PKCε appears to be the best-studied isoform for Hsp90 interaction. Hsp90 appears to regulate expression of PKCε, in that Hsp90 overexpression reduced PKCε expression (Coaxum et al. 2003). Kinase maturation for all conventional and novel (but not atypical) PKC isoforms, including PKCε, was shown to be mediated by Hsp90 and Cdc37 (Gould et al. 2009). Adenosine-induced mitochondrial targeting of PKCε, thought to be an important step in cardioprotection, was shown to be dependent on Hsp90 (Yang et al. 2012). Similarly, although with ischemic preconditioning in brain instead of heart, Hsp90 was shown to promote PKCε activity, increases in SIRT1 protein levels, and the targeting of both to the mitochondria,

leading to enhanced brain protection (Thompson et al. 2013). Lastly, Hsp90 was shown to specifically oppose the activity of Hsp70 to promote protein stability and activation by phosphorylation of PKCα (Lum et al. 2013).

9.2.4 Cyclins and Cyclin-Dependent Kinases (CDK)

Cyclins and CDKs are central and crucial regulators of cell cycle progression, and are thus primarily responsible for regulating cell proliferation. For this reason, Hsp90 and Cyclin/CDK interactions have been fairly well-studied due to the implications for cancer cell proliferation regulation. Hsp90 forms complexes with various cyclins and CDKs, which regulates activity and/or expression of the proteins, thus regulating cell cycle progression, proliferation, and apoptosis.

Molecular modeling has shown how intrinsic activity of the CDKs, notably CDK9, is destabilizing, necessitating chaperone intervention to maintain functional competency of the proteins (Stetz et al. 2017). Hsp90-Cdc37 forms complexes with CDK4 and CDK6 by different molecular mechanisms (Hallett et al. 2017; Verba et al. 2016); Hsp90 inhibition led to Cyclin D1 and CDK4 degradation, leading to cell cycle arrest, decreased cancer proliferation, and apoptosis (Haarberg et al. 2013; Pai et al. 2015). Hsp90 was also shown to maintain CyclinD/CDK6 complexes in the cytoplasm, where they were inactive, in contrast to the CDK4 results cited above (Mahony et al. 1998). However, in a separate study, Hsp90 inhibition led to loss of CDK6 activity (Georgakis et al. 2006). Similarly, Hsp90-Cdc37 was shown to stabilize CDK11, and Hsp90 inhibition degraded CDK11, slowing apoptosis (Mikolajczyk and Nelson 2004).

Hsp90 also was shown to stabilize the checkpoint protein Cdc2 (Watanabe et al. 2009), and Cdc2 loss was associated with decreased cancer proliferation. Hsp90 also interacts with and stabilizes Cyclin B1, with inhibition associated with Cyclin B1 loss and decreased proliferation (Samadi et al. 2011; Sisinni et al. 2017). However, one study did find that Hsp90 *represses* Cyclin B1 expression, which may be due to particular regulatory elements present in hepatocellular carcinoma vs. most other cells (Zhang et al. 2016).

The results cited above show two general roles for Hsp90 and Cyclin/CDKs. In one, Hsp90 stabilizes and promotes activity of these molecules, promoting proliferation in cells (i.e. cancer). However, in another set of studies, Hsp90 restrains activity of these molecules, preventing inappropriate cell cycle entry. Inhibition of Hsp90 in these cases leads to unwanted cell cycle activation, which would be procancerous (Chaklader et al. 2012). Which role Hsp90 takes is likely to be strongly dependent on particular cell regulatory contexts, although these details have yet to be worked out. Overall, the weight of the evidence suggests that Hsp90 promotes Cyclin/CDK activity in most contexts, and that Hsp90 inhibition leads to reduction of Cyclin/CDK activity and a reduction of proliferation. This activity underlies at least in part the efficacy of Hsp90 inhibitors in cancer therapy (Yamaki et al. 2011).

9.2.5 Glycogen Synthase Kinase-3α/β (GSK-3)

GSK-3 is another central node kinase with more than 40 identified target effectors. This diversity has implicated GSK-3 in a variety of physiological and disease roles, including cell proliferation/apoptosis (cancer), metabolism (diabetes), inflammation, and more. GSK-3 is also highly unusual for a kinase, in that it is constitutively active when unphosphorylated. Phosphorylation by upstream regulatory kinases *decreases* GSK-3 activity; thus, phosphorylation during signal transduction decreases GSK-3 signaling.

In light of the phosphorylation regulation above, GSK-3 also has an unusual kinase maturation process. Intramolecular autophosphorylation mediated by Hsp90 is necessary for full kinase maturation; after this step, GSK-3 does not require Hsp90 or other chaperones to maintain mature kinase activity (Jin et al. 2016; Lochhead et al. 2006). However, Hsp90 has at least been shown to have a role in promoting GSK-3β protein stability, if not directly on activity (Banz et al. 2009; Liu et al. 2012). Hsp90 also has a critical role in complexing GSK-3 with the Wnt/β--catenin signaling cascade; the Wnt/β-catenin/GSK-3 signaling cascade is important in many contexts, including development and cancer, meaning that Hsp90 regulation of GSK-3 in this cascade is highly impactful (Cooper et al. 2011). It is likely for this reason that inhibition of GSK-3 signaling is associated with the efficacy of Hsp90 inhibitors in cancer (Lee et al. 2017). Apart from proliferation and cancer, Hsp90 also has a crucial role in promoting GSK-3 signaling during the process of neuronal axon specification and polarization, making Hsp90/GSK-3 highly impactful in neuronal organization and physiology (Benitez et al. 2014).

9.2.6 Adenosine Monophosphate-Activated Protein Kinase (AMPK)

Much of the research in regards to Hsp90 and signaling regulation has focused on signaling physiology relevant to cancer. However, Hsp90 has roles in many other aspects of cell physiology. One such role is metabolic regulation via regulation of AMPK. AMPK is one of the primary metabolic sensors of the cell, and is activated by AMP in low energy conditions. In response, AMPK promotes lipogenesis, ketogenesis, glucose uptake, and similar metabolic processes to promote an increased energy state.

Hsp90 has been shown to be important in maintaining the kinase activity of AMPK, meaning that Hsp90 is implicated in the metabolic processes initiated by AMPK signaling (Zhang et al. 2012). There is also a small but interesting literature that connects Hsp90 and AMPK signaling to association of Hsp90 with endothelial nitric oxide synthase (eNOS) and the promotion of NO production (Davis et al. 2006; Fujimura et al. 2012; Schulz et al. 2005; Wang et al. 2009). Through this AMPK- and Hsp90-mediated association, increased NO promotes endothelial

cell function, vasodilation, and protects against endothelial cell injury. These associations demonstrate the importance of Hsp90 in AMPK specifically and in metabolism generally, and further research should be focused on these Hsp90 mechanisms.

9.2.7 IκB Kinase (IKK)

Continuing the theme of underappreciated Hsp90 roles, Hsp90 has also been shown to have a significant role in regulating IKK. IKK phosphorylates IκB, leading to ubiquitination and degradation of this protein. IκB is the primary inhibitory regulator of NFκB, meaning that IKK-mediated IκB degradation leads to NFκB activation and nuclear translocation. The transcription factor NFκB is a central node and master regulator primarily of inflammatory signaling, and NFκB activity is highly impactful and generally pro-inflammatory downstream of many immune-related receptors. Hsp90 thus has a large potential role in regulating inflammation through the regulation of IKK.

Hsp90 stabilizes IKK expression and promotes acute IKK signaling/activity, meaning that Hsp90 activity through IKK is primarily pro-inflammatory (Broemer et al. 2004; Hinz et al. 2007; Qing et al. 2006). Acute inflammatory signaling works in part by promoting an Hsp90-IKK complex to assist in NFκB activation; this complex also contains the vesicle protein clathrin (Chen et al. 2002; Gamboni et al. 2014). Direct Hsp90 inhibition through small molecule inhibitors blocks or reduces inflammatory signaling by dissociating Hsp90 from IKK, reducing NFκB activation (Crevecoeur et al. 2008; Shimp et al. 2012; Walsby et al. 2013). Intriguingly, physiological or pathological inhibition of the Hsp90-IKK complex can also block inflammatory signaling through reduction in IKK signaling, including stress (Pittet et al. 2005), bacterial products (Kim et al. 2008), and nitric oxide (Lee et al. 2015). Related to these findings, the Hepatitis B virus suppresses inflammatory IKK signaling and promotes its own survival through disrupting the Hsp90-IKK complex (Liu et al. 2014). Conversely, the KSH virus enhances IKK activity to promote lymphoma cell proliferation; treatment with Hsp90 inhibitor blocks the effects of the virus in this context (Gopalakrishnan et al. 2013).

The Hsp90/IKK interaction regulates physiology beyond direct inflammatory signaling. Hsp90 regulates the switch between autophagy and apoptosis via IKK/NFκB regulation (Jiang et al. 2011). Hsp90 promotion of IKK signaling is required to promote angiotensin-induced cardiac hypertrophy (Lee et al. 2010). There is also a small literature linking Hsp90/IKK to endothelial cell function – by balancing between eNOS (see above) and IKK, Hsp90 promotes endothelial dysfunction in response to high glucose (Mohan et al. 2009) and endothelial injury in response to 20-HETE (Cheng et al. 2010). The efficacy of Hsp90 inhibitors in cancer has also been linked in some studies to loss of IKK signaling with Hsp90 inhibition (Hertlein et al. 2010; Lin et al. 2015; Liu et al. 2012; Walsby et al. 2013).

9.3 Conclusions

The above review is not exhaustive, we've only sought to highlight some prominent signaling molecule families regulated by Hsp90 (see Table 9.1). Through hundreds of identified signaling regulators, Hsp90 can have an incredibly broad and impactful effect on signal transduction. Hsp90 also chaperones and regulates many receptors themselves, which we did not cover in this chapter. Hsp90 thus must be considered one of the master regulators of signal transduction in the cell, which we hope will receive greater focus in the coming years. We've also identified several broad mechanisms by which Hsp90 regulates signaling. Classically most investigators regard Hsp90 as a chaperone protein that assists in late protein folding and maturation. Certainly, Hsp90 is critical in this role, and assists in protein maturation and mature protein stability for many proteins discussed above (and many others). Through this mechanism Hsp90 would only have an indirect role in signal transduction by preserving protein expression. However, Hsp90 also has a role in regulating acute signaling in moment-to-moment activity. Hsp90 may associate with kinases like CDK9 and preserve the appropriate conformation for activity. Hsp90 also has a crucial scaffolding role in associating and targeting proteins for proper activity, such as Raf kinase complexes necessary for activation or targeting of PKCε to the mitochondria. These mechanisms impart a direct role for Hsp90 in signaling regulation that is also amenable to acute regulation by other factors. These mechanisms are also the most likely to strongly vary by protein context, meaning that the determinants of Hsp90 regulation remain a major future area of investigation. Through these mechanisms Hsp90 has an incredibly impactful role on a wide range of physiology and signaling, including inflammation, analgesia, proliferation/survival, neuronal development, and cardioprotection.

As a ubiquitous protein with many roles, Hsp90 could be a challenging therapeutic target. Most investigators have focused on cancer, where a poor side effect profile is more acceptable. It is true that first generation geldanamycin derivatives like 17-AAG were disqualified from clinical trials for liver toxicity (Sidera and Patsavoudi 2014). However, second generation and later compounds have not shown this toxicity, suggesting that the clinical effects seen with first generation compounds were scaffold-related and not target-related. Supporting this hypothesis, a new generation of novobiocin-derived C-terminal Hsp90 inhibitors have been developed which are neuroprotective, effective in treating diabetic peripheral neuropathy, and are currently in Phase I clinical trials for diabetic peripheral neuropathy (Ansar et al. 2007; Anyika et al. 2016; Burlison et al. 2006; Lu et al. 2009; Samadi et al. 2011; Urban et al. 2010). These findings suggest that in principle non-toxic Hsp90 inhibitors could be developed for chronic condition management. Ultimately however, Hsp90 itself is still a very broad target, and the inhibitors above target all four isoforms equally.

In the future, identifying the particular molecular isoform of Hsp90 involved in the target condition could increase treatment specificity. Dr. Brian Blagg and others have led the way in developing isoform-selective Hsp90 inhibitors (Liu et al. 2015; Mishra et al. 2017). Even more specifically, Hsp90 is directed and guided during its

interactions with client proteins by a network of chaperone proteins, including Hop and Cdc37 (Li and Buchner 2013; Odunuga et al. 2004). These chaperone proteins can be far more selective by cellular and disease context, meaning that targeting them could reduce the chances of side effects during therapy. Co-chaperone-selective inhibitors like celastrol have been developed, which could be used for therapy (Zhang et al. 2008). A similar approach has been employed for Hsp70 in conditions like cataract treatment, suggesting a way forward for selective Hsp90 therapy (Assimon et al. 2013, 2015). Other mitigation strategies like cell-type specific drug targeting with aptamers or peripherally-restricted compounds could also be employed (Luo et al. 2017; Xie et al. 2016).

As explored above, most fields have barely scratched the surface on the many roles that Hsp90 plays in regulating signal transduction. The future will involve expanding the known roles of Hsp90 by further exploring the clients outlined in Table 9.1. These interactions also need to be studied in roles beyond cancer, which has dominated most of the Hsp90 biomedical field. Nearly every molecular and physiological system will be impacted by Hsp90. Determining these roles and interactions will also elucidate a new wealth of impactful molecular mechanisms, which will not only expand our knowledge of Hsp90 regulation of signal transduction, but also provide novel future targets for clinical intervention.

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Chapter 10
Diverse Roles of Heat Shock Proteins in Immune Activation and Tolerance: A Comprehensive Review of Mechanisms and Therapeutic Relevance

Anjali Ramaswamy, Ping Wei, and Fan Pan

Abstract Heat shock proteins (HSP) are a broad set of proteins that are induced by a variety of cellular stresses. These proteins predominantly act as chaperones of other proteins in the cell. Since inflammation and infection are a source of physiologic cellular stress, it is unsurprising that the heat shock protein response and the immune response are closely linked. In this chapter, we explore ways in which HSP participate in diverse immune activities, as well as the therapeutic relevance of HSP-immune crosstalk. Firstly, HSP have been found to positively influence the process of immune activation by stimulating innate immune cells and aiding in antigen processing and presentation. Numerous vaccine strategies have been devised based on the finding that HSP can assist in entry of tumor antigens into antigen processing and presentation pathways. These vaccines, which largely consist of HSP-peptide-complexes, have produced striking therapeutic effects in animal tumor models and early clinical studies. In a seeming paradox, HSP have been shown to support immune tolerance and protect against various forms of autoimmunity in mouse models, possibly through the production of IL-10 by regulatory T-cells. These findings have similarly led to efforts to develop HSP-based therapeutic strategies to reduce inflammation associated with arthritis and other inflammatory conditions. We discuss these concepts in detail, and attempt to shed light on why and how HSP influence the immune system to shift towards activation or tolerance.

Keywords Antigen presentation · Autoimmunity · Cancer · Heat shock protein · Immunotherapy · Vaccine

A. Ramaswamy · P. Wei · F. Pan (✉)
Immunology and Hematopoiesis Division, Department of Oncology, Bloomberg-Kimmel Institute, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA
e-mail: fpan1@jhmi.edu

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Abbreviations

AA	adjuvant-induced arthritis
AHR	aryl hydrocarbon receptor
APC	antigen-presenting cell
APL	altered peptide ligand
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
HPV16	human papilloma virus 16
HSF1	heat shock factor 1
MHC	major histocompatibility complex
NK	natural killer
OVA	ovalbumin
PD-1	programmed death 1
RA	rheumatoid arthritis
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TLR	toll-like receptor
Treg	regulatory T-cell

10.1 Introduction

Heat-shock proteins (HSP) are highly conserved set of proteins whose expression is dramatically increased in response to cellular stresses including heat, acidosis, heavy metals, and hypoxia (Craig 1985). This system of proteins exists in organisms ranging from bacteria to mammals with a striking degree of conservation (Lindquist and Craig 1988). HSP were originally identified in the 1960s when accidental temperature changes induced unique chromosomal puffing patterns in *Drosophila* salivary gland cells that correlated with increases in transcription and protein synthesis (Ritossa 1962; Lewis et al. 1975). Over the next several decades, the roles of HSP in homeostasis and disease, including their roles in immunity, were steadily uncovered. The chief role of HSP in the cell is as chaperones: these factors aid in protein folding, translocation, and prevention of aggregation (Wegele et al. 2004). Therefore, the induction of HSP in response to environmental changes and cellular stress can be seen a mechanism for cells to retain normal functions and survive under trying conditions.

A significant amount of research is dedicated to the role of HSP in cancer biology. HSP are disproportionally upregulated in tumor tissues for a variety of reasons including environmental stresses within tumor tissue, aberrant signaling pathways leading directly to the upregulation of HSP, and perhaps most importantly, the need for tumor cells to continue to survive and proliferate despite the accumulation of possibly lethal or proteotoxic mutations (Takayama et al. 2003). HSP chaperone proteins and peptides in tumor cells as they would in other cells that are subject to

stress: however, unlike normal tissue, the HSP repertoire of client proteins in tumor cells includes mutated and abnormally expressed proteins in addition to normal proteins (Ishii et al. 1999). HSP form complexes with these tumor-specific peptides and persist through a variety of processes that include antigen processing and presentation to lymphocytes (Basu et al. 2001; Srivastava et al. 1994). This extraordinary phenomenon has formed the basis for vaccine strategies based on the purification of HSP-peptide complexes from tumor tissue and re-administration to tumor-bearing mice or patients. This therapeutic approach has shown promise in animal models and in early clinical studies, and has garnered a significant amount of excitement in the field of immunotherapy (Udono and Srivastava 1993). Interestingly, HSP are also known to bind receptors on innate immune cells, aid in class I and class II antigen presentation, and stimulate maturation and secretion of cytokines in a peptide-independent manner (Basu et al. 2000).

Heat-shock proteins have also been shown to mediate immune suppression and tolerance during homeostasis. HSP60 and HSP70 have been shown to promote suppressive immune responses, causing the induction of tolerogenic dendritic cells (DCs) and regulatory T-cells in vitro and in vivo. Interestingly, the prophylactic administration of HSP appears to protect from a variety of autoimmune and inflammatory disorders in animal models (Borges et al. 2012). The dual role of HSP in aiding antigen presentation of foreign or tumor-derived antigens; in inducing inflammatory responses in innate immune cells in some instances and tolerogenic innate cell phenotypes in others; and in mediating immunosuppression in vivo highlights the intricacies of HSP and their diverse roles in the immune system.

This chapter briefly introduces the history of heat-shock proteins and physiological roles, as well as their significance in cancer. More comprehensively, this chapter will cover the various effects that HSP have on innate and adaptive immunity, including recent and exciting findings that appear to reconcile or shed light on the seemingly opposing influences that HSP appear to have on the immune system. Finally, we will review the significance of HSP in cancer immunotherapies and the development of HSP-based vaccines from animal testing to human clinical trials.

10.2 Background on Heat Shock Proteins and Homeostatic Function

The heat shock proteins are part of a highly conserved system that is present to a great degree of similarity in the eukaryotic cells of mammals, bacterial organisms, and plants. HSP act as biochemical buffers that maintain the function and structural stability of proteins despite environmental conditions that may compromise normal protein and cellular functions. Such environmental stresses include high temperatures, ultraviolet exposure, harmful metabolites, and hypoxia among others. In the 1960s, an Italian geneticist named Ferrucio Ritossa discovered heat shock proteins. He noticed that incidental exposure of Drosophila salivary gland cells to high temperatures induced unique puffing patterns in the chromosomes of these cells. Upon

further study, these puffing patterns appeared to correspond to heat induced gene expression and protein synthesis. The group of proteins that were enhanced upon temperature increases came to be known as heat shock proteins (Ritossa 1962). Over the next several decades, HSP were discovered to have important chaperoning functions. Molecular chaperones are proteins that assist in the functions of other proteins, including proper folding, translocation, and prevention of aggregation of newly synthesized and assembled protein units (Hendrick and Hartl 1995). Heat shock proteins are a major component of molecular chaperones, and the induction of these proteins upon conditions of high heat and other stressors is thought to be a protective mechanism against aberrant protein folding. They typically act in large complexes consisting of other chaperones, accessory molecules, and substrates, or "client" proteins (Whitesell and Lindquist 2005). HSP typically bind non-covalently to hydrophobic resides of their client proteins.

The HSP can be divided into ten families based on size, amino acid content, and genetic sequence, with higher degrees of similarity between members of the same family than between families; while all HSP share the same nomenclature, there are enormous variations in form and function between the families of HSP. In contrast, HSP within families tend to be more closely related. HSP-assisted protein folding is largely ATP-dependent and proceeds through a variety of mechanisms depending on the family of protein. For example, HSP70 often interacts with newly synthesized proteins. When ATP bound, HSP70 adopts an open conformation, releasing its client peptide into solution for folding. When HSP40, which assists HSP70 in ATP synthesis or hydrolysis, hydrolyses ATP to ADP, HSP70 can bind its client peptide more stably. In contrast, HSP60 (also known as chaperonins, or GroEL in prokaryotes), together with HSP10, forms ring structures that enclose folding proteins to discourage aggregation or interaction with other proteins. Finally, HSP90 assists in downstream maturation and essential conformational changes in signaling molecules (Hartl et al. 2011). Interestingly, many of HSP90 client molecules have been demonstrated to have roles in cell survival, proliferation, and growth, which is important when considering the role of this chaperone in disease (Pratt 1998). The small HSP, including HSP27, are expressed in the cytoplasm and nucleus and are known to stabilize cytoskeletal dynamics. HSP27 is also known to have antioxidant and apoptotic properties. Other than binding a variety of client proteins in the cell and regulating their folding and translocation, HSP can also regulate their own expression: HSP70 contributes to the downregulation of HSF1, the gene responsible for inducing HSP expression (Pockley 2003).

10.3 HSP in Immunity: An Introduction

The connection between HSP and immunity was first established when bacterial HSP were found to elicit specific immune responses both with and without vaccination. In these foundational experiments, purified T-cells from mice that were initially immunized with mycobacteria were re-stimulated with a mix of killed

mycobacteria organisms, mycobacterial antigens, and accessory cells. The investigators of this study subsequently determined that approximately one-fifth of *Mycobacterium tuberculosis* reactive T-cells in this culture recognized an r64-kDa protein that was purified from mycobacterium bovis. This protein was subsequently shown to be HSP60. The same group further observed that human PBMCs exhibited cytotoxic lymphocyte reactivity against fragments of a MB 65 kDa HSP, following isolation and activation of these cells with killed *M. tuberculosis* (Kaufmann et al. 1987). Another group showed that IgG responses were elicited immediately in mice that were exposed to HSP70 of *M. tuberculosis*, indicating that these mice may have antibodies reactive to the HSP70 antigens already existing in their repertoire (Bonorino et al. 1998). As a whole, these early in vitro and in vivo studies seemed to indicate that HSP could elicit strong responses from both mouse and human immune cells, and more specifically, that T-cells in these organisms are reactive to HSP fragments from bacteria in an antigen specific manner.

10.4 HSP in Cancer

The presence of HSP in cancer and their role in cancer progression has held a great deal of interest during the evolution of the heat shock protein field. One of the first HSP families to be implicated in cancer was HSP90. HSP83a was observed in malignant breast tissue, and HSP90 also appeared to be expressed in leukemias. Soon, however, a number of additional HSP families came to be implicated in cancers. HSP27 is expressed in breast, prostate, ovarian, brain, and esophageal cancers (Fuller et al. 1994; Lianos et al. 2015). HSP70 expression has been correlated with cell proliferation rate, malignancy, and poor prognosis in cases of colon, breast, skin, and bladder cancers. The overexpression of HSP in cancers can be attributed to a variety of causes. Firstly, HSP are induced to protect cells against the harmful tumor microenvironment, which features stressors such as hypoxia and nutrient deprivation. Secondly, HSP stretch the limits of cancer cell survival and proliferation despite accumulation of mutations or aberrant signaling pathways that would otherwise be lethal (Whitesell and Lindquist 2005). One method by which HSP sustain cell survival despite external stresses and internal molecular changes is the inhibition of apoptosis. The anti-apoptotic activities of these chaperones are largely dependent on their chaperone function and ability to bind other proteins. For example, HSP70 can inhibit apoptosis through inhibiting the formation of death inducing signaling complex and blocking senescence. In addition, HSP70 and HSP27 prevent mitochondrial release of cytochrome-C, an important component of the apoptosome. HSP27 can also bind cytochrome-C, directly inhibiting apoptosome formation. Finally, HSP70 and HSP90 can bind to Apaf-1 as another way of inhibiting apoptosome formation. In some cases, the number of damaged client proteins can exceed HSP protein capacity and the cell will subsequently be destroyed (Mosser and Morimoto 2004; Mosser et al. 2000; Steel et al. 2004). The requirement of HSP for stressed-cell survival carries into a tumor setting, as inhibition of HSP70

isoforms in breast cancer cell lines causes cell death, while normal breast epithelial cells survive under the same experimental conditions. This is presumably through the loss of HSP-mediated anti-apoptotic function (Nylandsted et al. 2000).

HSP90 is the focus of a great deal of research on heat shock proteins in the context of cancer. Similar to HSP70, this protein has been implicated in inhibition of cancer cell apoptosis. Furthermore, signaling transducers, kinases, and hormone receptors comprise a great deal of HSP90 client proteins. HSP90 binds and assists mature signaling proteins known to be involved in cell proliferation, immortalization, angiogenesis and other characteristics that are often associated with cancers. Accordingly, clients of HSP90 include receptor tyrosine kinases, telomerase, and hypoxia-inducible factor 1-α, all of which are major players in cancer processes (Munster et al. 2002; Holt et al. 1999; Isaacs et al. 2002). Mutations in these important molecules can lead to subsequent abnormalities in signaling and activation of these proteins, which in turn leads to an increased threshold for HSP90 chaperoning function. The effects of HSP on the ability of cells to handle aberrant signaling pathways and mutational burden has caused HSP to be viewed as "biochemical buffers". This buffering phenomenon causes cancer cells to appear "wild-type" despite having an abnormal molecular makeup. Because HSP90 seems to support aberrant signaling pathways in cancer cells and maintain survival of these cells despite accumulation of intracellular instability and extracellular environmental stresses, this chaperone appears to be a promising target for cancer therapy. Because HSP are seen as biochemical buffers, however, careful consideration must be given to the potential of HSP inhibition to reveal mutations that would otherwise escape evolutionary pressure and selection. This pressure could enhance the progression of disease by causing certain mutations to be selected based on survival or proliferation (Whitesell and Lindquist 2005).

10.5 HSP in Induction of Immune Response

Very early on, the logical conclusion that could be made from the overexpression of HSP in cancer tissue, and the ability of these proteins to chaperone and carry tissue-specific peptides, was that T-cells may have the capacity to recognize tumor derived HSP-peptide complexes in an antigen-specific manner. Furthermore, it was thought that vaccination with these complexes could induce anti-tumor immunity. HSP-peptide complexes sourced from tumors were essential for protection from challenge with the same tumor type, lending support to the hypothesis that tumor-derived HSP-peptide complexes could bolster anti-tumor immunity (Udono and Srivastava 1993). When such complexes were isolated from normal tissue or tumors that were mismatched with the challenge tumor, no tumor protection was seen (Udono et al. 1994; Srivastava et al. 1986; Ullrich et al. 1986). These data formed a foundation for the hypothesis that HSP-peptide complexes could be used as vaccines to enhance anti-tumor immunity.

In these experiments, HSP-peptide complexes that were protective against tumors in mice included gp96, HSP90, and HSP70 (Udono and Srivastava 1993; Udono et al. 1994; Tamura et al. 1997; Yedavelli et al. 1999; Kovalchin et al. 2001; Sato et al. 2001). However, it was unclear which portion of these complexes was immunogenic or antigenic: was it the peptide that was non-covalently bound to the HSP, the HSP itself, or some motif or characteristic that was unique to the complex of the two? Interestingly, one group showed that no alterations could be seen in the DNA of the HSP used to mount tumor protection (Srivastava et al. 1998). Furthermore, HSP70 preparations that were isolated from tumor tissue and purified so as to remove associated peptides did not protect mice from subsequent tumor challenge (Udono and Srivastava 1993; Li and Srivastava 1993). These data undermine the hypothesis that HSP themselves are antigenic, and instead suggest that complexes of HSP with peptides are more likely to be responsible for tumor protection. In fact, both the HSP and peptide components of an HSP-peptide-complex are needed for immunogenicity. In 1997, Blachere et al. executed a powerful in vitro study in which gp96 and HSP70 were stripped of endogenous peptides and loaded with a panel of seven synthetic peptides. Upon exposure to T-cells in vitro, these authors showed that complexes of HSP and peptide could induce antigen-specific major histocompatibility complex (MHC) class-I-restricted CD8 T-cell responses, while either the peptides or HSP alone could not induce an immune response (Blachere et al. 1997). Importantly, this study showed that an MHC-class-I restricted response could be induced from an exogenous peptide, indicating that these HSP-peptide-complexes could be capable of cross presentation and induction of antigen specific CD8 T-cell response. Another finding was that mouse serum albumin loaded with peptides could not induce an immune response, pointing to the likelihood that HSP themselves have an immunogenic quality other than their ability to bind and chaperone peptides (Srivastava 2002). To confirm that HSP-peptide complexes could induce a robust antigen-specific CD8+ T-cell-mediated immune response in vivo, in keeping with the in vitro findings of Blachere et al., Suzue et al. fused mycobacterial HSP70 and a fragment of the ovalbumin (OVA) gene. The authors showed that immunization of mice with this fusion protein prior to challenge with an OVA-expressing tumor could induce an OVA-specific CD8+ T-cell response and induce tumor rejection (Suzue et al. 1997). As a whole, these studies suggest that HSP-peptide complexes are protective against tumors in mice, that this protection is due to immunogenicity of both the HSP and peptide components, and that HSP-peptide complexes can enter into the cross presentation pathway to induce antigen specific MHC class I-restricted CD8+ T-cell responses in vitro and in vivo.

In the 1980s and 1990s, as illustrated above, the bulk of the work to characterize the in vivo immune response to HSP-peptide complexes had been done using tumor models. Several groups, however, have recently harnessed this technique to target virus-infected cells. Human papilloma virus 16 (HPV16) is highly associated with the appearance of high-grade cervical intraepithelial neoplasia and cervical cancer (Olsen et al. 1995; Bosch et al. 1995). E7 is an early viral protein of HPV16 that is expressed all the way from viral integration into the cell, to virus-induced transformation of the cell. Because E7 is a foreign, antigenic protein, it is of interest to

develop vaccines or immunotherapeutic strategies to raise an immune response against cells expressing this protein. Due to aforementioned reports that fusion proteins incorporating portions of mycobacterial HSP sequences could elicit powerful immune responses, several groups, namely those led by T.C. Wu, have constructed recombinant proteins incorporating mycobacterial HSP65 sequences and E7 (HSP65-E7). HSP65-E7 was tested in the TC-1 murine tumor model for its efficacy as a therapeutic vaccine. In these experiments, therapeutic administration of HSP-E7 caused tumor regression and prolonged survival compared to the E7 protein alone or PBS controls. This effect was deemed CD8+ T-cell dependent, as administration of a CD8+ T-cell depleting antibody abrogated the efficacy of the HSP-E7 vaccine (Chu et al. 2000a, b). Another study by the same authors showed that prophylactic immunization with HSP-E7 protected mice against challenge with the TC-1 tumor cell line, and that these tumor-free animals are also resistant to challenge again with TC-1 (Chu et al. 2000a, b). Thus, strategies to use HSP-based vaccines in treatment of virally induced cancers have been successful in animal models and show promise for development as therapies.

Most recently, several groups have thought to incorporate HSP-peptide complexes or fusion proteins into autologous dendritic cells to enhance the immunogenicity of these as vaccines. One such strategy involved the construction of a fusion protein incorporating sequences from the mycobacterial HSP70 and the chloride intracellular channel 1 (CLIC1), which is a highly expressed but poorly immunogenic protein in ovarian cancer. Yu et al. pulsed human cortical blood-derived dendritic cells with this fusion protein, and resulting inflammatory and anti-tumor effects were observed in NOG mice. MT-HSP70-CLIC1 appeared to promote dendritic cell maturation, as measured by upregulation of CD40, CD80, and CD86, as well as pro-inflammatory cytokine production in vitro. Furthermore, these protein-pulsed dendritic cells seemed to promote an anti-tumor T-cell response in vivo, and this therapy significantly inhibited tumor burden compared with MT-HSP70 alone, CLIC1 alone, or the PBS control (Yu et al. 2017).

We have recently found that endogenous HSP can drive pro-inflammatory immune responses by destabilizing regulatory T-cells (Tregs). Tregs are a suppressive cell type that plays a key role in curbing excessive immune responses and sterilizing immune responses against cancers. These cells, which are characterized by the transcription factor Foxp3+, carry out their function by secreting soluble suppressive cytokines such as IL-10, TGF-b, and sequestering the pro-inflammatory cytokine IL-2 from other T-cells. The transcription factor Foxp3 is essential for the differentiation and function of regulatory T-cells (Sakaguchi et al. 2008). Interestingly, Treg cells are plastic and can adopt effector T-cell phenotype and function under inflammatory or other destabilizing conditions (Rubtsov et al. 2010). We have found that, in response to inflammatory stimuli, HSP70 mediates Foxp3 degradation via an E3 ubiquitin ligase called Stub1. This finding originated from the observation that Foxp3 is destabilized during exposure to pro-inflammatory cytokines or stress stimuli that included IL-1B, TNFa, heat shock, or lipopolysaccharide. This degradation of Foxp3 appeared to coincide with an upregulation of Stub1. Furthermore, HSP70, a known chaperone for recruiting E3 ligases to proteins

targeted for degradation, was found to be part of the Foxp3 complex under these conditions. Through further experimentation, we showed that Foxp3-Stub1 interaction leads to degradation of Foxp3 through the proteasome, and that knockdown of HSP70 mitigated this degradation process. Thus, the overall finding of this paper is that that endogenous HSP70 and Stub1 are both necessary for destabilization of Foxp3+ Treg cells in the setting of inflammation. These experiments suggest that not only are exogenous HSP-peptide complexes and fusion proteins able to induce immune responses, but endogenous HSP also seem to have a role in promoting inflammation (Chen et al. 2013). In sum, HSP-peptide complexes, fusion proteins, and even endogenous HSP appear to have potent effects on immune responses in vitro and in vivo, often strongly supporting anti-tumor immunity. These therapies can be administered and designed in a variety of ways, ranging from carefully constructed fusion proteins directed to one antigen, all the way to autologous HSP preparations from tumor tissue that incorporate an entire fingerprint of HSP client antigens. HSP-peptide complexes are the most clinically advanced version of this therapy, as will be explored in the next section; however, many variations on these therapies are being explored in recent years.

10.6 Effect of HSP on Innate Immune Responses

There is a great deal of research centering on the effects of heat shock proteins on innate immune cells, the receptors they bind, and the conditions in which they affect innate cells. A major consideration for ascertaining the impact of HSP on innate cells is the intracellular or extracellular localization of HSP, as this affects how cells of the innate immune system perceive these molecules. HSP are generally thought of as cytosolic, nuclear, or mitochondrial proteins that are sequestered inside of the cell except in the case of necrosis and subsequent release of HSP into the extracellular environment. This release of HSP from inside the cell is thought to constitute a danger signal and attract pro-inflammatory immune responses (Pockley 2003). In keeping with this hypothesis, human HSP60 has been shown to induce the expression of pro-inflammatory adhesion molecules such as intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 on vascular endothelial cells. Endothelial cells, smooth muscle cells, and macrophages were also shown to produce IL-6 in response to free HSP60 (Kol et al. 1999, 2000). These data substantiate the hypothesis that non-physiologic presence of HSP, perhaps as a result of cell damage or injury, can act as a danger signal and cause a local pro-inflammatory response.

On the other hand, HSP can be released from several types of viable cells, including cultured rat embryo cells, a human neuroblastoma cell line, and vascular smooth muscle cells, in a manner that is independent of necrosis and cellular damage (Pockley 2003). Hightower et al. stimulated rat embryo cells with a short heat shock and observed the rapid release of several HSP including HSP110, HSP73, and HSP71. Addition of monensin or colchicine, inhibitors of the common secretory

pathway of proteins, did not reduce HSP secretion, indicating that the release of HSP from viable cells must depend on a distinct, unique secretory pathway (Hightower and Guidon 1989). HSP can be released from cells as free protein, in association with an exosome or endosome, or as part of cholesterol rich microdomains (Gastpar et al. 2005; Mambula and Calderwood 2006; Broquet et al. 2003). Breuninger et al. recently developed an enzyme-linked immunosorbent assay targeted at detecting liposomal HSP70 in human serum. Since much more liposomal HSP70 was detected with this assay compared to detection of free non-lipid-associated HSP70, this assay suggested that the majority of extracellular HSP70 in the human associates with a lipid membrane (Breuninger et al. 2014). Hightower and colleagues propose that this rapid HSP secretion mechanism may serve as a homeostatic mechanism to mount a heat shock response to support surrounding cells that are unable to do so. This group also observed that the presence of nonionic detergents did not lead to more HSP70 release, indicating that cellular damage is not the basis for HSP release (Hightower and Guidon 1989; Pockley 2003). The data surrounding the intracellular or extracellular localization of HSP, and factors contributing to HSP release from cells is worth consideration, as these conditions will undoubtedly influence the physiologic impact of HSP on innate and adaptive immunity.

A number of groups have supported the hypothesis that HSP can induce activation and maturation of antigen presenting cells (APCs) and other innate cells. This is in line with the notion that HSP can act as danger signals or "danger associated molecular patterns" for tissue injury and a need for an inflammatory response. In the early 2000s, many groups observed that inflammatory cytokines such as IL-1b, IL-6, TNFa were induced in monocytes that were exposed to human HSP60, and that these effects were downstream of HSP60 binding to CD14 and Toll-like receptor 4 (TLR4). These signaling networks are notably also utilized by lipopolysaccharide, with the same effects on monocyte cytokine induction (Ohashi et al. 2000; Kol et al. 2000; Hoshino et al. 1999; Asea et al. 2002). However, major concerns emerged regarding the possibility of LPS contamination of HSP isolated from *E. coli*, and strong signaling of LPS through TLR4 and CD14 pathways among other signaling pathways commonly used by LPS, which would likely overwhelm the effects of HSP on innate cells (Gao and Tsan 2004). Several studies have upheld the findings that HSP can activate antigen presenting cells, being careful to remove endotoxin contamination from their HSP preparations, showing that HSP interaction with antigen-presenting cells including macrophages and dendritic cells lead to markedly pro-inflammatory and adjuvant-like effects. These experiments showed that cytokines such as TNFa, IL-1b, IL-12, and granulocyte-macrophage colony-stimulating factor were induced by macrophages upon exposure to HSP90, HSP70, and gp96. Furthermore, exposure of DC to these HSP induced expression of co-stimulatory and antigen-presentation molecules, effects that are indicative of DC maturation. Finally, activation of APCs by HSP corresponded with nuclear translocation of NF-kB with a kinetic signature that was distinct from LPS-induced APC activation (Basu et al. 2000). Despite the abundance of these data, some groups have demonstrated that removal of endotoxin contamination and exposure of innate

immune cells to HSP70 seemed to promote immunosuppressive and tolerogenic phenotypes in monocytes and dendritic cells (Borges et al. 2012). These results are discussed later in the chapter.

The effects of HSP on natural killer (NK) cells have also been studied in vitro and in the context of tumors. This connection was based on initial observations from Multhoff et al. that lysis of tumor cells that express HSP70 is mediated by NK cells. This group observed that HSP72 was expressed on the cell membrane of about 60% of human colon carcinoma cells under physiologic conditions. By separating HSP72 low and high populations and quantifying sensitivity to NK lysis in vitro, Multhoff et al. showed that high HSP72 expression correlates with sensitivity to NK lysis. Furthermore, when antibodies were used to block HSP72, the steepest reduction in NK-induced lysis was in the HSP72-high tumor cells (Multhoff et al. 1997). The same group also investigated the direct impact of other HSP70 family members on NK cells. This group noticed that rHSP70 an HSP70 homolog, called DnaK, could induce proliferation of NK and T-cells. In addition, IFNy was robustly upregulated in response to low levels of IL-2 and recombinant HSP70 (Multhoff et al. 1999). It is also of interest to consider the ligands or signals that are recognized by these tumor-reactive NK cells, and whether these cells can recognize and target the HSP molecules in the context of tumor cell killing. Recent papers have indicated that NK cells can attack tumor cells that express HSP70 at the cell plasma membrane. Interestingly, HSP70-activated NK cells have also been shown to recognize and kill tumor cells that express stress-induced NKG2D ligands at the cell surface (Dressel 2017). Finally, several groups have shown that NK cells are activated in cancer patients following treatment with HSP-peptide complexes, and that HSP70 and NK cell infiltration are prognostic indicators for post-radiochemotherapy patients with head and neck squamous cell carcinoma (Pilla et al. 2005; Stangl et al. 2017). The data above seem to suggest that HSP have adjuvant-like and pro-inflammatory effects on a variety of innate cells including macrophages, dendritic cells, and NK cells. If these findings hold true in vivo, these would have major implications in the ability of HSP to influence the immune response and effectiveness of HSP-based vaccine strategies.

The way in which innate immune cells can take up extracellular HSP and exogenous HSP-peptide-complexes has been of great interest. The first receptors proposed to bind extracellular HSP were TLR2 and TLR4 (Asea et al. 2002). However, this finding was disputed due to the aforementioned possible contamination of HSP preparations with endotoxin. Many studies have implicated CD91 and Lox-1, scavenger receptors that are expressed on APCs, as capable of binding and internalizing HSP-peptide complexes and facilitating subsequent antigen processing and presentation of these complexes. Basu et al. observed that upon exposure of HSP-peptide complexes to a macrophage cell line, RAW264.7, peptides chaperoned by HSP90, HSP70, and CRT are re-presented by MHC class I molecules. Introduction of anti-CD91 antibody blocked this re-presentation process, indicating that CD91 might be a shared receptor for HSP-peptide complexes and necessary for peptide re-presentation on MHC class I molecules (Basu et al. 2001). On the other hand, when Theriault et al. investigated the interactions between extracellular mammalian

HSP70 and Chinese hamster ovary cells expressing cDNA encoding the receptors TLR2, TLR4, CD40, CD91, LOX-1, SREC-1, and FEEL-1, it seemed that HSP70 could be internalized via binding with SREC-1, FEEL-1, and LOX-1, but not CD91 (Theriault et al. 2006).

A great deal of recent work has been done by Robert Binder's group to demonstrate that HSP and their associated peptides can engage CD91 and induce phenotypically differing T-cell responses depending on the type of HSP that CD91 engages (Pawaria and Binder 2011). In addition, the CD91 receptor appears to be essential for HSP-peptide-mediated anti-tumor immune responses, as the genetic knockout of this receptor in mice or inhibition of the HSP-CD91 signaling axis with endogenous receptor associated protein attenuates anti-tumor immune responses (Zhou et al. 2014). In general, there appear to be more than one type of receptor that binds HSP and subsequently internalizes these proteins. The choice of receptor may depend in part on the form and status of the HSP, including the source of the HSP used in these experiments and whether the HSP is bound to peptides, other HSP, or ATP (Borges et al. 2012). This complexity sheds a little bit of light on the context-dependency of the effects of HSP on immunity, a theme that continues to emerge in past and current HSP research.

10.7 Role of HSP in Antigen Processing and Presentation

In order to understand the immunologic impact of HSP and HSP-peptide complexes, it is important to understand whether HSP play a role in antigen processing and presentation, and if so, how HSP interact with the machinery involved in these processes. Initially, there were several possibilities proposed for the mechanism of HSP-peptide antigen presentation. One possibility was that altered or mutated tumor HSP themselves could act as tumor antigens and be presented in the context of MHC-I or MHC-II. However, the data above strongly suggests that HSP themselves are not the reason that HSP-peptide complexes produce strong immune responses. A second was that HSP could act as chaperones to aid in presentation of tumor-specific peptides, that is, mutated and overexpressed proteins that are aberrantly generated by the tumor (Fuller et al. 1994). Finally, some groups attested that HSP could travel to the cell surface and present peptides themselves in a manner similar to MHC proteins. These latter two hypotheses were based on the findings that preparations of HSP isolated from tumor tissue were shown to be in complex with tumor-specific peptides, and that HSP70 was shown to possess a peptide binding groove similar to the MHC binding grooves (Rippmann et al. 1991).

A number of investigators have pursued the question of whether HSP-peptide complexes are presented on MHC-I or MHC-II and how such complexes interact with each of these pathways, endogenously or exogenously. The experiments listed above show that exogenous HSP in complex with peptides can induce antigen-specific CD8+ T-cell responses through cross presentation to MHC class I. Udono et al. carried out a great deal of work to support the hypothesis that HSP participate

in MHC class I presentation, and characterized biochemical associations between HSP and MHC class I-restricted peptides at various stages of their processing inside of the cell. This group showed that HSP90 and gp96 associate with MHC class I-restricted mouse leukemia tumor peptides, not only with the peptides' final, truncated form that is presented to cytotoxic lymphocytes in the context of MHC class I, but also with precursors of these peptides that are present in the cytosol and endoplasmic reticulum (Ishii et al. 1999). These findings strongly support a physical interaction of HSP with MHC class I restricted tumor-specific antigens and sustained association of HSP with peptides during antigen processing steps.

To confirm the importance of HSP for MHC class I antigen presentation in the context of cancer cells, Malkovsky et al. transfected a plasmid expressing HSP70 into a B16 melanoma cell line and monitored antigen presentation via MHC class I. Where baseline levels of antigen presentation were very low in B16 melanoma cancer cells, HSP70 transfection seemed to robustly up-regulate the surface expression of folded MHC class I molecules and concomitant antigen presentation. These experiments seemed to attest that HSP, specifically HSP70, aid in MHC class I antigen presentation in cancer cells (Wells et al. 1998). It is very likely that endogenous HSP, due to their ability to bind to a large variety of intracellular proteins and peptides, aid in antigen presentation by MHC class I molecules, presumably by helping to transfer peptides to these molecules. Based on the notion that HSP can chaperone proteins through the ubiquitin-proteasome protein degradation system, one group has suggested that HSP chaperoning of degraded proteins and peptides is conducive to transfer of these peptides onto MHC class I molecules, and subsequent antigen presentation (Srivastava et al. 1994). This group suggested that a complex of heat shock proteins and antigen processing and presentation machinery could exist, which includes HSP70, HSP90, and HSP110, MHC class I, tapasin, ERP57, calnexin, calreticulin, transporter associated with antigen processing, and gp96. This complex, known as the "presentosome," forms the basis for HSP transfer of peptides to MHC class I molecules (Cresswell et al. 1999; Srivastava 2002).

MHC class I-restricted presentation of HSP-associated peptides is important for antigen-specific immunity in vivo to eradicate infections or cancer. However, this process can only partially support a full-fledged immune response, and a CD8+ T-cell response would likely require help and pro-inflammatory cytokine production from CD4+ T-cells. In 1992, DeNagel and Pierce postulated that a novel HSP70 family member 72/74 kDA chaperone called PBP72/74 could act to scavenge processed antigens and concentrate them at sites of MHC class II-peptide assembly. Supporting the role for this protein in antigen processing, PBP72/74 also appears to associate with fragments of antigen (DeNagel and Pierce 1992). However, at this point, it was still unclear what role this chaperone had, if any, in directly facilitating processing and MHC class II-peptide binding. However, a few years later it became clear that inhibiting HSP70 with a small molecule could reduce antigen presentation by MHC class II (Hoeger et al. 1994). These experiments were largely focused on endogenous HSP and their role in facilitating antigen processing and presentation. Because of the promise of HSP-peptide complexes in bolstering antigen-specific immunity, several groups were also interested in characterizing the way in which

these complexes could be internalized by antigen presenting cells and incorporated into the MHC class II pathway, ultimately inducing an antigen-specific CD4+ T-cell activation and response. In 2014, Murshid et al. showed that HSP90-peptide complexes could bind to SREC-1, a scavenger receptor expressed on antigen presenting cells, become internalized, and enter into the MHC class II pathway. Despite that SREC-1 appeared necessary for MHC class II restricted presentation of these HSP90 associated peptides, it was unclear what mechanistic role SREC-1 played in helping peptides load onto MHC molecules. The authors noticed that SREC-1 co-localized with MHC II on the membrane of these cells, and hypothesized that internalization by SREC-1 helped bring exogenous peptides in close proximity to MHC class II molecules and machinery required for MHC loading (Murshid et al. 2014). MHC class II presentation of HSP-derived peptides appeared to be important in vivo as well, as immunization of mice with gp96-peptide complexes induced proliferation of naïve CD4+ T-cells (Doody et al. 2004).

10.8 Development of HSP-Based Vaccines Through Clinical Testing

The concept of immune therapy for clinical treatment of cancer has been pursued in the forms of cytokine administration, cancer vaccines, and immune-modulating antibodies. Therapeutic vaccines consisting of autologous lysates, peptides, or dendritic cells pulsed with peptides, sometimes administered in combination with cytokines such as IL-2, have not had a great deal of success. This is likely due to immune suppression, immune evasion, and/or CD8+ T-cell exhaustion in the tumor microenvironment (Frankel et al. 2017; Zou 2005). One of the most successful immune therapies to date has been checkpoint blockade, which binds to and blocks inhibitory receptors on the antigen presenting cell in the case of anti-CTLA4 and the tumor cell in the case of anti-PD-1 (Pardoll 2012). Advanced melanoma is a devastating but markedly immunogenic cancer in humans, as specimens are often infiltrated with CD8+ T-cells; thus it is unsurprising that metastatic melanoma was one of the first indications for which checkpoint blockade was approved. However, these therapies have only been successful in a small percentage of patients, and alternative or complementary strategies are needed to produce an anti-tumor immune response in these patients (Topalian et al. 2014).

Based on findings that HSP-peptide complexes isolated from mouse tumors could protect against challenge with the same tumor, it was predicted that these complexes could be used in humans to provoke an anti-tumor immune response. The advantage to these vaccines is that HSP isolated from the patient's tumor tissue will include a wide-ranging fingerprint of various tumor-associated peptides that are representative of the patient tumor. This circumvents the need to find a particular immunogenic peptide of interest to form the basis of the vaccine strategy (Srivastava 2002). One of the first preclinical experiments to test whether HSP-peptide

complexes were effective as cancer therapeutics involved treatment of D122 tumor-bearing mice with D122-derived gp96, liver-derived gp96, or PBS. D122 is a non-immunogenic lung carcinoma model that metastasizes naturally to the lungs following subcutaneous injection. The authors injected 100,000 tumor cells in the footpads of each mouse, and allowed 11 days to pass before administering the first treatment with D122-gp96. Treatments were administered five times per week at a dose of 20 μg each. The authors observed that tumor burden substantially decreased upon treatment with D122-derived gp96 compared to the liver-derived control group and PBS group. This reduction in tumor volume seemed to depend on CD4+, CD8+, or NK cells, as the effect of the tumor-derived vaccine was eliminated when these cell types were systematically depleted (Tamura et al. 1997).

HSP peptide complexes have been tested for their ability to provide clinical benefit to patients with malignancies. Some of the earliest efficacy data emerged from a Phase I clinical pilot study that was run by Janetzki et al. in 2000. This study showed that administration of 25 ug of autologous tumor tissue-derived gp96 to 16 patients with a variety of advanced, treatment-refractory cancers resulted in 4 cases of disease stabilization. Notably, ELISPOT of IFNy on bead-isolated CD8+ T-cells from peripheral blood mononuclear cells showed that 6/12 cases displayed CD8+ T-cell immune responses. Furthermore, no serious toxicities were identified during the course of therapy and events such as pain and fever during treatment course were attributed to tumor progression (Janetzki et al. 2000). This initial study gave way to larger and more involved clinical trials to further test the efficacy of HSP peptide complexes in cancer patients. Many of these trials were in the context of advanced metastatic melanoma, given the severity of disease at this stage as well as the likelihood that an immune response might arise in these patients. In one Phase I clinical trial, 36 stage III/IV melanoma patients were administered the gp96-peptide vaccine at various doses for 4 weeks. For stage III and IV patients, the median overall survival was 31 months and 15.9 months, respectively. While ELISPOT cytokine assays did not display significant and lasting IFNy induction, 4/11 stage III patients showed no disease progression in a follow up 10 years after the study, and 9/11 stage IV patients treated with vaccine and adjuvant were alive at the 10-year follow up time point. Furthermore, no major toxicities were observed (Eton et al. 2010). A Phase II trial was later run which tested the effects of 5 ug or 50 ug gp96-peptide vaccine on 64 patients with metastatic melanoma. Vaccine administration resulted in two complete responses and three disease stabilizations in patients that had residual disease after surgery. This trial also showed some promising mechanistic data: around 48% of patients showed elevated antigen-specific T-cell counts after receiving vaccination. These immunologic responses seemed to correlate well with clinical outcome, as six out of seven patients with a clinical response also exhibited a T-cell response. By contrast, out of 16 patients without a clinical response, only 5 exhibited a T-cell response (Belli et al. 2002). These preclinical and clinical studies substantiate that autologous HSP peptide complexes are not only effective in protecting mice against tumors, but can also be conceived as a therapeutic vaccine that can eradicate existing tumors, produce significant clinical responses in some patients, and bolster anti-tumor immunity in mice and humans. It may be possible

that these therapeutic moieties may be even more effective when combined with modern, clinically validated immunotherapies such as checkpoint blockade or GVAX; however, to our knowledge, these data have not been published.

HSP-peptide complexes have been explored for a variety of cancer indications. Glioblastoma is a highly aggressive brain cancer that has been refractory to many therapies, including immunotherapy. In glioblastoma, HSP27, HSP72, HSP73, and HSP90 are constitutively elevated. In a phase I study, HSPP96 was investigated as a vaccination regimen in patients with high-grade glioma. Strikingly, this vaccination elicited a tumor specific peripheral immune response in 11 out of 12 patients. Ex vivo analysis of PBLs showed enhanced T-cell proliferation and production of IFNy upon re-stimulation with autologous HSP. Responders to the vaccine had a median OS of 47 weeks, while non-responders had a median OS of 16 weeks. This therapeutic strategy has also had success in later phase II trials compared to historical controls. Challenges, however, include the acquisition of sufficient brain tumor tissue for processing into a vaccine (Ampie et al. 2015).

10.9 HSP in Tolerance Induction and Protection from Autoimmunity

Based on early knowledge that the mammalian immune system could mount specific responses to bacterial HSP, coupled with the notion that bacterial and human HSP share a high degree of amino-acid similarity, it was predicted that bacterial HSP could provoke autoimmunity in humans (Borges et al. 2012). In this scenario, a microbial infection would initially provoke cellular and humoral immunity against bacterial HSP, and this immune response could ultimately become redirected against highly homologous human HSP (Schultz and Arnold 1993). However, in vivo studies showed that immunization of Lewis rats with DNA vaccines containing human HSP70 and HSP90 actually protected the animals against adjuvant-induced arthritis (Quintana et al. 2004). Another study showed that immunization with whole mycobacterial HSP65 protected rats against experimental arthritis, including adjuvant-induced arthritis. The authors of this study also observed that patients with a mild, remitting form of juvenile idiopathic arthritis possess a proliferative T-cell response to self-HSP60, and the patients with systemic or poly-articular disease did not. The phenotype of these T-cells was hypothesized to be tolerogenic and suppressive, as these cells were seen to express the Th2 marker CD30 and produce IL-10 (Vercoulen et al. 2009). These studies show a clear protective effect conferred by HSP in the context of autoimmunity, and have casted doubt upon the hypothesis that the presence of HSP may provoke autoimmunity due to homology. These early preclinical and clinical observations set forth above convincingly show that bacterial and human HSP of various families could protect against in vivo challenge with inflammation or autoimmunity, and that tolerogenic HSP-specific T-cell responses could be seen in patients with mild (as opposed to pathological) forms of arthritis.

Newer experiments have not only confirmed these observations using both human and bacterial HSP, but have also done a great deal to reveal mechanisms by which HSP can drive immune suppression. A major consideration regarding the mechanism by which HSP can induce tolerance is whether the effects of HSP on cells of the innate immune system could be characterized as pro-inflammatory or anti-inflammatory. In contrast to the experiments set forth above, where exposure to a variety of HSP seemed to induce pro-inflammatory cytokine production in monocytes and macrophages, and induce maturation of dendritic cells, several groups reported the opposite: that exposure to endotoxin-free HSP induced IL-10 production in peripheral blood mononuclear cells (PBMCs) from patients with and without arthritis (Detanico et al. 2004).

Furthermore, when bone marrow dendritic cells were exposed to endotoxin-free tuberculosis HSP70 for 24–48 h, these dendritic cells failed to mature and failed to induce MHC class II molecules and CD86 compared to controls. Furthermore these HSP70-exposed cells substantially increased IL-10 production (Motta et al. 2007). Human HSP70 could also skew monocyte derived dendritic cells towards a regulatory phenotype. Interestingly, to investigate the contribution of contaminating endotoxin on these in vitro results, this group made use of HSP preparations with high, medium, and low endotoxin levels. While the high and medium endotoxin groups induced maturation of monocyte-derived dendritic cells, the low endotoxin group reduced maturation and functional ability of these antigen-presenting cells to stimulate responder T-cells in vitro (Stocki et al. 2012). This work, which shows that dendritic cells can be made more tolerogenic by exposure to HSP70, is in sharp contrast to the aforementioned experiments done by Basu et al. in 2000, where exposure to HSP70 seemed to cause DC maturation and secretion of pro-inflammatory cytokines. The work of Stocki et al. and Motta et al. run contrary to previous experiments that suggested that HSP have a definitively pro-inflammatory effect on antigen-presenting cells and other cells of the innate immune system. Even more strikingly, the Stocki et al. paper quantitatively relates these observations of HSP-induced pro-inflammation to endotoxin contamination. It is possible that these discrepancies are due to factors such as differences in HSP preparations, which would influence the proteins that are in complex with these chaperones during in vitro studies (Borges et al. 2012).

On a mechanistic level, it was also of major interest to identify mechanisms that mediated increases in anti-inflammatory cytokines and decreases in pro-inflammatory cytokines from innate cells exposed to HSP. In particular, one group identified that NF-KB, a transcription factor that activates the transcription of several pro-inflammatory cytokines, can be inhibited by HSP70 in astroglial cells (Feinstein et al. 1996). Furthermore, heat shock factor 1 (HSF1), which is the major transcription factor for heat shock proteins, can directly inhibit TNFa gene expression in antigen presenting cells including monocytes, macrophages and dendritic cells (Ferat-Osorio et al. 2014). Interestingly, another group found that the presence of pro-inflammatory cytokines such as TNFa, IL-1a, and IL-6 induces HSF1, which induces HSP70 expression (Schett et al. 1998). This latter experiment was done in synovial fibroblasts, which are important for the pathogenesis and inflammation of

rheumatoid arthritis. While these experiments were not done in the same system, one can imagine a negative feedback mechanism wherein an initial inflammatory response to invasion or damage can directly induce HSF1 and HSP70. HSF1 and HSP70 may then directly inhibit pro-inflammatory cytokine expression, possibly acting to bring inflammation back to homeostatic levels. Finally, another group recently showed that inhibiting HSP90 could activate the transcription of interferon response genes, indicating that HSP90 may directly or indirectly suppress the transcription of these genes (Mbofung et al. 2017). The experiments and implications involved in this finding will be described later in the chapter.

Given that HSP appear to have protective effects when administered in vivo in advance of challenge with adjuvant-induced arthritis or acute allograft, and findings that endotoxin-free HSP can induce a suppressive phenotype in innate cells in vitro, it is of major interest to decipher the downstream process by which HSP can exert immune suppression in vivo. A large number of these studies have centered on the role for IL-10 and Tregs in enforcing immune suppression. As mentioned above, IL-10 seems to be a key cytokine that is induced in macrophages or dendritic cells upon exposure to HSP. IL-10 is a well-known suppressor of T-cell responses, and is secreted by a number of cell types, including suppressive dendritic cells, macrophages and regulatory T-cells. This cytokine constitutes a mechanism for dampening overactive immunity. In fact, the secretion of IL-10 by Treg cells appears to be an important mechanism by which Tregs exert immune suppression (Couper et al. 2008; Wang et al. 2016). IL-10 can also maintain Foxp3 expression in Tregs and support the function of these cells (Wang et al. 2016). Tolerogenic DCs, which are characterized by low expression of MHC Class II and co-stimulatory molecules and secretion of anti-inflammatory cytokines including IL-10, are known to affect immune microenvironments by facilitating the generation of peripheral Treg cells (Steinman et al. 2003; Rutella et al. 2006; Thomson and Morelli 2007). Thus it was predicted that HSP could induce a suppressive immune microenvironment in vivo by inducing a tolerogenic phenotype in dendritic cells, which would subsequently augment Tregs in the microenvironment (Borges et al. 2012).

Borges et al. tested the idea that Tregs are important mediators of HSP-induced immune suppression by incubating C57BL6 tumor cells and skin sections with endotoxin-free TB-HSP70 and then grafting onto a BALB/c host. This skin graft was tolerated for about 7–10 days longer than controls that had been immersed in PBS, and tumors successfully grew. This experiment showed that HSP70-conferred immune suppression could be mounted in the context of tumors and allografts. To ascertain the mechanism of this effect, the authors showed that depletion of Tregs with an anti-CD25 monoclonal antibody markedly reduced immune suppression and abolished the survival benefit that was seen in mice that were grafted with skin sections previously incubated with TB-HSP70. In this paper, the authors also showed that local injection of TB-HSP70 induced Treg in draining lymph node, which correlated with induction of IL-10 (Borges et al. 2010). In sum, these set of experiments confirmed that HSP70 induces a suppressive microenvironment that strongly manifests in the context of tumor and allograft. That this suppressive microenvironment is abolished when Tregs are depleted indicates that Tregs are

necessary for the suppression that allows graft survival. Having seen that HSP exposure often induces IL-10 in vitro in innate immune cells and in vivo in the context of tumor or allograft, it was of interest to many groups to investigate the cell signaling mechanism by which IL-10 is produced in innate immune cells exposed to HSP. ERK activation was considered as a possible signaling pathway leading to HSP-mediated IL-10 induction, as activation of ERK has been shown to be upstream of IL-10 production in diverse immune cells including DCs. Furthermore, TLR2 seemed to promote ERK signaling and downstream IL-10 production in these innate immune cells (O'Garra and Saraiva 2010; Yi et al. 2002; Dillon et al. 2004; Kaiser et al. 2009). These experiments point to a possible mechanism by which HSP induces a tolerogenic phenotype in innate immune cells. In addition to the model proposed where HSP cause IL-10 induction by suppressive dendritic cells, which then recruit Tregs and reinforce their function, it is also possible that HSP directly modulate Treg. One group clearly demonstrated that HSP60 enhances Treg suppressive function and causes upregulation of IL-10 and TGF-b secretion by these cells. These effects were dependent on TLR2, as introduction of a neutralizing antibody against TLR2 abrogated the enhancing effects of HSP60 on Treg suppressive function. Interestingly, use of p277, a peptide derived from HSP60 that was found to decrease progression of type 1 diabetes in mice and humans, recapitulated the effects that HSP60 had on regulatory T-cell function (Zanin-Zhorov et al. 2006).

For many reasons, the physiological basis for HSP-induced tolerance seems unclear and unintuitive. HSP are induced in response to stress, inflammation, and damage, so exogenous and endogenous HSP are often thought to be conducive to pro-inflammatory responses. Furthermore, as mentioned previously, heat shock proteins are highly conserved between bacterial and mammalian sources and seemed more likely to provoke autoimmune reactions than to induce tolerance. Therefore, it is surprising that heat shock proteins appeared to have such profound tolerogenic effects in these early studies. Besides HSP, other bacterial motifs have also been associated with tolerance. For example, a striking study done by Round et al. showed that polysaccharide A, a component of *B. fragilis*, induced the conversion of CD4+ T-cells to Foxp3+ Treg cells during commensal colonization of the gut, induced IL-10 production by these Treg cells, and cured animals of experimental colitis (Round et al. 2010). In an experiment exploring the relationship between microbial antigens and immune tolerance specifically relating to bacterial HSP antigens, Moudgil et al. evaluated the T-cell responses of mice with adjuvant-induced arthritis (AA) and found that rats kept behind barriers were more prone to arthritis than conventionally-housed rats. Both groups of rats exhibited similar T-cell responses directed at mycobacterial HSP65. However, unlike rats housed behind barriers, naive conventionally housed rats induced spontaneous T-cell responses to mycobacterial HSP65 c-terminal determinants (BCTD). Previous work by this group had showed that peptides associated with this BCTD region could be administered to rats prior to AA induction, and that these peptides had a protective effect against AA (Moudgil et al. 1997). Finally, the transfer of splenic cells from the conventionally housed rats to those that were behind barriers was protective against disease, indicating that BCTD-specific T-cells were unique to conventionally housed

mice and were sufficient to confer protection to mice under barrier conditions (Moudgil et al. 2001). This group did a great deal of work to show that HSP antigen-specific T-cell responses, the existence of which appears to be dependent on microbes in the environment, are important for protection in experimental autoimmune models. These experiments bring up many interesting points, namely that that microbes, particularly those that are commensal or environmental, can induce tolerance to mammalian HSP or other highly homologous sequences shared between bacteria and mammals, possibly due to the high cross reactivity of microbial-directed T-cell responses.

One group took these experiments a step further and aimed to identify and characterize microbial HSP antigen epitopes that were directly responsible for inducing tolerance in AA. In this experiment, Lewis rats were vaccinated with different epitopes corresponding to mycobacterial HSP65. Of nine epitopes that seemed to provoke specific T-cell responses, only one sequence (sequence 256-270) protected against AA, and this protection seemed to be mediated by T-cells specific to this sequence. Pre-immunization with this peptide induced T-cells that were protective against CP20961-induced arthritis, which importantly showed that this mycobacteria-derived peptide was protective against a variety of experimental arthritis models, and not just those that were dependent on mycobacteria. Interestingly, only mycobacterial HSP65, and not rat HSP65, was protective against arthritis in rats. The authors dissected this phenomenon further and found that mycobacterial 256-270-immunized T-cells could cross-react with rat 256-270 and rat 256-265 peptides, but rat 256-270-immunized T-cells could only react to rat peptides. Moreover, immunization of rats with mycobacterial 256-270 induced T-cells specific for the core epitope 256-65, which is common between mycobacterial and rat sequences, while immunization with rat 256-270 only induced T-cells specific for the rat core epitope 261-270. In this situation, the cross-reactivity of mycobacterial HSP65-specific T-cells to mammalian HSP65 confers tolerance and protection against mammalian HSP65-directed inflammation (Anderton et al. 1995).

The accumulating knowledge on the ability of heat shock proteins to confer tolerance to inflammatory models in vivo and induce immune-suppressive mechanisms in vitro has paved the way for the preclinical and clinical development and testing of HSP-based therapeutics for human autoimmune diseases. Similar to Anderton et al., one group recently identified an epitope of HSP70 that appears to act as a suppressor of inflammation. This epitope, which corresponded to an HSP70-derived peptide that the authors dubbed B29, was identified from a screen of dominant T-cell responses to synthetic peptides spanning regions of the mycobacterial HSP70 molecule. B29 was identified as a peptide that not only elicited a dominant T-cell response in immunized Balb/c mice, but that also had a high degree of similarity to the mammalian HSP70 peptide counterpart. In fact, T-cells from mice immunized with B29 were reactive with mammalian homologues and upon stimulation, these cells were observed to secrete suppressive cytokines such as IL-10. Furthermore, B29 appeared to enhance Treg suppressive capacity in vitro. In vivo experiments showed that transfer of Tregs from B29-immunized mice seemed to prevent induction and resolve ongoing proteoglycan induced arthritis, and that these

transferred Tregs were able to home to inflamed joints. Depletion of these transferred Tregs abrogated their anti-inflammatory effect, confirming that B29-induced Tregs were indeed responsible for this protection and/or resolution of inflammation (van Herwijnen et al. 2012). These authors subsequently showed that MHC II molecules preferentially load HSP70 sequences, including the aforementioned mycobacterial B29 sequence. This sequence appears to bind promiscuously to human MHC II molecules associated with rheumatoid arthritis (RA), including HLA-DR4 and HLA-DQ8. This is therapeutically significant, as the number of MHC molecules that this peptide binds is directly related to the number of patients for which a B29-based therapy would be available (de Wolf et al. 2016).

Finally, the concept that heat shock proteins can support immune tolerance has been explored for application in cancer immunotherapy. As mentioned previously, investigators have shown that HSP90 inhibition with ganetespib in cancer cells enhances T-cell mediated killing of patient-derived human melanoma cells by autologous cells in vitro, and that administration of ganetespib synergistically reduces tumor volume when combined with anti-PD-1 and anti-CTLA-4 in vivo. Interferon response genes were significantly upregulated in these tumor cells following ganetespib treatment, and silencing of these genes by shRHA abrogated the enhancement of T-cell mediated killing seen upon use of ganetespib. Based on these data, HSP90 appears to suppress interferon response genes, and this suppression is released by ganetespib-mediated HSP90 inhibition. Furthermore, this release of interferon response gene suppression is seen to enhance anti-tumor immunity. Interestingly, these experiments point to the notion that HSP can down-modulate transcription of pro-inflammatory cytokines in tumor cells. A similar phenomenon was described previously, where HSP can promote tolerance by repressing pro-inflammatory genes in antigen-presenting cells. Furthermore, these experiments also bring up an interesting concept that HSP inhibition can be utilized as a therapeutic strategy to break tolerance in the setting of a tumor (Mbofung et al. 2017).

10.10 Role of HSP in Gut Homeostasis

HSP have recently factored into the question of how the immune system maintains gut homeostasis, and tolerates the range of commensal microbiota that exist in the intestinal tract (Hua et al. 2017). It is known that intestinal antigen-presenting cells recognize and respond to gut microbiota through pattern recognition receptors including toll-like receptors and NOD-like receptors. These antigen-presenting cells are important in shaping the immune cell populations in the gut. For example, CD103+CD11b- DCs are essential for peripheral Treg induction during oral antigen exposure (Esterházy et al. 2016), while CX3CR1+ dendritic cells and macrophages are known to induce Th1 and Th17 differentiation in the gut (Varol et al. 2009; Liu and Nussenzweig 2010; Atarashi et al. 2008). Gp96, a member of the HSP90 family, is an essential chaperone for TLRs and is expressed in antigen presenting cells. However, the role of gp96 in APC function, specifically with respect to gut

immunity and tolerance, is unknown. Interestingly, when gp96 was knocked out specifically in CD11c+ dendritic cells in the mouse, an alteration in dendritic cell phenotype and loss of antigen specific Treg in the mesenteric lymph nodes is observed (Hua et al. 2017). In addition, these knockout mice developed spontaneous colitis with age, and high amounts of IgA were observed in the gut and fecal tissue. In addition, compared to wild-type mice, these knockout mice were more susceptible to chemical-induced colitis.

Hua et al. observed that gp96-knockout BM-derived dendritic cells failed to respond to microbial cues in the form of TLR2, TLR4, and TLR9 ligands, suggesting that gp96 is essential for proper TLR folding and function in DCs. These altered dendritic cells had broad effects on the immune populations of the gut in vivo: in the presence of gp96-knockout DCs, more CD4+ T-cells were found to colonize the lamina propia, with Th1 and Th17 cells highly increased, and Treg cells significantly decreased. Finally, to assess whether gp96 was important for inducing antigen-specific Treg in vivo, these investigators fed mice with ovalbumin (OVA) for 3 days, and subsequently adoptively transferred CFSE-labeled OVA-specific CD4+ T-cells isolated from OT-II T-cell reporter transgenic mice. While these adoptively transferred cells proliferated equally in the wildtype and gp96 conditional knockout mice, differentiation of these cells into Foxp3+ Treg cells and total number of Treg was markedly lower in the knockout recipients. These data indicate that gp96 is important for CD11c+ dendritic cells to prime antigen-specific Tregs in the gut. These experiments were important to establish a connection between heat shock proteins and the functionality of dendritic cells in the gut. Importantly, these findings seem to suggest that heat shock proteins are important for maintaining a pool of antigen-specific regulatory T-cells, which are in turn essential for maintaining oral tolerance and gut homeostasis.

10.11 Aryl Hydrocarbon Receptor and HSP in Immune Tolerance

Another story that appears to play into the story of HSP in immune tolerance is the aryl hydrocarbon receptor (AHR). AHR is a transcription factor that is controlled by endogenous and environmental small molecules. The most notable of these is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which binds to AHR with high-affinity that varies depending on species. Because of the nature of AHR ligands, this transcription factor appears to be part of a signaling pathway that responds to noxious substances. AHR is normally present in the cytoplasm in complex with HSP90 and c-SRC. Upon binding to TCDD in the cytoplasm, AHR dissociates from these other proteins and traverses into the nucleus, where it heterodimerizes with AHR nuclear translocator (ARNT) to act upon target genes through binding to dioxin response elements. AHR can also associate with a number of transcription factors to modulate transcription of other genes. These include STAT transcription factors,

NF-KB, and the estrogen receptor (ER) (Quintana 2013). AHR has been linked to carcinogenesis and is seen to have increased nuclear localization in a number of aggressive cancers (Murray et al. 2014). Interestingly, in a transgenic mouse model where AHR is expressed without the HSP90 binding domain, AHR is constitutively activated and exhibits high transcriptional activity. When exposed to the carcinogen diethylnitrosamine, these mice exhibited enhanced levels of liver tumors (Moennikes et al. 2004). This data indicates that HSP90 is essential for regulating AHR activation, and that constitutive transcriptional activation of AHR can enhance carcinogenesis. AHR has been implicated in several processes of the immune system. Activation of AHR by TCDD causes in vivo expansion of regulatory T-cells, which are functional and capable of suppressing experimental autoimmune encephalomyelitis, colitis, and spontaneous diabetes (Quintana et al. 2008; Benson and Shepherd 2011; Kerkvliet et al. 2009). In turn, ligands of AHR appear to inhibit Th17 differentiation (Benson and Shepherd 2011; Mezrich et al. 2010). However, AHR has also been seen to be upregulated in Th17 cells, which in vivo appears to boost the Th17 response and worsen CNS autoimmunity (Quintana et al. 2008; Marc Veldhoen et al. 2008). AHR activation also causes murine dendritic cells to skew to a more tolerogenic phenotype and produce fewer pro-inflammatory cytokines (Quintana et al. 2010). Thus, AHR appears to have a role in sensing environmental substances, and responding in a multitude of ways, including immunologically (Quintana 2013).

10.12 HSP-Based Immune Suppressive Therapies in the Clinic

The dnaJP1 is a 15-mer synthetic peptide based on the dnaJ family of HSP. This peptide was tested in early RA patients in early clinical trials evaluating immunologic activity, and then in later phase I/II trials evaluating the same parameters and clinical efficacy. In both of these studies, upon analysis of peripheral blood mononuclear cells isolated from treated patients, dnaJP1appeared to reduce T-cell production of pro-inflammatory cytokines such as TNFa, IL-2, and IFNy, and increase that of IL-4 and IL-10 (Prakken et al. 2004; Koffeman et al. 2009). In addition, a peptide based on the altered peptide ligand (APL) of a human HSP60 molecule, called APL1, has also been moving towards the clinic. APL1 was able to control progression of adjuvant-induced arthritis in Balb/c mice, and concurrently increase the percentage of Foxp3+ Treg cells in the spleen. Ex vivo addition of APL1 also increased Treg percentages, defined as CD4 $CD25^{hi}$ Foxp3, in PBMCs isolated from RA patients compared to healthy controls, and also seemed to enhance the suppressive function of these cells, as determined by an in vitro suppression assay (Barberá et al. 2016). When this peptide was administered to 18 patients with rheumatoid arthritis in a phase I/II study, a marked clinical improvement and reduction in inflammatory responses was seen in a number of patients (van Eden 2018).

In a similar vein to efforts taken to promote of antigen-specific immune responses with dendritic cells loaded with HSP-associated peptides, antigen-loaded DC vaccines are of interest to those seeking to promote tolerance in a clinical setting. The dendritic cells used in tolerance-promoting situations, however, are typically tolerized in vitro and loaded with self-peptides against which tolerance is desired in the setting of autoimmunity. For example, one group treated autologous DCs with an NF-kB inhibitor and loaded these with citrullinated peptide antigens. These peptides are known to be self-proteins that are recognized in the context of rheumatoid arthritis. These were then re-administered to patients (Benham et al. 2015). The cell therapy, dubbed Rheumavax, induced a suppressive immune response, increasing the ratio of regulatory T-cells to effector cells in the blood of these patients, and induced no toxic effects. Clinical efficacy, however, was not evaluated in this early study. In summary, HSP appear to be part of an important immune-regulatory network and this has been consistently established in vitro and in vivo. Several groups are interested in applying these principles to treat autoimmune disease and these efforts, though early, look very promising.

10.13 Conclusions

Heat shock proteins are part of a highly conserved system of chaperones, which are abundantly expressed upon physiologic and environmental stresses. Because of the role of HSP in maintaining cellular processes in times of physiologic insult, which include inflammation and infection, it is intriguing to think about the cross talk between the heat shock proteins and the immune system. It is also interesting to consider the ubiquity and structural similarity of these proteins across vastly diverse organisms, and what this might mean for the immune system. Do HSP induce pro-inflammatory responses because of the strong resemblances between human and bacterial HSP, or because of their ability to chaperone antigenic peptides? Do their chaperoning functions extend to antigen presentation processes? Conversely, do HSP help to down-regulate overactive immune responses? Based on the literature presented above, it seems likely that HSP have both pro-inflammatory and tolerance-inducing roles depending on context. In many instances, in both mice and humans, HSP appear to increase the immunogenicity of vaccines that would not be immunogenic if the same peptide(s) were used as vaccines in the absence of HSP. Furthermore, there is a great deal of evidence that HSP play into both MHC class I and MHC class II presentation pathways. Importantly, as described above, HSP can facilitate cross-presentation of their associated peptides by antigen presenting cells and subsequently induce CD8+ T-cell responses. In a seemingly opposing manner, HSP appear to induce tolerance in homeostasis or in the setting of inflammation, protecting against various types of autoimmunity in animal models. As mentioned above, heat shock factor 1, a gene that encodes HSP, can be induced to express HSP70 in response to inflammatory cytokines, but can also directly bind the TNFa promoter to repress the transcription of this pro-inflammatory cytokine. Finally, the impact of

HSP on cells of the innate immune system is highly disputed. Different groups have seen diverse effects of HSP on dendritic cells and macrophages among others. In some instances, HSP appear to enhance maturation and expression of pro-inflammatory cytokines in a manner that resembles the effects of an adjuvant. On the other hand, some groups have reported that HSP appear to promote a tolerogenic phenotype in innate cells, causing the secretion of IL-10. There have been many recent efforts to clarify these results and to shed light on which receptors are bound by different HSP. It is possible that the seemingly dual nature HSP in immunology can be attributed to context. Factors that affect whether HSP act to promote or down-regulate immune responses may include the stage of inflammation, the organ system in which inflammation takes place, and whether there is a homeostatic role for HSP in that organ system. Additionally, it is important to consider how cells of the immune system "perceive" HSP at a molecular level; i.e., we must consider that experimental HSP preparations differ substantially depending on the tissue or organism from which they are isolated, the immunogenicity of the peptides to which they are bound, the variety of gene products that HSP can bind, which include other HSP, and importantly, the possibility of endotoxin contamination (Borges et al. 2012). Interestingly, one group recently found that low dose immunization with gp96 has a very different effect on T-cell immune responses in vitro and in vivo when compared with high dose immunization of gp96. These authors found that low dose immunization with this chaperone primes a pro-inflammatory Th1 immune response, whereas high dose immunization primes a suppressive response mediated by regulatory T-cells. Upon further analysis of the mechanism, the authors observed that gp96 appears to differentially engage CD91+ antigen presenting cell populations in a manner that is dependent on its dosage, and subsequently drives different methylation patterns in these cell populations. On further investigation of the effects of the high-dosage regimen on immunosuppression, the authors found that gp96 exposure appeared to increase Nrp1+ plasmacytoid DC populations and promote prolonged interaction between these cells and Treg, which is an important process for bolstering Treg suppression (Kinner-bibeau et al. 2017). This finding is interesting because it takes steps towards understanding how HSP can enhance immune responses towards their associated peptides and drive tolerance under different conditions. Finally, it is helpful to note that "HSP" is a blanket term for chaperones that are induced upon stresses. Distinct families are diverse enough that it is erroneous to consider these proteins as though all share any given trait that one HSP member possesses. Possibly due to their shared chaperoning activities and ability to engage shared receptors on innate immune cells, many heat shock proteins of different families share similar immunologic traits, such as their ability to induce anti-tumor immunity in the context of a vaccine or activate antigen presenting cells. Nevertheless, as research in this area further matures, it will be exciting to understand which immunologic activities are shared and which are distinct among different heat shock proteins. Despite the many aspects that remain unknown, it is indisputable that HSP play a key role in immunity. Furthermore, therapeutic interventions based on the roles of HSP in both promoting antigen-specific immune responses and controlling overactive inflammation have been extended into patients.

These therapies appear to have encouraging results in broadly ranging conditions including glioblastoma, HPV, and rheumatoid arthritis. As we continue to improve our knowledge of the roles of these chaperones in immunity, it is likely that a greater number of HSP-based therapies for a broader range of immunologic disorders will emerge.

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Chapter 11
Cytosolic Heat Shock Protein 90 in Plant Hormone and Environmental Stress Response

Kenji Yamada and Etsuko Watanabe

Abstract From recent findings, it was revealed that cytosolic heat shock protein (HSP) 90 plays important roles in plant signal transduction during hormone sensing and environmental stress response. The various types of signaling proteins are identified as substrate for cytosolic HSP90 in plants; auxin receptor complex, auxin transporters, jasmonate receptor complex, brassinosteroid receptor complex, brassinosteroid-related transcription factors, heat shock transcription factors, receptors for pathogen infection, and components for circadian system. From the overview of these HSP90 substrates, cytosolic HSP90 works essentially in terms of sensing by receptor and output by transcription. It is also obvious that HSP90 facilitates SCF-type ubiquitin ligase complexes (e.g., auxin and jasmonate receptors) together with co-chaperone SGT1 in plants. This implies that cytosolic HSP90 regulates various SCF-type ubiquitin ligase complexes in other plant signaling systems. This chapter reviews recent advances in our understandings of cytosolic HSP90 function in plant hormones and environmental stress signaling.

Keywords Auxin · Environmental stress · Heat shock · HSP90 · Plant hormone · Receptor

K. Yamada (✉)
Małopolska Centre of Biotechnology, Jagiellonian University, Kraków, Poland
e-mail: kenji.yamada@uj.edu.pl

E. Watanabe
Department of Pathophysiology and Metabolism, Kawasaki Medical School, Kurashiki, Japan

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Abbreviations

AFB	auxin signaling F-box
ARF	auxin response factor
BAK1	BRI1-associated receptor kinase 1
BES1	*bin1*-EMS-suppressor 1
BR	brassinosteroid
BRI1	brassinosteroid insensitive 1
BZR1	brassinazole resistant 1
CHIP	carboxyl terminus of Hsc70-interacting protein
COI1	coronatine insensitive 1
EBF	EIN3 binding F-box protein
FAN	FKBP-associated NAC
FKBP	FK506-binding protein
GA	gibberellic acid
GID	gibberellin insensitive dwarf
HSF	heat shock transcription factor
HSP	heat shock protein
IAA	indole-3-acetic acid
JA	jasmonic acid
LOV	light oxygen voltage
NLR	nucleotide binding leucine-rich repeat receptor
PAS1	pasticcino 1
PGP	P-glycoprotein
PP2A	protein phosphatase 2A
PPIase	peptidyl-prolyl *cis*/*trans* isomerases
SCF	Skp, Cullin, F-box-containing
SNC1	suppressor of *npr1* constitutive 1
TIR1	transport inhibitor response 1
TWD	twisted dwarf
ZTL	zeitlupe

11.1 Introduction

HSP90 is a highly conserved chaperone that is essential for viability in eukaryotes. HSP90 fulfills a housekeeping function in contributing to the folding, maintenance of structural integrity, and proper regulation of substrate proteins (Picard 2002). The protein structure of HSP90 is highly conserved among homologues. HSP90 is a constitutive homodimer with its main inter-subunit contacts within the carboxyl-terminal region (Young et al. 2001). The amino-terminal domain of HSP90 contains an ATP-binding site, but its Bergerat-type ATP-binding fold is unique to diverse protein families, namely the GHKL superfamily (Dutta and Inouye 2000).

The small chemicals, such as geldanamycin and radicicol, specifically bind to the ATP-binding site of HSP90 (Picard 2002; Young et al. 2001). Because of the unique affinity of these chemicals to the ATP-binding site of HSP90, these drugs are thought to be pharmacologically specific inhibitors of HSP90. Indeed, HSP90 has ATPase activity, but the affinity of HSP90 for ATP is about ten times poorer than that of HSP70 in yeast (Picard 2002; Young et al. 2001). ATP is not always required in HSP90 chaperone activity; ATP binding and hydrolysis may only become important in more complex systems with an interplay of co-chaperones (Picard 2002; Young et al. 2001). Co-chaperons themselves do not have chaperone activity, but they directly interact with HSP90 to work as chaperones. Some of the co-chaperons do not have ATPase activity, but they negatively or positively regulate ATPase activity in HSP90 to regulate chaperone function. Others do not affect ATPase activity in HSP90, but they regulate chaperone function by possibly affecting the substrate loading to HSP90. In these co-chaperons, tetratricopeptide repeat (TPR) or CS domains are identified to bind with HSP90 (Röhl et al. 2013).

In higher plants, HSP90 is localized in the cytosol, ER, chloroplasts, and mitochondria. Although the analyses of HSP90 in plants are not as thorough as those in animals or yeasts, the researches using the plant model *Arabidopsis thaliana* (hereafter *Arabidopsis*) have increased our knowledge about the function of HSP90 in plants. In *Arabidopsis*, mutation in ER-type HSP90 causes expanding shoot apical meristems and floral meristem, disorganized root apical meristem, and defects in pollen tube elongation (Ishiguro et al. 2002). The mutant phenotype is similar to the *Arabidopsis clavata* (*clv*) mutants, suggesting that the ER-type HSP90 is involved in maturation of the CVL receptor complex in the ER prior to transport to the plasma membrane (Ishiguro et al. 2002). Chloroplast-type HSP90 is involved in chloroplast development and the mutant shows delays in leaf greening (Cao et al. 2003). Compared to ER-, mitochondria- and chloroplast-type HSP90, a lot of cytosolic (in precisely, nucleocytoplasmic) HSP90 functions were discovered within the last decade. Although cytosolic HSP90 is induced by heat stress, substantial levels of cytosolic HSP90 have already accumulated in non-stressed plants. Cytosolic HSP90 was found to selectively target substrate proteins to regulate their function in the various types of signal transduction system. In *Arabidopsis*, there are four cytosolic HSP90 homologues in chromosome 5 (Sangster et al. 2007). Three of them are tandem-replicated genes and it is extremely hard to produce multiple knockout mutants by crossing each T-DNA insertion knockout mutants that is available from the seed stock center (Sangster et al. 2007). However, a depletion of HSP90 activity by homologous sequences of HSPs with RNAi or overproduction of dominant negative forms induces a wide variety of morphological changes, suggesting the importance of cytosolic HSP90 in various cellular processes in plants (Sangster et al. 2007; Watanabe et al. 2016). Consistent with this, various protein substrates for cytosolic HSP90 are identified, including transcription factors, kinases, and receptors. In this chapter, we introduce the function of cytosolic HSP90 in terms of hormone signaling and environmental stress response.

11.2 Cytosolic HSP90 Is Essential for Auxin Receptor Function

Plants produce chemical hormones to regulate growth and environmental responses. These plant hormones are perceived by receptor proteins for the output. Importantly, some ubiquitin ligase complexes have hormone receptor activity in plants. In the case of auxin signaling, F-box-containing TIR1/AFB proteins are the auxin receptors and these proteins are a component of the SCF ubiquitin ligase complex (Salehin et al. 2015). The complex, namely $SCF^{TIR1/AFB}$, directs Aux/IAA proteins to the ubiquitin-dependent degradation pathway (Salehin et al. 2015). In the absence of auxin, AUX/IAA proteins bind to and inhibit transcription factor, ARFs. Auxin works as a molecular glue connecting between AUX/IAA proteins and TIR1/AFB proteins. Subsequently, AUX/IAA proteins are ubiquitinated and degraded by proteasome. The free ARFs activate auxin inducible genes.

Cytosolic HSP90 is known to bind to some SCF complexes in various organisms (Zhang et al. 2008). Indeed, cytosolic HSP90 binds TIR1 together with cochaperone SGT1 and regulates the function of auxin response in plants (Wang et al. 2016; Watanabe et al. 2016). In *Arabidopsis*, chemical inhibition of cytosolic HSP90 reduced the expression of auxin inducible genes after auxin treatment (Wang et al. 2016; Watanabe et al. 2016). The expression of dominant negative forms of cytosolic HSP90 caused growth defects, presumably inhibition of auxin response (Watanabe et al. 2016). The formation of lateral roots is stimulated by auxin treatment. Expression of dominant negative forms of cytosolic HSP90 reduced lateral root formation even after auxin treatment in *Arabidopsis* (Watanabe et al. 2016). These findings suggest that cytosolic HSP90 is crucial for auxin response by modulating $SCF^{TIR1/AFB}$ function in plants. TIR1/AFB proteins are normally localized in the nucleus, indicating that $SCF^{TIR1/AFB}$ works in the nucleus. The inhibitor treatment of HSP90 caused partial cytosolic localization of TIR1 and the degradation of TIR1 (Wang et al. 2016; Watanabe et al. 2016). These findings suggest that cytosolic HSP90 is required for the stable localization of $SCF^{TIR1/AFB}$ complex in the nucleus for auxin perception.

The morphology of plants can be changed depending upon the temperature in which they are grown. In *Arabidopsis*, increased growing temperature causes elongation of hypocotyl in seedlings and elongation of petioles in leaves. These morphological changes seem to be a consequence of both the increase of auxin synthesis and auxin response (Gray et al. 1998). On one hand, increased growing temperature increases the level of IAA in seedlings. In *Arabidopsis*, a transcription factor, PIF4 is responsible for the temperature-dependent increase of IAA by activating IAA synthesis genes (Franklin et al. 2011). On the other hand, TIR1 protein level is increased by high temperature in a cytosolic HSP90-dependent manner (Wang et al. 2016). The treatment of HSP90 inhibitor reduced TIR1 protein level along with the reduction of hypocotyl elongation in high temperatures in *Arabidopsis* (Wang et al. 2016). Thus, cytosolic HSP90 stabilizes the $SCF^{TIR1/AFB}$ complex to increase the auxin response in high temperature.

11.3 FKBP-Type HSP90 Co-chaperones Are Involved in Auxin Transport and Auxin Response

The co-chaperons of HSP90 include FKBP proteins that have PPIase activity. Although there is still no direct evidence, cytosolic HSP90 may participate in auxin response and transport through the regulation of FKBP proteins. *Arabidopsis* TWD1 (also known as UCU2 or AtFKBP42) encodes a FKBP protein and the *twd1* mutant shows defects in plant growth. The defect in growth in the *twd1* mutant is mainly caused by the disruption of auxin and BR function. TWD1 was found to interact with two ABC transporters, PGP1 and PGP19, which are involved in polar auxin transport on the plasma membrane (Geisler et al. 2003). Indeed, *twd1* showed the defect of root gravitropism response and reduced auxin polar transport, as well as the *pgp1 pgp19* double mutant (Bouchard et al. 2006; Geisler et al. 2003), suggesting that TWD1 is involved in the regulation of PGPs. Cytosolic HSP90 was found to interact with TWD1 (Kamphausen et al. 2002), implying the involvement of HSP90 in the polar auxin transport by regulating TWD1-PGP protein complexes.

Arabidopsis PAS1 gene encodes FKBP72, which is the potential co-chaperon of HSP90 (Vittorioso et al. 1998). The *pas1* mutant shows ectopic cell proliferation in cotyledons, extra layers of cells in the hypocotyl, and an abnormal apical meristem (Faure et al. 1998). The expression of auxin response genes is reduced in the *pas1* mutant, indicating that the auxin response is modified in the *pas1* mutant (Harrar et al. 2003). It was found that PAS1 interacts with FAN, a new member of the plant specific family of NAC transcription factors. Interestingly, auxin treatment induces the cytosol to nuclear relocation of PAS1 along with FAN (Smyczynski et al. 2006), suggesting that PAS1 regulates FAN localization during auxin response.

11.4 Cytosolic HSP90 Is Essential for Jasmonate Receptor Function

JA is a plant hormone that is responsible for transducing wound signals (Stratmann 2003). JA activates wounding-related genes and is involved in induced acquired resistance. Indeed, JA is modified to conjugate with amino acids, such as Ile to form JA-Ile, and the resultant metabolite is perceived by the COI1 receptor protein (Thines et al. 2007). COI1 is a F-box protein and a homolog of TIR1/AFB proteins (Gagne et al. 2002; Xie et al. 1998). Like the auxin receptor, COI1 also form an SCF-type ubiquitin ligase complex (Zhang et al. 2015). After the binding by JA, the SCF^{COI1} complex ubiquitinates JAZ proteins, which is a suppressor of JA signaling transcription factor AtMYC2 in *Arabidopsis*. SCF^{COI1} complex physically interacts with the SGT1-HSP70-HSP90 chaperone complex (Zhang et al. 2015). Chemical inhibition of cytosolic HSP90 reduced the expression of JA inducible genes after JA treatment (Zhang et al. 2015). These findings suggest that HSP90 is responsible for JA receptor function.

11.5 Cytosolic HSP90 Regulates Transcription Factor in Brassinosteroid Signaling

BRs are a group of steroidal hormones that control growth and development, as well as defense responses to abiotic and biotic stress in plants. BRs are perceived by cell surface receptor kinase complexes, namely BRI1 and BAK1, and the signal is transduced to transcription factors BES1 and BZR1 to activate BR response genes (Clouse 2011). When BR levels are high, BES1 and BZR1 are dephosphorylated and bind to DNA to induce BR response genes (Clouse 2011). When BR levels are low, BES1 and BZR1 are phosphorylated by BIN2 kinase, thereby losing DNA binding and degrading by proteasome (Clouse 2011). The localization of BES1 and BZR1 is also regulated by phosphorylation/dephosphorylation; dephosphorylated BES1 and BZR1 are localized in the nucleus, and phosphorylated forms are localized in the cytosol (Ryu et al. 2007). It turned out that cytosolic HSP90 interacts with BES1 and BZR1 (Lachowiec et al. 2013; Shigeta et al. 2014, 2015). Chemical inhibition of HSP90 results in high phosphorylation of BES1 and BZR1 even in exogenous treatment with BR (Lachowiec et al. 2013; Shigeta et al. 2015), suggesting that HSP90 positively regulates BR signaling by facilitating the dephosphorylation state of BES1 and BZR1 to nuclear localization and DNA binding.

The analysis of the *twd1* mutant also provides more evidence for the importance of HSP90 in BR signaling. In the *twd1* mutant, the inhibition of root growth by BR is reduced along with the increase of phosphorylation of BZR1, indicating that TWD1 is important for dephosphorylation of BZR1 during BR signaling. TWD1 binds to the cytosolic kinase domain of BRI1 and BAK1 (Chaiwanon et al. 2016). The molecular mechanism in which TWD regulates the BZR dephosphorylation by binding BRI1 and BAK1 is still unclear. The deficiency of TWD1 did not affect the subcellular localization of BRI1 (Chaiwanon et al. 2016). Considering that TWD1 is a co-chaperone of HSP90, one possibility is that HSP90 interacts with the BR receptor complex through TWD1 to regulate dephosphorylation of BES1 and BZR1.

11.6 Cytosolic HSP90 May Be Involved in Ethylene, Gibberellin, and Cytokinine Signalings

Considering the nature of the chaperon function of HSP90, it is highly possible that cytosolic HSP90 regulates various regulatory molecules in plant hormone signaling. Indeed, many F-box proteins are known to participate in plant hormone signaling (Lechner et al. 2006). Ethylene works as an airborne plant hormone, which is involved in plant developmental processes and environmental responses, such as fruit ripening, leaf senescence, and wounding response. EBF1 and EBF2 are F-box proteins that are down-regulating ethylene responses by degrading a transcription

factor, EIN3 in *Arabidopsis* (Gagne et al. 2004; Guo and Ecker 2003; Potuschak et al. 2003). Considering that cytosolic HSP90 binds with F-box proteins, $SCF^{EBF1/EBF2}$ will be candidates for substrates of HSP90. For the same reason, a SCF complex in GA signaling, namely $SCF^{SLY1/GID2}$ (Itoh et al. 2003), will be a substrate of cytosolic HSP90. GA mainly works as a growth hormone in various plant organs. When the GA receptor, GID1, perceives GAs, it binds to DELLA proteins that are negative regulators of GA signaling (Itoh et al. 2003). This GID1-DELLA complex is recognized by $SCF^{SLY1/GID2}$, and $SCF^{SLY1/GID2}$ directs DELLA proteins for a ubiquitin-dependent degradation pathway (Itoh et al. 2003). In the *Arabidopsis pas1* mutant, expression of cytokinin response genes are more enhanced after cytokinin treatment compared to that of the wild type (Harrar et al. 2003). Although the details of the molecular mechanism are still obscure, the findings indicate the importance of HSP90 co-chaperon PAS1 in the cytokinin signaling.

11.7 Cytosolic HSP90 Regulates Cytosolic Receptors for Pathogen Recognition

Many plants suffer the exposure to various bacterial or fungal pathogens. However, if the plants have the receptors to recognize these pathogens, the plants induce the resistance genes to cope with them. There are two types of receptors for the recognition of pathogen infection; one is a plasma membrane type and the other is a cytosolic type. It is known that some of the cytosolic-type receptors are the substrate of cytosolic HSP90. In *Arabidopsis*, RPM1 and RPS2 are NLR proteins that confer recognition to bacterial strains expressing type III effector genes, *avrRpm1*, or *avrRps2*, respectively (Spoel and Dong 2012). The recognition of the bacteria is the consequence of phosphorylation by AvrRpm1 of *Arabidopsis* RIN4 or degradation of RIN4 by AvrRps2 (Axtell and Staskawicz 2003). Cytosolic HSP90 forms a complex with RPM1 and RPS2 together with co-chaperone SGT1 and RAR1 to recognize the phosphorylation of RIN4 or absence of the RIN4 protein in *Arabidopsis* (Hubert et al. 2009; Hubert et al. 2003; Takahashi et al. 2003). This positive function of HSP90 toward NLRs seems widely distributed in plants because cytosolic HSP90 interacts with NLR proteins and stabilizes their function in tobacco (Liu et al. 2004; Lu et al. 2003). It was proposed that cytosolic HSP90 facilitates stabilization and accumulation of NLR proteins (Lu et al. 2003). However, cytosolic HSP90 also shows the opposite effect in some NLRs. The *Arabidopsis snc1* mutant contains a gain-of-function mutation in a NLR protein SNC1, and shows an autoimmune phenotype (Huang et al. 2014). The mutation of cytosolic HSP90 in the *snc1* causes enhancement of the phenotype because of the accumulation of the mutated form of SNC1 (Huang et al. 2014). These findings suggest that cytosolic HSP90 is required for not only for activation of NLRs, but also deactivation by the degradation of NLRs.

11.8 Cytosolic HSP90 Regulates Heat Shock Transcription Factor Function

Short-term or slightly longer heat stress induces the heat inducible genes, which are required to cope with high temperatures in plants. Various heat sensing/signaling systems are predicted in plants, including heat activation of transcription factors by dissociation from HSP90 (McLellan et al. 2007; Yamada et al. 2007). In plants, pharmacological and genetic inhibition of HSP90 induces heat inducible genes, suggesting that HSP90 negatively regulates the heat shock response (McLellan et al. 2007; Nishizawa-Yokoi et al. 2010; Yamada et al. 2007). HSFs are responsible for the induction of heat inducible genes (Nover et al. 2001, 1996). It is proposed that cytosolic HSP90 interacts with the HSFs in animal and yeast cells to down regulate its function in normal temperature (Zou et al. 1998). When the heat shock induces the denatured proteins, the denatured proteins occupy most of HSP90. This causes the dissociation of HSP90 from HSFs, and the free HSFs induce heat inducible genes. Indeed, some of HSFs interact with cytosolic HSP90 in plants (Hahn et al. 2011; Yamada et al. 2007; Yoshida et al. 2011), supporting the hypothesis that cytosolic HSP90 negatively regulates HSFs in plants. Cytosolic HSP90 seems also regulate the HSF protein localization. In *Arabidopsis*, three HSFs, namely AtHsfA1b, AtHsfA1d, and AtHsfA1e are predominantly localized in the cytosol, but are re-localized to the nucleolus after heat shock or HSP90 inhibitor treatment (Yoshida et al. 2011). More complex regulation mechanism of HSP90 for HSF is observed in tomato. In tomatoes, cytosolic HSP90 increases the binding of HsfB1 to a promoter region of heat inducible genes during heat shock, but at the same time is required for the degradation of HsfB1 after recovery from heat shock (Hahn et al. 2011). Taken together, these findings suggest that cytosolic HSP90 regulates HSF localization and activity during heat shock response.

11.9 A HSP90 Co-chaperone CHIP Is Involved in a Chilling Response

CHIP is a U-box-containing ubiquitin ligase that binds to the carboxyl terminal of HSP70 and HSP90. After the ubiquitylation by CHIP, the substrate of HSP70 or HSP90 goes to the proteasome-dependent degradation pathway. When CHIP is overproduced in *Arabidopsis*, the plants reduced chilling tolerance to 7 °C and enhanced ABA sensitivity compares to that of the wild type (Luo et al. 2006; Yan et al. 2003). In vitro experiments have shown that the *Arabidopsis* CHIP can ubiquitylate two A subunits of protein phosphatase 2A, namely PP2AA3 and RCN1 (Luo et al. 2006), suggesting that CHIP enhances the degradation of PP2A in *Arabidopsis*. However, in vivo experiments showed there is no difference in protein levels of these PP2As among CHIP overproducing lines and the wild type (Luo et al. 2006). Furthermore, contradictory to the fact that CHIP promotes the

ubiquitylation of PP2A, PP2A activity is increased in CHIP overproducing lines (Luo et al. 2006). Although the molecular mechanism is unclear regarding how CHIP integrates its function into chilling tolerance, those findings imply that enrollment of HSP90 in chilling and the ABA response through the interaction with CHIP.

11.10 Cytosolic HSP90 Is Involved in Circadian Clock Through Stabilization of ZEITLUPE

The circadian clock is a system sustaining 24-h rhythmicity in a day/night cycle accompanying the proper gene expression, and the system is required for the optical growth and flowering in plants (Dodd et al. 2005; Ni et al. 2009). In *Arabidopsis*, rhythmic expression of the F-box protein ZTL is necessary to sustain a normal circadian period (Kim et al. 2007). ZTL forms a SCF-type ubiquitin ligase complex, and the SCF^{ZTL} directs the proteasome-dependent degradation of clock proteins, TOC1 and PRR5. Cytosolic HSP90 is required for the maturation of ZTL; inactivation of HSP90 caused reduction of the ZTL protein level in *Arabidopsis* (Kim et al. 2011). GIGANTIA (GI), a plant unique protein, seems a co-chaperone of HSP90, and forms a complex with HSP90 for the refolding of the ZTL protein (Cha et al. 2017). The findings indicate that the HSP90 functions in the circadian clock. In Arabidopsis three homologues proteins, ZTL, LKP2, and FKF1 harbor the LOV domain, F-box, and KELCH repeats (Takase et al. 2011). These proteins all work as regulators in the circadian clock (Takase et al. 2011). Considering that ZTL is a substrate of HSP90, HSP90 may regulate these homologues to regulate the circadian clock.

Interestingly, ZTL was found to be responsible for the degradation of aggregated proteins generated by heat shock (Gil et al. 2017). The *Arabidopsis ztl* mutant exhibited a reduction in survival after heat shock (Gil et al. 2017). These findings suggest that HSP90 is involved in the heat resistance through the function of ZTL.

11.11 Conclusions

Knowledge about the function of plant cytosolic HSP90 has dramatically increased over the last decade. In particular, it was revealed that cytosolic HSP90 is indispensable in the function of SCF-type ubiquitin ligases that are engaged in plant hormone signaling and the circadian clock system. This finding suggests that the SCF complex is a common substrate of HSP90. There are many F-box proteins in plants; nearly 700 F-box proteins have been predicted in *Arabidopsis*, and they are involved in various aspects; e.g., development, hormone signaling, circadian clock, self-incompatibility, and defense response (Lechner et al. 2006). In addition, HSP90 regulates the function of transcription factors and cytosolic receptors for pathogen

infection. Therefore, one can easily estimate that HSP90 influences various cellular activity through the regulation of its substrates. It will be necessary to discuss carefully which substrate(s) is responsible for the observed phenomena *in planta* pharmacological inhibition of HSP90 activity. It is proposed that HSP90 buffers or hides phenotypic variations in plants, because the slight reduction of HSP90 activity by inhibitors or the RNAi method definitely increases the morphological changes in *Arabidopsis* (Queitsch et al. 2002; Sangster et al. 2008a, b). One molecular mechanism for the HSP90 buffering seems the consequence of its chaperon activity to mask a mutation in substrate proteins. Indeed, such an example has been identified recently by the analysis of the auxin receptor TIR1. The specific *tir1-1* mutant allele did not show any defect in root elongation; however, the root elongation is more sensitive to pharmacological inhibition of HSP90 compare to the wild type (Watanabe et al. 2016). Further analysis of HSP90 substrates and their function will provide great insights into the specific molecular mechanism in which HSP90 guides genome mutation during the course of evolution in plants. The number of identified cytosolic HSP90 substrates is still smaller in plants compared to animals and yeasts, in which the bulk interactome analyses of HSP90 substrates were performed (Millson et al. 2005; Taipale et al. 2012; Tsaytler et al. 2009; Zhao et al. 2005). Such bulk analysis is still waiting, and the results will surely provide new plant-specific functions of cytosolic HSP90.

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Part II
Oncology Signaling Pathways

Chapter 12
Targeting Heat Shock Proteins in Multiple Myeloma

Vijay P. Kale, Sangita Phadtare, Shantu G. Amin, and Manoj K. Pandey

Abstract The heat shock proteins (Hsp), the family of molecular chaperons, are key proteins in protein folding and maturation. The client proteins of Hsp are critical in number of biological processes including cellular proliferation, differentiation, survival, metastasis, invasion, and angiogenesis. Thus, Hsp family becomes one of the desirable targets for cancer treatment. It has been demonstrated that Hsp overexpress in multiple myeloma and linked in poor prognosis and relapse. This chapter describes about the Hsp and their possible link with the pathogenesis of multiple myeloma. It addresses the advancement and challenges in the development of Hsp inhibitors.

Keywords Chaperons · Chemo-resistance · Hsp27 · Hsp70 · Hsp90 · Multiple myeloma

Abbreviations

ADP	Adenosine diphosphate
Aha1	activator of HSP90 ATPase activity 1
ALT	alanine aminotransferase
Apaf-1	apoptotic peptidase activating factor 1
AST	aspartate aminotransferase
ATP	adenosine triphosphate

V. P. Kale
Clinical and Nonclinical Research, Battelle Memorial Institute, West Jefferson, OH, USA

S. Phadtare · M. K. Pandey (✉)
Department of Biomedical Sciences, Cooper Medical School of Rowan University, Camden, NJ, USA
e-mail: pandey@rowan.edu

S. G. Amin
Department of Pharmacology, Penn State College of Medicine, Hershey, PA, USA

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Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma-2
Cdc37	cell division cycle 37
Chip	carboxy terminus of Hsc70 interacting protein
Cns1	tetratricopeptide repeat domain 4
CR	connecting linker region
CTD	C terminal domain
ErbB	epidermal growth factor receptor
ER	Estrogen receptor
Her3	erb-b2 receptor tyrosine kinase 3
HIF-1α	hypoxia inducible factor 1 subunit alpha
Hip	Hsc70-interacting protein
Hop	Hsp70-Hsp90 organizing protein
HSF-1	heat shock transcription factor-1, Hsp, heat shock proteins
MAPK	Mitogen-activated protein kinase 1
MEEVD	Met-Glu-Glu-Val-Asp motif
Mek1/2	mitogen activated protein kinase kinase
MD	middle domain
NF-κB	nuclear factor kappa-light-chain- enhancer of activated B cells
NTD	N terminal domain
PP5	protein phosphatase 5
p23	prostaglandin E synthase 3
Smac	second Mitochondria-derived activator of caspases
Tom70	translocase of outer mitochondrial membrane 70
TPR	tetratricopeptide repeat domains
Unc45	unc-45 myosin chaperone B

12.1 Introduction

Heat Shock Proteins (Hsp) are the family member of highly conserved proteins (Wu et al. 2017). In addition to their most studied role in protein folding, Hsp are also involved in intracellular trafficking, signaling pathways, and immune responses. These functions enable Hsp to play critical roles in the regulation of protein homeostasis, cell survival, development and differentiation. Based on the molecular weight, mammalian Hsp have been classified into five families: Hsp100, Hsp90, Hsp70, Hsp60, and small Hsp such as Hsp27. These proteins expressed differentially in the cells depending on the cell conditions, though often times these proteins are constitutively expressed. These proteins can be found in different cellular compartments, for example, Hsp90 is produced in the nucleus and cytoplasm, while Hsp60 is produced in the mitochondria (Whitley et al. 1999). Based on the studies it has been illustrated that each family members are designated for specific function for example, Hsp90 family found to be involved in steroid receptor complex formation, while Hsp70 plays an important role in protein synthesis process including

folding, and Hsp60 provides protein stability. Along with the co-chaperones, Hsp90 protein forms a huge complex, which interacts with variety of proteins involved in various biological processes such as proliferation, survival, angiogenesis, cell cycle, invasion, and metastasis. Importantly, it has been reported that a complex of Hsp90 is required for activation of transcription factors (e.g. p53, NF-κB, HIF-1α), MAPK kinases (e.g. RAF-1), receptor tyrosine kinases (e.g. erbB) (Zagouri et al. 2012). Hsp70 interacts with a variety of proteins irrespective of their conformational stages, for example it may bind to unfolded, and natively folded proteins. Hsp70 protects the cells against cellular stresses, lethal injuries, and apoptosis, thus, promotes the cell survival. In cancer cells, it regulates intrinsic and extrinsic apoptosis by modulating caspase-independent apoptotic pathways (Kumar et al. 2016). Because of the critical role of Hsp70 in cancer cell survival and proliferation, mammoth efforts have been made to develop Hsp70 inhibitors for cancers mostly other than multiple myeloma (Kumar et al. 2016).

The small Hsp are conserved among species. This family of Hsp play very important role in autophagy, degradation of proteasomes, development, differentiation, and stress tolerance. In addition, small Hsp are anti-apoptotic in nature. The mutations in these proteins result in number of pathological conditions such as myopathies, neuropathies and cataract. It has been demonstrated that small Hsp bind with copper and suppress the generation of reactive oxygen species (Madamanchi et al. 2001). This property of small Hsp is implicated in copper homeostasis and neurodegenerative disorders, such as Alzheimer, Parkinson (Bakthisaran et al. 2015). All together small Hsp have both beneficial and detrimental effects on human health.

In most of the cancers, Hsp are found to be overexpressed, which is linked with the proliferation, survival, invasion, and metastasis (Lianos et al. 2015; Teng et al. 2012). Intracellularly, Hsp stabilize a number of oncogenic proteins, and facilitate the interaction of various signaling pathways associated with cancer. Because of their role in regulation and stabilization of a number of oncogenic proteins, the expression of Hsp is associated with poor prognosis of cancers including gastric, liver, prostate, osteosarcomas, breast, endometrial, uterine cervical, and bladder carcinomas. Hsp27 regulates the stability, nuclear shuttling and transcriptional activity of androgen receptor that are involved in prostate cancer (Azad et al. 2015; Cordonnier et al. 2015; Teng et al. 2012; Zagouri et al. 2012). Altogether, Hsp thus prove to be an excellent anti-cancer targets (Ciocca and Calderwood 2005; Zhang et al. 2014).

Multiple myeloma, is a plasma cell neoplasm. The hallmark of multiple myeloma is the production of monoclonal proteins and bone loss (Palumbo and Anderson 2011; Rajkumar and Kumar 2016). In last one decade the treatment options for multiple myeloma evolved beyond the expectations and now 5-year survival expectancy is more than 50%, which is remarkable. Nonetheless, almost every patient relapse after successful initial treatment, thus there is always a demand for novel drug development. Because Hsp regulate a number of key proteins which are integral in the pathogenesis of multiple myeloma, it has become attractive therapeutic target. Along these lines, number of Hsp inhibitors have been developed and shown

promise (Born et al. 2013; Duus et al. 2006; Richardson et al. 2011c). Importantly, studies have demonstrated that these inhibitors possess the potential to inhibit drug resistance. How Hsp inhibitors control drug resistance is not fully understood, though a number of mechanisms have been proposed, for instance, inhibitor of Hsp27 induces the release of mitochondrial protein, Smac, and inhibits dexamethasone resistance in multiple myeloma cells (Chauhan et al. 2003).

In this chapter, we briefly discussed the roles of Hsp family members and current advancement in the development of Hsp inhibitors, particularly in multiple myeloma. Moreover, the strategies and challenges in targeting Hsp90 in particular is discussed in detail.

12.2 Role of Heat Shock Proteins in Cancer Cell Signaling

Hsp proteins play critical role in cancer cell survival (Calderwood and Gong 2016), because they interact with a variety of client proteins involved in regulation of cell cycle and inhibition of apoptosis (Chatterjee and Burns 2017). Thus, the importance of Hsp become more relevant in cancer cells as compared to their normal counterpart, because malignant cells require more energy in order to survive in challenging hostile environment. Consequently, the high levels of Hsp are reported in variety of tumors including solid as well as hematological cancers including multiple myeloma (Ciocca and Calderwood 2005; Flandrin et al. 2008; Saluja and Dudeja 2008; Zhang et al. 2014).

12.2.1 Hsp90

The monomer Hsp90 contains three highly conserved domains: amino terminal domain (NTD), middle domain (MD), and carboxy terminal domain (CTD). Each domain is unique in function, for example, NTD mediates the binding of ATP, and MD facilitates the binding of Hsp90 to client proteins, and hydrolyzes ATP, while CTD mediates the dimerization of Hsp90, which is essential for its function (Fig. 12.1) (Ciglia et al. 2014; Colombo et al. 2008; Hawle et al. 2006; Nemoto et al. 1995; Ratzke et al. 2010). Both NTD and MD are connected by a long, flexible, charged linker. Monomer Hsp90 also contains peculiar motif at CTD site called MEEVD motif. The MEEVD motif facilitates the binding of Hsp90 to specific co-chaperons those contain TPR domains (Zuehlke and Johnson 2010). The ATP free Hsp90 exist in V-shaped open conformation. ATP binding induces conformation changes, which leads to a closed and active conformation. The overexpression Hsp90 has been observed in a variety of malignancies including multiple myeloma (Zhang et al. 2014). It has been demonstrated that proteins integral in cell cycle,

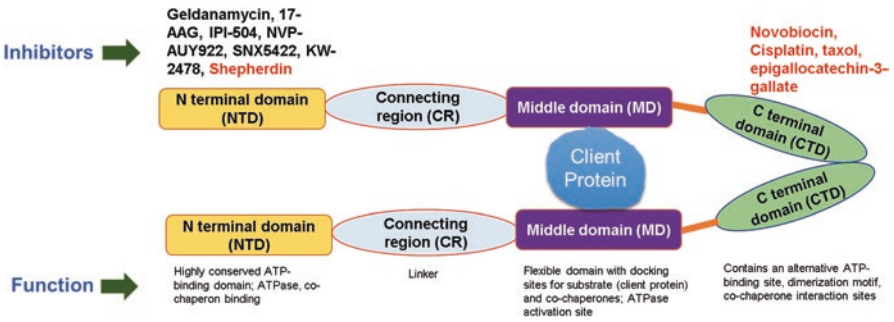

Fig. 12.1 Schematic presentation of Hsp90 protein structure. The homodimer of Hsp90 contains a N terminal domain (NTD), connecting linker region (CR), middle domain (MD), and a C terminal domain (CTD). Each domain has a specific function, for example NTD is responsible for ATPase and co-chaperone binding, MTD binds to client protein, and facilitates co-chaperon binding, and CTD is instrumental in dimerization. Targeting specific domains has developed various small molecule inhibitors. The inhibitors highlighted in red color have been identified in other than multiple myeloma

kinases, transcription factors, survival and anti-apoptotic proteins are the client of Hsp90 (Wayne et al. 2011). Thus Hsp90's client proteins regulate number of key processes linked with "hallmarks of cancer" (Chatterjee and Burns 2017; Miyata et al. 2013).

12.2.2 Hsp70

The chaperon Hsp70 family facilitates protein folding process of client proteins (Saibil 2013). The overexpression of Hsp70 is linked with poor prognosis and aggressiveness of cancers (Lee et al. 2013; Murphy 2013). The HSP70's client proteins play key role in anti-apoptosis, senescence, and autophagy (Mayer and Bukau 2005). It has been reported that Hsp70 is anti-apoptotic in nature and promotes the cancer cell survival and linked with drug resistance in a variety of cancers including multiple myeloma (Nimmanapalli et al. 2008; Reikvam et al. 2013; Yang et al. 2012). Number of mechanisms have been proposed regarding the role of Hsp70 in the regulation of apoptosis. Hsp70 protects the degradation of Bcl-2, suppresses Bax translocation to the mitochondria, and inhibits the recruitment of caspase 9 to apoptosome by binding to Apaf-1 (Li et al. 2000; Rerole et al. 2011). Overall, these studies demonstrate that targeting of Hsp70 is required in order to induce apoptosis and treatment of cancers including multiple myeloma (Bailey et al. 2015; Zhang et al. 2014).

12.2.3 Hsp27

Hsp27 is overexpressed in a variety of cancers including multiple myeloma, breast, colorectal, ovarian, and prostate that is correlated with poor prognosis, chemo-resistance, and tumor aggressiveness (Chauhan et al. 2003; Langdon et al. 1995; Voll et al. 2014; Wei et al. 2011; Yu et al. 2010; Zhang et al. 2014, 2016). Not only Hsp27 is critical in tumor survival, it is also instrumental in actin dynamics, cell migration and invasion. Studies have demonstrated that knocking down of Hsp27 leads to suppression of bone metastasis in a breast tumor model (Gibert et al. 2012). Elevated levels of Hsp27 transcripts have been observed in multiple myeloma cells (Zhang et al. 2014). Using oligonucleotide array, Chauhan et al. (2004) showed that Hsp27 mRNA is highly expressed in Dexamethasone-resistant multiple myeloma cells versus Dexamethasone -sensitive multiple myeloma cells, which further supports its role in chemo resistance.

12.3 Molecular Structural Sites to Target Hsp90

Hsp90 proteins are evolutionarily conserved molecular chaperons accounting for almost 1–2% of total protein under normal condition (non-stressful) and 5–6% under stressful condition (Usmani and Chiosis 2011). The protein Hsp90, regulated by more than ten genes, is mainly an intracellular non-secretary localized in the cytoplasm, endoplasmic reticulum, mitochondria and small amount in nucleoplasm (Chen et al. 2005; ProteinAtlas 2018). However, Hsp90 are also secreted and found in the extracellular environment of cancer cells to some extent with role in metastasis (Wong and Jay 2016). Considering intracellular abundance of Hsp90, small molecules and peptides may be ideal drug tools to target Hsp90 than the large molecules. Indeed, over a decade, a several synthetic molecules have been developed to inhibit Hsp90 and some also made to the clinical trials although none is yet approved by Food and Drug Administration (FDA). The possible drug-able sites on Hsp90 can be predicted by understanding the roles of different sites of Hsp90. As mentioned earlier the Hsp90 constitutes: NTD, connecting flexible linker region, MD, and CTD (Fig. 12.1) (Richardson et al. 2011b). The NTD is the site for nucleotide (ATP/ADP) binding and interaction with co-chaperons. It is the site where ADP-ATP exchange and hydrolysis of ATP to ADP occurs. The binding of ATP makes the conformational changes in the Hsp90 molecule which is also coordinated by co-chaperons such as Hip, Hop, Cdc37, p23, immunophilins, and Aha1 which bind at NTD, MD or CTD. These co-chaperons regulate the interaction of Hsp90 with nucleotides and substrate proteins (a.k.a. client proteins). Most of the client proteins bind to the MD during “immature” phase of the complex – just before ATP hydrolysis – and eventually becomes “mature” complex upon ATP hydrolysis (Sidera and Patsavoudi 2014). The set of required co-chaperons may differ with the client proteins which emphasizes the significance of the role of co-chaperons in Hsp90

activity. Hence, all three sites on Hsp90 play crucial role in the function of Hsp90 and therefore all three sites are plausible drug-able sites. However, each site poses challenges and benefits to target with the therapeutic. This basic understanding of mechanics of Hsp90 hints some possible targeting strategies: (1) Targeting ATP-binding site on Hsp90, (2) Targeting interaction of co-chaperons with Hsp90, and (3) Targeting interaction of Hsp90 with its client proteins.

12.3.1 Targeting ATP-Binding Site on Hsp90 (NTD and CTD)

Geldanamycin, radiciol and their derivatives bind to NTD and interfere with binding of ATP to NTD. However, it is important to note that geldanamycin and radiciol exhibit two different modes of binding within ATP-binding pocket. Although these drugs initially looked promising, they failed in clinical trials either due to toxicity or not having enough therapeutic effects, lowering the risk-benefit ratio. This might be due to ubiquitous nature of ATP binding sites on more than 500 kinases present in humans and hence more possibilities of off-target effects. However, this does not mean more specific ATP-competitive inhibitors cannot be developed. By rational drug design more selective inhibitors targeting cancer cells can be developed. The rational for development of cancer cell specific Hsp90 inhibitors is that cancer cell-derived Hsp90 shows 100-fold higher binding affinity to the inhibitors than Hsp90 from normal cells (Kamal et al. 2003). Moreover, two types of Hsp90 exists in cancer cells: major housekeeping Hsp90 which have low affinity for inhibitors and another type of stressed Hsp90 in complex with multi-chaperones with high affinity conformation (Moulick et al. 2011). The small molecule PU-H71, which was a result of rational drug design, showed binding specificity for stressed cancer specific Hsp90-oncoprotein complex in chronic myeloid leukemia cells (Bcr-Abl-Hsp90), melanoma cells (B-Raf-Hsp90) and breast cancer cells (Her3-Hsp90 and Raf1-Hsp90). Per http://www.ClinicalTrials.gov, as on April, 2018, PU-H71 is being studied ("recruiting" status) in clinical trials as a combination therapy for breast cancer (with Nab-Paclitaxel) and myelofibrosis (with rituximab). Additionally, Phase 1 clinical trial for PU-H71 alone in patients with advanced malignancies shows status as "active but not recruiting", while another clinical trial in patients with solid tumors and low-grade Hodgkin's lymphoma has been terminated. Phase 1 clinical trial data indicated that PU-H71 was well tolerated with some tumor regression in cervical squamous cell carcinoma (SCC; 22.6%), triple negative breast cancer (8.3%), ER positive breast cancer (25.6%), penile SCC (20.8%), and marginal zone lymphoma (20.6%) (Gerecitano et al. 2015). However, its effect in multiple myeloma is not yet clear. In this trial, 5 patients out of 40 exhibited dose limiting toxicities such as mucositis, liver enzyme elevation (ALT and AST), nausea/vomiting, intolerable myalgia, anemia, and intolerable headache (Gerecitano et al. 2015).

CTD of Hsp90 has another putative ATP-binding site which may be involved in allosteric regulation of conformational change of Hsp90 during activity. Novobiocin

was the first small molecule inhibitor to be identified as inhibitor of Hsp90 at CTD (Marcu et al. 2000). Eventually cisplatin, epigallocatechin-3-gallate (a green tea component), and taxol were also identified as CTD inhibitors (Donnelly and Blagg 2008). A novel CTD inhibitor, KU675, has shown to be effective in vitro in prostate cancer cell lines. KU675 selectively inhibited HSP90α (K_d = 191 μM) over HSP90β (K_d = 726 μM) (Liu et al. 2015). However, its activity in multiple myeloma cells is not known. One probable advantage with CTD-binding Hsp90 inhibitors is that they do not induce stress response in cells and hence less chances of developing resistance.

12.3.2 Targeting Interaction of Co-chaperons with Hsp90

Binding of co-chaperons is essential for the activity of Hsp90. The list of Hsp90 co-chaperons includes Aha1/Ch1 (stimulates Hsp90 ATPase), p23 (involved in maturation of client proteins at later stage), Cdc37 (involved in protein kinase folding), Cns1 (uncertain role), Unc45 (involved in myosin assembly), PP5 (protein phosphatase), Tom70 (mitochondrial preprotein import), Chip (ubiquitin ligase), and HOP (connects Hsp70 and Hsp90, and transfers client proteins) (Caplan 2003). Inhibition of Hsp90 at NTD has shown to induce heat shock response by upregulating Hsp70 and hence conferring resistance to Hsp90 inhibitors. In view of that, targeting interaction of co-chaperon with Hsp90 seems to be a viable alternate strategy to make Hsp90 dysfunctional. Novobiocin which was initially discovered to bind ATP binding site at CTD, also interferes with binding of p23 and Hsc70 with Hsp90 and hence its activity. The co-chaperon p23 helps in maturation of the client proteins. Hence, in absence of maturation, the client proteins will undergo proteasome-dependent degradation. Similarly, celastrol and gedunin were also identified to inactivate p23 and substrate maturation (Chadli et al. 2010; Patwardhan et al. 2013). Before identifying celastrol and gedunin as p23 inhibitors, these agents were identified as inhibitors of co-chaperon Cdc37 during gene-expression based target pathway identification analysis (Hieronymus et al. 2006). Mechanistic studies revealed that celastrol induces large conformational change in N-terminal 'kinase-binding' domain and middle 'Hsp90 (N-terminal)-binding domain' of Cdc37 resulting in disruption of Cdc37-Hsp90 complex formation and destabilization of numerous kinase client proteins (Sreeramulu et al. 2009). However, although optimistic, efficacy of these inhibitors need yet to be investigated in multiple myeloma cancer cell types. Another important co-chaperon Aha1 binds to NTD and MD of Hsp90 and stimulates ATPase activity at those sites. siRNA mediated selective inhibition of Aha1 decreased activity of C-Raf, and levels of Mek1/2 and Erk1/2 in colon cancer cells (Holmes et al. 2008). Recently identified small molecule HAM-1 binds to NTD of Hsp90 overlapping with Aha1 interacting site (Stiegler et al. 2017). Thus HAM-1 interferes with transient interaction of Aha1 and NTD of Hsp90, without dissociating Hsp90-Aha1 complex, but affects catalytic activity of Hsp90 and activity of Hsp90-Aha1 dependent client proteins in yeast.

12.3.3 Targeting Interaction of Hsp90 with Its Client Proteins

The main function of Hsp90 is to ensure proper folding of client proteins for their proper activity. Hence, targeting the interaction of Hsp90 and their substrate becomes a logical strategy to inhibit function of Hsp90. A considerable body of evidence from structural and functional analysis indicates that middle segment of Hsp90 plays a crucial role in binding of Hsp90 with the client proteins (Meyer et al. 2003). One of such client proteins is androgen receptor (AR). Targeting AR has been a promising strategy to treat prostate cancer. In vitro studies revealed that Hsp90 stabilizes AR in the inactive conformation and is responsible for the nuclear translocation and transcriptional activity of androgen bound-AR (Georget et al. 2002). Although these studies reveal necessity of Hsp90-AR interaction in prostate cancer, it is less doubtful that this mechanism will be applicable for other client proteins that are key to multiple myeloma. A topoisomerase-I inhibitor, camptothecin, has been shown to disrupt association between Hsp90 and AR, interferes in binding of androgen to AR leading to inhibition of cell growth in LNCaP prostate cancer cells (Liu et al. 2010). Another such example of Hsp90-client protein interaction is Hsp90-survivin. Survivin plays a key role in promoting cell proliferation and preventing apoptosis in cancer cells (Wheatley and McNeish 2005). Fortugno et al. reported that survivin proteins remain physically associated with Hsp90 in human cervical cancer HeLa cells (Fortugno et al. 2003). They further demonstrated that Hsp90 is required for stability of survivin and inhibition of Hsp90 by geldanamycin induced proteasome-dependent degradation of survivin. Plescia et al., creatively identified minimal amino acid sequence in survivin that can block Hsp90-survivin interaction by using synthetic peptidyl mimicry (Plescia et al. 2005). The resultant peptide from this sequence was named shepherdin. Shepherdin inhibited interaction of survivin and Hsp90 not by binding at the middle domain, but at N-terminal ATP binding site of Hsp90 which is slightly different than geldanamycin site in that shepherdin does not require geldanamycin-specific D93 site for its activity. Shepherdin selectively killed cancer cells (prostate carcinoma and HeLa cells) over normal cells indicating less probability of having off-target effects or overt toxicity. Moreover, shepherdin also showed promising anticancer effects in prostate and breast cancer mouse models. Altogether, these studies suggest that strategy to disrupt Hsp90-client protein interaction may well work in multiple myeloma as well.

12.4 Hsp90 Inhibitors

There are many Hsp90 inhibitors in the clinical trials, however none is approved by FDA or European Medicines Agency (EMA) so far for the treatment of multiple myeloma (Table 12.1). The Hsp90 inhibitors are being studied in clinical trials either alone or in combination with existing preferred therapeutics which

Table 12.1 Hsp inhibitors under development for multiple myeloma

Class	Drug; *Company*	Target	Developmental stage completed	Adverse events in clinical trials or toxicity in animals	Combination (if any)	Reference	Comments
Geldanamycin derivatives	17-AAG (Tanespimycin; KOS-953); *Bristol-Myers Squibb*	NTD (ATP-binding site)	Phase 3	Diarrhea, back pain, asthenia, nausea, hepatotoxicity and gastrointestinal complaints	Single; combination with Bortezomib or rapamycin	Usmani and Chiosis (2011)	Halted clinical development in Phase-3 by BMS
Second generation Geldanamycin derivatives	Retaspimycin (IPI-504); *Infinity*	NTD (ATP-binding site)	Phase 1	Injection site pain; no dose limiting toxicity up to 400 mg/m^2	Single	Siegel et al. (2011)	IV
Resorcinol-Isoxazole derivative	NVP-AUY922; *Novartis*	HSP90	Phase 1/1B	Ocular toxicities (night blindness, photophobia, visual impairment), diarrhea, and nausea	Alone and with Bortezomib and dexamethazone	Seggewiss-Bernhardt et al. (2015)	Eightfold more potent than17-AAG in vitro; IV
Indazol-4-one	PF-04929113 (SNX5422); *Pfizer/ Serenex*	HSP90	Phase 1	Pruritis, prolonged QTc interval, fatigue, nausea, diarrhea and thrombocytopenia	Alone	Reddy et al. (2013)	Oral
Other HSP90 inhibitors being developed for non- multiple myeloma therapy							
Second generation Geldanamycin derivatives	Alvespimycin (17-DMAG); *Bristol-Myers Squibb*	NTD (ATP-binding site)	Phase 1 (halted)	Peripheral neuropathy, renal dysfunction, cardiotoxicity, fatigue, ocular toxicity, thrombocytopenia, pneumonitis,	Not available	Jhaveri et al. (2012)	IV
Synthetic (Purine scaffold agent)	MPC-3100; *Myriad*	NTD (ATP-binding site)	Phase 1	Not available	Not available	Sidera and Patsavoudi (2014)	Oral

Synthetic (Resorcinol-Triazole derivative)	STA-9090; *Synta*	HSP90	Phase 1/2	Not available	Not available	Sidera and Patsavoudi (2014)	IV
Synthetic	XL888; *Exelixis*	HSP90	Phase 1	Not available	Not available	Sidera and Patsavoudi (2014)	Oral
Synthetic (Resorcinol derivative)	AT13387; *Astex*	HSP90	Phase 1/2	Not available	Not available	Sidera and Patsavoudi (2014)	IV, Oral
Synthetic (Resorcinol derivative)	NVP-HSP990; *Novartis*	HSP90	Phase 1	Not available	Not available	Sidera and Patsavoudi (2014)	Oral
Purine analog	CNF2024 (BIIB021) *Biogen Idec*	NTD (ATP-binding site)	Phase 1/2	Not available	Not available	Sidera and Patsavoudi (2014)	Oral
Purine analog	PU-H71; *Samus therapeutics*	NTD (ATP-binding site)	Phase 1	Dose limiting toxicity (mucositis, liver enzyme elevation, nausea/vomiting, intolerable myalgia, anemia, intolerable headache)	Alone	Gerecitano et al. (2015) and Jhaveri et al. (2012)	IV

predominantly include bortezomib and dexamethasone. However, the clinical trial results indicated that Hsp90 inhibitors are more effective in combination with standard multiple myeloma chemotherapy. The molecular mechanisms for its reasons are discussed in following section. The obvious reason for better efficacy in combination with bortezomib is by combined inhibition of Hsp90 and proteasomal degradation leads to increased accumulation of unfolded proteins and enhanced unfolded protein response (UPR) in cancer cells leading to activation of apoptotic pathways.

The 17-AAG was the first geldanamycin derivative to enter in the clinical trials. However, poor solubility, limited bioavailability, and overt toxicity limited its success. In these trials, 17-AAD was studied as a single agent with no/minimal efficacy in multiple myeloma patients. However, some adverse events like diarrhea, hepatotoxicity, gastrointestinal complaints, and nausea were observed in patients. However, combination of 17-AAG with bortezomib in Phase 1/2 and abbreviated Phase 2 clinical trials showed some promising response rate and diminished severity of bortezomib-induced peripheral neuropathy in multiple myeloma patients (Richardson et al. 2010, 2011a). A Hsp90 inhibitor, NVP-AU922, was studied in Phase 1/1B clinical trials either as a monotherapy or in combination with bortezomib for treatment of multiple myeloma. During the treatment, 12 out of 24 (50%) patients discontinued the NVP-AU922 treatment for various reasons while 8 out of remainder 12 showed adverse events without partial or complete response which may be due to compensatory upregulation of Hsp70, another chaperon like Hsp90. However, 66.6% patient showed disease stabilization. All the five patients enrolled in NVP-AU922 (50 mg/m^2) plus bortezomib (1.3 mg/m^2) combination therapy showed adverse events and 3 out of 5 showed dose limiting toxicities (Seggewiss-Bernhardt et al. 2015). Similar effect of disease stabilization was observed in multiple myeloma patients during Phase 1 clinical trial of PF-04929113, a Hsp90 inhibitor (Reddy et al. 2013). The treatment with PF-04929113 also showed some adverse events (Table 12.1). Both NVP-AU922 and PF-04929113 had shown significant anticancer activity in in vitro and in vivo models (Okawa et al. 2009; Stuhmer et al. 2008).

12.5 Challenges in Targeting Hsp90

It is intriguing to question that why none of the Hsp inhibitors have been yet approved by FDA in spite almost 20 such inhibitors have gone through clinical trials. The analysis of data published about Hsp90 inhibitors indicate two possible reasons for their failure: No better efficacy than existing treatment regime; and toxicity/adverse events. Clinical trials have revealed that inhibition of Hsp90 did not lead to a remarkable cure rather at the most it stabilized the disease (Ramanathan et al. 2007; Reddy et al. 2013; Seggewiss-Bernhardt et al. 2015). One of the main reasons for Hsp90 inhibitors for not achieving desirable efficacy in cancer patients is induction of heat shock response in cancer cells in response to Hsp90 inhibition and induction of other compensatory mechanisms. This compensatory phenomenon

is not new to be observed in cancer cells. To adhere to the Darwin's theory of evolution, cancer cells remodel themselves to survive the repeated attacks of chemotherapy. Normal eukaryotic cells (and cancer cells as well) have a huge incomprehensible network of cellular proteins and their signaling. Under normal conditions, there are some ancillary and "redundant" (not really!) pathways, the role of which may not be much appreciated in normal cells by the scientists. However, these "redundant" pathways drive the survival process in cancer cells when their "main" networks are disrupted by chemotherapy. The similar process has been indicated to be involved in targeting Hsp90. During clinical trials of Hsp90 inhibitors, expression of Hsp70 protein is monitored as pharmacodynamic biomarker for Hsp90-inhibitors and indeed, Hsp70 was found to be upregulated in the patients treated with Hsp90 inhibitor 17-AAG (Banerji et al. 2005; Goetz et al. 2005; Ramanathan et al. 2007; Solit et al. 2007). It is hypothesized from the molecular studies that the resistance to Hsp90 inhibition by 17-AAG and NVP-AUY922 may be a result of compensatory overexpression of Hsp70 and Hsp27 chaperons (McCollum et al. 2006; Seggewiss-Bernhardt et al. 2015). Hsp90 is a negative regulator of HSF-1 and inhibition of Hsp90 activates HSF-1 in homotrimer form. The activated HSF-1 in turn induces expression of chaperones such Hsp70 and Hsp27 and co-chaperons like Aha1 which have major pro-survival roles. Hsp70 binds to Bax (a proapoptotic protein) preventing it to localize in mitochondria and induce apoptosis (Guo et al. 2005). Considering anti-apoptotic role of Hsp70, no wonder that Hsp70 offers protection against neurodegenerative diseases and trauma by modulating inflammatory and apoptotic pathways (Magrane et al. 2004; Yenari 2002). This is considered as a plausible mechanism behind neuroprotective effect of tanespimycin, a Hsp90 inhibitor, in bortezomib-induced peripheral neuropathy (Argyriou et al. 2008; Cavaletti and Jakubowiak 2010; Flint et al. 2009; Richardson et al. 2011a). Clinically, other compensatory mechanisms conferring resistance to Hsp90 inhibitors needs to be shunted for their full effect. The other chaperones like Hsp70 and Hsp27 needs to be inhibited simultaneously either by another inhibitor or a single inhibitor with selective poly pharmacologic effect blocking these chaperones along with Hsp90 (McCollum et al. 2008). The siRNA mediated abrogation of Hsp70 along with Hsp90 inhibition has proved to be an effective strategy to overcome Hsp70 medicated resistance to 17-AAG treatment in acute myelogenous leukemia (AML) cells (Guo et al. 2005). Similarly, Hsp70 inhibition with VER155008 has also exhibited potentiation of Hsp90 inhibitor (17-AAG)-induced apoptosis in human colon cancer cells (Massey et al. 2010). The other small molecule inhibitors of Hsp70 such as A17, ADD70 and PES, have been developed although for non-multiple myeloma cancers (Jego et al. 2013). There is hope that these small molecule inhibitors of Hsp70 can be effective as a co-treatment with Hsp90 inhibitors in multiple myeloma as well. In 2015, Lu et al. had reported discovery of KU675, a selective small molecule inhibitor of Hsp90α (K_d = 191 μM) and Hsp70 (K_d = 76.3 μM) over Hsp90β (K_d = 726 μM), and anticancer activity in prostate cancer cells (Liu et al. 2015). It is intriguing to question whether this dual inhibitor of Hsp90 and Hsp70 will show activity in multiple myeloma cells. The natural flavonoid quercitin and synthetic small molecule inhibitor KNK437 have been found to inhibit Hsp70 expression at mRNA level mediated

by HSF-1 inhibition in HeLa human cervical cancer, HL-60 AML or COLO 320DM human colon carcinoma cells (Guo et al. 2005; Nagai et al. 1995; Yokota et al. 2000). Moreover, HSF-1 induction in response to Hsp90 inhibition with 17-AAD also induces activity of co-chaperons like Aha1 which increases the phosphorylation of pro-survival kinases like Mek1/2 and Erk1/2 and activity of c-Raf in HT-29 colon cancer cells (Holmes et al. 2008). However, selective silencing of Aha1 along with 17-AAD in these studies sensitized HT-29 cells to 17-AAD treatment with two- to threefold increase in apoptosis. Although, these results need to be confirmed in multiple myeloma cells, these strongly indicate that combination therapy of Hsp90 inhibitors and co-chaperon inhibitors may be effective in developing such a therapeutic strategy for multiple myeloma. Actually, in 2017, Stiegler et al. identified small molecule HAM-1 as an inhibitor of Hsp90-Aha1 complex (Stiegler et al. 2017). Co-inhibition of HSF-1, which is considered as main cause of developing 'heat shock response'-mediated resistance to Hsp90 inhibitors, may also seem an attractive strategy. But, as a word of caution, targeting HSF-1 with small molecule inhibitors may bring challenges of non-specific inhibition of HSF-1 in normal cells as well, which can lead to toxicity. This issue can be circumvented by synthesizing cancer specific antibody-drug conjugates which can be delivered specifically to tumors only and thus avoiding any untoward effects resulting from inhibition of HSF-1 in normal cells. The alternate strategy could be to inhibit the final product of these co-chaperones, like glutathione (GSH) of Hsp27, which is also responsible for conferring resistance to Hsp90 inhibitors (McCollum et al. 2006). The delivery of the drug to the inaccessible Hsp90 located in the mitochondria would be challenging, which may also confer some degree of resistance to Hsp90 inhibitors (Piper and Millson 2011).

Another challenge is to develop relatively safe Hsp90 inhibitors. Inherently, any therapeutic that binds to the ATP-binding pocket of kinases are promiscuous for the target with low selectivity. This results in increased toxicity and adverse events. This observation is supported by adverse events reported the clinical trials on Hsp90 inhibitors. These adverse events ranged from ocular toxicity (night blindness, photophobia, and visual impairment), diarrhea, nausea, prolonged QTc, thrombocytopenia, peripheral neuropathy, pneumonia, and hyponatremia. However, the ultimate decision for the development of such inhibitors targeting ATP-binding sites depends on continual efforts in improving their specificity and their risk-benefit ratio.

12.6 Conclusions

It has now been established that targeting one protein or signaling pathways would not be sufficient for multiple myeloma treatment. Therefore, targeting selective multiple proteins and signaling pathways concurrently is the future of cancer treatment, particularly multiple myeloma. Along these lines, combination therapies are now common practice in myeloma treatment. Furthermore, poly pharmacological agents have been also appreciated and got great amount of attention recently. It may

be worthwhile approach to target chaperone proteins because these proteins regulate a number of key events in carcinogenesis. As Hsp are highly expressed in cancer cells as compared to their normal counterpart, it makes them an attractive target. Importantly, early results from combination clinical trial are encouraging. Furthermore, targeting several Hsp together may be a good option as it has been observed that simultaneous inhibition of Hsp90 and Hsp70 is more effective compared to single targeting, nonetheless, further studies are required to fully validate this observation (Cavanaugh et al. 2015). Despite the progress in Hsp inhibitor development there are several challenges that need to be addressed. First, the complete understanding of the mechanism is required, because Hsp exist in several isoforms and perform similar functions. Dissecting the roles of individual Hsp and the effect of combined inhibition of multiple Hsp is the key in order to develop an effective treatment strategy against myeloma. Second, the development of suitable Hsp inhibitors for the clinic remains a major challenge. Overall, much more effort is needed for targeting Hsp and exploiting this protein for better treatment in multiple myeloma.

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Chapter 13
IER5 Is a p53-Regulated Activator of HSF1 That Contributes to Promotion of Cancer

Tatsuya Kawase, Yu Chen, and Rieko Ohki

Abstract The *p53* gene is one of the most frequently mutated genes in human cancer and functions as a tumor suppressor and transcriptional factor that regulates various genes involved in cancer. One p53 target gene is *IER5,* whose function was initially unknown, but we have shown facilitates the activation of the transcriptional activator HSF1 by recruiting the PP2A phosphatase to HSF1, leading to its hypophosphorylation and activation. HSF1 is the master transcriptional regulator of the *HSP* genes, which encode molecular chaperones essential for cellular homeostasis. HSP also exhibit anti-apoptotic functions by repressing pro-apoptotic factors, thereby protecting stressed cells from cell death. Although HSF1-HSP pathway is generally activated by cellular stress such as heat shock, this pathway is also hyperactivated in cancers independent of heat shock and contribute to promotion of cancer development and resistance to cancer treatments. We observed that *IER5* is overexpressed in several cancers in a p53-independent manner and contributes to tumor malignancy via activation of the HSF1-HSP pathway. We propose a model in which IER5 activates HSF1 in cancer as part of the p53-IER5-HSF1-HSP pathway, thereby providing stress resistance to cancer cells. This section briefly reviews the roles of HSF1, HSP, p53 and IER5.

Keywords Cancer · HSF1 · HSP · IER5 · p53 · Post-translational modification · PP2A

T. Kawase
Unit 1, Candidate Discovery Science Laboratory, Drug Discovery Research, Astellas Pharma Inc., Tsukuba-shi, Ibaraki, Japan

Laboratory of Fundamental Oncology, National Cancer Center Research Institute, Tokyo, Japan

Y. Chen · R. Ohki (✉)
Laboratory of Fundamental Oncology, National Cancer Center Research Institute, Tokyo, Japan
e-mail: rohki@ncc.go.jp

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Abbreviations

4C-seq	circular chromatin conformation capture sequencing
ABC	ATP binding cassette
ABCB1	ATP-binding cassette sub-family B member 1
AIF	apoptosis-inducing factor
Apaf-1	apoptosis protease activating factor 1
ASK1	apoptosis signal regulating kinase 1
BAG3	Bcl-2-associated athanogene domain 3
Bax	Bcl-2-associated X protein
CAD	caspase activated DNase
ChIP-seq	chromatin immunoprecipitation sequencing
GOF	gain-of-function
HSEs	heat shock elements
HSF1	heat shock factor 1
HSP	heat shock proteins
ICAD	inhibitor of CAD
IER5	immediate early gene response 5
LFS	Li-Fraumeni syndrome
MDM2	murine double minute 2
MDR	multidrug resistance
MDR-1	multidrug resistance protein 1
Pgp	P-glycoprotein
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homolog

13.1 Introduction

The p53 protein was independently discovered by several groups in 1979 as a molecular partner of SV40 large T antigen (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). Until the mid-1980s, the *p53* gene had been generally regarded as an oncogene based on several experimental observations: the p53 protein was bound to the major oncogenic protein of SV40; high levels of p53 expression were detected in various cancers; and overexpression of p53 transformed normal cells into cancerous cells (Eliyahu et al. 1984; Parada et al. 1984). Later, however, Hinds and colleagues showed that this ability to transform cells in these experiments was due to a mutation in the *p53* gene (Hinds et al. 1989). In 1988, genetic analyses of colorectal cancer cells revealed a very high rate of heterozygous loss of the short (p) arm of chromosome 17, which carries the *p53* gene (Vogelstein et al. 1988). Analysis of the *p53* gene sequence derived from tumor specimens showed that it often contains point mutations (Baker et al. 1989; Takahashi et al. 1989). Furthermore, germ-line mutations of the *p53* gene were found in patients with Li-Fraumeni syndrome, which is associated with a broad

spectrum of cancers including osteosarcomas, breast cancer, soft tissue sarcoma and leukemia (Malkin 2011; Malkin et al. 1990). Since then, *p53* has been regarded as a tumor suppressor gene. The p53 has two divergent functions in normal cells, both as a protector and as a killer (Aylon and Oren 2007; Harris and Levine 2005; Vousden and Prives 2009). p53 is a transcription factor that induces its target genes in a manner dependent on the type, intensity and tissue context of cell damage. Under conditions of mild stress, p53 induces genes involved in cell cycle arrest, DNA repair and antioxidation to protect and restore cells that are not damaged beyond repair. However, under severe stress conditions, p53 induces genes involved in cellular senescence or apoptosis to eliminate cells that are beyond repair and thus have the potential to become cancerous. Interestingly, it has recently been suggested that the protective function of wild-type p53 could also play a role in promoting cancer development or progression. For example, among the p53 target genes are several that adapt cells to metabolic changes such as nutrient deprivation and ROS. This function could enable cancer cells to survive under harsh conditions and thereby contribute to cancer progression (Vousden and Prives 2009).

The transactivation function of p53 is essential to its ability to suppress cancer since most of the mutant p53s found in human cancers have lost this transactivation function (Levine 1997). Therefore, identification of p53 target genes is an important goal of p53 research. Analysis of p53 target genes, such as *MDM2* and *PTEN*, have further led to identification of novel cancer biology and anti-cancer drug targets. *MDM2* encodes a negative regulator of p53 and functions as an oncogene when overexpressed in cancers such as osteosarcoma (Momand et al. 1992; Oliner et al. 1992; Barak et al. 1993). Suppression of Mdm2 expression induces the accumulation/activation of p53 and thereby inhibits the proliferation of cancers in which Mdm2 is overexpressed. Based on this, inhibitors of Mdm2 have been developed and tested in clinical trials as anti-cancer drugs (Fesik 2005). PTEN, a PIP_3 phosphatase, has also been shown to function as a tumor suppressor (Maehama and Dixon 1999; Stambolic et al. 2001). We have more recently undertaken the identification of novel p53 target genes using microarray and ChIP-seq analysis with the goal to discover novel cancer biology or anti-cancer drug targets. In particular, we have focused on target genes with previously unknown functions that we could analyze to try to understand their roles and potential as novel drug targets (Asano et al. 2016; Ezawa et al. 2016; Kawase et al. 2008, 2009; Ohki et al. 2007, 2014). One particular example is the *PHLDA3* gene, whose product we determined is a repressor of the Akt oncogene and plays a role as a novel tumor suppressor of pancreatic neuroendocrine tumors (Kawase et al. 2009; Ohki et al. 2014; Takikawa and Ohki 2017).

Another gene we have recently identified as a novel p53 target gene is *IER5* (Asano et al. 2016). Functional analysis of IER5 revealed that IER5 activates HSF1 in a heat shock-independent manner resulting in the induction of HSF1 target genes, including the *HSP* genes. In addition, we showed that transcription of *IER5* is highly activated in various cancer specimens and in cancer cell lines. Consistent with these observations, it has been also reported that HSF1 is aberrantly activated and HSP are overexpressed in various cancers (Santagata et al. 2011; Ciocca and Calderwood 2005). We would further suggest that IER5 could

contribute to the tumor-promoting effects of wild-type p53 due to the ability of IER5 to regulate the HSF1-HSP pathway, which has a cellular protective function. This section briefly reviews the roles of HSF1, HSP, p53 and the novel p53 target gene IER5.

13.1.1 HSF1 and HSP

HSF1 is an evolutionarily highly conserved transcriptional factor and a master regulator of the heat shock response (Akerfelt et al. 2010). HSF1 contains four functional domains including a DNA-binding domain, an oligomerization domain, a transactivation domain and a regulatory domain (Fig. 13.1a). Under normal conditions, HSF1 exists in the cytoplasm as an inactive monomer that is repressed by its interaction with a protein complex consisting of Hsp90 and Hsp70 (Baler et al. 1996; Zou et al. 1998). Proteotoxic stressors such as heat shock trigger the dissociation of this repressive protein complex and the release of inactive HSF1. Subsequently, HSF1 is activated through trimerization, nuclear translocation, and post-translational modifications including phosphorylation, acetylation, and sumoylation (Anckar and Sistonen 2011) (Fig. 13.1b). In particular, phosphorylation of HSF1 has been shown to be critical for modulating its activation (Fig. 13.1a). For instance, it has been reported that phosphorylation of HSF1 at S121, T142, S303, S307 and S363 inhibits HSF1 activation, whereas phosphorylation at S230, S320, S326 and S419 induces its activation (Chou et al. 2012; Chu et al. 1998; Holmberg et al. 2001; Kim et al. 2005; Soncin et al. 2003; Wang et al. 2004, 2006; Zhang et al. 2011). In the nucleus, activated trimeric HSF1 binds to specific heat shock elements (HSEs) in the proximal promoter region of its target genes, and consequently induces a number of these genes including Hsp27, Hsp70 and Hsp40 (Akerfelt et al. 2010; Anckar and Sistonen 2011).

HSP are a subset of molecular chaperons that are expressed in response to increased temperature or a variety of other cellular stresses. When activated they participate in the folding of denatured or aggregated client proteins, promote the assembly/disassembly of multiprotein complexes, regulate protein degradation and facilitate protein translocation (Richter et al. 2010). Thus, HSP are important to tissue homeostasis in normal cells. In addition, a number of studies have indicated that HSP possess multiple functions to suppress apoptosis (Takayama et al. 2003). For instance, Hsp27 interferes with apoptosis by directly binding to cytosolic cytochrome c and sequestering it from Apaf-1 (Bruey et al. 2000; Garrido et al. 1999). Hsp70 protects cells from apoptosis by interacting with p53 and other pro-apoptotic factors including Apaf-1, AIF, ASK1 and Bax (Beere et al. 2000; Park et al. 2002; Ravagnan et al. 2001; Saleh et al. 2000; Wiech et al. 2012; Yang et al. 2012). Hsp40, one co-chaperon of Hsp70, negatively regulates CAD, a factor that causes chromosomal DNA fragmentation during apoptosis, by interacting with Hsp70 and ICAD (Sakahira and Nagata 2002).

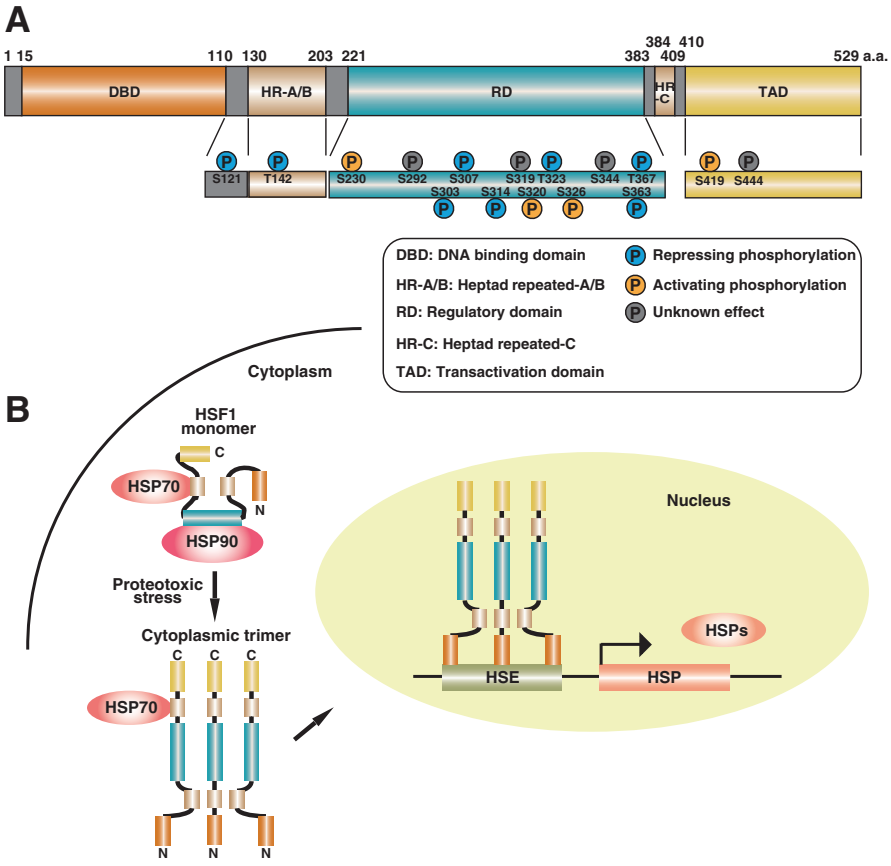

Fig. 13.1 Schematic view of HSF1 domain structure, post-translational modification and mechanism of activation. (**a**) HSF1 is comprised of four functional domains including a DNA-binding domain, an oligomerization domain (heptad repeated domain), a transactivation domain and a regulatory domain. Phosphorylation of HSF1 critically regulates HSF1 activation and inactivation. (**b**) The process of HSF1 activation

13.1.2 HSF1, HSP and Cancer

HSF1 is expressed at high levels in cancer cells and has recently been recognized as playing a significant role in cancer. HSF1 is constitutively activated in cancers even in the absence of heat-shock conditions and higher HSF1 activity has been reported to be associated with poor prognosis in breast cancer patients. However, the mechanism by which HSF1 is activated in cancer has remained unclear (Santagata et al. 2011). HSF1 has also been shown to transactivate a variety of genes involved in tumor progression and to promote tumorigenesis, a property that is critically dependent on its ability to upregulate HSF1 target genes including the HSP family of genes (Mendillo et al. 2012). As with HSF1, HSP are also highly expressed in

cancer and their overexpression is associated with poor prognosis in various types of cancer (Ciocca and Calderwood 2005). For instance, Hsp27 overexpression has been reported to be correlated with poor prognosis in gastric cancer, liver cancer, prostate cancer and osteosarcomas (Cornford et al. 2000; King et al. 2000; Takeno et al. 2001; Uozaki et al. 2000). Hsp70 overexpression is associated with poor prognosis in breast cancer, endometrial cancer, uterine cervical cancer and bladder cancer (Ciocca et al. 1993; Nanbu et al. 1998; Piura et al. 2002; Syrigos et al. 2003; Thanner et al. 2003). These data indicate that HSF1 and HSP are involved in cancer progression, and indeed their expression is regarded as useful diagnostic and prognostic markers of cancer in certain tissues.

A number of recent studies have identified HSF1 and Hsp70 as therapeutic targets for cancer treatment. For example, elimination of HSF1 has been shown to protect mice from tumors induced by mutation of the RAS oncogene or by a hot spot mutation in tumor suppressor p53 and from DEN-induced hepatocellular carcinoma formation (Dai et al. 2007; Jin et al. 2011). In addition, HSF1 knock-out or knock-down cancer cells have been shown to be more sensitive to Hsp90 inhibitors (Chen et al. 2013). As mentioned in the previous section, Hsp90 represses HSF1, and therefore, Hsp90 inhibitors confer resistance to stress in cancer cells via activation of the HSF1-HSP pathway. Simultaneous inhibition of both Hsp90 and HSF1 may be required for the effective elimination of cancer cells. On the other hand, RNAi silencing of Hsp70 has been shown to inhibit human gastric cancer growth by inducing apoptosis and enhancing the efficacy of radiotherapy (Du et al. 2009; Xiang et al. 2008). Cancer cells can acquire multidrug resistance to chemotherapy through overexpression of ABC transporters including Pgp, also known as MDR-1 or ABCB1, which results in the efflux of the therapeutic agents (Wang et al. 2017). HSF1 and Hsp70 play important roles in inducing MDR and resistance to therapies. For instance, HSF1 has been shown to induce an MDR phenotype by modulating the stability and/or the splicing of MDR-1 transcripts (Tchenio et al. 2006). In addition, Hsp70 have been reported to stabilize some mutant forms of p53, which in turn activates the MDR-1 gene, resulting in an MDR phenotype (Chin et al. 1992; Wiech et al. 2012). Thus, HSF1 and Hsp70 are thought to be promising therapeutic targets to suppress MDR and enhance the efficacy of therapeutic agents against chemoresistant cancers.

13.1.3 HSF1 Inhibitors and HSP70 Inhibitors

Since HSF1 and Hsp70 inhibitors are thought to be promising anti-cancer agents, a number of groups have tried to identify small-molecule inhibitors of HSF1 and Hsp70 (Chatterjee and Burns 2017; Dayalan Naidu and Dinkova-Kostova 2017; Evans et al. 2010). For example, Kim and colleagues showed that the natural compound cantharidin induces cancer cell death by blocking the binding of HSF1 to the promoters of HSF1 target genes, and resulting in the downregulation of Hsp70 and BAG3 expression (Kim et al. 2013). Rohinitib, also known as rocaglate, has been reported to indirectly inhibit HSF1 by interfering with ribosomal translation initiation (Dayalan Naidu and Dinkova-Kostova 2017). In addition, novel hybrid HSF1

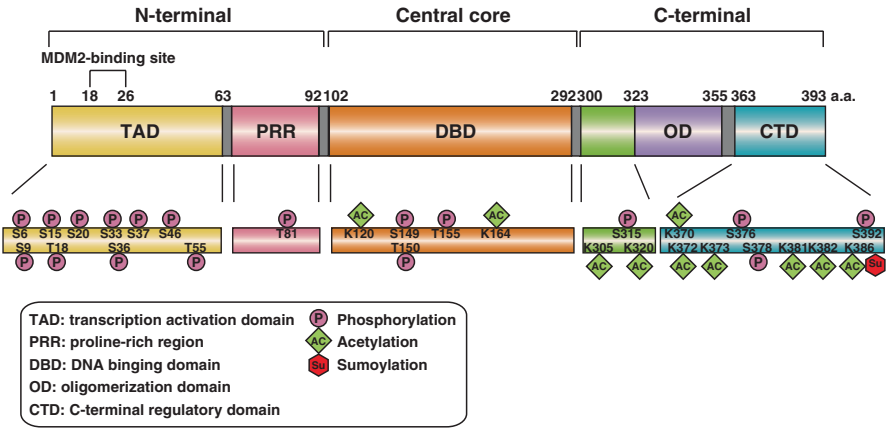

Fig. 13.2 Schematic view of the domain structure and post-translational modification of p53. The 393-amino acid p53 protein is comprised of several functional domains: a transactivation domain and proline-rich region in the N-terminus; a DNA-binding domain in the central region; and a tetramerization and regulatory domain in the C-terminus. Stabilization and activation of p53 require the post-translational modification of p53, including phosphorylation, acetylation and sumoylation

inhibitors based on rohinitib and cantharidin have been reported to have potent inhibitory activity against HSF1 (Agarwal et al. 2015). Other HSF1 inhibitors such as quercetin, KNK437, triptolide and KRIBB11 have been demonstrated to affect the transcriptional activity of HSF1 (Nagai et al. 1995; Ohnishi et al. 2004; Westerheide et al. 2006; Yoon et al. 2011). However, proof of concept that HSF1 inhibition can assist in the treatment of human cancers awaits future clinical trials. A number of HSP70 inhibitors such as MKT-077, pifithrin-μ, 15-DSG, MAL3-101 and VER155008 have been reported to inhibit Hsp70 in biological assays. In addition, a few compounds including minnelide have been tested in clinical trials (Evans et al. 2010). Minnelide, a prodrug of Triptolide, has been shown to suppress Hsp70 and inhibit in vivo growth in a xenograft model of mesothelioma (Jacobson et al. 2015). Given these results, we may expect that inhibitors of HSF1 and Hsp70 could be approved as cancer therapeutics in the future.

13.1.4 p53, a Tumor Suppressor and a Transcription Factor

The *p53* gene is the most frequently mutated tumor suppressor gene in human cancers. Both somatic and germline mutations of *p53* can lead to the development of various cancers, and the involvement of p53 in suppression of various types of cancers is one of its hallmarks. The *p53* gene encodes a transcriptional factor and its structure contains several functional domains. These include a transactivation domain and proline-rich region in the N-terminal region, a DNA-binding domain in its central region, and a tetramerization and regulatory domain in its C-terminal region (Bode and Dong 2004) (Fig. 13.2). Under normal physiological conditions, the p53 protein

has a short half-life and is maintained at low levels due to MDM2-mediated ubiquitin/proteasome degradation. However, p53 protein is stabilized and activated in response to various cellular stresses including DNA damage, hypoxia, oncogene activation and virus infection (Harris and Levine 2005). Stabilization and activation of p53 are regulated by various post-translational modifications including phosphorylation, acetylation, and sumoylation (Bode and Dong 2004; Meek and Anderson 2009) (Fig. 13.2). For example, p53 is phosphorylated by several kinases such as CHK2 at Thr18 and Ser20, which are located within the alpha-helix involved in MDM2 interaction. Phosphorylation of this region stabilizes p53 following DNA damage by disrupting the p53-MDM2 interaction (Chehab et al. 1999; Sakaguchi et al. 2000; Shieh et al. 1999; Unger et al. 1999). Additionally, phosphorylation at Ser15 has been shown to increase the association of p53 with p300/CBP and to stimulate its transactivation function (Dumaz et al. 1999; Lambert et al. 1998). Acetylation of p53 at Lys370/372/373/381/382/386 is also significant for p53 stabilization and activation as this facilitates the recruitment of CBP/p300, which interferes with MDM2-mediated p53 ubiquitination (Barlev et al. 2001; Rodriguez et al. 2000; Wei et al. 2006).

Activated p53 binds as a tetramer to p53 consensus binding sequences in the proximal promoter or intron region of its target genes. These binding sites consist of two copies of the ten base pair motif 5′-RRRCWWGYYY-3′ (R=G or A, W=T or A, Y=C or T) separated by 0–13 base pairs, (el-Deiry et al. 1992). The genes induced by p53 are involved in cell cycle arrest, DNA repair, senescence, metabolism, regulation of antioxidant generation and apoptosis, which collectively function to prevent normal cells from turning into cancerous cells. Under conditions of severe stress, p53 can induce target genes involved in apoptosis (e.g. *PUMA*, *NOXA*) or cellular senescence (e.g. *PAI-1*), which function to eliminate cells that have the potential to become cancerous (Kortlever et al. 2006; Kunz et al. 1995; Nakano and Vousden 2001; Oda et al. 2000). In addition, p53 induces genes related to cell cycle arrest (e.g. *p21WAF1/CIP1*), regulation of antioxidant generation (e.g. *TIGAR*, *Sestrins*), and DNA repair (e.g. *p53R2*) to protect and facilitate the recovery of cells from damage under conditions of mild stress (Bensaad et al. 2006; Bensaad and Vousden 2007; Budanov et al. 2004; el-Deiry et al. 1993; Tanaka et al. 2000). Together, these p53-mediated responses to various types of cellular stress suppress tumorigenesis in normal cells.

Most p53 mutations are missense mutations located in its DNA binding domain, whereas mutations found in many other tumor suppressor genes, for example *BRCA1/2* or *APC*, are mainly nonsense mutations. In particular, the residues R175, G245, R248, R249, R273 and R282 in the p53 protein are frequently mutated in cancer, and are therefore called hot spot mutations. Mutant p53 proteins found in human cancers can neither bind to the promoters of nor induce expression of the target genes normally induced by wild-type p53, indicating that the ability of p53 to prevent cancer development and progression depends on its gene-activation function. In addition, many mutant p53 proteins, including those resulting from hot spot mutations, acquire oncogenic gain-of-function properties that promote invasion, migration, angiogenesis and/or suppress apoptosis (Freed-Pastor and Prives 2012; Muller and Vousden 2014). Functional analysis of these mutant p53 proteins is an ongoing area of research that should further expand our understanding of p53 in the future.

13.1.5 p53 and Tumor Promotion

Although *p53* clearly plays an important role in tumor suppression, it has more recently been suggested that the protective functions of wild-type p53 such as DNA repair, cell cycle arrest and regulation of antioxidant generation could also contribute to tumor development in some contexts (Vousden and Prives 2009). Indeed, many cancers express wild-type p53, including sarcoma, breast cancer, cervical cancer, melanoma and neuroendocrine tumors. In sarcoma, breast cancer or cervical cancer, it has been reported that wild-type p53 is degraded or inactivated by several mechanisms. For example, p53 protein can be degraded by overexpression of MDM2 or MDMX (also known as MDM4) in sarcomas and breast cancers (Danovi et al. 2004; Oliner et al. 1992). In cervical cancers, the E6 protein derived from human papillomavirus interacts with p53 and abrogates p53 function (Scheffner et al. 1990). On the other hand, we have confirmed that p53 in neuroendocrine tumors has an apparently normal transactivation function and appears functional. To date, no negative regulators of p53 have been reported in neuroendocrine tumors. Therefore, we believe that analysis of wild-type p53 and its potential role in promoting tumor development such as in neuroendocrine tumors could be of great interest. Below we propose a model in which the p53-IER5-HSF1-HSP pathway may promote cancer development.

13.1.6 p53 and IER5

In order to identify genes involved in the regulation of tumorigenesis, and to dissect the functional relevance of p53 phosphorylation in the induction of its target genes, we undertook an exhaustive effort to identify p53 target genes that are induced in a manner dependent on p53 phosphorylation status. We found that induction of approximately 80% of the p53 target genes depends on p53 phosphorylation, illustrating the importance of this modification in p53's transactivation function. One of the genes identified by our screen was the immediate early response gene 5 (*IER5*), which can be induced by wild-type p53 but not a phosphorylation-deficient p53. To confirm that IER5 is a direct target of p53, we performed ChIP-seq analysis and identified a p53 binding site 46 kb downstream of the *IER5* gene containing sequences highly similar to the consensus p53 binding sequence. When this binding site was tested in a luciferase promoter reporter construct, we observed that p53 strongly activated the reporter. Because the p53 binding site was quite distant from the *IER5* gene promoter region, we performed circular chromatin conformation capture (4C-seq) to analyze potential three-dimensional chromatin interactions. We detected chromatin interaction between the downstream p53 binding site and the *IER5* promoter. From these analyses, we confirmed that *IER5* is a direct target of p53. In addition to our results, Wei et al. and Melo et al. have also identified p53 binding at the *IER5* gene by a comprehensive genome-wide analysis of p53 binding sites (Wei et al. 2006; Melo et al. 2013).

13.1.7 IER5 and Cancer

Although we determined that *IER5* is a p53 target gene, the molecular function of IER5 was totally unknown when we started our work. The *IER5* gene was originally characterized as an immediate-early gene induced by various growth-promoting stimuli (Williams et al. 1999). Consistent with this, we observed that *IER5* could be induced by various mitogenic stimuli such as PMA, serum and ionomycin. In addition, Yoon and his colleagues revealed that *IER5* was overexpressed in cervical cancers (Yoon et al. 2003). We also observed *IER5* upregulation and overexpression in a number of cancers using the Gene Logic SCIANTIS database and COSMIC database. These results collectively suggest that various growth-promoting and oncogenic signals may induce expression of *IER5*. Furthermore, we found so-called "super-enhancers" localized around the *IER5* gene locus, including the p53 binding site, in various cancer cell lines. It has been reported that many cancer cells acquire these super-enhancers at key driver oncogenes as well as other genes important for tumor pathogenesis. The presence of super-enhancers at the *IER5* gene locus suggests that *IER5* expression may be important for cancer cells (Hnisz et al. 2013; Loven et al. 2013). To analyze the function of IER5 in cancer cells, we first examined the effect of IER5 expression on their proliferation. First, we observed high expression of *IER5* in cells in which the *IER5* gene was associated with super-enhancers. Knock down of IER5 expression in these cancer cells resulted in a marked decrease in proliferation in suspension culture conditions and in soft agar, indicating that IER5 is required for anchorage-independent cancer cell growth. These results collectively indicate that IER5 expression is required for cancer cell proliferation.

13.1.8 IER5 and HSP

To further understand IER5 function, we undertook a comprehensive analysis of gene expression in cells expressing IER5. This analysis showed the *HSP* family genes (*HSPA1A*, *HSPA1B*, *HSPA6*, *DNAJB1*, *HSPB1* and *HSPH1*) were transcriptionally upregulated by IER5 expression. Furthermore, the *HSP* family gene promoters were strongly activated by IER5, indicating that IER5 induces transcription of *HSP* family genes. Next, using luciferase promoter reporter assays, we showed that the *HSP* family gene promoters were strongly activated by IER5. We then generated a series of serially deleted *HSPA1A* promoters and cloned these upstream of the luciferase reporter gene. We found that the region between – 310 and – 100 relative to the transcription initiation site of the *HSPA1A* gene was responsible for IER5-dependent expression (Fig. 13.3). Analysis of the sequences within this region revealed the presence of an HSF1 transcription factor binding site, i.e. a heat shock element (HSE). Given the presence of this HSE within the IER5-responsive promoter region, we expected that HSF1 would be required for induction of the *HSP*

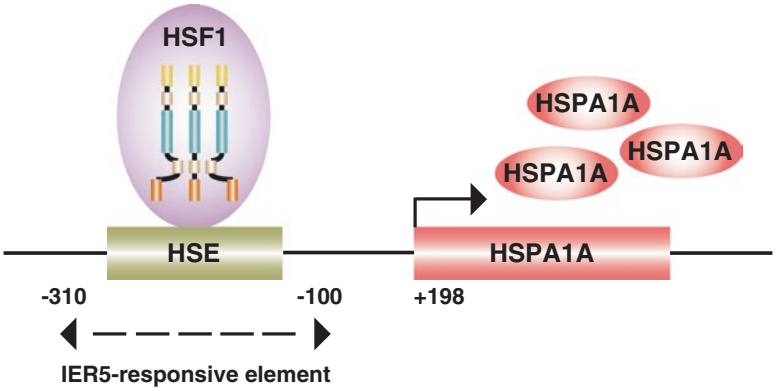

Fig. 13.3 Identification of the IER5 responsive region within the HSPA1A promoter. The region between −310 and −100 relative to the transcription initiation site of the *HSPA1A* gene mediates IER5 responsiveness. The IER5-responsive region contains an HSE (heat shock element) which is recognized by HSF1

family genes by IER5. Indeed, we found that knockdown of HSF1 suppressed IER5-mediated induction the *HSP* family genes, demonstrating that upregulation of the *HSP* family genes by IER5 is dependent on HSF1.

13.1.9 IER5 and Phosphorylation of HSF1

We found that expression of IER5 decreased the interaction between HSF1 and Hsp90 and resulted in increased HSF1 trimerization. IER5 also induced a greater accumulation of HSF1 in the nucleus. In addition, binding of HSF1 to HSE was strongly increased in cells overexpressing IER5. HSF1 trimerization, nuclear localization and acquisition of DNA binding ability are indicators of HSF1 activation, demonstrating that IER5 can activate HSF1. HSF1 activation involves various post-translational modifications, particularly phosphorylation, and we found that IER5 expression dramatically altered the post-translational modification of HSF1 (Fig. 13.4). Furthermore, LC-MS/MS analysis revealed that HSF1 phosphorylation is reduced at multiple sites in cells expressing IER5. In particular, strong reduction in phosphorylation was observed at five residues: Ser121, Ser307, Ser314, Thr323 and Thr367. It has been reported that phosphorylation at Ser121 represses HSF1 trimerization, while phosphorylation at Ser307 reduces transactivation by HSF1. These results suggested that IER5 induces HSF1 trimerization and activation through dephosphorylation of the residues involved in the repression of HSF1 activity (Wang et al. 2004, 2006). While we showed that dephosphorylation at Ser314, Thr323 and Thr367 was required for expression of *HSPA1A*, the mechanism by which this alters transactivation is unclear at present and would be an interesting subject for future study. The ability of IER5 to cause decreased phosphorylation of

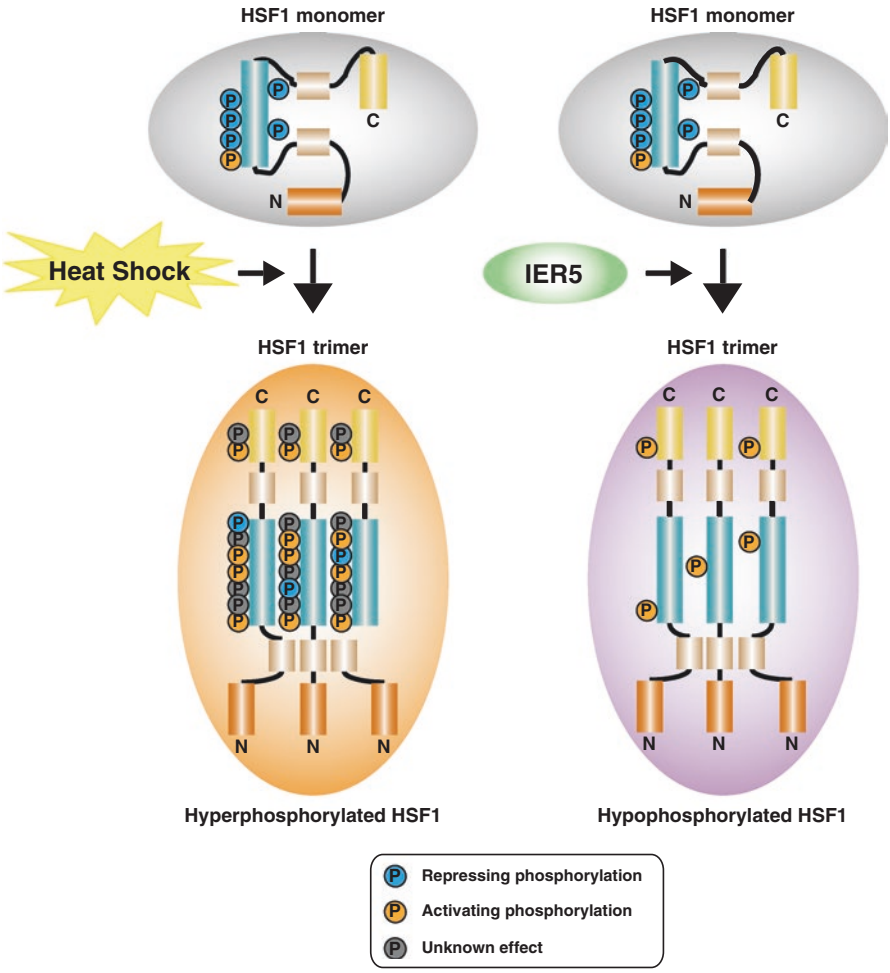

Fig. 13.4 IER5 generates a novel hypo-phosphorylated active form of HSF1. While heat shock generates a hyper-phosphorylated active form of HSF1, IER5 induces a dramatic dephosphorylation of HSF1

HSF1 suggests the potential involvement of a Ser/Thr protein phosphatase in HSF1 activation. Previous studies have reported that protein phosphatase 2A (PP2A) interacts with IER5 and the HSF family protein HSF2 (Glatter et al. 2009; Xing et al. 2007). We showed that treatment by okadaic acid, an inhibitor of PP2A, or knock down of PP2A compromised the ability of IER5 to dephosphorylate HSF1 and to induce HSP, indicating that PP2A is involved in IER5-mediated HSF1 dephosphorylation and HSF1 activation. Finally, co-immunoprecipitation experiments confirmed a physical interaction between IER5, HSF1 and PP2A. Collectively, these results demonstrate that IER5 functions as a scaffold protein to bring HSF1 and PP2A together, facilitating the dephosphorylation and activation of HSF1

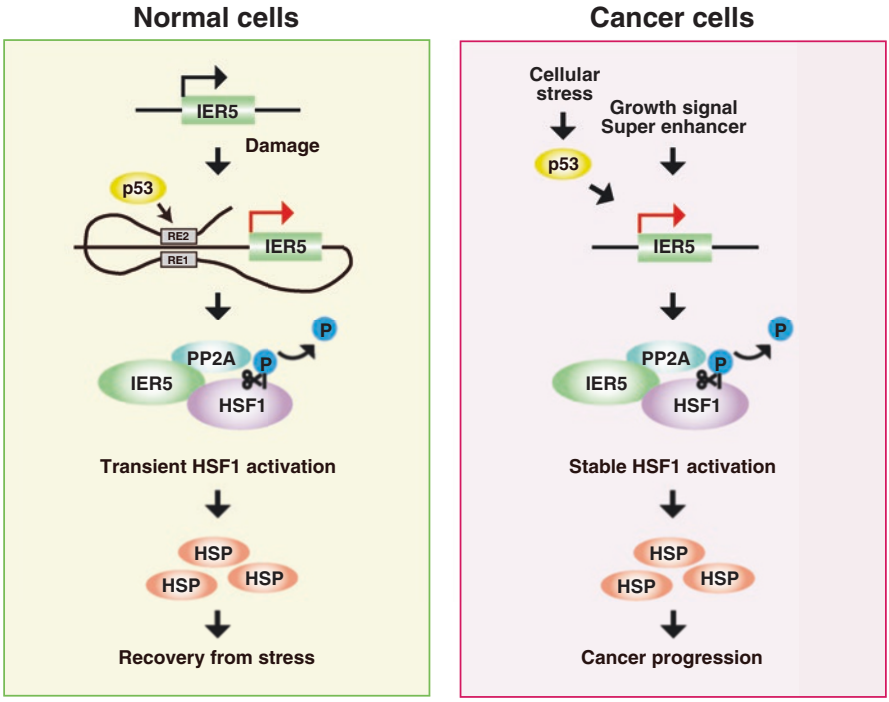

Fig. 13.5 p53/IER5/HSF1/HSP pathway in stressed cells and in cancer cells. Cellular stress transiently activates the IER5-HSF1-HSP1 pathway downstream of p53. On the other hand, IER5 is overexpressed and constitutively activates HSF1 in cancer cells. IER5 forms a complex with HSF1 and PP2A to dephosphorylate and activate HSF1

(Fig. 13.5). The involvement of IER5 and PP2A in HSF1 activation under heat stress conditions was also reported, confirming the importance of this pathway in HSF1 activation (Ishikawa et al. 2015).

13.1.10 p53/IER5/HSF1 Axis

To analyze the role of the IER5-HSF1 pathway in cancer, we introduced into cancer cells a constitutively active form of HSF1 (caHSF1), which has the ability to induce HSF1 target genes independent of an HSF1 activating signal (Uchiyama et al. 2007). In these cells, the HSP family of genes and their protein products were constitutively expressed independent of IER5. Knock down of IER5 expression resulted in poor cell proliferation in the parental cancer cells, but not in caHSF1-expressing cells, which showed anchorage-independent cancer cell growth. This indicates that the IER5-HSF1 pathway is required for anchorage-independent proliferation of cancer cells. Furthermore, a search of a publicly available cancer microarray

database (PrognoScan; Mizuno et al. 2009) showed that higher *IER5* expression is associated with poorer prognosis in various cancer patients. Additionally, higher *HSPA6* expression, the gene most highly induced by IER5, was also related to poorer prognosis and there was a positive correlation between *IER5* and *HSPA6* expression in these cancers. These data collectively suggest that the IER5-HSF1-HSP pathway may be involved in cancer progression.

Since *IER5* is a p53 target gene and its expression is induced under conditions of cellular stresses in a p53-dependent manner, we asked whether *HSP* family gene expression can be induced by DNA damage. Adriamycin treatment resulted in the coincidental upregulation of *IER5*, *HSPA6* and *HSPA1A* mRNAs in several cell lines containing wild-type p53. In addition, upregulation of HSPA1A protein by DNA damage was decreased when p53 or IER5 expression was knocked down. These results show that HSP family proteins are induced by p53 and IER5 following DNA damage, and collectively suggest a role of p53/IER5/HSF1/HSP in the recovery and protection of both normal cells and cancer cells having wild-type p53 (Fig. 13.5).

13.2 Conclusions

We have shown that IER5 is a mediator of wild-type p53 activation of the HSF1-HSP pathway. However, it has also been reported that HSF1 can be activated by mutant p53 (Li et al. 2014). Mutant p53 induces the phosphorylation of HSF1 at Ser326 via activation of MAPK and PI3K cascades, leading to HSF1 induction of Hsp70 expression. This, in turn, stabilizes the mutant p53, forming a positive feed-forward loop. It is interesting therefore that both wild-type and mutant p53 are linked to activation of the HSF1-HSP pathway, illustrating the importance and complexity of the HSF1-HSP pathways in the regulation of cancer. Extensive phosphorylation and activation of HSF1 following heat stress has been well documented. For example, heat stress induces phosphorylation of multiple residues, including Ser230, Ser320 and Ser326, and enhances the transcriptional activity of HSF1. Contrary to our expectations, we demonstrated that IER5 promotes the dephosphorylation of HSF1 at multiple residues including Ser121, Ser307, Ser314, Thr323 and Thr367, and this dephosphorylation critically mediates the activation of HSF1 by IER5. While the repressive role of phosphorylation at Ser121 and Ser307 has been reported previously, the precise roles of Ser314, Thr323 and Thr367 in the repression of HSF1 activity requires further investigation. Recently, a number of studies have implicated HSF1 in tumorigenesis. Mendillo and his colleagues have shown that HSF1 activation in cancer cells can occur in the absence of heat stresses, and that HSF1 induces the expression of different sets of genes from under heat stress conditions (Mendillo et al. 2012). However, the precise mechanisms by which HSF1 is activated in the absence of heat stress remain unclear. Since HSF1 activity is normally regulated by various post-translational modifications, overexpression of the HSF1 mRNA or protein alone would not be expected to cause enhanced HSF1

activity or tumorigenesis. We reported that the *IER5* gene is associated with super-enhancers in cancer cells and that high IER5 expression is correlated with poorer prognosis of cancer patients. Furthermore, we demonstrated a high correlation between mRNA expression of *IER5* and the HSF1 target gene *HSPA6* in several cancers. In addition, in cancer cells that express high amounts of *IER5*, knockdown of *IER5* by RNAi results in the suppression of HSF1 activity and cell proliferation. These findings collectively indicate that IER5 is responsible for HSF1 activation and cell proliferation in at least some cancers. While our results demonstrate the tumor-promoting potential of IER5, HSF1 and the HSP family of genes are well known to maintain protein homeostasis and modulate the survival of normal cells. Therefore, in normal cells, IER5 may function downstream of p53 to promote the recovery and survival in response to stress conditions via the activation of HSF1 and induction of HSP family genes. It is well known that approximately half of all human cancers carry a p53 mutation, illustrating the importance of p53 function in tumor suppression. However, the remaining half of all cancers retain wild-type p53, and we imagine that some cancer cells may actually select for wild-type p53 as induction of IER5 and the downstream activation of HSF1 may help to protect these cells from stress conditions, thus promoting cancer. It therefore will be of great interest and importance to examine the involvement of the p53/IER5/HSF1 pathway in the promotion of cancers retaining wild-type p53 in mouse models and human cancer specimens in future studies.

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Chapter 14
Heat Shock Proteins in Digestive Tract Cancer: Molecular Mechanism and Therapeutic Potential

Liang Wenjin, Li Zeming, Liao Yong, Wang Yan, and Tang Bo

Abstract Heat Shock Proteins (HSP) are a category of proteins for stress regulation being highly conservative in respect of evolutionary sequence, which mainly consist of HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, HSP110 and multiple sub-types thereof. The main function is to maintain the normal functional structure of proteins through the molecular chaperone role. Simultaneously, it can also play a role of cell protection and immunoregulation. The expression of HSP is extensively high in digestive tract cancer and closely related to multiple biological functions such as tumor cell proliferation, cell apoptosis, cell cycle, invasion and migration and drug resistance in chemical therapy, etc. Simultaneously, it has also verified that the inhibitor of HSP can resist the tumor promotion effect effectively in vitro and in vivo. However, the current researches are being concentrated on the inhibitors of HSP70 and HSP90. Further clinic research verification is also needed. In summary, HSP are not only potential tumor biomarkers in early diagnosis and prognosis monitoring to digestive tract cancer, but also a potential target for effective therapy to tumors.

Keywords Clinical applications · Digestive tract cancer · Heat shock proteins · Molecular mechanism · Therapeutics

L. Wenjin
Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Zhongnan Hospital of Wuhan University, Wuhan, China

L. Zeming · L. Yong · W. Yan · T. Bo (✉)
Department of Hepatobiliary Surgery, Guilin Medical University, Affiliated Hospital, Guilin, Guangxi, People's Republic of China

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Abbreviations

BDEC	bile duct epithelial cells
CCA	cholangiocarcinoma
CTL	cytotoxic T lymphocyte
ESCC	esophageal squamous cell cancer
HCC	hepatocellular carcinoma
HSP	heat shock proteins

14.1 Introduction

Heat Shock Proteins (HSP) is a category of proteins for stress regulations being activated in biological cells after stimulation by various stress factors, which are highly conservative in respect of evolutionary sequence. They mainly consist of HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, HSP110 and multiple sub-types thereof Tang et al. (2015). HSP can maintain the normal functional structure of proteins through the molecular chaperone function and participate in regulating and controlling cell proliferation and substance metabolism in vitro and in vivo. Simultaneously, they can also play a role of cell protection and immune-regulation and enhance the tolerance of cell organism to various stress factors (Kamoshida et al. 1999; Moseley et al. 1998). At present, many researches have found that in multiple tumor tissues and cells, the expression of HSP are high and they are closely related to tumor development (Wu et al. 2016). HSP can serve not only as an important indicator for disease development monitoring and patient clinic prognosis assessment, but also as an important target for tumor therapy (Schopf et al. 2008; So et al. 2012).

The digestive tract cancer include esophageal cancer, gastric cancer, liver cancer, cholangiocarcinoma, pancreatic cancer and intestinal cancer, etc. which would happen at various positions in digestive tract. They are malignant cancers being high in the rate of incidence and death in the world, wherein the fairly common ones are gastric cancer, liver cancer, esophageal cancer and carcinoma of large intestine (Chen et al. 2016; Siegel and Miller 2017). As the digestive tract cancer are not typical in respect of early symptoms, most of them have developed into medium or late disease stages in clinic definite diagnosis, meanwhile, invasion or migration of tumor cells to adjacent visceral organs are often accompanied, the current therapy mainly relies on surgical operations, radioactive therapy and chemical therapy. However, the general long-term therapy effects to patients are not satisfactory. To explore the molecular markers for early diagnosis and further tap the applications in multiple aspects of clinic prognosis monitoring and therapy, etc., HSP has become a hot topic currently (Kamoshida et al. 1999). Many researches have showed that the expression of HSP in multiple digestive tract tumors are high, while such the

high expression is closely related to tumor development, cytobiological characteristics of tumor cells, poor clinic prognosis and drug resistance in chemical therapy to tumors, etc. (Sawyer et al. 2006). Through a review of the outline research of HSP in digestive tract cancer, this paper summarizes the basic researches and clinic applications to provide theoretical basis for early diagnosis and effective therapy to digestive tract cancer.

14.1.1 HSP and Esophageal Cancer

14.1.1.1 Expression of HSP in Esophageal Cancer and the Influence of HSP on the Cytobiological Characteristics of Esophageal Cancer Cells

Esophageal cancer is the sixth major disease leading cause of death to cancer patients and also a common tumor in digestive system. Iqbal used immunohistochemical and Western blotting to detect the expression of esophageal squamous cell cancer (ESCC) specimens in 46 patients and found that the expression of HSP27 in the HSP family is the highest, while the next ones are HSP70 and HSP90. Lee used immunohistochemical to detect the expression of ESCC specimens in 157 patients and found that the expression of HSP47 in the tumor tissues were significantly higher and were also positively correlated to tumor cell proliferation and poor prognosis of patients. Zhang used qRT-PCR and Western blotting to detect the expression of ESCC specimens in 30 cases and found that the expression of HSPA2 in the tumor tissues were higher than peri-carcinomatous tissue. Then, the same used immunohistochemical to detect the ESCC specimens in 120 cases and also found that the expression of HSPA2 in the tumor rose and was closely correlated to poor tumor patient prognosis (Hang et al. 2013). Noguchi T used immunohistochemical to analyze the superficial esophageal cancer specimens in 37 cases and found that lymphatic metastasis and the expression of the CD68 positive macrophages in the tumor stroma are related to HSP70 expression. It is a prompt that HSP70 can participate in lymphatic metastasis of superficial esophageal cancer (Noguchi et al. 2003).

14.1.1.2 HSP and Drug Therapy to Esophageal Cancer

At present, most applied researches in HSP' inhibitors are in the experimental stage. Wang et al. (2015) used such carcinogenic substance as 4- nitroquinoline-1-oxide (4NQO) to induce C57BL/6 mice to catch ESCC in 16 weeks. Then the same gave an intraperitoneal injection of Hsp90's inhibitor SNX-2112 and observed for 2 weeks, in which the same found that after SNX-2112 inhibited the expression of

HSP90, it can further reduce the expressions of AKT and cyclin D1 and inhibit the development of esophageal cancer. Besides, the SNX-2112 therapy decreased the expression of proliferating cell nucler antigen (PCNA) in esophageal cancer tissues and promoted cell apoptosis. Farkas et al. (2011) used immnoblotting to compare 20 cases of ESCC patients. The same found before and after using new auxiliary radioactive therapy that after the radioactive therapy, the expressions of both HSP16.2 and HSP90 reduced significantly. Takeno et al. (2010) used immunohistochemical to detect the esophageal cancer specimens receiving new auxiliary chemical therapy combined with radioactive therapy (NAT) and found that the expression level of hsp27 has certain reference significance in forecasting the NAT therapy effect.

14.1.2 HSP and Liver Cancer

14.1.2.1 Expression of HSP in Hepatocellular Carcinoma (HCC) and the Influence of HSP on the Cytobiological Characteristics of Liver Cancer Cells

Current researches have showed that most members in the family of HSP are at a high expression level in liver cancer tissues and cells. The high expression of HSP is closely related to multiple biological characteristics of liver cancer cells such as the development, invasion, metastasis and etc. Zhou et al. (2015) have showed that MiR-625 can down-regulate HSP27 expression and inhibit the invasion and migration of multiple liver cancer cell lines such as BEL-7402, BEL-7404, Huh-7, QGY-7703 and SMMC-7721 through PTEN/HSP27 signal pathway. Liu et al. (2014) found that 14-3-3σ can promote the invasion and migration ability of Huh-7 and SK-Hep1 cells, they also found that after reducing the HSP70 expression, the invasion and migration ability of liver cancer cells becomes weakened markedly. It indicates that the invasion and migration ability of liver cancer cells are induced by 14-3-3σ through regulating HSP70. Matsushima-Nishiwaki et al. (2016) have showed that phosphorylated HSP20 can inhibit JNK signal pathway and then inhibit TGF-α so as to induce the invasion and migration ability of huh7 cell. Guo et al. (2009) have showed that in Hep3B, MHCC97L and MHCC97H, down-regulation of HSP27 can induce the cell apoptosis by NF-κB pathway. Guo et al. (2009) have found that in HepG2 cells, HSP90 can regulate the expression of survivin, cyclin D1 and NF-κB, and then inhibit the cell apoptosis. Wang et al. have showed that clusterin(CLU), through interaction with HSP70's sub-family member-GRP78 (78-kD glucose-regulated protein), protects HepG2, SMMC7721 and HCCLM3 cells from the cell apoptosis as induced by endoplasmic reticulum (ER) stress.

14.1.2.2 HSP and Drug Therapy to HCC

In recent years, more and more evidences have showed that HSP are important regulating factors in drug resistance in HCC therapy and invasion. Therefore, HSP have become a new target for HCC therapy (Lu et al. 2009; Wang et al. 2012; Wang et al. 2013a). Chen et al. (2011) have showed that HSP27 can activate autophagy to inhibit the chemical therapy of cisplatin to SMMC-7721, HepG2 and Hep3B cells. Sharma have showed that HSP27 can inhibit the chemical therapy effect of paraplatin and 5- fluorouracil in Hep3B and HepG2 cells. Wang have showed that HSP90 can inhibit the protective autophagy as mediated by ERK, enhance the efficacy of Bcl-2 inhibitor in HepG2 and Hep3B cells and finally improve the chemical drug therapy effect. Lang et al. (2009) have showed that reducing the expression of HSP90 can inhibit the activation of Akt and NF-κB as induced by mTOR inhibitor-rapamycin and reduce the expressions of PDGF-Rbeta and VEGFR-2 in vascular smooth muscle cell (VSMC) in endothelial cells and thus improve the therapy effect of rapamycin in HepG2 and Huh-7 cells.

14.1.3 HSP and Gastric Cancer

14.1.3.1 Expression of HSP in Gastric Cancer and the Influence of HSP on the Cytobiological Characteristics of Gastric Cancer Cells

The development of gastric cancer results from the interactions of multiple factors such as heredity, environment, etc., being a canceration process with multiple factors, multiple steps involved. Constantinos et al. have found that in 66 cases of clinic gastric cancer specimens, the expressions of HSP27, HSP60 and HSP90 significantly higher than para-carcinoma tissue, while the expression of HSP90 is closely related to the survival rate of patients and can be used as an independently predictive factor for gastric cancer, while Khaldon et al. have found that the expression of HSP27 not only related to gastric cancer differentiation, but also to the existence of helicobacter pylori infection in the patients. Kang et al. (2013) have found that in comparison with advanced gastric cancer, the expression of HSP70 in early gastric cancer is higher. This is a prompt that HSP70 may be a predictive factor for early diagnosis of gastric cancer. Wang et al. (2013b) used immunohistochemical technique detected advanced gastric cancer specimens in 322 cases and found that, the expression level of HSP90 is correlated to gastric cancer invasion and metastasis and poor prognosis. So it can be acted as a monitoring indicator for gastric cancer prognosis. Anna et al. have found that after heat shock pre therapy, gastric mucosal cells can resist the cell apoptosis as induced by H_2O_2, in which HSP70 and HSP90

play a key role. Deng et al. (2016) have found that HSP27 can regulate P38-PI3K/Akt signal pathway and reverse the role of melatonin to induce apoptosis of SGC-7901 cell. Aneta et al. (2012) have found that helicobacter pylori can activate COX-2 to inhibit the activity of HSP70 and result in apoptosis of MKN7 cell.

14.1.3.2 HSP and Drug Therapy to Gastric Cancer

Akiharu et al. (2016) have found that the expression of HSP110 is related to resistance to chemical therapy to gastric cancer and poor prognosis. In colorectal cancer, the functional loss and mutation of HSP110 (HSP110ΔE9) is something having been confirmed. In patients with gastric cancer, the carrying HSP110ΔE9 mutation are highly sensitive to the anticancer drugs of oxaliplatin, 5- fluorouracil, etc. YX, et al. have found that the expression of HSP27 in SGC-7901/VCR cell resistant to vincristine (VCR) is higher than that of gastric cancer cell line SGC-7901. It's stated that the expression of HSP 27 is related to the resistance drugs of tumor cells. Using antisense oligonucleotide (AON) to inhibit the expression of HSP27 can enhance the sensitivity of vincristine (VCR) and adriacin to SGC-7901/VCR cell in chemical therapy. Choi, et al. (2011) have found that to prepare DNA vaccines by constructing DNA recombinant plasmid with the extracellular region of Mucin1 genes fusing with human HSP70 genes can produce immune response from specific cell and inhibit the growth of B16/Muc1 cells. This verifies that HSP70 can be acted as an anti-tumor role. Wang et al. used gene reproduction technique to load HSP70 to oncolytic adenovirus, and then synthesized the adsurp-HSP70 virus therapy system to explore the effect of therapy to gastric cancer. The results have showed that the virus therapy synthesis can selectively dissolve survivin positive of BGC-823 and SGC-7901 cells and induced them apoptosis while have no effect to normal cells.

14.1.4 HSP and Pancreatic Cancer

14.1.4.1 Expression of HSP in Pancreatic Cancer and the Influence of HSP on the Cytobiological Characteristics of Pancreatic Cancer Cells

Pancreatic cancer is a kind of digestive tract cancer with high degree of malignance and being difficult for early diagnosis. Current researches have showed that the abnormal expressions of HSP in pancreatic cancer tissues and cells have been reported. Gress et al. (1994) firstly reported that the expression of HSP90 in pancreatic cancer tissues is high and the abnormal expression is positively correlated to poor prognosis of patients. Ogata et al. (2000) have demonstrated that the

expression of HSP90mRNA in pancreatic cancer tissues are significantly higher than normal tissues and it can promote the proliferation of pancreatic cancer cells. Okuno detected the expression of HSP27 in 49 cases of pancreatic cancer tissues and showed that the expression of HSP27 was high in pancreatic cancer and negatively correlated to the prognosis of pancreatic cancer patients (Mitsuru et al. 2016). Maitra et al. (2002) detected the expression of HSP47 in 57 cases of pancreatic cancer tissues and it showed that the expression of HSP47 in the pancreatic cancer tissue was significantly higher. Aghdassi et al. (2007) found that in Panc-1, BxPC-3 and MiaPaCa-2 cell lines, the expression levels of HSP70 were significantly higher than that of normal cells and HSP70 can inhibit the apoptosis of pancreatic tumor cells. For HSP70, the following mechanisms may exist in tumor development: (1) Combine with multiple proto-oncogene and anti-oncogene (c-fos, raf, p53, Rb) products to regulate cell proliferation. Over-expressing of HSP70 combines with p53 to inhibit the p53 signal pathway and promote cell survival. After down-regulated HSP70, the p53 signal pathway can be activated to induce apoptosis of pancreatic tumor cells. Ciocca and Calderwood (2005) (2) Being closely correlated to cell proliferation. Knockout of HSP70 gene can make tumor cell stop in G2-M phase. Knockout of HSP70-2 gene can make tumor cell stop in G0 phase. While simultaneous removal of HSP70 and HSP70-2 can inhibit in synergy the proliferation of tumor cells. Through the synergic functioning of HSP70 with Bcl-2, Bcl-xL, CrmA, etc., the expressions of cell apoptosis related genes and proteins are finally reduced so as to inhibit cell apoptosis.

14.1.4.2 HSP and Drug Therapy to Pancreatic Cancer

HSP90 plays a very crucial role in the development of pancreatic cancer. Therefore, HSP90 inhibitors have become a hot point in anti-tumor researches. Geldanamycin is the first type of Hsp90 inhibitor used in clinic test for anti-cancer therapy. It is a natural product separated from streptomycin and can inhibit the formation of HSP90 multi-chaperone complexes by combining with HSP90 directly and result in the extensive degradation of HSP90 receptor protein as mediated by ubiquitin. As a result, anti-tumor cells proliferate and the apoptosis thereof is promoted. However, geldanamycin is fairly high in liver toxicity and simultaneously the inactivation is rapid during in vivo metabolism. This limits its further applications. 17-allylamino-demethoxy-geldanamycin (17-AAG), an analogue of geldanamycin, has almost all of the characteristics of geldanamycin and the toxicity is even lower. Bauer et al. have found that 17-AAG together with gemcitabine can inhibit the proliferation of pancreatic tumor cells and liver metastasis. Phillips et al. (2007) used triptolide to treat the pancreatic cancer cells of PANC-1 and MiaPaCa-2 and found that the expression level of HSP70 decreased and simultaneously, the tumor organism size of pancreatic tumor cells was also reduced and the local tumor spreading occurrence rate became significantly less. The clinic application prospect is favorable.

14.1.5 HSP and Cholangiocarcinoma

14.1.5.1 Expression of HSP in Cholangiocarcinoma and the Influence of HSP on the Cytobiological Characteristics of Cholangiocarcinoma Cells

Cholangiocarcinoma (CCA) comes from bile duct epithelial cells (BDEC), being one of the most common malignant tumors globally and accounting for around 3% of digestive tract tumors and 10–25% of primary liver tumors. In liver tumors, the occurrence rate is only next to HCC. Depending on the anatomic position, CCA consists of intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC). (Gatto et al. 2010; Shaib and Elserag 2004). Lagana et al. (2013) used immunohistochemical method to detect the CCA specimens in 41 cases, finding that the HSP70 expression in 31 patients were significantly higher than in para-carcinoma tissue. Sato et al. (2012) used ELISA method to detect respectively the serum in 8 cases of CCA patients in and 48 cases of bile duct stone patients. The results showed that the HSP27 and HSP70 expressions of CCA patients significantly higher than in para-carcinoma tissue. Moreover, when combined with these two proteins, the sensitivity and specificity were 100% and 90% respectively. It's predicted that HSP27 and HSP70 can serve as new bio-markers. Kang et al. (2017) found in research that the mortalin protein in HSP70 family can promote the proliferation, invasion and migration ability of CCA cells. It plays a vital role by EMT pathway.

14.1.5.2 HSP and Drug Therapy to CCA

Chen et al. (2013) found that the combined action of HSP90 inhibitor NVP-AUY922 and PI3K/mTOR inhibitor NVP-BEZ235 is synergic with each other in inducing CCA cell death, while the combination of NVP-AUY922 and NVP-BEZ235 can cause the reduce tumor volume of rat model. Such a combination not only inhibits the PI3K/Akt/mTOR pathway, but also induces the ROS pathway. It's stated that the inhibitor of HSP may provide new thinking for the clinic therapy to CCA. Chen et al. (2014) found that both the inhibitors of HSP90, 17-AAG (tanespimycin) and NVP-AUY922 have a significant role to resist tumor proliferation during in vitro, while NVP-AUY922 can also inhibit the PIK3/AKT and KRAS/MAPK pathway.

14.1.6 HSP and Intestinal Cancer

14.1.6.1 Expression of HSP in Intestinal Cancer and the Influence of HSP on the Cytobiological Characteristics of Intestinal Cancer Cells

Researches have showed that in intestine cancer tissues, HSP10, HSP27, HSP60, HSP70 and HSP90 are all in high expression. The high expression of HSP in intestine cancer is not only related to tumor development, but also closely related to the prognosis of patients. In detecting the expressions of HSP10 and HSP90 in 20 cases of intestinal carcinoma patients, Francesca found that both of HSP10 and HSP90 are high expressed in cancer tissues. Simultaneously, Ghosh et al. thought that the high expression of HSP27 in primary intestinal cancer relies on the activation of the KRAS, PI3K/AKT pathway as well as the mutation of TP53. Therefore, the high expression of HSP27 can inhibit the ageing of tumor cells (Cercek et al. 2014). Through the serum analyses to 64 cases of patients, Meral found that the HSP70 expression in intestinal cancer patients was markedly higher than normal people. Simultaneously, the high expression of HSP70 is negatively correlated to the prognosis of patients. Through testing the Alpha B-crystallin (CRYAB) expression levels in 18 cases of intestinal cancer tissues and the intestinal cancer tissue chips about 100 cases, Chuanbing found that the expression of CRYAB in intestinal cancer tissues is higher than para-carcinoma tissue, meanwhile positively correlated to the distant metastasis and poor prognosis of intestinal cancer (Shi et al. 2014).

14.1.6.2 HSP and Drug Therapy to Intestinal Cancer

As HSP participate in the development of intestinal cancer extensively, Evans et al. (2010) put forth that T-cell activation mechanism as mediated by HSP70 can be used as a potential cancer immunotherapy method, and the anticancer therapy effect reaction can be forecasted through monitoring the expression level of HSP70. Rena found in test that DNAJB8 (DnaJ (Hsp40) homolog, subfamily B, member 8), as a sub-type of HSP40, is significantly high in intestinal cancer tissues and participates in targeted immune killing of intestinal cancer cells as mediated by the specific cytotoxic T lymphocyte (CTL). Therefore, they are effective target for immune therapy to tumors. He et al. (2013) thought that quercetin liposome could inhibit the increase in HSP70 expression as induced by thermal stress and chemical therapy reaction and result in CT26 cell apoptosis. The researches of Andrea have showed that HSP90's inhibitor Ganetespib has a significant therapy effect to KRAS mutation type intestinal cancer.

14.2 Conclusions

Digestive tract cancer, as a kind of disease being difficult for early diagnosis with a seriously malignance, affect human life and health to a great extent. To start with the molecular mechanisms of tumor development is one of the effective ways to solve this troublesome problem. The extensively high expression of HSP as an important category of stress-regulating proteins for organisms in digestive system has been verified and it can extensively participate in multiple biological processes in digestive tract cancer, including cell proliferation, invasion and migration, internal environment steady state regulation and drug resistance in chemical therapy, etc. Through reviewing and summarizing the expression of HSP family members in digestive tract cancer and participating in regulating the molecular mechanisms of tumor development, we have outlined simultaneously the applied researches into HSP (as shown in the table), it explained that HSP are not only the potential biomarker for early tumor diagnosis and prognosis monitoring, but also a potential target for effective tumor therapy. However, as the functions and roles of HSP have similarity and extensiveness and they are lack of specificity in tumor diagnosis, a comprehensive judgment in conjunction with other tumor markers is also needed. Besides, due to the complexity of molecular regulation and the individualized difference in genic mutation, the experimental drug sensitivity effect of HSP' inhibitors in vivo is greatly affected. Simultaneously, HSP, as crucial stress-regulating proteins in organisms, whether the application of its inhibitors in organism can cause other important biological function changes needs further clinic researches for exploration and verification (Table 14.1).

Table 14.1 Roles of major HSP family members in digestive tract cancer

Family	Biomarker	Cancer development	Drug target and resistance	References
HSP10	↑in intestinal cancer			Rappa et al. (2014)
HSP20	↑in HCC	Inhibit JNK, TGF-α; induce cell invasion and migration;		Guo et al. (2009)
HSP27	↑in esophageal cancer, HCC, gastric cancer, intestinal cancer	Promote the invasion and migration through PTEN/HSP27 signal pathway, induce the cell apoptosis by NF-κB, KRAS, PI3K/AKT	Predicting the NAT treatment effect, activate autophagy to inhibit cisplatin, oligonucleotide (AON) can inhibit HSP27,	Takeno et al. (2010)
				Zhou et al. (2015)
				Guo et al. (2009)
				Sharma et al. (2010)
				Yang et al. (2009)
HSP40	↑in intestinal cancer		Participates in targeted immune killing of intestinal cancer cells	Morita et al. (2014)

(continued)

Table 14.1 (continued)

Family	Biomarker	Cancer development	Drug target and resistance	References
HSP47	↑in esophageal cancer, pancreatic cancer,			Lee et al. (2016)
				Maitra et al. (2002)
HSP60	↑in gastric cancer			Giaginis et al. (2009)
HSP70	↑in esophageal cancer, HCC, gastric cancer, pancreatic cancer, intestinal cancer, CCA	Promote cell invasion and migration by 14-3-3σ, combined with Bcl-2, Bcl-xL, CrmA, resist the cell apoptosis, stop tumor cell in G2-M phase. Promote proliferation	Relate to helicobacter pylori infection, induced survivin positive of BGC-823 and SGC-7901 cells apoptosis, inhibit by triptolide, negative correlated with prognosis,	Iqbal et al. (2016)
				Liu et al. (2014)
				Bodoor et al. (2016)
				Leung et al. (2015)
				Sherman et al. (2007)
				Rohde et al. (2005)
				Phillips et al. (2007)
				Gunaldi et al. (2015)
HSP90	↑in esophageal cancer, HCC, gastric cancer, pancreatic cancer, intestinal cancer, CCA	Reduce AKT, cyclin D1, survivin and NF-κB inhibit cell apoptosis,	Inhibited by SNX-2112, rapamycin, 17-AAG, NVP-AUY922, promote activation of Akt and NF-κB, promote PI3K/Akt/mTOR pathway inhibit the protective autophagy	Wang et al. (2015)
				Leng et al. (2012)
				Wang et al. (2014a)
				Lang et al. (2009)
				Kamal et al. (2003)
				Chen et al. (2013)
HSP110	↑in gastric cancer		Resistance to oxaliplatin, 5- fluorouracil,	Akiharu et al. (2016)

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Chapter 15
Extracellular HSP90α Versus Intracellular HSP90β in Wound Healing and Cancer

Vadim Lincoln, Xin Tang, Mei Chen, and Wei Li

Abstract Of ~7000 gene products generated by an average cell, 2–3% of them are accounted for by the Hsp90 family of proteins. Mammals have two members of Hsp90, Hsp90α and Hsp90β, with which they respond to environmental stress, especially during tissue ischemia. Hsp90β fulfills the role of an important intracellular chaperone, whereas Hsp90α is dispensable inside the cell. Instead, Hsp90α gets secreted and the extracellular Hsp90α protects surrounding cells from hypoxia-induced cell death and promotes cell migration, during wound healing. The cell surface receptor, LRP-1, binds and mediates extracellular Hsp90α signaling by activating Akt kinases independently of Hsp90α's intrinsic ATPase. Topical application of recombinant Hsp90α promotes healing of acute, diabetic and burn wounds. Tumors take advantage of these protective functions of both intracellular and extracellular Hsp90 family proteins to cope with the constant paucity of oxygen and nutrients within the tumor microenvironment. Unlike intracellular Hsp90β which is equally critical to the survival of both tumor and normal cells, secreted Hsp90α is non-essential for maintaining physiological homeostasis. In contrast, tumor cells constitutively secrete Hsp90α and use secreted Hsp90α to prevent tumor cell death under hypoxic conditions and to promote tumor cell invasion and metastasis. Monoclonal antibodies that selectively target tumor-secreted Hsp90α may prove more effective and less toxic than those that target the ATPase of the intracellular Hsp90β.

Keywords Exosome · Hsp90α · Hsp90β · Microenvironment stress · Tissue repair · Tumor progression

V. Lincoln · X. Tang · M. Chen · W. Li (✉)
Department of Dermatology and the USC-Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, CA, USA
e-mail: wli@usc.edu

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Abbreviations

17-AAG	17-(Allylamino)-17-demethoxygeldanamycin
F-5	fragment-5
HIF-1	hypoxia-inducible factor-1
hsp90	heat shock protein-90
LRP-1	LDL receptor-related protein-1
PDGF	platelet-derived growth factor
PRAS40	proline-rich Akt substrate of 40 kDa

15.1 Introduction

The 90-kDa heat-shock protein (Hsp90) was first reported half a century ago and described as an intracellular protein whose cellular levels increases in response to heat (Ritossa 1996). In the decades that followed, Hsp90 was further characterized as an evolutionarily-conserved and ATPase-driven chaperone molecule that plays an important role in the homeostasis of a wide range of living organisms under both physiological and pathophysiological conditions. In vertebrate cells, Hsp90 is a family of constitutively expressed proteins that protect hundreds of intracellular client proteins from possible damage or deterioration secondary to extracellular stress (Young et al. 2001; Whitesell and Lindquist 2005). Many cancer cells express levels of Hsp90 family proteins that are two to tenfold higher than their normal cell counterparts (Isaacs et al. 2003; Banerji 2009), and even amongst cancer cell lines which express relatively normal levels of Hsp90 protein, the Hsp90 in the cancer cells was reported "more active" than the Hsp90 expressed in the overexpressing cancer cells (Kamal et al. 2003). Interestingly, for long time the Hsp90 family proteins were not sought out as a target for cancer therapeutics. The reason behind this previous lack of investigation is that the *HSP90AA1* and *HSP90AB1* genes, which code for Hsp90α and Hsp90β, respectively, do not show any of the conventional traits of oncogenes like gene translocation, amplification, and oncogenic mutations. Regardless, the status of Hsp90 began to change in early 1990s, largely because of considerable new challenges emerging in the field of cancer therapeutics. Many cancer drugs designed to target an individual oncogene product or pathway began to encounter a common barrier, drug resistance. Cancer drug resistance can manifest from additional mutations in the same gene or by activation of a totally independent pathway in the same cancer cells (Neckers and Neckers 2002; Workman 2004; Workman et al. 2007). Therefore, it became desirable to produce drugs that could not only target a specific oncogenic mutation, but also inhibit multiple key signaling pathways that support the hallmarks of cancer pathogenesis (Hanahan and Weinberg 2011). Since Hsp90's client proteins include many gene products that play critical roles in the regulation of a cell's life-cycle (such as ERKB2, MET, RAF, AKT, BCR-ABL, CDK4, and HIF-1α) and Hsp90 serves as a "nodal protein" during multi-molecular complex formation for transformation (McClellan et al. 2007),

it became widely accepted that inhibition of the Hsp90's intrinsic ATPase could simultaneously shut down multiple growth-controlling signaling pathways in cancer cells and, therefore, decrease the genetic plasticity and development of a drug resistance (Workman 2004). Since 1999, many ATPase inhibitors entered clinical trials as potential cancer therapeutics, but few have obtained approval for use in humans.

Two independent studies made the discovery that Hsp90, and especially Hsp90α, are secreted by cells involved in tumor progression (Eustace and Jay 2004) and wound healing (Li et al. 2007), thereby unearthing a potentially new realm of function for this ancient family of intracellular proteins. Over the following decade, tremendous progress has been made in elucidating the functions of secreted Hsp90, its mechanism of action, and its therapeutic value using various preclinical models. Results of those studies in both wound healing and oncology have been summarized in several excellent review articles and book chapters dated up to 2013 (Tsutsumi and Neckers 2007; McCready et al. 2010; Li et al. 2012, 2013; Sidera et al. 2004; Hance et al. 2014). Therefore, in this chapter, we will focus on the more recent, novel progress that has occurred over the past several years, namely the delineation of the distinct roles of Hsp90α and Hsp90β, the stress-mediated regulation of Hsp90α secretion via exosomes, and the latest therapeutic innovations in wound healing and tumor progression. We apologize for the exclusion of many earlier, excellent quality research articles from our references that we obligated to omit in order to comply with the space restrictions.

15.1.1 HSP90α Versus HSP90β

Vertebrates have two distinct Hsp90 genes that encode Hsp90α and Hsp90β with a shared 86% identity in amino acid sequences. In addition, two organelle-residing isoforms, Grp94 (a 94-kDa protein in the lumen of the endoplasmic reticulum) and TRAP1 (a 75-kDa protein within mitochondria), are related to the Hsp90 proteins. Voss et al. reported that knockout of Hsp90β caused a primary defect in the embryonic allantois (a sac-like structure involved in nutrition and excretion), resulting in loss of liquid waste collection and gas exchange functions and consequent embryonic lethality at day 9.0/9.5 in mice (Voss et al. 2000). This finding demonstrated that Hsp90β is an essential chaperone during embryonic development. In addition, it suggested two possible relationships between Hsp90α and Hsp90β: (1) the role of Hsp90β is distinct and cannot be replaced by Hsp90α or (2) Hsp90β and Hsp90α work cooperatively to reach a minimum threshold level of activity required for successful chaperone functions to ensue, and therefore, reduction of either Hsp90β or Hsp90α would cause the observed defect. However, the reported phenotypes of Hsp90α-knockout mice appeared to challenge the second possibility. Picard's group generated a mouse with an insertion in intron-10 of the Hsp90α gene using gene trapping techniques. This insertion was expected to produce a truncated Hsp90α protein lacking 36 amino acids in the C-terminal domain, however, the truncated

protein was not detectable (likely due to compromised stability of the mutant protein). This outcome resulted in an unexpected Hsp90α-knockout mouse model, as reported (Grad et al. 2010). Udono's group used a similar approach to generate conditional Hsp90α-knockout mice by floxing exons 9 and 10 in the Hsp90α gene (Imai et al. 2011). In contrast to the Hsp90β-knockout mice, lack of Hsp90α had little effect on embryonic mouse development, although these authors observed (1) lack of sperm in male mice due to an apparently higher rate of cell apoptosis in the testes and (2) a defect in translocation of extracellular antigens across endosomal membranes into the cytosol. Taken together, one may conclude that the two Hsp90 family members play unequal roles during development, in which the support provided by the ATPase-driven chaperone function of Hsp90β, not Hsp90α, is essential.

Hsp90α and Hsp90β have also shown distinct functions and mechanisms of action at cellular levels in vitro. Unmethylated CpG oligodeoxynucleotides (CpG ODNs) activate immune responses in a TLR9-dependent manner. Kuo et al. reported that only Hsp90β, and not Hsp90α, responded to CpG-B ODN stimulation and demonstrated a protective effect on serum starvation- and staurosporine- induced apoptosis in mouse macrophages and dendritic cells, although no RNAi experiments were carried out to explicitly address the role of Hsp90α (Kuo et al. 2007). Similarly, Chatterjee et al. reported that Hsp90β plays a more important role in control of multiple myeloma cell survival (Chatterjee et al. 2007). More convincingly, Jayaprakash and colleagues recently discovered a novel mechanism by which human dermal fibroblasts respond to environmental ischemia, in which Hsp90α and Hsp90β have distinct, non-interchangeable functions. They showed that within the ischemic environment of wounds, Hsp90α and Hsp90β work in conjunction to promote cell motility during wound healing. Under the stress, Hsp90β acts as a chaperone by binding to the cytoplasmic tail of the LDL Receptor-Related Protein-1 (LRP-1) and stabilizes the receptor at the cell surface. Hsp90α, on the other hand, is secreted into the extracellular space, where it signals through the Hsp90β-stabilized LRP-1 receptor to promote cell motility, leading to wound closure (Jayaprakash et al. 2015). Differences in the function of these two proteins have also been confirmed by Zou and colleagues during their work with the breast cancer cell line, MDA-MB-231. In their study, CRISPR/Case9 knockout of Hsp90α nullified the tumor cells' ability to migrate, invade, and metastasize, but did not affect cell survival or growth. In contrast, knocking out Hsp90β resulted in tumor cell death. Intriguingly, extracellular supplementation with recombinant Hsp90α protein, but not Hsp90β, restored the tumorigenicity of the Hsp90α-knockout cells (Zou et al. 2016). These studies indicate that, unlike Hsp90β whose chaperone function is of equal importance in both tumor and normal cells, extracellular Hsp90α is non-essential for normal cells to maintain homeostasis but is a critical factor in the tumor microenvironment that promotes invasion and metastasis. The take home message is illustrated in Fig. 15.1 and summarized here: (1) The window for inhibitors to do more harm to cancer cells than normal cells may not be as wide as originally hoped. In theory, membrane permeable ATPase inhibitors, such as 17-AAG, could penetrate cells and cell organelles to prevent all Hsp90 isoforms from acting as chaperones

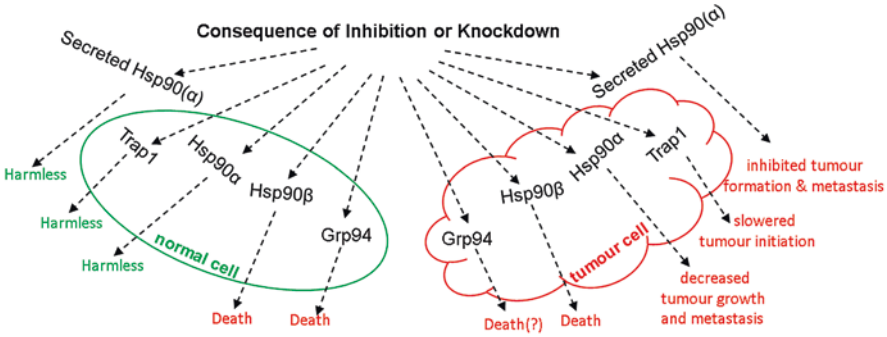

Fig. 15.1 Targeting tumor-secreted Hsp90α may be more effective and less toxic. Figure illustrates the consequences of inhibition or knockdown of Hsp90 family members depending on their intracellular or extracellular location and their cell of origin. ATPase inhibitors would indiscriminately inhibit all intracellular Hsp90 functions, however, inhibition of tumor-secreted Hsp90α would selectively target tumor progression pathways

in both normal and cancer cells. However, when this occurs, inhibition of Hsp90β or Grp94 is toxic to both cancer and normal cells in patients. (2) Isoforms and locations of distinct Hsp90 family members should be taken into consideration when designing potential therapeutics. Some of these isoforms are even secreted into the extracellular environment by certain cancer cells, especially those with constitutively elevated HIF-1α (hypoxia-inducible factor-1alpha) expression (detected in ~50% of all invasive human tumors). Unlike intracellular Hsp90β, secreted Hsp90α no longer depends upon an ATPase to function as (i) a survival factor for tumor cells under hypoxic conditions (Dong et al. 2015) and (ii) as a pro-motility factor promoting invasion (Cheng et al. 2008). Indeed, selective inhibition of secreted Hsp90α, which is nonessential for normal cells, could be an alternative therapeutic approach for certain cancers (Li et al. 2012, 2013). For the past decade, several studies have utilized this alternative approach to bypass targeting of intracellular Hsp90β, namely to selectively target the extracellular, tumor-secreted Hsp90α to inhibit tumor progression. In fact, the results from animal models employing this approach are encouraging (Stellas et al. 2007; Tsutsumi et al. 2008; Song and Lou 2010; Zou et al. 2016). This new approach remains to be tested in human trials.

15.1.2 Environmental Stress or Cellular Oncogene-Triggered, Exosome-Mediated Secretion of HSP90α

Yu and colleagues reported that γ irradiation induced exosome-mediated secretion of Hsp90β, but not Hsp90α, in a p53-dependent fashion, prompting proposal of the "DNA damage > p53 > Hsp90β secretion" pathway (Yu et al. 2006). Cheng et al. showed that in primary human keratinocytes, TGFα-induced Hsp90α membrane

translocation and secretion were sensitive to inhibitors of exosome protein trafficking, but not to inhibitors of the conventional ER/Golgi protein trafficking pathway (Cheng et al. 2008). Li and colleagues showed that hypoxia (1% O_2) induces Hsp90α secretion via HIF-1α and that blockade of the secreted Hsp90α functionality via neutralizing antibodies completely inhibits hypoxia-induced cell motility (Li et al. 2007). The identification of HIF-1α as a key upstream regulator of Hsp90α secretion also has an important implication for cancer pathogenesis. Hypoxia is a known micro-environmental stress that is connected to the growth, invasion, and metastasis of many solid tumors (Simon and Keith 2008). Under chronic hypoxic conditions, cancer cells are forced to adapt alternative and self-supporting mechanisms via HIF-1α for continued survival and expansion. It is likely why overexpression of HIF-1α has occurred in approximately 40% of the tumors in humans (Semenza 2007). Accordingly, surface expression and/or secretion of Hsp90α should become constitutive in those HIF-1α-overexpressing tumors. To support this notion, many tumor cell lines have been reported to secrete Hsp90α. Kuroita and colleagues reported purification of Hsp90α from the conditioned media of human hybridoma SH-76 cells (Kuroita et al. 1992). Eustace et al. reported presence of Hsp90α, but not Hsp90β, in the conditioned media of HT-1080 tumor cells (Eustace et al. 2004). Wang et al. reported secretion of Hsp90α by MCF-7 human breast cells (Wang et al. 2009). Suzuki and Kulkarni found Hsp90β secreted by MG63 osteosarcoma cells (Suzuki and Kulkarni 2010). Chen and colleagues reported secretion of Hsp90α by a colorectal cancer cell line, HCT-8 (Chen et al. 2010). Work by Tsutsumi and colleagues implied secretion of Hsp90α occurred in a variety of tumor cell lines (Tsutsumi et al. 2008). Finally, several recent studies from our laboratory demonstrated that seven breast cancer cell line constitutively secrete Hsp90α (Sahu et al. 2012; Dong et al. 2015; Zou et al. 2016). In these cells, HIF-1α plays a critical role in regulation of Hsp90α secretion, although the signaling steps between HIF-1α and the secretory machinery remain unknown.

What is known, however, is that Hsp90 proteins do not have the signal peptide (SP) required for secretion out of cells via the classical ER/Golgi protein secretory pathway. An alternative secretory pathway for molecules without SPs is mediated by secreted extracellular vesicles (EVs). EVs have several synonyms that are widely used in independent studies including “microvesicles”, “ectosomes”, “microparticles” and “exosomes” (Yáñez-Mó et al. 2015; Raposo and Stoorvogel 2013). Secretion of EVs by normal cells under stress is an evolutionarily conserved phenomenon observed in almost all cell types. Biologic origin and size variation account for the main distinctions among different kinds of EVs. Exosomes belong to a subtype of EVs with defined diameters between 30 and 150 nm and are derived from intraluminal vesicles (ILVs) within intracellular multivesicular bodies (MVB) (Harding et al. 1983; Pan et al. 1985; Johnstone et al. 1987). Nonetheless, due to technical limitations in purifying distinct EV populations, the term “exosome”, as currently used, refers to a population of EVs of varying sizes but with the majority being between 30 and 150 nm in diameter, instead of a single subtype of EV with a clearly defined population with respect to size and origin of production (Tkach and Théry 2016). In contrast to normal cells which secrete exosomes secondary to

extracellular environmental stress, tumor cells constitutively secrete exosomes in a process driven by intracellular oncogenes (Kucharzewska and Belting 2013). In addition to proteins and peptides, secreted exosomes also contain other cargo molecules including DNA, mRNA, miRNA, and lipids. Consequently, exosomes are the most complex extracellular signaling entity identified for cell-to-cell communication to date (Théry et al. 2002; Witwer et al. 2013; Colombo et al. 2014). This new and increasingly recognized mechanism of intercellular communication has been demonstrated to play critical roles in host immune responses (Nolte et al. 2012), tissue repair (Yuana et al. 2013; Yáñez-Mó et al. 2015) and tumor invasion and metastasis (Kucharzewska and Belting 2013; Hoshino et al. 2015; Zhang et al. 2015). Three lines of evidence for exosome-mediated secretion of Hsp90α currently exist. First, using two chemical inhibitors, brefeldin A (BFA), which selectively blocks the classical ER/Golgi protein secretory pathway, and dimethyl amiloride (DMA), which blocks the exosome protein secretory pathway, several groups have shown that DMA selectively inhibits membrane translocation and secretion of Hsp90α or Hsp90β in various cell types (reviewed in Li et al. 2012). In contrast, BFA resulted in little inhibition of either Hsp90 protein secretion (Cheng et al. 2008). Second, Clayton and colleagues detected the presence of Hsp90α, among other proteins, during proteomic analysis of isolated exosomes from B-cell conditioned media (Clayton et al. 2005). Third, Yu et al. presented electron microscopic evidence of the presence of Hsp90 co-localized with exosomes (Yu et al. 2006). A recent study by Gou and colleagues demonstrated that the majority of tumor-secreted Hsp90α is present in the 100,000 g ultracentrifugation fraction of the cells' conditioned media, together with exosome markers including flotillin, CD9, CD81, and Cd63 (Guo et al. 2017).

15.1.3 Regulation of HSP90α Secretion in Normal and Tumor Cells

While it is clear that environmental stress cues trigger release of exosomes in normal cells and intrinsic oncogenic signal(s) result in constitutive exosome secretion in many tumor cells (Raposo and Stoorvogel 2013), the mechanism by which the stress signals regulate exosome secretion remains elusive. In fact, there has been limited progress in this field until recently. First, the Rab27 small GTPases, Rab27a and Rab27b, were reported to regulate both exosome biogenesis and secretion. Rab27a and Rab27b regulate distinct steps of multi-vesicular endosome (MVE) docking to plasma membranes and exosome biogenesis; Rab27a regulates MVE breakdown and Rab27b regulates MVE distribution, formation, and secretion in various types of cells (Ostrowski et al. 2010; Zheng et al. 2013). However, Rab27a was also reported to regulate secretion of MMP9 and growth factors through the conventional ER/Golgi pathway (Kucharzewska and Belting 2013; Bobrie et al. 2012). Since the mechanisms of these two trafficking systems are completely

unrelated, these observations generate more questions than answers. An interesting study by Sinha and colleagues showed that knockdown or overexpression of cortactin in cancer cells resulted in a respective decrease or increase in exosome secretion, without altering exosome cargo content. These authors proposed that cortactin promotes exosome secretion by binding to Arp2/3 and stabilizing cortical actin-rich MVE docking sites (Sinha et al. 2016). Our laboratory has studied stress-regulated and exosome-mediated secretion of Hsp90α during wound healing and tumor invasion. Our approach was to first understand how environmental stress signals trigger the secretion of Hsp90α, an exosome cargo molecule, which will garner insight into the larger question of how stress regulates exosome secretion. By taking advantage of a unique property of human keratinocytes in response to transforming growth factor-alpha (TGFα) and epidermal growth factor (EGF) in culture**,** we have recently identified a critical signaling molecule that links microenvironmental cues to exosome secretion. We found that, while both TGFα and EGF are known to utilize the same cell surface EGF receptor for transmembrane signaling and the same, previously undistinguishable, intracellular signaling networks for regulation of gene expression, we surprisingly found that only TGFα triggers secretion of Hsp90α. By comparing activations of 43 intracellular signaling molecules/pathways in the same cells in response to TGFα or EGF stimulation, we identified PRAS40 as a TGFα-specific downstream target. More importantly, activated PRAS40 acts not only as a regulator of TGFα-triggered and exosome-mediated secretion of Hsp90α, but rather as a common regulator of distinct microenvironmental and oncogenic signal-triggered exosome secretion in both normal and tumor cell types (Guo et al. 2017). PRAS40 is the first regulator identified for stress-induced exosome secretion.

The proline-rich Akt substrate of 40 kDa (PRAS40) was initially identified as a direct substrate of Akt kinase and a binding partner for the 14-3-3 scaffolding molecule (Kovacina et al. 2003). Most studies focused on PRAS40's role in insulin, as well as NGF and PDGF, signaling to the mTOR (mammalian target of rapamycin) pathway (specifically mTORC1), which regulates cell metabolism, protein synthesis, and cell growth (Saito et al. 2004; Shimaya et al. 2004; Vander Haar et al. 2007; Sancak et al. 2010; Fonseca et al. 2007; Oshiro et al. 2007; Thedieck et al. 2007; Wang et al. 2007). In growth-arrested cells, PRAS40 was reported to bind, via the raptor subunit, to mTORC1 and inhibit mTOR kinase activity. Insulin stimulation activates Akt kinase mainly via threonine (Thr)-308 phosphorylation. The activated Akt kinase in turn phosphorylates PRAS40 on Thr-246. Thr-246-phosphorylated PRAS40 dissociates from mTORC1, resulting in activation of mTORC1, and (re-) associates with 14-3-3 (Vander Haar et al. 2007; Oshiro et al. 2007; Sancak et al. 2007). In addition to Akt, increased PIM1 kinase activity also correlated with increased PRAS40 phosphorylation. Activated mTORC1 phosphorylates PRAS40 at Ser-183, Ser-212 and Ser-221 (Wang et al. 2008; Zhang et al. 2009). Despite these reports, others showed that PRAS40 is not a common regulator of mTOR activation in response to extracellular signaling (Thedieck et al. 2007; Fonseca et al. 2008). Gou et al. demonstrated that stress signals activate Akt via phosphorylation at Thr-308. The Thr-308-phosphorylated Akt in turn activates PRAS40 via Thr-246 phosphorylation. More interestingly, Thr-246-phosphorylated PRAS40, even in the

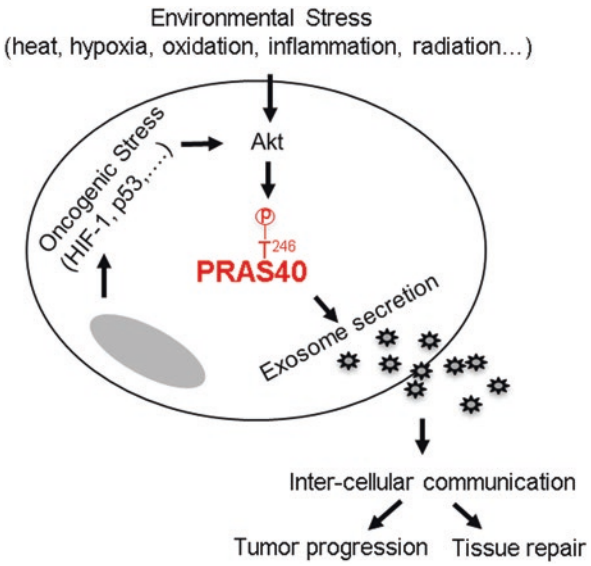

Fig. 15.2 A schematic representation of stress-triggered exosome secretion through PRAS40. Extracellular stress cues like cytokines, hypoxia, and H_2O_2 activate Akt kinase, which in turn phosphorylates PRAS40 at Thr-246. Activated PRAS40 communicates with a currently unknown intermediate(s), leading to exosome secretion. Secreted exosomes contain large quantities of DNA, RNA, and protein molecules, enabling much more efficient cell-to-cell communication than a single secreted molecule, like a peptide hormone, would alone. PRAS40 is the first linker identified between stress and exosome secretion. (Taken from Guo et al. 2017 with permission)

absence of stress, is both necessary and sufficient to cause exosome secretion, without affecting the ER/Golgi pathway. These findings are schematically depicted in Fig. 15.2.

Identification of PRAS40 as a linker protein not only paves the way for understanding how stress regulates exosome secretion under pathophysiological conditions, but also provides direct support for previous studies that show PRAS40 has also a stimulating role in both normal and cancer cells. In those studies, PRAS40 (i) prevents stress-induced normal and tumor cell apoptosis and (ii) supports tumor progression in vitro and in vivo (Saito et al. 2004; Madhunapantula et al. 2007; Kazi and Lang 2010; Huang et al. 2012; Havel et al. 2015). Similar findings were also made in normal cells. Yu et al. reported that elevated PRAS40 levels protect motor neurons from spinal cord injury-induced cell death (Yu et al. 2008). Shin et al. showed that overexpression of PRAS40 prevents ischemic insults in the brain and oxidative stress-induced brain cell death (Shin et al. 2016). All these findings cannot be explained by the reported role of PRAS40 as an inhibitor of the mTOR pathway and leave the question of how PRAS40 exerts these "positive" functions unanswered. Our finding that Thr-246-phosphorylated PRAS40 regulates exosome secretion provides a possible mechanism for how PRAS40 protects cells from

apoptosis and supports tumor progression – via secreted exosomes and their cargo protein, Hsp90α.

15.1.4 Secreted HSP90α Is Not Enclosed Inside Exosomes

There have been several lines of experimental support suggesting that secreted Hsp90α is not enclosed within the interior of exosomes. First, Eustace et al. reported that DMAG-N-oxide, a cell-impermeable version of geldanamycin that targets Hsp90's ATPase, blocks tumor cell invasion (Eustace et al. 2004). Tsutsumi and colleagues reported similar findings with respect to in vitro cancer cell invasion and lung colonization by melanoma cells in mice (Tsutsumi et al. 2008). The results of these studies also implied that the Hsp90α's intrinsic ATPase is still required for the extracellular functions of secreted Hsp90α. Second, Cheng and colleagues reported that the pro-motility activity of secreted Hsp90α from cell-conditioned medium could be completely blocked by neutralizing antibodies (Cheng et al. 2008). Furthermore, a specific anti-Hsp90α neutralizing monoclonal antibody, 1G6-D7, blocks the pro-motility and pro-invasion activities of isolated exosomes in vitro (Guo et al. 2017) and in vivo (Zou et al. 2016). Finally, RAP (LRP-1-associated protein), which competitively inhibits ligand binding and signaling through the extracellular domain of LRP-1 (the receptor for secreted Hsp90α), blocks enhanced cell motility by isolated exosomes (Guo et al. unpublished). The action of exosome-associated Hsp90α does not appear to be due to exosome rupturing or a slow release of cargo molecules, since the high stability of exosomes is widely reported. Nonetheless, these observations raised several questions. How does secreted Hsp90α anchor to the lipid membrane of exosomes? Does membrane anchoring occur before or after exosome secretion? If the membrane anchoring occurs intracellularly, how could exosomes diffuse through the plasma membrane with a highly hydrophilic polypeptide on the surface? If the anchoring occurs after exosomes are secreted into the extracellular environment, potential mechanisms are limited to speculation. All things considered, more research, especially work utilizing imaging and biochemistry approaches, needs to be done to gain insights into these puzzles.

15.1.5 Extracellular HSP90 Is Not a Chaperone Protein and Does Not Require Intrinsic ATPase or Dimerization to Function

One of the central debates in Hsp90 research was whether secreted Hsp90α still acts in an ATPase-dependent manner. Recent studies have provided several lines of convincing evidence that extracellular Hsp90α is no longer an ATPase-driven chaperone and instead acts as a novel class of signaling proteins that directly binds their

receptors to triggers cellular responses. Taking advantage of the previous report that the Hsp90α-wt, the Hsp90α-E47D mutant, and the Hsp90α-E47A and Hsp90α-D93N mutants have 100%, 50%, and undetectable ATPase activity, respectively (Young et al. 2001), Cheng et al. made comparisons among the recombinant proteins encoded by these Hsp90α genes assessing their abilities to stimulate cell migration, a main function of secreted Hsp90α. They reported that all the ATPase mutant proteins retained similar degrees of pro-motility activity compared to Hsp90α-wt (Cheng et al. 2008). These authors then used sequential deletion mutagenesis to narrow down the pro-motility domain to a 115-amino acid fragment, called F-5 (aa-236 to aa-350), between the linker region (LR) and the middle (M) domain of the human Hsp90α. F-5, alone, was found to promote skin cell migration in vitro under serum-free conditions and wound healing in vivo as effectively as full-length Hsp90α-wt (Cheng et al. 2011). These findings demonstrate that the N-terminal ATPase domain and the C-terminal dimer-forming and co-factor-binding domain are dispensable with regard to extracellular Hsp90α's ability to promote cell migration.

The next mystery elucidated was the identity of the molecular entity which determines the extracellular functions of secreted Hsp90α. Zou and colleagues took a clever approach by comparing amino acid substitutions between Hsp90α and Hsp90β (the former has extracellular functions and the latter does not). The authors first utilized the deletion mutagenesis approach to further narrow down the functional fragment from F-5 fragment to a 27-amino acid peptide fragment, called F-8. They then identified 8 amino acids within Hsp90α's F-8 peptide that are substituted by variant amino acid residues within the corresponding F-8 peptide from Hsp90β. Sequential site-directed mutagenesis allowed them to identify two evolutionarily conserved lysine residues, lys-270 and lys-277, in the Hsp90α subfamily that determine extracellular Hsp90α functions. The Hsp90β subfamily lacks the dual lysine motif and extracellular functions. Substitution of lys-270 and lys-277 in Hsp90α with the two corresponding gly-262 and thr-269 from Hsp90β completely nullified the extracellular functions of Hsp90α. The reverse is also true. Substitutions of gly-262 and thr-269 in Hsp90β with lysines converted Hsp90β to an Hsp90α-like protein, at least in vitro. Intriguingly, these authors found that the dual lysine motif is conserved evolutionarily in all Hsp90α subfamily members and, similarly, the gly-262 and thr-269 are conserved in the Hsp90β subfamily proteins (Zou et al. 2016). A schematic representation of the structure and functional component requirements for intracellular Hsp90 and secreted Hsp90α is shown in Fig. 15.3.

15.1.6 Two Biological Functions of Extracellular HSP90α

Conventional wisdom dictates that locally released growth factors in an injured tissue constitute the principal driving force initiating wound healing (Werner and Grose 2003; Grose and Werner 2004). Under this assumption, growth factors are responsible for both inducing wound closure by promoting the lateral migration of

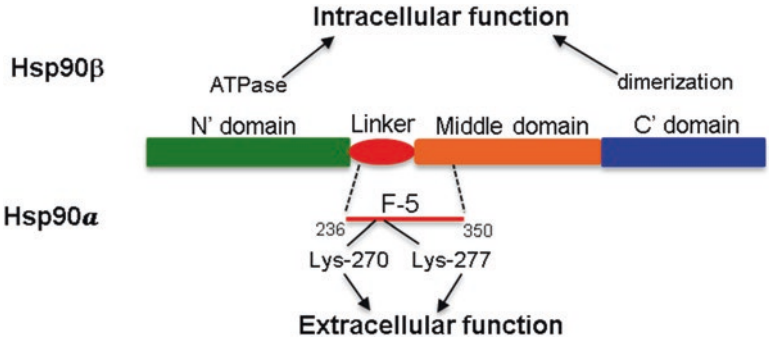

Fig. 15.3 Hsp90β is an intracellular chaperone, whereas Hsp90α is an extracellular factor. Hsp90β gene knockout is embryonic lethal in mice and causes cell death in culture dish. Absence of functional Hsp90α has little impact on mouse development and causes certain tumor cells to selectively lose their tumorigenicity. The action of intracellular Hsp90β is driven by an intrinsic ATPase, whereas action of secreted Hsp90α is determined by the dual lysine (Lys-270 and Lys-277) motif within the F-5 region. These lysine residues are present in all Hsp90α family members, but they are replaced by glycine-262 and Thr-269 in the Hsp90β family

epidermal keratinocytes and for remodeling and revascularizing the neodermis of the wounded space by inducing the inward migration of dermal fibroblasts and microvascular endothelial cells. Since the first report of a clinical trial using EGF to stimulate wound healing in 1989 (Brown et al. 1989), more than a dozen trials utilizing growth factors to promote wound healing have been conducted. However, only the human recombinant protein, platelet-derived growth factor-BB (PDGF-BB), has received US Food and Drug Administration (FDA) approval only for the treatment of diabetic limb ulcers (Regranex™/becaplermin gel 0.01%, Ortho-McNeil Pharmaceutical, Raritan, NJ). Following its FDA approval in 1997, multicenter and randomized clinical evaluations showed an overall ~15% improvement in wound closure by becaplermin gel at 100 μg PDGF-BB/g vesicle, a dose already more than a thousand-fold higher than the physiological range of PDGF-BB in human circulation (Steed 1995; Wieman et al. 1998; LeGrand 1998; Mandracchia et al. 2001; Embil and Nagai 2002). In 2008, the FDA added a black box warning to becaplermin gel due to an increased risk of cancer mortality in patients who required extensive treatments (≥3 tubes). This side-effect may not be altogether surprising, since autocrine release of PDGF-BB (c-sis), or its viral form (v-sis), at concentrations as low as 15–30 ng/ml can trigger cell transformation (Bejcek et al. 1992).

Li and colleagues concluded that the primary driving force behind skin wound closure does not originate from growth factors in circulation and subsequently began to search for novel factors secreted from the keratinocytes at the wound edge under stressful conditions like hypoxia and paucity of nutrients. Protein purification with the sensitive individual cell-based pro-motility assay, the colloidal gold motility assay, allowed them to identify Hsp90α as a possible candidate (Li et al. 2007; Cheng et al. 2008). Using purified recombinant Hsp90α protein, two important

biological functions of extracellular Hsp90α in both in vitro cell culture and in vivo animal models were established: (1) promotion of cell-survival under stress and (2) stimulation of cell motility for tissue repair. First, using hypoxia as an in vitro model and burn wounds as an in vivo model, cells were observed to secrete Hsp90α to protect themselves from hypoxia- and heat- induced apoptosis (Dong et al. 2015; Bhatia et al. 2016). This finding is logically consistent with observed cellular behaviors in both physiological wound healing and cancer pathogenesis. During wound healing, cells in the hypoxic environment of the wound edge must remain viable, at least temporarily, prior to engaging in the repair process. During tumor growth, most solid tumors outgrow the nearest blood vessels and, therefore, are constantly challenged with a severely hypoxic microenvironment and possible cell death. To remain viable under these conditions, almost 50% of solid tumors have managed to maintain a level of constitutively expressed HIF-1α. As previously mentioned, hypoxia and HIF-1α are presently the best-characterized upstream stress cues that trigger Hsp90α secretion in both normal and tumor cells (Li et al. 2007; Woodley et al. 2009; Sahu et al. 2012). In addition to protecting cells from hypoxia-induced apoptosis, the second important biological function of secreted Hsp90α protein is its role as a novel class of motogen that promotes cell motility to "escape" the hazard microenvironment. Recombinant Hsp90α promotes cell migration, but not proliferation, of a wide range of cell types in the total absence of serum factors or any other exogenously supplemented large molecules (Cheng et al. 2008, 2011). Since migrating cells do not proliferate and proliferating cells cannot do motility at the same time, Hsp90α being a motogen, nut not mitogen, makes a perfect sense. Both the pro-survival and pro-motility functions of extracellular Hsp90α utilize a common signaling transduction pathway. Briefly, Hsp90α binds to subdomain II in the extracellular portion of the low-density lipoprotein receptor-related protein-1 (LRP-1). The NPVY, but not NPTY, motif in the intracellular tail of LRP-1 in turn connects Hsp90α signaling to serine-473 (but not threonine-308) phosphorylation in Akt kinases, leading to enhanced cell migration. Individual knockdown in cell culture or knockout of Akt1, Akt2, or Akt3 gene in mice demonstrates the importance of Akt1 and Ak2 in extracellular Hsp90α signaling and control of cell motility both in vitro and in vivo. Most convincingly, these two biological functions of secreted Hsp90α, promotion cell survival and cell motility, have been verified in wound healing models. Li's group showed that topical application of recombinant Hsp90α protein strongly stimulates wound closure by promoting keratinocyte migration-driven re-epithelialization in mouse and pig wounds (Cheng et al. 2011; O'Brien et al. 2015). The pro-survival effects of extracellular Hsp90α were assessed using a burn wound healing model which undergoes a unique pathological process called "secondary burn wound progression", in which the wound expands horizontally and vertically from the initial site of trauma. If left untreated, the cells in the expanded areas soon die of necrosis, apoptosis, or both, due to ischemia, infection, and accumulation of toxic metabolites. Bhatia et al. showed that topical application of recombinant Hsp90α dramatically reduced the degree of secondary burn wound progression by preventing heat-induced apoptosis of the surrounding cells (Bhatia et al. 2016).

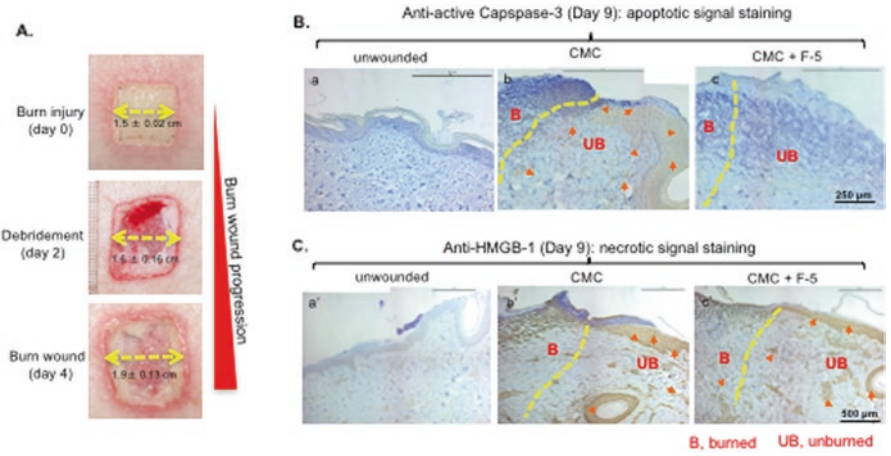

Fig. 15.4 Extracellular Hsp90α treatment prevents burn-induced cell apoptosis, but not necrosis. (**a**) "Secondary burn wound progression" from day 0 to day 4 following initial injury burn (120 °C, 30 s); (**b**) Human Hsp90α F-5 peptide treatment (panel **c**) prevents burn-induced cell apoptosis (brown stain) in cells surrounding the burn wound compared to no treatment (panel **b**); (**c**) F-5 treatment (panel **c**) demonstrates little rescuing effect on burn-induced cell necrosis (brown stain) compared to no treatment (panel **b**). (Taken and modified from Bhatia et al. with permission)

The secondary burn wound progression, surrounding cell death and prevention of apoptosis by topically applied Hsp90α protein in a pig wound model are as shown in Fig. 15.4. Identification of these two functions of extracellular Hsp90α provides direct support for independent studies from many laboratories (reviewed by Li et al. 2012, 2013).

15.1.7 Keratinocyte-Secreted HSP90α During Wound Closure

The 1-year long skin wound healing process is divided into (1) an inflammatory phase, (2) a proliferation phase and (3) a maturation phase. Following a brief period of inflammation in response to skin injury, the proliferation phase begins and lasts a few weeks. During this phase, granulation tissue takes form and serves as the pavement support for epidermal keratinocytes at the wound edge to attach and migrate, ultimately resulting in resurfacing of the open wound. Therefore, this phase is also known as the re-epithelialization or wound closure phase. After wound closure, the time-consuming maturation phase, which involves extracellular matrix remodeling and neovascularization in the dermis, can last months to a year to complete. Most previous studies have primarily focused on the initial weeks of the inflammation and re-epithelization/wound closure phases due to lack of reliable animal models that could allow for analysis of the year-long wound maturation process (Singer and

Clark 1999; Gurtner et al. 2008; Sen et al. 2009). However, despite years of studies and investment, there are few topical or systemic medications which effectively promote skin wound closure.

Since it has been widely accepted that identification of a natural "driver gene" for a given pathological process is the foundation for successful therapeutic development, we hypothesized that the natural driver factor(s) for wound closure was still at large. Proof of *HSP90AA1*'s driver gene nature during wound closure could facilitate development of a new and effective treatment of wounds. Bhatia and colleagues found that when skin is injured, there is a massive increase of Hsp90α protein in the wound bed (Bhatia et al. 2016). Using a unique mouse model which expresses a carboxyl terminal deletion mutant, Hsp90α-Δ, to prevent the dimerization and chaperone functions of Hsp90α, but spares the extracellular F-5-supported pro-motility function (Imai et al. 2011), allowed these authors to specifically test the role of the non-chaperone functions of secreted Hsp90α in normal wound closure. They showed that the chaperone-defective Hsp90α-Δ mutant mice had similar wound closure rates when compared to the Hsp90α wild type mice. Topical application of recombinant Hsp90α-Δ mutant protein promoted wound closure as effectively as full-length Hsp90α wild type protein. Finally, selective functional inhibition of secreted Hsp90α-Δ protein, via monoclonal antibodies targeting the F-5 region, disrupted normal wound closure in both Hsp90α wild type and Hsp90α-Δ mice. Thus, this study provides evidence that non-chaperone, extracellular Hsp90α is a potential driver for normal wound closure. An earlier study by Song and Luo which used regular nude mouse model and neutralizing antibodies against Hsp90 reported similar findings (Song and Luo 2010). As previously mentioned, within the 732-amino acid polypeptide of human Hsp90α, the fully functional therapeutic entity which promotes wound closure is located within a 115-amino acid fragment, F-5, between the linker region and middle domain of Hsp90α (Cheng et al. 2011). Topical application of F-5 peptide strongly promotes traumatic (full-thickness, excisional) wound closure, burn wound closure, and diabetic wound closure in mouse and porcine models. In side-by-side comparisons, FDA-approved becaplermin gel showed either minimal effects on acute wound (traumatic and burn wounds) closure or a much weaker effect on chronic wound (diabetic wounds) closure (Cheng et al. 2011; O'Brien et al. 2015; Bhatia et al. 2016). The effect of F-5 on burn wounds is especially encouraging since it is the first topically applied peptide that prevents secondary burn wound progression, a significant barrier for developing burn therapeutics. Currently, F-5 has entered industrial development as a new topical treatment for skin wounds.

But what qualifies secreted Hsp90α a driver for wound closure, rather than growth factors? Studies from our laboratory have identified three unique properties of extracellular Hsp90α, which are absent from conventional growth factors. First, extracellular Hsp90 is a common pro-motility factor for all three types of human skin cells involved in wound healing. Following skin injury, keratinocytes migrate laterally to cover the open wound and dermal fibroblasts and microvascular endothelial cells migrate inwardly to remodel the damaged tissue and reestablish a blood supply. Ideally, a single factor-based wound-healing agent should recruit all three

types of skin cells into the wound bed. Secreted Hsp90α is exactly such an agent; it promotes motility in all three major types of human skin cells because each type of skin cell expresses a compatible level of LRP-1 for Hsp90α to bind (Cheng et al. 2008). In contrast, PDGF-BB only acts on dermal fibroblasts and not on keratinocytes or dermal microvascular endothelial cells due to lack of both PDGFRα and PDGFRβ on the latter two cell types (Cheng et al. 2011). Second, conventional growth factors are unable to override the inhibition of cell migration and proliferation caused by TGFβ3, which is co-present in the wound bed and present at especially high levels in inflammatory wounds (Bandyopahdhay et al. 2006). In contrast, even in the presence of TGFβ3, extracellular Hsp90α remains equally effective in promoting migration of all three types of human skin cells (Cheng et al. 2011). Third, extracellular Hsp90α is able to promote healing in diabetic wounds. All forms of diabetes are characterized by chronic hyperglycemia, which is believed to be one of the major factors delaying wound closure in diabetic patients (Brownlee 2001). A reported mechanism of wound-healing impairment mediated by hyperglycemia is destabilization of HIF-1α protein, the key regulator of Hsp90α secretion in the wound (Catrina et al. 2004; Botusan et al. 2008). While hyperglycemia blocks hypoxia-induced and serum-stimulated human dermal fibroblast migration, extracellular Hsp90α not only enhances hypoxia-driven migration in normal glycemic conditions, but also "rescues" migration of cells cultured in hyperglycemic conditions. In this case, extracellular Hsp90α promotes diabetic wound closure possibly by bypassing HIF-1α down-regulation induced by hyperglycemic conditions and jumpstarting migration of cells that otherwise are unable to raise HIF-1α level to respond to the environmental hypoxic cue. Finally, the notion that extracellular Hsp90α is a motogen, and not a mitogen (i.e. it does not stimulate cell proliferation) also makes sense from a physiologic perspective. Keratinocyte migration occurs almost immediately following skin injury, whereas the inward migration of dermal cells is not detected until 4 days afterward (Singer and Clark 1999). During wound healing, cell migration precedes cell proliferation and when a cell is actively migrating, it cannot proliferate simultaneously. Then, when and where does cell proliferation take place to replenish the lost tissue in wounded skin? We speculate that, as the cells at the wound edge move toward the wound bed, they leave "empty space" between themselves and the cells behind them. The cells behind the migrating cell front subsequently begin to proliferate after losing contact inhibition with the travelling cells. The stimuli responsible for this cell proliferation are likely plasma growth factors which have diffused from surrounding unwounded blood vessels, where TGFβ levels are low or undetectable (Bandyopahdhay et al. 2006). Thus, the role of cell proliferation in wound closure is to refill the space generated by the migrating cell front. The role for secreted Hsp90α in wound closure is to achieve initial wound closure as quickly as possible to prevent infection, water loss, and impact of severe environmental stress. After initial wound closure, many other factors (including growth factors and TGFβ) will participate in the remaining time-consuming wound remodeling processes for up to 1 year.

15.1.8 Tumor Cells Secrete HSP90α to Gain Invasive and Metastatic Advantages

It is clinically relevant to determine which tumors secrete and utilize secreted Hsp90α to gain invasive and metastatic advantages. So far, the best-characterized upstream oncogene that triggers Hsp90α secretion is the *HIF1A* gene which encodes HIF-1α (Li et al. 2007; Sahu et al. 2012). This finding is significant, since HIF-1α overexpression is associated with increased patient mortality in approximately 40% of solid tumors in humans (Semenza 2007, 2012a). Using breast cancer as a model, Dales et al. carried out anti-HIF-1α immunohistochemical assays on the frozen sections of 745 breast cancer samples and found that elevated levels of HIF-1α expression correlated with poor prognoses, lower overall survival, and higher risk of metastasis among both node-negative and node-positive patients (Dales et al. 2005). By using HIF-1α expression as a marker, it was estimated that approximately 25–40% of all invasive breast cancer samples are hypoxic, suggesting that HIF-1α can be used as a broader marker for breast cancer (Dales et al. 2005; Semenza 2012b). Sahu et al. have shown that down-regulation of endogenous HIF-1α in the breast cancer cell lines, MDA-MB-231 and MDA-MB-468, completely blocked the constitutive secretion of Hsp90α, and that secretion could be rescued by re-introducing the genes encoding WT-HIF-1α and CA-HIF-1α (constitutively active), but not DN-HIF-1α (dominant-negative) (Sahu et al. 2012). These data establish that HIF-1α is a direct upstream regulator of Hsp90α secretion.

The important role of tumor-secreted Hsp90α has been demonstrated in tumor cell-derived xenograft models. Tsutsumi and colleagues showed that the membrane impermeable 17-AAG inhibitor, DMAG-N-oxide, blocked the action of tumor-secreted Hsp90 and decreased tumor cell lung colonization in nude mice (Tsutsumi et al. 2008). Stellas et al. reported that the anti-Hsp90α monoclonal antibody, 4C5, inhibited breast cancer cell "deposits" in the lungs of nude mice (Stellas et al. 2010). A similar observation was made with an independent monoclonal antibody by Song and colleague (Song and Luo 2010). Sahu et al. showed that down-regulation of the LRP-1 receptor in MDA-MB-231 cells dramatically reduced lung colonization of the cancer cells in nude mice (Sahu et al. 2012). However, these studies suffered from several technical limitations, among the most notable being uncertainty regarding the specificity of the antibodies and their untested effects on tumors that had already formed, similar to cancer patients who initially come to see doctors. A recent study by Zou and colleagues took a systematic approach to address these issues. First, they showed that CRISPR/Case9 knockout of Hsp90α nullified the tumor cells' ability to migrate, invade, and metastasize without affecting cell survival or growth. As expected, knockout of Hsp90β, the critical intracellular chaperone, led to tumor cell death. Extracellular supplementation with recombinant Hsp90α, but not Hsp90β, protein fully recovered tumorigenicity of the Hsp90α-knockout cells. Sequential mutagenesis identified two evolutionarily conserved lysine residues, lys-270 and lys-277, in the Hsp90α subfamily that determine extracellular Hsp90α function. The Hsp90β subfamily lacks the dual lysine motif and

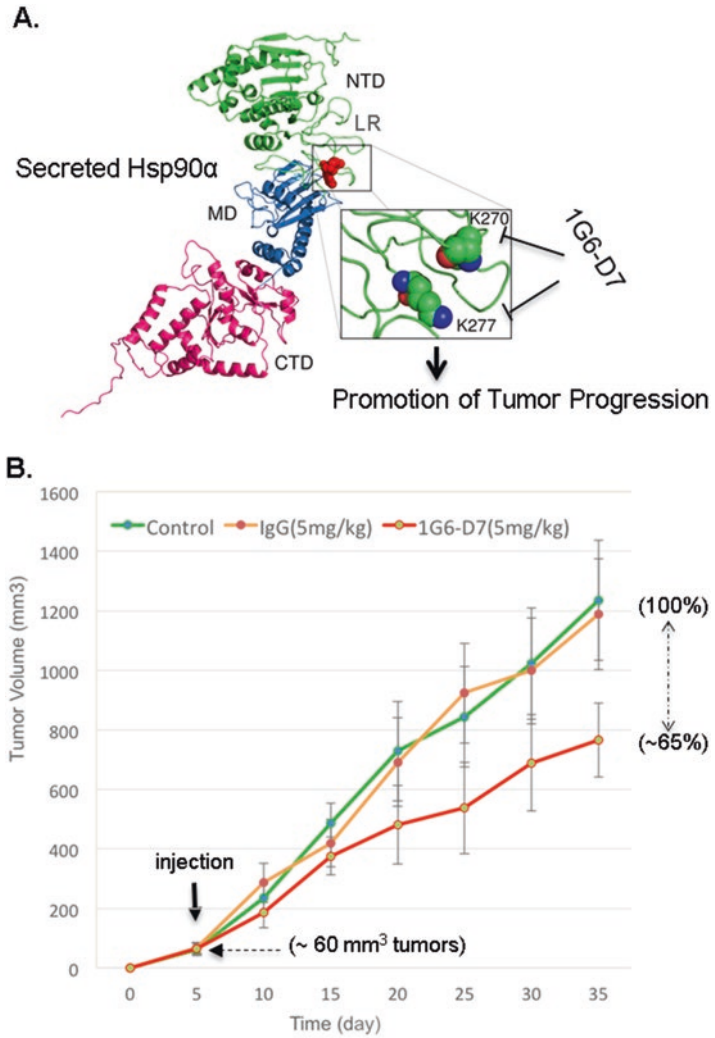

Fig. 15.5 1G6-D7 inhibits expansion of pre-formed tumors in vivo. (**a**) Based on the predicted crystal structure of Hsp90α, lysine-270 and lysine-277 are located in the unstructured linker region (LR) between the NTD and MD domains. F-5 inhibitors, such as mAb 1G6-D7, targeting the dual lysine residues (enlarged box) block secreted Hsp90α-triggered tumorigenesis; (**b**) A representative experiment demonstrating the effects of mAb 1G6-D7 on tumor progression over 35 days in mice injected with MDA-MB-231 cells (5×10^6). On day 5, with an average tumour size of 60 mm^3, either vehicle, control mouse IgG, or 1G6-D7 was injected via IV (5 mg/kg) and around the tumour site (125 μg/injection). Measurement of tumor volumes in live mice (n = 5) were recorded every 5 days. (Taken from Zou et al. 2016 with permission)

extracellular function. Zou et al. then constructed a monoclonal antibody, 1G6-D7, that targets the dual lysine region of secreted Hsp90α. 1G6-D7 inhibited both de novo tumor formation and expansion of pre-existing tumors in mice. As schematically shown in Fig. 15.5, this study suggests an alternative therapeutic approach to targeting tumor-secreted Hsp90α, instead of intracellular Hsp90α or Hsp90β, in cancer therapy. When these findings (the number of HIF-1α-overexpressing tumors, the biological functions of tumor-secreted Hsp90α, and Hsp90α's effects on in vivo tumor progression), are taken together, one can extrapolate that tumor-secreted Hsp90α plays an important role in HIF-1α-overexpressing malignancies, which are approximately 40% of all tumors in humans.

15.1.9 Is Targeting Tumor-Secreted HSP90α More Effective and Less Toxic Than Targeting the Intracellular HSP90β Chaperone When Treating Tumors?

In contrast to the critical chaperone function of intracellular Hsp90β in normal cell homeostasis, no physiological function has been reported for secreted Hsp90α. Instead, all studies over the past decade have clearly shown that secretion of Hsp90α by normal cells is an emergency response to environmental insults, such as inflammation, heat, oxidation, and hypoxia, among others. Furthermore, the functions of secreted Hsp90α no longer require its intrinsic ATPase, which is the target site for small molecule inhibitors and the principle cause of cytotoxicity in clinical trials. Therefore, the F-5 epitope of tumor-secreted Hsp90α may represent an excellent target for design of safer and more effective inhibitors for treatment of tumors in which *HIF1A* is the upstream driver gene. We propose that this new type of anticancer drug should (i) selectively inhibit tumor-secreted Hsp90α (and not intracellular Hsp90β) and (ii) specifically target the dual lysine residues within the F-5 region. At least in theory, drugs that bear both properties should achieve high efficacy and pose minimal toxicity to patients.

15.1.10 Wound Healing and Tumor Progression: Similar Mechanisms with Opposite Outcomes

Wound healing has fixed beginning and end points, whereas cancer, once initiated, may continue to progress until a patient's demise. In his analytical article published in the New England Journal of Medicine, Dvotak outlined the similarities and distinctions between wound healing and tumor stroma generation. He suggested that tumor stroma formation is a subversion of the normal wound healing process. He called tumors the "wounds that do not heal" (Dvorak 1986). When considering design of drugs which promote healing or inhibit tumorigenesis, it is critical to

remember that targeting molecules which affect one process, will likely affect the other. This is especially important to consider when discussing Hsp90α as a drug target. For instance, when formulating a topical application of F-5 peptide to promote wound healing, a drug developer would have to consider whether or not the peptide could gain access to peripheral circulation and travel to sites where an early-stage tumor is already in progress. Under these circumstance, F-5 may aid the tumor's progression and accelerate invasion and metastasis. On the other hand, administration of F-5 inhibitors, such as monoclonal antibodies to block tumor-secreted Hsp90α activity, might interfere with the wound healing process and negatively impact patients with surgical or chronic wounds. For example, numerous studies have shown that patients with type II diabetes are more likely to die from cancer than non-diabetics. Therefore, if a diabetic patient who was simultaneously suffering from a malignancy and a foot ulcer was treated with inhibitors of F-5 by an oncologist to slow cancer progression, the administered inhibitor could interfere with the healing of the foot ulcer. The reverse is also true. If the diabetic ulcer was treated by a wound specialist with F-5 peptide to promote healing, the peptide may travel through blood circulation to the tumor site and aid tumor invasion and metastasis. It is critical to know when and why patients should receive these potential treatments.

15.1.11 Is Extracellular HSP90α a "General Repair Molecule"?

Studies over the past few years have clearly indicated the previously contended notion that Hsp90α is not a critical intracellular chaperone, but Hsp90β is. First, the absence of Hsp90α, or at least its intracellular chaperone form, has a limited impact on mouse development (Grad et al. 2010; Imai et al. 2011), whereas Hsp90β knockout is lethal to developing embryos (Voss et al. 2000). These findings indicate that Hsp90α is less important than Hsp90β for homeostasis during development. Similarly, at the cellular level, Zou et al. have recently shown that CRISPR/Case9 knockout of Hsp90β in the MDA-MB-231 human breast cancer cell line led to tumor cell death, whereas knockout of Hsp90α in the same cells only eliminated the cancerous properties of the cells (Zou et al. 2016). Second, at the signaling level, Jayaprakash and colleagues reported discovery of a novel ischemia-response mechanism by which the two Hsp90 isoforms, Hsp90α and Hsp90β, work together, but have distinct and non-exchangeable functions involving LRP- receptor signaling during wound healing. Under hypoxic conditions or in paucity of nutrients, Hsp90β, but not Hsp90α, binds to the cytoplasmic tail of LRP-1 and stabilizes the receptor at the cell surface. Hsp90α, but not Hsp90β, is then secreted by the same cells into the extracellular space, where it binds and signals through the LRP-1 receptor to promote cell motility, leading to wound closure (Jayaprakash et al. 2015). This study provides a mechanistic support for the outcomes of the genetic studies in mice.

Currently, the hypothesis that Hsp90α was designed by nature as a "general repair" molecule is limited to indirect support and speculation. To proceed forward, this hypothesis needs to be tested in multiple tissue injury animal models. However, there is a preexisting narrative suggesting a role of Hsp90α in healing that has been conserved through evolution. Due to its unusual abundance of 2–3% of the total cellular protein in all types of normal cells, Csermely et al. have long argued that evolution would not have tolerated such a "waste" if the function of Hsp90 had only been as an intracellular chaperone (Csermely et al. 1998). Instead, we can view stockpiles of Hsp90α all over our bodies as fire stations dispersed around a city. When tissue is injured, the pre-stored Hsp90α will guarantee a quick response to repair signals immediately released into the wound environment. Likewise, it would not make sense for a city to only start building fire stations after the fire already took place. While Csermely's hypothesis is a bold one, our studies have provided three unique qualifications that secreted Hsp90α has, whereas growth factors do not, as previously described. Extracellular Hsp90α's apparent superiority over growth factors in promotion of wound closure has been reflected in experiments in which topical application of recombinant Hsp90α protein dramatically shortened the time of diabetic mouse wound closure from 35 days to ~18 days, a ~17 day reduction in healing time compared with the 5 day reduction achieved via treatment with becaplermin gel (Cheng et al. 2011)

Cancer cells have an interesting, love-hate relationship with the TGFβ protein family, whose members are regarded as both tumor suppressors and tumor promoters (Bachman and Park 2005). The inhibitory effects of TGFβ on cancer progression are reflective of their hindering effects on wound healing. To bypass the tumor suppressing effects of TGFβ, some tumors mutate either their type II (TβRII) or type I (TβRI) TGFβ receptor, whereas other tumors achieve bypass of TGFβ inhibition by eliminating its downstream signaling molecule, Smad4, which forms complexes with activated Smad2/3 to regulate gene expression in the nucleus. These alterations in the TGFβ signaling pathway presumably result in cancer cells which are insensitive to the anti-proliferation and anti-migration signals of TGFβ. However, aside from these examples, the majority of cancers do not appear to harbor mutations in TGFβ signaling components. How then do most cancers continue to proliferate and migrate without mutation-mediated bypass of TGFβ's inhibitory signals? If we put the importance of HIF-1α in tumor progression and the recent discovery of the "HIF-1 > Hsp90α secretion > LRP-1 > tumor cell invasion" axis into perspective, one can extrapolate a possible answer to this puzzle from the following facts: (1) Approximately 40% of all human tumors has constitutively elevated expression of HIF-1α, the critical subunit of the master transcription factor for tissue oxygen homeostasis (Semenza 2007); (2) HIF-1α is a central upstream activator of Hsp90α secretion (Li et al. 2007; Sahu et al. 2012); and (3) secreted Hsp90α is required for cancer cell invasion in vitro (Eustace et al. 2004) and tumor formation in vivo (Stellas et al. 2007; Tsutsumi et al. 2008; Song and Lou 2010; Zou et al. 2016). Therefore, it is conceivable that secretion of Hsp90α is an alternative strategy for cancer cells to bypass the anti-motility effects of TGFβ without mutating TGFβ signaling components.

15.2 Conclusions

Between 2006 and 2015, the FDA's overall likelihood of approval (LOA) from Phase I clinical trials for all therapeutic candidates was 9.6%, with the highest LOA in hematology (26.1%) and the lowest LOA in oncology (5.1%). Two critical features attributed to the success of advancing cancer trials were (i) targeting driver genes of the diseases and (ii) employing human disease-relevant animal models during preclinical studies. Mammals have two isoforms of heat shock protein-90 (Hsp90α and Hsp90β) with which they respond to environmental stress, especially during tissue ischemia. Hsp90α is not a critical chaperone like Hsp90β inside the cells. Instead, Hsp90α is secreted into the extracellular space by cells under stress, such as tissue injury. Secreted Hsp90α signals through the LRP-1 receptor to protect cells from hypoxia-induced apoptosis and to promote cell motility during wound closure. Tumors take advantage of these "useful" functions of secreted Hsp90α to cope with the constant paucity of oxygen and nutrients within the tumor microenvironment. Therefore, Hsp90α appears to be designed by nature as a "general repair" molecule with an exceptional large stockpile throughout the body of mammals. This design guarantees a quick first response by avoiding the travel delays of obstructed and damaged blood vessel "highways" and arrives equipped to extinguish TGFβ and hypoxia-induced inhibition. We look forward to exploring these new questions together, alongside the scientific community, with optimism and excitement.

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Chapter 16
Regulation of Calcineurin Signaling Through Blocking of the Chaperone Function of Hsp90 by HDAC Inhibitors

Yoichi Imai and Arinobu Tojo

Abstract Many heat shock proteins act as molecular chaperones to regulate the stability of proteins related to cell growth and survival. Histone deacetylases (HDAC) regulate gene transcription through deacetylation of histones and are involved in cell apoptosis, senescence, differentiation, and angiogenesis. HDAC can enhance protein stability through deacetylation of Hsp90. Aberrant expression of HDAC is reported in many kinds of malignancies, so HDAC inhibitors may have utility as anti-cancer agents. Panobinostat, a pan-HDAC inhibitor, blocks Hsp90 function by inhibiting HDAC6 and shows anti-leukemic effects through degradation of CXCR4 and/or AML1-ETO protein. We found that calcineurin signaling plays an important role in the pathogenesis of multiple myeloma (MM). PPP3CA, a catalytic subunit of calcineurin, was revealed to be a target of panobinostat, which shows an anti-myeloma effect by inducing PPP3CA protein degradation through Hsp90 inhibition. The anti-myeloma effect of panobinostat was enhanced on addition of FK506 and supported the importance of PPP3CA in the pathogenesis of MM. Blocking calcineurin signaling also inhibited differentiation of osteoclasts, that is essential for lytic bone lesions frequently found in patients with MM. Thus, the usefulness of calcineurin signaling regulation by HDAC inhibitors through Hsp90 chaperone blocking was revealed.

Keywords Bortezomib · Calcineurin · HDAC inhibitor · Hsp90 · Multiple myeloma · Panobinostat

Y. Imai (✉)
Department of Hematology/Oncology, IMSUT Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
e-mail: imaiyo-tky@umin.ac.jp

A. Tojo
Department of Hematology/Oncology, IMSUT Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Division of Molecular Therapy, Department of The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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Abbreviations

17-AAG	17-(allylamino)-17-demethoxygeldanamycin
AML	acute myeloid leukemia
CsA	cyclosporine A
CXCR4	C-X-C-chemokine receptor type 4
ER	estrogen receptor
GA	geldanamycin
GEO	gene expression omnibus
HDAC	histone deacetylases
MAPK	mitogen-activated protein kinase
MM	multiple myeloma
NFATc1	nuclear factor of activated T cells, cytoplasmic calcineurin-dependent 1
PKC	protein kinase C
PPP3CA	protein phosphatase 3 catalytic subunitα isozyme
RANKL	receptor activator nuclear factor-κ-B ligand
ROS	reactive oxygen species
SDF-1	stromal cell-derived factor 1
T-ALL	T cell acute lymphoblastic leukemia
Tam	tamoxifen

16.1 Introduction

Histone deacetylases (HDAC) are enzymes with multiple functions. They induce numerous biological effects, including cell apoptosis, senescence, differentiation, and angiogenesis (West and Johnstone 2014). In addition to transcriptional regulation, some HDAC enhance protein stability through deacetylation of Hsp90. Aberrant expression of HDAC has been reported in many kinds of malignancies, so HDAC inhibitors are potentially useful as anti-cancer agents. We found that calcineurin signaling plays an important role in the pathogenesis of multiple myeloma (Imai et al. 2016b). Here, we demonstrate the mechanism underlying the anti-myeloma effects mediated by HDAC inhibitors through blocking of Hsp90 chaperone function. In addition, we discuss the role of Hsp90 as a molecular chaperone in many biological functions and the possible utility of Hsp90 inhibition as an anti-cancer therapy.

16.1.1 Role of Hsp90 as a Molecular Chaperone in Biological Functions

Heat shock proteins are constitutively expressed in normal cells, and many of them act as molecular chaperones that regulate folding, intracellular localization, and turnover of many regulators of cell growth and survival, which is mediated by

proteasomal degradation (Whitesell and Lindquist 2005). Hsp90, a member of the heat-shock protein family, comprises 1–2% of the total cellular protein content, an amount that increases under stress. Hsp90 binds to a variety of client signal transducers and forms heterocomplexes (Pratt 1998). Many proteins are Hsp90 clients, including molecules related to uncontrolled proliferation, immortalization, impaired apoptosis, angiogenesis, and invasion/metastasis. Their complex organization is presumed to be involved in carcinogenesis.

HER2 is a member of the EGFR family. High expression of HER2 is associated with aggressive breast cancers with poor disease prognosis (Münster et al. 2002). 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) is an analogue of geldanamycin (GA) that binds to a highly conserved pocket in the Hsp90 chaperone protein and inhibits its function (Stebbins et al. 1997); (Prodromou et al. 1997). HER2 is one of the most sensitive targets of 17-AAG. 17-AAG reduces growth and induces apoptosis of breast cancer cells expressing high levels of HER2 by allowing its degradation (Münster et al. 2002).

The serine/threonine kinase Raf-1 is a signal transducer of the Ras-Raf-1-MEK-mitogen-activated protein kinase (MAPK) signaling pathway and forms a complex with Hsp90 and other proteins (Schulte et al. 1996). GA binds to Hsp90 and disrupts the complex formation between Raf-1 and Hsp90, leading to the destabilization of Raf-1. GA blocks phorbol 12-myristate 13-acetate-induced proliferation and activation of MAPK-sensitive transcription factors. Thus, the interaction between Raf-1 and Hsp90 is assumed to be essential for the stability and function Raf-1 as a signal transducer.

Mammalian $p50^{Cdc37}$ associates with Hsp90 and targets Cdk4, which is involved in cell cycle progression (Stepanova et al. 1996). Newly synthesized Cdk4 is unstable in the absence of functional Hsp90. Pharmacological inactivation of Cdc37/Hsp90 function induces Cdk4 degradation.

Estrogen receptors are one of the therapeutic targets in hormone-responsive breast cancer. The antiestrogen tamoxifen (Tam) has been used for therapy in estrogen receptor (ER)-positive breast cancers (Beliakoff et al. 2003). However, resistance to Tam develops frequently and a ligand-independent approach could be useful in overcoming this resistance compared to hormonal therapy that depletes ER levels. ER forms complexes with multiple proteins, including Hsp90. It can be depleted by GA treatment in Tam-resistant breast cancer cell lines. Tam inhibits GA-induced depletion of ER levels, and it is probable that Tam might reduce the anti-cancer activity of 17-AAG.

Telomerase provides the enzymatic activity that compensates for chromosome shortening during meiosis (Holt et al. 1999). In the absence of this compensatory activity, the progressive shortening of chromosomes will result in the inability of cells to proliferate. Molecular chaperones, p23 and Hsp90, were shown to bind to the catalytic subunit of telomerase, and this interaction is essential for assembly of active telomerase. Thus, Hsp90 is thought to perform various biological functions by protecting the interacting proteins from degradation.

16.1.2 Hsp90 Is One of the Targets of HDAC Inhibitors

HDAC inhibitors target class I, II, and IV HDAC. Hematological malignancies are treated with three HDAC inhibitors, including vorinostat, romidepsin, and panobinostat (West and Johnstone 2014; Richardson et al. 2013). Eleven HDAC, differing in subcellular location, substrate specificity, and enzymatic activity, are divided among three classes (I, II, and IV). Class I HDAC (1, 2, 3, and 8) are generally nuclear proteins, and HDAC 1, 2, and 3 regulate gene expression through deacetylation of histones. Class II HDAC (4, 5, 6, 7, 9, and 10) can shuttle between the nucleus and the cytoplasm. Among class II HDAC, HDAC6 is uniquely involved in enhancement of the stability of proteins that are members of signaling pathways through deacetylation of tubulin and Hsp90 (Sadoul and Khochbin 2016; Boyault et al. 2007). Thus, each HDAC shows different biological functions depending on its substrate.

Acetylation of histones is generally associated with upregulation of transcription. HDAC regulate gene expression through enzymatic removal of the acetyl group from histones. Genome-wide changes in histone acetylation, including a global loss of monoacetylation and trimethylation of histone H4, are reported to be related to cancer onset and progression (Fraga et al. 2005). Furthermore, aberrant expression of HDAC is reported in many kinds of hematological and non-hematological malignancies (West and Johnstone 2014). Expression of HDAC1, −5, and −7 serves as a useful biomarker to differentiate between tumor and normal tissue (Ozdağ et al. 2006). Epigenetic suppression of tumor suppressor genes, like *CDKN1A* encoding the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ by overexpression of HDAC is reportedly linked to tumorigenesis (Glozak and Seto 2007). Thus, epigenetic change is hypothesized to be a mechanism underlying the anti-cancer action of HDAC inhibitors.

The activity of HDACs against non-histone proteins, such as tubulin and Hsp90, is one of the biological functions of HDACs (Spange et al. 2009). Panobinostat is a pan- HDAC inhibitor that blocks proliferation and induces apoptosis of acute myeloid leukemia (AML) cells through inhibition of Hsp90 function (Spoo et al. 2007). C-X-C-chemokine receptor type 4 (CXCR4) is a receptor for stromal cell-derived factor 1 (SDF-1), and SDF-1-CXCR4 signaling is essential for the maintenance of viability of leukemic cells. Correlation between its elevated expression in the blasts of AML patients and poor disease prognosis implies that CXCR4 could be a therapeutic target (Mandawat et al. 2010). CXCR4 binds to Hsp90 as its client protein, and this binding protects CXCR4 from protein degradation by the 20S proteasome. Panobinostat-induced acetylation of Hsp90 reduces the chaperone function of Hsp90 and prompts protein degradation of CXCR4; this degradation is assumed to contribute to an anti-leukemic effect.

AML1-ETO fusion protein, generated from a chromosomal translocation t(8;21), is assumed to cause leukemia in conjunction with secondary mutagenic events involving genes, such as *FLT3*, c-*KIT*, N-*RAS*, and K-*RAS* (Müller et al. 2008). AML1-ETO recruits various kinds of transcription factors and epigenetic regulatory

agents, including HDAC (Bots et al. 2014). This complex formation also plays an important role in leukemogenesis in AML patients with t(8;21). Thus, HDAC is a potential therapeutic target for AML patients with t(8;21). An AML mouse model was generated by enforced expression of AML1-ETO9a, a shorter isoform of AML1-ETO, and $Nras^{G12D}$. Treatment of the mice with panobinostat demonstrated a robust antileukemic response (Bots et al. 2014). This response was neither dependent on p53 function nor activation of the conventional apoptotic pathway. Instead, myeloid differentiation of blast cells was induced by proteasomal degradation of AML1-ETO9a. These results suggest that oncogenic protein is protected from protein degradation by Hsp90 and that Hsp90 could be a good target in anti-cancer therapy,

16.1.3 Mechanism Underlying the Action of HDAC Inhibitors in the Treatment of Multiple Myeloma

Multiple myeloma (MM) originating from plasma cell, a mostly differentiated hematopoietic cell, is an incurable hematological malignancy. In many cases, myeloma cells respond poorly to treatment with conventional chemotherapy. Introduction of novel drugs, including proteasome inhibitors (bortezomib and carfilzomib) and immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide) with mechanisms of action different from conventional chemotherapy has led to great advancements in treatment of patients with MM. However, many patients become resistant to proteasome inhibitors and immunomodulatory drugs, and development of novel therapies that target molecules other than proteasome inhibitors and immunomodulatory molecules is expected. In this context, the possible utility of HDAC inhibitors in the treatment of MM has been revealed by several preclinical studies (Mitsiades et al. 2003; Pei Dai and Grant 2004).

Suberoylanilide hydroxamic acid (vorinostat) is a class I and II HDAC inhibitor and its use for treatment of cutaneous T-cell lymphoma was approved by the FDA (Duvic and Dimopoulos 2016). The cyclin-dependent kinase inhibitor $p21^{WAF1}$ plays the role of a tumor-suppressor through mediation of p53-dependent cell cycle arrest (Gartel 2007). Treatment of myeloma cells with vorinostat led to modification of the acetylation and methylation status of promoter regions and induction of $p21^{WAF1}$ expression (Gui et al. 2004). Vorinostat treatment also inhibited interleukin-6 secretion from bone marrow stromal cells and made it possible to overcome cell adhesion-mediated drug resistance (Mitsiades et al. 2003; Noborio-Hatano et al. 2009). Furthermore, oxidative injury caused by reactive oxygen species (ROS) generation due to mitochondrial dysfunction and apoptosis induced by vorinostat is enhanced by pretreatment with bortezomib. The effectiveness of this combination therapy was demonstrated in dexamethasone- and doxorubicin-resistant myeloma cells (Pei et al. 2004).

Combination therapy with bortezomib and panobinostat, the other HDAC inhibitor, is effective for relapsed and refractory patients, who have been previously treated with bortezomib (Richardson et al. 2013). In myeloma cells, various kinds

of proteins, including immunoglobulins are produced and protein homeostasis is maintained through ubiquitination and degradation of misfolded and unfolded proteins (Adams 2004). An excessive accumulation of misfolded and unfolded proteins disturbs normal cell function, and the dependence of cancer cells on the proteasome for clearance of such cytotoxic proteins is stronger than that of normal cells (Plemper and Wolf 1999). Bortezomib is a reversible inhibitor of chymotrypsin in the 20S subunit of proteasomes (LeBlanc et al. 2002). Blocking the clearance mechanism increases cell stress due to accumulation of misfolded and unfolded proteins. This is supposed to be the mechanism underlying the action of proteasome inhibitors.

An aggresome is a microtubule-based structure made from protein aggregates transported in a retrograde manner (Johnston et al. 1998; Kopito 2000). When production of misfolded and unfolded proteins is beyond the proteasome's capacity for degradation, an aggresome is formed to degrade the excess. This aggresome formation is supposed to weaken the cytotoxic effects of proteasome inhibitors. Aggresomes formed in cell peripheries travel to microtubule organizing centers along microtubules (García-Mata et al. 1999); this movement is mediated by dynein and HDAC6, a microtubule-associated deacetylase. Thus, clearance of misfolded and unfolded proteins in bortezomib-treated myeloma cells is blocked by HDAC6 inhibition mediated by panobinostat. Panobinostat and bortezomib act synergistically and induce apoptosis by inhibiting protein degradation (Hideshima et al. 2011). This synergistic action may partly explain the effectiveness of combination therapy using bortezomib and panobinostat in patients previously treated with bortezomib.

16.1.4 PPP3CA a New Therapeutic Target for MM

As discussed above, combination therapy using proteasome and HDAC inhibitors showed enhanced effectiveness compared with a single agent alone that mediates its effects through inhibition of aggresome formation or induction of apoptosis via ROS generation. However, the pathogenic molecules in myeloma cells are not identified in either study, so we tried to find the molecule responsible for MM progression, which could be targeted by proteasome and HDAC inhibitors (Imai et al. 2016a, b). For this purpose, we evaluated the data collected by the high-resolution analysis of recurrent copy number alterations and expression profiles in myeloma cell lines and MM patient samples (Carrasco et al. 2006). In this study, there were 179 possible genes located on minimal common chromosomal regions with gains or amplifications that were postulated to be potential oncogenes. We compared the expression of these genes in different disease stages using the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) analysis of gene expression data (Agnelli et al. 2005). The disease stage of each patient was diagnosed based on the Durie-Salmon staging classification system, which divides myeloma patients into three groups – stage I, asymptomatic and not advanced; stage II, intermediate; and stage III, advanced (Durie and Salmon 1975). Those genes with more

expression in stage III patients than stage I and II are supposed to be related to MM progression. Among the 179 candidate genes, mRNA expression of 8 genes (*BCS1L*, *POLR2H*, *PPP3CA*, *H2AFZ*, *ASK*, *RBM28*, *MRPL13*, and *WDR18*) was significantly higher in myeloma cells from stage III patients as compared to than in cells from stage I patients. To elucidate the effects of HDAC inhibitors on lymphoid malignancies, we selected *PPP3CA (protein phosphatase 3, catalytic subunit, α isozyme)* which encodes the catalytic subunit of calcineurin for further analysis. Calcineurin is a calcium-calmodulin-dependent serine/threonine protein phosphatase that plays a critical role in T cell activation following T cell receptor engagement (Gachet and Ghysdael 2009). Calcineurin inhibitors, including FK506 and cyclosporine A (CsA), act as immunosuppressive drugs through suppression of nuclear translocation of the nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), a dephosphorylation target of calcineurin. NFATc1 is a transcription factor essential for activation of gene transcription during the immune response (Rao et al. 1997). Calcineurin is engaged in regulation of the immune response through activation of pre-T-cell receptor signaling (Neilson et al. 2004). The relevance of calcineurin activation in the pathogenesis of hematological malignancies has been reported for T cell acute lymphoblastic leukemia (T-ALL) (Medyouf et al. 2007). Treatment with FK506 and CsA induced T-ALL regression in a T-ALL mouse model and prolonged mouse survival. Although studies on calcineurin have been performed mainly using T cells, defective B cell activation caused by decreased calcineurin activation suggests the possible pathogenesis of calcineurin activation in B cell malignancy (Bhattacharyya et al. 2011). In fact, activation of NFATc1 promotes proliferation and induces survival of diffuse large B cell lymphoma-derived cell lines (Pham et al. 2005; Fu et al. 2006). In this context, we investigated whether overexpression of *PPP3CA* could be related to progression of MM, a B cell malignancy. We found high *PPP3CA* expression in MM cell lines compared to that in non-MM cell lines. Furthermore, examination of 42 MM patient samples from our institute revealed higher expression of *PPP3CA* in stage III compared to stage I.

16.1.5 Calcineurin Degradation Induced by HDAC and Proteasome Inhibitors Through Hsp90 Inhibition in MM and Its Clinical Significance

Next, we investigated the effects of panobinostat and bortezomib on PPP3CA protein expression in MM cell lines. PPP3CA protein expression in myeloma cell lines was reduced on treatment with both panobinostat and bortezomib. A co-immunoprecipitation assay revealed the physical interaction between PPP3CA and Hsp90 in myeloma cells, and treatment of myeloma cells with HDAC inhibitor induced the release of Hsp90 from PPP3CA. Furthermore, PPP3CA expression was reduced by the Hsp90 inhibitor, 17-AAG while lactacystin, a proteasome inhibitor, recovered the decrease in PPP3CA levels induced by panobinostat. These results

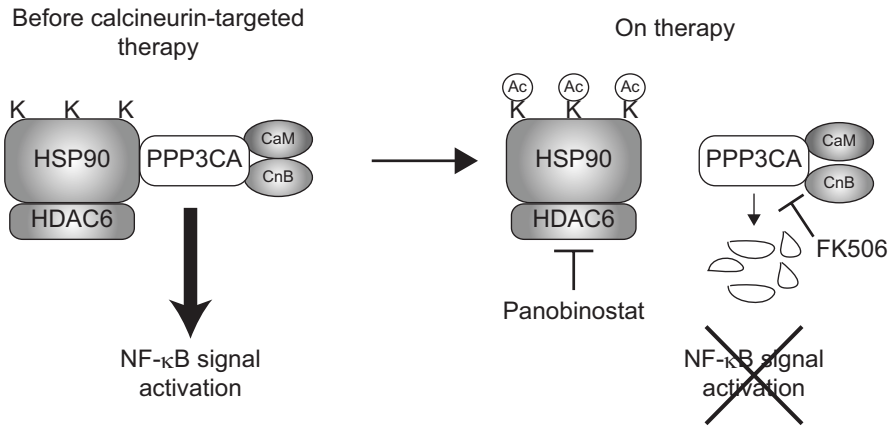

Fig. 16.1 Molecular mechanisms of calcineurin-targeted therapy for patients with MM. Calcineurin complex is composed of PPP3CA, calmodulin (CaM), and calcineurin B (CnB). Before calcineurin-targeted therapy, PPP3CA is protected from proteasome degradation through binding to Hsp90. Treatment with panobinostat induces acetylation of the lysines of Hsp90 by HDAC6 inhibition. PPP3CA is released from hyperacetylated Hsp90 and its protein degradation is induced. Addition of FK506 enhances this protein degradation by blocking the interaction between PPP3CA and CnB. Thus, NF-κB signal activation in MM cells is inhibited and the viability of myeloma cells is reduced. *K* lysine, *Ac* acetylation

support the possibility that PPP3CA is protected from protein degradation by binding to Hsp90 and that its expression is reduced through protein degradation after Hsp90 is inhibited by hyperacetylation due to panobinostat's action (Fig. 16.1). Calcineurin signaling was assumed to be modified by HDAC inhibitors through inhibition of Hsp90. In contrast, bortezomib reduced PPP3CA expression by two ways, namely, transcriptional repression and HDAC6 inhibition. Treatment of myeloma cell lines with bortezomib reduced expression of *PPP3CA* mRNA in a dose dependent manner. At the same time, mRNA of *HDAC6* was also reduced on bortezomib treatment, and PPP3CA protein level was possibly reduced through Hsp90 inhibition. NFATc1 is one of the dephosphorylation substrates of PPP3CA. In addition to mediating NFATc1 activation, PPP3CA was shown to act synergistically with protein kinase C (PKC) to activate NF-κB signaling in T cells (Trushin et al. 1999). The involvement of NF-κB signaling in the pathogenesis of MM has been reported (Keats et al. 2007). In fact, knockdown of *PPP3CA* using shRNA in myeloma cell lines revealed the important roles of PPP3CA in the maintenance of the viability of myeloma cells; PPP3CA exerts these effects by activation of the NF-κB signaling pathway (Fig. 16.1). Treatment of myeloma cell lines with FK506 alone did not affect PPP3CA expression or cell viability. However, combination therapy using panobinostat and FK506 enhanced the reduction of both PPP3CA expression and cell viability compared to that induced by panobinostat alone. PPP3CA heterodimerizes with calcineurin B, the regulatory subunit of calcineurin. Calcineurin B has been shown to protect PPP3CA from protein degradation (Gachet and Ghysdael 2009; Cheng et al. 2011). FK506 inhibits the association between

calcineurin B and unstable PPP3CA released from Hsp90, and this dissociation is supposed to promote the degradation of PPP3CA (Fig. 16.1) (Hemenway and Heitman 1999; Brewin et al. 2009). The enhanced anti-myeloma effect of panobinostat mediated by FK506 was also demonstrated in vivo using a mouse xenograft model. As described above, PPP3CA was a common target of panobinostat and bortezomib. Examination of patient samples revealed that there is a correlation between expression of *PPP3CA* mRNA and resistance to bortezomib. Expression of *PPP3CA* mRNA was higher in samples from patients resistant to bortezomib compared to that in samples from sensitive patients. Furthermore, progression-free survival in bortezomib-containing therapies was shorter in patients with high *PPP3CA* mRNA expression than in patients with low expression. MM patients often suffer from lytic bone lesions generated by activated osteoclasts (Abe et al. 2004). The induction of NFATc1 expression by receptor activator nuclear factor-κ-B ligand (RANKL) is essential for osteoclast differentiation, which FK506 inhibits (Zawawi et al. 2012). The previous study demonstrated that panobinostat reduced bone density loss in a disseminated MM xenograft mouse model (Ocio et al. 2010). In our study, panobinostat and FK506 each inhibited osteoclast differentiation, while treatment with both drugs together increased the blockade of osteoclast formation compared to treatment with either agent alone. Thus, calcineurin-targeted therapy is supposed to effectively inhibit lytic bone lesions and MM cell viability and is expected to halt the vicious cycle induced between MM cells and osteoclasts.

16.2 Conclusions

Among its many biological functions, Hsp90 also plays a role as a molecular chaperone. It protects proteins related to carcinogenesis, intracellular signaling, cell cycle progression, and telomerase from degradation and maintains them in their active form. Panobinostat, a pan HDAC inhibitor, blocks Hsp90 function via HDAC6 inhibition and shows an anti-leukemic effect by allowing degradation of CXCR4 and AML1-ETO, which are related to leukemogenesis. Combination therapy of panobinostat with bortezomib, a proteasome inhibitor, was approved for treatment of relapsed and refractory MM patients, and we revealed that PPP3CA, a catalytic subunit of calcineurin, is a common target of panobinostat and bortezomib. Panobinostat shows an anti-myeloma effect by inducing PPP3CA protein degradation through Hsp90 inhibition, which leads to inhibition of the NF-κB signaling pathway in myeloma cells. Enhancement of the anti-myeloma effect of panobinostat by addition of FK506 would support the importance of PPP3CA in the pathogenesis of MM. Blocking of calcineurin signaling will also be effective for treating lytic bone lesions, which frequently co-occur with MM.

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Chapter 17
Signaling Functions of Extracellular Hsp90 (eHsp90) in Cancer Metastasis

Aaron S. Bernstein and Daniel G. Jay

Abstract Heat shock protein 90 (Hsp90) is a highly conserved eukaryotic molecular chaperone. Hsp90 is abundantly expressed in normal cells, and even more abundant in cancer cells, from which it is also secreted. Homeostatic and cancer-supporting roles for intracellular Hsp90 have both been identified. Hsp90 in the extracellular space (extracellular Hsp90, or eHsp90) has been shown to support each step during cancer progression, and could potentially be targeted in order to safely prevent metastasis without affecting critical intracellular functions of Hsp90. This chapter will highlight pro-metastatic signaling functions of eHsp90.

Keywords Cancer · eHsp90 · Extracellular Hsp90 · Metastasis · Signaling

Abbreviations

CAF	cancer-associated fibroblasts
CCM	conditioned culture media
ChIP	chromatin immunoprecipitation
DAMP	damage-associated molecular patterns
EGFR	epidermal growth factor receptor
eHsp90	extracellular Hsp90
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-related protein kinase
EZH2	enhancer of Zeste homolog 2
FAK	focal adhesion kinase

A. S. Bernstein
Graduate Program in Cellular & Molecular Physiology, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, MA, USA

D. G. Jay (✉)
Department of Developmental, Molecular & Chemical Biology, School of Medicine, Tufts University, Boston, MA, USA
e-mail: daniel.jay@tufts.edu

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FALI	fluorophore-assisted light inactivation
GBM	glioblastoma multiforme
HB-EGF	heparin-binding epidermal growth factor
HIF-1α	hypoxia-inducible factor-1 alpha
IL	interleukin
LOXL2	lysyl oxidase-like protein 2
LRP1	lipoprotein-related protein
MMP	matrix metalloproteinase
MVB	multivesicular body
PAMP	pathogen associated molecular patterns
PKCδ	protein kinase Cδ
PRC2	repressive Polycomb complex 2
rHsp90	recombinant heat shock protein 90
SMA	smooth muscle actin
SRF	serum response factor
TCF12	transcription factor 12
TLR	Toll-like receptor 4
TNFα	tumor necrosis factor alpha
tPA	tissue plasminogen activator
TRP	tetratricopeptide repeat

17.1 Introduction

Metastasis, or the spread of cancer from one part of the body to another, is the major cause of death from cancer. However, no current therapies target metastasis. Hence, there is a pressing need for safe drugs that can prevent this process. Heat shock protein 90 (Hsp90) is a chaperone protein that activates, stabilizes, and processes proteins that function in cancer progression/metastasis, and may serve as a viable target for preventing the spread of cancer.

Hsp90 is a highly-conserved, 90 kDa eukaryotic molecular chaperone with over 200 identified intracellular client proteins (Woodford et al. 2017). In higher eukaryotes, there are two distinct Hsp90 isoforms that share 86% amino acid conservation, Hsp90α and Hsp90β (Pratt and Toft 1997). Hsp90β is constitutively expressed, while the expression of Hsp90α is stress-inducible (Buchner and Li 2013). Hsp90α accounts for 2–3% of total cellular proteins in normal cells, and up to 7% in certain tumor cell lines (Li et al. 2012).

For the past 24 years, since the discovery that geldanamycin, a natural Hsp90 inhibitor, has antitumor activity (Whitesell et al. 1994), Hsp90 has been a target of interest for cancer therapy (Barrott and Haystead 2013). Since multiple Hsp90 clients have been identified as oncoproteins, targeting Hsp90 for cancer therapy has the potential to disrupt multiple cancer pathways. In this way, Hsp90 inhibition may also increase tumor cell sensitivity to chemotherapeutic intervention (Kim et al. 2017). To date, over 100 clinical trials examining more than 25 different Hsp90

inhibitors, or formulations of Hsp90 inhibitors, either alone or in combination with other drugs or anti-cancer therapies have been conducted in the United States (clinicaltrials.gov). Unfortunately, the results of these trials have been largely disappointing (Chatterjee et al. 2016; Modi et al. 2013).

One of the major roadblocks for anti-Hsp90 therapy has been dose-limiting toxicity due in part to inhibition of Hsp90's many intracellular functions. In 2004, using a fluorophore-assisted light inactivation (FALI) screen, our lab first found that Hsp90α was released by fibrosarcoma and breast adenocarcinoma cells where it has essential roles in cell invasion. Mechanistically, we showed that eHsp90α enhances the activity of matrix metalloproteinase 2 (MMP2), an extracellular matrix-remodeling enzyme (Eustace et al. 2004). Later that same year, it was shown that Hsp90 localizes to the surface of cells from the nervous system of rats and that this extracellular Hsp90 (eHsp90) was necessary for lamellipodia formation and cell migration (Sidera et al. 2004). Since these initial findings, eHsp90 released from stressed and/or tumor cells has been implicated in multiple processes that contribute to cancer progression and ultimately metastasis (Li et al. 2012; Wong and Jay 2016).

Normal cells secrete Hsp90α when triggered by environmental stress, such as heat shock or hypoxia. Tumor cells constitutively secrete Hsp90α in a hypoxia-inducible factor-1 alpha (HIF-1α)-dependent fashion. Although the signaling steps between HIF-1α and Hsp90α secretion, as well as the precise mechanism of Hsp90α secretion remain to be fully elucidated, it has been demonstrated that cleavage of a C-terminal EEVD motif that interacts with proteins containing tetratricopeptide repeat (TRP) domains is required for secretion of Hsp90α. Hsp90α secretion is also dependent upon phosphorylation at Thr-90 (Wang et al. 2009). Heat shock proteins lack the N-terminal signal sequence required for the canonical endoplasmic reticulum/Golgi apparatus pathway of protein secretion (Cheng et al. 2008). Instead, it appears that Hsp90α exits cells via the endocytic pathway, on the surface of exosomes, which are a subset of microvesicles originating in the multivesicular body (MVB). When an MVB fuses with the cell's plasma membrane, exosomes are released from cells. McCready et al. (2010) demonstrated that blocking exosome release from breast cancer, fibrosarcoma, or glioblastoma cells decreased eHsp90α. However, further work is still needed to more completely define the mechanism of Hsp90α secretion and to understand if it plays a role in exosomal cell-cell communication, which itself has been shown to promote the spread of cancer (Christianson et al. 2013). Understanding the pathways that control the post-translational modifications and interactions necessary for the translocation of Hsp90 to the extracellular space has the potential to point researchers toward upstream activators of this process, which could yield new targets for preventing the secretion of this protein. Reducing tumor-released Hsp90 would be one way to abrogate its pro-metastatic effects.

Targeting eHsp90 with molecules that block its pro-metastatic effects is an alternative approach. While global inhibition of Hsp90 disrupts normal, critical intracellular functions of this chaperone, leading to significant toxicity complications, specific inhibition of extracellular Hsp90 could avoid these adverse events and might reduce metastasis in humans (Wong and Jay 2016). Several papers have

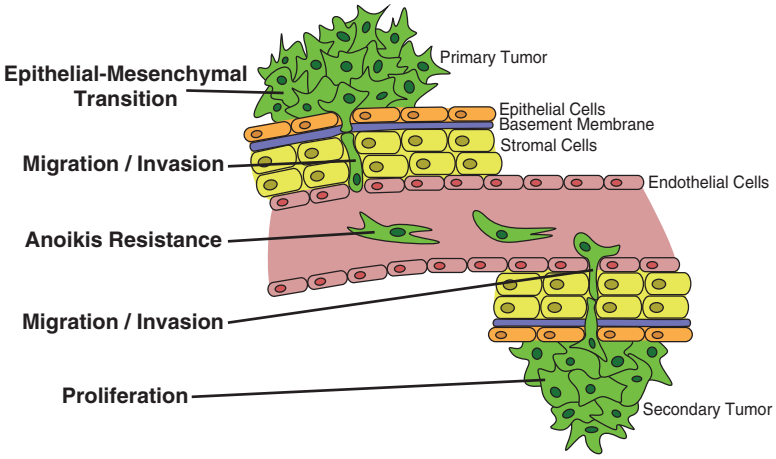

Fig. 17.1 The process of cancer metastasis. There are multiple steps in the metastasis of a carcinoma. In a process termed epithelial-mesenchymal transition, cancer cells disengage cell-cell and cell-matrix connections in order to break away from the primary tumor. The cells then up-regulate motility programs and invade through the basement membrane. Once in the surrounding stromal tissue, cells intravasate blood or lymphatic vessels by further invasion through endothelial cell linings. Circulating tumor cells must next survive traveling to a distant site in the body by avoiding anoikis, or apoptotic cell death brought about by the loss of cell attachments. Cancer cells that extravasate blood or lymphatic vessels, again by invading through the surrounding stromal tissue, can go on to form secondary tumors. The final step of metastasis occurs when a cancer cell takes root and begins to proliferate in a distant site

shown that inhibitors of eHsp90 improve survival and/or reduce metastasis in tail vein or mammary fat pad injection animal models of metastasis (Stellas et al. 2010; Tsutsumi et al. 2008; Zou et al. 2016). Zou et al. (2016) examined the blockade of particular eHsp90α epitopes in an effort to more precisely target the pro-metastatic functionality of this protein.

Metastasis of a carcinoma, or epithelial cancer, which will be the main focus of this chapter, is a multi-step process (Fig. 17.1). Cancer cells must first disengage cell-cell and cell-matrix connections holding them in place in order to break away from the primary tumor. Activation of a cell program known as epithelial-mesenchymal transition can promote these changes. After breaking away from the primary tumor, cancer cells invade through the basement membrane, and into the surrounding stromal tissue, where they then intravasate a blood or lymphatic vessel by invading through endothelial cell linings. Pro-motility and pro-invasion behaviors aid in a cell's ability to move from the site of a primary tumor to circulatory vessels. Once in circulation, a cancer cell must survive traveling to a distant site in the body, avoiding anoikis, or apoptotic cell death brought about by the loss of cell attachments. Cancer cells that go on to form secondary tumors must then exit the blood or lymphatic vessels in a process called extravasation, again by invading through the surrounding stromal tissue. The final step of metastasis occurs when a cancer cell takes root and begins to proliferate in a distant site. (Chaffer and Weinberg 2011).

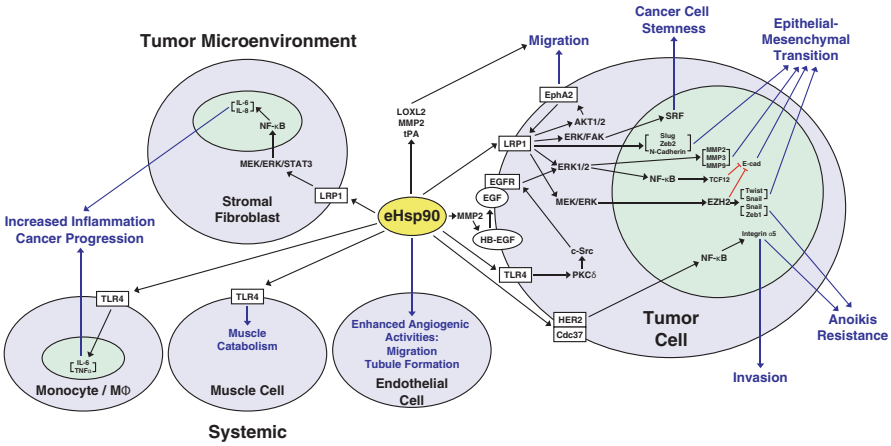

Fig. 17.2 Signaling functions of eHsp90 promote cancer metastasis. eHsp90 signaling promotes cancer metastasis by increasing cancer cell epithelial-mesenchymal transition (EMT), migration, invasion, stemness, and anoikis resistance. eHsp90 signaling also promotes cancer progression by increasing inflammatory cytokine production by immune cells and stromal fibroblasts in the tumor microenvironment, stimulating muscle cell catabolism, and promoting angiogenic activities in endothelial cells

This chapter will examine the signaling functions of eHsp90 that contribute to each step of cancer metastasis (Fig. 17.2). Because signaling by eHsp90 contributes to each step of metastasis, specific inhibition of eHsp90 could be a safe way to prevent the spread of cancer.

17.1.1 Pro-EMT Signaling Functions of eHsp90

As a carcinoma transitions from cancer in situ to metastatic disease, epithelial-mesenchymal transition (EMT), "a complex molecular and cellular programme by which epithelial cells shed their differentiated characteristics, including cell–cell adhesion, planar and apical–basal polarity, and lack of motility, and acquire instead mesenchymal features, including motility, invasiveness and a heightened resistance to apoptosis," often takes effect (Polyak and Weinberg 2009). EMT is a transdifferentiation pattern recognized for its utility for cell dispersal in vertebrate embryo development (Hay 1995) and the creation of fibroblasts in the context of injury repair (Kalluri and Neilson 2003). However, understanding the mechanisms underlying cancer's utilization of this program and the impact of these mechanisms on disease progression could point to new targets for the prevention of metastasis. Multiple signaling pathways involving eHsp90 have been implicated in epithelial-mesenchymal transition in the context of cancer.

Chen et al. (2010) demonstrated that eHsp90α promotes colorectal cancer cell migration/invasion via the cell surface receptor low density lipoprotein-related

protein (LRP1, also called CD91). eHsp90 signaling through an LRP1/IκB kinase (IKK)/NF-κB signaling cascade induces transcription factor 12 (TCF12) expression, which then leads to down-regulation of epithelial cadherin (E-cadherin), a change that is a hallmark of EMT. Here, Hsp90α facilitates complex formation between the cell surface receptor LRP1 and IκB kinases (IKKs) α and β, and leads to increases in phosphorylated (active) IKKs α/β and NF-κB. Physical association between NF-κB and the TCF12 promoter, which was seen by chromatin immunoprecipitation (ChIP) in rHsp90α-treated colorectal cancer cells, is the likely mechanism underlying the enhancement of TCF12 expression (Chen et al. 2013). TCF12 functions as a transcriptional repressor of E-cadherin (Lee et al. 2012). Again, loss of this calcium-dependent cell-cell adhesion protein is a classical indication of EMT (van Roy and Berx 2008).

Hance et al. (2012) found that eHsp90 signaling through LRP1 promoted prostate cancer cell motility and EMT, in part by up-regulating extracellular signal-related protein kinase (ERK) and focal adhesion kinase (FAK) activation. ERK activity following eHsp90 exposure up-regulated transcription of the extracellular matrix-remodeling matrix metalloproteases (MMPs) 2, 3 and 9. MMPs are, "zinc-dependent endopeptidases that degrade components of the basement membrane, promote EMT events, and support metastatic spread" (Hance et al. 2012). Corroborating this finding, Tsen et al. (2013) reported that activating phosphorylation of ERK1/2 following eHsp90α signaling was blocked in LRP1 down-regulated cells. Hance et al. (2012) also demonstrated that physiologically relevant expression of secreted eHsp90 in phenotypically epithelial cells down-regulated transcript expression of the epithelial marker E-cadherin, while up-regulating expression of N-cadherin, Snail, Slug, Zeb1, and Zeb2 transcripts, all of which are indicative of EMT. In this study, inhibition of eHsp90 blocked pro-motility signaling, prevented cell migration and promoted an epithelial phenotype. Inhibition of pan-MMP or ERK activity attenuated the pro-motility effects of eHsp90, which indicated that these intermediaries are critical for the pro-EMT effects of eHsp90 in the context of prostate cancer cells.

Following-up on this work, the same group demonstrated that in vivo, eHsp90 was sufficient to promote prostate tumor growth and cellular invasion and that in a cell culture model, inhibition of eHsp90 led to increased expression of the epithelial marker E-cadherin (Nolan et al. 2015). Here, a novel epigenetic function of Hsp90 was uncovered. eHsp90 signaling, likely through the cell surface receptor LRP1, was seen to up-regulate MEK/ERK activation. This signaling promoted up-regulation of methyltransferase enhancer of Zeste homolog 2 (EZH2) transcription. EZH2 was then recruited to the E-cadherin promoter and served as the catalytic element of the repressive Polycomb complex 2 (PRC2), which initiates gene silencing by inducing histone H3-K27 trimethylation. Up-regulation of EZH2 by eHsp90 signaling also caused increased expression of Snail and Twist, transcription factors which have both been reported as crucial for EMT activation (Wang et al. 2016). Inhibition of eHsp90 reduced activating phosphorylation of ERK as well as EZH2 expression, and led to an increase in E-cadherin expression (Nolan et al. 2015).

eHsp90 signaling through LRP1 up-regulates ERK activity that increases MMP2 and MMP9 transcription (Hance et al. 2012). eHsp90 also appears to play a crucial role in mediating the enhancement of MMP2 activation. Eustace et al. (2004) showed that inhibiting eHsp90 reduced pro-MMP2 in the extracellular medium by 15%, while reducing active extracellular MMP2 by 80%. Sims et al. (2011) demonstrated that Hsp90α-mediated activation of MMP2 is assisted by a complex of co-chaperones including Hsp70, Hop, Hsp40, and p23, which were all previously determined to be present outside of cancer cells. In addition to degrading gelatin and type IV collagen, which is "the basic component of the basement membrane…the main barrier separating in situ and invasive carcinoma," (Stellas et al. 2010) MMP2 and MMP9 were shown to be involved in the shedding of heparin-binding epidermal growth factor (HB-EGF) and its subsequent induction of constitutive epidermal growth factor receptor (EGFR, also called ErbB1 and HER1) phosphorylation in human prostate cancer cells (Lue et al. 2011). This EGFR phosphorylation further enhanced ERK signaling, which promotes EMT.

Targeting the pro-EMT effects of eHsp90 signaling through LRP1 in order to prevent the spread of cancer is an appealing concept. However, this interaction has also been shown to play an important role in the migration of skin cells during normal wound healing (Cheng et al. 2008). Future work aimed at developing eHsp90-targeting therapeutics in order to prevent EMT in cancer cells will also need to account for this protein's protective role in wound repair in order to avoid the emergence of deleterious side effects.

After transitioning from an epithelial to a mesenchymal phenotype, cancer cells become more motile and can begin to invade the stromal tissue surrounding a tumor. As MMPs work to degrade the basement membrane, signaling by eHsp90 further enhances cell motility and invasiveness, which allows cancer cells to begin their journey toward blood and lymphatic vessels that are then used for cancer cell travel to distant sites in the body (Chaffer and Weinberg 2011).

17.1.2 Pro-motility and Pro-invasion Signaling Functions of eHsp90

Following the induction of EMT, cell migration and invasion are critical steps in the metastatic process. These cellular behaviors allow cancer cells to migrate away from a primary tumor and invade the surrounding stromal tissue. They come into play again when metastatic cancer cells extravasate blood or lymphatic vessels and invade a metastatic niche at a distant site. eHsp90 increases cancer cell motility and invasiveness (Eustace et al. 2004; McCready et al. 2010; Sidera et al. 2008).

In addition to activating matrix proteases and interacting with tissue plasminogen activator (tPA) to promote the activation of plasmin- activities that contribute to remodeling of the extracellular matrix (ECM), cell migration, and potentially to further signaling events (Eustace et al. 2004; McCready et al. 2010; Sims et al.

2011)-eHsp90 also directly affects pro-migratory signaling. Gopal et al. (2011) demonstrated that eHsp90 enhances the motility of cancer cells by interacting with cell surface LDL receptor-related protein 1 (LRP1). In the context of wound healing, this interaction makes eHsp90 a *bona fide* pro-motility signaling molecule for both epidermal and dermal cells (Cheng et al. 2008). Interaction between eHsp90 and subdomain II in the extracellular part of LRP1 leads to the sustained activating phosphorylation of AKT kinases 1 and 2 by the NPVY motif in the cytoplasmic tail of LRP1 (Tsen et al. 2013). Activated AKT then causes the activating phosphorylation of the receptor tyrosine kinase EphA2, which is overexpressed in the majority of glioblastoma multiforme (GBM) specimens. Phosphorylated EphA2 contributes to GBM cell motility and invasiveness via interaction with LRP1. This interaction sustains LRP1 signaling promoting lamellipodia formation, which is a phenotype that enhances GBM aggressiveness (Gopal et al. 2011). Sahu et al. (2012) demonstrated that a fragment from the linker region and middle domain of Hsp90α, termed F5 and consisting of amino acids 236–350, retained the pro-motility function of full length Hsp90α, but failed to stimulate migration and invasion in LRP1-downregulated breast cancer cells. Following-up on this work, Zou et al. (2016) showed, via mutagenesis, that within the F5 fragment, two evolutionarily conserved lysine residues, lys-270 and lys-277, are crucial for eHsp90's ability to enhance breast cancer cell invasion (but not its ability to bind LRP1) in vitro, as well as mammary fat pad tumor formation and lung metastasis in vivo. In this study, a blocking monoclonal antibody against an epitope just downstream from lys-270 and lys-277 inhibited in vitro breast cancer cell invasion in a dose dependent manner. This antibody also prevented fat pad tumor formation and lung metastasis, and significantly slowed the growth of already formed tumors in vivo.

eHsp90 also contributes to glioblastoma cell migration by signaling through Toll-like Receptor 4 (TLR4) and a protein kinase Cδ (PKCδ)/c-Src pathway, which transactivates the Epidermal Growth Factor Receptor (EGFR). This activation leads to ATP release and an increase in cytosolic Ca^{2+}, which promotes cell migration (Thuringer et al. 2011).

McCready et al. (2014) demonstrated that eHsp90 interacts with the precursor form of lysyl oxidase-like protein 2 (LOXL2) in the conditioned culture media (CCM) of MDA-MB-231 breast cancer cells. Lysyl oxidase proteins can modify the extracellular matrix by crosslinking collagen, which can enhance the migration of cancer cells. The findings that LOXL2 both increases wound closure in a tissue culture model of cell migration, and rescues a decrease in wound closure resulting from inhibition of eHsp90 support a role for LOXL2 in eHsp90-stimulated cancer cell migration (McCready et al. 2014; Wong and Jay 2016). It has also been shown that secreted LOXL2 can enhance gastric tumor cell invasion and metastasis via the Src/FAK pathway (Peng et al. 2009).

The importance of the interaction between eHsp90 and LOXL2 in the context of metastasis is still currently being investigated since extracellular LOXL2 contributes to cancer progression in a number of ways. In the tumor stroma, LOXL2 activates fibroblasts, i.e. leads them to express α-smooth muscle actin (α-SMA), via engagement of Integrin β3 and subsequent FAK/AKT signaling. Activated

fibroblasts, in turn, secrete increased levels of growth factors, chemokines, extracellular matrix proteins, and matrix remodeling enzymes (Barker et al. 2013). This is a mechanism by which invasive cancer cells can, "orchestrate stromal responses in favor of malignant progression" (Tlsty and Coussens 2006). Whether eHsp90 affects these mechanisms has yet to be determined.

Finally, eHsp90 enhances the invasiveness of cancer cells by interacting with the extracellular domain of the receptor tyrosine kinase HER2 (also known as Erb-B2, CD340, and Neu), which is known to play important roles in cell proliferation, differentiation, and migration, as well as cancer progression. Disruption of this interaction inhibits cell invasion and alters actin dynamics in human breast cancer cells under ligand stimulation conditions (Sidera et al. 2008). El Hamidieh et al. (2012) demonstrated that eHsp90 is crucial for recruitment of cell division cycle protein 37 (Cdc37) to the ErbB receptors HER2 and EGFR- interactions that appear to promote breast cancer cell migration. eHsp90α signaling through HER2 (and LRP1) in colon cancer cells was shown to enhance invasiveness by inducing integrin α5 expression via NF-κB activation, a pathway also relevant in the context of EMT (Chen et al. 2010).

eHsp90 signaling through the surface receptors LRP1, TLR4, and HER2 has been shown to promote cancer cell migration and invasion. Although epitope mapping has begun to identify particular amino acid residues critical to the eHsp90-LRP1 interaction, the precise mechanisms underlying eHsp90's activation of surface receptor signaling cascades are still incompletely understood. While eHsp90 interaction with extracellular LOXL2 has also been shown to enhance cell migration, it is currently unclear if eHsp90 participates in LOXL2's activation of fibroblasts, which itself promotes cancer progression. Future work to identify other residues and epitopes involved in eHsp90's pro-migratory and pro-invasion signaling functions could allow for the design of novel inhibitors with increased specificity for the prevention of pro-metastatic functions.

17.1.3 Functions of eHsp90 That Promote Anoikis Resistance

After escaping the primary tumor microenvironment, traversing the surrounding stromal tissue, and invading blood or lymphatic vessels, cancer cells must then survive the journey to a distant site in the body in order for a carcinoma to metastasize. Once inside the circulatory system, cancer cells face a variety of threats to their survival, including hemodynamic shear forces and destruction by the immune system (Gupta and Massagué 2006). Although loss of cell-matrix attachments causes normal cells to die of apoptosis, metastatic cancer cells avoid this fate, displaying a characteristic known as anoikis resistance. Nolan et al. (2015) demonstrated that this phenotype is bestowed upon prostate cancer cells by the same eHsp90-EZH2 signaling axis that affects EMT. The transcription factors Snail, Twist, and Zeb1, all of which are known to be up-regulated by eHsp90 signaling, are also crucial to cancer cell anoikis resistance mediated by the neurotrophic tyrosine

kinase receptor TrkB (Hance et al. 2012; Jie et al. 2017; Nolan et al. 2015; Smit and Peeper 2011; Smit et al. 2009). Haenssen et al. (2010) showed that integrin α5, which in colon cancer cells is up-regulated by an eHsp90α-HER2-NF-κB signaling axis (Chen et al. 2010), is necessary for HER2-mediated anoikis resistance in human mammary epithelial cells. Mechanistically, integrin α5 is requisite for the activating phosphorylation of Tyrosine 877 on HER2, which is associated with increased Src activity that is critical for anchorage-independent cell survival.

eHsp90 has been implicated in a number of signaling pathways that promote the survival of tumor cells in circulation. However, it remains to be determined if eHsp90 signaling is a critical effector of anoikis resistance, or merely a contributor. Future studies specifically examining the role of eHsp90 in anchorage-independent cancer cell survival could help to determine if this phenotype is a prime target for anti-eHsp90 intervention.

17.1.4 Pro-growth and Pro-survival Signaling Functions of eHsp90

Cancer cells that are able to survive the journey to a distant site in the body, colonize a metastatic niche, and proliferate can become metastatic secondary tumors. Pro-growth and pro-survival signals received by these cells contribute to metastasis. Signaling by eHsp90 can enhance cell growth and survival. After traveling to a distant site in the body, cancer cells must then proliferate in order to form a secondary tumor. It has been shown that pro-growth and pro-survival signaling by eHsp90 aids in the ability of metastatic cancer cells to proliferate once they have settled at distant sites in the body.

eHsp90 signaling through LRP1 has been shown to enhance ERK activation (Hance et al. 2012; Tsen et al. 2013). ERK signaling promotes activation of the transcription factor serum response factor (SRF), which leads to the transcription of immediate early genes, and has been shown to promote mammary stem cell-like properties in basal-like breast cancer via an SRF-YAP-IL6 signaling axis. Promoting cancer cell stemness is relevant to the initiation of secondary tumors, as stem cells and tumor-initiating cells have many overlapping characteristics (Calderwood et al. 2006; Kim et al. 2015). Cancer stem cells possess a capacity for sustained self-renewal, which contributes to tumor development (Chang 2016).

eHsp90 may play an important role in multiple activities of LOXL2, which has been shown to promote fibroblast activation. Activated fibroblasts express extracellular matrix proteins and growth factors that promote the survival and proliferation of carcinomas in a paracrine manner (Bhowmick and Moses 2005). Combining cancer-associated fibroblasts with normally non-tumorigenic human prostate epithelial cells induces malignant transformation and increased proliferation in the epithelial cells (Hayward et al. 2001). Thus, eHsp90 released by cancer cells may also alter the tumor microenvironment.

eHsp90 signaling through LRP1 has been shown to promote cancer cell stemness and proliferation, as well as tumor development. Although it has not yet been established if this protein participates in the formation of secondary tumors, eHsp90 activity in the pre-metastatic niche and on cancer cells settling at distant sites in the body could be major contributors to metastasis. It is possible that eHsp90 also plays a role in the fibroblast-activating function of LOXL2, which further stimulates cancer cell growth. Preventing cancer cell proliferation by targeting eHsp90 could be an effective way to halt cancer progression and spread.

17.1.5 eHsp90 as a Systemic Signal Affecting the Body's Response to Cancer and Its Treatment

Cancer cells surviving the journey to a distant site in the body and forming metastatic secondary tumors are the basis of cancer spread and disease progression. However, cancer is a systemic condition that dysregulates metabolism in the body, and also affects the cardiovascular system, and the immune system. It has been shown that eHsp90 promotes cancer progression by directly affecting the molecular and phenotypic properties of cancer cells and stromal cells in the tumor microenvironment. However, eHsp90 also contributes to cancer progression by acting on muscle cells, immune cells, and endothelial cells.

Decreased body mass resulting from muscle wasting, and systemic inflammation are both hallmarks of cancer cachexia, and often major contributors to cancer-related death. Tumor-released Hsp90 and Hsp70, recognized as damage-associated molecular patterns (DAMPs), activate Toll-like receptor 4 (TLR4) on muscle cells, leading to muscle catabolism. Zhang et al. (2017) hypothesize that this effect occurs primarily due to activation of the TLR4-p38β MAPK-C/EBPβ catabolic signaling pathway.

Tumor-released eHsp90 and extracellular Hsp70 also contribute to activation of TLR4 on monocytes and immune cells. This activation leads to elevated serum tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) levels. Since these molecules both stimulate the innate immune system, Zhang et al. (2017) propose that circulating Hsp90 and Hsp70 are the source of tumor-induced systemic inflammation and should be examined as targets for the prevention of cancer cachexia.

Tumor-secreted eHsp90α was shown to promote a more motile and reactive inflammatory stroma in the tumor microenvironment of prostate cancer by acting on prostate stromal fibroblasts. Inducing phenotypes characteristic of myofibroblasts or reactive cancer-associated fibroblasts (CAFs) in normal stromal fibroblasts increased the production of inflammatory cytokines that are known contributors to metastasis. Signaling, likely through LRP1, and by activating the extracellular effectors MMP2 and MMP9, eHsp90 enhanced MEK/ERK and STAT3 activation, which promoted NF-κB-dependent transcription of IL-6 and IL-8 in prostate stromal

fibroblasts. These interleukins are both multifunctional chemokines that play causative roles in prostate cancer progression. eHsp90 also induced the expression of MMP3, which is known to further promote the myofibroblast phenotype (Bohonowych et al. 2014).

Song and Luo (2010) demonstrated that secreted Hsp90α localizes to the leading edge of human dermal microvascular endothelial cells and promotes angiogenic activities such as cell migration, and tubule formation. They also showed that treatment with exogenous recombinant Hsp90α increased blood vessel densities in an in vivo wound healing model. All of these effects were blocked by Hsp90α neutralizing antibodies. These results support a role for eHsp90 in angiogenesis, which has been extensively studied as a therapeutic target for preventing cancer progression (Carmeliet and Jain 2000).

Cell-surface Hsp90 also plays a critical role in the normal functioning of human monocytes and macrophages (MΦs). Bzowska et al. (2017) recently showed that Hsp90α and Hsp90β are expressed on the surface of human monocytes and monocyte-derived MΦs, and are critical for normal cytokine response by these cells, following either soluble or particulate insult in the form of pathogen associated molecular patterns (PAMPs). Specifically, blocking cell surface Hsp90 reduced TNF production by these cells. The authors propose that these cell surface Hsp90 molecules serve as, "signaling complex chaperones," for monocytes and MΦs. This finding suggests that even specifically targeting eHsp90, or cell-surface Hsp90 for the prevention of metastasis has the potential for complications.

Beyond its well studied pro-metastatic effects on cancer cells, themselves, eHsp90 has been shown to promote cancer spread by acting on muscle cells, immune cells, fibroblasts, and endothelial cells. However, eHsp90 activity has also been shown to function in normal processes such as wound repair and immune function. Epithelial-mesenchymal transition, enhanced cell motility, stimulated inflammation, extracellular matrix remodeling, and increased angiogenesis are all critical to the wound healing process (Hance et al. 2014; Wong and Jay 2016). As future studies aim to determine if eHsp90 is an effective target for preventing cancer spread, researchers must remain mindful of the fact that this protein also has beneficial roles in normal tissue and homeostatic processes.

17.2 Conclusions

Signaling by eHsp90 contributes to each step of cancer metastasis. eHsp90 serves as a potent stimulator of cancer progression by promoting epithelial-mesenchymal transition (EMT) in cancer cells, enhancing cancer cell motility and invasiveness, aiding in anoikis resistance, enhancing cancer cell growth, and acting on stromal cells, muscle cells, immune cells, and endothelial cells. The extracellular pool of this protein is thus an appealing therapeutic target for preventing the spread of cancer. One caveat of targeting eHsp90 in cancer is that this treatment could negatively impact the immune system in cancer patients being treated with

chemotherapy, who are already more susceptible to infection than the general population (CDCBreastCancer 2017). Although MΦs normally respond to pathogen-associated molecular patterns (PAMPs), the MΦ inflammatory cytokine response is suppressed by selective inhibition of cell-surface Hsp90 (Bzowska et al. 2017). As the search to identify better cancer biomarkers and prognostic indicators continues, new diagnostic approaches are being used and novel technologies are being developed to identify patients best suited for particular treatment approaches. An ELISA kit has been validated for invasive non-small lung cell carcinoma and approved as a diagnostic by the Chinese FDA (Shi et al. 2014). Studies are currently being conducted in China to detect Hsp90α levels in the plasma of patients with breast, colorectal, and liver cancer (clinicaltrials.gov identifiers: NCT02324101, NCT02324114, NCT02324127). Measuring plasma eHsp90α levels, as opposed to eHsp90α only in the tumor microenvironment not only simplifies collecting patient samples, but also identifies patients most at risk of the systemic effects of eHsp90α. The results of these studies have not yet been released, but if elevated plasma Hsp90α levels correlate with disease progression in some form of cancer, then those patients with the highest plasma eHsp90 burden may be the best candidates for an intervention targeting this molecule. eHsp90 could prove to be an excellent target for the current personalized/precision medicine revolution. eHsp90 serves as a signaling hub for the metastatic niche. Although there are still gaps in the understanding of its numerous pro-metastatic activities, it has been shown to play roles in each step of metastasis and may have additional, as-yet unidentified roles in other pro-metastatic pathways. Blocking eHsp90 without affecting its intracellular functions may have benefit in reducing metastasis. Together, these studies on cells near the tumor and beyond the tumor microenvironment raise the possibility that circulating eHsp90 may have complex effects beyond the activation of pro-invasive proteins in the invasive niche. Additional research is needed to elucidate these mechanisms and their pathological relevance. Such studies may contribute to a richer understanding of eHsp90's role in metastasis to better inform preclinical and clinical studies going forward.

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Chapter 18
Role of the Molecular Chaperones Hsp70 and Hsp90 in the DNA Damage Response

Laura E. Knighton and Andrew W. Truman

Abstract Heat Shock Protein 70 (Hsp70) and Heat Shock Protein 90 (Hsp90) are well-conserved, highly expressed molecular chaperone proteins, which assist in the folding and stabilization of the human proteome. It is not surprising that chaperones regulate and fine-tune many important signal transduction pathways. An important example of this is the DNA Damage Response Pathway (DDR), which is critical for DNA repair after creation of single or double strand breaks. An increasing body of work suggests that many DDR proteins are kept active by Hsp70 and Hsp90 proteins. Detailed understanding of these interactions may lead to the development of novel anti-cancer therapies.

Keywords Cancer · DNA damage · Hsp70 · Hsp90 · Proteins

Abbreviations

AP	apurinic
ATM	ataxia-telangiectasia-mutated kinase
BER	Base Excision Repair
DDR	DNA damage response
DSB	double strand break
HR	homologous recombination pathway
HS	Heat Shock
HSF	Heat Shock Factor
Hsp	Heat Shock Protein
HU	Hydroxyurea
IR	Ionizing Radiation
MRN	MRE11–RAD50–NBS1 complex

L. E. Knighton · A. W. Truman (✉)
Department of Biological Sciences, The University of North Carolina at Charlotte, Charlotte, NC, USA
e-mail: atruman1@uncc.edu

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MRX Mre11p–Rad50p–Xrs2p
NBD Nucleotide-binding domain
NER Nucleotide Excision repair
NHEJ non-homologous end joining pathway
MMR Mismatch repair pathway
MMS methylmethanesulphonate
SBD Substrate-binding domain

18.1 Introduction

Accurate transmission of genetic information is vital to the survival of all organisms. For this to happen, the replication of DNA needs to be tightly regulated and precise. However, cells are constantly being challenged by both spontaneous and induced DNA damage (Houtgraaf et al. 2006; Martin 2008; McGowan and Russell 2004). The continued cell viability and cell proliferation depend on the effectiveness of the DNA damage response pathway (Houtgraaf et al. 2006; Martin 2008; McGowan and Russell 2004). When the system fails to repair the DNA, it may lead to the pathogenesis of multiple human diseases including cancer (Hakem 2008; Houtgraaf et al. 2006; Truman et al. 2015).

DNA damage is caused by both endogenous and exogenous sources. The majority of endogenous DNA damage results from errors during DNA replication (Boulianne and Feldhahn 2017). Insertions or deletions of bases during replication lead to frameshift mutations in coding DNA and must be rapidly repaired to maintain genome integrity. Internal metabolic by-products can also damage DNA. For example, oxygen-derived free radicals can cause non-specific binding of Fe (Halliwell and Aruoma 1991; Santivasi and Xia 2014). Sources of exogenous DNA damage include alkylating drugs such as methylmethanesulphonate (MMS) and the anticancer drugs cisplatin and carboplatin. These drugs typically function by binding to guanine, crosslinking the two strands together (Pfuhler and Wolf 1996). UV and ionizing radiation are also common sources of external DNA damage. UV light can cause thymine base pairs next to each other in DNA to bond together into pyrimidine dimers. In contrast, Ionizing radiation (IR) normally causes single or double strand breaks in DNA. Double strand breaks are the most detrimental form of DNA damage and must be repaired to maintain cell viability (Krokan and Bjoras 2013; Santivasi and Xia 2014).

Given the deleterious effects of DNA damage, DNA must be repaired in a timely fashion. The response to DNA damage is coordinated through a well-characterized signaling pathway known as the DNA damage response (DDR pathway). Initially sensor molecules recognize and bind to the DNA lesion. Transducer molecules are activated and become recruited to the site of damage forming a platform DNA repair enzymes to fix any lesions (McGowan and Russell 2004). Transducers are typically kinases including the ATM/ATR that phosphorylate multiple downstream targets to initiate DNA repair (McKinnon 2012). Many important cellular processes are

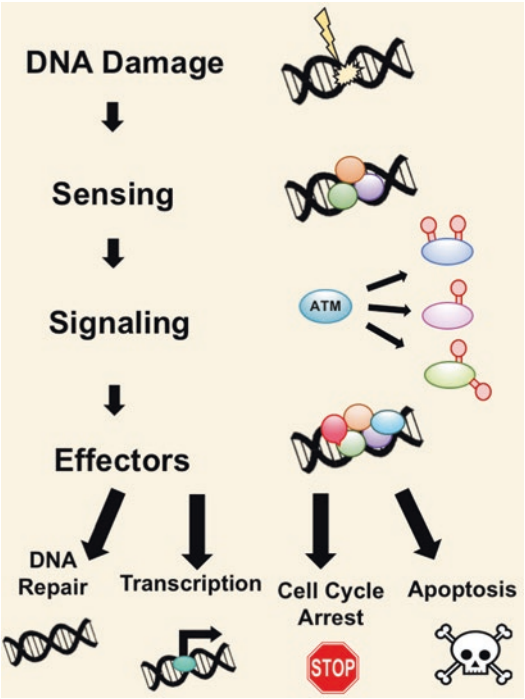

Fig. 18.1 General overview of the DNA damage response. When cells are exposed to DNA damage, sensor molecules localize to the site of damage. Recruitment of signal transducer proteins occur, typically with phosphorylation of multiple DDR proteins. Finally, effector molecules function to temporarily arrest the cell cycle, transcribe DNA repair proteins and repair the site of damage. If repair cannot be achieved, apoptosis may be activated to terminate the damaged cell

regulated by the DNA damage response including cell cycle arrest, apoptosis, transcription and DNA repair (Fig. 18.1).

The specific repair pathway activated depends on the nature of the DNA damage (Hakem 2008). There are three excision repair pathways known; the Nucleotide Excision repair (NER), the Base Excision Repair (BER) and the Mismatch repair pathway (MMR) (Hakem 2008; Houtgraaf et al. 2006). The NER is activated when UV light causes bulky adducts on DNA. After recognition, the DNA segment contacting the pyrimidine dimer will be eventually removed and the gaps filled in with DNA polymerase and sealed by DNA ligase (de Laat et al. 1999). The base excision repair pathway is used when there is a small DNA lesion such as 8-oxoguanine lesions. BER is initiated by glycosylases which recognize damage or inappropriate bases and work to remove them. An apurinic site (AP) is formed, meaning neither a purine or pyrimidine, and is cleaved by AP endonuclease causing a single strand break in the DNA (Krokan and Bjoras 2013). The BER pathway is able to respond to single strand breaks by either the short patch repair, replacing a single nucleotide, or by the long-patch BER where two to ten new nucleotides must be replaced (Krokan and Bjoras 2013). The mismatch repair pathway is used to correct insertions, deletions or wrongly paired nucleotide bases. All DNA damage repair pathways are extremely complex and have been simplified in this introduction. The most complex and harmful DNA damage is double strand breaks (DSB) (Bronner et al. 1994). There are two pathways that can be followed after DSBs occur, either the

non-homologous end joining pathway (NHEJ) or the homologous recombination pathway (HR). The repair pathways for double strand breaks will be discussed in full detail in the following pages (McGowan and Russell 2004).

The DNA damage response pathway regulates several major checkpoints that are able to arrest the cell cycle if damage is detected. This process comprises of signal traducers, mediators and eventually effector proteins which work to phosphorylate targets that arrest the cell cycle at one of three major check points; G1/S, intra-S, or G2/M (Houtgraaf et al. 2006; Zhou and Elledge 2000). If cell cycle arrest is accomplished at one of these checkpoints, DNA repair machinery has time to repair the damage properly. If cell cycle arrest is not effective, this could lead to genomic instability and oncogenesis. In the cell cycle directly after Mitosis, G1 phase begins, in which the cell increases in size and both transcription and translation starts. Followed by the S phase, where DNA replication occurs producing an exact copy of genome for future daughter cells (Houtgraaf et al. 2006; Zhou and Elledge 2000). The third major phase is G2, during which the cell will grow and proteins are made for the daughter cells to ensure viability. From G2 the cell will move into M phase producing two identical daughter cells. Although the G1/s, intra-S and G2/M checkpoints are separate, they share many proteins in the response to DNA damage. The intra-S checkpoint does differ slightly from the other two in that the signal to block cell progression at the check point is unreplicated DNA rather than actual damage (Bakkenist and Kastan 2003; Houtgraaf et al. 2006; Zhou and Elledge 2000). Intra-S phase is important because it blocks mitosis from occurring when DNA replication is either unfinished or inhibited, preventing incorrect DNA from being passed on. DNA damage repair pathways and checkpoints are not turned “on” and “off” with the presence of damaged DNA but are constant in cells. Sensor proteins bind to normal DNA under unstressed conditions in search of damage (Jackson and Bartek 2009; Martin 2008; Zhou and Elledge 2000). It is important that the aforementioned repair pathways work to correct DNA damage so mutations are not replicated and passed on. Unrepaired damage can lead to genomic instability and cell death (Blackford and Jackson 2017; Hakem 2008; Jackson and Bartek 2009). Accumulated mutations can result in tumorgenesis. Many identified carcinogens are known DNA modifiers (Blackford and Jackson 2017; Hakem 2008; Jackson and Bartek 2009).

18.2 DNA Damage Response (DDR) Pathway

Double strand breaks are the primary damage caused by ionizing radiation and radiomimetic chemicals. DSBs are frequent in cells, an estimated ten occurrences per day (Hakem 2008). DSBs, if left uncorrected will likely lead to genomic instability from the generation of dicentric or acentric chromosomal fragments (Chiruvella et al. 2013; Santivasi and Xia 2014). A dicentric chromosome is one with two centromeres, while acentric translates to no centromere. Either of these causes uneven distribution of genetic material to daughter cells. Because of the complexity of DSBs and the need to repair them accurately and efficiently there are

two separate pathways possible to repair the damage; non-homologous end joining pathway (NHEJ) or the homologous recombination pathway (HR) (McGowan and Russell 2004).

Cell cycle stage plays an important role in determining whether NHEJ or HR is activated after DNA damage. For HR to occur, there needs to be an available template strand to repair the DNA. This is possible when the organism is diploid, including "transient diploids" such as replicating bacteria and yeast (McGowan and Russell 2004). In non-dividing haploid organisms or in diploid organisms that are not in S phase, there is no template strand available. In this case, NHEJ will be followed (McGowan and Russell 2004). Before repair can take place, the damaged DNA must be sensed and signaled to other proteins to be recruited. MRE11–RAD50–NBS1 (MRN) complex recognizes the DSB and starts the response pathway (Burma et al. 2001). MRN has been shown to sense and localize to DSBs in vitro and has known exonuclease, endonuclease and DNA unwinding activities. The analogue complex Mre11p–Rad50p–Xrs2p (MRX) in yeast has been studied more extensively. Next, the protein kinase protein ataxia-telangiectasia-mutated kinase (ATM) self phosphorylates resulting in its singular activated state as opposed to its inactivated dimeric state under normal cellular conditions (Hakem 2008; Jackson and Bartek 2009; McGowan and Russell 2004). Once activated through DNA damage, ATM phosphorylates the histone variant H2AX, creating yH2AX which will serve as a docking site for recruited transducer and effectors. yH2AX serves as a biomarker for DSBs (Caldecott 2008). ATM will then phosphorylate several more transducers including two very important kinases; checkpoint kinase 1 (Chk1), checkpoint kinase 2 (CHK2) (Burma et al. 2001; Shiloh and Ziv 2013). The transducers then work to recruit mediators such as 53BP1, BRCA1. If the DNA cannot be repair, the effector p53 is activated along with either cell cycle arrest, apoptosis or the senescence pathway (Kastan et al. 1991). For single strand break, the closely ATM related kinase ATR is activated which works with Chk1 to activate single strand repair (Caldecott 2008).

18.3 Non-homologus End-Joining Pathway (NHEJ)

NHEJ is one possible pathway that can be utilized to repair damaged DNA. Unlike most other biochemical processes, NHEJ is known to be mechanistically flexible by accepting a diverse group of substrates that it can accept and convert to joined products (Fell and Schild-Poulter 2012; Lieber 2010; McGowan and Russell 2004). In many organisms (including yeast and humans), the central protein in the NHEJ pathway is the Ku protein, a heterodimer consisted the subunits Ku70 and Ku80 (Fell and Schild-Poulter 2012; Lieber 2010; McGowan and Russell 2004). Through crystallography studies it has been determined that the protein forms an open ring structure around DNA, both protecting and binding to the DSB but also allowing recruited proteins access. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) then binds to the Ku protein forming the DNA-PK holoenzyme which then

displays serine/threonine protein kinase activity (Fell and Schild-Poulter 2012; Lieber 2010; McGowan and Russell 2004). Although the studies that elucidated these mechanisms utilized predominantly in vitro technologies it is thought that this accurately represents the situation in vivo. Presumably, phosphorylation of the MHEJ factors XRCC4 and replication factor A2 that facilitate repair. It is also assumed that DNA-PKcs works to stabilize the two ends of DNA (McGowan and Russell 2004). DNA-PKcs has only been discovered in vertebrates, it is possible that other kinases in the phosphatidylinositol-3-kinase protein kinase-like (PIKK) family can carry out the same function in lower Eukaryotes (Blackford and Jackson 2017; McGowan and Russell 2004) The Artemis protein is a factor in mammalian cells that has been seen to have endonuclease activity on activity on the 5′ and 3′ overhangs of the broken DNA (Zhang et al. 2004). Finally, DNA ligase IV is loaded onto the break by DNA-PKcs and joins the two strands together in a tight complex with X-Ray Repair Cross Complementing 4 (XRCC4) protein. Because there is no template strand require, NHEJ tends to be error prone. To minimize error, the DNA is most likely processed before NHEJ (Chiruvella et al. 2013). In mammalian cells this is accomplished by the (MRN) complex (van den Bosch et al. 2003). Cells that impaired for MRX complex activity typically also have deficient NHEJ capabilities (Bakkenist and Kastan 2003; Boulton and Jackson 1998; Moore and Haber 1996).

18.4 Homologus Recombination Pathway (HR)

The HR pathway is highly conserved in organisms from bacteria to humans. This pathway uses a template strand so it is less error prone than NHEJ. First, the (MRN) complex (MRX in yeast) recognizes and senses the DSB. MRN complex activates the protein kinase ATM, which then activates the full DNA damage response (Moore and Haber 1996; Uziel et al. 2003). The protein CtIP is required for its nuclease activity on the end resection from 5 to 3′, leading to the formation of a single strand DNA. Then DNA replication protein A (RPA) coats the exposed single strand DNA and stabilizes it (Noguchi et al. 2006; Peng and Lin 2011; Sancar et al. 2004). The DNA is then loaded on the homologous DSB region by the strand exchange protein RAD51 and by BRCA2, forming the Holliday junction intermediates. The DNA is then ligated and endonuclease and resolvase proteins are resolve the Holliday junction intermediates (Lieber 2010; Noguchi et al. 2006).

18.5 Regulation of DNA Damage Sensing by Chaperones

The highly conserved MRN complex consisting of MRE11, RAD50 and Nbs1 is involved in both modes of DSB repair (van den Bosch et al. 2003). Before the repair pathway can begin the broken ends must be recognized and processed. The MRN complex binds to the damaged DNA and is able to process it through its nuclease

activity. This occurs from the Mre11 protein's reaction with the Rad50 ATPase. Nbs1 role is thought to be involved in nuclear localization and assembly of the broken ends through its interaction with the histone protein H2AX (McGowan and Russell 2004; van den Bosch et al. 2003). Shortly after DSB induction occurs, H2AX is phosphorylated. Recently, it has been shown that the MRN complex is necessary for proper activation of the transducer protein kinase ATM (Uziel et al. 2003). The chaperone Hsp90 is involved in many levels of the DSB response. In the sensing portion of the pathway, it has been shown that Hsp90 stabilizes Nbs1 protein of the MRN complex. Inhibition of Hsp90 with the drug 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) disrupts the essential Hsp90-Nbs1 interaction, resulting in loss of ATM activity (Dote et al. 2006). In healthy mammalian cells, Hsp90 and Nbs1 remain in a complex with Nbs1 disassociating becoming recruited to the MRN complex after damage is repaired (Dote et al. 2006; Uziel et al. 2003).

18.6 Chaperone Regulation of Signal Transduction in the DNA Damage Response

ATM is an important global regulator of the DNA damage response, phosphorylating and recruiting many effector proteins after exposure to ionizing radiation exposure (Lieber 2010; McKinnon 2012; Pennisi et al. 2017; Uziel et al. 2003). ATM phosphorylates Nbs1 and regulates Hsp90 phosphorylation indirectly (see below) causing them to dissociate, allowing Nbs1 to be recruited to the MRN complex (Pennisi et al. 2015; Quanz et al. 2012). ATM activity is further promoted by binding the MRN complex through direct interaction of Nbs1 (McKinnon 2012; Pennisi et al. 2017; Uziel et al. 2003). It is interesting to note the co-regulation relationship between Hsp90, ATM and Nbs1. These interactions provide insight to the complexity of the DNA damage response proteins. Upon 17-AAG treatment, ATM levels drop rapidly impacting signaling throughout the DDR pathway simultaneously (Bakkenist and Kastan 2003; Pennisi et al. 2017).

The DNA-PK complex is another known client of Hsp90. This interaction was seen in the epithelioid cervix carcinoma cell line (HeLa) but not in normal embryonic kidney human cell line (HEK293). Interestingly, cytosolic but not the nuclear DNA-PK levels are reduced in HeLa cells after treatment with the Hsp90 inhibitor radicicol (Blackford and Jackson 2017; Pennisi et al. 2015). After cells are treated with ionizing radiation, DNA-PK interacts with ErbB1, an epidermal growth factor receptor. When cells were treated with an Hsp90 inhibitor, ErbB1 activity was reduced, resulting in reduced interaction between DNA-PK and ErbB1 under ionizing radiation compromising DSB repair (Pennisi et al. 2015; Zhou and Elledge 2000). Checkpoint kinase 1 (Chk1) is a crucial regulator of the signaling pathway activated by DNA replication stress and DNA damage. Inhibition of Hsp90 by 17-AAG results in degradation of the Chk1 protein and ultimately disruption of its interaction with Cdc25C and Cdc25 (Arlander et al. 2003). Notably, the degradation

of Chk1 by 17-AAG sensitizes tumor cells arrested in the S-phase by gemcitabine treatment (Arlander et al. 2003).

18.7 Chaperone Control of DNA Damage Response Effector Proteins

Several DNA damage effectors have been identified as Hsp90 clients including BRCA1, BRCA2, RAD51, CHK1. BRCA1 is an important nuclear tumor suppressor and is also essential for DSB repair in the HR pathway (Stecklein et al. 2012). After DNA damage BRCA1 is phosphorylated by ATM, ATR and CHK2 kinases. Phosphorylated BRCA1 coordinates multiple distinct protein complexes that recognize and repair damaged DNA and activate cell cycle checkpoints (McGowan and Russell 2004; Pennisi et al. 2015; Stecklein et al. 2012). Since BRCA1 is directly stabilized by Hsp90, BRCA1 levels are very sensitive to Hsp90 inhibitors (Stecklein et al. 2012). These client interactions appear to be conserved in other organisms. For example, in the budding yeast *Saccharomyces cerevisiae* the homologues of HSC70 and Hsp72 (Ssa1 and Ssa2) interact with Rad9, the yeast homologue of BRCA1 (Hunter et al. 1989). Loss of both Hsp70 isoforms in yeast results in sensitivity to UV radiation and loss of Rad9 stability and phosphorylation (Hunter et al. 1989).

P53 is an important tumor suppressor protein that is involved in activation of either cell cycle arrest, apoptosis or the senescence pathway (Sherman et al. 2007). Previous studies have demonstrated that inactivation of Hsp70-2, a member of the Hsp70 family results in the senescence of cancer cells through depression of p53 function (Sherman et al. 2007).

Rad51 is the major strand-transferase involved in the HR pathway. Rad51 is amplified in many cancers and Rad51 inhibition results in sensitization of cancer cells to a variety of chemotherapeutic agents (Ko et al. 2012). The Hsp90 inhibitor 17-AAG promotes down-regulation of Rad51 implicating that Hsp90 stabilizes Rad51. In support of this idea, overexpression of Rad51 rescued cell viability in cells treated with 17-AAG (Ko et al. 2012).

There are multiple clients in other DNA damage pathways other than DSB. For example, the MSH2 is a vital protein in the mismatch repair pathway. MSH2 plays a role in the recognition of DNA mismatches and recruits other repair proteins to the lesion (Pennisi et al. 2015). In studies combining the treatment of tamoxifen (a breast cancer drug) with 17-AAG promotes the cytotoxic effect and growth inhibition of tamoxifen, resulting in the decreased expression of MSH2 in human lung carcinoma cells (Pennisi et al. 2015). A summary of Hsp70/90-interacting DDR proteins can be found in Fig. 18.2.

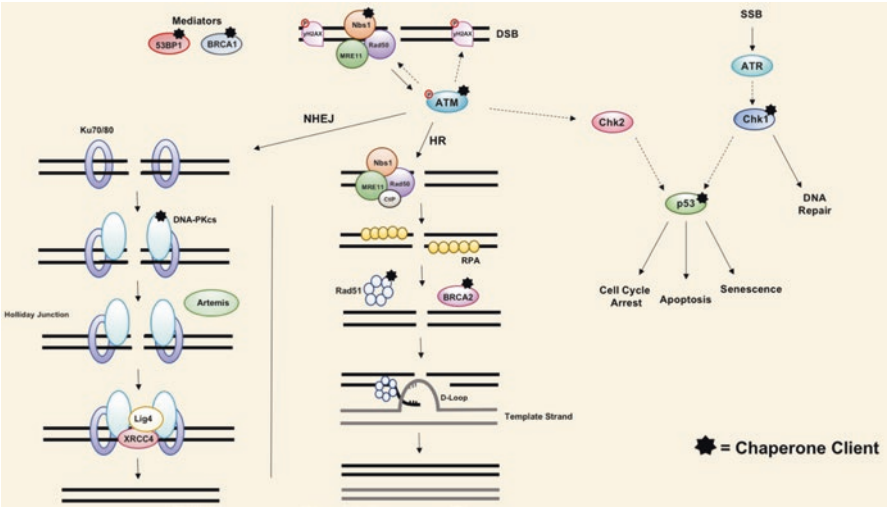

Fig. 18.2 Hsp90 and Hsp70 client proteins of the DDR pathway. The ATM pathway is activated by DSBs while ATR is activated by SSBs. Either the Non-homologous end-joining (NHEJ) or the Homologous recombination (HR) pathways can be activated to repair a DSB. If the DNA cannot be repaired, p53 is activated promoting cell cycle arrest, apoptosis or senescence. All chaperone clients in the pathway are indicated with a black star

18.8 Interplay Between Chaperone Phosphorylation and the DNA Damage Response

Chaperone activity can be regulated through abundance, localization and co-chaperone binding. Recently the post-translational modifications on both Hsp70 and Hsp90 (known as the chaperone code) have been identified as being key players in chaperone function (Nitika and Truman 2017; Woodford et al. 2016). Hsp90 is directly phosphorylated by DNA-PK on Thr5 and Thr7 upon exposure to ionizing radiation (Hunter et al. 1989). Phosphorylation of these sites alters Hsp90-MRN binding and it is interesting to note that several cancer cell lines display higher basal and stress induced levels of Thr5/7 phosphorylation (Nitika and Truman 2017). It has been suggested that ATM also directly phosphorylates these sites as loss of kinase activity correlates with loss of Hsp90 phosphorylation (Nitika and Truman 2017). It should be noted however that Hsp90 phosphorylation is only marginally reduced upon treatment of cells with the ATM inhibitor Ku60019 and Thr5/7 do not exist as part of the well-characterized SQ/TQ phosphorylation motif of ATM, suggesting that ATM is an indirect regulator of Hsp90 phosphorylation. Hsp90 phosphorylation status can mediate cellular resistance to anticancer therapeutics and will be interesting to see whether combinations of small molecules targeting Hsp90 Thr5/7 and the DDR pathway are synergistic in reducing cancer cell proliferation (Woodford et al. 2016).

Hsp90 is also phosphorylated on Ser164 during replication stress by the CDC7-DBF4 kinase. This phosphorylation is required for the stability of the Hsp90-HCLK2-MRN complex and activity of ATM and ATR and directly regulates the HR repair response (Cheng et al. 2017). The authors propose that a new anticancer therapy consisting of combined Hsp90 and CDC7 could be used to treat patients that have cancers expressing hyperactive ATM signaling (Cheng et al. 2017).

18.9 Chaperone Control of Ribonucleotide Reductase (RNR)

Ribonucleotide reductase (RNR) is a key enzyme in deoxyribonucleotides (dNTP) formation. Loss of RNR activity results S-phase arrest and disruption of DNA synthesis and repair (Truman et al. 2015). RNR is comprised of two subunits; large (R1) and small (R2). Both subunits are essential for viability in the cell. R1 (RRM1 in vertebrates, Rnr1/Rnr3 in yeast) forms the catalytic domain while R2 (p53R2/RRM2 in vertebrates, Rnr2/Rnr4 in yeast) is the regulatory subunit (Truman et al. 2015). Recent proteomic studies in yeast examining the global interactions of Ssa1 and Hsp82 under DNA damage stress identified Rnr2 and Rnr4 as novel interacting proteins. Inhibition of chaperone function through point mutation or small molecule inhibition led to destabilization of both Rnr2 and Rnr4 (Truman et al. 2015), suggesting they are bona fide of clients Ssa1 and Hsp82. This interaction appears to be conserved in mammalian cells as Hsp70 and Hsp90 bind RRM2. In addition, treatment of MCF7 breast cancer cells with chaperone inhibitors 17-AAG or VER15008 promoted rapid degradation of RRM2 and sensitized cells to gemicitabine, a clinically utilized RNR inhibitor (Sluder et al. 2018; Truman et al. 2015).

18.10 Global Proteomic Analysis of Chaperones in DNA Damage

Recent efforts to identify novel clients of Hsp70 and Hsp90 have utilized comprehensive proteomic approaches. Instead of focusing on single chaperone-client interactions, mass spectrometry analysis can be used to obtain an exhaustive list of all possible clients. One such effort examined global protein abundance changes upon treatment of bladder cancer cells with two different Hsp90 inhibitors, AUY922 and ganetespib (Li et al. 2017). Interestingly, there were 41 DNA damage pathway related proteins found to be either significantly up or down regulated in the treated cells. These included proteins from base excision, nucleotide excision, non-homologous excision and mismatch excision repair pathways and others such as chromatin structure and modification (Li et al. 2017). Clearly, there is still much to be understood about Hsp control of the DNA response, particularly in cancer.

18.11 Translational Use of Chaperone Inhibitors to Impair DDR Function

The knowledge that many DDR proteins are clients of chaperone proteins can be used in a clinical setting. Destabilization of DDR proteins should render cancer cells unable to repair DNA damage caused by common chemotherapeutic agents such as ionizing radiation, cisplatin and RNR inhibitors. Currently, there are multiple Hsp90 inhibitors that are in late stage clinical trials for cancer therapy (Pennisi et al. 2015; Stecklein et al. 2012). Cancer cells are "addicted" to chaperone function because of the inherently unstable nature of the oncoproteins they express. In addition, the relatively unstable genome of cancer cells and rapid pace of cell cycle renders cancer cells especially vulnerable to genotoxic agents. Taken together, this suggests that combination therapies of chaperone and DDR protein inhibitors should be particularly selective for cancer cells over non-tumorgenic tissue (Fig. 18.3).

A clear example of this can be seen in cells expressing high levels of BRCA1-they are resistant to both IR and other types of chemotherapeutic agents. Targeting BRCA1 for degradation via Hsp90 inhibition restores sensitivity to anti-tumor agents, implicating a possible cancer therapeutic strategy (Pennisi et al. 2015; Stecklein et al. 2012). Additionally, the innate sensitivity of BRCA1 mutated or deficient cells to 17-AAG suggests that this agent might also show potency in both primary BRCA1 mutant tumors but also in sporadic tumors that have lost BRCA1 expression by non-mutational means (Pennisi et al. 2015). Stability of RNR

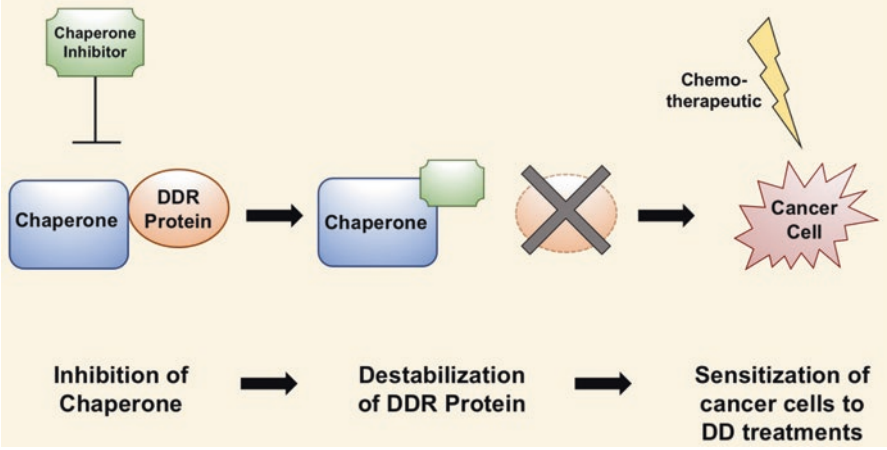

Fig. 18.3 Combinations of chaperone inhibitors and genotoxic agents may be a novel, highly synergistic anticancer therapy. Chaperone inhibitors typically prevent client protein interaction, causing folding errors, destabilization and client protein degradation. If the client protein is present in the DDR, inactivation should render cells sensitive to genotoxic agents

components rely on chaperone function which explains why treatment of cancer cells with Hsp70 and Hsp90 inhibitors sensitized breast cancer cells to both hydroxyurea and gemicitabine, RNR-targeting agents (Niu et al. 2006; Truman et al. 2015).

18.12 Conclusions

The DNA damage response is a vast and highly complex system. Understanding the interactions of this system is essential to understand the pathogenesis of many disease and also possibly treatment of diseases, especially cancer (Houtgraaf et al. 2006; Pennisi et al. 2017; Zhou and Elledge 2000). Heat shock proteins are consistently seen to play a vital role in human health and physiology, assisting in the folding of newly synthesized proteins and denatured proteins, as well as protein transport and stabilization. The recent discoveries of sensitizing cancer cells by using chaperone inhibitors could lead to novel cancer therapies (Houtgraaf et al. 2006; Pennisi et al. 2017; Truman et al. 2015; Zhou and Elledge 2000). Moreover, since chaperones are vital to normal cells as well as cancer cells, inhibiting them may make a person very sick. Once the co-chaperones are determined, they may provide novel anti-cancer therapy targets as well.

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Chapter 19
Heat Shock Protein 90 Inhibitors in Lung Cancer Therapy

Suman Chatterjee and Timothy F. Burns

Abstract Heat shock protein 90 (HSP90) plays crucial roles in intracellular quality control mechanisms leading to cytoprotection against variety of stressors including hypoxia, oxidative and thermal and oncogenic stress. The chaperoning activity of the evolutionary conserved and ubiquitously expressed HSP90 is adenosine triphosphate (ATP)–dependent and is essential for the folding, maturation, stabilization, activation or proteolytic degradation of its diverse array of client proteins, many of which are products of driver oncogenes in multiple cancers. Hence, tumorigenesis regulation by HSP90 chaperonage function has been the subject of extensive investigation for decades. Targeted HSP90 inhibition has shown promise and may provide an effective and alternate therapeutic approach to treat patients with lung cancer, especially non-small cell lung cancer (NSCLC) with specific mutational background or that have been characterized to show acquired resistance to other drugs targeting different signaling proteins. Although development of HSP90 inhibitors has spanned decades, both preclinically and clinically, the promise is far from being reached. In this chapter, we discuss the potential of HSP90 inhibition, and the preclinical and clinical development and future of important HSP90 small molecule inhibitors that have been or will be critical for lung cancer therapeutics.

Keywords 17-AAG · EGFR · Ganetespib · HSP90 · KRAS · Lung cancer · NSCLC

Abbreviations

ADC	adenocarcinoma
ADP	adenosine diphosphate
ATP	adenosine triphosphate

S. Chatterjee · T. F. Burns (✉)
Department of Medicine, Division of Hematology Oncology, UPMC Hillman Cancer Center, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: burnstf@upmc.edu

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CTD	C-terminal domain
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FDA	US Food Administration
GM	geldanamycin
HSF	heat shock factor
HSP90	heat shock protein 90
HSP	heat shock protein gene
MAPK	mitogen-activated protein kinase
MD	middle domain
NGS	next generation sequencing
NSCLC	non-small cell lung cancer
NTD	N-terminal domain
ORR	objective response rate
OS	overall survival
PFS	progression free survival
RD	radicicol
ROS	reactive oxygen species
SCC	squamous cell carcinoma
TKI	tyrosine kinase inhibitor
TRP	tetratricopeptide repeat

19.1 Introduction

Worldwide, 1.8 million patients are diagnosed with lung cancer each year and 1.6 million patients will die from their disease annually (Ferlay et al. 2015; Siegel et al. 2016). As lung cancer is most frequently diagnosed in the metastatic setting, the 5-year survival rate is only 17% (Siegel et al. 2016). Clearly there is an urgent need for new and innovative therapeutics targeting lung cancer. The last couple of decades has witnessed many advancements in the development of tyrosine kinase inhibitors which can target distinct oncogenic drivers in lung cancer (Hirsch et al. 2017). Unfortunately, resistance to such traditional TKI therapies in frequently observed (Sequist et al. 2011). Advancement of science and better understanding of cancer biology has been providing us with many potential novel targets that if inhibited could serve as alternative therapeutic approaches. One such important target is the cellular protective machinery that consists of multiple proteins commonly known as chaperones. These proteins, acting in concert, perform critical functions in all mammalian cells including facilitation of correct folding and maturation of nascent peptides as well as refolding of the ones that are denatured due to different cellular stress factors, hence being named as heat shock protein (HSP). The clientele of these HSP group represents many signaling proteins that are critical mediators of transformation and stabilization of malignant features. Therefore, targeting HSP is an attractive way forward in many cancer treatments. Among the different players

of the chaperone machinery, HSP90 is often considered an important target as HSP90 inhibition can lead to the blockade of several cellular signaling pathways required for cancer. Based on this rational, several HSP90 small molecule inhibitors has been synthesized and characterized pre-clinically in the last 20 years, but only a few have progressed into the clinic. In this chapter, we will describe how HSP90 represents an effective therapeutic target in lung cancer and how pharmacological inhibition of HSP90-dependent chaperone machinery has evolved over the time in response to the unmet need for lung cancer targeted therapeutics.

19.1.1 The Chaperone, HSP90

The heat shock protein 90 (HSP90), an important member of a well-studied family of molecular chaperones, is abundantly expressed in both eukaryotes and prokaryotes. HSP90s are indispensable for eukaryotic cell survival, however they typically are not essential in bacteria (Borkovich et al. 1989; Versteeg et al. 1999). Based on transcriptome and genome analyses, human HSP90 family includes 17 genes that are broadly grouped into four classes – 7 genes in HSP90AA, 6 in HSP90AB, 3 in HSP90B, and finally, 1 gene in TRAP (Chen et al. 2005). Among the 17 genes, 6 genes are considered functional (*HSP90AA1*, *HSP90AA2*, *HSP90N*, *HSP90AB1*, *HSP90B1*, and *TRAP1*), whereas the other 11 genes were found to be pseudogenes (Chen et al. 2005). *HSP90N* was originally described as Hsp89-α-δ-N (Schweinfest et al. 1998) and was later found to be almost identical to *HSP90AA1* (Chen et al. 2005). The five most important members in the human HSP90 protein family that are encoded by the *HSPC* gene family (Chen et al. 2005; Kampinga et al. 2009) are predominantly abundance in cytoplasm and the nucleus, but localization to other organelles such as mitochondria and endoplasmic reticulum (ER) is also common (Table 19.1) (Langer et al. 2003). Like other large heat shock proteins (HSP), HSP90 is also an adenosine triphosphate (ATP)-dependent chaperone with ATPase activity (Bepperling et al. 2012). Although, HSP90 is functionally active when it forms a homo-dimer, each of the monomers consists of three highly conserved functional domains, the N-terminal domain (NTD) that contains a nucleotide (ATP or ADP) binding pocket which mediates ATP binding and its hydrolysis to ADP (Prodromou et al. 1997), a charged middle domain (MD) that regulates the ATP hydrolysis and the binding and interaction with specific co-chaperones and substrates, and the C-terminal domain (CTD), which is essential for dimerization (Krukenberg et al. 2011; Pearl and Prodromou 2006). There is also a consensus sequence in CTD, Met-Glu-Glu-Val-Asp (MEEVD) which is involved in the interaction with co-chaperones that have a tetratricopeptide repeat (TRP) domain (Buchner 1999). HSP90 is involved in the regulation of functions of hundreds of substrate proteins often referred to as clients that are critical members of multiple signaling pathways (Rohl et al. 2013; Zhao et al. 2005).

The fidelity of HSP90 chaperonage activity, which is critical for cell's survival, is also aided by a great number of co-chaperones (Table 19.1) (Rohl et al. 2013).

Table 19.1 Brief summary of human HSP90 family members and their cellular location and functions

Family	Protein members	Peptide length (amino acid)/ molecular weight (kDa)/encoding gene	Cellular location	Co-chaperones	Function	References
HSP90	HSPC1	732/86/*HSP90AA1* or *HSPC1*	Cytosol	p23, PP5, TTC4, AHA1, CYP40, CDC37, HOP, SGTA, Tah1, CHIP, Unc45, FKBP51, FKBP52	Molecular chaperone	Chatterjee and Burns (2017), Chen et al. (2005), Fuller et al. (1994), Kopecek et al. (2001), and Sreedhar et al. (2004)
	HSPC2	757/87/*HSP90AA2* or *HSPC2*	Cytosol			
	HSPC3	724/84/*HSP90AB1* or *HSPC3*	Cytosol			
	HSPC4	803/92/*HSP90B1* or *HSPC4*	Cytosol, ER			
	HSPC5	704/75/*TRAP1* or *HSPC5*	Mitochondria			

Among the many functions of the co-chaperones involved in regulation of HSP90 ATPase activity, are modulating the affinity of HSP90 for client proteins, or directing HSP90 to a particular cellular pathway (Rizzolo et al. 2017). Based on recent studies, it is evident that some co-chaperones compete among each other for the HSP90 binding, whereas binding by other co-chaperones is not mutually exclusive and this simultaneous binding leads to enhanced function of the co-chaperones. As such, the constituents of the HSP90 complex with different co-chaperones forming the same complex likely determines client selection (Schopf et al. 2017). Examples of HSP90 co-chaperones are listed in Table 19.1. Several biochemical and genetic analyses have indicated that HSP90 does not interact with unfolded or nascent proteins, rather it prefers significantly folded protein substrates, further suggesting its involvement in protein structure remodeling or in protein stabilization (Pearl and Prodromou 2006; Wandinger et al. 2008). As HSP90 clientele plays crucial roles in several biological mechanisms, HSP90 can be considered a master regulator of vast cellular activities including signal transduction, telomere maintenance, vesicle transport, transcriptional regulation, cell cycle regulation, steroid signaling, viral infections, immune response, and finally cancer development (Echeverria et al. 2010; Makhnevych and Houry 2012), ultimately earning its status as a validated anticancer drug target (Neckers and Workman 2012).

Ubiquitous expression of HSP90 in normal cells often counts up to 1–2% of the total cellular protein content (Soga et al. 2013). As the primary function of the HSP90 chaperone complex is proper folding and stabilization of its clients, proper step-wise assembling of different components of the complex is important for formation of client's biological active conformation. According to one hypothesis, the client protein first binds to a specific co-chaperone complex HSP70/HSP40 followed by uploading onto the ADP-bound HSP90 homodimer. Functional activity of

HSP90 is highly dependent on its association with specific co-chaperones such as HSP70, HSP60, HSP40, or p23, which is mediated by the ATP/ADP binding of HSP90. It has been shown that pharmacological inhibition of HSP90 led to release of the p23 co-chaperone from the HSP90 complex following ATP hydrolysis to ADP and promoted HSP70/P60^{HOP} association with the ADP-bound HSP90 complex facilitating the degradation steps of many client substrates by ubiquitination (Fig. 19.1) (Soga et al. 2013).

19.1.2 Oncogenic Targets of HSP90

The evolutionary conserved HSP90 family members are extensively studied for their important roles associated with maturation, stabilization, activation and proteolytic degradation of several proteins, popularly known as clients. These client proteins are involved in a variety of biological processes ranging from stress response to DNA repair, development, immune response, proliferation and cell survival which are critical for tumorigenesis (Chatterjee et al. 2016; Chatterjee and Burns 2017; Echeverria et al. 2011). Although, compared to the other chaperones, the characteristics that can define an HSP90 client are poorly understood, recent

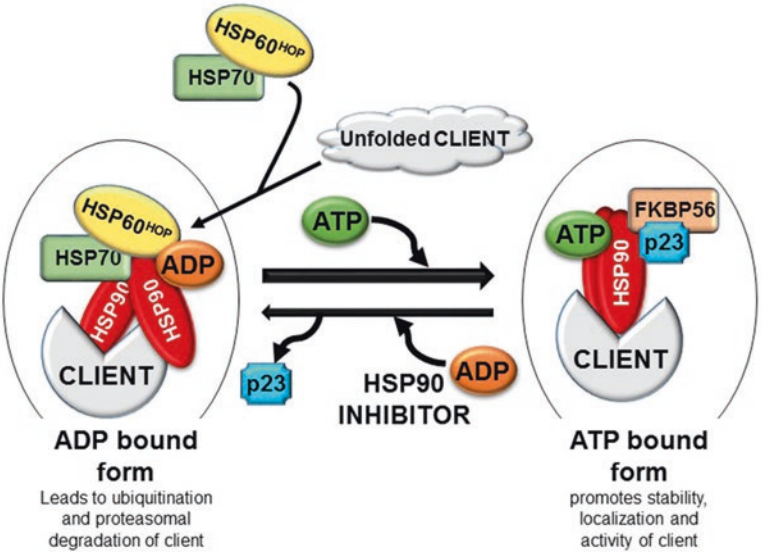

Fig. 19.1 Co-chaperone selectivity of HSP90 decides fate of client protein. In ATP bound state, HSP90 forms chaperone complex with the FKBP56/p23 co-chaperones that binds to a client protein to promote its maturation and stability leading to proper client activity. HSP90 inhibition leads to hydrolysis of HSP90 bound ATP to ADP leading to the release of p23 co-chaperone allowing HSP70/HSP60^{HOP} to form a different chaperone complex with HSP90 that promotes ubiquitination of client protein that is fated to the proteasomal degradation of the later

high throughput studies have been successful to generate a sizable list of clients (Taipale et al. 2012). The clientele chart is dominated by oncoproteins including oncogenic kinases such as AKT, MET, MEK, CDK4, BRAF, CRAF, EGFR, ERBB2 (HER2), and BCR-ABL, critical transcription factors such as p53 and HIF1α, steroid hormone receptors including the estrogen and androgen receptors (Chatterjee et al. 2016; Chatterjee and Burns 2017; Schopf et al. 2017; Workman et al. 2007). The oncoprotein clientele of HSP90 has been shown to contribute to all six "hallmarks of cancer" – evasion of apoptosis (e.g., Apaf-1, p53, AKT), self-sufficiency in growth signaling (e.g., EGFR, HER2, members of MAPK pathway), insensitivity to anti-growth signals (e.g., CDK4, Myt1, Wee1), tissue invasion and metastasis (e.g., c-MET), sustained angiogenesis (e.g., VEGFR, Fit-3), and finally, potential of limitless replication (e.g., hTERT) (Hanahan and Weinberg 2000, 2011) . For the convenience of the readers a concise summary of HSP90 clients related to cancer has been provided in Table 19.2 (for more comprehensive list, please visit https://www.picard.ch/downloads) and a depiction of the key oncogenic signaling pathways regulated by HSP90 is provided in Fig. 19.2.

Table 19.2 Brief summary of oncogenic client proteins of HSP90 and their functions

Client	Function
Kinases	
AKT (PKB)	Mitogen signaling
BRAF	Mitogen signaling
ERBB2	EGF receptor
BCR-ABL	Constitutively active Tyr kinase
SRC	Constitutively active Tyr kinase
CDK4	Cell cycle control
JAK1 and/or KAJ2	Cytokine signaling
HCK	Immune response
Transcription factors	
p53	Tumor suppressor protein
HIF-1α	Angiogenesis
OCT4	Embryonic development
STAT2, STAT3, STAT5	Cytokine signaling
Steroid hormone receptors	
Progesterone receptor	Response to progesterone
Estrogen receptor	Response to estrogens
E3 ubiquitin ligases	
MDM2	p53 degradation
UHRF1	DNA methylation
Others	
TERT	Telomere maintenance
RAD51 and/or RAD52	DNA repair

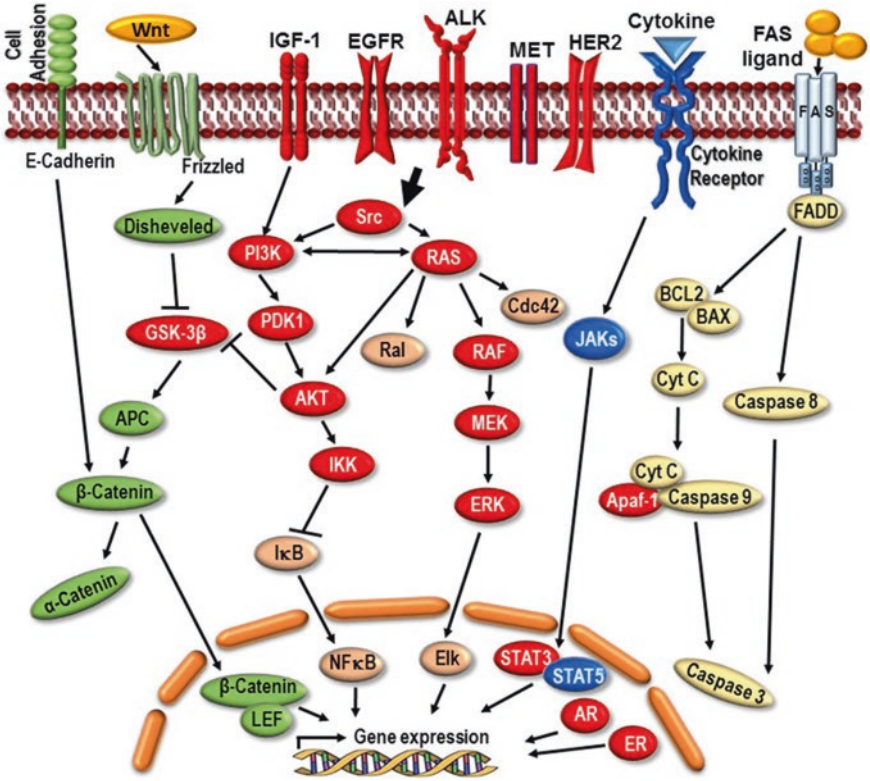

Fig. 19.2 Hsp90 function is important for the stability of oncoproteins belong to diverse signaling pathways. HSP90 chaperones the regulation of functional activity of variety of client proteins including receptors as well as other signaling molecules that are bona fide oncoproteins (*red*) that are key modulators of oncogenic signaling pathways. Green proteins represent the cell-adhesion and Wnt-signaling, whereas blue proteins constitute the JAK/STAT pathway and yellow proteins belong to apoptotic pathway

Importantly, HSP90 has been reported to be overexpressed in many cancers (Burrows et al. 2004; Chatterjee et al. 2016; Chatterjee and Burns 2017; Patel et al. 2014) and its overexpression is generally associated with poor prognosis, especially in melanoma, leukemia, bladder, lung and esophageal cancers (Huang et al. 2014; McCarthy et al. 2008; Tian et al. 2014; Zackova et al. 2013). As such, suppression of HSP90 expression, genetically or pharmacologically, can simultaneously co-inhibit a wide range of client proteins, which ultimately antagonize all the hallmark features of malignancy (Chiosis et al. 2004; Neckers 2006; Workman 2004; Zhang and Burrows 2004).

19.1.3 Lung Cancer, a Significant Health Problem and the Need for a New Approach

Although in nineteenth century, lung cancer was one of the rarest diseases, in the current century it has become the major cause of cancer related death in both men and women in United States and worldwide, with approximately 160,000 deaths in United States alone each year and the 5-year survival rate is still below 20%. Non-small cell lung cancer (NSCLC) account for almost 85% of lung cancer cases and the 5-year survival rate is only 17% (Siegel et al. 2016). Although, NSCLC is the most common type of lung cancer, it is only in the past decade, when technological advancements including introduction of next-generation sequencing (NGS), generation of multiple genetically engineered mouse models (GEMMs) and production of large databases featuring the molecular profiles of human tumors have changed our view of NSCLC. The histopathological description of NSCLC can now be further refined with division of NSCLC into distinct targetable molecular subsets (Gridelli et al. 2015).

There are two predominant pathologic types of NSCLC, adenocarcinoma (ADC, about 40%) and squamous cell carcinomas (SCC, about 25%) of the lung (Davidson et al. 2013; Langer et al. 2010; Rosell and Karachaliou 2016). Although the histopathological features and immunohistochemical protein expression still serve as the clinical basis for tumor diagnosis, recent technological advancements provided us with a new approach to categorize NSCLCs, which is based on oncogenic driver mutations within lung tumors. Based on several recent reports, a summary of incidence and variety of oncogenic drivers in lung adenocarcinomas is provided in Fig. 19.3 (Davidson et al. 2013; Kris et al. 2014; Langer et al. 2010; Reck and Rabe 2017; Rosell and Karachaliou 2016). Expansion of our knowledge in past decade, especially on the characterization of NSCLCs based on oncogenic driver mutations, has led to some impressive new treatments for subsets of patients. These include the US Food Administration (FDA) approval of EGFR tyrosine kinase inhibitor (TKIs) in *EGFR*-mutant NSCLC, crizotinib and others in echinoderm microtubule-associated protein-like 4 (*EML4*)-ALK fusion and ROS1 fusions (Camidge et al. 2012; Mok et al. 2009; Shaw et al. 2011; Somasundaram et al. 2014) as well as other targeted agents for less common oncogenic drivers (BRAF, MET, RET, NRKT).

Despite the fact that these targeted agents were able to produce impressive response and improved survival rates among the patients, acquired resistance to chronic treatment inevitably develops within 9–12 months in majority of the patients who received these kinase inhibitors as their targeted therapies (Katayama et al. 2012; Kobayashi et al. 2005; Mok et al. 2009). Furthermore, targeted therapies for patients with *KRAS*-mutant NSCLC is still lacking. Recent therapeutic approaches have mostly focused on inhibiting KRAS downstream signaling pathways including the RAF-MEK-ERK or PI3K-mTOR pathway, and have shown limited promise both preclinically as well as clinically (Chen et al. 2012; Engelman et al. 2008; Janne et al. 2013) due to toxicity and the inability to determine the key pathways to

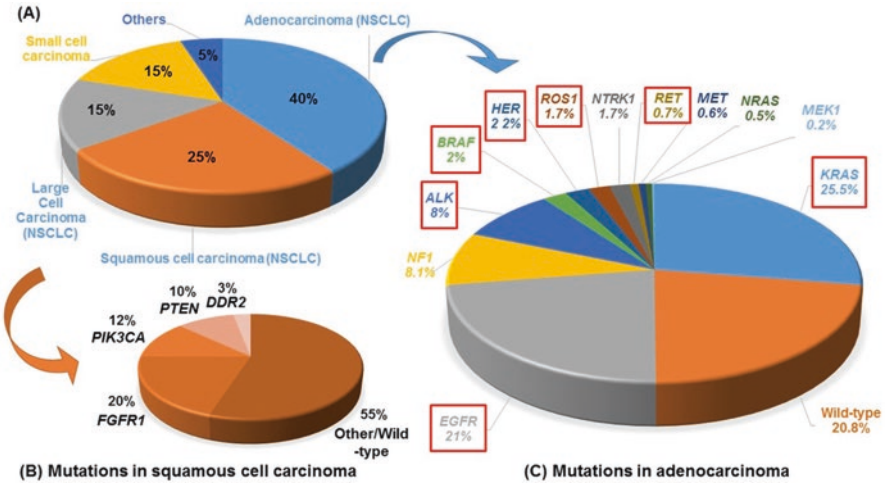

Fig. 19.3 Lung cancer histologic subtypes contain distinct targetable oncogenic drivers. (**a**) Pie chart depicting the relative frequency of each histologic subtype. (**b**) Pie chart depicting the molecular subtypes found in squamous cell carcinoma based on oncogenic driver mutations. (**c**) Pie charts depicting the molecular subtypes found in adenocarcinomas. Oncogenic driver mutations that are RED boxed are known HSP90 client proteins and/or oncogene driver molecular subtypes in which HSP90 inhibition that have been tested

inhibit simultaneously. As HSP90 inhibitors can inhibit multiple of the key downstream signaling pathway of mutant KRAS, many speculated that HSP90 inhibition may be an effective therapy strategy for *KRAS* mutant NSCLC (Chatterjee et al. 2016).

19.1.4 HSP90 and Lung Cancer

Inhibition of HSP90 expression may prove to be effective in NSCLC as it clearly overexpressed in NSCLC and its expression has prognostic significance. A recent investigation has revealed that 44% of NSCLC contained a deletion on chromosome 14 (14q32.2-33), which contains the *HSP90A* gene, and this incidence correlated with a statistically significant survival benefit (Gallegos Ruiz et al. 2008). Patient stratification by HSP90α expression in tumors also revealed a significant correlation between prolonged survival and HSP90 level, which was further validated in an additional 307 NSCLC patients (Trepel et al. 2010). Whether HSP90 expression can be used as a predictive measure of the response of NSCLC patients to HSP90 inhibitors remains to be determined. As most of HSP90 client proteins are validated oncogenic drivers, it represents an attractive therapeutic target. As such NSCLC cells and cancer cells in general are more dependent on HSP90 for proliferation and survival compared to normal cells, as the oncoproteins are often misfolded and HSP90

overexpression is required for repair (Neckers 2006; Neckers and Workman 2012) This rationale led to the development of many HSP90 inhibitors which have shown promise both preclinically as well as in the clinical treatment of NSCLC patients.

Clinical studies revealed strong efficacy of small molecule inhibitors specific for HSP90 in combination with EGFR-TKIs in patient with *EGFR*-mutant NSCLC, even in those who have been pre-treated with TKI therapy (Sequist et al. 2010). Preclinical data strongly indicates that pharmacological inhibition of HSP90 can attenuate the oncogenic switch, which promotes the malignant cells to opt for other receptor tyrosine kinases when one is blocked. This type of switching is a known resistance mechanism to TKIs. Other alternative kinases that are induced, altered or mutated include HER2, MET, BRAF and ALK. Of note, these are all HSP90 clients sensitive to HSP90 inhibitors (Shimamura and Shapiro 2008). Moreover, it has also been reported that HSP90 inhibitors are effective against rare *EGFR* mutations that are resistant to TKIs (Shimamura et al. 2008; Xu et al. 2007). These preclinical and clinical investigations will be described in more detail in section 19.1.4.2 and 19.1.5, respectively. These findings strongly advocate HSP90 inhibitors as useful agents to combine with TKIs or other kinase inhibitors to overcome acquired resistance to drugs that may have been generated by oncogenic switch in NSCLC and other malignancies.

19.1.4.1 HSP90 Inhibition, Structural and Functional Evolution

As HSP90 has been implicated in cancer initiation and progression, over the last decade, a significant amount of investment has been made in the discovery of HSP90 inhibitors starting with natural products followed by, their first generation derivatives and finally fully synthetic second generation small molecule inhibitors. In this section, we will focus on discussing the different inhibitors and their development over time.

Inhibitors Targeting ATP Binding Site of N-Terminal Domain

Natural Inhibitors and Their Derivatives

HSP90 therapeutic targeting in cancer treatment was established with the use of two natural products, geldanamycin (GM) and radicicol (RD) (Fig. 19.4). GM, a benzoquinone ansamycin, was extracted from *Streptomyces hygroscopicus* and showed potential as an anticancer agent (Ochiana et al. 2014; Patel et al. 2011). RD, on the other hand, is a macrocyclic lactone antibiotic that was first extracted from *Monocillium nordinii* and *Monosporium bonorden* with strong antitumor efficacy, in vitro as well as in vivo (Soga et al. 2003). Both GM and RD were able to mimic the structure adopted by ATP in the NTD of HSP90, therefore leading to selective inhibition of ATP binding and hydrolysis, ultimately leading to degradation of HSP90 client oncoproteins (Mimnaugh et al. 1996; Roe et al. 1999). Although, both

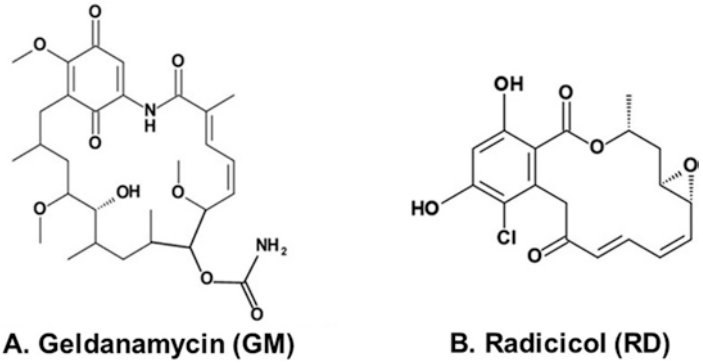

Fig. 19.4 Chemical structures of naturally available HSP90 inhibitors. (**a**) geldanamycin and (**b**) radicicol are two natural products, which were the first Hsp90 inhibitors to be investigated in preclinical studies

were able to produce potent cytotoxicity, structural instability, poor water solubility and severe hepatotoxicity led to cessation of clinical development (Soga et al. 2003; Supko et al. 1995). Despite their toxicity and structural instability, these two compounds played crucial roles as structural scaffolds for the development of superior inhibitors to come in time.

The first GM derivative to proceed to clinical trial was tanespimycin or 17-allylam ino-17-demethoxygeldanamycin (17-AAG; Bristol-Myers Squibb) (Fig. 19.5a), which showed superiority over GM as it was less toxic and exhibited potent in vivo efficacy (Solit and Chiosis 2008; Workman et al. 2007). However, water solubility was still a persistent issue with 17-AAG leading to a need for better formulation. Next was alvespimycin or 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; Kosan), a water soluble GM analogue, which could be administered both orally as well as intravenously. 17-DMAG (Fig. 19.5b) has an ionizable functional group at C-17 position and similar to GM, it showed potent cytotoxicity by attacking the NTD ATP-binding pocket of HSP90 (Jez et al. 2003). Compared to 17-AAG, 17-DMAG showed better potency, improved bioavailability and much lower cytotoxicity which led to several phase I clinical studies. However, the dose limiting side effects of 17-DMAG lead to the discontinuation of its clinical development (Kummar et al. 2010; Lancet et al. 2010). The next generation GM derivative developed was retaspimycin hydrochloride or 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504; Infinity Pharmaceuticals, Inc.) (Fig. 19.5c), which is a reduced analogue of 17-AAG with improved water solubility (Sydor et al. 2006). Favorable properties of IPI-504 lead to clinical investigation in multiple clinical trials (Hanson and Vesole 2009; Neckers and Workman 2012; Ochiana et al. 2014; Song et al. 2008). However, due to several setbacks including poor pharmacological properties, IPI-504 development was halted. Another derivative generated as a major metabolite of IPI-504 and 17-AAG was 17-AG or 17-animo-17-demethoxygeldana-

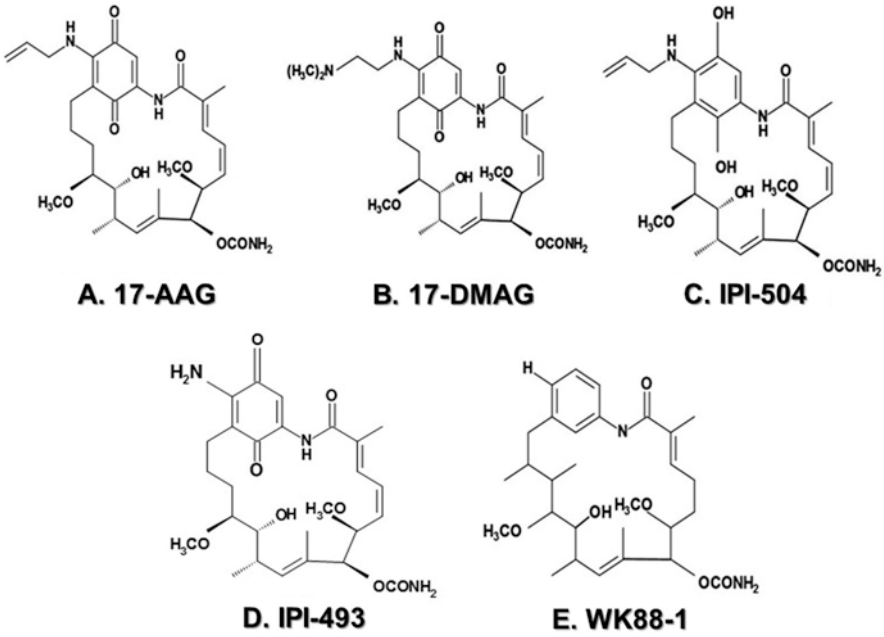

Fig. 19.5 Chemical structures of Geldanamycin derived HSP90 inhibitors. (**a**) 17-AAG, (**b**) 17-DMAG, (**c**) IPI-504, (**d**) IPI-493 (17-AG), and (**e**) WK88-1

mycin (IPI-493; Infinity Pharmaceuticals, Inc.) (Fig. 19.5d). It was an orally administered HSP90 inhibitor that was able to reach a phase I study (NCT00724425). Among the nonquinone GM derivatives, WK88-1 (Fig. 19.5e) was an important inhibitor that was generated by mutasynthesis in genetically engineered *Streptomyces hygroscopicus* and was found to be most efficacious both in vitro as well as in mouse xenograft models (Jang et al. 2014).

Many oxime- and cyclopropane analogues of RD were derived that minimized in vivo metabolism and displayed better cytotoxic activities in preclinical animal models as well as was characterized by much less toxic side effects. (Shiotsu et al. 2000; Soga et al. 2003; Yamamoto et al. 2003). KF55823 (Fig. 19.6a) and KF25706 (Fig. 19.6b) are two examples of oxime-derivatives of RD, which showed potent anti-tumor activity in vitro (Soga et al. 2003). They were also reported to show potent anti-tumor activity in human tumor xenografts at well tolerated doses with no severe hepatotoxicity (Agatsuma et al. 2002; Kurebayashi et al. 2001). Despite the promising anti-tumor activity, they did not progress to clinical trials due to the presence of reactive epoxide moiety in them. To address this issue, cyclopropyl analogue RD was derived. Radester (Fig. 19.6c) was developed as a hybrid of RD's resorcinol and GM's benzoquinone that showed potent in vitro anti-tumor activity but also was not further developed (Shen and Blagg 2005).

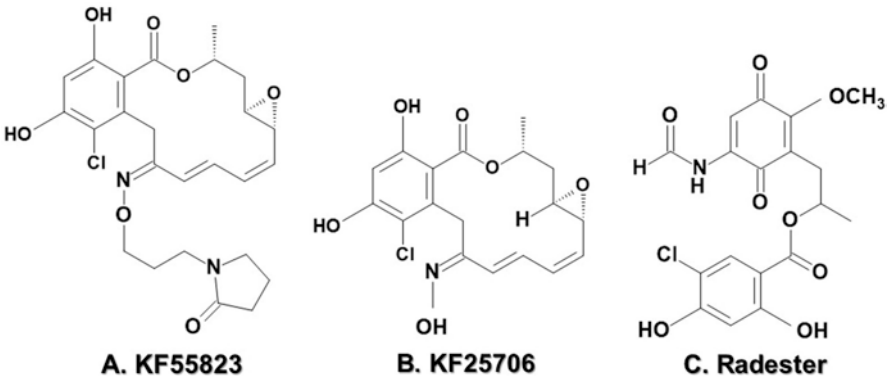

Fig. 19.6 Chemical structures of Radicicol derived HSP90 inhibitors. (**a**) KF55823, (**b**) KF25706, and (**c**) Radester

Synthetic Small Molecule and Peptide Derivative Inhibitors

The clinical failure of natural products and their derivatives led to the development of the second generation synthetic small molecule HSP90 inhibitors, which would overcome the limitations faced by the previous group of compounds. A wide range of chemical scaffolds was generated in last decade covering purines, resorcinols, pyrimidines, aminopyrimidines, azoles and other chemotypes (Messaoudi et al. 2011). In the following sections, we will be discussing the most preclinically/clinically relevant ones.

Purine Scaffold Series

Based on the fact that GM and RD when bound to HSP90 acquires a C-shaped conformation, Chiosis et al. developed the first purine scaffold, synthetic HSP90 inhibitor, PU3 (Fig. 19.7a) (Chiosis et al. 2001, 2002), which was later optimized by various strategies to increase its potency and pharmaceutical properties. CNF2024/BIIB021 (Fig. 19.7b) was the first purine-based HSP90 inhibitor to enter phase I/II clinical trials, but despite being efficacious it caused severe side effects in patients including dizziness, fatigue, hyponatremia, and hypoglycemia (Von Hoff et al. 2007). The next notable member of this group is MPC-3100 (Fig. 19.7c), which completed a phase I trial in 2011 (NCT00920205) (Kim et al. 2015; Sidera and Patsavoudi 2014). Two other important members are Debio 0932 and PU-H71. Debio 0932, also known as CUDC-305 (Fig. 19.7d), was generated as a purine-like derivative by replacing the N3 of the purine with a carbon and was evaluated in a phase I/II studies but was reported to show dose limiting toxicities (Sidera and Patsavoudi 2014). PU-H71 (Fig. 19.7e) is being tested in the clinic, in a phase I study involving patients with advanced lymphoma and solid tumors, and myeloproliferative disorders (Jhaveri et al. 2012b; Sidera and Patsavoudi 2014).

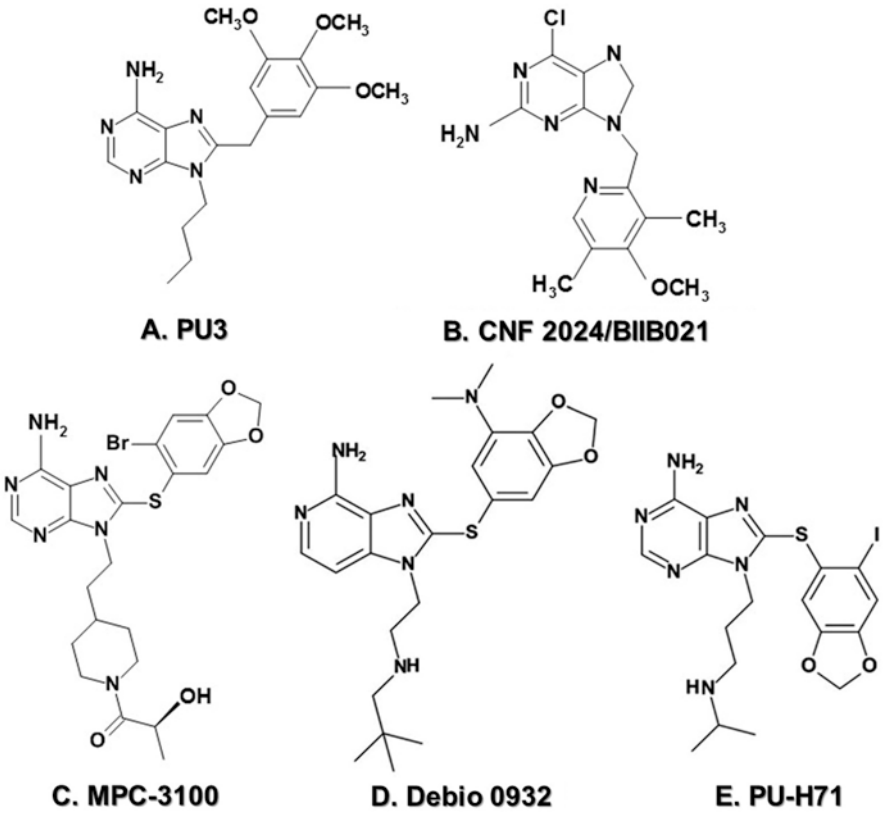

Fig. 19.7 Chemical structures of Purine scaffold HSP90 inhibiting agents. (**a**) PU3, (**b**) CNF2024/BIIB021, (**c**) MPC-3100, (**d**) Debio 0932, and (**e**) PU-H71

Resorcyclic Pyrazole/Isoxazole Series

The Workman group identified diarylpyrazole-scaffold inhibitor, CCT018159 that contains the resorcinol anchoring unit which is critical for radicicol binding (Fig. 19.8a). This compound showed stronger potency to inhibit both yeast and human HSP90 N-terminal ATPase activity (Sharp et al. 2007). This compound served as a template and was optimized using a structure based approach to develop a more potent resorcinylic isoxazole amide named NVP-AUY922/VER52296 (Fig. 19.8b) (Eccles et al. 2008; Gaspar et al. 2010). This compound is considered to be the most potent small molecule HSP90 inhibitor and has showed strong efficacy both pre-clinically as well as clinically (Brough et al. 2008; Shiotsu et al. 2000; Ueno et al. 2012). A follow up to NVP-AUY922, was the development of NVP-HSP990 (Fig. 19.8c) (Lamottke et al. 2012; Menezes et al. 2012), the development of which was eventually stopped as it failed to produce effective clinical responses at the maximum tolerated dose.

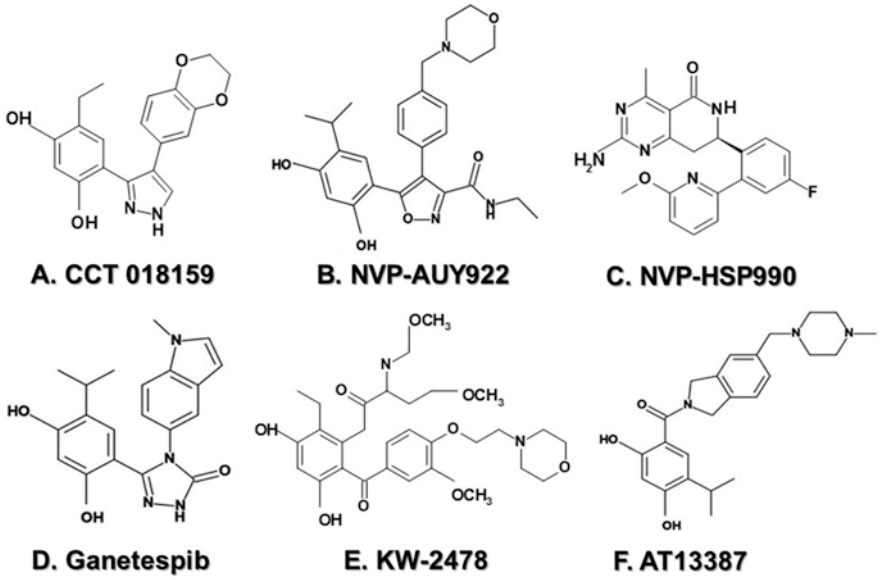

Fig. 19.8 Chemical structures of resorcyclic pyrazole/isoxazole HSP90 inhibitors. (**a**) CCT 018159, (**b**) NVP-AUY922, (**c**) NVP-HSP990, (**d**) Ganetespib, (**e**) KW-2478, and (**f**) AT13387

So far, ganetespib (STA-9090) (Fig. 19.8d), a recorcinol-based second generation synthetic small molecule appeared to be the most promising. Ganetespib binds to the N-terminal ATP-binding pocket to disrupt the chaperonage function. It contains a triazole moiety (Chatterjee et al. 2016; Kummar et al. 2010). The preclinical success of ganetespib led to several clinical studies. Ganetespib has showed not only strong single agent activity, but strong efficacy in combination with other drugs in various cancer types characterized by distinct mutations in driver oncogenes such as mutant *EGFR* (Shimamura et al. 2012), mutant *KRAS* NSCLCs (Acquaviva et al. 2012; Gomez-Casal et al. 2015; Proia et al. 2012b). Significant single agent activity by ganetespib was also reported in *ALK*-driven disease, however responses were only transient in patients with *KRAS*-mutant NSCLC due to rapid onset of resistance (Socinski et al. 2013). Unfortunately, clinical development of this compound has now ceased (2015). Two more important resorcinol analogs were KW-2478 (Fig. 19.8e) and AT13387 (Fig. 19.8f). KW-2478 was discovered by a novel lead optimization strategy that included microbial screening, X-Ray crystallography, cell-based assays and in vivo animal models (Sidera and Patsavoudi 2014). It is currently being evaluated in clinic (NCT01063907). AT13387 (also known as Onalespib) is a radicicol derived agent and the most important feature of this inhibitor is its extended pharmacodynamic activity (Graham et al. 2012). It has produced enough promising pre-clinical data to be evaluated in several phase I/II clinical studies, some of which completed (NCT01294202, NCT01685268, NCT00878423, NCT01246102), whereas others are still recruiting.

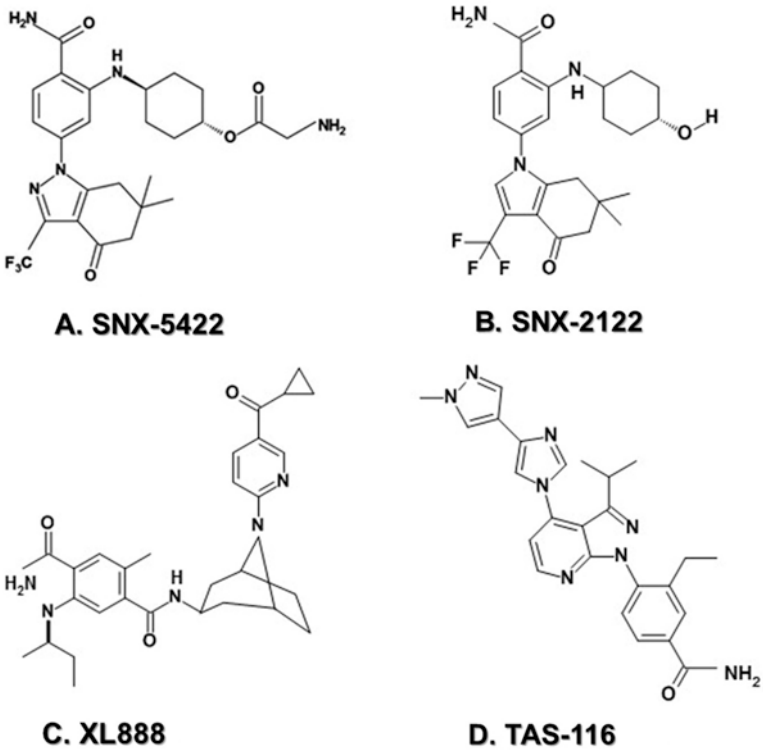

Fig. 19.9 Chemical structures of Benzamides and other N-terminal inhibitors. (**a**) SNX-5422 and (**b**) SNX-2122 represent two benzamides, whereas the structural classes of (**c**) XL888 and (**d**) TAS-116 are still not disclosed

Benzamides

SNX-5422 mesylate (Fig. 19.9a), a pro-drug of SNX-2122 (Fig. 19.9b), is an indazolone 2-aminobenzamide analog (Chandarlapaty et al. 2008), was able to enter clinical trial in 2007 as an orally bioavailable HSP90 inhibitor. Owning to ocular toxicity and the potential of causing irreversible retinal damage, its development was discontinued.

Other Inhibitors

Among other N-terminal HSP90 inhibitors, XL-888 (Fig. 19.9c) is notable. It is a tropane derivative without any recognizable structural pattern. A collection of preclinical analyses suggests that this inhibitor was able to induce significant degradation of HSP90 client proteins and inhibition of proliferation of a wide range of human tumor cell lines (Bussenius et al. 2012). This compound was examined in a phase 1 clinical trial (NCT00796484) but did not proceed beyond early phase trials. A multiparameter lead optimization campaign was utilized to discover the latest

candidate, TAS-116, to join the growing list of inhibitors (Fig. 19.9d) (Ohkubo et al. 2015). It has shown efficacy both in vivo and in vitro. It possesses favorable bioavailability and an improved metabolic stability in rodent and non-rodent animal models which was accompanied by decreased ocular toxicity and increased antitumor activity in several xenograft models (Ohkubo et al. 2015; Suzuki et al. 2015). Preclinical studies demonstrated that TAS-116 could sensitize human cancer cells to radiation such as to X-rays and carbon ion radiation (Lee et al. 2017). Furthermore, there are many more inhibitors, which targets the CTD of the linker region of HSP90, but they are still in early developmental stages. Finally, some inhibitors in early stages of development have also been generated to target the HSP90 and co-chaperone interaction or HSP90-client interactions.

19.1.4.2 Preclinical Studies

The basis of the preclinical success of HSP90 inhibition in cancer therapeutic studies is the fact that HSP90 activity is significantly elevated in malignancy as a means of cytoprotection (Kamal et al. 2003). The continuous and augmented HSP90 chaperone activity is essential for the oncogene-driven neoplastic growth as genetic and/or pharmacological inhibition of HSP90 leads to tumor growth inhibition and apoptosis. Although, the preclinical studies on HSP90 inhibitors covers many different types of cancers, in this section, we will be discussing the preclinical assessment of different HSP90 inhibitors with reference to different oncogenic targets in lung cancer.

EGFR Mutant Non-small Cell Lung Cancer

EGFR mutations are detected in about 21% (Fig. 19.3) of adenocarcinomas (Kris et al. 2014), the majority of which are the L858R point mutation and exon 19 internal deletions. EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib and later afatinib have been in use to target this specific type of cancer, however, development of acquired resistance to these TKIs within 9–12 months of treatment initiation is often observed (Pao et al. 2005). In some cases, the resistance is mediated by a second mutation, T790M (Kobayashi et al. 2005; Pao et al. 2005). As mutant *EGFR* is an HSP90 client oncoproteins, use of HSP90 inhibition was proposed as a strategy to overcome such resistance (Shimamura et al. 2005). The first HSP90 inhibitor to be used was the ansamycin antibiotics such as geldanamycin that was able to inhibit tumor growth in *EGFR*-mutant NSCLC carrying L858R and/or deletions such as delL747-S752, P753S and the T790M mutation which confers resistance to first and second generation EGFR TKIs (Shimamura et al. 2005). Compared to wild-type controls, *EGFR*-mutant NSCLC cell lines (NCI-H3255, NCI-H1975, and NCI-H1650) were found to be more sensitive to gendanamycin-induced HSP90 degradation, which also led to significant reduction in expression of phospho-AKT and cyclin D1 in the same set of cell lines (Shimamura et al. 2005).

These initial studies suggested that HSP90 inhibition could represent a novel therapeutic strategy to treat *EGFR*-mutant NSCLC. The next set of HSP90 inhibitors that were being used both in *EGFR*-mutant NSCLC cell lines as well as in animal models, were 17-AAG, 17-DMAG and IPI-504 (Ge et al. 2006; Nguyen et al. 2000). These inhibitors were able to produce strong preclinical efficacy in vitro and in mouse models harboring *EGFR*-mutant NSCLC that showed resistance to EGFR-TKIs mediated by the second mutation T790M (Kobayashi et al. 2005; Shimamura et al. 2008; Yang et al. 2006). The nonquinone GM derivative, WK88-1, has also been preclinically investigated in *EGFR* T790M mutation NSCLC harboring the (Hong et al. 2014). Hong et al. (2014) showed that pharmacological HSP90 inhibition by WK88-1 was able to degrade EGFR in the gefitinib-resistant NCI-H1975 cells, ultimately leading to growth arrest and apoptosis. Moreover, WK88-1 was also able to inhibit proliferation, migration and invasion in H1975 cells significantly. WK88-1 evaluation in vivo xenograft assay also reduced tumor growth in the H1975 xenografts markedly, advocating the potent efficacy of WK88-1 to overcome gefitinib-resistance in *EGFR*-mutant NSCLCs harboring T790M mutation (Hong et al. 2014). Among the resorcinol derivatives, both NVP-AUY922 as well as ganetespib had marked preclinical efficacy in *EGFR*-mutant NSCLCs. In addition to in vitro inhibition of *EGFR*-mutant NSCLC cell growth, NVP-AUY922 also significantly reduced EGFR protein expression in H1975 xenografts (Garon et al. 2013). Stronger efficacy, both in vitro as well as in mice xenografts bearing NCI-H1975 cells with L858R and T790M mutations was observed with ganetespib compared to 17-AAG (Shimamura et al. 2012). Prolonged pharmacodynamics action of AT13387 made it an even more effective choice to target *EGFR*-mutant NSCLC. It was shown to suppress EGFR signaling for prolonged period of time both in vitro and in vivo (Graham et al. 2012), which ultimately led it to enter multiple ongoing clinical trials. TAS-116 [4-(1H-pyrazolo[3,4-b]pyridine-1-yl)benzamide], a selective inhibitor of cytosolic HSP90α and β, is so specific that it does not inhibit HSP90 paralogs such as mitochondrial TRAP1 or endoplasmic reticulum GRP94. In a preclinical study involving in vitro NCI-H1975 cells as well as a rat model bearing a NCI-H1975 xenograft, TAS-116 showed great potential to maximize antitumor activity with much lesser adverse side effects such as visual disturbances (Ohkubo et al. 2015).

ALK-Positive Non-small Cell Lung Cancer

Eight percent of adenocarcinomas of the lung contain rearrangements in anaplastic lymphoma kinase (ALK) that is often associated with echinoderm microtubule-associated protein-like 4 (EML4) (Kwak et al. 2010; Soda et al. 2007). The *ALK-EML4* rearrangement was first described as an oncogenic driver by Soda et al. as they demonstrated strong transforming activity of the ALK-EML4 fusion protein (Soda et al. 2007). Although, several ALK inhibitors including crizotinib, ceritinib, and alectinib were developed and evaluated preclinically as well as clinically, acquired resistance to these ALK-inhibitors is inevitable (Katayama et al. 2011). As

ALK-EML4 fusion protein is a bona fide HSP90 client (Neckers and Workman 2012), HSP90 inhibition was utilized in order to overcome the ALK TKI acquired resistance. 17-AAG was the first HSP90 inhibitor to be tested and exhibited promising growth inhibitory activity in NSCLCs expressing ALK-EML4 fusion protein (Chen et al. 2010). It was able to induce depletion of ALK-EML4 fusion protein and its downstream signaling molecules such as phospho-AKT, phospho-ERK1/2 and phospho-S6 (Chen et al. 2010). 17-AAG was also effective in inducing significant growth inhibition of crizotinib-resistant NSCLCs (Katayama et al. 2012). 17-DMAG (Chen et al. 2010) and IPI-504 were also able to cause significant tumor regression in mouse xenograft models carrying NSCLCs with ALK-EML4 fusion protein expression (Normant et al. 2011). IPI-504 was also shown to degrade ALK-EML4 fusion protein specifically, which in turn induced rapid depletion in the expression of downstream signaling molecules like phospho-ERK, phospho-STAT3 and phospho-AKT ultimately leading to inhibition of cell growth and apoptosis (Normant et al. 2011). Ganetespib is among the second generation HSP90 inhibitors that showed notable potency against *ALK*-positive NSCLC. Sang et al. demonstrated that ganetespib attenuated EML4-ALK expression leading to depletion of multiple proteins in *ALK*-driven NSCLC cells, which was enough to establish ganetespib's greater in vitro potency and better antitumor efficacy compared to its predecessors (Sang et al. 2013). Additionally, ganetespib also demonstrated prolonged animal survival when compared to the data generated with crizotinib (Sang et al. 2013). Not only that, but ganetespib either in monotherapy or in combination with an ALK inhibitor other than crizotinib was able to show strong efficacy in treating *ALK*-positive NSCLC cells (Sang et al. 2013). Other notable HSP90 inhibitors that has been used in *ALK*-positive NSCLC are NVP-AUY922 (Ueno et al. 2012) and AT13387 (Wallis et al. n.d.). Due to the strong in vitro and in vivo efficacy shown by AT13387 in *ALK*-positive diseases (Wallis et al. n.d.), it is being now evaluated in the clinic.

MET-Amplified Non-small Cell Lung Cancer

To analyze the mechanisms of acquired drug resistance to EGFR TKIs, Sequist et al. performed systematic genetic and histological analyses of tumor biopsies and reported that 5% of cancers that showed EGFR TKI resistance, also showed *MET* amplification as the mechanism of resistance (Sequist et al. 2011). In addition, 3–8% of NSCLCs are characterized to carry *MET* mutations and/or amplification (Cancer Genome Atlas Research 2012, 2014). Given the significant efficacy of a MET inhibitor, crizotinib that has been shown in patients with *MET*-amplified or *MET*-mutant tumors (Camidge et al. 2014; Frampton et al. 2015), MET mutations and possible *MET* amplification are targetable oncogenic drivers in NSCLC. HSP90 inhibition has been evaluated in the NSCLC with acquired resistance to EGFR TKI due to multiple mechanism including *MET*-amplification. NVP-AUY922 treatment was shown to effectively suppress the cell proliferation followed by cell death in *EGFR*-mutant NSCLC cell line HCC827 that is resistant to gefitinib and erlotinib

mediated via MET activation (Choi et al. 2015). The NVP-AUY922 induced down-regulation of EGFR and MET was followed by decreased AKT activation (Choi et al. 2015). NVP-AUY922 was also found efficacious in vivo, where it was able to control the tumor growth in xenograft mouse models containing the resistant HCC827 cells (Choi et al. 2015). These results established NVP-AUY922 as a promising therapeutic agent for MET-mediated resistance to EGFR-TKI in NSCLCs. The benzamide, SNX-2112 has also been used to inhibit HSP90 in an *EGFR*-mutant NSCLC, where it was able to block the EGFR cross-talk and MET receptor activation via MET degradation (Rice et al. 2009). SNX-2112, as monotherapy or in combination with erlotinib, was able to inhibit EGF activation of phospho-AKT, phospho-STAT3, phospho-ERK1/2 and pS6 (Rice et al. 2009). In NCI-H1975 xenograft models, SNX-5422 showed potent cytotoxic activity both as a single agent as well as in combination with erlotinib and extended the animal survival significantly, supporting the advanced investigation of SNX-2112 as well as it's derivative, SNX-5422 as a treatment for *EGFR*-mutant NSCLC with acquired resistance to EGFR-TKIs, especially in cases where *MET*-amplification serves as the resistance mechanism. HSP90 inhibitors such as 17-DMAG can also overcome HGF-mediated resistance to EGFR-TKIs which could lead to increase response rate in treatment naïve *EGFR*-mutant NSCLC. Although erlotinib could not induce growth inhibition in *EGFR*-mutant NSCLC cell lines in the presence of HGF, 17-DMAG was able to induce apoptosis and significant growth inhibition of these cell lines, even in the presence of HGF (Koizumi et al. 2012). Moreover, the 17-DMAG induced growth inhibition was associated with decreased EGFR and MET expression. 17-DMAG also reduced tumor growth by inhibiting angiogenesis and inducing apoptosis in an in vivo model of HGF-triggered erlotinib-resistance harboring Ma-1/HGF cells (Koizumi et al. 2012). Jang et al. (2014) evaluated the preclinical anticancer efficacy of WK88-1 in a gefitinib-resistant *EGFR*-mutant NSCLC cell line with *MET* amplification as well as its parental *EGFR*-mutant HCC827 cell line. WK88-1 was able to decrease cell viability in both HCC827 and HCC827GR cells, which was demonstrated to be associated with significant reduction in expression of HSP90 client oncoproteins such as EGFR, ERB2, ERBB3, MET and AKT (Jang et al. 2014). Moreover, overall, this WK88-1 preclinical works demonstrated HSP90 inhibition caused by WK88-1 could affect the activation of EGFR- or c-MET-mediated survival of *MET*-amplified NSCLCs and overcome gefitinib resistance in HCC827GR cells.

HER2/BRAF/ROS1/RET Mutations and Translocations in Non-small Cell Lung Cancer

HSP90 inhibition has shown potent antitumor activity in *HER2*-mutant NSCLC as well as in novel targetable oncogenic drivers such as *ROS1* and *RET* translocations. HER2, also denoted as ERBB2, is a member of ERBB family. It has been shown that *HER2/ERBB2* overexpression could regulate the heat shock factor 1 (HSF1) activity, the crucial transcription factor for the subsequent stabilization of several

oncogenic HSP90 clients such as AKT, MIF, and HSF1 itself, which in turn promotes tumor growth in *HER2*-positive breast cancer (Schulz et al. 2014). Trastuzumab emtansine (T-DM1, Herceptin, Genentech), an anti-HER2 humanoid antibody coupled with vinca-alkaloid, has been used preclinically and clinically in patients with *HER2*-positive NSCLCs and despite being effective in suppressing *HER2*-positive small-cell lung cancer in preclinical models (Morimura et al. n.d.), it failed to produce promising results due to low response rates (Harada et al. n.d.; Ivanova et al. n.d.). Based on the fact that there are currently no effective targeted therapies for *HER2* mutant lung cancer (Ivanova et al. n.d.), and HER2 being a critical oncogenic client of HSP90, inhibition of HSP90 chaperonage may be a reasonable approach. In addition to efficacy of GM against HER2 overexpressing breast cancer cell lines (Xu et al. 2007), it has been demonstrated that *HER2* exon 20 insertion mutants are dependent on HSP90 for stability and signaling functions, and showed strong sensitivity to 17-AAG (Xu et al. 2007) advocating the use of HSP90 inhibitors in NSCLC bearing exon 20 insertions in *HER2*. NVP- AUY922 has also been reported to be efficacious in *HER2*-mutant NSCLCs (Nogova et al. 2014). The potent antitumor activity of ganetespib, the non-GM derivative, has been demonstrated in vitro as well as in vivo models of *HER2*-mutant NSCLCs (Shimamura et al. 2012).

About 1.7% of NSCLCs are characterized by aberrant *ROS1* gene fusions/rearrangements (Davies et al. 2012). It has been reported that multiple proliferative pathways are triggered by oncogenic ROS1 kinase activity (Rikova et al. 2007), which defines a specific category of molecularly defined NSCLC (Takeuchi et al. 2012). On the other hand, chromosomal rearrangements in *RET* gene has been identified in an estimated 0.7–1% of NSCLCs (Ju et al. 2012; Kohno et al. 2012; Lipson et al. 2012; Takeuchi et al. 2012). To date, only ganetespib that has shown potent cytotoxic activity in NSCLC carrying oncogenic rearrangements of *ROS1* (Proia et al. 2012a; Sang et al. 2013) or *RET* kinase genes (Sang et al. 2013). About 2% of adenocarcinomas are characterized by the presence of activating *BRAF* mutations, especially V600E (da Rocha Dias et al. 2005; Kris et al. 2014; Millington 2013). As BRAF is an HSP90 client that is a bona fide oncoprotein, several preclinical studies have been perform, but AT13387 (Smyth et al. 2014) and XL888 (Paraiso et al. 2012) are the two HSP90 inhibitors that delivered promising preclinical results suggesting that HSP90 inhibition could be an effective alternate strategy against *BRAF*-mutant NSCLCs.

DNA Repair and/or Cell-Cycle Checkpoint Mechanisms

As many HSP90 clients are involved in controlling the genomic instability in many cancer types via aberrant cell-cycle regulation, causing persistent DNA damage and promoting other epigenetic changes, HSP90 inhibition is an effective approach to sensitize the malignant cells to the compounds that are effective in causing cell-cycle arrest and irreversible DNA damage. Many investigators have examined the pre-clinical efficacy of several HSP90 inhibitors either as monotherapy, or in

combination of taxanes and found that they could enhance cell-cycle arrest. One such inhibitor is 17-AAG, which alone or in combination with paclitaxel, was found to increase the sensitivity of NSCLC cells characterized by overexpression of EGFR or HER2 to paclitaxel, both in vitro and in vivo (Nguyen et al. 2001; Sawai et al. 2008). In vitro as well as in vivo NSCLC xenograft model studies have also demonstrated the synergistic potency of IPI-504 in combination with docetaxel (O'Connell et al. 2014). Unfortunately, our recent data suggests that KRAS-mutant NSCLC cells that were resistance to ganetespib, also demonstrated cross-resistance to the taxane, docetaxel (Chatterjee et al. 2017a) which will likely limit the clinical efficacy of this combination. 17-AAG was also shown to specifically down-regulate the expression of critical cell-cycle regulators such as CHK1 and CDC25A leading to G_2/M arrest (Arlander et al. 2003). Dysregulation of CHK1 expression by 17-AAG also led to sensitization of NSCLCs to gemcitabine, which is known nucleoside analogue and antimetabolite that causes cell-cycle progression (Arlander et al. 2003). Combination therapy of 17-AAG and irradiation in lung cancer cell lines also led to reduction in CDC25C and CDC2 expression causing G_2/M arrest (Senju et al. 2006). 17-DMAG could also sensitize NSCLC cells radiotherapy via disruption of their DNA repair machinery (Koll et al. 2008). Similar efficacy was also observed with ganetespib. It was able to disrupt the DNA repair mechanisms triggered by irradiation leading to senescence (Gomez-Casal et al. 2015) in addition to showing additive or synergistic effect in combination with etoposide or other related agents used for advanced cancer treatments (Bansal et al. 2010).

KRAS-Mutant Non-small Cell Lung Cancer

Oncogenic *KRAS* mutations are present in an estimated 25% of the NSCLC patients with no current effective therapies (Chatterjee et al. 2016; Park et al. 2016). Many attempts over the years to develop small molecules and target the activating mutant KRAS failed miserably due to its structural characteristics literally earning it the status "undruggable" (Cox and Der 2002; Garassino et al. 2011; Young et al. 2009). KRAS is not an HSP90 target, but many critical signaling oncoproteins that are downstream targets of KRAS require HSP90 chaperonage for their stability and functions. Thus, HSP90 inhibition was explored as a possible therapy for *KRAS*-mutant NSCLC.

The preclinical studies began with 17-AAG, which produced potent cytotoxic effects in KRAS-mutant NSCLCs via disruption of expression of several important KRAS downstream HSP90 clients such as CRAF and AKT (Sos et al. 2009). In addition, similar efficacy was also reported for 17-DMAG in vivo (Sos et al. 2009). IPI-504 has been shown to suppress HSP90 activity and promote endoplasmic reticulum (ER) stress in *KRAS*-mutant NSCLC (De Raedt et al. 2011). Moreover, in combination with mammalian target of rapamycin (mTOR) inhibitor – rapamycin, IPI-504 augmented excessive oxidative stress by activating ROS and simultaneously inhibiting the G6PD/glutathione antioxidant pathway and promoted tumor regression in a xenograft model of *KRAS/p53* mutant NSCLC (De Raedt et al.

2011). NVP-AUY922 was also able to induce strong anti-tumor activity in *KRAS*-mutant NSCLC, either as a single agent (Garon et al. 2013; Ueno et al. 2012), or synergistically in combination with other agents such as PI3K inhibitor, GSK458 (Park et al. 2016). Similarly, ganetespib was able to induce potent cytotoxicity in *KRAS*-mutant NSCLC cell lines via destabilization of several KRAS downstream signaling effectors (Acquaviva et al. 2012; Chatterjee et al. 2017b). Combinations ganetespib at low-dose with other small molecule inhibitors such as that of MEK or PI3K/mTOR generated superior cytotoxic activity compared to single agent therapies in a subset of *KRAS*-mutant NSCLC cells (Acquaviva et al. 2012). Moreover, the ganetespib antitumor activity was further augmented when administered with the PI3K/mTOR inhibitor BEZ235 in vivo *KRAS*-mutant NSCLC model (Acquaviva et al. 2012). Although these results support the potential of ganetespib as monotherapy or combination treatment in *KRAS*-mutant NSCLC, only transient and unconfirmed responses were observed in patients with *KRAS*-mutant tumors due to rapid emergence of acquired resistance to ganetespib (Socinski et al. 2013). Therefore, significant clinical efforts focused on testing ganetespib with docetaxel in both *KRAS*- wild type and mutant NSCLC. Unfortunately, a phase III clinical trial in the second line setting (Galaxy-2), which tested the combination of ganetespib and docetaxel failed to meet its primary endpoint (Ramalingam et al. 2014). We performed series of preclinical analyses to determine the mechanism(s) of the ganetespib resistance in *KRAS*-mutant NSCLC (Chatterjee et al. 2017a, b). We established that the acquired ganetespib resistance is mediated via hyperactivation of oncogenic ERK1/2-p90RSK-mTOR signaling pathways leading to the subsequent bypassing of G_2/M checkpoint arrest through CDC25C. In addition, we also found that ganetespib resistance led to concomitant cross resistance to the anti-microtubule agent, docetaxel providing an explanation for the Galaxy-2 trial failure (Chatterjee et al. 2017a). The studies strongly support that the combination of ganetespib with an inhibitor of ERK1/2, p90RSK or CDC25C could be an efficacious therapeutic strategy to overcome the ganetespib resistance (Chatterjee et al. 2017a). Although the development of ganetespib has been stopped, these prior studies provide preclinical evidence for rationale combinations with other second generation HSP90 inhibitors that are currently being evaluated (e.g., AT13387 and TAS-116) and may lead to overcome or prevent acquired resistance to HSP90 inhibitor (Chatterjee et al. 2017a).

19.1.5 HSP90 Inhibitor in the Clinic

In the last decade, the successful preclinical evaluation of many HSP90 inhibitors in oncogene driven lung cancers led several of them to enter clinical trials. Although, the outcome of these trails is mixed at best, there is still hope that these agents may be effective in NSCLC. In this section, we will be focusing on the clinical development of relevant HSP90 inhibitors. Although, 17-AAG and 17-DMAG were among the first set of GM-derivatives to enter clinical trials, they were not involving lung

cancer patients. Despite some early promising results, due to poor response rates and presence of adverse side effects including hepato- and ocular toxicities among patients, the trials were terminated (Jhaveri et al. 2012a; Saif et al. 2013). Following an initial small phase I trial involving patients with gastrointestinal stromal tumors or soft-tissue sarcoma (Wagner et al. 2013), IPI-504 was the first HSP90 inhibitor to be evaluated in multiple clinical trials involving patients with NSCLC.

19.1.5.1 IPI-504

IPI-504 was tested in a phase II trial involving 76 patients with advanced, molecularly defined NSCLC carrying *EGFR*-mutations and *ALK*-rearrangements who have already received EGFR-TKI pretreatment (Sequist et al. 2010). The overall study revealed an objective response rate (ORR) of 7% (5 of 76) in all subjects, 10% (4 of 40) in patients with wild-type *EGFR*, and 4% (1 of 28) in patients with *EGFR*-mutant NSCLC. Although the observed results were below the target ORR of 20%, there were three patients with an *ALK* gene rearrangement, two patients showed partial responses and a third patient showed prolonged stable disease (7.2 months) with 24% reduction in tumor size. The overall median progression free survival (PFS) was observed to be 2.86 months. This study revealed that IPI-504 possesses potent clinical activity NSCLC patients, especially the ones with *ALK*-rearrangements (Sequist et al. 2010). The combinatorial efficacy of IPI-504 and the standard chemotherapeutic agent docetaxel was tested in a phase Ib clinical trial involving lung cancer patients (Riely et al. 2009) and in a subsequent expansion with patients with pretreated metastatic NSCLC. Compared to the overall response rate (26%) for all patients, higher response rates were detected in patients with squamous histology (43%), former smoking habit (33%) and in those with wild-type KRAS (36%). These rates were significantly higher than the 8% historical response rate with docetaxel. Following this, a phase II study was conducted involving patients with NSCLC (NCT01362400), which was completed in 2014, but the results were not published. Due to treatment related patient mortalities caused by side effects such as hepatotoxicity and lack of efficacy, further development of IPI-504 was ceased (Modi et al. 2013).

19.1.5.2 NVP-AUY922

Before being evaluated in patients with lung cancer, NVP-AUY922 was clinically tested in a phase I study involving patients with solid organ malignancies primarily including breast, ovaries and colon cancers with a primary objective of determining the maximum tolerated dose (MTD) of AUY922 in addition to the characterization of the safety and pharmacokinetic/pharmacodynamic profiling. Based on the acceptable tolerability of NVP-AUY922, multiple phase II single-agent and combination studies have been initiated in patients with NSCLC (Sessa et al. 2013). In one such phase II study (NCT01124864), patients were grouped based on the oncogenic

driver mutations in *EGFR*, *KRAS*, and *ALK* rearrangement and wild-type NSCLCs (Garon et al. 2012). The objective response rate detected to be highest for patients with ALK rearrangement. Compared to other HSP90 inhibitors, a higher response rate was observed in patients with *EGFR*-mutant NSCLCs (18%) than in those with wild-type *EGFR* (13%) with no response in patients with *KRAS*-mutant NSCLC. Overall findings revealed an acceptable safety profile of NVP-AUY922 and showed preliminary activity in heavily pretreated NSCLC patients. This study was followed by several clinical trials involving patients with *EGFR*-mutant NSCLC. Efficacy of the combination of NVP-AUY922 with erlotinib in order to overcome acquired erlotinib resistance was evaluated in a phase I/II trial involving 37 patients with *EGFR*-mutant NSCLC (Johnson et al. 2015). A response rate of 16% was observed at the maximum tolerated dose. In addition to observed side effects of diarrhea, skin rash, hyperglycemia and night blindness, no complete responses were observed and the phase II segment didn't meet its expected endpoints. The clinical efficacy of AUY922 is also being evaluated in patients with *EGFR* exon 20 insertions that are TKI-resistant (NCT01124864). Based on the preliminary results including the partial responses and prolonged stable disease, it is possible that Hsp90 inhibition could be an effective strategy for this patient subset (Piotrowska et al. 2015). Unfortunately, further development of this compound was stopped by Novartis in 2014.

19.1.5.3 Ganetespib

Ganetespib is the only HSP90 inhibitor that has been tested in a phase III trial in NSCLC. Ganetespib was first clinically evaluated in a phase I trial involving 53 patients with pretreated metastatic solid organ malignancies. Ten out of the 53 patients had NSCLC, 6 of which possessed stable disease for a minimum of 8 weeks. The ganetespib side effects observed in this trial included diarrhea, fatigue, and nausea/vomiting (Goldman et al. 2013). Next, Socinski et al. evaluated the activity and tolerability of ganetespib in previously treated 99 NSCLC patients in this phase II study (Socinski et al. 2013). Patients were grouped into three cohorts based on the mutational background – mutant *EGFR*, mutant *KRAS* and wild-type *EGFR*/*KRAS*. At 16 weeks, after ganetespib treatment, progression-free survival (PFS) rates for mutant *EGFR*, mutant *KRAS* and wild-type *EGFR*/*KRAS* were found to be 13.3%, 5.9% and 19.7% respectively. Interestingly, significant single agent activity was noted in four of the wild-type *EGFR*/*KRAS* patients who were positive for the *ALK* gene rearrangement. Although the most common side effects observed were diarrhea, fatigue, nausea and anorexia, serious adverse effects were evident in eight patients, two of which finally died of cardiac arrest and renal failure (Socinski et al. 2013). Although, ganetespib monotherapy demonstrated a manageable side effects as well as clinical efficacy in heavily pretreated NSCLC patients, particularly inducing improved survival in those with *ALK* gene rearrangement, it failed to produce any significant activity in patients with *EGFR*-mutant or *KRAS*-mutant NSCLC. This led to two further ganetespib clinical trials involving patients with *ALK*-driven

NSCLCs. The first one was evaluating the efficacy of ganetespib alone in a phase II study in subjects with advanced *ALK*-positive NSCLC (NCT01562015), which was completed in May, 2015 with no results published yet. The other one is specifically investigating the combinatorial activity of ganetespib and crizotinib (NCT01579994) undertaken by Memorial Sloan Kettering Cancer Center (New York). This study is ongoing, but not recruiting new participants.

The most important ganetespib clinical trials in NSCLC were the phase IIB/III GALAXY and GALAXY2 trials, the purpose of which were to determine whether combining ganetespib with docetaxel was more effective than docetaxel alone in subjects with advanced stage IIIb or IV NSCLC. Three hundred and eighty-five enrolled patients were pretreated with docetaxel alone at the dose of 75 ng/m^2 on day 1 or in combination with ganetespib at 150 mg/m^2 on day 1 and 15 of a 3-week cycle (NCT01348126). As early results demonstrated elevated hemoptysis and lack of efficacy in non-adenocarcinoma patients, only patients with adenocarcinoma histology were subsequently enrolled the trial moved forward. The combination therapy generated positive results, but only in the adenocarcinoma group with respect to PFS and overall survival (OS). The PFS was improved in adenocarcinoma patients (4.5 months in combination arm vs. 3.2 months in docetaxel arm). Combination therapy also increased the OS to 7.6 months from 6.4 months with docetaxel only treatment in adenocarcinoma group (Ramalingam et al. 2015). Based on these results, this particular subpopulation was further investigated in a large scale, randomized phase III study named GALAXY-2 (NCT01798485). The patients participating in this study had pretreated advanced (stage IIIB/IV/metastatic) NSCLC and were diagnosed with more than 6 months prior to the joining the study. Patients were randomly assigned into two groups – docetaxel and combination of docetaxel and ganetespib in 1:1 ratio. Unfortunately, based on preliminary results, the interim report concluded that the combination of docetaxel and ganetespib was not likely to provide with a statistically significant improvement in OS compared to docetaxel alone (Ramalingam et al. 2014) and was finally terminated by Synta Pharmaceutical in 2016 (2015).

19.1.5.4 AT13387

Promising preclinical evaluation of AT13387 led to its involvement in a phase I study to determine the maximum tolerated dose, which was found to be 260 mg/m^2 per week for 3 weeks of a 4-week cycle. Notable side effects included gastrointestinal effects, fatigue and infusion-related symptoms (Mahadevan et al. 2012). Currently, two more phase I/II studies have been undertaken. The purpose of one of them is to evaluate safety and efficacy of AT13387 monotherapy and in combination with crizotinib in NSCLC patients (NCT01712217). The other one is also a phase I/II trial (NCT02535338), which studies the side effects and best dose of AT13387 in combination with erlotinib hydrochloride in treating patients with recurrent or metastatic *EGFR*-mutant NSCLC. The rational for this study was that erlotinib hydrochloride and AT13387 could inhibit the growth of tumors by hindering the enzymes or other proteins required for the cancer cell growth.

19.1.6 Emerging Inhibitors

In this section, we will briefly mention the currently active trials that are active, and either recruiting or the recruitment has not yet been started. A notable first-in-human phase I/IIa study (NCT03221400) is currently recruiting that evaluates PEN-866 (Terveda Therapeutics), a novel HSP90 small molecule inhibitor in patients with advanced solid malignancies whose disease has progressed after treatment with previous anticancer therapies. It is an open-label, multicenter study to evaluate the preliminary anti-tumor efficacy and the safety and tolerability, pharmacokinetics as well as pharmacodynamics of PEN-866 in patients with advanced solid malignancies. A first-in-human phase I trial of TAS-116 in patients with advanced solid tumors, first initiated in Japan in April 2014, has been ongoing (NCT02965885). This phase I study is also planned to enroll with advanced solid tumors in UK to confirm the MTD, safety, tolerability, and pharmacokinetics of TAS-116 in a Western patient population in the dose expansion phase. In addition to other types of cancers, patients with NSCLCs harboring *EGFR* mutations (T790M+) or *EGFR* mutations (T790M-) will be further evaluated for efficacy, safety and tolerability at recommended doses of TAS-116 in 3 separate cohorts. In addition to direct Hsp90 inhibition, a small-molecule inhibitor of histone deacetylase (HDAC) – vorinostat (suberoylanilide hydroxamic acid [SAHA]), which has been shown to inhibit acetylation and disruption of HSP90 leading to reduction in activity of pro-growth and prosurvival client proteins (Bali et al. 2005), is currently being tested in the clinic in combination with gefitinib. Vorinostat induces cell differentiation, cell cycle arrest, and apoptosis in several tumor cells. There is a strong synergistic antiproliferative effect of vorinostat in combination with gefitinib in NSCLC cells. These preclinical results lead to the evaluation of the combination of vorinostat and gefitinib in relapsed/ or refractory subjects with advanced NSCLC in a phase I/II study (NCT01027676).

19.2 Conclusions

As mentioned earlier, the first-generation GM and its derivatives were mostly abandoned clinically due to significant toxicities and other related problems, lead to the development of the second generation HSP90 inhibitors. Compared to their predecessors, these second generation small molecules showed better bioavailability, stronger cytotoxicity, and specific inhibition of HSP90 chaperonage and improved toxicity profiles. Although prematurely terminated, ganetespib did advanced to a phase III clinical evaluation state. Even after a decade of successful preclinical run by these compounds, unfortunately no HSP90 inhibitor has been FDA-approved. In this chapter, we attempted to shed light into several major HSP90 inhibitors and their preclinical and clinical development a novel and promising cancer therapeutic approach. The unfilled promise (Chatterjee et al. 2016) has raised many doubts for

the HSP90 inhibition therapeutics. One prominent question is how tumors are developing the de novo or acquired resistance to these compounds? One possible explanation is that the inhibition of HSP90 could lead to overexpression of another chaperone such as HSP70 as a compensatory mechanism for survival. Our preclinical studies on HSP90 inhibitor ganetespib (Chatterjee et al. 2017a, b) and many similar studies involving other HSP90 inhibitors (Piper and Millson 2011) strongly advocates the ration that the use of HSP90 inhibitors in combination with targeted agents in patients subtyped on the basis of distinct molecular background may help overcome or prevent these acquired resistance. Another major question is centered on overcoming or preventing the serious side effects, including organ-specific toxicities (liver or ocular) caused by these inhibitors. Understanding the mode of action of HSP90 and building on the growing preclinical knowledge will advance the development of novel synthetic HSP90 small molecule inhibitors that will inhibit HSP90 with more specificity and potency. In addition to the development of small molecule HSP90 inhibitors, much focus has been shed on targeting the HSP90/co-chaperone interactions. As we have mentioned earlier that the co-chaperone binding and activity is extremely important for proper HSP90 chaperone function, blockage of the co-chaperones such as CDC37, AHA1 or p23 leading to the multi-complex interaction can achieve the same consequences produced by HSP90 inhibition (McDowell et al. 2009). There are HSP90 inhibitors that can also target the client-HSP90 interactions which would offer the ultimate selectivity. Series of compounds have been reported that are potent inhibitors of HSP90 interactions with many negative regulators of apoptosis including survivin, XIAP, cIAPI and cIAP2 (Sidera and Patsavoudi 2014). Proper and specific delivery of HSP90 inhibitor could utilize the use of nanomedicine (Sauvage et al. 2017), which could incorporate the HSP90 inhibitor into an appropriate delivery system, the nanocarriers. Nanocarrier drug delivery system comes with manifold advantages, but is currently beyond the scope of discussion. A growing sub-field in HSP90 targeting is the utilization of HSP90 and antigen cross-presentation to develop HSP90 antigens for cancer vaccine (Murshid et al. 2011). The immunogenicity of HSP90 is being explored to stimulate the innate as well as the adaptive immunity promoting them as a candidate tumor antigen for not only lung cancer but overall cancer vaccine development. Although, the combination therapies in the field of lung cancer are impeded by elevated treatment-related toxicities and sometimes acquired resistance to the agents, the task moving forward will be to find synthetic and novel agents that are either active in monotherapy, or efficient in combination with other targeted therapeutics. This can only be achieved by a more in-depth understanding of the intricate biology that drives these tumors.

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Chapter 20
HSP90 Inhibitors Blocking Multiple Oncogenic Signaling Pathways for the Treatment of Cancer

Fen Jiang, Xiao-Li Xu, and Qi-Dong You

Abstract Heat shock protein 90 (HSP90) is an ATP-dependent molecular chaperone which plays important roles in the development of cancer. Inhibition of the HSP90 chaperone function can disrupt multiple cancer dependent signaling pathways and result in potent anti-cancer effects, which has been a promising anti-cancer strategy. Up to now, HSP90 inhibitors with different mechanisms have been developed, including HSP90 N-terminal inhibitors (pan-isoform and isoform selective), C-terminal inhibitors and HSP90-cochaperone protein-protein interaction (PPI) inhibitors. In this chapter, we will review the current development of HSP90 inhibitors as anti-cancer agents.

Keywords Cancer · GRP94 · HSP90-CDC37 PPI · HSP90 inhibitor · Isoform selective · TRAP1

Abbreviations

BTZ	bortezomib
CR	complete response
CTD	C-terminal domain
DHPM	3,4-dihydropyrimidin-2-(1H)-one
DLTs	dose-limiting toxicities
EGCG	epigallocatechin gallate
ER	endoplasmic reticulum
ESCC	esophageal squamous cell carcinoma
FU	5-fluorouracil
GRP94	glucose-regulated protein 94

F. Jiang · X.-L. Xu · Q.-D. You (✉)
State Key Laboratory of Natural Medicines and Jiang Su Key Laboratory of Drug Design and Optimization and Department of Medicinal Chemistry, School of Pharmacy, China Pharmaceutical University, Nanjing, China
e-mail: youqd@cpu.edu.cn

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HGF	hepatocyte growth factor
HSP	Heat shock protein
HTRF	homogeneous time-resolved fluorescence
IGFs	insulin-like growth factors
IRI	irinotecan
l-OHP	oxaliplatin
MD	middle-domain
MM	multiple myeloma
MTD	maximum tolerance dose
NSCLC	non-small cell lung cancer
NTD	N-terminal domain
PPI	protein-protein interaction
PR	partial response
RDA	radamide
SD	stable disease
SDH	succinate dehydrogenase
SPR	surface plasmon resonance
TEAEs	treatment-related adverse events
TRAIL	TNF-α–related apoptosis-inducing ligand
TRAMP	transgenic adenocarcinoma of the mouse prostate
TRAP1	tumor necrosis factor receptor-associated protein-1

20.1 Introduction

Heat shock proteins (HSP) are molecular chaperones that are curial for the maturation, stability and function of other proteins named “client proteins”. Hsp include large Hsp, HSP90, HSP70, HSP60, HSP40 and small HSP. Among which, HSP90 is the most well studied one in the last decades (Garcia-Carbonero et al. 2013). As the most abundant intracellular protein in mammalian cells, HSP90 comprises approximately 1–2% of total cell protein under normal conditions. While under stressed conditions (heat, hypoxia and nutrient deprivation), HSP90 is upregulated to maintain the cellular homeostasis (Barrott and Haystead 2013). There are four HSP90 isoforms in higher eukaryotes, cytosolic HSP90α and HSP90β, endoplasmic reticulum (ER) paralog glucose-regulated protein 94 (GRP94) and mitochondria paralog tumor necrosis factor receptor-associated protein-1 (TRAP1). Despite the high homologies, the four isoforms possess different cellular functions and have different client profiles (Gewirth 2016). In mammalian cells, HSP90 exists as a homodimer, and each monomer is consisted of three domains: N-terminal domain (NTD), middle-domain (MD), and C-terminal domain (CTD). The NTD incorporates an ATP binding site, which hydrolyzes ATP to provide energy for the chaperone function. The MD provides binding sites for clients and cochaperones. The CTD is important for the dimerization. Recent studies suggest that another ATP

binding pocket is located at CTD (Miyata et al. 2013). The chaperone function of HSP90 relies on an ATP triggered chaperone cycle. ATP binding leads to transient dimerization of the NTD, then several cochaperones (CDC37, HSP70, P23, HOP, etc.) form a chaperone complex with HSP90 to regulate the folding and release of the client proteins (Calderwood 2013). HSP90 plays important roles in the development of cancer, and HSP90 inhibition is proved to be a promising anti-cancer strategy. To data, HSP90 inhibitors with different mechanisms have been developed, including HSP90 N-terminal inhibitors, C-terminal inhibitors and inhibitors blocking the PPI between HSP90 and cochaperones. These inhibitors block multiple cancer dependent signal pathways and exhibit potent pre-clinical anti-cancer effects both in vitro and in vivo. Moreover, several HSP90 N-terminal inhibitors have entered clinical trails. In this chapter, we will review the development progress of HSP90 inhibitors as anti-cancer agents.

20.2 HSP90 and Cancer

HSP90 is reported to be over-expressed in tumors two to threefold higher than corresponding non-tumorigenic tissue (Barrott and Haystead 2013). Cancer cells are addictive to the HSP90 chaperone function to prevent the mutated and overexpressed oncoproteins from degradation (Trepel et al. 2010). Among the over 500 client proteins of HSP90 (https://www.picard.ch/downloads/Hsp90interactors.pdf.), many are notorious oncogenic proteins, including tyrosine-kinase receptors (EGFR, HER2, SRC, MEK, etc.), signal-transduction proteins (BCR-ABL, ALK, BRAF, AKT, etc.), transcription factors (AR, OR, HIF1α, P53, etc.), cyclin-dependent proteins (CDK4, CDK6, Cyclin D, etc.), antiapoptotic proteins (BCL2, Survivin), matrix metalloproteinases (MMP-2, MMP-9) and telomerase (hTERT) (Garcia-Carbonero et al. 2013; Verma et al. 2016). These oncogenic client proteins are involved in multiple cancer dependent signaling pathways.

GRP94 is the ER paralog of HSP90. Clinically, high expression of GRP94 is associated with the advanced stage and poor prognosis of some types of carcinomas, including hepatocellular carcinoma, multiple myeloma, gallbladder cancer, et al. (Chen et al. 2015; Chhabra et al. 2015; Rachidi et al. 2015). GRP94 has a limited client protein profile, and some client proteins correlate with the development and metastasis of cancer. Integrins are a major class of GRP94 client proteins. They mediate cell–cell, cell–extracellular matrix communication and are important for cell adhesion and metastasis (Desgrosellier and Cheresh 2010). GRP94 based α-helix peptide blocking the interaction between GRP94 and integrins inhibited cell invasion (Hong et al. 2013). In addition, GRP94 knockdown led to reduce migration and metastasis potential of MDA-MB-231 cells and ROS resistant MCF-7 cells (Dejeans et al. 2012). The maturation and secretion of Insulin-like growth factors (IGFs) are also dependent on GRP94 (Barton et al. 2012; Marzec et al. 2016; Ostrovsky et al. 2009; Wanderling et al. 2007). Both IGF1 and IGF2 play important

roles in cancer, and IGF pathway inhibition has been considered as a novel anti-cancer strategy (Brahmkhatri et al. 2015; King and Wong 2016). Bei Liu and coworkers discovered that GRP94 bound and promoted the exportation of Wnt coreceptor LRP6 from ER to cell surface (Liu et al. 2013). Their further study demonstrated that GRP94 deficiency blocked Wnt-LRP-Survivin pathway in human multiple myeloma cells, and induced cell apoptosis and tumor growth inhibition in xenograft models (Hua et al. 2013). Recent studies showed that surface GRP94 interacted with HER2 and facilitated HER2 dimerization. Purine-scaffold GRP94 specific inhibitor **PU-WS13**, GRP94 based α-helix peptide or anti-GRP94 monoclonal antibody treatment led to decreased HER2 level in SKBr3 cells and exhibited selective therapeutic effects in HER2 positive breast cancers (Li et al. 2015a, b; Patel et al. 2013, 2015).

TRAP1, the mitochondrial paralogue of HSP90, is also reported to be elevated in various human carcinomas, including colorectal carcinoma, prostate cancer, ovarian carcinoma, breast cancer, non-small cell lung cancer (NSCLC) and esophageal squamous cell carcinoma (ESCC). The high TRAP1 expression is associated with the progress and prognostic of these carcinomas (Agorreta et al. 2014; Costantino et al. 2009; Landriscina et al. 2010; Leav et al. 2010; Maddalena et al. 2013; Tian et al. 2014). TRAP1 is involved in the proliferation and apoptosis of cancer cells. In ESCC cells, knockdown of TRAP1 inhibited the proliferation, induced cell cycle arrest and apoptosis. While re-expression of TRAP1 promoted cell proliferation and reduced cell apoptosis (Tian et al. 2014). TRAP1 also promoted neoplastic growth by binding and inhibiting succinate dehydrogenase (SDH). TRAP1 dependent SDH inhibition led to respiratory down-regulation and succinate accumulation. Succinate then stabilized HIF-1α and prompted neoplastic growth (Sciacovelli et al. 2013). Studies in Matteo Landriscina group suggested that TRAP1 was involved in drug resistance of cancer cells. TRAP1 up-regulation was observed in HT-29 colorectal carcinoma cells resistant to 5-fluorouracil (FU), oxaliplatin (l-OHP) and irinotecan (IRI), and was responsible for the multi-drug resistance (Costantino et al. 2009). As the mitochondrial paralogue of HSP90, TRAP1 was also demonstrated locating in ER and involving in ER stress protection and protein quality control of tumor cells. The ER-associated TRAP1 protected MCF7 and MBA-MB-231 cells from paclitaxel-induced apoptosis and was relevant in paclitaxel resistance of these breast carcinoma cells (Maddalena et al. 2013). ER-associated TRAP1 was also responsible for the anthracyclins resistance of breast carcinoma cells (Sisinni et al. 2014). In general, HSP90 isoforms are involved in multiple cancer dependent signaling pathways. HSP90 inhibition is a promising anti-cancer strategy.

20.3 HSP90 N-Terminal Inhibitors

Inhibitors competitively occupy the NTD ATP binding pocket can block the HSP90 chaperone function. These inhibitors are named HSP90 N-terminal inhibitors.

20.3.1 Pan-HSP90 N-Terminal Inhibitors

Up to now, more than 20 distinct N-terminal HSP90 inhibitors have entered clinical trails. These are pan-HSP90 inhibitors that exhibit similar affinities to the four isoforms. As shown in Fig. 20.1, these inhibitors can be divided into three classes. Geldanamycin analogues are the first-generation inhibitors, including **17-AAG**, **17-DMAG**, **IPI-493** and **IPI-504**. To solve the unfavorable drug-like properties of the geldanamycin analogues, the second-generation synthetic small molecule inhibitors are developed, including purine derivatives, resorcinol derivatives and benzamide derivatives. The pre-clinical properties and clinical performance of these inhibitors have been thoroughly reviewed. Despite the favorable pre-clinical anti-tumor activity, all these inhibitors exhibit poor clinical efficacy in mono-therapy. Some inhibitors only show moderate anti-cancer effects in specific cancer types when combined with other anti-cancer agents. In 2015, the highly anticipated phase III study of **STA-9090** combined with docetaxel for the treatment of advanced stage lung adenocarcinoma was failed. The **STA-9090** and docetaxel combination regimen did not result in improved efficacy compared with docetaxel mono-therapy. In clinical trials, these inhibitors exhibited many treatment-related adverse events (TEAEs). The

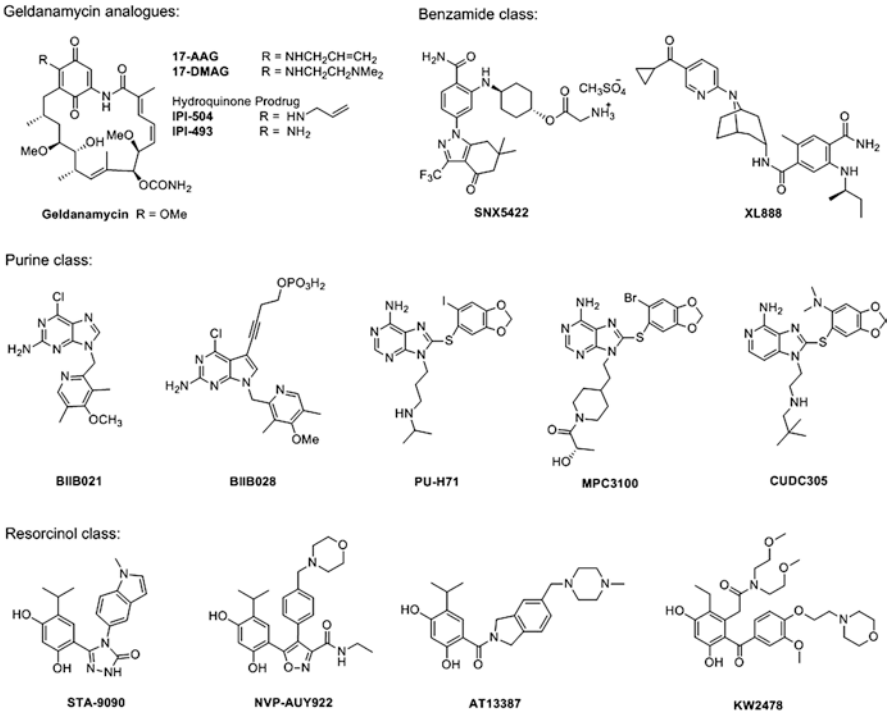


Fig. 20.1 Structures of representative pan-HSP90 N-terminal inhibitors

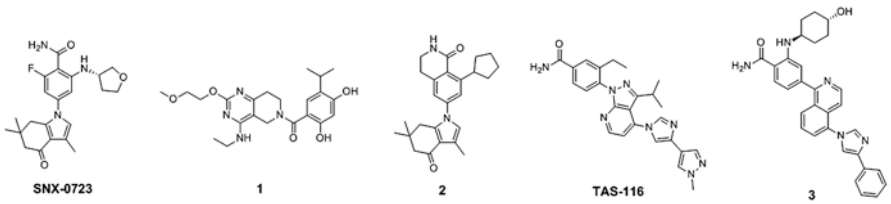


Fig. 20.2 Structures of HSP90α/β selective inhibitors

most common side effects were fatigue, visual disorders, gastrointestinal disorders (nausea, vomiting and diarrhoea), anaemia and neutropenia (Bhat et al. 2014b; Biamonte et al. 2010; Garcia-Carbonero et al. 2013). Because of the disappointing clinical performance, the clinical studies of most inhibitors are terminated. Now, only the resorcinol derivative **AT-13387** and the benzamide derivative **XL888** are active in clinical trials (Fig. 20.2).

The disappointing clinical performance of these pan-HSP90 N-terminal inhibitors can be partly attributed to the following reasons. (1) The clinical dosages of these inhibitors are limited because of the various adverse events, which lead to the drug exposure levels in patients are insufficient to effectively inhibit tumor cell HSP90. HSP90 four isoforms have different functions and chaperone different client proteins. Pan-HSP90 N-terminal inhibitors inhibit the four isoforms simultaneously, which may lead to mechanism related adverse events. Thus, isoform selective HSP90 N-terminal inhibitors may reduce the adverse events and improve the clinical performance. (2) The pan-HSP90 inhibitors trigger significant heat shock response. The increased cytoprotective HSP (HSP70 and HSP27) partly neutralize the anti-cancer effects. Studies have shown that GRP94 selective inhibitors, TRAP1 selective inhibitors and HSP90 C-terminal inhibitors don't induce heat shock response. These inhibitors may have more promising usage as anti-cancer agents.

20.3.2 Isoform Selective HSP90 N-Terminal Inhibitors

HSP90 isoform selective inhibitors only influence partial isoform specific client proteins. Theoretically, isoform selective inhibitors will have reduced adverse effects compared with the pan-inhibitors. In addition, GRP94 selective inhibitors and TRAP1 selective inhibitors don't induce heat shock response. Though isoform selective inhibition is attractive, it used to be considered very difficult. HSP90 four isoforms are highly homologous. HSP90α possess 93%, 59%, and 47% sequence similarity with HSP90β, GRP94, and TRAP1, respectively. Even worse, the residues in ATP-binding pocket are more heavily conserved (Ernst et al. 2014a). However, isoform selective inhibitors have been developed recently. The isoform selective inhibition can be attributed to the conformation flexibility of the HSP90 ATP-binding pocket.

20.3.2.1 HSP90α/β Selective Inhibitors

Some HSP90 inhibitors exhibit moderate selectivity for the cytosolic HSP90α/β, such as the benzamide-based **SNX2112** and purine-based **BIIB021**. While some other inhibitors exhibit no selectivity, such as the resorcinol-based **NVP-AUY922**. As the paralogue of **SNX2112**, **SNX0723** exhibits similar HSP90α/β selectivity with **SNX2112**. It was used as the probe molecule to analyze the structural basis for the HSP90α/β selective inhibition. In **SNX0723** bound HSP90α (Fig. 20.3b), the hydrophobic tetrahydroindolone moiety targeted into the big hydrophobic pocket (Site 1) formed by Leu107, Ile110, Ala111, Phe138, Tyr139 and Trp162. To accommodate this binding mode, the flexible loop sequence 104–111 was induced to form an extended α-helix conformation with the H3 and H4 helix. The residues Leu107, Ile110 and Ala111 covered on the tetrahydroindolone moiety and formed hydrophobic interactions with it. In **SNX0723** bound GRP94 (Fig. 20.3c), the equivalent sequence of HSP90α 104–111 in GRP94 (160–167) were disordered. The hydrophobic interactions between tetrahydroindolone moiety and the equivalent residues of Leu107, Ile110, Ala111 in GRP94 were missing, which led to the lower affinity to GRP94 compared with HSP90α/β. The different ligand-bound conformations of HSP90α/β and GRP94 can be attributed to the minimal primary sequence difference. Compared with HSP90α/β, five amino acids (QEDGQ) are inserted in GRP94 at position 126 (HSP90α numbering). For TRAP-1, two amino acid (AE) are inserted at position 129. The amino acid insertions are unfavorable for the ligand induced extended α-helix conformation. The pan-HSP90 inhibitors (such as **NVP-AUY922**, Fig. 20.3a) can't induce the extended α-helix conformation, so they exhibit no discrimination for the four isoforms (Ernst et al. 2014a; Gewirth 2016). Compound **1** developed in our lab could conduct both flexible loop conformation and extended α-helix conformation to bind with HSP90α, so **1** possessed moderate HSP90α selective inhibition against GRP94 (Jiang et al. 2016b). These analyses

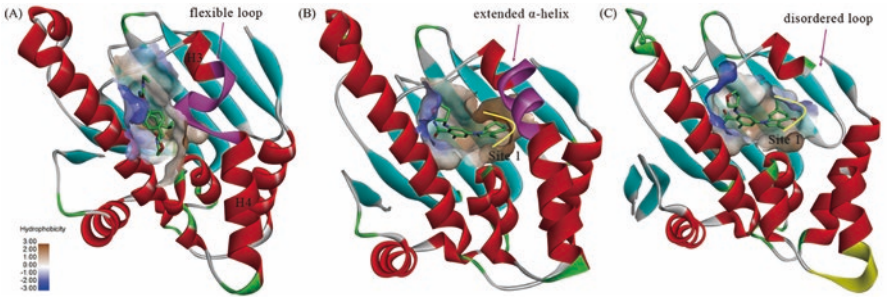


Fig. 20.3 Structural insights of the HSP90α/β selective inhibition. (**a**) NVP-AUY922 bound HSP90α (PDB ID: 2VCI), the flexible loop sequence 104–111 is colored in magenta. (**b**) SNX0723 bound HSP90α (PDB ID: 4NH8), the sequence 104–111 on the extended α-helix is colored in magenta. (**c**) SNX0723 bound GRP94 (PDB ID: 4NH9), the inserted five amino acids (QEDGQ) are colored in yellow. Carbon atoms of the compounds are shown in green. The active sites of the proteins are surfaced according to the hydrophobic state

provide structural insights for the design of inhibitors with improved HSP90α/β selectivity.

Inspired by the above analyses, researchers in Vertex Pharmaceuticals conducted extensive SAR study focused on **SNX0723** to discover more potent and selective HSP90α/β inhibitors. Structure optimization led to cyclopentyl analogue **2**, which exhibited improved HSP90α/β selective inhibition activity, with Ki value of 5 nM for HSP90α/β and >10 μM for GRP94 and TRAP1. In human HD patient derived fibroblasts, **2** induced significant up-regulation of HSP70 and degradation of the HSP90α/β clients htt, CDK4 and AKT (Ernst et al. 2014b). The goal of this research was to develop HSP90α/β selective inhibitors for the treatment of CNS disorders, so the anti-cancer effects of **2** were not evaluated. Considering the significant oncogenic clients degradation effects and the HSP90α/β addiction of tumor cells, **2** should have potent anti-cancer effects.

TAS-116 is another HSP90α/β selective inhibitor which has already entered clinical trials. This compound was developed by Taiho Pharmaceutical Co. LTD. through fragment based drug design. **TAS-116** possessed excellent HSP90α/β selective inhibition activity with Ki values of 34.7 nM, 21.3 nM, >50 μM, and >50 μM for HSP90α, HSP90β, GRP94, and TRAP1, respectively (Ohkubo et al. 2015). The accurate binding mode of **TAS-116** was not reported, while the crystal structure of **TAS-116** homolog **3** complexed with HSP90α was released. Considering the similar structure of the two compounds, **TAS-116** and compound **3** should take same binding modes. As shown in Fig. 20.4, compound **3** also induced an extended α-helix conformation. In addition to the Site 1 pocket, **3** also targeted into a specific hydrophobic pocket (Site 4) which extended toward the opposite side of the ATP pocket. The excellent HSP90α/β selective inhibition could be partially attributed to the occupation of this pocket (Ohkubo et al. 2012).

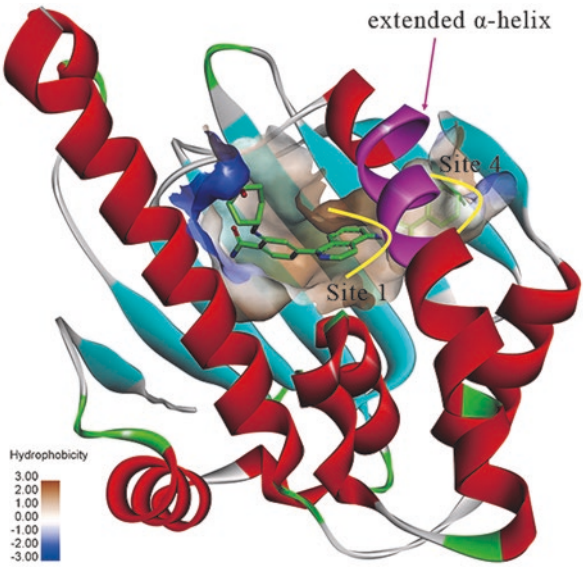


Fig. 20.4 Binding mode analysis of compound 3 complexed with HSP90α (PDB ID: 3WQ9). The sequence 104–111 on the extended α-helix is colored in magenta. Carbon atoms of the compounds are shown in green. The active sites of the proteins are surfaced according to the hydrophobic state

The specific HSP90α/β inhibition of **TAS-116** was confirmed at cellular level. In HCT116 cells, **TAS-116** treatment induced the degradation of DDR1 (client of HSP90α) and the up-regulation of HSP70, while exhibited no influence on the LRP6/GRP94 complex formation and LRP6 expression level (LRP6 is the client of GRP94). In pre-clinical study, oral administration of **TAS-116** exhibited potent anti-tumor activity in various xenograft models. In HER2-expressing NCI-N87 human gastric cancer xenografts, **TAS-116** inhibited tumor growth significantly in a dose dependent manner. The T/C value was 47%, 21%, and 9% for dose of 3.6 mg/kg, 7.1 mg/kg, and 14.0 mg/kg, respectively. **TAS-116** treatment downregulated the HSP90 clients HER2, HER3, and AKT in tumor tissues, indicting the potent anti-tumor activity was HSP90 inhibition dependent. As results, the PI3K/AKT and MAPK/ERK signaling pathways were inhibited. In FLT3-ITD-expressing MV-4-11 human acute myeloid leukemia xenografts, 14.0 mg/kg/day **TAS-116** administration led to tumor shrinkage, accompanied with FLT3-ITD degradation in tumors. In an orthotopic lung tumor model expressing EGFR (L858R/T790M), **TAS-116** administration also inhibited tumor growth significantly (Ohkubo et al. 2015).

Multiple myeloma (MM) was reported to be sensitive to HSP90 inhibitors in both pre-clinical models and clinical trails (Kaiser et al. 2010; Patterson et al. 2008; Richardson et al. 2010a, b, 2011; Stuhmer et al. 2008). As a potential therapeutic agent, the pre-clinical anti-MM activity of **TAS-116** alone or in combination with other agents was well studied. **TAS-116** exhibited potent cytotoxicity in a series MM cell lines and patient derived MM cells, including RAS-mutated MM cells (Suzuki et al. 2015a, b). Moreover, **TAS-116** could overcome **17-AAG** resistance mechanisms and was more effective than 17-AAG in N-RAS mutated MM cell lines, associated with lower HSP27 induction and more significant degradation of the clients p-C-RAF and p-MEK1/2. In addition, **TAS-116** augmented the bortezomib (**BTZ**) cytotoxicity against MM cells. The synergistic activity was due to inhibition of **BTZ**-triggered canonical NF-κB activation and enhanced unfolded protein response (UPR). In murine xenografts, both **TAS-116** alone and combination with BTZ exhibited potent anti-MM activities. The combination treatment inhibited tumor growth more potently than **TAS-116** and **BTZ** monotherapy. The OS of combination treated animals was significantly prolonged. (Suzuki et al. 2015a). Considering that **TAS-116** induced significant degradation of key RAS-RAF-MEK-ERK pathway regulators (p-C-RAF, p-MEK1/2, and p-ERK), the synergistic anti-MM activities of **TAS-116** in combination with RAS-RAF-MEK-ERK signaling pathway inhibitors were studied. In RAS mutated MM cell lines, **TAS-116** significantly enhanced the cytotoxicity and apoptosis induction effects of tipifarnib (farnesyltransferase inhibitor), dabrafenib (RAF inhibitor), and **AZD6244** (MEK inhibitor). In B-RAF-mutated U266 MM cell line, synergistic cytotoxicity of **TAS-116** combination with dabrafenib was also observed (Suzuki et al. 2015b). All these results indicting **TAS-116** is a potent anti-MM agent, either alone used or combined with other agents.

As a favorable property, **TAS-116** exhibited no ocular toxicity in pre-clinical study. In human retinal pigment epithelial ARPE-19 cells that were essential for the support of photoreceptors, **TAS-116** was less toxic than **17-AAG** (Suzuki et al.

2015a). In NCI-H1975 rat xenografts, **TAS-116** exhibited low retina exposure and high tumor/retina drug exposure ratio. In addition, administration of **TAS-116** in SD rats didn't induce cell apoptosis and histologic change in the outer nuclear layer of the retina (Ohkubo et al. 2015).

Inspired by the excellent pre-clinical anti-tumor effects and safety profile, **TAS-116** entered clinical trials in 2014 for the phase I dose escalation study enrolled patients with advanced solid tumors. Preliminary clinical data reported in 2015 revealed that two patients (GIST and NSCLC) had partial response (PR) and five patients had stable disease (SD) more than 12 weeks in the qd dose regimen (n = 16) (Shimomura et al. 2015). During the whole study, patients (n = 52) received **TAS-116** according to three dose regimens, were 4.8–150.5, 107.5–295.0 and 160 mg/m^2 day in qd, qod and qd×5, respectively. The maximum tolerance dose (MTD) for qd and qod regimen was 107.5 and 210.7 mg/m^2 day, respectively. Anorexia, AST/ALT/gamma-GTP elevation, night blindness and visual disorders, platelet decrease (grade 3), septic shock, respiratory failure, pneumonia (grade 4) and febrile neutropenia (grade 3) were the observed dose-limiting toxicities (DLTs). For efficacy assessment, in qd, qd×5 and qod groups, PR was achieved by 12.5, 0 and 5.6%; SD was achieved by 43.8, 61.5 and 38.9%; disease control rate (>/=12 weeks) was 31.1%, 46.2% and 22.2%, respectively (Yanagitani et al. 2017). This phase I study revealed the preliminary clinical anti-cancer activity of **TAS-116**. In the further phase II study in patients with GIST (n = 40), **TAS-116** was orally administrated on a 5-days-on/2-days-off schedule. TEAEs were observed in all patients, and 52.5% were grade 3 or higher. Gastrointestinal disorders were the most common TEAEs and could be resolved with dose interruption. Other TEAEs including diarrhea, anorexia, nausea, ALT/AST increase, anemia, fatigue, vomiting, rash and bladder infection. Of the 40 patients enrolled, the median progression-free survival was 4.4 months and no complete response (CR) and PR was achieved. Thirty-four (85%) patients had SD lasting >/= 6 weeks (Kurokawa et al. 2017). Another phase I study is ongoing in US and UK to evaluate the MTD, safety, tolerability and efficacy of **TAS-116** in patients with advanced solid tumors (Takanami 2005). Based on these reported clinical data, though **TAS-116** was well-tolerated, it still manifested many TEAEs. In addition, no significant anti-tumor activity was observed during the treatment. The clinical safety and efficacy of **TAS-116** should be further studied.

20.3.2.2 GRP94 Selective Inhibitors

In consideration of the correlation between GRP94 and cancer, many GRP94 selective inhibitors have been developed and exhibited promising pre-clinical anti-cancer effects. As shown in Fig. 20.5, many GRP94 selective inhibitors have been developed. Same as the pan-HSP90 N-terminal inhibitors, GRP94 selective inhibitors can also be divided into three types. As a typical adenosine receptors agonist, purine scaffold **NECA** was the first discovered GRP94 selective inhibitor. It bound to purified GRP94 with a dissociation constant of 200 nM while exhibited no binding to purified HSP90 (Rosser and Nicchitta 2000). Simple structure modification focused

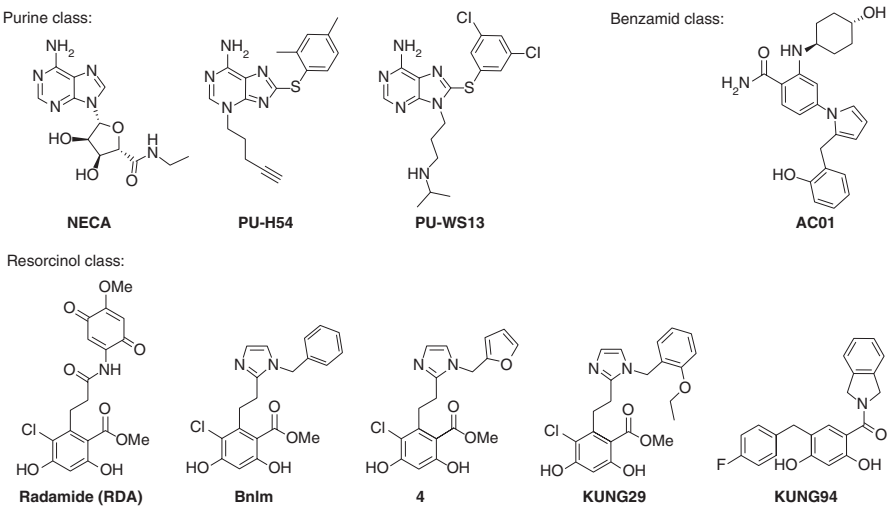


Fig. 20.5 Structures of GRP94 selective inhibitors

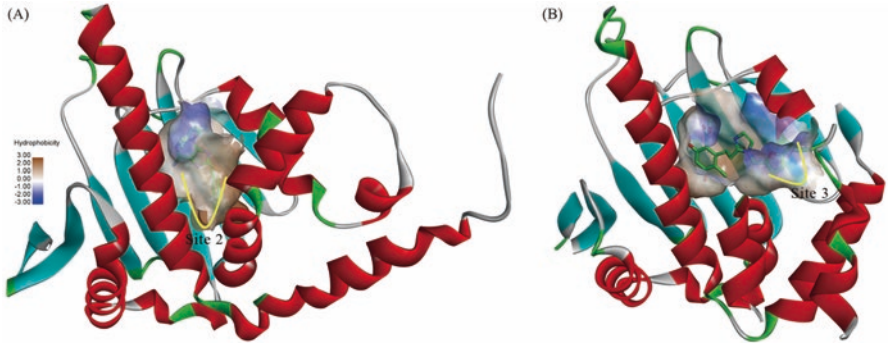


Fig. 20.6 Structural insights of the GRP94 selective inhibition. (**a**) GRP94-PU-H54 complex (PDB ID: 3O2F). (**b**) GRP94-4 complex (PDB ID: 5IN9). Carbon atoms of the compounds are shown in green. The active sites of the proteins are surfaced according to the hydrophobic state

on N-ethyl moiety of **NECA** didn't lead to derivatives with higher binding affinity (Immormino et al. 2009). Considering the off-target adenosine receptor pathway activation effect of the purine scaffold and the limited structure modification feasibility, **NECA** is not a proper lead compound for further development. The cellular GRP94 inhibition effect of **NECA** has not been reported. In 2013, combined compound library screening with structural and computational analyses led to the discovery of another purine scaffold GRP94 selective inhibitor **PU-H54**. As shown in Fig. 20.6a, crystal complex showed that **PU-H54** induced rearrangements of the GRP94 lid region, and occupied the newly exposed hydrophobic Site 2 pocket. Further structure modification led to **PU-WS13**, which exhibited more potent and

selective GRP94 inhibition activity. **PU-WS13** inhibited IGF-II secretion and TLR9 trafficking in cells in a dose dependent manner, and exhibited no effects on HSP70 induction and AKT degradation, indicting GRP94 specific inhibition in cells. The discovery of **PU-WS13** provided a new tool to investigate the cellular functions of GRP94. **PU-WS13** exposure led to membrane but not cytosolic HER2 reduction in SKBr3 cells, while no same effect was observed in HER2 low-expressing MCF-7 cells. The researchers further demonstrated that GRP94 was important for maintaining the architecture of high-density HER2 formations at the plasma membrane in high HER2-expressing cells. **PU-WS13** treatment induced sub-G1 cell cycle arrest, apoptosis and impaired cell viability in various HER2-overexpressing breast cancer cell lines (Patel et al. 2013). In addition, tumor specific retention effect was also observed for **PU-WS13**, same as the pan-HSP90 inhibitors (Patel et al. 2015). All these results indicated that GRP94 was a promising therapeutic target for HER2-overexpressing breast cancer. The therapeutic effects of **PU-WS13** in multiple myeloma and hepatocellular carcinoma were also observed. In various multiple myeloma cell lines, **PU-WS13** induced both apoptosis and necrosis, and inhibited cell growth. This was believed to be caused by the Wnt-LRP-Survivin pathway blockade effect of **PU-WS13** (Hua et al. 2013). In human HepG2 and PLC hepatocellular carcinoma cell lines, **PU-WS13** treatment exhibited significant growth inhibition effect. In HepG2 cells, **PU-WS13** lowered the levels of total β-catenin and its downstream target cyclin D1, indicating Wnt signaling pathway blockade. **PU-WS13** treated cells also expressed lower levels of hepatocyte growth factor (HGF) and its downstream targets, including p-ERK1/2, p-AKT, and p-STAT3. Further gene expression profile analysis revealed that nine genes involved in cell cycle modulation were down-regulated (Rachidi et al. 2015). All these studies provided proof-of-concept for the anti-cancer effects of GRP94 selective inhibitor **PU-WS13** at cellular level. Further pharmacodynamic evaluation in animal models should be conducted to study the anti-cancer effects of **PU-WS13**.

Another major class of GRP94 selective inhibitors containing resorcinol pharmacophore were developed in Blagg group. Radamide (**RDA**) is a radicicol-geldanamycin chimera that hybrids the resorcinol moiety from radicicol and the quinone moiety form geldanamycin. Further crystal study in Daniel T. Gewirth group showed that **RDA** adopted different conformations to bind with HSP90α and GRP94. In the **RDA**-GRP94 complex, the amide linkage exhibited two orientations. In the cis-amide orientation, the quinone moiety inserted into the GRP94 specific Site 3 and formed van der Waals interactions. In the trans-amide orientation, the quinone moiety was targeted into the solvent area. In the **RDA**-HSP90α complex, the amide linkage only exhibited a trans-amide orientation and the quinone moiety formed tight hydrogen-bonding network with the surrounded residues. These results indicting GRP94 selective inhibitors could be designed based on the cis-amide orientation **RDA** (Duerfeldt et al. 2012; Immormino et al. 2009). In the following study of Blagg group, imidazole was chosen as the bioisoster of cis-amide. Hydrophobic phenyl ring was introduced to form π-interactions with Phe199, Tyr200, and Trp223 in the GRP94 specific Site 3 pocket. These efforts led to **BnIm**, which exhibited 12-fold selectivity for GRP94. **BnIm** treatment inhibited Toll

receptor trafficking and IGF-II secretion in cells. **BnIm** exhibited no cytotoxicity in MCF7, SKBr3, and HEK293 cells even at 100 μM (Duerfeldt et al. 2012). This phenotype was different with the purine-type GRP94 selective inhibitor **PU-WS13**, which exhibited selective anti-proliferative activity against the HER2-overexpressing breast cancer cells. In Gabriela Chiosis group's study, **BnIm** showed little effect on the HER2 expression in SKBr3 cells. The researchers explained that these different phenotypes between **BnIm** and **PU-WS13** might contribute to the different binding modes to GRP94 (Patel et al. 2015). The discovery of **BnIm** provided further insights for the design of GRP94 selective inhibitor, but the low activity and selectivity limited its usage as a probe to study the GRP94 cellular function. Further modification of **BnIm** led to the more potent and selective compound **4** and **KUNG29**. As shown in Fig. 20.6b, crystal structure analysis showed that **4** bound into the Site 3 pocket of GRP94, confirmed the binding mode of this series. **KUNG29** exhibited an IC_{50} value of 0.2 μM for Grp94 and a 41-fold selectivity over HSP90α. In MDA-MB-231 cells, **KUNG29** exhibited potent antimigratory activity without cytotoxicity. Further mechanism study showed that **KUNG29** induced integrin α2 degradation, indicting the antimigratory activity was GRP94 dependent. **KUNG94** was another resorcinol-based GRP94 selective inhibitor. Inspired by the binding mode of **PU-WS13**, hydrophobic moieties were introduced at the 5-position of resorcinol to occupy the GRP94-specific hydrophobic site 2 pocket. SAR study led to **KUNG94** incorporating a 4-fluorobenzyl moiety manifested ~10-fold selectivity for GRP94. The IC_{50} value of **KUNG94** for GRP94 and HSP90α was 8 nM and 77 nM, respectively. In RPMI8226 multiple myeloma cell line, **KUNG94** manifested a GI_{50} value of 1.4 μM. The level of GRP94 specific client LRP6, a Frizzled coreceptor, was reduced after treatment. In addition, the antiapoptotic protein Survivin was reduced, indicting **KUNG94** activated apoptosis through decreasing the Wnt signaling. Considering that **KUNG94** possesses a low selectivity profile and still high inhibition activity to HSP90α, it is difficult to distinguish the cellular effects of GRP94 inhibition and HSP90α inhibition. The selectivity profile of **KUNG94** should be further improved (Khandelwal et al. 2017).

Recently, Blagg group developed an benzamide-containing GRP94 selective inhibitor called **ACO-1** through structure-based design. In this study, aminocyclohexanol-based clinical HSP90 N-terminal inhibitor **SNX2112** was chosen as the rational design template. In order to access the GRP94 specific Site 2 pocket and form additional π interactions with Phe-195, tetrahydroindazolone fragment in **SNX2112** was replaced with a pyrrole, and further SAR study led to **ACO-1**. **ACO-1** manifested an IC_{50} value of 0.44 μM for Grp94 while had no inhibition for HSP90α at 100 μM, displaying over 200-fold selectivity. In PC3-MM2 cells, **ACO-1** treatment led to reduced levels of GRP94 specific clients, including integrin α2, integrin αL, Syne2, VAMP2, and Rab10. These clients are important for the migration process of cancer cells. As significant characteristics during cell migration process, filamentous actin (f-actin) localization and integrin trafficking were inhibited in **ACO-1** treated cells. In further wound healing scratch assay, **ACO-1** potently inhibited the migration of the highly metastatic MDA-MB-231 and

PC3-MM2 cells. These results confirmed the anti-migratory activity of GRP94 selective inhibitors (Mishra et al. 2017).

20.3.2.3 TRAP1 Selective Inhibitors

As TRAP1 involved in multiple processes of cancer development, TRAP1 inhibition has been a promising anti-cancer strategy. Considering that TRAP1 mainly located at mitochondria, mitochondria specific delivery of pan-HSP90 inhibitor is a feasible strategy to inhibit TRAP1. In 2009, Byoung Heon Kang and coworkers developed the first mitochondria-targeted HSP90 inhibitor. Combination of the pan-HSP90 inhibitor **17-AAG** with mitochondrial targeting moieties led to Gamitrinibs (**G-G1**−**G-G4**, **G-TPP**). Gamitrinibs significantly accumulated in the isolated tumor mitochondria and induced immediate loss of inner mitochondrial membrane potential and release of cytochrome c. While the unconjugated **17-AAG** didn't exhibit these properties. Gamitrinibs exhibited potent anti-cancer effects. In lung adenocarcinoma H460 cells, Gamitrinibs treatment significantly induced cell death, mitochondrial apoptosis and decreased colony formation. Unlike **17-AAG**, Gamitrinibs didn't induce degradation of HSP90α clients AKT and Chk1. The HSP70 expression level was also not affected, indicating TRAP1 selective inhibition didn't trigger heat shock response. Gamitrinibs exhibited broad spectrum anti-cancer activity against various cancer cell lines, while exhibited low effects in normal cells, indicting they were selective anti-cancer agents. The anti-cancer effects and safety of Gamitrinibs were further demonstrated in vivo. Gamitrinib (**G-G4**) administration potently inhibited the growth of human leukemia, breast and lung xenograft tumors without significant body weight loss. In the tumors harvested from Gamitrinib-treated animals, extensive apoptosis and release of cytochrome c in the cytosol was observed. In addition, organs of the Gamitrinib-treated animals exhibited similar histological properties compared with the vehicle group, confirmed the safety of Gamitrinib (Kang et al. 2009). Inspired by these preliminary in-vivo anti-cancer effects and safety profile, the researchers then conducted pre-clinical study of gamitrinibs in advanced prostate cancer. In vitro, Gamitrinibs potently inhibited cell viability in prostate cancer cells. In PC3 xenograft tumors, 10 mg/kg i.p. administration of Gamitrinib (**G-TPP**) completely inhibited the tumor growth, while 10 mg/kg **17-AAG** administration exhibited no effect. Meanwhile, no significant weight loss and histological change of organs were observed in the Gamitrinib-treated group. In bone metastatic prostate cancer models, administration of Gamitrinib significantly reduced bone loss compared with the vehicle group (Kang et al. 2010). The anti-cancer effects of Gamitrinib against advanced prostate cancer were further demonstrated in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) models. Long-term treatment TRAMP mice with Gamitrinib (**G-G4**) suppressed the formation of localised prostate tumors of neuroendocrine or adenocarcinoma origin, while had no effect on prostatic intraepithelial neoplasia or prostatic inflammation. In addition, the metastasis of prostate cancer to liver and abdominal lymph nodes was also inhibited. No significant animal weight loss or organ toxicity was observed

during treatment (Kang et al. 2011). These results indicated that Gamitrinibs were effective and safe agents for the treatment of advanced prostate cancer. Synergistic anti-cancer activities of Gamitrinib with other agents were observed in various models. In glioblastoma cells, noncytotoxic concentrations of **G-TPP** sensitized tumor cells to TNF-α–related apoptosis-inducing ligand (**TRAIL**). In nude mice carrying intracranial U87-Luc glioblastomas, combination of **TRAIL** and **G-TPP** exhibited potent in-vivo antiglioma activity. Mechanically, **G-TPP** treatment triggered UPR effect. The synergy between **TRAIL** and **G-TPP** was attributed to the UPR-related suppression of NF-κB–dependent gene expression (Siegelin et al. 2011). **G-TPP** also exhibited synergistic anti-cancer effects with doxorubicin. In vitro, combination of **G-TPP** with doxorubicin exhibited enhanced cytotoxicity and apoptotic inducing effect. This synergistic effect was associated with increased expression of pro-apoptotic CHOP and Bim, and the enhanced mitochondrial accumulation of Bim and Bax. In prostate and breast xenograft models, the combination treatment exhibited more potent tumor growth inhibition compared with single treatment with **G-TPP** or doxorubicin. In addition, no aggravating cardiotoxic side effect of doxorubicin was observed during treatment (Park et al. 2014a). **G-TPP** could activate the calcium-mediated UPR in the ER lumen and impair the stress adaptation of cancer cells. Combination treatment with **G-TPP** and ER stressor thapsigargin augmented interorganelle stress signaling, and exhibited synergistic anti-cancer effects. In relapsed prostate cancer xenograft models, **G-TPP** and thapsigargin combination potently inhibited tumor growth (Park et al. 2014b). In general, Gamitrinibs manifested potent in-vitro and in-vivo anti-cancer effects either single used or combined with other agents. They are potential anti-cancer agents and provide proof-of-concept for mitochondrial HSP90 inhibition as novel anti-cancer strategy (Fig. 20.7).

In 2015, Byoung Heon Kang group demonstrated that TRAP1 was the major HSP90 paralogue located in cancer cell mitochondria, with an approximately ten-

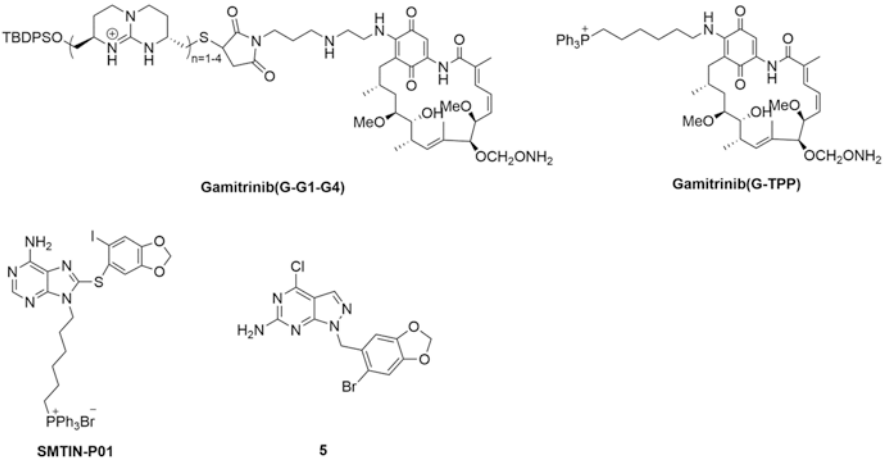


Fig. 20.7 Structures of TRAP1 selective inhibitors

fold higher level than HSP90α/β. In addition, TRAP1 possessed an approximately 32-fold higher ATPase activity. Specific inactivation of TRAP1 alone by siRNA was enough to induce ER UPR in cancer cells. These results indicated that TRAP1 was the predominant target of Gamitrinibs and TRAP1 inhibition was responsible for the potent anti-cancer effects of Gamitrinibs. To design novel TRAP1 selective inhibitor, the researchers resolved the crystal structure of TRAP1 complexed with the pan-HSP90 inhibitor **PU-H71**. Under the guidance of the crystal structure, another mitochondrial TRAP1 inhibitor named **SMTIN-P01** was developed. In **SMTIN-P01**, the solvent-expose isopropyl amine moiety of the **PU-H71** was replaced with the mitochondria-targeting moiety TPP. **SMTIN-P01** exhibited dramatic mitochondria accumulation effect compared with the parent molecule **PU-H71**. In Hela cells, **SMTIN-P01** treatment induced significant mitochondrial membrane depolarization. In addition, the expression levels of HSP90α clients (AKT and Chk1) and HSP70 were not affected, indicating no inhibition on the cytosolic HSP90. In various cancer cell lines, **SMTIN-P01** exhibited more potent cytotoxicity than the parent molecule **PU-H71**. These results confirmed that TRAP1 selective inhibition is an effective anti-cancer strategy (Lee et al. 2015a).

Gamitrinibs and **SMTIN-P01** inhibit TRAP1 via mitochondrial delivery of pan-HSP90 inhibitors, and provide favorable probes to study the function of TRAP1. Alternatively, TRAP1 selective inhibitors with higher affinity to TRAP1 than the cytosolic HSP90α/β may have better applications. Considering the high sequence homologies and the almost superimposable nucleotide-binding sites of the HSP90 four paralogues, development of TRAP1 selective inhibitors is difficult. Recently, the TRAP1 crystal structures complexed with different ligands were resolved, which provided structural guidance for the rational design of TRAP1 selective inhibitors. As shown in Fig. 20.8a, b, some differences were observed in the ligands bound HSP90α and TRAP1. The lid structure (residues 171–201) in TRAP1 was disordered, and

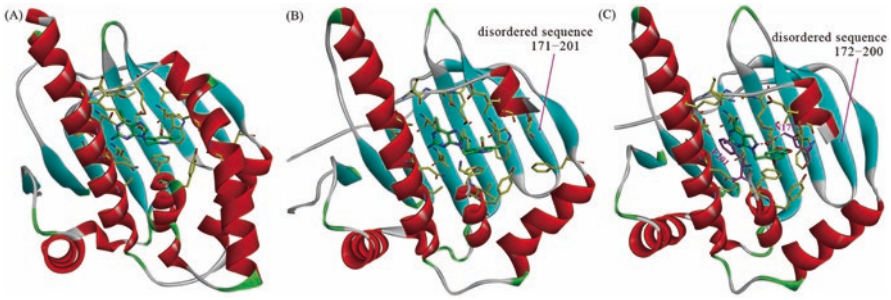


Fig. 20.8 Structural insights of the TRAP1 selective inhibition. (**a**) HSP90α-**BIIB021** complex (PDB ID: 3QDD). (**b**) TRAP1-**BIIB021** complex (PDB ID: 4Z1G), the NTD is intercepted from the $TRAP1_{NM}$. (**c**) TRAP1-5 complex (PDB ID: 5Y3N), the NTD is intercepted from the $TRAP1_{NM}$. The conserved water molecule is shown as red spheres, hydrogen bonds are indicated by magenta dashed lines. Carbon atoms of the compounds are colored in green. N171 and F201 in (**c**) are shown in magenta sticks, other residues are shown in yellow sticks. The active sites of the proteins are surfaced according to the hydrophobic state

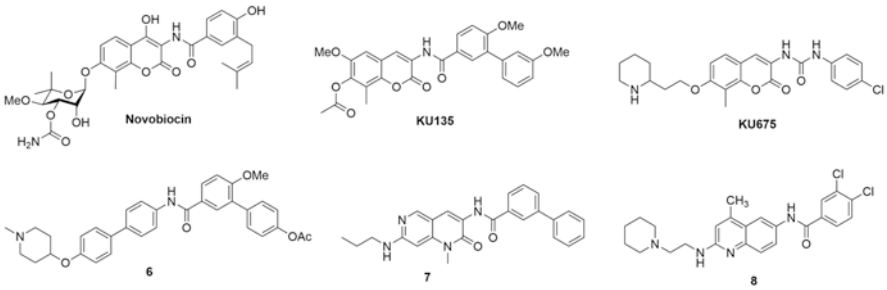


Fig. 20.9 Structures of novobiocin and representative derivatives

residues Asn171 and Gly202 in the two proteins adopted different configurations. These differences indicate compounds that can stabilize the flexible lid structure may manifest TRAP1 selectivity (Lee et al. 2015a; Park et al. 2017). Based on these analyses, Byoung Heon Kang group conducted structure modification focused on **BIIB021** to generate TRAP1 selective inhibitors. The purine scaffold which closed to the disordered lid was changed into pyrazolopyrimidine, then bromopiperonyl was introduced on N-1 position. These efforts led to compound **5**, which exhibited modest TRAP1 selective inhibition (IC_{50} = 79 nM) compared with HSP90α (IC_{50} = 698 nM). Crystal structure analysis confirmed the structural basis for the selectivity. As shown in Figs. 20.8c, compared with the **BIIB021** bound TRAP1, the pyrazolopyrimidine core in **5** formed π-π stacking interaction with the residue Phe201, water-mediated hydrogen bonds were formed between N-2 and Asn171 amide. These additional interactions made great contribution to the TRAP1 selectivity of **5**. In Hela cells, **5** treatment induced loss of mitochondrial membrane potential, overproduction of mitochondrial ROS, elevation of CHOP expression and discharge of cytochrome c, which were all signatures of TRAP1 inhibition. **5** possessed potent cytotoxicity against various cancer cells lines. In PC3 xenograft models, 30 mg/kg daily treatment inhibited tumor growth significantly without toxicity. Both in cancer cells and tumor tissues, **5** treatment had no influence on the HSP70 expression level, indicating no heat shock response was induced (Park et al. 2017). TRAP1 selective inhibitors may overcome the heat shock response effect of pan-HSP90 inhibitors (Fig. 20.9).

20.4 HSP90 C-Terminal Inhibitors

Another ATP-binding pocket is located at the HSP90 CTD. Some inhibitors are reported to occupy this C-terminal ATP-binding pocket and exhibit promising anticancer effects.

20.4.1 Novobiocin and Derivatives

As the first discovered HSP90 C-terminal inhibitor, novobiocin provides a proof-of-concept for HSP90 C-terminal inhibition. In 2000, Neckers and coworkers discovered that novobiocin exhibited HSP90 inhibition activity in a different manner compared with the HSP90 N-terminal inhibitors geldanamycin and radicicol (Marcu et al. 2000b). Further studies demonstrated that novobiocin bound to a second ATP-binding pocket which located at the HSP90 CTD (Marcu et al. 2000a; Soti et al. 2002). In SKBr3 cells, novobiocin treatment reduced $p185^{erbB2}$, mutated P53, and RAF-1 protein levels in a dose-dependent manner (Marcu et al. 2000b). To improve the anti-proliferative effects and simplify the structure of novobiocin, Blagg group conducted extensive SAR study and discovered many derivatives with potent anti-cancer effects. As representative, **KU-135** exhibited dramatically improved anti-proliferative activity against Jurkat T Lymphocytes and melanoma cells compared with novobiocin. **KU135** treatment induced significant mitochondria-mediated apoptosis and G2/M cell cycle arrest in these cells. Moreover, HSP90 client degradations were observed without obvious HSP70 induction (Samadi et al. 2011; Shelton et al. 2009). **KU675** is another novobiocin derivative with potent anti-cancer effects. In both androgen-dependent and -independent prostate cancer cell lines, **KU675** exhibited potent anti-proliferative activity and cytotoxicity. Interestingly, **KU675** exhibited some HSP90α selectivity compared with HSP90β (191 vs 726 μM, K_d in an intrinsic-fluorescence-spectra based binding assay). In prostate cancer cells, **KU675** induced significant degradation of HSP90α specific client proteins (Survivin, B-RAF), but had no obvious effect on the HSP90β specific CXCR4, which confirmed the HSP90α selectivity. In addition, **KU675** manifested Hsc70 binding affinity with a K_d value of 76.3 μM, which associated with the degradation inducing effects of the Hsc70 client proteins Myb and Sp1. **KU675** didn't trigger heat shock response in PC3-MM2 cells, and exhibited a modest down-regulation of HSP (Hsc70 and HSP27) in the LNCap-LN3, LAP C-4, and C4-2 cells (Liu et al. 2015). Compound **6** was a recently developed novobiocin derivative in Blagg group. **6** potently inhibited the proliferation of SKBr3 and MCF-7 cells with IC_{50} of 0.14 and 0.64 μM, respectively (Zhao et al. 2015). David Montoir and coworkers developed another novobiocin derivative **7**, which showed potent anti-proliferative properties in MCF-7 and MDA-MB-468 breast cancer cells (Montoir et al. 2016). Though the above novobiocin derivatives showed potent anti-proliferative activity in cancer cells, the in-vivo anti-tumor efficacy was not evaluated. Recently, our group developed a novobiocin analogue **8** incorporating a 6-acylamino-2-aminoquinoline scaffold. **8** potently inhibited the proliferation of MCF-7 and SKBr3 cells. In MCF-7 cells, **8** inhibited cell migration and induced significant apoptosis. We further evaluated the in-vivo anti-tumor effects of **8** in 4T1 mice breast xenografts. **8** potently inhibited tumor growth and lung metastasis potential without obvious toxicity, indicating **8** was an effective and safe anti-tumor agent

(Jiang et al. 2017). All the above novobiocin derivatives inhibited HSP90 without heat shock response.

20.4.2 Deguelin and Derivatives

Deguelin is a natural rotenoid isolated from several plant species, such as *Mundulea sericea*. In recent years, the potent cancer chemo-preventive and anti-tumor activities of Deguelin against various cancer types have been demonstrated (Lee et al. 2008; Thamilselvan et al. 2011; Udeani et al. 1997). Though some studies indicate that the potent anti-cancer effects of Deguelin is related to the inhibition of several cell signaling pathways, such as PI3K-AKT and GSK-3β/β-catenin, its accurate binding target is still unclear (Chun et al. 2003; Thamilselvan et al. 2011). In 2007, to explore the anti-cancer mechanisms of Deguelin, Oh and coworkers observed that Deguelin could reduce the expression of HIF-1α in a HSP90 inhibition-mediated mechanism. Additionally, Deguelin could induce the degradation of HSP90 clients (CDK4, AKT, eNOS, MEK1/2, and mutant P53) in a time- and dose-dependent manner. ATP-Sepharose assay suggested that Deguelin could bind to HSP90 competitively with ATP, and no activity of Deguelin and its analogues was observed in HSP90 N-terminal inhibitor based fluorescence polarization assay, indicating Deguelin might bind to the HSP90 C-terminal ATP binding pocket (Chang et al. 2012; Oh et al. 2007). In order to elucidate the essential skeleton for the potent HSP90 inhibition activity and discover novel and safe C-terminal inhibitors, Chang and coworkers conducted extensive SAR studies for Deguelin. These efforts led to the discovery of some simplified derivatives with potent anti-proliferative activity (Chang et al. 2012). In the further pre-clinical evaluation, the C-ring truncated **L80** and B, C-ring truncated **SH-1242** exhibited potent anti-tumor effects in various cancer cell lines and xenograft tumor models (Lee et al. 2015b, 2016). It is reported that the potential Parkinsonism-like neurotoxicity of Deguelin may limit its clinical application (Caboni et al. 2004). Compared with Deguelin, **L80** and **SH-1242** displayed significantly reduced cytotoxicity against normal cells. In addition, **SH-1242** didn't produce obvious Parkinsonism-like toxicity in rat brains. These results indicated that **L80** and **SH-1242** were safe anti-cancer agents. In the mechanism study, results of ATP-Sepharose assay and fluorescence-based equilibrium binding assay indicated that the HSP90 inhibition activity of the derivatives was mainly mediated by the direct binding with HSP90 CTD. Docking modeling suggested that the derivatives could bind into the C-terminal ATP-binding pocket and form key interactions with the residues Ser677 and Lys615. Further modification of **L80** and **SH-1242** led to the discovery of the third generation Deguelin derivatives (compound **9** and **10**) (Kim et al. 2015, 2016). The discovery of these novel Deguelin derivatives enriched the structural diversity of HSP90 C-terminal inhibitors and verified the druggable ability of HSP90 C-terminal domain.

20.4.3 Dihydropyrimidinones

Drug design based on the endogenous ligand is an efficient strategy to discover specific inhibitors of the targets. As a successful case, the purine class HSP90 N-terminal inhibitors were designed based on the endogenous ligand ATP (Biamonte et al. 2010). It is reported that the HSP90 CTD nucleotide binding pocket preferred to interact with GTP and UTP (Soti et al. 2003). Therefore, GTP and UTP can serve as the leads to design novel HSP90 C-terminal inhibitors. Inspired by the structural analogy between UTP and the privileged heterocyclic core 3,4-dihydropyrimidin-2-(1H)-one (DHPM), Maria Strocchia and coworkers synthesized several DHPM-based compounds. In Surface Plasmon Resonance (SPR) based binding analysis, seven compounds possessed high binding affinity with the immobilized HSP90α. Compound **11** exhibited moderate cytotoxic effects, which was in line with the SPR affinity. Western-blot analysis showed that compound **11** could induce the HSP90 clients RAF-1 and p-AKT degradation, while the HSP90 and HSP70 levels were not affected. These results verified that compound **11** was an inhibitor of HSP90. Limited proteolysis and oligomerization assays confirmed compound **11** interacting with the C-terminal domain of HSP90α. Further docking analysis showed that compound **11** bound with the region located at the dimerization site interface. Compound **11** was the first reported non-nature-inspired HSP90 C-terminal inhibitor (Strocchia et al. 2015). In order to explore the SARs systematically and discover more potent HSP90 C-terminal inhibitors, researchers conducted further structural modifications and developed the second (**12**) and third generation (**13**) derivatives based on the DHPM core (Terracciano et al. 2016a, b). The two derivatives exhibited improved anti-proliferative activity, especially compound **13**. It inhibited the growth of A375 cancer cells potently with an IC_{50} value of 2.1 ± 0.3 μM. These DHPM-based HSP90 C-terminal inhibitors provided new options for the treatment of cancer and the discovery process provided reference for the design of novel HSP90 C-terminal inhibitors.

20.4.4 EGCG and Derivatives

Epigallocatechin gallate (**EGCG**) is the major polyphenol component in green tea which exhibits broad anti-cancer and chemo-prevention effects. Previously study showed that **EGCG** could inhibit proliferation and induce apoptosis via blocking multiple essential survival pathways in human cancer cells, including JAK/STAT, MAPK, PI3K/AKT, Wnt and Notch (Singh et al. 2011). In 2005, Gasiewicz group first reported that **EGCG** exhibited chemo-preventive effects through directly binding to HSP90 (Palermo et al. 2005). Then they further demonstrated that **EGCG** could inhibit the activity of HSP90 through proteolytic footprinting and immunoprecipitation. Further ATP-agarose pull-down assay declared **EGCG** bound to the

C-terminal ATP binding pocket and interacted with amino acids (538–728) same as novobiocin (Yin et al. 2009). In pancreatic cancer cells, **EGCG** could impair the HSP90 super-chaperone complex and downregulate the HSP90 clients (AKT, Cdk4, RAF-1, Her-2, and pErk) (Li et al. 2009). In addition, **EGCG** inhibited the expression of HSP70 and HSP90 in MCF-7 cells and CT26 xenograft models by inhibiting the expression of the transcription factors HSF1 and HSF2 (Tran et al. 2010). As results, **EGCG** inhibits HSP90 chaperone function with no heat shock response.

To improve the anti-proliferative activity and drug-like properties, Anuj Khandelwal and coworkers conducted structure modification on **EGCG** and illustrated the SARs. The systematical investigation of the four rings and the linker between C- and the D-rings led to discovery of some derivatives with enhanced anti-proliferative activity, such as **14** (IC_{50}, 4 μM in MCF-7 cells). **14** exhibited more than 18-fold improved anti-proliferative activity against MCF-7 cells compared with **EGCG**. It could also induce the HSP90 clients HER2, RAF, and p-AKT degradation in a dose-dependent manner, indicating the potent anti-proliferative activity was HSP90 inhibition dependent (Bhat et al. 2014a). Though the accurate binding modes of **EGCG** and **14** are unclear, they provide novel scaffolds for HSP90 C-terminal inhibitors.

20.4.5 Derrubone and Derivatives

Derrubone is a natural isoflavonoid isolated from the Indian tree *Derris Robusta* (East et al. 1969). It was found to inhibit HSP90 in 2007 by Hadden and coworkers through high-throughput screening based on luciferase refolding inhibition. Derrubone inhibited HSP90-dependent refolding of denatured firefly luciferase with an IC_{50} value of 0.23 ± 0.04 μM. It also exhibited low micro-molar inhibition activity against various cancer cell lines (IC_{50} in MCF-7 and SkBr3 cancer cell lines are 9 and 12 μM, respectively). Meanwhile, the HSP90 clients HER2, RAF-1, AKT and ERα were dose-dependently degraded. Additionally, Derrubone could stabilize the HSP90/CDC37/kinases complex and then prevent the chaperone cycle from progressing (Hadden et al. 2007). Through docking study, Khalid and coworkers predicted that Derrubone might bind to the HSP90 C-terminal ATP-binding pocket and interact with the residues Leu665, Leu666, and Leu694 (Khalid and Paul 2014).

Hastings and coworkers reported the SAR study of Derrubone as HSP90 inhibitors and got three derivatives (**15**–**17**) with improved anti-proliferative activity. Derivative **17** possessed the most potent anti-proliferative activity. Meanwhile, the derivatives could induce the degradation of the clients HER2 and RAF dose-dependently (Hastings et al. 2008). In some extent, further modification can be conducted on Derrubone to improve the HSP90 inhibition and anti-proliferative activity.

20.4.6 Silybin and Derivatives

Silybin, a flavonoid natural product, is the major active constituent in the silymarin that is extracted from milk thistle seeds. In recent years, the anti-cancer effects and chemo-preventive efficacy of Silybin was demonstrated in various cancer types, such as skin, prostate, breast, lung, etc. (Deep and Agarwal 2007; Ting et al. 2013). Encouraged by the promising effects in pre-clinical models, silybin entered clinical trials for safety and pharmacodynamic study (Flaig et al. 2007, 2010; Hoh et al. 2006). The anti-cancer and chemo-preventive mechanisms of silybin have been well studied, including growth inhibition, angiogenesis inhibition, chemo-sensitization, and metastasis inhibition (Desgrosellier and Cheresh 2010). Considering that silybin could block the cell cycle and decrease the Cyclin D1, CDK4 and CDK6 levels (well known HSP90 clients), Zhao and coworkers hypothesized silybin might be a HSP90 inhibitor (Zhao et al. 2011; Zi and Agarwal 1999). The following study showed that silybin could inhibit HSP90-dependent refolding of denatured firefly luciferase (IC_{50}, 0.23 ± 0.04 μM) and induce HSP90 clients (HER2, RAF-1, and AKT) degradation in a dose dependent manner. Riebold and coworkers demonstrated that silybin could bind to HSP90 C-terminal through an ELISA assay. Further NMR spectroscopy analysis suggested that silybin bound to the C-terminal ATP-binding pocket, same as novobiocin (Riebold et al. 2015).

Zhao and coworkers conducted SAR study of silybin. The phenol-removal derivatives **18**, **19**, and **20** exhibited significant improved cytotoxicity against MCF-7 and SKBr3 cancer cell lines. Western blot analysis verified these derivatives induced HSP90 client proteins degradation without heat shock response (Zhao et al. 2011).

20.5 HSP90 Inhibitors Blocking HSP90-CDC37 Protein-Protein Interactions (PPI)

Cochaperones are important components of the HSP90 chaperone machine. They form HSP90-cochaperone complex and regulate the HSP90 chaperone function. Inhibition of the HSP90-cochaperone PPI is a promising strategy to block the HSP90 chaperone function. CDC37 is the kinases specific HSP90 cochaperone. Theoretically, inhibiting the HSP90-CDC37 PPI might specifically block the maturation of the kinase clients, while have no influence on other client proteins. Thus, HSP90-CDC37 PPI inhibitors are speculated to reduce the mechanism related side effects of HSP90 N-terminal inhibitors (Trepel et al. 2010).

Celastrol is the first discovered HSP90-CDC37 PPI inhibitor. In 2008, Duxin Sun lab demonstrated that celastrol could disrupt the HSP90-CDC37 PPI via immunoprecipitation assay. In Panc-1 cells, celastrol treatment induced HSP90 client proteins (CDK4 and AKT) degradation significantly. As results, celastrol inhibited Panc-1 cell growth and induced apoptosis. In Panc-1 xenograft mice, celastrol treatment inhibited tumor growth potently. These studies provided proof-of-concept for

HSP90-CDC37 inhibitors as anti-tumor agents (Kannaiyan et al. 2011). Further study revealed that celastrol covalently bound to CDC37 via a conjugate addition between the Michael reaction acceptor substructure and cysteine residues on CDC37, and then blocked the HSP90-CDC37 interaction (Sreeramulu et al. 2009a). Recently, our lab studied the SARs of celastrol derivatives as HSP90-CDC37 PPI inhibitors and discovered **CEL20**, which exhibited improved HSP90-CDC37 disruption activity and anti-proliferative effects (Jiang et al. 2016a). Some other agents are reported to be HSP90-CDC37 blockers, including Withaferin A, FW-04-806, Apigenin, Sulforaphane and Kongensin A (Wang et al. 2017a). All these agents are natural products incorporating covalent-binding moieties and the accurate HSP90-CDC37 blocking mechanisms are unclear and their further applications are limited. Thus, rational designed HSP90-CDC37 PPI inhibitors with high affinity and clear mechanisms are urgently needed (Figs. 20.10, 20.11, 20.12, 20.13 and 20.14).

The crystal structures of $HSP90_N$-$CDC37_{M/MC}$ complex have been resolved and provides structural insights for the rational design of HSP90-CDC37 PPI inhibitors. As shown in Figs. 20.15, two interaction regions are observed on the $HSP90_N$-$CDC37_M$ interface. The residues on $CDC37_M$ α4 and α5 helix form hydrophobic interactions with the $HSP90_N$ H4 helix. Mutation results suggest that Leu205 is critical for the HSP90-CDC37 PPI. Additionally, the $CDC37_M$ α1 and α2 helix and the connecting loop interact with the H5 helix of $HSP90_N$ (Roe et al. 2004; Sreeramulu et al. 2009b). Recent calculation results in our lab suggest that residues Lys160, His161, Met164, Leu165, Arg166 and Arg167 contributed the most to the binding free energy of the complex, especially the Arg167 (Song et al. 2015).

Peptides derived from the PPI interface are favorable leads for the development of PPI inhibitors. Based on the crystal structures and calculation analysis, our group developed several peptides derived from CDC37 that could block the HSP90-CDC37 PPI. **Pep1** was an 11-peptide directly extracted from CDC37 (sequence 160–170). **Pep1** bound to $HSP90_N$ with K_D value of 6.90 μM and inhibited the HSP90 ATPase with IC_{50} of 3.0 μM. In addition, **Pep1** interfered the HSP90-CDC37

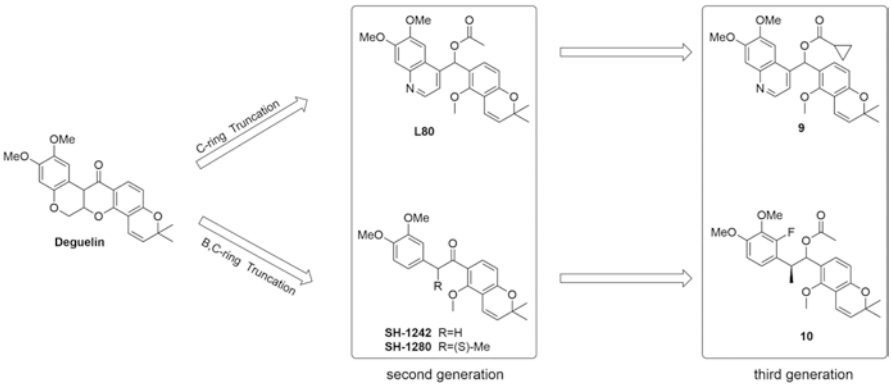


Fig. 20.10 Structures of Deguelin and representative derivatives

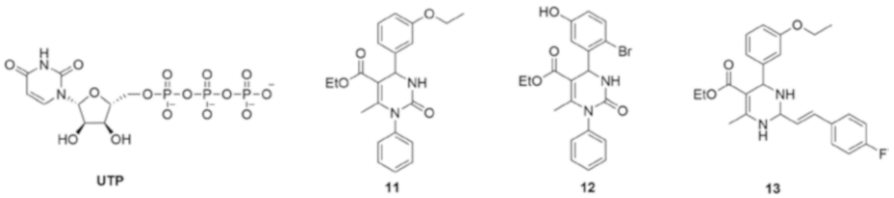
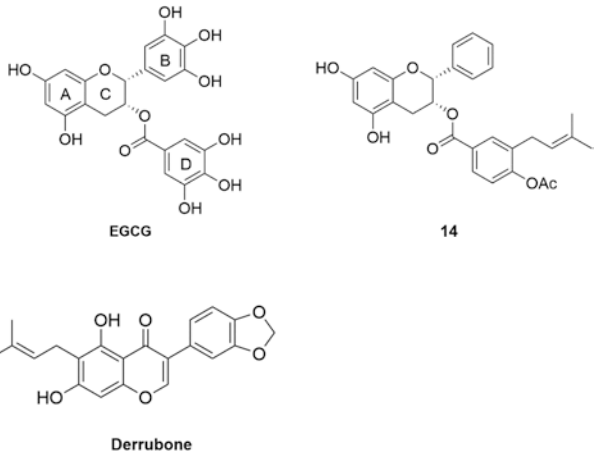


Fig. 20.11 Structures of dihydropyrimidinones as HSP90 C-terminal inhibitors

Fig. 20.12 Structures of EGCG and representative derivative



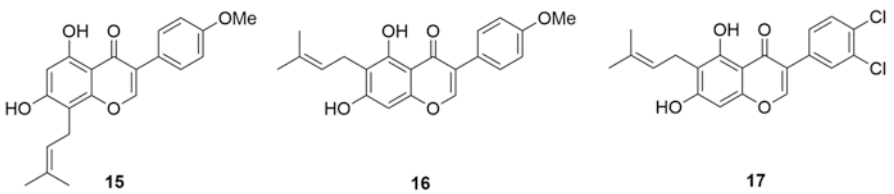


Fig. 20.13 Structures of Derrubone and representative derivatives

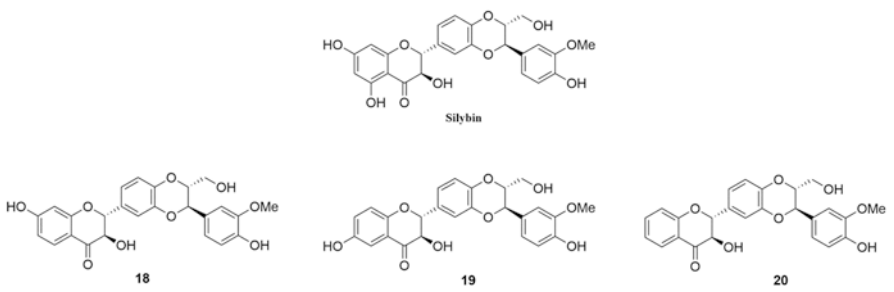


Fig. 20.14 Structures of Silybin and representative derivatives

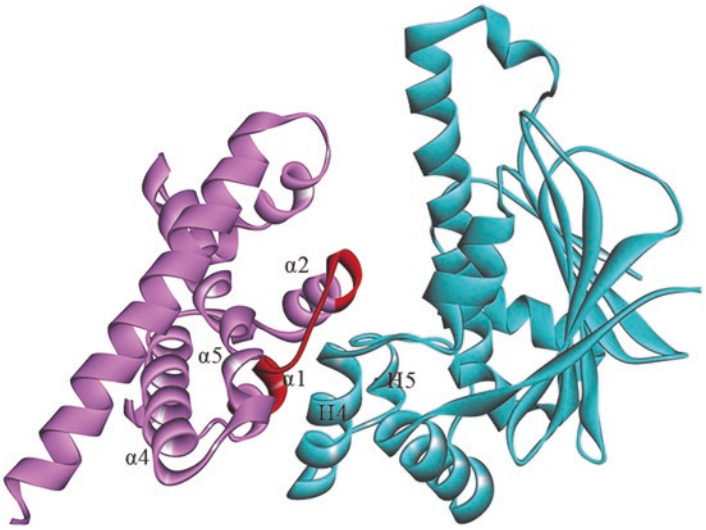


Fig. 20.15 HSP90-CDC37 PPI interface (PDB ID: 2K5B). $HSP90_N$ and $CDC37_M$ are colored in blue and magenta, respectively. Sequence 160–170 on $CDC37_M$ is shown in red, α-helix numbers are indicated

PPI in a biolayer interferometry (BLI) based competitive assay (Wang et al. 2015). Further optimization led to the pentapeptide **Pep5**, which exhibited slightly improved $HSP90_N$ binding affinity with K_D value of 5.99 μM. GST-$HSP90_N$ pull down assay further demonstrated that **Pep5** could block HSP90-CDC37 PPI (Wang et al. 2017b). The discovery of **Pep5** further confirmed that the residues His161, Met164, Leu165, Arg166 and Arg167 on CDC37 were critical for the HSP90-CDC37 PPI. Thus, small molecules disrupting the interactions between these residues and HSP90 can block the HSP90-CDC37 PPI. Based on these results, a pharmacophore model was generated and used for virtual screening to discover HSP90-CDC37 PPI inhibitors. Fortunately, we discovered compound **VS-8** exhibited moderate HSP90-CDC37 PPI inhibition activity with IC_{50} value of 76.85 μM in Homogeneous Time-Resolved Fluorescence (HTRF) assay. Further optimization of **VS-8** led to compound **21**, which exhibited threefold improved activity with IC_{50} value of 27.40 μM. Compound **21** bound to HSP90 with K_D value of 40.4 μM. In GST-pull down assay, **21** blocked HSP90-CDC37 interaction in a dose dependently manner. Moreover, **21** possessed moderated anti-proliferative activity in cancer cells and induced HSP90 kinase clients AKT and CDK4 degradation (Wang et al. 2017b). As the first reported non-natural small molecule HSP90-CDC37 PPI inhibitor, **21** can serve as a probe to study the HSP90-CDC37 PPI (Fig. 20.16).

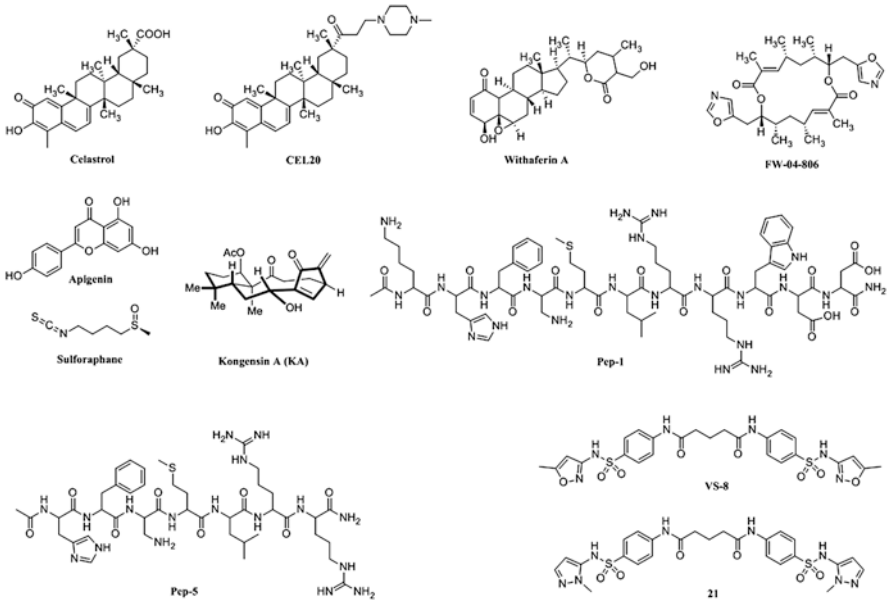


Fig. 20.16 Structures of HSP90-CDC37 PPI inhibitors

20.6 Conclusions

HSP90 isoforms are involved in multiple processes of cancer development, and HSP90 inhibition has been a promising anti-cancer strategy. Till now, HSP90 inhibitors with different mechanisms have been developed. Many pan-HSP90 N-terminal inhibitors have entered clinical trials. Despite the potent pre-clinical anti-cancer effects, these inhibitors exhibit disappointing clinical efficacy. In addition, many serious adverse effects are occurred during the treatment, such as the ocular toxicity. The adverse effects may limit the clinical dosage, which is a possible reason for the poor clinical efficacy. Heat shock response triggered by the pan-HSP90 inhibitors may be another reason. Thus, inhibitors with reduced adverse effects and no heat shock response may bring breakthrough for the HSP90 inhibitor development. HSP90 four isoforms have different functions and client protein profiles, so isoform selective inhibitors may have reduced adverse effects. **TAS-116** is a HSP90α/β selective inhibitor which has entered clinical trials, and moderate clinical anti-cancer effects are observed in patients with advanced solid tumors. For further clinical studies, **TAS-116** should be evaluated in more cancer types to find the most sensitive patient populations. Moreover, the heat shock response effect should be a concern of **TAS-116**, which may neutralize the anti-cancer effects. GRP94 selective inhibition and TRAP1 selective inhibition don't trigger heat shock response, may be more promising. GRP94 inhibitor **PU-WS13** exhibited favorable anti-proliferative

activity in HER2-overexpressing breast cancer cells and multiple myeloma cells. Additionally, some GRP94 inhibitors possess anti-migratory potential. The anti-cancer effects of GRP94 selective inhibitors should be further evaluated in pre-clinical study. Studies of Byoung Heon Kang and coworkers demonstrate TRAP-1 selective inhibition is a promising anti-cancer strategy. Though Gamitrinibs and **SMTIN-P01** possess potent anti-cancer effects, their clinical application may be limited. Compound **5** exhibits moderated TRAP1 selectivity compared with cytosolic HSP90, the selectivity profile should be improved in further modification. HSP90 C-terminal inhibition doesn't induce heat shock response, is another promising HSP90 inhibition strategy. Most reported HSP90 C-terminal inhibitors are natural product analogues, and their accurate binding modes are still unclear. In future study, more efforts should be done to reveal the binding modes of the C-terminal inhibitors. This will promote the development of HSP90 C-terminal inhibitors. HSP90-cochaperone PPI inhibition is an attractive strategy to inhibit the HSP90 chaperone function. CDC37 is a cochaperone which specifically regulate the maturation of kinase client proteins. Theoretically, HSP90-cochaperone PPI inhibition will only block the maturation of kinase clients and have no influence on other client proteins. Thus, HSP90-CDC37 PPI inhibitors can reduce the mechanism related adverse effects of the pan-HSP90 inhibitors. All previously reported HSP90-CDC37 PPI inhibitors incorporate a covalent-binding moiety and the HSP90-CDC37 PPI inhibition activity is covalent-binding based. Thus, rational designed HSP90-CDC37 PPI inhibitors are urgently needed. Our group have explored the hot-spots of the HSP90-CDC37 interface and discovered compound **21** as the first small-molecule HSP90-CDC37 inhibitor. **21** manifests moderate HSP90-CDC37 PPI blocking activity, and the activity should be further improved. Moreover, the anti-cancer effects of HSP90-CDC37 PPI inhibitors should be further studied. In general, HSP90 chaperone function inhibition is a promising anti-cancer strategy, but there is still a long way to go.

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Part III
Neurological Signaling Pathways

Chapter 21
Heat Shock Proteins Involved in Neuromuscular Pathologies

Candelaria Merino-Jiménez, César García-Cruz, Jorge Aragón, Lourdes Siqueiros-Márquez, and Cecilia Montañez

Abstract Heat shock proteins (Hsp) have been widely studied for many years. These proteins are part of a large family of proteins with multiple functions that include chaperone activity, responding to cellular stress and anti-apoptotic activity. Additionally, Hsp participate in many cellular processes such as cell homeostasis, F-actin-dependent processes, differentiation and proliferation. Different Hsp are expressed in the skeletal muscle and are important for muscular and neurological functions. These proteins serve as indicators of muscle stress and play a role in muscle disease or injury processes. In this chapter, we summarize the evidence reported about the role of some of these protein family members in neuromuscular disorders, such as the following: muscular dystrophies, myotonic dystrophy, myopathy, hereditary neuropathies and inflammatory myopathy.

Keywords Chaperone · Heat shock proteins · Mutation · Neuromuscular disorders · Stress response

Abbreviations

AD	autosomal-dominant inheritance
APP	amyloid precursor protein
AR	autosomal-recessive inheritance
BMD	Becker muscular dystrophy
CASA	chaperone-assisted selective autophagy
CHIP	carboxy terminus of heat shock protein 70-interacting protein
CMs	congenital myopathies

C. Merino-Jiménez · C. García-Cruz · J. Aragón · L. Siqueiros-Márquez · C. Montañez (✉)
Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del IPN, México City, México
e-mail: cecim@cinvestav.mx

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CMT	Charcot-Marie-Tooth disease
CMT1	Charcot-Marie-Tooth type 1
CMT2	Charcot-Marie-Tooth type 2
DAPC	dystrophin-associated protein complex
DCM	dilated cardiomyopathy
dHMN	distal hereditary motor neuropathy
DM	dermatomyositis
DMD	Duchenne muscular dystrophy
DRM	desmin-related myopathy
FSHD	facioscapulohumeral muscular dystrophy
HIMD	hypertonic infantile muscular dystrophy
HSF1	heat shock factor-1
HSP	heat shock protein family
Hsp	heat shock proteins
IMs	inflammatory myopathies
INI	intranuclear inclusions
JDM	juvenile dermatomyositis
LGMD	limb-girdle muscular dystrophy
LGMD1D	limb girdle muscular dystrophy type 1D
MBLN	muscle bling-like proteins
MKBP	myotonic dystrophy kinase binding protein
MM	myofibrillar myopathy
MMD	myotonic muscular dystrophy
MMD1	myotonic muscular dystrophy type 1
MMD2	myotonic muscular dystrophy type 2
NAM	necrotizing autoimmune myositis
OPMD	oculopharyngeal muscular dystrophy
PM	polymyositis
ROS	reactive oxygen species
sHsp	small heat shock protein
sIBM	sporadic inclusion body myositis
2DE	two-dimensional gel electrophoresis
UPP	ubiquitin proteasome pathway
UTR	untranslated regions

21.1 Introduction

Heat shock protein family (HSP) are a group of highly conserved proteins that are mainly expressed in response to different types of stress and whose main functions include cytoprotection and the prevention of protein denaturation and aggregation through chaperone-like activity (Haslbeck et al. 2005; Sun and MacRae 2005). HSP are involved in other important cellular functions such as ischaemia, oxidative damage, anti-apoptotic activity and cytoskeletal rearrangement (Garrido et al. 2001; Gusev et al. 2002). In addition, these proteins participate in many other cellular processes

including inflammatory signalling, cell proliferation, and cell differentiation (Senf 2013). Hsp contain a WDPF domain in their N-terminal end and have a conserved sequence called the α–crystallin domain and a non-conserved flexible domain at the C-terminal end (Juo et al. 2016; Launay et al. 2006; Thériault et al. 2004).

Hsp are classified according to their molecular weight, ranging from 8 to 110 kDa, and include the larger HSP such as HSP110, HSP90, HSP70, HSP60 and HSP40, as well as the small heat shock proteins (sHsp) comprising 11 members, designated HSPB1 to HSPB11, according to the HUGO Gene Nomenclature Committee (Bellyei et al. 2007; Kappé et al. 2003). Skeletal muscle expresses several HSP; among these, HSP90 and HSP70 have an important role in muscle physiology (Thakur et al. 2018), and the most studied is HSP70 (Chung et al. 2008; McArdle et al. 2004; Senf 2013). In normal conditions, HSP70 functions as a chaperone that participates in protein transport and the proper three-dimensional configuration folding and refolding of proteins (Henderson 2010), which can be summarized as: (1) protecting against muscle damage, (2) promoting muscle regeneration and recovery, and (3) maintaining skeletal muscle mass and integrity (Senf et al. 2013). Importantly, it has been described that the HSP70 family is highly regulated in skeletal muscle function by many factors. HSP70 expression is rapidly upregulated in response to muscle damage and is decreased during muscle inactivity (Senf et al. 2013), suggesting that this family plays a key role in the homoeostasis of skeletal muscle function.

HSPB1, HSPB5, HSPB6 and HSPB8 are ubiquitously expressed, while HSPB2, HSPB3, HSPB4, HSPB7, HSPB9 and HSPB10 are tissue specific. The best studied members of sHsp are HSPB1 (HSP27), HSPB4 (αA crystallin), and HSPB5 (αB-crystallin) (Kampinga et al. 2009). It has been estimated that the cellular distribution of sHsp changes in response to development, physiological stressors and oxidation and in relation to the pathological status. Interestingly, some sHsp members are exclusively expressed in skeletal and cardiac muscle (Kampinga et al. 2009). The sHsp expressed in muscle are HSPB1, HSPB5, HSPB6 (HSP20), and HSPB8 (H11 kinase, HSP22), where their overexpression is associated with reduced myocardial damage in various cardiac ischaemia models (Danan et al. 2007; Fan et al. 2005; Hollander et al. 2004; Ray et al. 2001). It is well known that HSP mutations are related to severe pathologies such as myopathy, distal heredity motor neuropathy and neurodegenerative diseases (Boncoraglio et al. 2012). The term myopathy refers to a muscle disease characterized by muscular weakness or muscle atrophy (Dimachkie and Barohn 2014; Liu and Steinacker 2001).

Myopathies may have different origins, including inflammatory, infectious, drug/toxin-induced, congenital, metabolic and genetic origins (Dimachkie and Barohn 2014), and may also affect other tissues (Lollo et al. 2013; Salo et al. 1991). In some cases, muscular (myopathy) as well as cognitive (neuropathy) impairments are present in diseases commonly referred as neuromuscular disorders, affecting both the skeletal muscle and cognitive/neuronal functions (Dowling et al. 2017; Nelson et al. 2017). Currently, many types of myopathies have been described that affect different tissues and organs (Dimachkie and Barohn 2014). This chapter focuses on the role of Hsp in muscle physiology and the relationship between Hsp and different neuromuscular disorders.

21.2 Role of HSP in Muscle

21.2.1 Muscle Myogenesis

Several studies have shown that the HSP are expressed during muscle differentiation; in this process the generation of multinucleated muscle fibres occurs through the fusion of mononucleated myoblasts (Buckley and Konigsberg 1974). Skeletal muscle uses two independent chaperone systems for muscle maintenance and differentiation; one system includes HSPB2 and HSPB3, and the other includes HSPB1, HSPB5 and HSPB6 (Sugiyama et al. 2000). The function of HSPB2 and HSPB5 in muscle is supportive because the loss of these proteins leads to skeletal muscle degeneration (Brady et al. 2001). In addition, it was reported that HSPB6 may play multiple roles in the muscle contractile processes associated with troponin complexes (Dreiza et al. 2010). On the other hand, HSPB5 is synthesized during myogenesis, and its expression is associated with muscle structure development (Benjamin et al. 1997). Furthermore, it is known that HSP activity increases during myotube maturation, where the production of these proteins is regulated by the most important transcriptional molecules: Heat shock factor 1 and 2 (HSF1 and HSF2) (Maglara et al. 2003). Mouse C2C12 myoblasts have been used as a model for elucidating the role of Hsp during muscle differentiation. In this model, an accumulation of HSPB1 and HSPB5 was observed during C2C12 cell differentiation into myotubes (Singh et al. 2010). Additionally, it was reported that HSPB1 is a target gene for the MEF2 transcription factors, which regulate advanced stages of myogenesis (Huang et al. 2007).

21.2.2 Muscle Physiology

HSP are expressed in many cell types including striated skeletal muscle; however, the specific role of Hsp in muscle physiology is unknown, and several studies have implicated that Hsp interact with microfilaments, intermediate filaments and actin filaments to modulate cytoskeleton structure and contribute to the organization of muscle protein complexes (Dreiza et al. 2005; Lavoie et al. 1993; Liang and MacRae 1997; Nicholl and Quinlan 1994; Nishimura and Sharp 2005; Perng et al. 1999). HSP90, HSP100, HSPB1 and HSPB5 are the main Hsp expressed in skeletal muscle and are associated with actin (Iwaki et al. 1993; KoYASU et al. 1986; Wilkinson and Pollard 1993). HSPB6, similar in structure to HSPB1 and HSPB5, may interact with the cytoskeleton and can promote smooth muscle relaxation (Gusev et al. 2005; Kato et al. 1994). Moreover, non-phosphorylated HSPB6 stabilizes actin filaments during muscle contraction (Brophy et al. 1999). Other studies have suggested that Hsp play an important role in maintaining the physiological function of striated muscles and motor neurons. In addition, fibre type distribution, fibre diameter,

myosin heavy chain and mitochondrial distribution are factors associated with HSP70 up- or downregulation.

21.2.3 Muscle Protection

The skeletal muscle is an important tissue that regulates mechanical mobility. Hsp are rapidly expressed in response to different stresses in skeletal muscle from a variety of species (Flanagan et al. 1995; Khassaf et al. 2001; McArdle et al. 2001; Salo et al. 1991). It has been reported that HSPB2 and HSPB3 are abundant in this tissue and are associated with maintaining myofibril integrity (Sugiyama et al. 2000). On the other hand, HSPB7 deficiency results in muscle fibrosis and sarcolemma integrity loss, which indicates that this protein is essential for maintaining muscle integrity (Juo et al. 2016). Interestingly, HSPB5 mutations disrupt the chaperone activity and intermediate filament interaction and are related to human muscle diseases (Bova et al. 1999; Kumar et al. 1999; Kumarapeli and Wang 2004).

Skeletal muscle damage occurs from various situations, such as excessive exercise, reperfusion following ischaemia and disease states (McArdle and Jackson 1997). Several studies have shown that cells activate an endogenous mechanism to prevent subsequent damage through increased Hsp synthesis (Morimoto et al. 1996; Hightower 1991); however, the mechanism of this protection is not clearly understood. Many studies have used exercise to simulate muscle damage, in which Hsp expression is rapidly upregulated indicating a stress response (Henstridge et al. 2016). Exercise is a major stressor on muscles; during intense muscle activity, a muscle fibre injury may occur as a consequence of increased reactive oxygen species (ROS). ROS contribute to muscle damage by denaturing proteins, disrupting membranes, and increasing the temperature (Birch et al. 1997; Sen 1995). Therefore, the upregulation of some Hsp is related to a defence mechanism for maintaining cytoskeletal organization in hyperthermic conditions. In this sense, HSPB5 prevents thermal unfolding and myosin II aggregation, which enables myosin enzymatic properties and muscle contractile activity to be maintained (Melkani et al. 2006). Additionally, HSP70 has been associated with muscle protection from exercise-induced oxidative stress (Smolka et al. 2000). In general, the significant Hsp levels induced with exercise imply that major protective and protein chaperone roles exist in muscle (Nishimura and Sharp 2005).

Oxidative stress, due to ROS accumulation, has been associated with age-associated muscle dysfunction (Beckman and Ames 1998; Marber et al. 1995; Warner 1994). For example, HSP70 overexpression in mouse protects against skeletal muscle damage and muscle dysfunction. Compared to control mice, transgenic mice expressing HSP70 presented with less muscle damage and higher muscle force and function (McArdle et al. 2004) as well as morphological recovery (Miyabara et al. 2006). All these findings support the hypothesis that HSP70 plays an important role in proper muscle function and protects against muscle damage.

21.3 Role of HSP in Neuromuscular Disorders

Some HSP members have been associated with severe pathologies, including neurological and muscular disorders, which are characterized by the accumulation of aggregate-prone proteins. It has been shown that mutations in some Hsp lead to neurological and muscular disorders, which may be due to a loss of function in protein quality control and/or to a toxic gain of function, resulting from the propensity of mutants to aggregate (Boncoraglio et al. 2012). Muscular dystrophies are a group of progressive and hereditary disorders principally involved in muscular weakness. The aetiology of these diseases is related to the disruption of genes located on different chromosomes and loci. In recent years, the molecular pathogenesis pathway for several muscular dystrophies has been described. Of these, the most studied dystrophies are Duchenne and Becker muscular dystrophy (DMD/BMD), myotonic muscular dystrophy (MMD), oculopharyngeal muscular dystrophy (OPMD), facioscapulohumeral muscular dystrophy (FSHD) and limb-girdle muscular dystrophy (LGMD), each of which has different genetic origins, such as duplications, deletions, repeated sequences or punctual changes. Muscular dystrophies affect skeletal muscle function, which is due to muscle damage producing abnormal muscle contraction/relaxation and ischaemia, among other factors, which are derived from several internal or external stress factors. The major response to these stress factors is HSP expression (Liu and Steinacker 2001; Senf 2013). A brief review of the scientific literature on the most representative diseases will be described.

21.3.1 Duchenne and Becker Muscular Dystrophy

Duchenne and Becker muscular dystrophy (DMD/BMD) are characterized by progressive muscular weakness, visual alterations and cognitive impairment. All these symptoms are more severe in DMD; however, both diseases can present different levels of cognitive disorders. DMD and BMD are dystrophinopathies linked to the X chromosome, which arise from mutations (including duplication, deletion, punctual mutation) along the *DMD* gene located on the locus Xp21.3-p21.2 (Darras et al. 2015). The incidence of these diseases has been reported to be between 1:3500 and 1:5000 male births for DMD and between 1:18,000 and 1:31,000 male births for BMD (Emery 1991; Moat et al. 2013). The mutations can affect the muscle function of dystrophin, which participates in the dystrophin-associated protein complex (DAPC) (Winder 1997), where it is speculated to work as a bridge between the cytoskeleton and extracellular matrix to prevent muscle fibre damage during muscular contraction (Gawor and Prószyński 2017). Although the first studies on Hsp began in the 1960s, the relation between Hsp and DMD was not proposed until 1995; this association was reported through immunohistochemical studies using antibodies against some Hsp (65, 72, 73 and 90), where muscle samples of DMD patients had stronger staining than healthy muscle samples (Bornman et al. 1995).

Several studies that used two-dimensional gel electrophoresis (2DE) and proteomic analyses in DMD patients have reported that Hsp levels are upregulated (Brinkmeier and Ohlendieck 2014).

One of the reasons for DMD/BMD patient death is the weakness of the respiratory tract diaphragm and other muscles associated with this system. Proteomic analyses have identified that the diaphragm of *mdx* mice of varying ages present a differential expression profile compared to healthy mice, where HSPB7 (cardiovascular HSP, also known as cvHSP) is upregulated (Doran et al. 2006). Although in another study, in the hearts of 9-month-old *mdx* mice, a slight upregulation in the HSPB1 and HSP60 level was reported (Lewis et al. 2010). Equally, using fluorescence 2DE with samples from 22-month-old *mdx* mice, an increase in HSPB7, HSP70 cognate, HSPB8 and 78-kDa glucose-regulator protein (GRP78; also considered a Hsp) was observed, but otherwise, the HSPB6 level was downregulated in the same report (Carberry et al. 2013). Additionally, a different Hsp expression profile was reported in a comparative study between the tibialis anterior muscle samples of *mdx* mice of different ages, in which a decrease in the HSPB1 (HSP25 in mouse) level was found (Carberry et al. 2012). This finding suggests that Hsp upregulation may be a signal of muscle damage or a protective mechanism during the first steps of DMD/BMD, similar to the change observed in damaged muscle fibres during exercise (Hageman et al. 2011; Morton et al. 2008). Interestingly, the protective effect of Hsp have been validated by the use of a pharmacological HSP72 inducer (known as BGP-15). This treatment improved the structure and contractile function of the diaphragm muscles of *mdx* mice (Gehrig et al. 2012).

It has been reported that dystrophin Dp71 protein expression plays an essential role in brain development and function (Daoud et al. 2009; Waite et al. 2012). Recently, through proteomic analyses, it was shown that overexpression of the dystrophin mutant called Dp71$\Delta_{78–79}$ stimulates neurite outgrowth in PC12 cells (a neuronal cell model) through upregulating and phosphorylating HSPB1 (Aragón et al. 2011; Merino-Jiménez et al. 2016). In addition, the overexpression of Dp71e$_{\Delta 71}$ also stimulates the neuronal differentiation of PC12 cells and upregulates the expression of HSPB1, suggesting that both dystrophin Dp71 isoforms and HSPB1 are involved in the neuronal functions (García-Cruz et al. 2019). Interestingly, it has been shown that HSPB1 phosphorylation is important for interactions with cytoskeletal elements that participate in neurite outgrowth (Clarke and Mearow 2013; Williams et al. 2006). Despite these findings, more studies are required to understand the role of Hsp in the cognitive deficit of DMD/BMD patients.

21.3.2 Oculopharyngeal Muscular Dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is a hereditary autosomal dominant disease that arises late in life and is characterized by a decreased capacity for ingesting liquid and solid nutrients, muscular weakness in proximal limbs and a permanent eyelid decline. According to studies reported in Canada and some regions of

America, the incidence of OPMD ranges from 1:600 to 1:1000 individuals (Brais 2003). It has been reported that this effect is due to the abnormal expansion of 8–13 trinucleotide GCN repeats (N could be any nucleotide) in the *PABPN1* gene located on the loci 14q11.2-q13 (Brais et al. 1998; Richard et al. 2015). Since 1980, it has been reported that the aggregates of filaments in muscle samples is a relevant characteristic of patients with OPMD (Tomé and Fardeau 1980). It was speculated that the translation of GCG trinucleotides, that code to the amino acid alanine, generate stable structures under different stress conditions, such as pH, temperature or denaturing solutions, that avoid the degradation mechanism (Forood et al. 1995); because of this avoidance, a protective role has been suggested for Hsp in OPMD.

Studies carried out by Abu-Baker and collaborators (2003) have shown that the intranuclear inclusions (INIs) associated with OPMD may be related to the ubiquitin-proteasome pathway (UPP). Because of the transitory transfection of the expanded alanine-17 stretch (GCG repeats), HeLa and COS-7 cell lines generate INIs. In addition, treating with the UPP (lactacystin) inhibitor and expanded alanine-17 (originated from a mutated PABPN1-ALA17 protein) increased protein aggregation and toxicity. Additionally, increased constitutive HSP73 and inducible HSP72 (isoforms of HSP70) levels were observed in HeLa cells. Interestingly, the number of INIs was decreased when HSP40 and HSP70 were co-transfected into COS-7 cells. Coupled with this finding, it was reported that the increased solubility of PABPN1-ala17 mRNA in the presence of HSP40 and HSP70 did not affect the level of the mutated PABPN1-ALA17 protein (Abu-Baker et al. 2003). On the other hand, HSP90 inhibition through an inhibitor known as 17-AAG decreased the presence of the mutated PABPN1-ALA17 protein in the C2C12 muscular cell line. These results were verified in vivo through the electroporation of a plasmid coding for the mutated PABPN1-ALA17 protein in mouse muscle fibres and subsequent treatment with a 17-AAG inhibitor, which increased the degradation of the mutated protein. This effect possibly increases the interaction with CHIP (carboxy terminal of heat shock protein 70-interacting protein), which promotes the ubiquitination of the mutant PABPN1-ALA17 protein (Shi et al. 2015). This finding suggests that a degradation mechanism exists in the absence of HSP90, meaning that the chaperone function block the appropriate degradation of the mutated protein.

Models that replicate the effect of repeated sequences have been useful for clarifying the participation of Hsp in mutated mRNA or protein degradation in OPMD. Therefore, these findings make it possible to focus on researching compounds that can efficiently restore mutated mRNA or protein degradation when Hsp are involved. However, the proposal to use Hsp as a target for treating OPMD patients must be analysed carefully because HSP are involved in many cellular processes.

21.3.3 Myotonic Muscular Dystrophy

Myotonic muscular dystrophy (MMD) is an autosomal dominant hereditary disease that is divided into two subtypes: type 1 (MMD1) and type 2 (MMD2). These diseases are characterized by progressive muscular weakness and variable

multisystemic manifestations, such as disruptions in smooth, skeletal and heart muscle, in brain and eye tissue, and in immune and endocrine systems, during adult life (Darras et al. 2015; Udd 2012). The aetiology of MMD1 stems from the CTG trinucleotide repeated expansion in the *DMPK* gene that codes for myotonic dystrophy protein kinase, which maintains muscle function. The repeated expansion can be located in two regions: in the 3′ untranslated region of the *DMPK* gene, in the 19q13.3 position of the human genome (Brook et al. 1992), and in the promoter region of the *SIX5* gene (Boucher et al. 1995). In the case of MMD2, the repeated CCTG tetranucleotides are located on intron 1 of the *ZNF9* gene, in the 3q21.3 position of the human genome (Liquori et al. 2001).

The origin of this disease is similar to OPMD; in addition, when repeated nucleotides are present in the untranslated regions (UTR), both diseases have muscular weakness in common and possibly a similar pathogenic mechanism. Studies carried out in *C. elegans* that contain CUG repeats of different lengths in the 3′ UTR of a reporter gene, demonstrated animal death during embryogenesis or delayed growth at larval stages due to damaged muscle development (Chen et al. 2007). In MMD1 and MMD2, the accumulation of mRNA containing repeated sequences affects the normal function of RNA-binding proteins and reduces the activity of alternative splicing regulators, such as muscle bling-like proteins (MBLN), through sequestration into ribonuclear foci (Mankodi et al. 2003; Ranum and Cooper 2006). At this moment, it is speculated that sequestering splicing regulators by CTG or CCTG repeats may modify the alternative splicing of other proteins and generate disrupted proteins that promote the expression of Hsp such as HSPB2 (also known as MKBP; Myotonic dystrophy kinase binding protein), which are increased in MMD patients (Suzuki et al. 1998). Additionally, it has been shown that HSPB2 is able to interact with HSPB1 and HSPB5 to form a complex (Forner et al. 2010) and that it has a chaperone activity depending of target when it is induced by dithiothreitol or heat (Prabhu et al. 2012).

21.3.4 Distal Myopathy

Distal myopathy is a group of clinically and pathologically heterogeneous diseases that are caused by different autosomal dominant or recessive genetic disorders and are characterized by muscle weakness and/or progressive muscular atrophy that mainly affects the hands, legs and feet, which may have an early or late onset (Dimachkie and Barohn 2014; Malicdan and Nonaka 2008; Palmio and Udd 2016; Udd 2007, 2012); motor neuropathy may also be observed in some patients (Benndorf et al. 2014; Ghaoui et al. 2016; Lewis-Smith et al. 2016). Distal myopathies are caused by the lack or deficiency of at least one protein with an essential role in muscle function; some of these proteins have been genetically determined, while others are under identification (Dimachkie and Barohn 2014; Udd 2012). To date, a large list of distal myopathies has been described, including Welander distal myopathy, tibial distal myopathy, Nonaka distal myopathy, Miyoshi distal myopathy, Laing distal myopathy, and Udd distal myopathy (Dimachkie and Barohn 2014; Malicdan and Nonaka 2008; Palmio and Udd 2016; Udd 2007, 2012).

It is well known that Hsp expression is mainly related to the stress response; therefore, it is hypothesized that in different types of distal myopathies, one or several Hsp are expressed due to the muscle damage. Despite only a few studies having tested the expression and function of Hsp in the different types of distal myopathies, several studies have shown the direct involvement of these proteins in distal myopathy and motor neuropathy. Importantly, it has been reported that HSPB5 mutations may result in desmin-related or myofibrillar myopathy (DRM, MM), dilated cardiomyopathy (DCM), and hypertonic infantile muscular dystrophy (HIMD) (Benndorf et al. 2014; Mitzelfelt et al. 2016). Furthermore, the loss of HSPB2, HSPB5 and HSPB8 in mouse models is associated with progressive skeletal myopathy and distal hereditary motor neuropathy and myopathy (Bouhy et al. 2017; Brady et al. 2001). In contrast, HSPB8 overexpression removes misfolded proteins (Crippa et al. 2010), and HSPB1, HSPB5, HSPB8 and HSP70 overexpression or the drug-induced expression of Hsp prevents protein aggregation (Chávez Zobel et al. 2003). All these finding suggest that these Hsp play an essential role in the refolding or degradation of misfolded proteins.

Recent works have reported that mutations in HSPB1 and HSPB8 are involved in distal motor neuropathy (Echaniz-Laguna et al. 2017; Ghaoui et al. 2016; Lewis-Smith et al. 2016). In addition, mutations in HSP40 (DNABJ6) are associated with Limb girdle muscular dystrophy type 1D (LGMD1D) (Sarparanta et al. 2012), and multiple DNAJB6-related myopathies have also been described (Ruggieri et al. 2016). Moreover, an increase in HSPB1, HSPB3, HSPB6, HSP40 (DNAJB1) and HSP60 (HSPD1) expression has been described in abnormal muscle. Interestingly, these proteins were mainly localized within protein aggregates and chaperone-assisted selective autophagy (CASA) components HSPB8, HSP70 and BAG3 (Beclin2-Associated Anathogen 3, a co-chaperone protein that allows the interaction with HSP70) (Kley et al. 2012, 2013). Therefore, these HSP regulate the homeostasis of both the peripheral nerve and muscle throughout the CASA complex, in which HSPB1, HSPB3, HSPB8 and BAG3 are the key components in the pathology of distal myopathies (Adriaenssens et al. 2017). In addition, mutations in these Hsp may also alter mitochondrial dynamics, affecting the energetic requirements of peripheral sensory and motor neurons and resulting in several myopathy and neuropathy disorders (Pareyson et al. 2015).

On the whole, distal myopathy and motor neuropathy have mainly been associated with mutations in HSPB1, HSPB3, HSPB5 and HSPB8 and other non-HSP genes (Adriaenssens et al. 2017; Benndorf et al. 2014). The role of these Hsp is related to their multiple functions as chaperones or co-chaperones for preventing the aggregation or refolding of misfolded proteins throughout the CASA complex (Kley et al. 2012, 2013), which play important roles in maintaining or correcting muscle functions in response to muscle damage (Arndt et al. 2010). HSP loss or mutation may alter the interaction with this complex; thus, alternative methods for trying to recover muscle functions include the overexpression or drug-induced expression of these proteins as therapeutic targets.

21.3.5 Congenital Myopathy

Congenital myopathies (CMs) are a heterogenous group of muscular disorders; the most important diagnostic indicator of CMs is the presence of abnormal muscle fibre architecture on muscle biopsies (histopathological features such as cores or rods) (Romero and Clarke 2013). There are seven types of CM: nemaline myopathy, myotubular myopathy, centronuclear myopathy, central core disease, multiminicore disease, congenital fibre-type disproportion myopathy and hyaline body myopathy. Many genes have been associated with these diseases including mutations in HSP; the list of Hsp mutations that lead to pathologies is growing (Boncoraglio et al. 2012; Datskevich et al. 2012; Gerasimovich et al. 2017).

Among the CMs that present with inclusion bodies, increased desmin expression within the muscular fibres (Desmin-related myopathy, DRM) has been associated with cytoplasmic and sarcoplasmic bodies, where the inclusion body and granulofilamentous types showed an accumulation of other proteins such as HSPB5, a chaperone protein necessary for desmin intermediate filament stabilization that binds to both desmin and actin and helps to maintain cytoskeletal integrity. A missense mutation in HSPB5 leads to HSPB5 accumulation with desmin (Bornemann and Goebel 2001); even more interestingly, a French family that presents with late onset disease, in which the desmin mutation is excluded, has a functional mutation in the *HSPB5* gene (R120G) as the cause of their muscular disease; apparently, this mutation has a deleterious effect on muscle after a long period of contractile activity, consistent with the late onset (Vicart et al. 1998). The R120G mutation leads to a decrease in β-sheet secondary structure and an altered aromatic residue environment of α-β-Crystallin (CryAB), resulting in defective chaperone activity, which leads to DRM and cataracts (Bova et al. 1999).

Furthermore, increased HSPB1 and HSPB5 immunoreactivity have been reported in central and minicore lesions as well as in target fibres; however, no increase in protein expression was detected. In addition, removing actin from muscle cryosections, markedly decreased HSPB1 and increased HSPB5 staining, suggesting that these sHsp have different roles: HSPB1 is closely related to the actin microfilament system, while HSPB5 is primarily associated with the intermediate filament system. During cell stress, HSPB1 acts as a storage tank for misfolded polypeptides until they are processed (Arrigo et al. 2007; Fischer et al. 2002). It has been shown that dexamethasone increases HSPB1 and HSPB5 expression and membrane localization in differentiated myotubes from DRM cells (both HSP can form heterocomplexes); it has been suggested that HSPB1 acts as a molecular chaperone for solubilizing mutant αB-crystallin-R120G and reducing inclusion bodies (Ito et al. 2003). After dexamethasone treatment, a decrease in the survival of the DRM satellite cell population is observed, suggesting that when the R120G mutation is present, dexamethasone negatively affects survival during oxidative stress, making it important to be cautious with the clinical use of this drug in DRM patients with HSPB5 mutations (Nédellec et al. 2002).

The mutation in the *HSPB5* gene (R120G) is involved in cardiomyopathy and heart failure in DRM patients, causing similar structural changes in the cardiomyo-

cyte cytoskeleton. Mitochondrial organization is highly controlled in muscle and in cardiac myocytes, and cytoskeletal integrity is important for this organization (Sanbe et al. 2004). When the R120G mutation is present in cardiomyocytes, it leads to visible aggregates, and this accumulation results in deficient contractile behaviour. Mitochondrial permeability transition is clearly affected in R120G-transfected cardiomyocytes and precedes an increase in the levels of apoptotic markers (Maloyan et al. 2005). Two types of aggregates have been described when the mutated HSPB5 (R120G) is overexpressed in a mouse model: type 1 aggregates are HSPB5-positive and contain only traces of desmin, whereas type 2 aggregates are both desmin- and CryAB-positive (as desmin aggregates). Hearts carrying desmin mutations show that the stable expression of a mutated chaperone in the heart leads to compensated hypertrophy and eventually heart failure (Wang et al. 2001). The loss of HSPB5 chaperone function subsequently leads to desmin misfolding and eventually the formation of characteristic aggregates. During this congenital myopathy, the R120G mutation seems to act synergistically with functional losses or gains. In summary, HSPB1 and HSPB5 are targets for the development of future therapeutic strategies against myopathies and a diverse group of other pathologies including neurodegenerative diseases, asthma, cataracts and cancers (Arrigo et al. 2007).

21.4 Role of HSP on Muscle Inflammatory Disorders

Inflammatory myopathies (IMs) constitute a heterogeneous group of immune-mediated inflammatory muscle diseases, which all have the presence of moderate to severe muscle weakness and inflammation in the muscle or associated tissues, such as in the blood vessels that supply the muscles. The main subtypes of IM are dermatomyositis (DM), polymyositis (PM), necrotizing autoimmune myositis (NAM) and sporadic inclusion body myositis (sIBM), which are clinically, histologically and pathogenically distinct (Amato and Greenberg 2013; Dalakas 2011; Dalakas and Hohlfeld 2003). These disorders present with primary and autoimmune pathogenesis, mediated by cytotoxic T-cells, complement-mediated microangiopathy or macrophages, and there has been reports that autoantibodies are also involved. In all IM forms, patients develop a myopathy characterized by muscle weakness; in advanced disease, respiratory muscles may be affected (especially in NAM and aggressive forms of DM and PM) (Dalakas 2011). Several studies have associated Hsp with IMs at different levels, and in this section, the role of Hsp in the main subtypes of this pathology will be highlighted.

21.4.1 Dermatomyositis

The term “dermatomyositis” (DM) describes patients with an inflammatory myopathy that is associated with dermatological findings. DM is a multisystemic autoimmune disease that mainly involves skin and skeletal muscle but may affect internal

organs, such as the coronary arteries and heart. DM can be classified into juvenile DM (JDM), when the onset age is below to 16 years, or adult form (Mammen 2010). The characteristic muscle pathology of DM is the presence of capillary abnormalities, perifascicular atrophy, and abnormal muscle fibres appearing around the periphery of some muscle fascicles bordering the perimysial connective tissue. DM differs from the other IMs due to a characteristic red or heliotrope rash (typically a blue-purple discoloration) accompanying or, more often, preceding muscle weakness (Amato and Greenberg 2013; Malik et al. 2016; Salajegheh et al. 2010). In DM, regenerating muscle fibres and perifascicular atrophic muscle fibres present with increased HSP70 and HSP90 levels compared with those of normal control muscle with aggregates present. The perifascicular inflammatory aggregates presented with diffuse HSP70 staining, while the scattered inflammatory cells are mostly HSP90 negative. In inflammatory DM and PM tissues, an increased HSP70:HSP90 ratio has been observed, and a generally protective role for HSP90 and HSP70 in damaged muscle fibres and a specific cytotoxic role for HSP90 in muscle fibre invasion have been suggested (De Paepe et al. 2009). HSP70 and HSP90 have an important but not complete overlap in muscle fibre regeneration, showing the general but also individual roles of Hsp in muscle fibre regeneration (Paepe et al. 2012). The heat shock 70 kDa protein 5 (HSPA5) levels are significantly higher in serum from DM patients than in control patient serum (Xiao et al. 2015); muscle fibres from MHC-I-positive patients also present with an increase in HSP70 chaperone expression, and the Hsp facilitate the cross-presentation of major histocompatibility complex class I and II (MHC-I and MHC-II) (Li et al. 2009). Moreover, the serum HSP70 levels in patients with clinical remission after steroid treatment decrease significantly, suggesting reduced endoplasmic reticulum stress in the muscle; therefore, the quantification of HSP70 levels may be useful for estimating prognoses (Xiao et al. 2015). On the other hand, in JDM, HSP60 expression is increased in affected muscle tissue; HSP60-specific T-cells have a potential immune regulatory role in JDM with a proinflammatory cytokine profile (IL-1β, TNFα and IL-10). HSP60 plays an active role in controlling inflammation in JDM patients (Elst et al. 2008).

21.4.2 Polymyositis and Sporadic Inclusion Body Myositis

PM affects skeletal muscles on both sides of the body. It is rarely seen in persons under 18 years old; most cases are in people between the ages of 31 and 60. PM patients present with progressive muscle weakness that leads to difficulty swallowing, speaking, standing from a sitting position, climbing stairs, lifting objects, or reaching overhead; PM mimics many other myopathies. On the other hand, sIBM is a progressive myopathy characterized by proximal and distal muscle weakness and atrophy. The onset age is over 50 years old, and the electrophysiological symptoms resemble a motor neuronal disease. New evidence suggests that some inherited types of sIBM not only affect skeletal muscles but also motor neurons. Ageing might be a synergistic factor in sIBM progression, and this factor might emphasize

the degenerative aspect of the pathophysiological mechanism of this disease (Hori et al. 2014).

An important hallmark for PM and sIBM is the invasion of autoaggressive immune cells (macrophages, CD8+ cells, CD4+ cells, myeloid dendritic cells) into non-necrotic muscle fibres; interestingly, HSP90 is highly expressed by actively invading cells (mostly $CD68^+$ some $CD3^+$) that target non-necrotic muscle fibres, and upregulated HSP90 levels were coupled with high chemokine levels. HSP70 is weakly expressed in most inflammatory cells, and its expression does not increase during active invasion. HSP90, but not HSP70, levels were increased in macrophages, and the expression level was positively correlated with the inflammation grade (De Paepe et al. 2009; De Paepe and De Bleecker 2013). The vacuolated fibres of biopsies from sIBM patients express high levels of HSP90 throughout the fibres, while HSP70 expression in primarily localized to the inclusions and rims of vacuoles. The presence of HSP70 in aggregates is probably due to an attempt to clear unfolded or misfolded proteins from the dysfunctional myocyte; thus, HSP70 overexpression may serve as a treatment for improving the degradation of misfolded proteins (De Paepe et al. 2009). Additionally, it has been shown that HSP90 has an important role in regulating HSP70; inhibiting HSP90 induces HSP70 expression (Elo et al. 2005). Furthermore, HSPB5 is associated with cell stress and amyloid clearance and colocalized with APP (amyloid precursor protein) and MHC-I before the appearance of amyloid deposits and before T-cell invasion, indicating that HSPB5, together with pro-inflammatory markers, are an early event associated with the cell stress response that precedes the β-amyloid accumulation associated with APP overexpression.

For treating the pathology described above, there is an experimental approach that modulates Hsp activity with the aim of dampening the detrimental aspects of both degeneration and inflammation; for example, arimoclomol (NCT00769860, phase 2a, double-blind randomized controlled trial; 24 patients), a safe and well-tolerated drug that prolongs the activity of heat shock factor-1 (HSF1), co-inducing *Hsp* gene expression, only acts on unstressed cells (where HSF1 is already activated). Arimoclonol ameliorates degenerative and inflammatory factures in IBM patients and showed neuroprotective effects; therefore, this drug may potentially be therapeutically beneficial for patients with sIBM; however, further investigation is needed (Ahmed et al. 2016; Haq and Tournadre 2015; Malik et al. 2016; Needham and Mastaglia 2016). The prominent HSP90 expression observed in patients with PM and sIBM suggests a new therapeutic approach, leading to decreased IBM-associated protein aggregation (Paepe et al. 2012).

21.5 Role of HSP on Hereditary Neuropathies

Hereditary neuropathies were first described in 1886, and they are classified into four groups: motor and sensory neuropathy, sensory neuropathy, motor neuropathy, and sensory and autonomic neuropathy. The symptomatology varies according to

the disease type; however, patients may develop feet and hand pain, muscle weakness, numbness, and tingling among other symptoms. Some hereditary neuropathies have mild symptoms and may be undiagnosed for years, but others are more severe. Currently, more than 200 forms of hereditary neuropathies exist, and some of them, such as Charcot-Marie-Tooth disease, have been associated with Hsp mutations (Weis et al. 2017).

21.5.1 Charcot-Marie-Tooth Disease

Charcot-Marie-Tooth disease (CMT) is a group of hereditary polyneuropathies with a common clinical phenotype; CMT is the most common inheritable neuromuscular disorder (1 per 2500) and is characterized by distal atrophy of the lower limbs, pes cavus (cavoid foot), hammer toes, difficulty running, twisting of the ankle and tripping, difficulty walking, foot drop, sensory loss of the distal lower segments and then upper limbs, difficulties with hand manipulation and reduced or absent deep-tendon reflexes (Barisic et al. 2008). CMT is classified by hereditary, autosomal-dominant inheritance (AD) or autosomal-recessive inheritance (AR), and the pathology is either demyelinating or axonal (Pareyson and Marchesi 2009). CMT is subdivided into two main groups: type 1 is the demyelinating form that is characterized by low nerve conduction (less than 38 m/s), and type 2 is the axonal form, in which HSP have an important role. Finally, there is an extra small group, in which motor axons are exclusively affected, named distal hereditary motor neuropathy (dHMN). Further CMT classification is based on the causative genes and assigned loci (Gentil and Cooper 2012; Pareyson and Marchesi 2009).

21.5.2 Charcot-Marie-Tooth 2 Disease

Charcot-Marie-Tooth type 2 (CMT2) is a genetically heterogeneous, autosomal-dominant axonal neuropathy. CMT2 occurs in approximately 20% of all CMT patients. CMT2 has a late onset and usually a less severe course. The important role of HSP has been described in the CMT2 phenotype development, where HSPB1 and HSPB8 protein mutations are involved (Reilly 2009). A mutation in the locus 7q11-q21 has been reported in a Russian family with autosomal dominant CMT2 and is associated with a missense mutation (S135F) in exon 2 of *HSPB1* gene (404C→T) (Ismailov et al. 2001). Moreover, 6 more unrelated families, from Belgium, Russia, UK, Croatia and Austria, were reported to have CMT2 or dHMN with HSPB1 mutations, and these mutations were absented in 200 control individuals. Four of the mutations occurred in the Hsp20α-crystallin domain, and the fifth mutation was in the variable C-terminus of HSPB1. Importantly, the mutation in *HSPB1* S135F (404C→T) is the only mutation reported that can cause either CMT2 or dHMN (Evgrafov et al. 2004). In addition, other mutations (*HSPB1*, 379C→T)

have been reported in patients with CMT2 disease with a late onset (35–60 years) and mild sensory disorder (Tang et al. 2005b), and although this mutation is not common, it also causes dHMN (Evgrafov et al. 2004). Many Hsp mutations have been identified in both diseases; however, the mechanism that leads to the same mutation causes the clinical manifestation of either CMT2 or dHMN remains unclear (Amornvit et al. 2017; Capponi et al. 2011; Evgrafov et al. 2004; Luigetti et al. 2010; Solla et al. 2010; Tang et al. 2005a, b). Mutations in the *HSPB8* gene have also been found in patients with CMT2 and dHMN (Irobi et al. 2004). The fact that the same mutation can lead to both clinical manifestations and the fact that some patients are classified as intermediate types, led to the hypothesis of a continuum between CMT2 and dHMN, suggesting that pathological classification should be reconsidered (Irobi et al. 2006; Solla et al. 2010).

HSPB1^{S135F} transgenic mice present with a CMT2 phenotype, including affected mitochondrial transport and axonal transport deficits due to decreased acetylated α-tubulin levels in the sciatic nerve (d'Ydewalle et al. 2011). Another *hspb1* gene mutation (146C→T) in the α-crystallin domain of HSPB1 is expressed in SH-SY5Y cells and substantially increased apoptotic gene levels, suggesting cell death, and the same mutant expressed in HeLa cells resulted in an accumulation of misfolded proteins (Amornvit et al. 2017). The mutation that affects the N-terminus of HSPB1 is associated with hereditary motor neuron diseases, increases the stability of large HSPB1 homooligomers, prevents homooligomer phosphorylation-dependent dissociation, modulates the interaction with HSPB6 and decreases the chaperone capacity, preventing normal HSPB1 function (Muranova et al. 2015; Nefedova et al. 2015).

21.5.3 Charcot-Marie-Tooth Distal Hereditary Motor Neuropathy Form II

Distal hereditary motor neuropathy (dHMNs) are characterized by peroneal muscular atrophy without sensory symptoms; dHMN type II displays normal or modestly reduced nerve conduction velocity and is associated morphologically with axonal degeneration, without conspicuous segmental demyelination. Patients develop progressive distal weakness and lower limb atrophy, which progresses through the upper limbs, and foot deformations can appear. CMT2 presents with only minor sensory symptoms, making this disease difficult to distinguish from dHMNs; importantly, both conditions can be caused by mutations in the same gene, showing some genetic overlap (Lupo et al. 2016).

In dHMNs, different cellular functions are affected, including RNA metabolism, axonal transport, cation channel activity, transcriptional control and protein folding or misfolding, in which sHsp are involved. HSPB1 (ubiquitously expressed), HSPB3 and HSPB8 (tissue-specific expression) are ATP-independent chaperons associated with dHMNs (Gentil and Cooper 2012; Nefedova et al. 2015). Mutations in *hspb1* gene have been identified in many dHMN families. These mutations are located in three domains (N-terminus, α-crystallin and C-terminus); the majority induce frameshifts or premature stop codons that affect chaperone activity and cyto-

skeletal functions. The overexpression of mutated HSPB1 produces protein aggregates that modify neurofilament transport in the axon. It has been reported that transgenic mice overexpressing the human mutant HSPB1, P182L ($HSPB1^{P182L}$), in neurons mimics a dHMN phenotype (pure motor neuropathy); this transgenic mouse line has several defects such as axonal loss and decreased innervated neuromuscular junctions without axonal transport deficits. General motor coordination, muscle force, and the hind paw angle are more affected in the $HSPB1^{P182L}$ mice than in the $HSPB1^{S135F}$ mice, which mimics a CMT2 phenotype (d'Ydewalle et al. 2011; Evgrafov et al. 2004).

HSPB3 is a tissue-specific protein that is expressed in the heart, brain, skeletal and smooth muscle; it is strongly expressed in muscle (Sugiyama et al. 2000). An HSPB3 mutation (R7S) involves the loss of a conserved arginine, associating this HSPB3 mutations with axonal motor neuropathy (Kolb et al. 2010). Moreover, two mutations described in HSPB8 (K141N and K141E) that affect the structural and functional activity of the central α-crystallin domain of this protein have been reported to be associated with dHMN; both missense mutations were found in Czech, Bulgarian and English nonrelated families. These mutations do not abolish the interaction between HSPB1 and HSPB8 but strengthen it; in an immunoprecipitation assay using transiently EGFP-tagged wild-type and mutant HSPB8, both mutated HSPB8 proteins pulled down more HSPB1 than wild-type HSPB8. Nonetheless, both mutants increased aggregate formation, even when the expression levels of mutant and wild-type HSPB8 were similar (Irobi et al. 2004). Another HSBP8 protein mutation found in a Korean CMT2 patient involves lysine 141 (K141T), which seems to be a mutational hot spot and can lead to different phenotypes (Nakhro et al. 2013). This mutation affects axonal transport function, causing motor neuron death in dHMNs. Even if HSPB8 is ubiquitously expressed, only motor neurons seem to be significantly affected by this mutation, which explains the phenotype: mutant HSPB8 does not affect sensory or cortical neurons (Irobi et al. 2004, 2010). It has been demonstrated that Dm-HSP67Bc (*Drosophila melanogaster* HSP67Bc), the closest functional orthologue of human HSPB8, can decrease the formation of mutant HSPB1 aggregates, whereas the overexpression of HSPB8 activity might defend against misfolded or aggregate protein-associated diseases (Carra et al. 2010). Changes in protein structure, gain-of-function effects, aggregate formation, and alterations in apoptotic pathway regulation are some of the possible effects of Hsp mutations that lead to some neuropathies, but the definitive pathomechanism of *hspb1* gene mutations remains unclear and cannot be fully explained by altered chaperone function (Lin et al. 2011).

21.6 Conclusions

Many different cell processes are affected as a consequence of Hsp mutations, leading to clinically and pathologically heterogeneous manifestations, including the following: aggregate formation, disrupted cytoskeletal function, axonal structure, functional alterations, and impaired protein degradation processes. As we

mentioned above, the mutations present in some Hsp members, specifically mutations found in HSP70, HSP60 and HSP40, and in sHsp members, such as HSPB1, HSPB3, HSPB5 and HSPB8, have been associated with the development of several diseases, such as muscular dystrophies, skeletal muscle myopathy, inflammatory myopathies and hereditary neuropathies. Nevertheless, the precise pathological mechanism by which Hsp mutations lead to clinical symptoms remains unclear. Therefore, more research is needed to clarify the mechanism of Hsp action and to propose and test an effective treatment against these diseases. Finally, the multifactorial origin of myopathies makes proposing or developing a single and/or specific treatment for these diseases difficult. Therapies may use several strategies, including physical therapy, drugs, and surgery, and all these therapies are available to ameliorate muscle pain and weakness. However, further studies need to be developed to find the best way to treat these diseases. Collectively, the data presented here support Hsp as a key target for treating various skeletal muscle disorders. In summary, HSP are involved in maintaining muscle function; however, although much has been discussed about the expression, function and cellular location of HSP in skeletal muscle damage or injury, many studies have not been able to specifically determine the functions of these proteins in skeletal muscle and neuromuscular disorders.

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Chapter 22
Heat Shock Proteins in Neural Signaling: Implications in Health and Disease

Pooja-Shree Mishra and Aditi Singh

Abstract Much of the recent work has given insights into the non-canonical roles of heat shock proteins, strongly indicating a potential avenue for exploring the contribution of these proteins towards the normal physiology as well as various pathological conditions apart from hyperthermia-related stress. In this chapter, the role of intracellular as well as extracellular heat shock proteins in regulating various cellular signalling pathways has been discussed, with an emphasis on the biochemical and functional aspects of signalling in the central nervous system throughout various developmental stages. Further, their influence in regulating various pathological pathways including apoptosis and immune responses has been highlighted concerning different acute and chronic neurological disorders. Finally, the possibility of heat shock proteins as potential neuroprotective targets and the clinical relevance of their intracellular as well as extracellular forms has been explored.

Keywords Clinical modulation · Heat shock proteins · HSP release · Neural signalling · Neuroinflammation · Neuroprotection

Abbreviations

AIF	apoptosis-inducing factor
Akt	protein kinase B
APCs	antigen presenting cells
CNS	central nervous system
CTL	cytotoxic T lymphocytes
eHSP	extracellular/exogenous HSP
Hsc	constitutive isoform of Hsp

P.-S. Mishra (✉)
Centre de Recherche CERVO, Quebec City, QC, Canada
e-mail: pooja-shree.mishra.1@ulaval.ca

A. Singh
Department of Biotechnology, TERI School of Advanced Studies, New Delhi, India

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Hsf1	heat shock factor1
Hsp	heat shock protein
HSP	heat shock protein family
IKK	IκB kinase
IL	interleukin
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PrPC	cellular prion protein
ROS	reactive oxygen species
STI1	stress-inducible protein 1
TGFβ	tumor growth factor beta
TLRs	toll-like receptors
TNFα	tumor necrosis factor alpha

22.1 Introduction

The serendipitous discovery of heat shock proteins five decades ago gave a new impetus to cellular biology. Heat shock proteins (HSP) are highly conserved across the living forms and comprise a heterogeneous group consisting of six major families, based on their molecular weights. These include small HSP/ HspB(12–43 kDa), Hsp40(DNAJ), Hsp60 (HspD), Hsp70 (HspA), Hsp90 (HspC), and Hsp110 (HspH) (Kampinga et al. 2009). The conventional properties of the heat shock proteins (HSP) to act as chaperons and facilitate protein folding or prevent protein aggregation are well known. However, more and more studies increasingly provide evidence towards the contribution of HSP in various other processes in normal and pathological physiology, ranging from autophagy, vesicle fusion, signal transduction, apoptosis and proteasomal degradation (Muchowski and Wacker 2005). Many or all of these properties of HSP find their application in the maintenance of vital functions in the physiology or pathology of living forms ranging from prokaryotes to mammals. In this chapter, we will walk through the role of HSP in influencing various signalling pathways, focussing on their impact on the central nervous system (CNS) and its disorders.

Apart from the chaperoning functions, HSP are known to influence various cellular processes by modulating a myriad of signalling pathways (Basu et al. 2000; Mehlen et al. 1996; Murashov et al. 2001; Stokoe et al. 1992). Hsp70/HspA is the most widely studied family of heat shock proteins, which perform housekeeping roles in cells and either the depletion or overstimulation of their functions may regulate the signalling circuit of numerous cellular pathways. Studies conducted on Hsp70 demonstrate its ability to inhibit p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK)-dependent signalling pathways (Gabai et al. 1997; Park et al. 2001). Moreover, intracellular overexpression of Hsp70 or its

pathological induction could reduce the production of inflammatory markers like nitric oxide, iNOS, TNFα, IL1, IL12 and Reactive Oxygen Species (ROS) by inhibiting NFκB activation (Ding et al. 2001; Feinstein et al. 1996; Heneka et al. 2000; Tang et al. 2007; Van Molle et al. 2002; Zheng et al. 2008). Interestingly, the mechanism of NFκB pathway inhibition by Hsp70 was found to be multifaceted, acting either directly or through the regulatory proteins, depending on the nature of the stimulus. For instance, in an experimental model of stroke, Hsp70 acted by preventing IκB phosphorylation by IκB kinase (IKK); while in a TNFα mediated insult, Hsp70 inhibited the IKKγ activity directly, thus impairing NFκBmediated survival signalling (Ran et al. 2004; Zheng et al. 2008). In addition, a recent study proposed a combinatorial mode of NFκB regulation by Hsp72 overexpression in the TNFα model, by reducing NFκB DNA binding activity, as well the number of RelA/p65 NFκB subunits in the cells, along with modulating the IκBα inhibitor phosphorylation (Sheppard et al. 2014). Moreover, increased levels of Hsp72were found to be associated with reduced nuclear NFκB translocation (Guzhova et al. 1997).

Additionally, HSP including Hsp70, Hsp90 and small heat shock proteinHsp27 have been shown to modulate Akt kinase activity by promoting its active, phosphorylated form, thus possibly controlling apoptosis during normal development as well as the diseased state (Beere 2001; Murashov et al. 2001; Rane et al. 2003; Sato et al. 2000; Stetler et al. 2010).HSP can also effectively function as apoptosis suppressors by sequestering apoptosis-inducing factor (AIF) and preventing caspase activation, as well as the formation of a functional apoptosome (Beere 2001; Beere et al. 2000; Matsumori et al. 2005).

22.2 Extracellular HSP in Cellular Signalling

The discovery of heat shock proteins in the extracellular space nearly three decades ago provided newer insights into the already broad spectrum of roles played by HSP. It is logical to assume a possible role of this extracellular/exogenous HSP (eHSP) in signalling. Indeed, these exogenous proteins play a crucial role in various cellular processes, with a major contribution towards immune homeostasis by influencing both, the innate and adaptive immunity (Henderson 2010; Matsumori et al. 2005). For instance, eHSP are known to interact with macrophages and Antigen presenting cells (APCs), e.g. dendritic cells mediated by surface receptors, mainly Toll-like receptors (TLRs). This interaction further leads to inflammation by upregulating the pro-inflammatory cytokines and other inflammatory mediators like iNOS, via the NFκB pathway (Beere et al. 2000; Matsumori et al. 2005; Srivastava 2002). Hsp70 and Hsp90 B1(gp96) generate adaptive, MHC-specific CD8(+) cytotoxic T lymphocytes (CTL) response in-vitro (Robert et al. 2002). In-vivo, the chaperoned minor histocompatibility antigenic peptides have been shown to cause graft rejection by Hsp90 B1 in the class I-deficient, immunocompetent Xenopus larvae, in the absence of chaperoned peptides, thus also highlighting the role of HSP in MHC independent innate immunity (Robert et al. 2002). Hsp70, in its capacity to

act as a TLR agonist, has been shown to act as a CD8(+) CTL and TH-1 adjuvant, as well as to elicit pro-inflammatory responses in the DCs by binding to the surface TLR4 and activating the MAPK and NFκB pathways (Fang et al. 2011; Wan et al. 2004; Wu et al. 2005). In what appears to be a relevant terminology, Asea termed Hsp70 a 'chaperokine,' emphasising the ability of eHSP to be able to modulate cellular activity by stimulating the production of pro-inflammatory cytokines and chemokines, as well as promoting expression of co-stimulatory molecules, thus regulating immune responses (Asea 2008; Fleshner and Johnson 2005).

Apart from TLRs, other less common signalling receptors known to interact with eHSP are scavenger receptors, CD40 and CD91 receptors present on the APCs (Floto et al. 2006; Pawaria and Binder 2011; Sedlacek et al. 2017; Wang et al. 2001; Zhou and Binder 2014). Here, the interaction of eHSP including Hsp90 B1, Hsp70 and calreticulin (CRT) with the CD91 receptors is mention-worthy, as it has been shown to result in the maturation of DCs and initiation of diverse inflammatory cytokine responses via the NFκB pathway, further leading to the priming of unique and specific subsets of Th-1 (Pawaria and Binder 2011).

HSP signalling functions can also be modulated by their extracellular co-chaperone interactions where together they form a network which has been known to cause malignancy (Moulick et al. 2011; Rodina et al. 2016; Workman et al. 2007) as well as neurodegeneration (Lindberg et al. 2015), when altered.Stress-inducible protein 1 (STI1) performs an intracellular co-chaperonic function by transferring client proteins from Hsp70 to Hsp90 and is known to inhibit Hsp90 ATPase activity. However, it was also found to suppress apoptosis through its interaction with PrPCat the cell surface via modulation of PI3K and/or MAPK/ERK signalling pathways, a process mediated by mTOR (Lackie et al. 2017; Roffé et al. 2010).

22.3 eHSP Release and Uptake

Since the majority of cytosolic HSP lack the 'leader sequences' carrying signals to be secreted through the classical ER-Golgi trafficking, it is likely that the process of HSP releases adopts unconventional alternative pathways for the active transport of these molecules (Nickel and Seedorf 2008). An explanation would be the protein release following necrotic cell death, which was indeed found to be true in a study, whereHsp90B1, calreticulin, Hsp90 and Hsp70 were found to be secreted in soluble forms post necrosis, and were able to interact with the APCs, as well as stimulate macrophages to induce cytokine production through NFκB pathway (Basu et al. 2000).

One of the proposed pathways is a lysosome–endosome pathway described by Mambula and colleagues where they discuss the translocation of Hsp70 to lysosomes, possibly via an ATP binding cassette (ABC) transport-like system to export the HSP outside the cell via the endocytic process (Mambula and Calderwood 2006). Another interesting study indeed did find Hsp70 in the lysosomal lumen of tumour cells, possibly functioning to extend survival by inhibiting the death-associated permeabilization of lysosomes (Nylandsted et al. 2004). A different

study highlighted the association of HSP with lipid-less, chromogranin-A positive secretory-like granules (Evdonin et al. 2006). Moreover, Rabouille et al. proposed the extracellular release via protein pore across the lipid membranes (Rabouille et al. 2012). However, the most accepted mechanism of HSP release is the vesicular export. Vega et al. (2008) proposed the mechanism similar to the lysosomal pathway where Hsp70 was seen inserted in the vesicular membrane and was able to potentially induce TNF-α production, thus signalling the inflammatory pathways. This has been evidentially supported by other studies reporting the presence of various HSP including Hsp70, Hsc70, Hsp27, and Hsp90 in the lumen of the exosomes, during a B-cell response to heat stress (Clayton et al. 2005). Additionally, a two-step active mechanism of eHSP release has been hypothesized, in which firstlytheHSP gets inserted into the lipid membrane followed by its release within the extracellular vesicles (De Maio 2011). It has been corroborated by the presence of HSP in extracellular vesicles obtained from different sources (De Maio 2011; Théry et al. 2009). Further, Multhoff et al. showed the presence of Hsp70 on the cell surface with their true insertion in the plasma membrane (Multhoff 2007; Multhoff and Hightower 1996). Human Hsp70 (HspA1) and bacterial Hsp70 (DnaK) have been studied for their interaction with liposomes, and both were confirmed to be inserted into the lipid membrane (Lopez et al. 2016). Hsp70 is highly specific for negatively charged phospholipids like phosphatidylserine and is majorly inserted into the membrane with only a small N-terminal region exposed outside the liposome. The c-terminal end is the region proposed to be containing the oligomerization domain which is initiated only after conformational changes, possibly due to the lipid-protein interaction induced upon insertion of HSP in the lipid membrane (Armijo et al. 2014).

While most of the leaderless proteins are released directly in the extra-cellular milieu following lysis of vesicular membrane;HSP are reported to be present in both, membrane-bound, as well as free-form and may signal different processes.The membrane-bound form of Hsp70 was found to be dramatically potent in increasing TNFα production in macrophages as compared to the recombinant form of HSP (Vega et al. 2008). In another study, membrane-bound Hsp70 elicited an intracellular calcium flux that could modulate pro-inflammatory cytokines in monocytes by inducing NFκB activity via at least two different signalling pathways depending on its interaction with CD14 (Asea et al. 2000).Hence, membrane-bound HSP offer potential avenues and need to be further explored for their possible implications in health and disease.

22.4 HSP and Neural Signalling: Implications in Normal Physiology

Several in-vitro, as well as in-vivo studies, document significant roles of intracellular and extracellular HSP in neurophysiological events. In a Drosophila model, mutations in E(sev)3A (HSP83) were shown to impair signalling by the sevenless receptor tyrosine kinase, thus negatively affecting the differentiation of the R7 photoreceptor neuron in Drosophila (Cutforth and Rubin 1994). Constitutive expression

of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp60, has been reported in neural and non-neural rat tissues during postnatal development (D'Souza and Brown 1998). Further, investigations in mice models revealed spatially and temporally distinct patterns of the HSP family proteins including Hsp90, Hsp25, Hsc70 and Hsp70 during E9.5 to E17.5, a period of active neuronal differentiation during the embryonic development (Loones et al. 2000). Moreover, molecular interactions of Hsp90 with protein kinases and transcription factors including steroid receptors (Picard et al. 1990), basic helix-loop-helix (bHLH) factors (Shaknovich et al. 1992) and heat shock factor 1 (Hsf1)(Ali et al. 1998) have been shown to influence various signalling pathways, eg., the morphogenesis of the nervous system.(Kageyama and Nakanishi 1997). Conclusively, these studies provide evidence of the HSP' ability to regulate early CNS development.

In a study conducted on murine cells, constitutive expression of Hsp27 was able to block apoptosis by regulating Fas/APO as well as protein kinase C pathway (Mehlen et al. 1996). In line with these observations, the transient accumulation of Hsp27 could regulate the process of differentiation and apoptosis in the mammalian olfactory neurons in-vitro, possibly by promoting differentiation over apoptosis (Mehlen et al. 1999). Akt pathways, which are a common target of HSP, have been associated with controlling apoptosis in the differentiating neuronal population along with neurotrophins and shown to induce neuritogenesis in PC12 cells (Crowder and Freeman 1998; Eves et al. 1998; Philpott et al. 1997). Further, in a study investigating the effects of axotomy on the cultured DRG neurons, upregulation of Hsp27 was observed, which also hints towards its role in neurite outgrowth and axon elongation (Gabai et al. 1997). Interestingly, the observation of negative regulation of apoptosis has been observed in the hippocampal neurons as a result of the interactions between co-chaperone STI1 and PrPC at the cell surface. These results provide interesting leads into the non-conventional signalling role of the conventional chaperoning attribute of HSP in relation toPI3K and MAPK/ERK-mediated signalling in neuronal pathophysiology (Roffé et al. 2010). Moreover, the influence of HSP on the development of CNS may not be limited only to the early stages. eHsp70 has been shown to be involved in reversing ageing effects by preventing ageing-related molecular alterations to the synaptic structure, thus ameliorating cognitive functions and delaying senescence (Bobkova et al. 2015).

It is interesting to note that Hsp70 can interact with membranes and modulate their conductance properties (Alder et al. 1990). Hsc70 /Hsp70 have been shown to integrate into the lipid bilayer and open ionic conductance across the membrane in various independent studies, thus highlighting the possible role played by these proteins in neuronal functioning (Arispe and De Maio 2000; Vega et al. 2008). Another remarkable phenomenon is the contribution of HSP in preserving synapse strength and maintaining the homeostatic synaptic plasticity as observed in the neuromuscular junction (NMJ) in Drosophila and locusts (Davis 2013; Dawson-Scully and Meldrum Robertson 1998; Karunanithi et al. 2002; Karunanithi et al. 1999). Hsp70 was able to induce structural plasticity of axonal terminals in Drosophila motor neurons thus upregulating neurotransmitter release at NMJ in response to heat shock (Xiao et al. 2007). Similar results were observed in mammalian CNS

when Hsp27 and Hsp32 were induced in the Bergmann glial cells in response to hyperthermia and found translocated into the synaptic elements as well as perisynaptic glial processes projecting into the 'synaptic-enriched' molecular layer of the cerebellum (Bechtold and Brown 2000). Additionally, Hsp70 was also found to be chiefly associated with the pre and postsynaptic elements in response to hyperthermia (Bechtold et al. 2000).

Here, the ability of eHsp70 to activate membrane potassium channels is mentionworthy (Negulyaev et al. 1996). Investigations on the motor neurons and visual interneurons in locust demonstrated increased action potential amplitude at elevated temperatures, possibly due to the prevention of the inactivation of voltage-gated Na+ channels, thus maintaining homeostatic intrinsic plasticity (Karunanithi and Brown 2015). Further, HSP were also found to interact with Voltage-gated calcium and potassium channels (Gao et al. 2013a, b; Krieger et al. 2006). These observations hint towards a potential role of HSP in altering membrane properties, thereby modulating the synaptic transmission across the nervous system. Furthermore, there are several reports that trace glial cells as the major source of eHSP in the neuronal milieu. Some of the earlier studies reported an HSP cross-talk between glial cells and neurons when glial cells were found to release HSP like proteins that were taken up by the axon of the giant squid neuron (Hightower and Guidon 1989; Tytell et al. 1986). Further experiments conducted on Human T98G glioma cells and differentiated LA-N-5 neuroblastoma cells could provide evidence for the neuronal uptake of glial Hsp70 in the normal conditions. During stress conditions, an increase in the levels of secreted Hsp70 was reported, and it was shown to confer stress tolerance to the mature neurons deficient in Hsp70 (Guzhova et al. 2001). Although mammalian neuronal cells have been shown to release Hsp70 and Hsp110 in response to a heat shock in-vitro (Hightower and Guidon 1989), it is also known that most neurons fail to express adequate amounts of HSP owing to transcriptional stringency (Tonkiss and Calderwood 2005). This leads to an HSP deficit in neurons, particularly throughout the long axonal distances stretching to the synapses, due to inadequate transportation (Tytell 2016). The glial HSP thus uptaken by neurons may indeed aid in rapid and efficient neuronal functioning or neuroprotection while helping the neurons maintain the synaptic homeostasis.

22.5 HSP and Neural Signaling: Implications in CNS Pathophysiology

There is an increasing evidence of the involvement of HSP in various neurological disorders by the virtue of their ability to repair and/or regulate various physiological and pathological processes cellular processes including innate and immune responses, apoptosis, protein misfolding and proteasomal degradation (Basu and Srivastava 2000; Shevtsov et al. 2014; Turturici et al. 2014; Wyttenbach and Arrigo 2009).Studies concerning the tissue-specific inhibition and induction of these

proteins demonstrate the influence of HSP as immunoregulatory agents (Coelho and Faria 2012; Dubey et al. 2015). What makes HSP interesting molecules to study is their dual role in eliciting either pro or anti-inflammatory responses based on the nature of the stimulus received. It could partly be explained by the fact that HSP can interact with multiple receptors engaged in signalling a multitude of divergent pathways that may cross-talk with each other. Further, HSP contain alternative promoter recognition sites which allow their transcriptional activation via multiple transcriptional pathways and cellular conditions, thus making it an intriguing molecule in the multifactorial conditions like immune responses (Stetler et al. 2010).

Moreover, the functional relevance of HSP mediated responses can significantly be realised, but not limited to the immune modulation effects. For instance, investigations carried out on an animal model for intracerebral haemorrhage reported an attenuation of Blood Brain Barrier (BBB) disruption and oedema formation, as well as amelioration of the neurological deficits in response to the overexpression of Hsp70, along with a downregulation in TNF-α expression (Manaenko et al. 2010). Further, in line with the in-vitro observations of eHSP, increased levels of HSP have been found in the biological fluids including serum and Cerebrospinal fluid (CSF) of patients suffering from neurological diseases (Table 22.1) (Ce et al. 2011; Hecker and McGarvey 2011). The specific roles of various HSP with relevance to different neurological disorders are discussed in detail below.

22.5.1 Brain Tumours

In a study by Graner et al. (2007), it was found that the brain tumour cell lines expressed high levels of HSP intracellularly as well as on their cell surfaces. Further, the HSP were released in the extracellular environment, possibly through secretory vesicles, which could be engaged in modulating the tumorigenicity (Graner et al. 2007). Moreover, increased intracellular expression of HSP including Hsp27, Hsp40, Hsp60, Hsp70, and Hsp90 was found in gliomas and meningiomas; and Hsp27 was shown to correlate with total-AKT, thus regulating the cell survival pathways (Alexiou et al. 2014).

22.5.2 Epilepsy

An extensive study carried out on two different models of epilepsy shed light on yet another non-conventional role of HSP in neurotransmission (Ekimova et al. 2010). Intracerebroventricularly administered Hsp70/Hsc70 not only relocated to different brain areas including cortex, hippocampus, thalamus, hypothalamus and pontine reticular formation but could also rescue their functions by acting as an anticonvulsant, as well as attenuating the severity of chemically induced seizures. The study linked the effect to the ability of Hsp70 to regulate GABAergic neurotransmission,

Table 22.1 Changes in HSP levels in serum and/or cerebrospinal fluid (CSF) of the patients suffering from various neurological diseases, along with their plausible biological/clinical relevance in these biofluids

Disease	HSP class	Source	Effect	References
AD	Hsp90, Hsp70,	Elevated CSF levels choroid plexus	Pro-inflammatory cytokine production; phagocytosis and clearance of Aβ peptides	Anthony et al. (2003), Kakimura et al. (2002). and Maarouf et al. (2009)
	HSP32	Extracellular matrix		
Ageing	Hsp70	Elevated serological levels, possible inflammatory involvement	Unknown	Njemini et al. (2004)
ALS	Hsp70, Hsp90	Elevated serological levels	Unknown	Miyazaki et al. (2016)
Ischemia	Hsp27	Elevated CSF levels	Unknown	Hecker and McGarvey (2011)
MS	Hsp27	Elevated serological levels	Unknown	Ce et al. (2011)
	Hsp70	Elevated CSF levels	Pro-inflammatory cytokine production	Chiba et al. (2006) and Yokota et al. (2010)
	Elevated CSF levels	Elevated CSF levels	Localized HSP antibody secretion	Gao et al. (1994) and Prabhakar et al. (1994)
PD	Hsp65,	Elevated CSF levels	Immunogenicity	Fiszer et al. (1996)
	Hsp70			
	Antibodies			
Tuberculous meningitis	Hsp65	Elevated CSF levels, diagnostic marker	Immunogenicity	Mudaliar et al. (2006)
Trauma	Hsp70	Elevated serological levels	Mortality prediction- biomarker	Da Rocha et al. (2005)

thus indicating the potential of these molecules in modulating the excitatory-inhibitory balance in CNS (Ekimova et al. 2010).

22.5.3 Neurodegeneration

The role of HSP in neurodegeneration has been multi-faceted. Neuroinflammation is believed to be a major contributor to neurodegenerative disorders and involves activation of both, astrocytes as well as microglia (Cherry et al. 2014; Glass et al. 2010; Mishra et al. 2016, 2017; Suzumura 2013; Zhao et al. 2013). Moreover, as previously discussed, HSP also modulate several signalling pathways including

MAPK, JNK and NFκB pathways, which appear to be significantly involved in many neurodegenerative disorders (Harper and Wilkie 2003).

In case of Alzheimer's disease (AD), it was found that several eHSP including Hsp90, Hsp70, and Hsp32could induce neuroinflammation in-vitro, followed by microglial activation and clearance of Aβ peptides, possibly via the TLR-4 mediated activation of NFκB and p38 MAPK pathways (Kakimura et al. 2002).The same study also reported that the levels of cytosolic as well as membrane-bound Hsp90 were upregulated in AD brains and the eHsp90 accumulated in the extracellular senile Aβ plaques, facilitating their phagocytosis by microglia and conferring neuroprotection. Interestingly, a recent study reported the therapeutic potential of HSP using intranasal delivery of eHsp70, that resulted in a significant amelioration of the pathology in a mice model mimicking AD. Further, the study reported a concomitant modulation of various pathways including those involved in amine transport and neurotransmission (Bobkova et al. 2014; Evgen'ev et al. 2017).

Further, Hsp27, Hsp40, Hsp60, Hsp70 and Hsp90 have also been found to be associated with Lewy neurites or Lewy bodies, the distinct protein aggregates found in Parkinson's Disease (PD) (Auluck et al. 2002; Leverenz et al. 2007; McLean et al. 2002; Uryu et al. 2006). HSP have been reported to play a neuroprotective role in PD by mitigating the α-synuclein toxicity. In one study, Hsp70 and α-synuclein co-expression have been shown to avert dopaminergic neurodegeneration in cases of α-synuclein aggregates mediated toxicity in Drosophila model (Auluck et al. 2002). Hsp70 binds to the α-synuclein filaments and alleviates their effect on the proteasome-mediated neurodegenerative pathway (Lindersson et al. 2004).In addition, Hsp70 was found to reduce the α-synuclein oligomer formation in extracellular spaces (Danzer et al. 2011). It binds to the hydrophobic residues of α-synuclein and induces an open conformation, reducing the formation of toxic α-synuclein intermediates and increasing their clearance, thereby alleviating toxicity (Klucken et al. 2006; Outeiro et al. 2008). Further, the occurrence of Hsp27 in the Lewy body positive neurons, as well as the functional impairment of their chaperonic activity due to sequestration into α-synuclein aggregates and mature Lewy bodies has been found to be overlapping with the severity and regional spread of Lewy body pathology (Ebrahimi-Fakhari et al. 2011; Iwaki et al. 1992). These results indicate that the enhancement of HSP functions in neural cells seems to be a viable approach to alleviate PD progression. Pharmacological upregulation of heat shock protein function via Hsp90 inhibitors or HSF-1 modulators holds promise as a therapeutic strategy.

Neuroprotective effects of HSP have also been reported in case of Amyotrophic Lateral Sclerosis (ALS). The inability of motor neurons to enhance the endogenous production of HSP following stress may partly explain their vulnerability status. In such a scenario, eHSP might play a neuroprotective role by acting against neuronal insults. Indeed, administration of eHsp70 in-vivo was not only able to protect the motor neurons from experimentally induced oxidative stress but could also inhibit the motor and sensory neuronal degeneration during sciatic axotomy (Robinson et al. 2008; Tidwell et al. 2004). Further, the ability of spinal astrocytes to release

Hsp70, as well as that of eHsp70 to promote motor neuronal survival against apoptosis establish a neuroprotective role of exogenous supplementation of HSP in ALS, which appears to be a promising therapeutic approach (Robinson et al. 2005).A multifold potent, anti-apoptotic effect of HSP was observed when Hsp27 and Hsp70 overexpression was carried out simultaneously (Patel et al. 2005). Interestingly, exogenous administration of Hsp70 could delay the onset and ameliorate the motor neuron pathology in a mutant SOD-1 model, further corroborating its therapeutic potential (Gifondorwa et al. 2007).

A similar effect was observed in case of Huntington's disease (HD), when eHsp70 /Hsc70 could prevent human neuroblastoma expressing pathogenic polyglutamine repeats from apoptotic cell death, while co-localising with, and reducing the pathological inclusions (Novoselova et al. 2005). Overexpression of HSP including Hsp70, Hsp40and Hsp27 was shown to rescue the normal phenotype in-vitro, by reducing the amount of pathological inclusions, as well as suppressing apoptosis and ROS mediated damage (Sakahira et al. 2002; Wyttenbach et al. 2000, 2002; Zhou et al. 2001). However, the neuroprotective effects of HSP could not be realised in-vivo in the mice models (Hansson et al. 2003; Zourlidou et al. 2007). However, the role of HSP in case of Multiple Sclerosis (MS) is controversial. Autoantibodies against Hsp70 found in the CSF of MS patients were able to enhance the production of pro-inflammatory cytokines and chemokines, thus aggravating Inflammation (Yokota et al. 2010). Extracellular HspA1A has been shown to exacerbate the immune response by acting as an adjuvant for myelin peptides and as a proinflammatory cytokine (Fleshner and Johnson 2005). In contrast, a neuroprotective role of eHspA1A has also been documented in the experimental autoimmune encephalomyelitis (EAE), (Galazka et al. 2006). Moreover, this effect was seen in response to immunisation with the HSP obtained from EAE models, and not from the healthy controls, thus igniting a debate over the differences in the interactions of HSP in normal versus pathological CNS. There is evidence of the neuroprotective role of intracellular HSP as well. Apart from the reports of its chaperoning functions for MBP, the ability of HspA1A to inhibit NFκB further renders it capable of ameliorating the pathology in MS (Turturici et al. 2014).

22.5.4 Other Neurological Disorders

The importance of HSP can be realised in many other neurological disorders including ageing, sleep disturbances and cognitive disorders, where these proteins appear to play a protective role (Bobkova et al. 2015; Hashimoto-Torii et al. 2014; Naidoo et al. 2008; Su et al. 2009). Bardsen and colleagues found that eHsp90α signalling plays an important role in chronic inflammation seen in primary Sjögren's syndrome (Bardsen et al. 2016).Moreover, the role of Hsp90 and its co-chaperone has been investigated in prion diseases, and strikingly, Hsp90 and STI1 interaction were reported to decrease PrPC dependent STI1 neuroprotection (Maciejewski et al. 2016) which suggests the interfering roles of Hsp90 in STI1 and PrPC. Thus, small

molecule inhibitors of Hsp90 and STI1 interaction may give a new perspective towards prion pathology treatment and advance the current drug development scenario.

22.6 Conclusion

In view of the regulatory roles of heat shock proteins in various pathophysiological conditions, tissue-specific inhibition and induction of HSP confer them as promising therapeutic drug candidates (Coelho and Faria 2012; Dubey et al. 2015). Controlling/manipulating the heat shock protein response seems to have a potent effect on extracellular stress response in neurological disease conditions. HSP are gaining clinical importance primarily for being cancer target (Renton et al. 2011) but also for regulation of various signalling processes in cell pathology. Targeted therapies against HSP in neurophysiological disorders are not yet available, which is mainly due to the complex action and interplay of several HSP families in neural physiology. In some cases, HSP overexpression mediates pathology while expression of others below the normal physiological levels can trigger a pathophysiological response. On the other hand, it may be the antagonistic role of different HSP families that work in coherence at differential concentrations, and during certain stress conditions, disruption of this balance leads to pathological symptoms and diseased state. For example, Hsp90 inhibition via DMAG has been reported to protect against atheroprogression and renal damage under diabetic stress. The mechanistic principal behind this protective effect is induction of Hsp70 by inhibiting the Hsp90 ATP binding site and in turn inactivating the Hsp90 associated proteins as NFκB and STAT (signal transducers and activators of transcription) thereby reduced gene expression levels of proinflammatory cytokine- TNF-α and T cell lymphokines- CCL2 & CCL5 (Lazaro et al. 2015). Hsp90 inhibitors also interfere with the TGF-β signalling pathway and ameliorate systemic sclerosis and other fibrotic diseases (Tomcik et al. 2013). HSP also interact with 14-3-3 hub proteins, one of the major players in various controlled cellular processes including cell division, apoptosis, hormonal production, signal transduction, ion channel trafficking and cytoskeleton rearrangements and consequently participating significantly in neurodegenerative diseases signalling (Sluchanko et al. 2017). 14-3-3 proteins contain an amphipathic groove by which they interact with their partners like HspB6 (Sluchanko et al. 2015; Sluchanko et al. 2011) by their phosphorylated (Ser/Thr) segments. Human Hspβ (HspB6) is phosphorylated at Ser16 position (Beall et al. 1999) which binds to the 14-3-3 groove (Sluchanko et al. 2017). Specifically targeting the phosphorylated residues of HSP interacting with 14-3-3 hub proteins may significantly contribute to the therapeutic approaches in neural pathologies. HSP are known for their neuroprotective roles proved by several studies in which the modulation towards increase HSP expression showed lesser neuronal damage (Zhong et al. 2008). Thus, not only HSP inhibitors but also the inducers of HSP expression are clinically relevant in neurodegenerative disease treatments. HSP are expressed

by the interplay of cellular signalling pathways and then regulate a network of signalling pathways themselves to control other cellular processes (Kiang and Tsokos 1996). In one study estrogen has been studied for its neuroprotective and neuromodulatory roles by induction of HSP expression where estrogen supplementation restored the impeded heat stress-induced expression of Hsp70in the brain of aged ovariectomized mice model (Valentine 2010). Till now, HSP inhibitors have been targeting the primary active sites and HSP interacting partners to modulate their chaperoning functions and the signalling functions as well. Recently, a new approach to screening was investigated in which it was shown that most of the proteins contain a biologically relevant secondary site able to bind fragments (Ludlow et al. 2015). The sequence analysis of these secondary sites revealed higher conservation and much similar physicochemical properties with primary ligand binding pockets. These secondary sites may probably work via allosteric mechanism and targetting these druggable secondary sites may be considered as potential therapeutic targets for allosteric modulation of primary sites and protein primary response in chaperoning and signalling as well.

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