**Heat Shock Proteins 13**
*Series Editors*: Alexzander A. A. Asea · Stuart K. Calderwood

Alexzander A. A. Asea
Punit Kaur *Editors*

# Regulation of Heat Shock Protein Responses

Springer

# Heat Shock Proteins

Volume 13

**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, United States of America

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, United States of America

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.

More information about this series at http://www.springer.com/series/7515

Alexzander A. A. Asea • Punit Kaur
Editors

# Regulation of Heat Shock Protein Responses

Springer

*Editors*
Alexzander A. A. Asea
Department of Medicine and 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
The University of Texas MD Anderson Cancer Center
Houston, TX, USA

ISSN 1877-1246 ISSN 1877-1254 (electronic)
Heat Shock Proteins
ISBN 978-3-319-74714-9 ISBN 978-3-319-74715-6 (eBook)
https://doi.org/10.1007/978-3-319-74715-6

Library of Congress Control Number: 2018935126



Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

# Preface

The heat shock response (HSR) is a key homeostatic mechanism that all cellular organisms utilize for resisting extracellular insult. The intracellular mediators of the HSR including the transcription factor heat shock factor 1 (HSF1) and the heat shock protein (HSP) have profoundly anti-inflammatory effects. HSF1 can be induced by the elevated temperatures encountered in inflamed tissues and in fever as well as by anti-inflammatory bioactive mediators.

The book *Regulation of Heat Shock Protein Responses* provides the most comprehensive review on contemporary knowledge on the regulation of HSP responses and its consequences to human diseases and disorders. Using an integrative approach to understanding the regulation of HSP responses, the contributors provide a synopsis of novel mechanisms by which HSP responses are regulated under normal physiological and pathophysiological conditions.

To enhance the ease of reading and comprehension, this book has been subdivided into various sections: Section I reviews current progress on the HSP and stress responses; Section II evaluates the chaperone function of HSP, including cellular proteostasis, disaggregation, protein folding, and calcium binding; Section III focuses the reader on the role of HSP in human diseases.

Key basic and clinical research laboratories from major universities and academic medical hospitals around the world contribute chapters that review present research activity and importantly project the field into the future. The book is a must read for researchers, postdoctoral fellows, and graduate students in the fields of Translational Medicine, Human Physiology, Biotechnology, Molecular Medicine, Infectious Diseases, and Pathology.

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

# Contents

# Editors Biography

**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. Asea's initial findings studying the effects of Hsp72 on human monocytes led to the proposal of a novel paradigm that Hsp72, previously known to be as intracellular molecular chaperones, can be found in the extracellular milieu where it has regulatory effects on immunocompetent 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 Publications) and is an editorial board member of 13 other scientific peer-reviewed journals. Currently, Prof. 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. Dr. Kaur's main research focus is on the use of heat-induced nanotechnology in combination with radiotherapy and chemotherapy in the cancer stem cell therapy. Dr. Kaur 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*. Dr. Kaur is an editor of five books in the highly successful *Heat Shock Proteins* book series by Springer Nature Publishers. Currently, Dr. Kaur is a Visiting Scientist Professor at the University of Texas MD Anderson Cancer Center in Houston, USA.

# Part I
# HSP and Stress Responses

# Chapter 1
# Regulation of Mammalian HSP70 Expression and Stress Response


**Kamalakshi Deka and Sougata Saha**

**Abstract** Abnormal environmental and physiological conditions can damage protein structures creating a toxic state in the cell due to loss of protein function and homeostasis. In many disease conditions the effect is so profound that interaction of structurally damaged proteins and aggregates with cellular macromolecules leads to cell and tissue damage as observed in protein misfolding related neurodegenerative disorders like Alzheimer's, Parkinson's and others. Thus structurally damaged proteins bring an organizational and functional challenge for the cells and tissue which need to be resolved very quickly and efficiently to prevent cell and tissue damage. One of the ways cell senses and mounts protective response to proteotoxic stress is by heat shock response (HSR) which constitutes high expression of chaperone proteins also called heat shock proteins (HSP) to tackle sudden increased demand for chaperones in a cell. HSR induces HSP70, one of the major chaperones, which protect cells from proteotoxic stress by prevention of misfolding and aggregation of polypeptides. Thus, a rapid and potent stress response depends on quick supply of large amount of HSP70 proteins. This extraordinary demand of HSP70 proteins is satisfied by an efficient gene expression programme which regulates HSP70 expression at every step from chromatin modification during transcriptional activation to stability of translated protein molecules. Stress dependent regulation of mammalian HSP70 expression is focus of this chapter and regulation at each of these steps will be discussed in detail.

**Keywords** Heat shock proteins · HSF1 · HSP70 · Regulation of HSP70 expression · Stress response

K. Deka · S. Saha (✉)
Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, Assam, India
e-mail: sougata@tezu.ernet.in



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_1

**Abbreviations**

| | |
|---|---|
| AD | Activation domain |
| AMP | Adenosine monophosphate |
| AMPKα | AMP-activated protein kinase α |
| ARE | AU rich element |
| ATE1 | Arginyl transferase 1 |
| ATF1 | Activating transcription factor 1 |
| Atxn | Attexin |
| BAG2 | BCL2 associated athanogene 2 |
| CBP | CCAAT binding proteins |
| CCDC127 | Coiled-coil domain-containing protein 127 |
| CCT | Cytosolic chaperonin containing *t*-complex |
| CHBF | Constitutive HSE binding factor |
| CHIP | Carboxy terminus of Hsp70-binding protein |
| COX | Cyclooxygenase |
| CPSF | Cleavage and polyadenylation specificity factor |
| CREB | cAMP response element binding protein |
| CRM1 | Chromosomal maintenance 1 |
| CSF-1 | Colony stimulating factor 1 |
| CstF | Cleavage stimulatory factor |
| CTD | C-terminal domain |
| CTF | CCAAT box transcription factor |
| DBD | DNA binding domain |
| eEF1A1 | Eukaryotic elongation factor 1A1 |
| eIF4F | Eukaryotic translation initiation factor 4 |
| ELAV | Embryonic lethal abnormal vision |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal–regulated kinase |
| GSK-3β | Glycogen synthase kinase 3 β |
| HBP | HSF1 binding protein |
| HLE | Human limbo-corneal epithelial |
| HR | Heptapeptide repeats |
| HS | Heat shock/ heat stress |
| HSE | Heat shock element |
| HSF | Heat shock factor |
| HSP | Heat shock protein |
| HSPBP | Heat shock protein binding protein |
| HSR | Heat shock response |
| HuR | Human antigen R |
| INFγ | Interferon γ |
| IRES | Internal ribosome entry site |
| JAK | Janus tyrosine kinase |
| JNK | c-Jun N-terminal kinase |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MEF | Mouse embryonic fibroblast |
| miRNA | micro RNA |
| MRPL18 | Mitochondrial ribosomal protein L 18 |
| NAD | Nicotinamide adenine dinucleotide |
| NF | Nuclear transcription factor |
| NF-IL6 | Nuclear factor Interleukin 6 |
| Nmi | N-myc and Stat interactor |
| OLA1 | Obg-like ATPase 1 |
| PGC-1α | Peroxisome proliferator-activated receptor γ coactivator 1-alpha |
| PIC | Pre transcription initiation complex |
| PKCα | Protein kinase C α |
| PKR | RNA-dependent protein kinase |
| PN | Proteostasis network |
| PP2A | Protein phosphatase 2A |
| SIRT | Sirtuin |
| SKI1 | Snf1-related kinase interacting protein |
| SNRPE | Small nuclear ribonucleoprotein polypeptide E |
| SSBP1 | Single strand DNA binding protein |
| STAT | Signal transducer and activator of transcription |
| StIP1 | Stress induced phosphoprotein 1 |
| SUMO | Small ubiquitin-like modifier |
| *SWI/SNF* | SWItch/sucrose non-fermentable |
| TNFα | Tumor necrosis factor α |
| TRiC | TCP-1 Ring *Complex* |
| UTR | Untranslated region |

## 1.1 Introduction

During our evolution, environment acts as an important selecting pressure and thus every organism lives in a habitat which has a specific environment. Such adjustment to a particular environment is achieved by establishing a homeostasis in its internal physiological components such as transcriptome, proteome, metabolome and intercellular signaling in case of metazoans. Thus, changes in the environment beyond a tolerable level or changes in the internal physiological balance put living systems under stress. To sense such changes and overcome the stressful conditions several protective pathways exist, commonly termed cellular stress response, which is aimed to reestablish homeostasis among cellular components. One such cellular stress response module is heat shock response (HSR) which is induced during many stressful conditions like temperature stress, oxidative stress, heavy metal stress and many disease conditions which causes imbalance in protein homeostasis (proteostasis) (Morimoto 2011). Thus HSR mainly comprises of induction of molecular

chaperones which help to reestablish proteostasis and recover the cells from the stress induced damage by modulating protein folding, activity and stability. One of the key chaperones in HSR and the focus of this book is heat shock protein 70 (HSP70). HSR and HSP70 are highly conserved across the evolutionary history from bacteria to mammals with some variations. In this chapter we will focus on regulatory mechanisms which govern the induction and expression of cytosolic HSP70 during stress in mammals.

The HSP70 is a family of molecular chaperones with molecular weight ranging from 66–78 kDa. HSP70 is highly conserved largest family of HSP comprising of as many as 7 genes in mouse and 13 genes in human (Hunt and Morimoto 1985; Radons 2016). HSP70 family members are monomeric proteins with diverse localizations like cytosol, nucleus, ER, mitochondria, exosomes in tissue fluids, and extracellular space in mammals (Asea et al. 2008; Asea et al. 2000; Lindquist and Craig 1988; Radons 2016; Welch and Feramisco 1984). For example in human while, HSPA1A, HSPA1B and HSPA8 (Hsc70) are predominantly cytosolic protein, HSPA5 (Grp78) and HSPA9 (Grp75/mortalin) is localized in ER and mitochondria respectively. In addition these HSP are known to translocate in different subcellular or extracellular locations at different physiological conditions. Another important regulation which differentiates HSP70 family members is ability to be induced in stress and diseases. The induction of HSP70 gene expression under same stress condition varies between tissue and cell types. For example, neuronal cells show very poor induction of HSP70 during proteotoxic stresses and thus higher susceptibility to protein aggregation disorders as seen in many neurodegenerative diseases like Alzheimer's, Parkinson's and others (Turturici et al. 2011). Many of the HSP70 family members like HSPA8, 5 and 9 are constitutively expressed and perform housekeeping function in proteostasis network (PN). While constitutively expressed HSPA8 accumulates in cytosol and nucleus and acts as a major component in PN by preventing protein aggregation and promoting protein folding, ER specific HSPA5 helps in transport and folding of nascent polypeptides inside ER. Mitochondria specific constitutive HSPA9 helps in transport of protein across mitochondrial membrane. On the other hand HSP70 family members HSPA1A (HSP70–1) and HSPA1B (HSP70–2) acts as a sensor of proteotoxic stress and show a very quick induction during temperature and other types of stresses to counteract the proteostasis imbalance by helping in protein folding, stabilization and degradation if the damage is unrepairable. In recent time, HSP70 is also implicated in coupling proteostasis to mRNA metabolism (ribostasis) (Walters and Parker 2015) which help in minimizing gene expression other than the stress response pathways. Three other HSP70 family member in human, HSPA6, HSPA7 and HSPA14 are also inducible genes, with HSPA7 considered as a pseudogene by many (Brocchieri et al. 2008; Parsian et al. 2000; Radons 2016). To deal with extreme proteotoxic condition caused by environmental and physiological stress, the regulation of HSP70 induction and expression also has to be fast and robust. HSP70 gene expression during stress represent one of the unique example where a strong induction in gene transcription is coupled with posttranscriptional, translational and posttranslational regulation to ensure a robust protein output in a critical condition when gen-

eral transcription translation machinery is halted (Morimoto 2011). In this chapter each of these aspects of HSP70 induction and expression will be discussed in detail.

## 1.2 Inducible HSP70 Genes in Mammals

Mouse inducible HSP70 genes HSP70.1 (HSPA1A) and HSP70.3 (HSPA1B) encode almost identical protein of 68 kDa. HSPA1A and HSPA1B ORF differs in only six single nucleotides encoding proteins that only differ by two amino acids and are thought to be functionally interchangeable proteins (Daugaard et al. 2007). However two genes show certain sequence differences in promoter region and 3'UTR which do play a role in transcriptional and post transcriptional regulation of both the transcripts during stress. These two genes located ~8 kb apart within the MHC class III locus in chromosome 6 in mouse genome (Milner and Campbell 1990). Two major inducible HSP70 proteins in human, HSPA1A and HSPA1B, are also highly identical proteins differing only by two amino acids and map to same locus in human chromosomes 6 (Harrison et al. 1987). The other stress inducible gene HSPA6 is highly homologous to HSPA1A (Leung et al. 1992) and located in human chromosome 1. Nearby resides another stress inducible gene, HSPA7, which is homologous to HSPA6 but the ORF is half in size compared to other HSP70s and thought to be a pseudo gene. However HSPA7 promoter shows stress specific regulation and can be induced by nutritional stress, but not by oxidative stress or change in pH (Siddiqui et al. 2008). A dendritic cell specific inducible HSP70 gene is HSPA14 (located in human chromosome 10) which produces little smaller protein compared to other HSP70s and play important role in immune cell regulation, cell transformation and metastasis (Wan et al. 2004; Wu et al. 2011; Yang et al. 2015).

## 1.3 HSP70 Promoter Organization and Activation

The promoter region of HSPA1A and HSPA1B genes are highly conserved in mammals. The key regulatory element which makes these promoters unique is the presence of conserved sequences known as the heat shock elements (HSEs) which binds the heat shock factor 1 (HSF1) complex upon heat stress causing Transcriptional activation of HSP70 promoters. HSEs are located upstream of the basal promoter elements, TATA box, and human promoter has two of such elements. HSEs are made up of conserved sequence: 5'NAGAANNTTCNNGAANN- 3′, where N is any nucleotide (Amin et al. 1988). Several other key transcription factor binding sites are also present in HSP70 promoter. These include NF-Y (nuclear transcription factor Y), NF-κB (nuclear factor kappa B), CREB (cAMP response element binding protein), CCAAT, sp1 and STAT3 Table 1.1.

Basal transcription form mammalian HSP70 promoters are mediated by Sp1, CCAAT binding proteins (CBP) and CCAAT box transcription factor (CTF)

**Table 1.1** Transcriptional regulators of inducible HSP70 genes in mammalian system

| Transcriptional regulators | |
|---|---|
| Positive | SP1, CIF, HSF1, NF-kB, STAT1 and 3, NF-IL6, SIRT1, p300, mTOR1, Attexin 3, eEF1A1, ATF1-BRG1, HSP105b |
| Negative | CHBF/Ku antigen, MAPK/ERK, JNK/p38, ATF1-p300 complex, GSK-3b |

(Bevilacqua et al. 1997; Morgan 1989). Atxn3, a deubiquitinase, is also shown to improve basal HSP70 expression by affecting HSF1 in brain tissue and fibroblast cells (Reina et al. 2012). In absence of stress, robust activity of these promoters is inhibited by many events. Among them four events at the HSP70 promoter level play crucial role. 1) Absence of active HSF1 in normal temperature. 2) Absence of proper chromatin modifications. 3) A factor called constitutive HSE binding factor (CHBF, or the Ku autoantigen) which remains constitutively bound to HSE elements in HSP70 promoters and inhibit HSF1 binding in normal temperature. The CHBF mediated regulation is shown to be specific for HSP70 promoter (Yang et al. 1996). 4) Pausing of RNApol II in pre transcription initiation complex (PIC) at the promoter in absence of activated HSF1. Mild HS (41 °C) causes activation of HSF1 but HSP70 induction does not takes place as CHBF still remains associated to the promoter region. Only severe HS (>42 °C) causes CHBF to leave the promoter region allowing the activated HSF1 to bind HSP70 promoters (Yang et al. 1996). During stress, activation and binding of HSF1 to HSEs triggers RNApol II to escape HSP70 promoter within a minute to initiate sustained elongation of HSP70 transcripts and robust induction of HSP70 gene expression which is tens of fold higher than the basal level. In mammals dephosphorylation and phosphorylation of CTD (C terminal domain) of the RNA polymerase II large subunit is important for assembly of transcription initiation complex and transcription elongation respectively. However during HS CTD is hyperphosphorylated by stress induced CTD kinase and MAPK p42/p44. While hyperphosphorylated RNApol induces transcription elongation of HSP70 genes, fails to interact with majority of the other genes ensuring a global transcription shut down (Dubois et al. 1999; Venetianer et al. 1995).

## 1.4 HSF1 and its Activation by Trimerization

Heat shock factor 1 (HSF1) is one of the major stress activated transcription factor which shows genome wide occupancy during stress and is responsible for expression of large number of stress induced genes including HSP70s (Trinklein et al. 2004). Among HSF family members HSF1 is mainly activated during stress and active DNA binding form of HSF1 is a homo-trimer. However among four other HSF isoforms present in mammals (HSF2, HSF3 and HSF4, HSF Y), in certain

acute stress conditions HSF2 can also form hetero-trimer complex with HSF1and modulate HSF1 mediated expression of HSP (Åkerfelt et al. 2010; Loison et al. 2006; Östling et al. 2007; Sandqvist et al. 2009; Vihervaara and Sistonen 2014). Induction of HSP70 gene by HSF1/HSF2 heterotrimer is reported during heat shock as well as in hemin-induced differentiation of K562 erythroleukemia cells. In both the cases HSF1 is needed for DNA binding activity of HSF2 (Östling et al. 2007). Across species, mammalian HSF domain organization is quite conserved. At the N-terminus it has a helix-turn-helix DNA binding domain (DBD) responsible for binding to HSE. It also has three hydrophobic heptapeptide repeats, two at the N-terminus (HR-A/B) and one at the C-terminus (HR-C). HRs are capable of interacting with each other forming leucine zipper coiled-coil. While during normal condition, intra molecular interaction between HR-A/B and HR-C keeps HSF in monomeric form, during stress HSF1 trimerize by inter-molecular interaction of HR-A/B (Sorger and Nelson 1989). A bipartite transcription activation domain (AD1 and AD2) present at the extreme C-terminal end of the molecule is responsible for transcription activation. A negative regulatory domain (RD) present in between HR-A/B and HR-C is responsible for suppression of activation domain in normal condition (Green et al. 1995; Newton et al. 1996). Trimerazation is a key step in the activation event of HSF1. In normal condition along with intramolecular interactions, interaction of monomeric HSF1 with other chaperone like HSP90 and HSP70 forms an inactive HSF1complex. It is interesting to note that due to interaction with proteastasis network proteins, this complex takes a center stage in sensing proteotoxic stress. Due to higher affinity of HSP70 and HSP90 towards denatured proteins, these molecules interact with denatured protein in the condition of proteotoxic stress and leave HSF1 free to be trimerize and translocate from cytosol to the nucleus (Guo et al. 2001; Zou et al. 1998).

## 1.5 Regulation of HSF1 by Posttranslational Modifications

Activation and functioning of HSF1 is regulated by three types of protein modification: phosphorylation, SUMOylation and acetylation at different positions. While some of the modifications acts as positive regulator others acts as negative regulator. HSF1 is constitutively phosphorylated at residues S303, S307, and S308 which inhibit its function at normal condition. These inhibitory phosphorylations are mainly mediated by MAPK/ERK, p38/JNK, and GSK-3β protein kinases. Stress dependent phosphorylation at residues S230, S326, and S419 positively regulate HSF1 activity (Åkerfelt et al. 2010; Chu et al. 1996; Guettouche et al. 2005; Holmberg et al. 2001). It is thought that activation event of HSF1 not only require phosphorylation but dephosphorylation of constitutive phosphorylation is also required (Xavier et al. 2000). HSP70 gene expression is regulated in such a way that it is not an all or none phenomena. Rather amplitude of HSP70 expression is proportional to level and duration of stress. Thus attenuation of HSP70 expression at the recovery period from stress is also regulated at different level. During recovery

period phosphorylation of Ser303 provides signal for sumolytion at Lys298 position of HSF1 mediated by SUMO1 and SUMO2/3 (Anckar and Sistonen 2007) which inhibits HSF1 transcriptional activities. Another co-chaperone HSP27 is important for interaction of HSF1 with SUMO2/3 (Simioni et al. 2009). Phosphorylation of Ser303 also causes binding of HSF1 with the protein 14–3-3ε, causing export of the HSF1 from nucleus to the cytosol (Wang et al. 2004). HSF1's DNA binding activity is also regulated by its acetylation/deacetylation state. Acetyltransferase p300/CBP acetylates HSF1 at K80 in the DBD and inhibits HSF1's DNA binding during recovery period after stress. However, NAD-dependent deacetylase SIRT1 (sirtuin) deacetylates HSF1 and thereby exerts an opposite effect, prolonging HSF1 activity and the HSR (Westerheide et al. 2009). As NAD level is a direct measure of cell's energy homeostasis, HSR is thus connected to nutritional level and metabolic state of a cell. mTOR is a protein kinase which play a central role in coordinating mitogen signaling to nutrition availability and possibly link nutritional state of the cell to HSR as well. mTOR1 induces HSP70 expression by activating phosphorylation of HSF1 on ser326 and inhibition of PI3K/mTOR signaling inhibit HSP70 induction by blocking HSF1 nuclear translocation (Acquaviva et al. 2014; Chou et al. 2012). A unique mechanism regulating HSR was reported in Nb2 lymphoma cells where proteolysis of HSF1 likely by a caspase family member decreases the HSP70 level (Zhang et al. 1999). In extreme conditions if HSR cannot resolve the stress, kinases like p38 and JNK rapidly inactivates HSF1by inhibitory phosphorylation there by lowering expression of HSP70 and activation of apoptosis (Anckar and Sistonen 2007; Chu et al. 1996).

## 1.6 Factors that Coordinate with HSF1 for HSP70 Gene Expression

Many factors directly or indirectly interact with HSF1 in regulation of inducible HSP70 expressions Table 1.1. One key regulation of HSP70 expression is mediated by HSP70 itself. In a feedback loop, during higher accumulation of HSP70 in the cell, it interacts with transactivation domain of HSF1 and thus attenuates HSP70 expression during recovery phase after stress. At this stage HSP90 also interacts with HSF1 which promote monomerization and stabilization of monomeric HSF1 (Åkerfelt et al. 2010; Shi et al. 1998). At the recovery phase, another co-chaperone Hdj1/HSP40 also interacts directly with the transactivation domain of HSF1 inhibiting its activity in spite of the presence of inducible phosphorylations on HSF1 (Shi et al. 1998). These auto-regulatory mechanisms again ensure HSP70 expression proportional to the proteotoxic stress in the cell. Another regulator of HSP70 expression, translation elongation factor eEF1A1, has recently being implicated in coordinating transcriptional expression of the gene to the need of HSP70 protein in the cell. It plays role in every steps of HSP70 expression from the mRNA synthesis to stability to translocation from nucleus to cytoplasm. eEF1A1 helps in efficient

loading and activation of HSF1 at its promoter region and thus promote efficient induction of HSP70 transcription (Vera et al. 2014).

HSP70 is induced during diverse stress conditions like temperature, metal as well as oxidative stress. However pathways that induce HSP70 expression varies as cell senses different types of stress in different way. It has been shown that phosphorylated AMP-activated protein kinase α subunit (AMPKα) inhibit HSF1 activity by ser303 phosphorylation (Zhu et al. 2014). At two different stress conditions, heat shock and heavy metal stress, it was also observed that protein phosphatase 2A (PP2A) can inhibit AMPKα by dephosphorylation indicating role of this pathway in induction of HSP70 during stress (Wang et al. 2010; Zhu et al. 2014). Inhibition of PP2A or activation of AMPKα in presence of heavy metals leads to severely dampened HSP70 induction resulting in poor clearance of ROS and heightened oxidative stress (Zhu et al. 2014). Subcellular compartments like mitochondria are also found to participate in sensing proteotoxic stress during heat shock and regulation of cytosolic nuclear and mitochondrial chaperones. Mitochondrial DNA replication protein, mitochondrial SSBP1 (single strand DNA binding protein), translocates from mitochondria to nucleus upon heat stress via mitochondrial permeability transition pore and interacts with HSF1 through its oligomerization domain. The SSBP1-HSF1 complex promotes HSF1 dependent transcriptional activation by recruitment of chromatin remodeling factor BRG1 (Tan et al. 2015). This pathway was shown to be specific to proteotoxic stress conditions as hypoxia and other stresses did not induce SSBP1 translocation. Another pathway that links proteotoxic stress sensing and HSF1 is direct interaction of HSF1 with key cytosolic chaperonin complex TRiC/CCT which inhibits transcriptional activity of HSF1 and dampen HSP70 induction at stress conditions like ER stress (Neef et al. 2014). HSP70 is induced during oxidative stress and both HSF1 and HSP70 play role in oxidative stress sensing. HSF1 and HSP70 both contain cysteine residues which are regulated by redox state of the cell. HSF1 has two cysteine residues in DBD which forms disulfide bond which helps in dimerization and DNA binding during oxidative stress as well as heat stress (Ahn and Thiele 2003). Inducible HSP70 but not the constitutive HSC70 has Cys at 306 position which undergo oxidation further exposing Cys267 for oxidation. Both the modifications abolish ATP binding ability of HSP70 and thought to promote HSF1 activation during oxidative stress (Miyata et al. 2012).

A connection between metabolic state of the cell and HSR is argued by in many studies (Kaur et al. 2010). One such regulator discussed earlier in the chapter is, NAD-dependent deacetylase SIRT1 (sirtuin). Another such potential regulator is cold-inducible PPARγ coactivator 1α (PGC-1α) which regulate transcription of a large number of metabolism related genes. In hepatic cells, PGC-1α directly interacts with HSF1 on HSP70 promoter and inhibits its activity (Minsky and Roeder 2015). Interestingly in a recent study, an opposite effect of PGC-1α - HSF1 interaction on HSP70 promoter has been shown in fibroblast cells resulting in induction of HSP70 genes (Xu et al. 2016). These observations reemphasize the fact that regulation of HSP70 expression is diverse in diverse cell types possibly depending on the transcriptome and proteome of that cell.

## 1.7 Role of Other Transcription Factors in Regulation of HSP70 Expression

Other than HSF1 several other transcription factors also modulate HSP70 expression during stress and also non-stressful conditions Table 1.1. Many times they work on HSP70 promoter in association with HSF1. Many studies suggested an interaction between immunomodulatory signaling with HSR. In non-stressful condition, treatment with immunostimulatory cytokine INFγ induces HSP70 expression in transcription factor STAT1 mediated manner. STAT1 interacts with HSP70 promoter at a region overlapping with HSE. STAT1 also physically interacts with HSF1 and induce HSP70 expression in presence of INFγ (Stephanou et al. 1999). Janus tyrosine kinase (JAK) which can be activated by INFγ promotes binding of STAT-1/3 to HSP70 promoter sequence and induces HSP70 expression in rat smooth muscle cells (Madamanchi et al. 2001). IL6 which can act as pro and anti-inflammatory cytokine induces two distinct gene regulatory pathways, JAK/STAT3 and MAPK/NF-IL6 which show opposing effect on HSP90 promoter (Stephanou et al. 1998). In agreement to that, unlike STAT and HSF1 positive cooperation on HSP70 promoter, study using HSP70B (HSPA7) promoter construct showed that NF-IL6 inhibit HSF1 activity on HSP70 promoter (Xie et al. 2002). Recently STAT3 activation by StIP1 (elongator protein 2) is also implicated in HSP70 expression (Suaud et al. 2011). Another signaling molecule related to immune and stress response, NF-κB, cooperates with HSF1 along with NF-Y to induce HSPA1A promoter in response to heat and chemical stress (Sasi et al. 2014; Taira et al. 1999). NF-κB and CREB also positively regulate HSP70 induction in cell culture model of ischemia-like conditions. However, human HSP70 promoter does not contain a strong CREB binding element and hence non responsive to CREB (Sasi et al. 2014; Tranter et al. 2010). Few other activators of monocyte function and differentiations can also activate HSF1 and induce HSP70 expression. These include CSF-1, LPS, and 1,25-dihydroxyvitamin $D_3$ (Polla et al. 1987; Teshima et al. 1996). TNFα which acts as proinflammatory at the same time proapoptotic factor transiently inhibit HSF1 activation and HSP70 expression during heat stress (Schett et al. 2003).

Over expression of HSP70 is associated with a large proportion of human cancers. While HSP70 is helpful in neutralization of proteotoxic stress it also makes cells resistant to apoptosis. Due to positive correlation of expression of HSP70 with poor prognosis and drug resistance of cancer cells, HSP70 is heavily pursued as target for anticancer therapy (Calderwood and Asea 2002; Kaur et al. 2011; Murphy 2013). Interestingly an inhibitory role of HSP70 on cancer cell metastasis has also been argued (Bausero et al. 2004). Apart from major transcription factors of HSP70 expression, other factors are also reported to induce HSP70 in cancer cells. The oncogene ΔNp63α, a p53 family member, induces HSP70 gene expression by interaction with NF-Y and CCAAT binding factor. Elevated level of ΔNp63α reported to have positive correlation with increased expression of HSP70 in cancer cell (Wu et al. 2005). p63α is also required to induce HSP70 expression in adult stem cells like human limbo-corneal epithelial (HLE) cells which has a high proliferation

capability (Ma et al. 2011). In pancreatic cancer cells, SP1 dependent NF-κB activation is required for HSP70 expression. Inhibition of SP1 activation by drug treatment in these cells inhibits HSP70 expression and promotes apoptotic cell death (Banerjee et al. 2013). Tumor suppressor gene p53 has been shown to inhibit HSP70 expression by interaction with CBP (Agoff et al. 1993). HSF3 which is involved in expression of HSP70 in unstressed proliferating cells is activated by interaction with proto-oncogene *c-Myb*. p53 inhibits HSF3 mediated HSP70 expression by disrupting this interaction (Tanikawa et al. 2000).

In a series of studies in recent years, another chaperon, HSP105, with similar function of HSP70 is identified as an inducer of HSP70 during mild heat shock (42 °C) in mammalian cells (Saito et al. 2007; Yamagishi et al. 2009). A differentially spliced isoform HSP105β is induced during mild heat stress localizes to nucleus and induces HSP70 expression in a STAT3 dependent manner. This interaction found to induce STAT3 phosphorylation at Tyr705 and promotes nuclear localization of the transcription factor (Yamagishi et al. 2009). A series of HSP105β interacting proteins are identified with potential role in regulation of HSP70 expression. Interaction of HSP105β and 'N-myc and Stat interactor' (Nmi) induces STAT3 phosphorylation and HSP105β induced HSP70 expression is abolished in absence of Nmi (Saito et al. 2014). Two other interacting partners, splicing factor SNRPE and transcriptional co-activator AF9, also found to help or enhance HSP105β mediated expression of HSP70 (Saito et al. 2016). Whereas another interacting protein, CCDC127, somewhat dampens the HSP70 induction. Interestingly HSF1 has not been identified as an interacting protein of HSP105β may suggest that it is one of the stress induced pathway which operate independent of HSF1.

## 1.8 Chromatin Remodeling and HSP70 Induction

Chromatin remodeling plays an important role in transcriptional regulation of HSP70 gene. In this aspect so far majority of the work is done in Drosophila (Garbuz 2017). The information available on mammalian HSP70 promoter indicates that similar to Drosophila mammalian HSP70 gene is also regulated by histone acetylation (Chen et al. 2002; Thomson et al. 2004). Histone modification in mammalian HSP70 gene is also stress specific. While heat shock induces H4 histone acetylation, arsenite treatment induces H3 histone phosphorylation along with H4 histone acetylation (Thomson et al. 2004). HSF1, the key transcription factor for HSP70 expression, plays an important role in chromatin modification as well. Recruitment of SWI/SNF (hSWI/SNF) remodeling complex to the HSP70 gene is mediated by direct interaction of HSF1 activator domain with BRG1 subunit of hSWI/SNF (Sullivan et al. 2001). Recently ATF1/CREB family members are identified as another factor in this complex along with lysine acetyl transferase p300. ATF1 phosphorylated at ser36 and ser41 residues promotes ATF1-BRG1-HSF1 complex which establishes active chromatin state by histone acetylation and transcriptional

elongation. Another phosphorylation of ATF1 at ser 63 residue promotes ATF1-p300/CBP-HSF1 complex formation which attenuates HSP70 expression in the recovery phase of the stress response by HSF1 acetylation at Lys80 residue leading to inhibition of HSF1 DNA binding (Takii et al. 2015). A recent study also linked lower level of HSP70 induction with lower level of H3 and H4 acetylation in neuronal cells in spite of activation and nuclear translocation of HSF1 (Gómez et al. 2015). It has also been shown that histone modifications on HSP70 gene do act as epigenetic memory of exposure to stress or environmental acclimation. Cross tolerance to ischemic stress in heart after heat acclimation shown to be mediated by high level of acetylated H4 histone and maintenance of euchromatin state of HSP70 locus (Tetievsky et al. 2008; Tetievsky and Horowitz 2010).

Subnuclear localizations of chromosomal locus also play important role in gene expression as different subnuclear locations has been identified with high transcriptional and RNA processing activity. In a recent study it has been shown that HSP70 locus relocates itself to nuclear speckles in a stress dependent manner. Localization of HSP70 genes in speckles correlate with rapid induction of HSP70 transcription. This localization is mediated by nuclear actin polymerization and inhibition of actin polymerization inhibits speckle localization of the chromosomal loci and expression of HSP70 gene. Requirement of promoter region, not the coding region of the gene for localization in speckles indicate close association of stress induced transcriptional activation and actin dependent speckle localization (Khanna et al. 2014).

## 1.9 Posttranscriptional Regulation of HSP70 Expression

After synthesis of nascent mRNA it is subjected to polyadenylation, splicing and export of mature mRNA to cytoplasm. Efficiency at all these steps is important for rapid and robust gene expression. Stability of the mRNA and efficient translation also boost gene expression and help to sustain it for a longer duration. Given the key role of HSP70 in HSR, stress induced mammalian HSP70 mRNAs happens to be regulated at all these steps except at splicing as mammalian inducible HSP70 genes are intron less. This also provides an advantage to HSP70 expression as splicing remains inhibited globally during many stress conditions to inhibit fresh mature mRNA production.

Efficient polyadenylation is important to stabilize a nascent mRNA. Polyadenylation of HSP70 mRNA is promoted by none other than HSF1. Thus it not only regulates the transcriptional activation, it is a key factor in post-transcriptional event as well. HSF1 interacts with symplekin, which forms a complex with polyadenylation factor CstF and CSPF and co-localized in punctate structures in nucleus of stressed cells (Xing et al. 2004). HSF1 also found to interact with 3'procesing factor CstF-64 during stress. Abolition of these interactions affected HSP70 expression severely. This suggested a critical role played by HSF1 in recruiting polyadenylation machinery and efficient polyadenylation of nascent HSP70 mRNAs (Xing et al. 2004). Stress regulated polyadenylation of

HSP70 mRNA was earlier reported in Drosophila. It was shown that, heat stress induces rapid deadenylation of the existing HSP70 mRNAs along with accumulation of new polyadenylated HSP70 mRNAs over time. It was also reported that deadenylated mRNAs were translated inefficiently (Dellavalle et al. 1994). Role of 3'UTR in stress dependent induction of HSP70 expression was also reported for human HSP70 (Moseley et al. 1993). Recent studies in mammalian system have shown that heat stress induces an alternate polyadenylation event in which a proximal polyadenylation signal is used at the 3'UTR of HSP70 gene. This results in stress induced HSP70 mRNAs with a shorter 3'UTR than the HSP70 mRNA expressed in basal condition (Tranter et al. 2011). The transcript with shorter UTR found to be translated much efficiently due to better loading to the polysomes (Kraynik et al. 2015). The reason for such elegant regulation is the alternate polyadenylation which removes target site for a micro RNA, miR-378*, which inhibits HSP70 expression. Interestingly ischemic stress in cardiac tissue has suppressed the expression of miR-378* and induced expression of HSP70 (Tranter et al. 2011). Many other miRs also target HSP70 mRNA (Place and Noonan 2014). While mIR-1, miR-133b-5p, and miR-142-3p negatively regulate HSP70, expression of miR-21 and miR-24 show positive correlation with HSP70 expression. miR mediated regulation of HSP70 expression is observed not only in stress conditions but also in disease conditions. miR-1 is up-regulated in the cell culture model of muscle atrophy and suppressed HSP70 expression (Kukreti et al. 2013). miR-133b-5p is up-regulated in apoptotic rat cortical neurons exposed to HIV-1 gp120 V3 loop peptide and suppressed HSP70 expression (Xia et al. 2016). Induction of miR-142-3p in cancer cells by drug treatment also suppressed HSP70 expression (MacKenzie et al. 2013). Whereas ischemic stress in mouse heart induced expression of miR-21, and miR-24 along with HSF1 and HSP70 (Yin et al. 2009).

Next event in the path to HSP70 gene expression is to transport HSP70 mRNA out of the nucleus. It has been earlier reported that heat stress inhibits global export of mRNA from nucleus. However stress induced mRNAs escape such inhibition (Carmody et al. 2010). Similarly it was also reported that human HSP70 family mRNAs can escape transport block during adenovirus infection (Moore et al. 1987). Recent studies on nuclear export of HSP70 mRNA during stress reveled that it is a highly coordinated event in which along with nuclear export factors, transcription as well as translation factors participate to achieve a smooth delivery of mature mRNA from site of synthesis to the site of translation. Key transcription factor on HSP70 promoter, HSF1, interacts with TPR protein in a stress dependent manner. TPR is a nuclear pore interacting protein and its interaction with HSF1 is thought to bring transcribed mRNA to the nuclear pore complex. Inhibition of TPR-HSF1 interaction inhibits HSP70 mRNA transport (Skaggs et al. 2007). Another HSF1 interacting protein eEF1A1 which helped earlier in the recruitment of HSF1 to the HSP70 promoter interacts with heat shock induced hyper-phosphorylated form of RNA Pol II and 3'UTR of HSP70. At the same time eEF1A1 also interacts with TPR1, the same nuclear pore interacting protein with which HSF1 interacts. eEF1A1 and its interaction with TPR1 promote nuclear export of HSP70 mRNA (Vera et al. 2014). Thus it is possible that the transcriptional assembly of HSF1 and eEF1A1 along

with HSP70 mRNA interacts with nuclear pore complex through TPR1 for an efficient export of HSP70 mRNA and delivery to ribosomes. Major nuclear export receptor CRM1 plays important role in HSP70 mRNA export. RNA binding protein HuR/ELAV which binds to ARE (AU reach element) and stabilize ARE containing mRNAs binds to HSP70 mRNA and interacts with CRM1 during heat stress to promote HSP70 mRNA transport (Gallouzi et al. 2001). Interaction of nuclear export receptor Tap-p15, which is also involved in CRM1 pathway, along with adaptor protein Aly and Thoc5 is shown to be crucial for heat stress induced HSP70 mRNA transport. As Thoc5 is not required for majority of the mRNA transport, it might explain the escape of transport block by HSP70 mRNAs (Black et al. 2001; Katahira et al. 2009).

Stability of HSP70 mRNA plays a very important role in potent HSR. As the stable mRNA can support prolonged protein expression, stability of mammalian HSP70 mRNA is much higher during stress compared to normal condition (Theodorakis and Morimoto 1987). Existing data suggest that stress dependent stabilization of HSP70 mRNA is also a multi factorial event. Interestingly HSP70 and cochaperone HSP110 interacts with 3'UTR of HSP70 mRNA and may play regulatory role in stability of the transcript (Henics et al. 1999). eEF1A1 is also suggested to stabilize HSP70 mRNA by interacting with it at the 3' UTR (Vera et al. 2014). As mentioned earlier, HSP70 mRNA contains an ARE which regulates mRNA stability. There are proteins which binds to ARE and can destabilize or stabilize an mRNA. Multiple studies identified HuR, an ARE binding protein which protects mRNA from degradation, binds to HSP70 mRNA and promote HSP70 mRNA expression (Amadio et al. 2008; Gallouzi et al. 2001; Kraynik et al. 2015). Oxidative stress induced phosphorylation of HuR in neuroblastoma cells also indicated role of this RNA binding protein in HSP70 mRNA stability (Amadio et al. 2008). Double-stranded RNA-dependent protein kinase (PKR), which remains inhibited by binding to chaperones like $P58^{IPK}$, HSP40, and HSP70, gets activated upon stress may be in the same way HSF1 does (Melville et al. 1999). Knock out of this kinase in mouse fibroblasts decreased the stability of HSP70 mRNA (Zhao et al. 2002). However target of this kinase during stress resulting in stabilization of HSP70 mRNA is not yet clear. Recent study from our laboratory showed that knock out of arginyl transferase 1 (*Ate1*) affects HSP70 mRNA stability in MEF cells. ATE1 mediates posttranslational protein arginylation which affect activity and stability of the target protein (Saha and Kashina 2011). Our study showed that protein arginylation is important for stress induced stabilization of HSP70 mRNA and loss of Ate1 makes MEF cells susceptible to heat stress (Deka and Saha 2017; Deka et al. 2016). However the target of such modification during stress is yet to be identified.

## 1.10 Regulation of HSP70 Expression at the Translation and Post-Translation Level

During HSR, global translation is inhibited to restrict proteotoxic stress by preventing accumulation of new denatured protein molecules (McCormick and Penman 1969). Heat shock inhibits cap dependent translation by inhibiting eukaryotic translation initiation factors including eIF4F which along with 40S ribosomal subunit helps in 5' UTR scanning for start codon (Duncan and Hershey 1984). Interestingly HSP mRNAs including HSP70 mRNA escape such inhibition (Hickey and Weber 1982). This is mainly due to presence of two elements in 5'UTR of HSP70 mRNA which makes it less dependent on eIF4F complex. Presence of a unique sequence in the 5'UTR of HSP70 mRNA complementary to the 18S rRNA 3′-terminal hairpin sequence helps in translation initiation by a process called ribosome shunting which bypasses 5'URT scanning (Yueh and Schneider 2000). HSP70 mRNA also has a 216 nt long strong internal ribosome entry site (IRES) which is essential for translation of HSP70 and also helps in escaping global translation inhibition (Rubtsova et al. 2003). IRES positions start codon on the ribosome by interacting with preinitiation complex containing 40S ribosomal subunit along with eIF2 and eIF3. This also bypasses the requirement of cap dependent 5'UTR scan event. These alternate modes of translation initiation ensure high expression of HSP70 protein in spite of a global translation inhibition during stress. Recently it has been shown that mitochondrial ribosomal protein L18 (MRPL18) plays a crucial role in selective stress induced translation of HSP70 mRNA and loss of it suppress HSR. A stress induced N-terminally truncated MRPL18 is produced due to usage of a downstream CUG start codon resulting in a cytosolic form of the protein. Cysolic MRPL18 associates with 80S ribosome and helps in HSP70 translation in an eIF2α dependent manner (Zhang et al. 2015). eEF1A1 interacting with transcription and transport machinery may also play important role in efficient translation of HSP70 mRNA (Vera et al. 2014).

Stability of a protein greatly affects its overall abundance in a cell. HSP70 protein is no exception and several regulatory pathways exist which stabilizes HSP70 protein by protecting from ubiquitination mediated degradation. Dual function co-chaperon, CHIP (carboxy terminus of Hsp70-binding protein), which also acts as E3 ubiquitin ligase modulates HSP70 level differently at different stage of stress response. While it promotes HSP70 expression during acute stress, it also targets HSP70 for ubiquitination mediated degradation at the recovery phase of the stress response. During acute stress, CHIP ubiquitinates HSP bound substrates. In absence of enough denatured proteins CHIP ubiquitinates HSP70 for degradation (Jiang et al. 2001; Qian et al. 2006). This is another way how cell ensures HSP70 expression proportional to the stress on the cell. Ubiquitin ligase activity of CHIP can be inhibited by binding of cochaperones like HSPBP1 and BAG2 to HSP70/CHIP complex (Alberti et al. 2004; Arndt et al. 2005; Dai et al. 2005). Recently it is shown that HSPBP1 knock out severely affects stability of HSP70 protein during stress in testis resulting in male sterility in mouse. It is shown that HSBP1P stabilizes

inducible HSP70 protein and testis specific HSP70 protein by inhibition of ubiquitination and proteasome targeting of HSP70. BAG2 also stabilizes HSP70 protein in the same way in tissues other than testis (Rogon et al. 2014). Another protein which inhibits function of CHIP is a cytosolic ATPase, OLA1 (Obg-like ATPase 1), which improves stability of HSP70 proteins by inhibiting CHIP dependent ubiquitination. OLA1 inhibits recruitment of CHIP to the HSP70 by interacting with carboxyl terminus variable domain of HSP70 (Mao et al. 2013). Finally posttranslational proteolysis mediated by subtilisin family protease SKI-1 found to down regulate functional HSP70 protein in endometriosis patients (Chehna-Patel et al. 2011).

## 1.11 Conclusions

Proteotoxic stress is induced in a cell or tissue due to structural damage to protein molecules at different stress conditions like elevated temperature, oxidative stress, and exposure to heavy metals. The heat stress response (HSR), which plays central role in resolving stress induced protein damages, is launched by the cell upon sensing proteotoxic stress. Thus HSR constitutes induction of large number of chaperone proteins including HSP70 which prevents misfolding and protein aggregation. The induction of HSP70 is very rapid and can be observed within a minute of exposure to the heat stress. Efficient neutralization of proeotoxic stress requires two things: a robust induction of HSP70 protein expression and coordination between induction and attenuation of HSP70 expression. Cellular protein level can be boosted by efficient transcription, better stability of the transcript, efficient translation and better stability of the protein. Robust induction of HSP70 is so important for the cell that, HSP70 expression is boosted at each of these levels to achieve high level of protein for an extended period of time. At the time when global transcription, mRNA transport and translation are inhibited, HSP70 expression relies on unique regulation to escape all of that to express at a high level. To improve efficiency of gene expression, coordination between different steps of HSP70 expression exists at several points. While key transcription factor HSF1 participates in transcriptional activation, polyadenylation and mRNA transport, translation elongation factor eEF1A1 participates in transcriptional activation, mRNA transport, stability and translation. Thus different steps of HSP70 expression is seamlessly integrated producing a robust HSP70 expression. A robust expression requires an efficient attenuation to protect cells from potential imbalance. HSP70 protein itself acts as an attenuator in a feedback loop where it inhibits HSF1 activity to suppress transcription at the time excess of HSP70 accumulates in the cell. Each of the events in HSP70 expression is highly regulated and integrated with other cellular conditions like nutritional and metabolic state of the cell. A large number of factors have been identified to regulate HSP70 expression at different levels. While bulk of the studies addressed transcriptional regulation, regulation at posttranscriptional, translational and posttranslational level is not addressed adequately. Recent discoveries

in these aspects suggest that our understanding of HSP70 gene expression regulation is far from complete and requires further investigation.

**Acknowledgements** Authors thank funding agencies for research supports and fellowships. Research in laboratory of SS is supported by SERB-India (EEQ/2016/000772, and EMR/2016/004001). KD is supported by fellowship from SERB, India (EEQ/2016/000772). Department of MBBT, TU is supported by UGC-SAP, DST FIST, DBT Strengthening, DBT Hub, and DBT-BIF.

## References

Acquaviva, J., He, S., Sang, J., et al. (2014). mTOR inhibition potentiates HSP90 inhibitor activity via cessation of HSP synthesis. *Molecular Cancer Research, 12*, 703–713.

Agoff, S. N., Hou, J., Linzer, D., & Wu, B. (1993). Regulation of the human hsp70 promoter by p53. *Science, 259*, 84–87.

Ahn, S.-G., & Thiele, D. J. (2003). Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. *Genes & Development, 17*, 516–528.

Åkerfelt, M., Morimoto, R. I., & Sistonen, L. (2010). Heat shock factors: Integrators of cell stress, development and lifespan. *Nature Reviews Molecular Cell Biology, 11*, 545.

Alberti, S., Böhse, K., Arndt, V., Schmitz, A., & Höhfeld, J. (2004). The cochaperone HspBP1 inhibits the CHIP ubiquitin ligase and stimulates the maturation of the cystic fibrosis transmembrane conductance regulator. *Molecular Biology of the Cell, 15*, 4003–4010.

Amadio, M., Scapagnini, G., Lupo, G., Drago, F., Govoni, S., & Pascale, A. (2008). PKCβII/HuR/VEGF: A new molecular cascade in retinal pericytes for the regulation of VEGF gene expression. *Pharmacological Research, 57*, 60–66.

Amin, J., Ananthan, J., & Voellmy, R. (1988). Key features of heat shock regulatory elements. *Molecular and Cellular Biology, 8*, 3761–3769.

Anckar, J., & Sistonen, L. (2007). *Heat shock factor 1 as a coordinator of stress and developmental pathways* (pp. 78–88). NY: Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks.

Arndt, V., Daniel, C., Nastainczyk, W., Alberti, S., & Höhfeld, J. (2005). BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP. *Molecular Biology of the Cell, 16*, 5891–5900.

Asea, A., Kraeft, S.-K., Kurt-Jones, E. A., et al. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature Medicine, 6*, 435.

Asea, A., Jean-Pierre, C., Kaur, P., et al. (2008). Heat shock protein-containing exosomes in mid-trimester amniotic fluids. *Journal of Reproductive Immunology, 79*, 12–17.

Banerjee, S., Sangwan, V., McGinn, O., et al. (2013). Triptolide-induced cell death in pancreatic cancer is mediated by O-GlcNAc modification of transcription factor Sp1. *Journal of Biological Chemistry, 288*, 33927–33938.

Bausero, M. A., Page, D. T., Osinaga, E., & Asea, A. (2004). Surface expression of Hsp25 and Hsp72 differentially regulates tumor growth and metastasis. *Tumor Biology, 25*, 243–251.

Bevilacqua, A., Fiorenza, M. T., & Mangia, F. (1997). Developmental activation of an episomic hsp70 gene promoter in two-cell mouse embryos by transcription factor Sp1. *Nucleic Acids Research, 25*, 1333–1338.

Black, B. E., Holaska, J. M., Lévesque, L., et al. (2001). NXT1 is necessary for the terminal step of Crm1-mediated nuclear export. *The Journal of Cell Biology, 152*, 141–156.

Brocchieri, L., De Macario, E. C., & Macario, A. J. (2008). hsp70 genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evolutionary Biology, 8*, 19.

Calderwood, S., & Asea, A. (2002). Targeting HSP70 induced thermotolerance for design of thermal sensitizers. *International Journal of Hyperthermia, 18*, 597–608.

Carmody, S. R., Tran, E. J., Apponi, L. H., Corbett, A. H., & Wente, S. R. (2010). The mitogen-activated protein kinase Slt2 regulates nuclear retention of non-heat shock mRNAs during heat shock-induced stress. *Molecular and Cellular Biology, 30*, 5168–5179.

Chehna-Patel, N., Warty, N., Sachdeva, G., & Khole, V. (2011). Proteolytic tailoring of the heat shock protein 70 and its implications in the pathogenesis of endometriosis. *Fertility and Sterility, 95*, 1560–1567. e3.

Chen, T., Sun, H., Lu, J., et al. (2002). Histone acetylation is involved in hsp70 gene transcription regulation in Drosophila melanogaster. *Archives of Biochemistry and Biophysics, 408*, 171–176.

Chou, S.-D., Prince, T., Gong, J., & Calderwood, S. K. (2012). mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. *PLoS One, 7*, e39679.

Chu, B., Soncin, F., Price, B. D., Stevenson, M. A., & Calderwood, S. K. (1996). Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *Journal of Biological Chemistry, 271*, 30847–30857.

Dai, Q., Qian, S.-B., Li, H.-H., et al. (2005). Regulation of the cytoplasmic quality control protein degradation pathway by BAG2. *Journal of Biological Chemistry, 280*, 38673–38681.

Daugaard, M., Rohde, M., & Jäättelä, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Letters, 581*, 3702–3710.

Deka, K., & Saha, S. (2017). Arginylation: A new regulator of mRNA stability and heat stress response. *Cell Death & Disease, 8*, e2604.

Deka, K., Singh, A., Chakraborty, S., Mukhopadhyay, R., & Saha, S. (2016). Protein arginylation regulates cellular stress response by stabilizing HSP70 and HSP40 transcripts. *Cell Death Discovery, 2*, 16074.

Dellavalle, R. P., Petersen, R., & Lindquist, S. (1994). Preferential deadenylation of Hsp70 mRNA plays a key role in regulating Hsp70 expression in Drosophila melanogaster. *Molecular and Cellular Biology, 14*, 3646–3659.

Dubois, M.-F., Nguyen, V. T., Bonnet, F., et al. (1999). Heat shock of HeLa cells inactivates a nuclear protein phosphatase specific for dephosphorylation of the C-terminal domain of RNA polymerase II. *Nucleic Acids Research, 27*, 1338–1344.

Duncan, R., & Hershey, J. (1984). Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. *Journal of Biological Chemistry, 259*, 11882–11889.

Gallouzi, I.-E., Brennan, C. M., & Steitz, J. A. (2001). Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock. *RNA, 7*, 1348–1361.

Garbuz, D. (2017). Regulation of heat shock gene expression in response to stress. *Molecular Biology, 51*, 352–367.

Gómez, A. V., Córdova, G., Munita, R., et al. (2015). Characterizing HSF1 binding and post-translational modifications of hsp70 promoter in cultured cortical neurons: Implications in the heat-shock response. *PLoS One, 10*, e0129329.

Green, M., Schuetz, T. J., Sullivan, E. K., & Kingston, R. E. (1995). A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. *Molecular and Cellular Biology, 15*, 3354–3362.

Guettouche, T., Boellmann, F., Lane, W. S., & Voellmy, R. (2005). Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochemistry, 6*, 4.

Guo, Y., Guettouche, T., Fenna, M., et al. (2001). Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. *Journal of Biological Chemistry, 276*, 45791–45799.

Harrison, G. S., Drabkin, H. A., Kao, F.-T., et al. (1987). Chromosomal location of human genes encoding major heat-shock protein HSP70. *Somatic Cell and Molecular Genetics, 13*, 119–130.

Henics, T., Nagy, E., Oh, H. J., Csermely, P., Von Gabain, A., & Subjeck, J. R. (1999). Mammalian Hsp70 and Hsp110 proteins bind to RNA motifs involved in mRNA stability. *Journal of Biological Chemistry, 274*, 17318–17324.

Hickey, E. D., & Weber, L. A. (1982). Modulation of heat shock polypeptide synthesis in HeLa cells during hyperthermia and recovery. *Biochemistry, 21*, 1513–1521.

Holmberg, C. I., Hietakangas, V., Mikhailov, A., et al. (2001). Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *The EMBO Journal, 20*, 3800–3810.

Hunt, C., & Morimoto, R. I. (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proceedings of the National Academy of Sciences, 82*, 6455–6459.

Jiang, J., Ballinger, C. A., Wu, Y., et al. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase identification of Hsc70 as a target for ubiquitylation. *Journal of Biological Chemistry, 276*, 42938–42944.

Katahira, J., Inoue, H., Hurt, E., & Yoneda, Y. (2009). Adaptor Aly and co-adaptor Thoc5 function in the Tap-p15-mediated nuclear export of HSP70 mRNA. *The EMBO Journal, 28*, 556–567.

Kaur, P., Reis, M. D., Couchman, G. R., Forjuoh, S. N., Greene, J. F., Jr., & Asea, A. (2010). Role of heat shock proteins in obesity and type 2 diabetes. In *Heat shock proteins and whole body physiology* (pp. 19–29). Dordrecht: Springer.

Kaur, P., Hurwitz, M. D., Krishnan, S., & Asea, A. (2011). Combined hyperthermia and radiotherapy for the treatment of cancer. *Cancer, 3*, 3799–3823.

Khanna, N., Hu, Y., & Belmont, A. S. (2014). HSP70 transgene directed motion to nuclear speckles facilitates heat shock activation. *Current Biology, 24*, 1138–1144.

Kraynik, S. M., Gabanic, A., Anthony, S. R., et al. (2015). The stress-induced heat shock protein 70.3 expression is regulated by a dual-component mechanism involving alternative polyadenylation and HuR. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1849*, 688–696.

Kukreti, H., Amuthavalli, K., Harikumar, A., et al. (2013). Muscle-specific microRNA1 (miR1) targets heat shock protein 70 (HSP70) during dexamethasone-mediated atrophy. *Journal of Biological Chemistry, 288*, 6663–6678.

Leung, T. K., Hall, C., Rajendran, M., Spurr, N. K., & Lim, L. (1992). The human heat-shock genes HSPA6 and HSPA7 are both expressed and localize to chromosome 1. *Genomics, 12*, 74–79.

Lindquist, S., & Craig, E. (1988). The heat-shock proteins. *Annual Review of Genetics, 22*, 631–677.

Loison, F., Debure, L., Nizard, P., Le Goff, P., Michel, D., & Le Dréan, Y. (2006). Up-regulation of the clusterin gene after proteotoxic stress: Implication of HSF1–HSF2 heterocomplexes. *Biochemical Journal, 395*, 223–231.

Ma, D. H., Lai, J.-Y., Chen, H.-C. J., Yeh, L.-K., & Chen, J.-K. (2011). Up-regulation of Heat Shock Protein 70-1 (Hsp70-1) in human Limbo-corneal epithelial cells cultivated on amniotic membrane: A proteomic study. *Investigative Ophthalmology & Visual Science, 52*, 6678–6678.

MacKenzie, T. N., Mujumdar, N., Banerjee, S., et al. (2013). Triptolide induces the expression of miR-142-3p: A negative regulator of heat shock protein 70 and pancreatic cancer cell proliferation. *Molecular Cancer Therapeutics, 12*, 1266–1275.

Madamanchi, N. R., Li, S., Patterson, C., & Runge, M. S. (2001). Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*, 321–326.

Mao, R., Rubio, V., Chen, H., Bai, L., Mansour, O., & Shi, Z.-Z. (2013). OLA1 protects cells in heat shock by stabilizing HSP70. *Cell Death & Disease, 4*, e491.

McCormick, W., & Penman, S. (1969). Regulation of protein synthesis in HeLa cells: Translation at elevated temperatures. *Journal of Molecular Biology, 39*, 315–333.

Melville, M. W., Tan, S.-L., Wambach, M., Song, J., Morimoto, R. I., & Katze, M. G. (1999). The cellular inhibitor of the PKR protein kinase, P58IPK, is an influenza virus-activated co-chaperone that modulates heat shock protein 70 activity. *Journal of biological chemistry, 274*, 3797–3803.

Milner, C. M., & Campbell, R. D. (1990). Structure and expression of the three MHC-linked HSP70 genes. *Immunogenetics, 32*, 242–251.

Minsky, N., & Roeder, R. G. (2015). *Direct link between metabolic regulation and the heat-shock response through the transcriptional regulator PGC-1α* (Vol. 112, pp. E5669–E5E78). Proceedings of the National Academy of Sciences.

Miyata, Y., Rauch, J. N., Jinwal, U. K., et al. (2012). Cysteine reactivity distinguishes redox sensing by the heat-inducible and constitutive forms of heat shock protein 70. *Chemistry & Biology, 19*, 1391–1399.

Moore, M., Schaack, J., Baim, S. B., Morimoto, R. I., & Shenk, T. (1987). Induced heat shock mRNAs escape the nucleocytoplasmic transport block in adenovirus-infected HeLa cells. *Molecular and Cellular Biology, 7*(12), 4505.

Morgan, W. D. (1989). Transcription factor Sp1 binds to and activates a human hsp70 gene promoter. *Molecular and Cellular Biology, 9*, 4099–4104.

Morimoto, R.I. (2011). *The heat shock response: Systems biology of proteotoxic stress in aging and disease*. Paper prresented at the Cold Spring Harbor symposia on quantitative biology.

Moseley, P., Wallen, E., McCafferty, J., Flanagan, S., & Kern, J. (1993). Heat stress regulates the human 70-kDa heat-shock gene through the 39-untranslated region. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 264*, L533–LL37.

Murphy, M. E. (2013). The HSP70 family and cancer. *Carcinogenesis, 34*, 1181–1188.

Neef, D. W., Jaeger, A. M., Gomez-Pastor, R., Willmund, F., Frydman, J., & Thiele, D. J. (2014). A direct regulatory interaction between chaperonin TRiC and stress-responsive transcription factor HSF1. *Cell Reports, 9*, 955–966.

Newton, E. M., Knauf, U., Green, M., & Kingston, R. E. (1996). The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. *Molecular and Cellular Biology, 16*, 839–846.

Östling, P., Björk, J. K., Roos-Mattjus, P., Mezger, V., & Sistonen, L. (2007). Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. *Journal of Biological Chemistry, 282*, 7077–7086.

Parsian, A. J., Sheren, J. E., Tao, T. Y., et al. (2000). The human Hsp70B gene at the HSPA7 locus of chromosome 1 is transcribed but non-functional. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1494*, 201–205.

Place, R. F., & Noonan, E. J. (2014). Non-coding RNAs turn up the heat: An emerging layer of novel regulators in the mammalian heat shock response. *Cell Stress and Chaperones, 19*, 159–172.

Polla, B., Healy, A., Wojno, W., & Krane, S. (1987). Hormone 1 alpha, 25-dihydroxyvitamin D3 modulates heat shock response in monocytes. *American Journal of Physiology-Cell Physiology, 252*, C640–CC49.

Qian, S.-B., McDonough, H., Boellmann, F., Cyr, D. M., & Patterson, C. (2006). CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. *Nature, 440*, 551.

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress and Chaperones, 21*, 379–404.

Reina, C. P., Nabet, B. Y., Young, P. D., & Pittman, R. N. (2012). Basal and stress-induced Hsp70 are modulated by ataxin-3. *Cell Stress and Chaperones, 17*, 729–742.

Rogon, C., Ulbricht, A., Hesse, M., et al. (2014). HSP70-binding protein HSPBP1 regulates chaperone expression at a posttranslational level and is essential for spermatogenesis. *Molecular Biology of the Cell, 25*, 2260–2271.

Rubtsova, M. P., Sizova, D. V., Dmitriev, S. E., Ivanov, D. S., Prassolov, V. S., & Shatsky, I. N. (2003). Distinctive properties of the 5′-untranslated region of human hsp70 mRNA. *Journal of Biological Chemistry, 278*, 22350–22356.

Saha, S., & Kashina, A. (2011). Posttranslational arginylation as a global biological regulator. *Developmental Biology, 358*, 1–8.

Saito, Y., Yamagishi, N., & Hatayama, T. (2007). Different localization of Hsp105 family proteins in mammalian cells. *Experimental Cell Research, 313*, 3707–3717.

Saito, Y., Yukawa, A., Matozaki, M., et al. (2014). Nmi interacts with Hsp105β and enhances the Hsp105β-mediated Hsp70 expression. *Experimental Cell Research, 327*, 163–170.

Saito, Y., Nakagawa, T., Kakihana, A., et al. (2016). Yeast two-hybrid and one-hybrid screenings identify regulators of hsp70 gene expression. *Journal of Cellular Biochemistry, 117*, 2109–2117.

Sandqvist, A., Björk, J. K., Åkerfelt, M., et al. (2009). Heterotrimerization of heat-shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. *Molecular Biology of the Cell, 20*, 1340–1347.

Sasi, B. K., Sonawane, P. J., Gupta, V., Sahu, B. S., & Mahapatra, N. R. (2014). Coordinated transcriptional regulation of Hspa1a gene by multiple transcription factors: Crucial roles for HSF-1, NF-Y, NF-κB, and CREB. *Journal of Molecular Biology, 426*, 116–135.

Schett, G., Steiner, C., Xu, Q., Smolen, J., & Steiner, G. (2003). TNF [alpha] mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response. *Cell Death and Differentiation, 10*, 1126.

Shi, Y., Mosser, D. D., & Morimoto, R. I. (1998). Molecularchaperones as HSF1-specific transcriptional repressors. *Genes & Development, 12*, 654–666.

Siddiqui, F., Avery, P. R., Li, C.-Y., et al. (2008). Induction of the human heat shock promoter HSP70B by nutritional stress: Implications for cancer gene therapy. *Cancer Investigation, 26*, 553–561.

Simioni, M. B., De Thonel, A., Hammann, A., et al. (2009). Heat shock protein 27 is involved in SUMO-2/3 modification of heat shock factor 1 and thereby modulates the transcription factor activity. *Oncogene, 28*(37), 3332–3344.

Skaggs, H. S., Xing, H., Wilkerson, D. C., et al. (2007). HSF1-TPR interaction facilitates export of stress-induced HSP70 mRNA. *Journal of Biological Chemistry, 282*, 33902–33907.

Sorger, P. K., & Nelson, H. C. (1989). Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell, 59*, 807–813.

Stephanou, A., Isenberg, A. D., Akira, S., Kishimoto, T., & Latchman, S. D. (1998). The nuclear factor interleukin-6 (NF-IL6) and signal transducer and activator of transcription-3 (STAT-3) signalling pathways co-operate to mediate the activation of the hsp90β gene by interleukin-6 but have opposite effects on its inducibility by heat shock. *Biochemical Journal, 330*, 189–195.

Stephanou, A., Isenberg, D. A., Nakajima, K., & Latchman, D. S. (1999). Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90β gene promoters. *Journal of Biological Chemistry, 274*, 1723–1728.

Suaud, L., Miller, K., Panichelli, A. E., Randell, R. L., Marando, C. M., & Rubenstein, R. C. (2011). 4-Phenylbutyrate stimulates Hsp70 expression through the Elp2 component of elongator and STAT-3 in cystic fibrosis epithelial cells. *Journal of Biological Chemistry, 286*, 45083–45092.

Sullivan, E. K., Weirich, C. S., Guyon, J. R., Sif, S., & Kingston, R. E. (2001). Transcriptional activation domains of human heat shock factor 1 recruit human SWI/SNF. *Molecular and Cellular Biology, 21*, 5826–5837.

Taira, T., Sawai, M., Ikeda, M., Tamai, K., Iguchi-Ariga, S. M., & Ariga, H. (1999). Cell cycle-dependent switch of up-and down-regulation of human hsp70 gene expression by interaction between c-Myc and CBF/NF-Y. *Journal of Biological Chemistry, 274*, 24270–24279.

Takii, R., Fujimoto, M., Tan, K., et al. (2015). ATF1 modulates the heat shock response by regulating the stress-inducible heat shock factor 1 transcription complex. *Molecular and Cellular Biology, 35*, 11–25.

Tan, K., Fujimoto, M., Takii, R., Takaki, E., Hayashida, N., & Nakai, A. (2015). Erratum: Mitochondrial SSBP1 protects cells from proteotoxic stresses by potentiating stress-induced HSF1 transcriptional activity. *Nature Communications, 6*, 6964.

Tanikawa, J., Ichikawa-Iwata, E., Kanei-Ishii, C., et al. (2000). p53 suppresses the c-Myb-induced activation of heat shock transcription factor 3. *Journal of Biological Chemistry, 275*, 15578–15585.

Teshima, S., Rokutan, K., Takahashi, M., Nikawa, T., & Kishi, K. (1996). Induction of heat shock proteins and their possible roles in macrophages during activation by macrophage colony-stimulating factor. *Biochemical Journal, 315*, 497–504.

Tetievsky, A., & Horowitz, M. (2010). Posttranslational modifications in histones underlie heat acclimation-mediated cytoprotective memory. *Journal of Applied Physiology, 109*, 1552–1561.

Tetievsky, A., Cohen, O., Eli-Berchoer, L., et al. (2008). Physiological and molecular evidence of heat acclimation memory: A lesson from thermal responses and ischemic cross-tolerance in the heart. *Physiological Genomics, 34*, 78–87.

Theodorakis, N. G., & Morimoto, R. I. (1987). Posttranscriptional regulation of hsp70 expression in human cells: Effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. *Molecular and Cellular Biology, 7*, 4357–4368.

Thomson, S., Hollis, A., Hazzalin, C. A., & Mahadevan, L. C. (2004). Distinct stimulus-specific histone modifications at hsp70 chromatin targeted by the transcription factor heat shock factor-1. *Molecular Cell, 15*, 585–594.

Tranter, M., Ren, X., Forde, T., et al. (2010). NF-κB driven cardioprotective gene programs; Hsp70. 3 and cardioprotection after late ischemic preconditioning. *Journal of Molecular and Cellular Cardiology, 49*, 664–672.

Tranter, M., Helsley, R. N., Paulding, W. R., et al. (2011). Coordinated post-transcriptional regulation of Hsp70. 3 gene expression by microRNA and alternative polyadenylation. *Journal of Biological Chemistry, 286*, 29828–29837.

Trinklein, N. D., Murray, J. I., Hartman, S. J., Botstein, D., & Myers, R. M. (2004). The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. *Molecular Biology of the Cell, 15*, 1254–1261.

Turturici, G., Sconzo, G., & Geraci, F. (2011). Hsp70 and its molecular role in nervous system diseases. *Biochemistry Research International, 2011*, 1.

Venetianer, A., Dubois, M. F., Nguyen, V. T., Bellier, S., Seo, S. J., & Bensaude, O. (1995). Phosphorylation state of the RNA polymerase II C-Terminal Domain (CTD) in heat-shocked cells. *The FEBS Journal, 233*, 83–92.

Vera, M., Pani, B., Griffiths, L. A., et al. (2014). The translation elongation factor eEF1A1 couples transcription to translation during heat shock response. *eLife, 3*, e03164.

Vihervaara, A., & Sistonen, L. (2014) HSF1 at a glance: The Company of Biologists Ltd.

Walters, R. W., & Parker, R. (2015). Coupling of ribostasis and proteostasis: Hsp70 proteins in mRNA metabolism. *Trends in Biochemical Sciences, 40*, 552–559.

Wan, T., Zhou, X., Chen, G., et al. (2004). Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant. *Blood, 103*, 1747–1754.

Wang, X., Grammatikakis, N., Siganou, A., Stevenson, M. A., & Calderwood, S. K. (2004). Interactions between extracellular signal-regulated protein kinase 1, 14-3-3ϵ, and heat shock factor 1 during stress. *Journal of Biological Chemistry, 279*, 49460–49469.

Wang, T., Yu, Q., Chen, J., Deng, B., Qian, L., & Le, Y. (2010). PP2A mediated AMPK inhibition promotes HSP70 expression in heat shock response. *PLoS One, 5*, e13096.

Welch, W. J., & Feramisco, J. (1984). Nuclear and nucleolar localization of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. *Journal of Biological Chemistry, 259*, 4501–4513.

Westerheide, S. D., Anckar, J., Stevens, S. M., Sistonen, L., & Morimoto, R. I. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science, 323*, 1063–1066.

Wu, G., Osada, M., Guo, Z., et al. (2005). ΔNp63α up-regulates the Hsp70 gene in human cancer. *Cancer Research, 65*, 758–766.

Wu, C.-Y., Lin, C.-T., Wu, M.-Z., & Wu, K.-J. (2011). Induction of HSPA4 and HSPA14 by NBS1 overexpression contributes to NBS1-induced in vitro metastatic and transformation activity. *Journal of Biomedical Science, 18*(1), 1.

Xavier, I. J., Mercier, P. A., McLoughlin, C. M., Ali, A., Woodgett, J. R., & Ovsenek, N. (2000). Glycogen synthase kinase 3β negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. *Journal of Biological Chemistry, 275*, 29147–29152.

Xia, C., Cai, Y., Lin, Y., Guan, R., Xiao, G., & Yang, J. (2016). MiR-133b-5p regulates the expression of the heat shock protein 70 during rat neuronal cell apoptosis induced by the gp120 V3 loop peptide. *Journal of Medical Virology, 88*, 437–447.

Xie, Y., Chen, C., Stevenson, M. A., Hume, D. A., Auron, P. E., & Calderwood, S. K. (2002). NF-IL6 and HSF1 have mutually antagonistic effects on transcription in monocytic cells. *Biochemical and Biophysical Research Communications, 291*, 1071–1080.

Xing, H., Mayhew, C. N., Cullen, K. E., Park-Sarge, O.-K., & Sarge, K. D. (2004). HSF1 modulation of Hsp70 mRNA polyadenylation via interaction with symplekin. *Journal of Biological Chemistry, 279*, 10551–10555.

Xu, L., Ma, X., Bagattin, A., & Mueller, E. (2016). The transcriptional coactivator PGC1α protects against hyperthermic stress via cooperation with the heat shock factor HSF1. *Cell Death & Disease, 7*, e2102–e2e02.

Yamagishi, N., Fujii, H., Saito, Y., & Hatayama, T. (2009). Hsp105β upregulates hsp70 gene expression through signal transducer and activator of transcription-3. *The FEBS Journal, 276*, 5870–5880.

Yang, S.-H., Nussenzweig, A., Li, L., et al. (1996). Modulation of thermal induction of hsp70 expression by Ku autoantigen or its individual subunits. *Molecular and Cellular Biology, 16*, 3799–3806.

Yang, Z., Zhuang, L., Szatmary, P., et al. (2015). Upregulation of heat shock proteins (HSPA12A, HSP90B1, HSPA4, HSPA5 and HSPA6) in tumour tissues is associated with poor outcomes from HBV-related early-stage hepatocellular carcinoma. *International Journal of Medical Sciences, 12*, 256.

Yin, C., Salloum, F. N., & Kukreja, R. C. (2009). A novel role of microRNA in late preconditioning. *Circulation Research, 104*, 572–575.

Yueh, A., & Schneider, R. J. (2000). Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. *Genes & Development, 14*, 414–421.

Zhang, M., Blake, M. J., Gout, P. W., Buckley, D. J., & Buckley, A. R. (1999). Proteolysis of heat shock transcription factor is associated with apoptosis in rat Nb2 lymphoma cells. *Cell Growth & Differentiation: The Molecular Biology Journal of the American Association for Cancer Research, 10*, 759–767.

Zhang, X., Gao, X., Coots, R. A., Conn, C. S., Liu, B., & Qian, S.-B. (2015). Translational control of the cytosolic stress response by mitochondrial ribosomal protein L18. *Nature Structural & Molecular Biology, 22*, 404–410.

Zhao, M., Tang, D., Lechpammer, S., et al. (2002). Double-stranded RNA-dependent protein kinase (pkr) is essential for thermotolerance, accumulation of HSP70, and stabilization of ARE-containing HSP70 mRNA during stress. *Journal of Biological Chemistry, 277*, 44539–44547.

Zhu, X.-n., Chen, L.-p., Bai, Q., et al. (2014). PP2A–AMPKα–HSF1 axis regulates the metal-inducible expression of HSPs and ROS clearance. *Cellular Signalling, 26*, 825–832.

Zou, J., Guo, Y., Guettouche, T., Smith, D. F., & Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell, 94*, 471–480.

# Chapter 2
# Heat Shock Proteins and Pesticide Stress


**Prem Rajak and Sumedha Roy**

**Abstract** Heat shock proteins (HSP) are highly conserved bio-molecules found almost in all taxa from protozoa to higher vertebrates. HSP mainly function as chaperones to protect proteins from unfolding, aggregation and destruction. They also help to cope with a range of environmental stresses. Pesticides in majority are synthetic chemicals used in agricultural fields to kill pests, but when ingested by non-target organisms, they generate reactive oxygen species and oxidative stress (OS). OS catalyzes unfolding and aggregation of native proteins. In response, HSP are synthesized within cells and block protein degradation. Therefore, HSP are produced to counteract pesticide stress and hence they are designated as potent biomarkers of OS. Several techniques are available that can monitor HSP level following pesticide insult. Reporter gene assay (HSP70-LacZ or HSP70-GFP) is a common practice and it provides qualitative information. Northern and Western Blot analysis presents quantitative measures of HSP at both mRNA and protein levels. Therefore, HSP constitute a part of cell protection machinery as well as they belong to first tier of biomarkers considered in risk assessment of pesticides.

**Keywords** Apoptosis · Heat shock factors · Heat shock protein · Pesticide · ROS

## Abbreviations

| | |
|---|---|
| AChE | Acetyl cholinesterase |
| AIF | Apoptosis inducing factor |
| ALS | Amyotrophic Lateral Sclerosis |
| BAG | BCL2 associated athanogene 1 |
| CHIP | Carboxy terminus of Hsc70 interacting protein |
| FR | Free radicals |

P. Rajak
Post Graduate Department of Zoology, A. B. N. Seal College,
Cooch Behar, West Bengal, India

S. Roy (✉)
Toxicology Research Unit, Department of Zoology, The University of Burdwan,
Burdwan, West Bengal, India



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_2

| | |
|---|---|
| GFP | Green fluorescent protein |
| HIP | Hsp70-interacting protein |
| HOP | Hsp70-Hsp90 organizing protein |
| HSE | Heat shock element |
| HSF1 | Heat shock factor 1 |
| HSP 20 | Heat shock protein 20 |
| HSP 26 | Heat shock protein 26 |
| HSP 40 | Heat shock protein 40 |
| HSP 70 | Heat shock protein 70 |
| HSP 83 | Heat shock protein 83 |
| HSP 84 | Heat shock protein 84 |
| HSP 90 | Heat shock protein 90 |
| NOS | Nitric oxide synthase |
| ONPG | O-nitro phenyl-β-d-galactopyranoside |
| OS | Oxidative stress |
| PCR | Polymerase chain reaction |
| ROS | Reactive oxygen species |

## 2.1 Introduction

Heat shock proteins (HSP) are molecular chaperons found almost in all living organisms and offer first line of defense mechanism against heat and other stresses (Lindquist 1986). HSP are highly conserved bio-molecules, reported to exist from single-celled animals to higher vertebrates. Genetic components for HSP were first distinguished in 1962 as chromosomal puffs in *Drosophila* after heat stress (Ritossa 1962), hence the name heat shock proteins. However, in 1973, heat shock response was found to coincide with synthesis of a number of new proteins (Tissières et al. 1974). These proteins quickly gained much attention in research and hence considerable numbers of such proteins belonging to different families have been recognized till date.

Mammalian HSP belong to two groups according to their molecular weight: high molecular weight HSP and low molecular weight HSP. High molecular weight HSP include HSP90, HSP70 and HSP60 (chaperonin). Among these, some are expressed constitutively whereas others are expressed strongly in stressful condition and become abundant in the cytosol and nucleus. HSP90 is constitutively expressed and is particularly involved in signal transduction. HSP70 family is highly conserved and best studied family of HSP and comprises proteins ranging from 66–78 kD molecular weight. HSP70 is encoded by multi-gene family consisting of at least 11 genes in humans. Some of HSP family like HSP70 is localized in cytosol, some are restricted to mitochondria (mtHSP70) where as others are localized in endoplasmic reticulum (GRP78/Bip) (Jaattela et al. 1998). Eukaryotic HSP70s have two functional domains: one is $NH_2$-terminal ATP binding domain (ABD) whereas the other one is COOH-terminal peptide binding domain (PBD). Under normal or

stressed condition, HSP70 proteins act in ATP-dependent manner and require co-chaperones like HSP40, CHIP, HOP, HIP, BAG-1 and BAG-3 to correctly fold nascent proteins or refold stress modified proteins. HSP60 is also constitutively expressed but its expression can slightly increase under stress. HSP60 facilitate folding of mitochondrial proteins and promotes degradation of misfolded proteins in an ATP-dependent manner.

Small molecular weight HSP like HSP27 are ATP-independent and do not require co-chaperone for their activity. Both HSP70 and HSP27 are most strongly induced during oxidative stress. HSP are supposed to perform a variety of functions within the cellular machinery. But particularly, they help organisms to cope with extrinsic and intrinsic stresses. The term chaperone for HSP is adopted as they block inappropriate aggregations of vital proteins. Besides this function, molecular chaperones also participate in folding, transport, assembly and degradation of misfolded or aggregated proteins. Some families of HSP having constitutive expression in living system indicate their possible cellular functions under normal state.

HSP play pivotal roles in reducing harm resulting from a wide range of stressed conditions such as exposure to insecticides, heavy metals, desiccation, diseases and parasites and are important for recovery and survival of organisms (Lindquist 1986). To evaluate the toxic potential of various chemicals and drugs, toxicologists nowadays use HSP, more specifically HSP70, because of its conserved nature and induction by environmental stressors (Steinmetz and Resing 1997). In this chapter we will restrict our discussion on the various known interactions between Heat Shock Proteins and Pesticide stress in different organisms and the plausible reasons behind such responses.

## 2.2 Pesticides

Pesticides are the chemical or biological agents formulated to control or kill insects, weeds, rodents, fungi and other organisms that can threaten public health and economy. Pesticides belong to several categories like insecticides (organochlorines, organophosphates, carbamates, pyrethroids), herbicides (paraquat, diquat, 2,4-dichlorophenoxyacetic acid, gyphosate), fungicides (dithiocarbamates, captan), fumigants (ethylene dibromide, methyl bromide), rodenticides and miticides to control pests. They have several remarkable effects like crop protection, preservation of food stuffs and prevention of vector-borne diseases. Though the usage of pesticides is fruitful and worldwide, some existing drawbacks include environmental contamination and potential hazards to humans and other ecologically important animals. Several reports are available confirming pesticide-based environmental pollution and health hazards (Alavanja et al. 2004; Shukla et al. 2006). It has been indicated that, approximately 98% of sprayed insecticides and about 95% of herbicides reach sites like air, water, soil, food and non-target organisms other than their destinations (Miller 2004). As they are biocides and affect enzymes and different physiological systems in pests which may be identical or very similar to biological machinery in human beings, therefore they pose potential risks to human health.

## 2.3 Pesticide Stress

Exposures to pesticides leave overwhelming effects on living creatures in the environment. Pesticides and many other industrial chemicals are known to generate free radicals (FR) inside the biological systems. FRs having unpaired electron, show various degrees of chemical configurations such as superoxide, hydroxyl, nitric oxide and lipid peroxyl radicals. These radicals constitute reactive oxygen species (ROS). Seeking stability, FRs attack nearby molecules to occupy required electron and therefore induces oxidative stress by destroying proteins, carbohydrates, lipids and nucleic acids. Additionally, chronic exposure to pesticides may lead to several diseases such as cancers, diabetes, neurodegenerative disorders like Parkinson, Alzheimer, and amyotrophic lateral sclerosis (ALS), birth defects, and reproductive disorders in human beings.

## 2.4 Heat Shock Proteins and Pesticide Stress

Several studies have indicated a positive correlation between pesticide stress and HSP expression in considerable number of organisms. Study conducted by Bagchi et al. (1996) demonstrated that, exposure to alachlor, endrin, chlorpyrifos and fenthion triggers expression of HSP89α and HSP89β in hepatic and brain tissues of female Sprague-Dawley rats as well as in cultured PC-12 cells. Eder et al. (2009) reported expression of HSP60, HSP70 and HSP90 at 1.2 and 7.2 μg/L chlorpyrifos (CP), and at 0.01 and 0.1 μg/L esfenvalerate (EV) respectively in Chinook salmon. Authors of the same finding suggested that, HSP are sensitive indicators of sublethal exposure to CP and EV. Ceyhun et al. (2010) analyzed effect of deltamethrin on rainbow trout. They showed increased expression of HSP70 with significant fold-chance values at 0.25, 1 and 2.5 μg/L deltamethrin. Reporter gene assay was performed by Sarkar et al. (2015) to investigate effect of flubendiamide on HSP70 expression of 3$^{rd}$ instar larvae of *Drosophila melanogaster* (transgenic to lacZ-HSP70). They reported that, when *D. melanogaster* were exposed to 5, 10, 20 and 40 μg/L of flubendiamide, HSP70 expression was induced in all concentrations, but reached plateau at 20μg/mL concentration after 24h of exposure. This indicated dose and time dependent factors that control HSP70 expression under pesticide stress. Rajak et al. (2017) also demonstrated enhanced HSP70 expression along with increased activities of antioxidants - SOD and catalase in transgenic *Drosophila melanogaster* following exposure to 5μg/mL acephate for 24 h.

## 2.5 Putative Mechanism of HSP Induction Under Pesticide Stress

Majority of pesticides have strong potential to induce ROS inside the cell (Kumar et al. 2011; Rajak et al. 2017; Dutta et al. 2017; Khatun et al. 2017). ROS in turn activates Heat-Shock factors (HSFs). ROS directly activate HSF1 to induce hsp expression inside the cells. A study by Gorman et al. (1999) has demonstrated that, treatment with antioxidants prior to heat shock results in attenuated hsp expression, suggesting that ROS may be necessary for HSF activation and hsp expression. HSF1 is activated under heat shock and other stressed conditions whereas HSF2 is thought to be activated during embryonic development and cell differentiation. In unstressed cells, HSF1 having DNA-binding activity is present as a non-active form. Stress generated following pesticide insult catalyzes phosphorylation, oligomerisation and redistribution of HSF1within the nucleus. Activated HSFs bind with the heat shock elements (HSEs) present upstream of all heat shock genes, to initiate transcription. HSP90 binds and prevents the activation of HSF1. Immediately following stress exposure, denatured proteins are thought to bind HSP90, causing dissociation of HSP90 from HSF1. This leads to the phosphorylation of HSFs, leading to HSF activation (Fig. 2.1). The active HSFs form trimers that bind to the promoter of the hsp70 gene to initiate transcription.

Pesticide induced oxidative stress may result in protein misfolding and aggregation by shifting the conformational equilibrium towards more aggregation-prone states. Misfolding exposes hydrophobic regions which interact with other exposed hydrophobic regions of peptides leading to aberrant protein-protein interactions and aggregations (Vabulas et al. 2010). Molecular chaperones such as HSP can recognize non-native conformations of proteins and act upon them to prevent aggregation (Kim et al. 2013). HSP may also facilitate degradation of denatured proteins. Hydrophobic stretches of unfolded proteins are the targets of chaperones like HSP70 and are blocked to prevent protein-protein binding and thus aggregations. HSP40, a cofactor assists in recruitment of HSP70 to substrates and then induces ATPase activity to drive protein refolding (Fig. 2.2). Some small classes of HSP are ATP independent and bind to misfolded proteins to prevent protein aggregation.

## 2.6 HSP70 as a Blocker of Pesticide Induced Cell Death

Apoptosis is a programmed cell death induced by a variety of factors like hypoxia, DNA damage, protein damage and oxidative stress. It is well known that, exposure to pesticides generates ROS and therefore oxidative stress within cellular

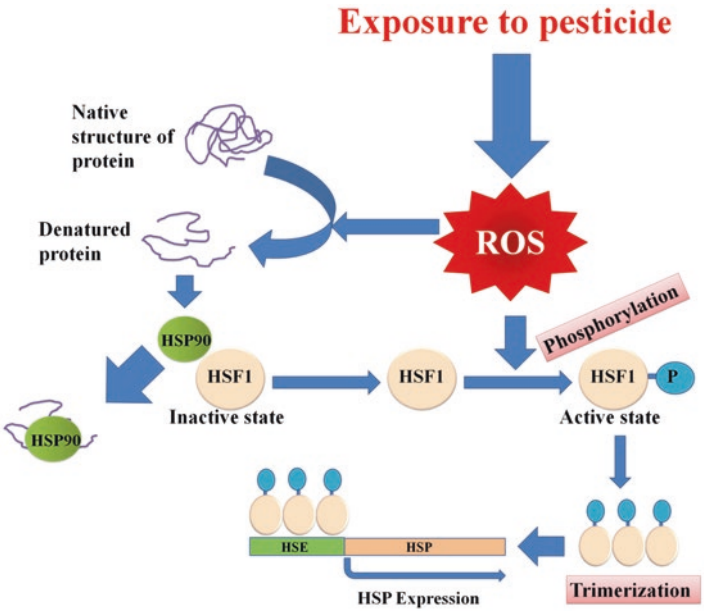


**Fig. 2.1** Probable mechanism of expression of Heat Shock Proteins (HSP) under pesticide stress. Pesticide exposure causes ROS production within the organism. ROS attacks functional proteins and catalyzes denaturation. Denatured proteins form aggregates with HSP90 and releases Heat Shock Factor 1 (HSF1) from HSP90-HSF1 complex. ROS induces phosphorylation and trimerization of HSF1 within the nucleus. Phophorylated HSF1 recognizes Heat Shock Element (HSE) located upstream of all hsp genes and triggers transcription

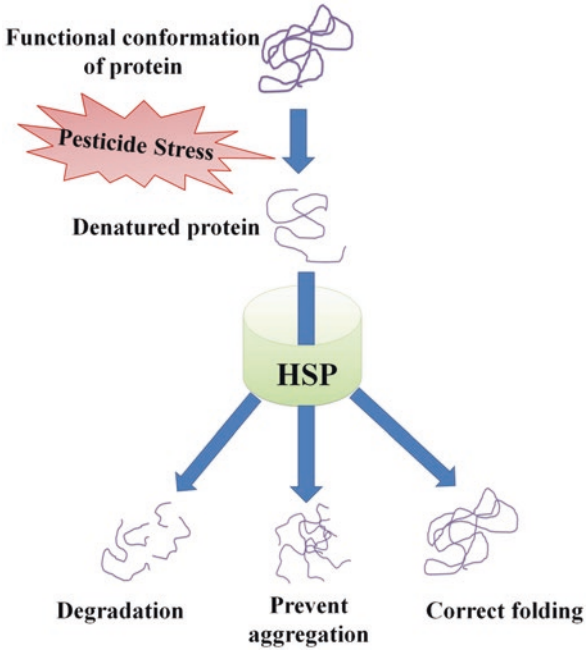


**Fig. 2.2** Major functions of HSP under pesticide stress. HSP show 3 major functions upon pesticide insult: **(a)** It may facilitate degradation of denatured proteins **(b)** aggregation of proteins is prevented **(c)** refolding of denatured protein into their functional confirmation

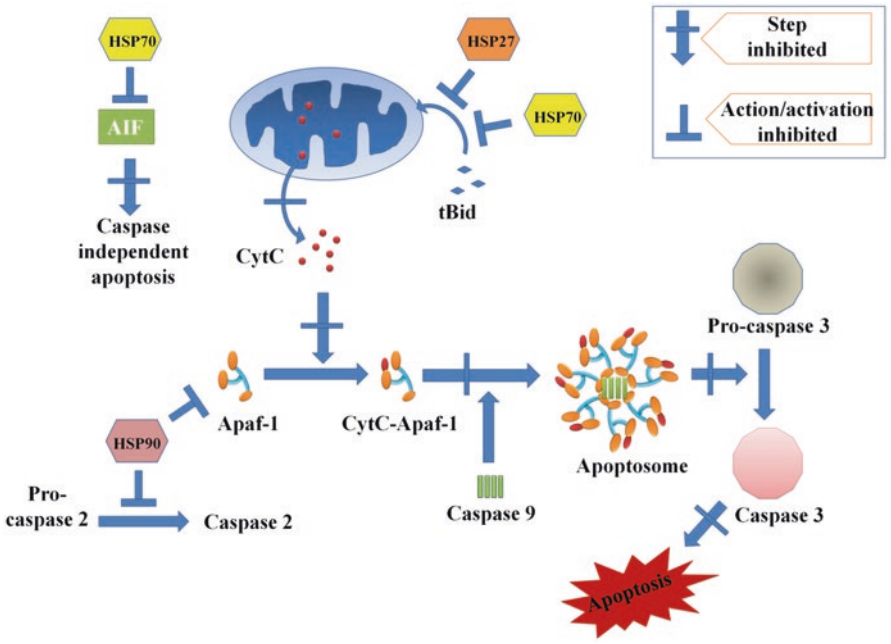


**Fig. 2.3** HSP as blockers of pesticide induced apoptosis. Some heat shock proteins modulate both extrinsic and intrinsic pathways of apoptosis. Translocation of tBid is important for release of cytochrome C from mitochondria. Released cytochrome C forms a complex with apaf-1 and with the help of caspase 9, apoptosome is formed. Apoptosome activates caspase 3 and execute apoptosis. Both HSP70 and HSP27 blocks translocation of tBid into the mitochondria. As a result, cytochrome C release is prevented and apoptosome formation is blocked. HSP70 also inhibits apoptosis inducing factor (AIF) to suppress caspase independent cell death. HSP90 binds with apaf-1 and prevent assembly of a functionally competent apoptosome. Simultaneously, caspase 2 activation can also be blocked by HSP90 upon its binding with procaspase 2

machinery. ROS triggers p53 mediated apoptosis in organisms (Li et al. 1999). HSP can block both extrinsic and intrinsic pathways by inhibiting keystone proteins of apoptotic cascades (Fig. 2.3). Recent evidences have suggested that, HSP27 induces intracellular level of glutathione that detoxify ROS and regulate apoptosis (Mehlen et al. 1996). HSP70 also sequesters apoptosis inducing factor (AIF) released from mitochondria and blocks caspase-independent pathway (Matsumori et al. 2005). Interestingly, both HSP27 and HSP70 can hinder translocation of tBid into the mitochondria to prevent cytochrome C release from mitochondria. HSP90 can bind apaf-1 and therefore downstream apoptotic cascades are inhibited. Besides, HSP90 is also found to block caspase-2 activation (Garrido and Solary 2003). Therefore HSP70, HSP90 and HSP27 are facilitating cell survival by blocking apoptotic cascades which may be activated by pesticide stressors.

## 2.7 HSP as a Biomarker of Pesticide and Other Stresses

Exposure to pesticide generates free radicals ($^{\cdot}O_2$ and $^{\cdot}OH$) and resultant oxidative stress. Free radicals are harmful and can damage protein moieties of plasma membranes. Hence antioxidant enzymes are expressed under pesticide stress to scavenge free radicals. Superoxide dismutase changes free radicals into $H_2O_2$ which is toxic. Hence, $H_2O_2$ is converted into $H_2O$ and molecular oxygen with the help of catalase. Thus, these two enzymes show considerable higher activities during oxidative stress and hence are promising bio-indicators of stress triggered by pesticides or other agents. Oxidative stress triggers protein unfolding and aggregations and hence families of HSP show increased expression to cope the problem. Therefore, HSP may also be considered as a potent biomarker of pesticide toxicity. In a study (Rajak et al. 2017), *e*xposure to organophosphate acephate enhanced SOD and catalase activities in transgenic *Drosophila melanogaster.* In addition, HSP70 expression was also significantly increased indicating its role in maintaining protein homeostasis. Doganlar and Doganlar (2015) reported that, exposure to a mixture of pesticides like molinate, thiobencarb, linuron etc enhances cellular SOD, catalase and glutathione synthetase antioxidant enzymes along with a significant hike in expression of HSP families like HSP70, HSP60, HSP83 and HSP26. Therefore, HSP are responsive to even minor pesticide insult and hence considered as sensitive biosensor of pesticide stress (Ait-Aissa et al. 2003). But, prolonged treatment with pesticides such as organophosphates cause cellular mortality and hence declined HSP expression may be observed due to unavaibility of viable cells (Stringham and Candido 1994). Similar finding has also been reported in case of fluoride containing pesticide (Dutta et al. 2017).

Interestingly, expressions of HSP in insects have been reported to elevate under various environmental stimuli. It is believed that, HSP protect proteins in eukaryotic cells from damage. Sahebzadeh and Lau (2017), have shown elevated levels of expression in HSP genes (hsp40, hsp70, and hsp90) in case of *Apis mellifera* L (Hymenoptera: Apidae) after exposure to sub-lethal concentrations of thymol, eucalyptol, α-pinene, trans-anethole, diallyl disulfide and infestation with Varroa mites. They further showed a dose-dependent up-regulation in the levels of HSP tested after the bees were treated with thymol, eucalyptol and α-pinene. Conversely, significant down-regulated expressions of the hsp genes were recorded after diallyl disulfide treatment. Transcriptions of all the HSP tested were significantly compromised when pupae were infested with different numbers (0–5) of Varroa mites. Thus, HSP can be used as biomarkers of survival in case of honey bees under toxic and pathogenic stress.

Organophosphorus compounds and oximes, very commonly used insecticides are known to interfere with molecules like Acetyl cholinesterase (AChE) in the living systems thereby affecting various cellular processes underlying normal biological mechanisms. These non-cholinergic effects might be responsible for clinical status in OP poisoning. Workers like Katalinić et al. (2013) have shown that, oximes affect IL-6 release and also regulate HSP. IL-6 was found to stimulate muscle regeneration,

which followed well known OP-induced myopathy. The HSP were found to have cytoprotective effect against various stressors including xenobiotics. Their experiments carried out on cultured human myoblasts as the precursors of muscle regeneration detected unchanged AChE mRNA level following treatment with oximes. This suggested that, the transcription or stability of such mRNA is not affected, even if AChE catalytic activity was significantly altered. On the other hand, significant changes in the protein levels of HSP27 and in secretion of IL-6 were observed. The secretion of IL-6 was decreased to 53% and the level of HSP 27 increased by 34% compared to the control level. Thus their study predicted that, OPs and oximes might play an important role in muscle regeneration through the altered expression of IL6 and HSP27.

Chan et al. (2007) in their study with Sprague-Dawley rats demonstrated the neuroprotective role of HSP in the rostral ventrolateral medulla (RVLM) during brain stem death caused by exposure to organophosphate pesticide mevinphos (Mev). Western blot, real-time PCR and proteomic analyses illustrated Mev induced *de novo* synthesis of HSP60 and HSP70 in the RVLM without affecting HSP90 level. Induced loss-of-function of HSP60 and HSP70 in brain using anti-serum and antisense oligonucleotide demonstrated Mev-elicited cardiovascular depression along with reduced nitric-oxide synthase I (NOS I)/protein kinase G signaling and enhanced NOS II/peroxynitrite cascade which further results in intensified nucleosomal DNA fragmentation, elevated cytoplasmic histone-associated DNA fragments and activated caspase-3. Cytochrome C/ caspase-3 cascade of apoptotic signaling was also augmented in the RVLM. Thus, loss-of-function of HSP60 and HSP70 reflects significance of NOS I activation and NOS II inhibition to down-regulate mitochondrial apoptotic cascade which is important for neuroprotection.

Furthermore in another study, Eder et al. (2009) demonstrated rapid expression of heat shock proteins in response to exposure to variety of stressors like individual pesticides, virus, and both stressors combined in fish (juvenile Chinook salmon). On study of Acetyl cholinesterase (AChE) inhibition and recovery in response to applied stressors, it was found that enzyme inhibition levels were co-relatable with imminent mortality, and other observed sub-lethal physiological effects were concurrent with depressed AChE activity. Interestingly, both pesticides (pyrethroids and organophosphate) as well as virus successfully induced hsp expression, but highest HSP levels were observed after the combined treatments. This suggests an additive inductive effect of virus and pesticides as a stressor since highest virus level was found to cause strongest HSP induction, indicating a positive correlation between virus concentration and HSP expression. Thus, HSP are found to be quite significant in busting the stress. In an invasive coleopteran weevil, eight genes encoding HSP90, HSP70 and small HSP are found to be up-regulated under heat stress where as only one gene encoding for HSP70 and one encoding for HSP90 are up-regulated under cold stress. Thus in their work, Yuan et al. (2014) demonstrated that, HSP from all families except HSP60 are up-regulated by temperature stress.

In a review article, Mahmood et al. (2014) has discussed on the anomalous nature of expression of HSP70 in different cases of exposures. Ni at a concentration of 600 M has no effect on HSP70 expression at the transcriptional level in HeLa cells,

whereas manifests sufficient expression in black sea bream fibroblast cell line at concentration of 0.01 M. In a study on *Mytilus coruscus* after exposure to fuel and heavy metals, HSP expression was found to be induced, showing steady increase with the passage of time and reached the maximum (about 6-fold increase) after 25 days of the treatment, but soon the expression started decreasing after gaining the peak level, and at day 30, expression was about two fold of the control (Liu et al. 2014). Similarly both $Cu^{2+}$ and $Cd^{2+}$ enhanced the expression of HSP70 by 10- fold and 11-fold, respectively. However, time of maximum expression was different; incase of $Cu^{2+}$, peak expression was achieved at day 15 while in the case of $Cd^{2+}$ it was at day 9 (Liu et al. 2014). Hence HSP70 can be taken as biomarker, however not in isolation. Instead it would be more logical to use it as biomarker along with other parameters like oxidative stress and ultrastructural changes. Few reports have also shown the down-regulation of HSP70 gene in response to heavy metal stress. In a recent study, Luo et al. (2014) have observed the effect of long-term heavy metal stress on *Crassostrea hongkongensis,* where instead of usual up-regulation, HSP70 was found to be down regulated. Differentially expressed proteins were identified in oyster exposed to heavy metals such as zinc, copper, manganese, and lead. This event of unusual expression was attributed to prolonged exposure to heavy metals. Thus, the anomalous behavior throws some challenges on the authority of HSP claiming it as a biomarker of stress.

## 2.8 Common Techniques Used to Monitor Hsp Expression After Pesticide Exposure in *Drosophila melanogaster*

Earlier toxicological assessment of pesticides were dependent upon histological, hematological and biochemical tests which were lengthy and time consuming procedures. At present, several molecular techniques are available that provide qualitative or quantitative measurements of HSP in organisms undergone pesticide exposure. Common techniques (Fig. 2.4) that are utilized in case of non-target model organism, *Drosophila melanogaster* include the followings:

### *2.8.1 Reporter Gene Assay*

It is a simple practice for measurement of HSP levels following pesticide exposure. In transgenic animals, hsp sequence is tagged with a reporter gene (lacZ or GFP) and analyzed for expression of the conjugated form after chemical insult. LacZ encodes for β-galactosidase which reacts with X-gal to produce a blue colored product. Appearance of blue color thus is an indicative of co-expression of both target hsp and lacZ under chemical stress. GFP stands for green fluorescence protein and gets co-expression with the target hsp when aligned under same promoter. Expression of

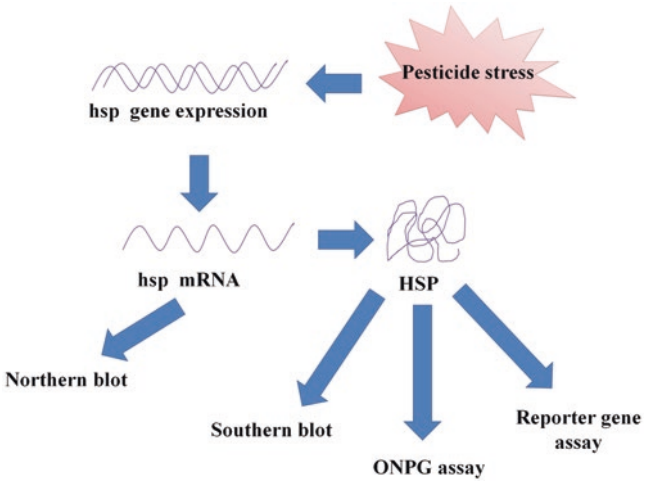


**Fig. 2.4** Common techniques used to monitor HSP expression after pesticide stress. Transcription of hsp genes can be triggered/enhanced upon pesticide exposure. Transcription is then followed by translation to produce functional HSP. Changes in mRNA levels are easily monitored by the northern blot technique. Functional HSP can be detected and in some cases quantified by southern blot, ONPG assay as well as reporter gene assay procedures

GFP can be monitored under fluorescence microscopy. Therefore, expression of lacZ or GFP can be detected easily to trace expression profile of a number of HSP. Widely accepted models, such as *Drosophila* transgenic for different stress protein encoding genes, like HSP70, HSP83, and HSP26, and reporter genes like β-galactosidase or GFP have been in use from several decades to detect cellular stress caused by environmental chemicals. Recent studies have implemented reporter gene assay to examine response of HSP70 following exposure to pesticides like NaF and flubendiamide in transgenic *Drosophila melanogaster* (hsp70-lacZ) (Dutta et al. 2017; Sarkar et al. 2015).

### *2.8.2 Soluble O-Nitro Phenyl-β-d-Galactopyranoside (ONPG) Assay*

It is a spectrophotometric technique utilized to get a quantitative measure of HSP expression in transgenic organisms (lacZ-hsp) under pesticide stress. Studies are available (Rajak et al. 2017; Dutta et al. 2017) that implicated soluble ONPG assay to investigate effect of different pesticides on HSP70 expression in transgenic *Drosophila melanogaster* (hsp70-lacZ) under exposure to different pesticides. lacZ encodes for β-galactosidase that hydrolyze ONPG into galactose and o-nitrophenol. ONPG is colorless compound where as O-nitrophenol is yellow and can be measured spectrophotometrically at 420 nm.

### *2.8.3 Northern and Western Blot Techniques*

Northern and Western Blot are sensitive techniques used to detect HSP in vivo and in vitro systems. They have been used in several studies during toxicological assessments of pesticides. Primarily, both techniques are used for HSP detection and quantification. But some researchers emphasized that, northern blot is highly sensitive and is initial step in the detection of HSP expression after environmental stress. Other scientists emphasized on western blot because of the fact that changes in mRNA expression do not necessarily correspond to changes in protein levels. Hence a simple measure of HSP mRNA may yield a doubtful result. Hence, it is better to consider both aspects in any proposed model organism.

## 2.9 Conclusions

The available literatures indicate the expression and functional roles of heat shock proteins during pesticide stress. HSP play important roles in mitigating pesticide-induced ROS and cellular damages. They are also considered as promising biomarkers of pesticide stress. The differential expression of HSP particularly HSP27, HSP70 and HSP90 helps to block apoptotic cascade which can be triggered following pesticide insult. More reliable and less time consuming methodologies such as reporter gene assay and northern and western blot techniques are now available that are in use to monitor pesticide-stress response of several families of HSP. Some HSP are investigated at high pesticide exposure and their roles during pesticide stress have been investigated in details but several other HSP like HSP84, HSP20 are available in cellular environment whose potential roles in stress response are yet to be explored.

**Acknowledgements** Authors show deep sense of gratitude towards the Head, Department of Zoology (DST-FIST, UGC-DRS sponsored), The University of Burdwan for infrastructural facilities. Continuous support obtained from other members of our research group during writing this book chapter is thankfully acknowledged.

## References

Ait-Aissa, S., Pandard, P., Magaud, H., Arrigo, A. P., Thyband, E., & Porcher, J. M. (2003). Evaluation of an in vitro hsp70 induction test for toxicity assessment of complex mixture: Comparison with chemical analysis and ecotoxicity tests. *Ecotoxicology and Environmental Safety, 54*, 92–104.

Alavanja, M. C. R., Hoppin, J. A., & Kamel, F. (2004). Health effects of chronic pesticide exposure: Cancer and neurotoxicity. *Annual Review of Public Health, 25*, 155–197.

Bagchi, D., Bhattacharya, G., & Stohs, S. J. (1996). In vitro and in vivo induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides. *Toxicology, 112*, 57–68.

Ceyhun, S. B., Senturk, M., Ekinci, D., Erdogan, O., Erdogan, O., Ciltas, A., & Koceman, E. M. (2010). Deltamethrin attenuates antioxidant defense system and induces the expression of heat shock protein 70 in rainbow trout. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology, 152*, 215–233.

Chan, J. Y. H., Cheng, H. L., Chou, J. L., Li, J., Faith, C. H., Dai, K. Y., Chan, S. H. H., & Chang, A. Y. W. (2007). Heat shock protein 60 or 70 Activates Nitric-oxide Synthase (NOS) I- and inhibits NOS II-associated signaling and depresses the mitochondrial apoptotic cascade during brain stem death. *The Journal of Biological Chemistry, 282*, 4585–4600.

Doganlar, O., & Doganlar, Z. B. (2015). Responses of antioxidant enzymes and heat shock proteins in *Drosophila* to treatment with a pesticide mixture. *Archives of Biological Sciences Belgrade, 67*(3), 869–876.

Dutta, M., Rajak, P., Khatun, S., & Roy, S. (2017). Toxicity assessment of sodium fluoride in *Drosophila melanogaster* after chronic sub-lethal exposure. *Chemosphere, 166*, 255–266.

Eder, K. J., Leutenegger, C. M., Kohler, H., & Werner, I. (2009). Effects of neurotoxic insecticides on heat-shock proteins and cytokine transcription in Chinook salmon (*Oncorhynchus tshawytscha*). *Ecotoxicology and Environmental Safety, 72*, 182–190.

Garrido, C., & Solary, E. (2003). A role of HSP in apoptosis through "protein triage"? *Cell Death and Differentiation, 10*, 619–620.

Gorman, A. M., Heavey, B., Creagh, E., Cotter, T. G., & Samali, A. (1999). Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS Letters, 445*, 98–102.

Jaattela, M., Wissing, D., Kokholm, K., Kallunki, T., & Egeblad, M. (1998). Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *The EMBO Journal, 17*, 6124–3614.

Katalinić, M., Miš, K., Pirkmajer, S., Grubič, Z., Kovarik, Z., & Marš, T. (2013). The cholinergic and non-cholinergic effects of organophosphates and oximes in cultured human myoblasts. *Chemico-Biological Interactions, 25*, 144–148.

Khatun, S., Rajak, P., Dutta, M., & Roy, S. (2017). Sodium fluoride adversely affects ovarian development and reproduction in *Drosophila melanogaster*. *Chemosphere, 186*, 51e61.

Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., & Hartl, F. U. (2013). Molecular chaperone functions in protein folding and proteostasis. *Annual Review of Biochemistry, 82*, 323–355.

Kumar, V., Ara, G., Afzal, M., & Siddique, Y. H. (2011). Effect of methyl methanesulfonate on hsp70 expression and tissue damage in the third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) $Bg^9$. *Interdisciplinary Toxicology, 4*, 159–165.

Li, P. F., Dietz, R., & Von Harsdorf, R. (1999). p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. *The EMBO Journal, 18*, 6027–6036.

Lindquist, L. (1986). The heat-shock response. *Annual Review of Biochemistry, 55*, 1151–1191.

Liu, H. H., He, J. Y., Chi, C. F., & Shao, J. (2014). Differential HSP70 expression in *Mytilus coruscus* under various stressors. *Gene, 543*, 166–117.

Luo, L., Ke, C., Guo, X., Shi, B., & Huang, M. (2014). Metal accumulation and differentially expressed proteins in gill of oyster (*Crassostrea hongkongensis*) exposed to long-term heavy metal-contaminated estuary. *Fish & Shellfish Immunology, 38*, 318–329.

Mahmood, K., Jadoon, S., Mahmood, Q., Irshad, M., & Hussain, J. (2014). Synergistic effects of toxic elements on heat shock proteins. *BioMed Research International*. https://doi.org/10.1155/2014/564136*, 2014*, 1.

Matsumori, Y., Hong, S. M., Aoyama, K., Fan, Y., Kayama, T., Sheldon, R. A., et al. (2005). Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. *Journal of Cerebral Blood Flow and Metabolism, 25*, 899–910.

Mehlen, P., Schulze-Osthoff, K., & Arrigo, A. P. (1996). Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *The Journal of Biological Chemistry, 271*, 16510–16514.

Miller, G. T. (2004). *Sustaining the Earth: An integrated approach* (pp. 211–216). Thomson/Brooks/Cole.

Rajak, P., Dutta, M., Khatun, S., Mandi, M., & Roy, S. (2017). Exploring hazards of acute exposure of Acephate in *Drosophila melanogaste*r and search for l-ascorbic acid mediated defense in it. *Journal of Hazardous Materials, 321*, 690–702.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia, 18*, 571–573.

Sahebzadeh, N., & Lau, W. H. (2017). Expression of heat-shock protein genes in *Apis mellifera meda* (Hymenoptera: Apidae) after exposure to monoterpenoids and infestation by Varroa destructor mites (Acari: Varroidae). *European Journal of Entomology, 114*, 195–202.

Sarkar, S., Podder, S., & Roy, S. (2015). Flubendiamide-induced HSP70 expression in transgenic *Drosophila melanogaster* (hsp70-lacZ). *Current Science, 108*, 2044–2050.

Shukla, G., Kumar, A., Bhanti, M., Joseph, P. E. and Taneja, A. (2006). Organochlorine pesticide contamination of ground water in the city of Hyderabad. Environment International 32, 244–247.

Steinmetz, U. N., & Resing, L. (1997). Heat shock protein induction by certain chemical stressors is correlated with their cytotoxicity, lipophilicity and protein denaturing capacity. *Toxicology, 123*, 185–195.

Stringham, E. G., & Candido, P. M. (1994). Transgenic hsp16 lacZ strains of the soil nematode *Caenorhabditis elegans* as biological monitors of environmental stress. *Environmental Toxicology and Chemistry*, (8), 1211–1220.

Tissières, A., Mitchell, H. K., & Tracy, U. M. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster*: Relation to chromosome puffs. *Journal of Molecular Biology, 84*, 389–398.

Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M., & Hartl, F. U. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harbor Perspectives in Biology, 2*, a004390.

Yuan, X., Zhou, W. W., Zhou, Y., Liu, S., Lu, F., Yang, M. F., Cheng, J., Gurr, G. M., & Zhu, Z. R. (2014). Composition and expression of heat shock proteins in an invasive pest, the rice water weevil (Coleoptera: Curculionidae). *Florida Entomologist, 97*(2), 611–619.

# Chapter 3
# Heat Shock Proteins and Abiotic Stress Tolerance in Plants


**Divya Mishra, Shubhendu Shekhar, Deepika Singh, Subhra Chakraborty, and Niranjan Chakraborty**

**Abstract** Abiotic stresses restrict plant growth and development, and reduce harvest index of many crop species worldwide. Maintenance of native conformation of proteins and reducing the accumulation of non-native proteins are imperative for survival under stress conditions as such stresses frequently lead to protein aggregation causing metabolic dysfunction. Heat shock proteins (HSP) play a key role in conferring abiotic stress tolerance. Plants protect themselves from numerous stresses by inducing HSP, besides some stress-responsive proteins, suggesting analogous response mechanisms. A close association between the HSP and ROS also co-exists, indicating that plants have evolved to gain a higher degree of regulation over ROS toxicity and can use ROS as elicitor to induce HSP for better adaptations through activating an array of molecules. Therefore, unraveling the mechanisms of plant response against various stress and the role of HSP in acquired stress tolerance is utmost important to delineate their specific function as a part of stress-responsive module. The HSP have been well characterized in different crop species, albeit the knowledge about their correlation with genome sequence information as well as their functional plasticity is limited.

**Keywords** Abiotic Stress · Chaperones · Co-chaperones · Heat shock factor · Heat shock protein · Protein folding · Stress tolerance

## Abbreviations

| | |
|---|---|
| ACD | α-crystalline C-terminal domains |
| HLS | High light stress |
| HMS | Heavy metal stress |
| HOP | Hsp70-Hsp90 organizing protein |

D. Mishra · S. Shekhar · D. Singh · S. Chakraborty · N. Chakraborty (✉)
National Institute of Plant Genome Research, Jawaharlal Nehru University Campus, New Delhi, India
e-mail: nchakraborty@nipgr.ac.in



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_3

| | |
|---|---|
| HSE | Heat shock element |
| HSF | Heat shock factor |
| HSP | Heat shock protein |
| HTS | High temperature stress |
| LTS | Low temperature stress |
| NMD | Nonsense-mediated mRNA degradation |
| PCD | Programmed cell death |
| PTM | Post-translational modification |
| ROS | Reactive oxygen species |
| sHSP | Small heat shock protein |
| UPS | Ubiquitin proteasome system |

## 3.1 Introduction

Plants are sessile, and are continuously exposed to environmental stresses, both biotic and abiotic. Abiotic stresses alone or most often their combinatorial effect/s leads huge yield loss worldwide, as they cause severe threat to plant's survival (Mittler 2006). However, plants have evolved a variety of mechanisms to acclimatize to changing environmental conditions. They undergo drastic adjustments and alterations in physiological as well as molecular programs with efficient molecular machineries to perceive and overcome such stresses (Ahuja et al. 2010). Plants induce different stress-responsive biomolecules as a part of their tolerance mechanisms. One of the most important such biomolecules is the molecular chaperones, which act in reducing cells from the adverse effects of stress. Heat shock proteins (HSP) are one of the significant classes of molecular chaperones, which act in response to various stresses viz., extreme temperature, dehydration, salinity, oxidative, heavy metals, high intensity irradiations and wounding, among others (Swindell et al. 2007; Al-Whaibi 2011; Xu et al. 2011). The roles of HSP are apparently more in response to high temperature stress (HTS) when compared with other stresses. The heat shock response and the HSP are predicted to be evolutionary conserved. There is an intimate association between expressions of HSP with that of resistance to HTS, but in-depth mechanism through which HSP work to increase thermotolerance is yet to be fully understood (Singh et al. 2016). In general, regulation of protein folding and unfolding, in conjunction with their subcellular localization and eventually the degradation of unfolded and denatured proteins is the principal function of HSP (Singh et al. 2016). Several studies revealed the assorted functions of HSP and/or their homologues through their constitutive or temporal expression under the tight regulation of cell cycle, cell growth and development. These studies further suggested the role of HSP in plant growth and development particularly in embryogenesis, seed and fruit development (Siddique et al. 2008; Al-Whaibi 2011; Koo et al. 2015). The role of HSP has also been predicted in tuber development (Lehesranta et al. 2006; Agrawal et al. 2008; Agrawal et al. 2013), and nutrient acquisition during tuberization (Shekhar et al. 2016).

Several HSP have been identified and characterized in organisms across the species (Bharti and Nover 2002). Presence of conserved heat-shock domain (~70 amino acids) at carboxylic terminal is the main distinctive feature of all HSP (Helm et al. 1993), while little homology is observed in the amino-terminal regions (Chen and Vierling 1991; Vierling 1991). It has, therefore, been hypothesized that the heat-shock domain favors the accumulation of low molecular weight aggregates (sHSP), and their diversity in amino termini might be responsible for either precise substrate specificities or separate functions (Helm et al. 1993). Altogether, HSP in a range of 10–200 kDa molecular weights are distinguished as chaperones, which play a key role in stress-responsive signal transduction (Schöffl et al. 1999). It has been increasingly evident that HSP act in association with heat stress transcription factors (HSFs). The HSFs, in plant, are considered to be one of the vital components of signal transduction cascade mediating the expression and regulation of genes implicated in several abiotic stress responses (Guo et al. 2016). The increased expression of HSP under the control of HSFs is thought to play a significant role in thermotolerance (Kotak et al. 2007). The coordinated role/s of HSP and HSFs in the development of tolerance against various abiotic stresses is depicted in Fig. 3.1.

Our existing knowledge about the role of HSP and HSFs has been recapitulated in several recent reports (Baniwal et al. 2004; Wang et al. 2004; Nakamoto and Vigh 2007; Guo et al. 2016). Previous attempts to enhance thermotolerance via ectopic expression of a solitary HSP or HSF gene exhibited limited impact owing to the intricacy and genetic complexity of the HTS response (Vinocur and Altman 2005; Fragkostefanakis et al. 2015). Genome-wide expression analysis as well as the comparative proteomics in Arabidopsis subjected to HTS, or the mutants with impaired thermotolerance significantly extended our understanding of HTS responses (Larkindale et al. 2005; Echevarría-Zomeño et al. 2016). Comparative expression data analyses revealed a similar transcript accumulation pattern in different plant species subjected to HTS. Interestingly, ~ 2% of the genome has been predicted to be affected by HTS. (Rizhsky et al. 2004; Rensink et al. 2005; Lim et al. 2006).

## 3.2 Hsp as Chaperones

The HSP have long been anticipated to function as molecular chaperones, which actively participate in protein quality control to maintain cellular homeostasis in stressed as well as in unstressed conditions. Besides their function as molecular chaperones, detail information as to how some HSP and sHSP contribute to stress tolerance in plants from HTS in particular and other stresses in general. Are still unclear. Several molecular chaperones are identified as stress-associated proteins, most of which are originally categorized as HSP (Wang et al. 2004). In plants, five major classes of HSP have been catalogued based on their estimated molecular weight, amino acid sequence and functions; (I) Hsp100, (II) Hsp90, (III) Hsp70, (IV) Hsp60, and (V) sHSP. Some other proteins have also been identified having

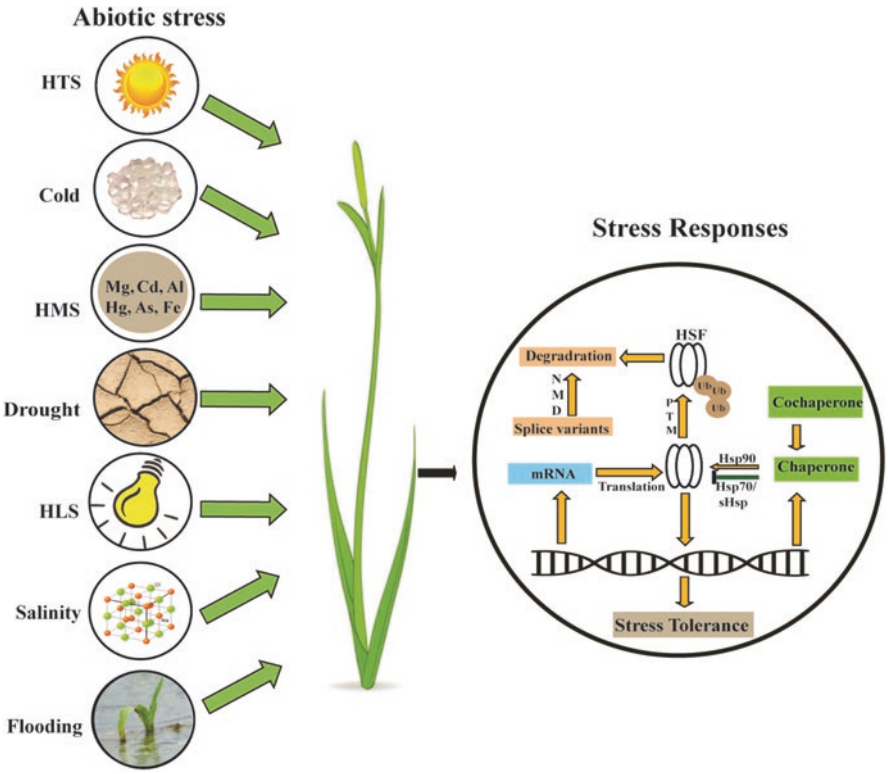


**Fig. 3.1** Abiotic stress responses in plants and molecular adaptation. Abiotic stress elicits myriad of responses in plants that include the induction of various stress-responsive biomolecules. Increased expression of heat shock transcription factors (HSFs) bind to promoter regions of stress-responsive genes, eventually leading to the expression of stress-inducible HSFs, chaperones, co-chaperones and downstream HTS-responsive genes. The network of HTS-inducible HSFs is tightly regulated by the chaperone and their co-chaperones. The regulation of HSF-chaperone/co-chaperone complex is orchestrated by post-translational modification (PTM) and also, by chaperone-mediated ubiquitination and degradation by the ubiquitin proteasome system (UPS). The HSP also regulate the activities of HSFs by the association or disassociation with HSF complex, besides alternative splicing and nonsense-mediated mRNA degradation (NMD). HTS, high temperature stress; HMS, heavy metal stress; HLS, high light stress

chaperone-like functions, for examples, calnexin/calreticulin and protein disulfide isomerase since they help in protein folding (Wang et al. 2004; Al-Whaibi 2011). The HSP are localized in cytoplasm as well as in organelles, for instances, nucleus, chloroplasts, mitochondria and ER (Vierling 1991; Waters et al. 1996; Boston et al. 1996).

### *3.2.1 Hsp100*

These chaperones are the members of the large AAA ATPase superfamily, which are implicated in multivariate stress responses. The reactivation of aggregated proteins is one of the exclusive functions of this class through re-solubilization of protein aggregates. Additionally, they help irreversibly damaged proteins to undergo degradation pathway (Wang et al. 2004; Al-Whaibi 2011). Some members of this class also play a key role in maintaining the housekeeping functions and chloroplast biogenesis Lee et al. 2007). Furthermore, these HSP contribute in expediting the post-stress normal conditions (Gurley 2000). To evade the aggregation of proteins, Hsp100 works cooperatively with Hsp70. The aggregated proteins are solubilized by the Hsp100 family proteins, which with the help of Hsp70 system could be subsequently refolded in the earlier conformations. Chaperones of Hsp100 family, in plants, are often constitutively expressed similar to several other HSP/chaperones. Their expression is induced by various environmental stresses, and is tightly regulated for developmental and metabolic cues (Queitsch et al. 2000; Keeler et al. 2000; Adam et al. 2001; Adam and Clarke 2002).

### *3.2.2 Hsp90*

Similar to Hsp100, the chaperones of Hsp90 also work in association with Hsp70, and constitute a major part of chaperone complexes. While the major role of Hsp90 is protein folding, it also acts as the key component in signal-transduction networks, cell-cycle control, protein trafficking and in the regulation of glucocorticoid receptor activity (Pratt and Toft 2003; Pratt et al. 2004). Furthermore, the role of Hsp90 has long been acknowledged in phyto-pathogen resistance (Hubert et al. 2003; Liu et al. 2004; Thao et al. 2007). A study by Yamada et al. (2007) suggested its role in regulation of HSF in the absence and presence of HTS (Yamada et al. 2007). Expression of Hsp90 is developmentally regulated and shows differential expression in response to different abiotic stresses (Wang et al. 2004). Organelle-specific Hsp90 have been isolated and characterized from different plant species, which showed 63–71% homology at amino acid level with that of yeast and animal. In Arabidopsis, seven Hsp90 family proteins has been annotated with cytosolic subfamily AtHsp90–1 to AtHsp90–4, whereas plastid, mitochondria and ER comprising AtHsp90–5, AtHsp90–6 and AtHsp90–7, respectively (Krishna and Gloor 2001).

### 3.2.3 Hsp70

The Hsp70 chaperones function in association with co-chaperones, for example, DnaJ/Hsp40 and GrpE etc., which prevent aggregation of misfolded proteins and facilitate refolding of non-native proteins under stressed as well as unstressed conditions (Sung et al. 2001; Su and Li 2008). They play a distinct role in protein import and translocation, particularly in the chloroplast and mitochondria. The Hsp70 act as the major components of driving irreparable proteins to lysosomal or proteasomal degradation pathway thereby preventing unfolded protein response (UPR) (Kaufman 1999; Huang et al. 1999). Constitutive expression of several members of Hsp70 family has been reported and often referred to as 70-kDa heat-shock cognate (Hsc70), while rest of the members usually express during the stressful conditions. Moreover, some of them are associated to regulating the biological function of folded regulatory proteins, which presumably act as negative repressors of HSF-mediated transcription. There exists a large number of Hsp70 family chaperones in plants, for instance, 18 Hsp70 in Arabidopsis genome of which 14 are represented by DnaK and 4 are represented by Hsp110/SSE subfamily (Wang et al. 2004). Available data suggest their role in transcriptional regulation of heat-shock genes by preventing transcriptional activation by their HSFs. Interaction of Hsp70 with HSF inhibits trimerization and thereby binding of HSF to heat-shock elements (Kim and Schöffl 2002). Additionally, Hsp70 acts as a key module of guidance complex import i.e. translocon, which co-operatively binds to protein precursor and drives into target organelles (Jackson-Constan et al. 2001; Soll 2002). It is apparent that Hsp70B in the stroma of chloroplasts contribute throughout and/or after the photoinhibition to the photo-protection and/or restoring the function of photosystem II. Further, stromal Hsp70 is needed for the differentiation of germinating seeds and tolerance against HTS (Schroda et al. 1999; Su and Li 2008).

### 3.2.4 Hsp60

The members of Hsp60 family are also known as chaperonins, which evolutionarily shows homology with GroEL from E. coli. This class of molecular chaperones are ubiquitously found in prokaryotes, but in eukaryotes they are predominantly found in mitochondria and plastids. These proteins are crucial for assisting the Rubisco and other plastid proteins (Hartl 1996; Wang et al. 2004). An earlier study indicated the role of Hsp60 in folding and aggregation of many chloroplast and mitochondrial resident proteins (Lubben et al. 1989). The chaperonins act as post-transcriptional regulator of many proteins as their cooperative binding helps to prevent aggregation of the target proteins (Parsell and Lindquist 1993). Information pertaining to the plant chaperonins are restricted and it seems that the stromal chaperones (Hsp70 and Hsp60) are associated in managing functional conformation of those proteins, which have chloroplast-mediated import signal (Jackson-Constan et al. 2001).

## 3.3 sHSP Family

The sHSP family chaperones are omnipresent and evolutionally conserved having molecular weight ranging from 12–42 kDa. Since the molecular weight of most of the sHSP are in the range of 15–22 kDa, they are also named as Hsp20. The sHSP are different with respect to the other HSP as they encompass extremely conserved 80–100 long amino acid sequences known as α-crystalline C-terminal domains (ACD). The sHSP function in an ATP-independent manner and bind co-operatively to the non-native protein substrates. This protein complex subsequently interacts further with other chaperones such as ClpB and Hsp70/Hsp40 for reactivation of the denatured proteins. Similar to other classes, sHSP also play crucial role in cellular protection via avoiding stress-induced protein aggregation (Muthusamy et al. 2017). In addition, sHSP are the decisive components for different developmental processes in plants (Siddique et al. 2008; Al-Whaibi 2011; Koo et al. 2015). Considering sequence similarity, cellular location and functions, sHSP constitute a more diverse family when compared with the other HSP/chaperones. Recent studies indicate that all sHSP are encoded by nuclear genomes. The sHSP are reported to be localized to the cytoplasm, nucleus, mitochondria, chloroplast, ER and peroxisome (Waters 2013). The defensive role of sHSP against a wide range of biotic and abiotic stresses has been increasingly evident in different crop species including rice (Sarkar et al. 2009), wheat (Muthusamy et al. 2017), tomato (Yu et al. 2016), soybean (Lopes-Caitar et al. 2013), maize (Hu et al. 2010), barley (Reddy et al. 2014) and pepper (Guo et al. 2015). Studies related to the roles of HSP in several crops in different abiotic stresses is enlisted in Table 3.1.

## 3.4 Role of Hsp in Abiotic Stress

### *3.4.1 High Temperature Stress*

The escalating earth temperature has far-reaching effects and any increase in the optimum temperature imparts negative impact on plant growth and productivity. Over the past decade, the omics studies revealed several HSP to be differentially regulated under HTS. Several previous investigations suggested the role of Hsp70 in various crops (Hu et al. 2009; Rollins et al. 2013; Zhang et al. 2014a, b; Singh et al. 2016; Zhang et al. 2017). Its role/s in protein translation, translocation, folding and preventing protein aggregates have been well explored. The members of Hsp70 has been classified into three categories: cytosolic, mitochondrial (mtHsp70) and chloroplastic Hsp70 (cpHsp70) (Yu et al. 2015). While cytosolic Hsp70 is the key regulator of HTS-mediated response (Jungkunz et al. 2011), the role of cpHsp70 has been found in chloroplast development (Sung et al. 2001, Kim and An 2013). The member of Hsp70 and Hsp90 are often found to be upregulated in response to HTS (Majoul et al. 2004; Hu et al. 2009; Li et al. 2013). Previous investigation showed

**Table 3.1** Studies on the roles of HSP in different crops

| Type of HSP | Stress type | Organism | Reference |
|---|---|---|---|
| Hsp70 | Heat stress | Rice, wheat, foxtail millet, *Chrysanthemum*, barley | Hu et al. (2009), Zhang et al. (2017), Singh et al. (2016), Zhang et al. (2014), and Rollins et al. (2013) |
| Hsp90 | Heat stress | Wheat, alfalfa, soybean | Majoul et al. (2004), Li et al. (2013), and Xu et al. (2013) |
| Hsp100 and 101 | Heat stress | Rice, maize, *Brassica* | Singla et al. (1998), Lee et al. (2007), Young et al. (2001), and Young et al. (2004) |
| Hsp60 | Heat stress | Grasses | Xu et al. (2011) |
| Hsp17.8 and Hsp 17.2 | Heat stress | *Rosa chinensis Camellia sinensis* | Wang et al. (2017), Jiang et al. (2009) |
| Hsp17.6 | Heat stress | *Brassica napus* | Young et al. (2004) |
| Hsp17.7 | Cold stress | Peach | Zhang et al. (2011) |
| Hsp17.5 | Cold stress | Chestnut | Soto et al. (1999) |
| Hsp17.4 and 17.6 | Cold stress | Tomato | Sanchez-Bel et al. (2012) |
| Hsp26 | Cold stress | Tomato, sweet pepper | Sanchez-Bel et al. (2012), Guo et al. (2007) |
| Hsp70 | Cold stress | Wheat, barley, maize, pea, tobacco and Arabidopsis | Vítámvás et al. (2012), Kosová et al. (2013), Kollipara et al. (2002), Dumont et al. (2011), Jin et al. (2011), and Bae et al. (2003) |
| Hsp90 | Cold stress | Wheat, tobacco, maize, sunflower and Arabidopsis | Jin et al. (2011), Kollipara et al. (2002), Balbuena et al. (2011), and Reddy et al. (1998) |
| Hsp21, 25, 95 and 75 | Cold stress | Rice | (Hahn and Walbot 1989) |
| Tom 111 and tom 66 | Cold stress | Tomato | Sabehat et al. (1998) |
| Hsp18.1, 18.2 and 22 | Cold stress | Grapefruits | Rozenzvieg et al. (2004) |
| sHSP1 | Cold stress | Plum | Sun et al. (2010) |
| Hsp94, 89, 75, 60, 58, 37 and 21 | Dehydration stress | *Gossypium hirsutum* | Burke et al. (1985) |
| Hsp70 | Dehydration stress | *Zea mays, Cicer arietinum, Aphanothece, Oryza sativa* | Benešová et al. (2012), Jaiswal et al. (2013), Subba et al. (2013a, b), Bhushan et al. (2011), Choudhary et al. (2009), Pandey et al. (2008), Sugino et al. (1999) |
| Hsp26 | Dehydration stress | *Zea mays* | Benešová et al. (2012) |
| Hsp70 and binding protein (BiP) | Dehydration stress | *Nicotiana tabacum* | Alvim et al. (2001), Ono et al. (2001) |

(continued)

**Table 3.1** (continued)

| Type of HSP | Stress type | Organism | Reference |
|---|---|---|---|
| Hsp90, Hsp20, CPN60, DnaJ | Dehydration stress | *Oryza sativa, Cicer arietinum* | Bhushan et al. (2011), Pandey et al. (2010) |
| Hsp19, chaperonin 21 precursor | Dehydration stress | *Cicer arietinum* | Subba et al. (2013a, b) |
| Hsp24.1 | Dehydration stress | *Oryza sativa* | Agrawal et al. (2016) |
| Hsp17.7 | Dehydration stress | *Oryza sativa* | Sato and Yokoya (2008), Sun et al. (2001) |
| Hsp17.5 | Dehydration stress | Barley | Reddy et al. (2014) |
| Hsp70 | Heavy metal | *Lycopersicon,* poplar, *Lotus corniculatus, Arabidopsis* and soybean | Neumann et al. (1994), Navascués et al. (2012), Yang et al. (2015), Sarry et al. (2006), Duressa et al. (2011) |
| Hsp90 | Heavy metal (Cadmium) | *Oryza sativa* | Ogawa et al. (2009) |
| Hsp26 | Heavy metal (Cadmium) | Soybean | Czarnecka et al. (1988) |
| Hsp17.7 | Heavy metal (lead and arsenic) | Carrot | Lee et al. (2011) |
| Hsp 20,22,23.1 | Heavy metal | Poplar | Yang et al. (2015) |
| Hsp90.3 | Heavy metal | Arabidopsis | Song et al. (2012) |
| Hsp90 | Salt stress | Soybean, Arabidopsis, *Glycine max* | Pi et al. (2016), Song et al. (2009a, b), and Xu et al. (2013) |
| Hsp70 | Salt stress | Tomato, rice | Manaa et al. (2011), Hoang et al. (2015) |
| Hsp110 | Salt stress | *Oryza sativa* | Singla et al. (1997) |
| Hsp100/ clpB2,B4 & D2 | Salt stress | Wheat | Muthusamy et al. (2017) |
| Hsp100/clpB1 | Salt stress | *Oryza sativa* | Mishra et al. (2016) |
| Hsp16.9 | Salt stress | *Oryza sativa* | Jung et al. (2014) |
| Hsp16.45 | Salt stress | *Lilum davidii* | Mu et al. (2013) |
| Hsp 17.8 | Salt stress | *Rosa chinensis* | Jiang et al. (2009) |
| Hsp101,80 and 70 | Light stress | *Arabidopsis thaliana, Chlamydomonas* | Rossel et al. (2002), Giacomelli et al. (2006) |
| Hsp70 | Flood stress | Maize, soybean, rice, Arabidopsis | Chen et al. (2014a, b), Komatsu et al. (2013), Qi et al. (2011), and Banti et al. (2010) |
| Cpn60 | Flood stress | Soybean | Komatsu et al. (2011) |
| Hsp 101 | Flood stress | *Arabidopsis thaliana* | Banti et al. (2010) |

(continued)

**Table 3.1** (continued)

| Type of HSP | Stress type | Organism | Reference |
|---|---|---|---|
| Hsp23.6 | Flood stress | *Solanum lycopersicum* | Hüther et al. (2017) |
| Hsp90, 90.2, 90.5, 90.7 | Oxidative stress | *Arabidopsis thaliana* | Song et al. (2009a, b), Nishizawa-Yokoi et al. (2010) |
| ClpB-cyt, ClpC2 and ClpD1 | Oxidative stress | *Oryza sativa* | Singh et al. (2010) |
| Hsp70 | Oxidative stress | *Oryza sativa* | Chankova et al. (2014) |
| Hsp17.6 | Oxidative stress | *Arabidopsis thaliana* | Scarpeci et al. (2008) |
| Hsp18.6, Hsp16.9B and Hsp23.7 | Combined stress | *Oryza sativa* | Zou et al. (2012), Wang et al. (2015), and Jung et al. (2014) |
| Hsp17.6A | Combined stress | *Arabidopsis thaliana* | Sun et al. (2001) |
| Hsp90 | Combined stress | Soybean | Xu et al. (2013) |
| Hsp17.5 | Combined stress | *Nelumbo nucifera* | Zhou et al. (2012) |

maximum induction of OsHsp90.1, all forms of GmHsp90 (A, B & C) and AtHsp90 under HTS (Hu et al. 2009; Prasad et al. 2010; Xu et al. 2013). Hsp90.2 inhibits the expression of HSF under unstressed condition, while HSFs are induced under HTS via inhibition of Hsp90.2 (Yamada et al. 2007). A number of Hsp100 (Hsp 97, 100, 101, 103, 108, 110, 114 and 118) were found to be upregulated in response to HTS (Singla et al. 1998; Young et al. 2001; Lee et al. 2007). Of the Hsp100 members, Hsp101 was found to be very critical for thermotolerance (Queitsch et al. 2000). The Hsp100 class not only responds to HTS, but also helps in organelle development such as chloroplast and mitochondria (Pyatrikas et al. 2014; Merret et al. 2017). Inhibition of Hsp101 in Arabidopsis showed reduced growth under extreme high temperature and constitutive expression of Hsp100 could provide better adaptation (Queitsch et al. 2000; Nieto-Sotelo et al. 2002). Increased transcript accumulation of Hsp17.6 and Hsp101 were observed in reproductive organs of Brassica under HTS. Similar observation was also observed in maize (Dupuis and Dumas 1990; Young et al. 2004). Since reproductive stages are more thermosensitive than the vegetative stage of plants, the HSP are induced in various reproductive stages under the HTS (Duck and Folk 1994; Sung et al. 2001). Conversely, constitutive overexpression of AtHsp90.3 compromised tolerance to HTS in Arabidopsis via delayed expression of HSFs and some HSP (Xu et al. 2010).

Hsp60 behaves very similar to Hsp70 as it shares some functions such as protein folding. The organellar Hsp60 is structurally very different from bacterial Hsp60 (Hartl 1996; Wang et al. 2004). Chloroplastic Hsp60 is crucial for assembling the Rubisco enzyme in native conformation and showed constitutive expression under normal condition, but little upregulation is observed in response to HTS (Xu et al.

2010). The mutation in cpHsp60 leads to the improper development of chloroplast (Wang et al. 2004). While mtHsp60 remains inactive during normal condition, induced expression is observed only in elevated temperature, presumably protect the mitochondria (Xu et al. 2010).

Several earlier studies, based on the proteomic analysis under HTS, showed differential expression of different types of sHSP (Hsp18.1, 17.9, 17.4, 22.3, 26 and 16.9) (Majoul et al. 2004; Lin et al. 2005; Lin et al. 2010; Liao et al. 2014; Kumar et al. 2017a, b). Expression of several sHSP (16.9, 17.7, 18, 18.9, 23.5 and 26.6) were shown to be upregulated in thermotolerant cultivar, while downregulated in sensitive cultivar (Chandel et al. 2013; Mishra et al. 2017). This clearly indicates that the expression of sHSP are cultivar-specific. Genome-wide analysis of sHsp 15, 25, 26 and 27 of foxtail millet substantiated these results as they were highly upregulated during HTS (Singh et al. 2016). Additionally, sHsp16.9 and variants of Hsp17 were reported to be elevated in various crops in response to HTS (Süle et al. 2004; Zhang et al. 2013). Overexpression of chloroplastic chaperone DnaJ/Hsp40 also showed increased tolerance in response to HTS by promoting synhesis of antioxidants and reduced accumulation of ROS (Wang et al. 2017). Similarly, heterologous expression of Hsp17.8 and Hsp17.2 displayed improved adaptation under HTS in Rosa chinensis and Camellia sinensis, respectively (Talamè et al. 2007). The increase in exposure of hydrophobic sites of dodecameric complex of NtHsp18.3 under HTS suggest fact that HTS induces the conformational change in 3-D so that it can protect other cellular proteins (Maimbo et al. 2007). The overexpression of sHsp17.7 in rice and carrot exhibited improved thermotolerance (Malik et al. 1999; Murakami et al. 2004). Similar to these reports, overexpression of Hsp21.4 showed upregulation of Hsp101 and Hsp70, suggesting the positive effect of sHSP in alleviating the damage caused by HTS in P. forrestii (Zhang et al. 2014a, b). Interaction studies of sHSP indicate that the sHSP may aggregate through ACD and form heteromeric complex, and promote stress adaptation (Chen et al. 2014a, b).

### *3.4.2 Cold Stress*

Low temperature or cold stress, a major factor of abiotic stresses, decreases the rate of uptake of water and nutrients, leading to cell desiccation and starvation. Cold stress can be divided into two subgroups, chilling stress (temperature less than 20 °C) and freezing stress (temperature less than 0 °C). To acclimatize to low temperature stress (LTS), plant induces the synthesis of variety of new proteins and also provide protection to existing proteins. Induction of HSP is the crucial part of cold acclimation. The members of Hsp70 are highly induced in various crops viz., wheat (Vítámvás et al. 2012; Kosová et al. 2013), rice (Lee et al. 2009), barley (Hlaváčková et al. 2013), maize (Kollipara et al. 2002), pea (Dumont et al. 2011), besides model plants tobacco (Jin et al. 2011) and Arabidopsis (Bae et al. 2003) in response to LTS.

The expression of Hsp90 under LTS is dependent on the developmental stages and crop species. Expression of Hsp90was shown to be downregulated under LTS in

wheat (Vítámvás et al. 2012), while upregulated in tobacco (Jin et al. 2011), maize (Kollipara et al. 2002), rape seed (Reddy et al. 1998) and sunflower (Balbuena et al. 2011). The expression of few HSP such as Hsp95 and Hsp75 are highly induced in response to LTS in rice (Hahn and Walbot 1989). The stability of Rubisco is also maintained in LTS via induced expression of Hsp60 and Hsp21 (Rinalducci et al. 2011; Kosová et al. 2013). However, expression of Hsp60 and Hsp21 was shown to be downregulated in sunflower (Balbuena et al. 2011). Importantly, the sHSP not only protect the protein structure and folding, but also maintain the membrane fluidity and electrolyte leakage, thereby contributing to cold tolerance. There have been several investigations on LTS-responsive sHSP in tomato (Sanchez-Bel et al. 2012), chestnuts (Soto et al. 1999), rice (Hahn and Walbot 1989) and plum (Sun et al. 2010). Many sHSP were shown to be expressed in LTS only when subjected to pre-heat treatments. Two sHSP, tom66 and tom111 in tomato, exhibiting limited or no expression under LTS were found to be induced when subjected to pre-heat treatment followed by low temperature (Sabehat et al. 1998). This phenomenon is not restricted to tomato, but exists in different crops such as sweet pepper (Guo et al. 2007), peach (Zhang et al. 2011) and grapefruits (Rozenzvieg et al. 2004). It seems that when plants are subjected to low temperature after pre-heat treatment, the induction of sHSP is higher as compared to high molecular weight HSP, indicating the recruitment of diverse HSP to generate cold tolerance (Zhang et al. 2011).

### *3.4.3 Dehydration Stress*

During dehydration, the cellular machinery adjusts itself to maintain water potential, which leads to improved antioxidant activity that reduces the production of reactive oxygen species (ROS) and prevents oxidative damage. Under such stress, many HSP which are mainly present in the cytoplasm, are involved in transferring the cellular signals to the nucleus that dictate the cell fate decision (Breiman 2014). HTS is generally accompanied by dehydration, but these aspects are rarely studied. One such investigation in cotton involved comparison of transcript abundance of HSP, which revealed steady state level in non-irrigated plants, but were not detected in irrigated plants (Burke et al. 1985). In a similar attempt, synthesis of HSP in dehydration- and heat-sensitive, and dehydration- and heat-resistant lines of maize, was examined under two environmental stress treatments, dehydration and high temperature, and independently at high temperature (Ristic et al. 1991). In both the lines, the pattern of synthesis of HSP was similar for high as well as low molecular weight proteins, indicating intraspecific differences in the synthesis of HSP. Pareek et al. (1998) reported different levels of these proteins in several dehydration-responsive wild species of rice. In yet another study, Benešová et al. (2012) showed induced expression of Hsp26 and Hsp70 isoforms in maize exposed to dehydration. Induction of HSP was found to be more in dehydration-tolerant than dehydration-sensitive cultivar. Similarly, expression of Hsp70 was shown to be downregulated in early stages in dehydration-tolerant chickpea cultivar as against dehydration-sensitive one, which showed high abundance in initial stage and

reduced expression in later stages. This indicates that Hsp70 expression is dependent on genotypes as well as growth stages (Jaiswal et al. 2013). There have been numerous studies demonstrating improved tolerance to environmental stress including dehydration, albeit the underlying mechanism is not fully understood (Sugino et al. 1999; Alvim et al. 2001; Ono et al. 2001). Subba et al. (2013a, b) observed similar trend in the case of sHSP; the expression was higher in dehydration-tolerant, while lower in dehydration-sensitive chickpea cultivar. The expression of sHSP was also found to be genotype-specific in various other crops, for instance, poplar (Bonhomme et al. 2009) and Kentucky bluegrass (Xu and Huang 2010). The proteomic landscape of the extracellular matrix unraveled that Hsp90, Hsp70, GroEL Hsp20, peptidyl-prolyl cis-trans isomerase and chaperonin 60 are upregulated in response to dehydration (Pandey et al. 2010; Bhushan et al. 2011). Similarly, dehydration-responsive nuclear proteome displays upregulation of Hsp70, co-chaperone DnaJ, Grp chaperonin 60β and several sHSP (Pandey et al. 2008; Choudhary et al. 2009). It is evident that the status of HSP are modified covalently through the phosphorylation of residues under dehydration. In addition, phosphorylation of chaperonin 21 precursor and Hsp19 activate the defense pathway in response to dehydration (Subba et al. 2013a, b). A complex response of chaperone was also observed in which isoforms of stress-induced protein (sti1) showed differential dehydration-responsive expression (Agrawal et al. 2016). Cruz de carvalho et al. (2014) demonstrated distinct dehydration response in bryophytes and showed association of many HSP with dehydration adaptation (Cruz de carvalho et al. 2014).

Increasing evidence suggest that there is a strong correlation between accumulation of sHSP and stress tolerance in plant The sHSP, usually undetectable under normal physiological conditions, are induced upon stress treatment (Low et al. 2000). Overexpression of sHsp17.7 in transgenic rice displayed increased dehydration tolerance (Sato and Yokoya 2008). The function of sHsp17.7 has been demonstrated during osmotic stress, when proteins are prone to be denatured upon dehydration (Sun et al. 2001). A recent genome-wide sequence survey in barley led to the identification of several sHSP and HSFs putatively involved in dehydration response (Reddy et al. 2014).

### *3.4.4 Metallic Stress*

Metals are crucial for plant growth and development in the optimum concentration, but when the concentration increases in the natural environment causes heavy metal stress (HMS). It is evident that Hsp70 is activated when exposed to cadmium and aluminum stress in Arabidopsis (Sarry et al. 2006), tomato (Neumann et al. 1994), poplar (Yang et al. 2015), bird's-foot trefoil (Navascués et al. 2012) and soybean (Duressa et al. 2011). The time-dependent gene expression analyses revealed high abundance of Hsp80 and Hsp17.9 in rice when exposed to cadmium stress (Ogawa et al. 2009). Hsp90 was previously shown to be differentially regulated in poplar (Ogawa et al. 2009) and bird's-foot trefoil (Yang et al. 2015) under HMS. Czarnecka et al. (1988) showed that cadmium could partially inhibit the intron processing by

favoring the expression of Hsp26 in soybean (Czarnecka et al. 1988). Constitutive expression of AtHsp90.3 has been shown to impair tolerance to Cd-stress causing lower germination potential and shorter root length possibly via reducing the activities of antioxidative enzymes (Song et al. 2012). Expression of Hsp17.7 was found to be increased in carrot under lead and arsenic stress (Lee and Ahn 2013). The HMS-responsive proteomic analysis of poplar revealed induction of diverse sHSP, for instances, sHsp20, 22 and 23.1 (Yang et al. 2015).

### *3.4.5 Salt Stress*

More than 20% of the cultivated land worldwide are affected by salinity stress, which has been increasing in every successive year posing serious threats to agriculture. The proteomic landscape of soybean showed differential expression of Hsp70-Hsp90 organizing protein (HOP), Hsp90, chaperonin 20, chloroplastic Hsp70 and chaperonin 60 under hypersalinity (Pi et al. 2016). Overexpression of Hsp90.2, 90.5 and 90.7 in Arabidopsis showed tolerance towards hypersalinity (Song et al. 2009a, b; Xu et al. 2013). Salinity-induced high expression of Hsp70 was evident in both sensitive and tolerant genotypes of soyabean, though the abundance was higher in the sensitive genotypes (Manaa et al. 2011). Transgenic plants expressing Hsp70 were shown to modulate the programmed cell death (PCD) under hypersalinity wherein Hsp70 acts as anti-apoptotic protein (Hoang et al. 2015). Higher induction of Hsp110 was also observed in rice when exposed to hypersalinity (Singla et al. 1997). Additionally, ClpB/Hsp100 B2, B3 and ClpD2 are predicted to function as molecular chaperone, and their expressions are highly increased under salt stress (Muthusamy et al. 2016). Overexpression of ClpD1 and sHSP has also been shown to cause better adaptation to salt stress (Jiang et al. 2009; Mu et al. 2013; Jung et al. 2014; Mishra et al. 2016). A previous study on mitochondrial electron transport in maize indicated a strong association between accumulation of sHSP and tolerance to hypersalinity (Hamilton and Heckathorn 2001).

### *3.4.6 Light Stress*

Light is an essential component for plant to carry out photosynthesis, but excessive light damages the photosynthetic apparatus and affects plant growth and development. Plant uses several mechanisms to overcome the photooxidative damage, the most common being the HSP, which are highly induced by high light stress (HLS). Rossel et al. (2002) demonstrated that Arabidopsis seedlings, upon exposed to HLS, activates HSP-chaperone pathway and induces the expression of different forms of HSP and sHSP. The increased expression of HSP and co-chaperones reduces the photooxidative damage and helps protein folding (Rossel et al. 2002). The upregulation of chloroplastic Hsp70 reaffirms the role of HSP in protection of photosystem

II during photoinhibition (Giacomelli et al. 2006). Kropat et al. (1997) reported the similar observation, the HLS-induced overaccumulation of the nuclear Hsp70, in Chlamydomonas.

Photoperiodism plays an important role in chloroplast development. In a recent report, Wang et al. (2016) showed that high abundance of HSP-associated proteins such as FKBP19, FKBP16–1, FKBP16–4 and CYP20–3 in dark-adapted chloroplast, which help in proper folding of the unfolded proteins (Wang et al. 2016). It has previously been observed that the exposure of marine ecosystem, particularly seagrasses to HLS induces the synthesis of new HSP (Hsp70, Sti and ClpB1) and chaperonin 60 (Kumar et al. 2017a, b). The HLS-induced posttranslational regulation of mitochondrial sHsp23 has also been observed in the cell suspension of Chenopodium rubrum (Korotaeva et al. 2001).

### *3.4.7 Flooding Stress*

Flooding is the major abiotic stress, which negatively affects plant growth and crop yield worldwide. Pathways involving various HSP, chaperones and co-chaperones are triggered by flooding stress-responsive. Largescale omics analyses in maize emphasized the role of Hsp70 in such stress (Chen et al. 2014a, b) via flooding stress-induced PCD through the maintenance of photosynthesis. Increased accumulation of Hsp70 in flooding-stressed soybean also indicated its pivotal role in flooding stress tolerance (Komatsu et al. 2013). These results were supported by the observation in rice protoplasts wherein ectopic expression of mtHsp70 led to inhibition of heat- and $H_2O_2$-induced PCD (Qi et al. 2011). Hypoxic or anoxic condition is the consequence of prolonged flooding stress. The transcript abundance of HSP were found to be upregulated under flooding stress in rice sensitive genotype, but few of them in the tolerant genotype. This indicated that even though HSP have a vital role against anoxia, these are not the principal components for the tolerance (Mertz-Henning et al. 2016). It unlikely that the HSP might act in association with other molecular signatures and help plants to endure the flooding stress. This is further substantiated by a proteometabolomic study of soybean under flooding stress wherein the acidic form of 60-kDa chaperonin was shown to be differentially regulated (Komatsu et al. 2011). It has been demonstrated that the expression of HsfA2 induces high abundance of Hsp70 and Hsp101, besides Sti1 and protect plants against anoxic condition (Banti et al. 2010; Hüther et al. 2017).

### *3.4.8 Overlapping and Secondary Stress*

Plant response to environmental stress is controlled and regulated by a complex network of genes. Gene expression database of Arabidopsis, AtGenExpress, encompasses the consequences of multivariate abiotic stresses (Kilian et al. 2007). There

exists a strong association of stress-responses and involvement of HTS and HSF, albeit the degree of interaction seems to be different suggesting a cross-talk among the networks. A recent global HTS-responsive gene expression profiling of rice suggested that HSP and their corresponding HSFs might be crucial in crosstalk of different stress signaling pathways (Hu et al. 2009). It is reasonable to deduce that the synchronized exposure to various abiotic stresses would simultaneously activate different stress-responsive pathways. Hence, there might be a synergistic and/or antagonistic impact on each other. The distinct pathways exclusively for the particular stress combinations might also be stimulated (Mittler 2006). Therefore, it is imperative to understand the role of HSP in relation to the combined stress responses. It has been increasingly evident that several HSP are responsive to multiple stresses (Al-Whaibi 2011), though there are instances wherein a particular stress-responsive HSP may not function in other stress or even differs crop-wise (Jin et al. 2011; Vítámvás et al. 2012). Investigations on combined stresses suggest increased expression of the genes encoding Hsp100, Hsp90, Hsp70 and sHSP. The combined as well as individual stress response include numerous commonly regulated genes. The expression of stress-responsive genes is fine tuned to combined stress condition, for instances, plants subjected to dehydration and HTS showed higher induction of HSP when compared individually to either dehydration or heat stressed plants (Rizhsky et al. 2002). Ectopic expression of OsHsp18.6 and OsHsp16.9B in rice were shown to have improved tolerance towards multiple stresses (Zou et al. 2012; Wang et al. 2015). Overexpression of OsHSP16.9B and OsHsp23.7 were also reported to induce increased tolerance to salt and dehydration (Zou et al. 2012). Similar outcome was observed by overexpression of Oshsp16.9 (Jung et al. 2014). In Arabidopsis, overexpression of AtHsp17.6A were shown to effect enhanced tolerance to hypersalinity and dehydration, whereas no effect was observed against HTS (Sun et al. 2001). Ectopic expression of GmHsp90 in Arabidopsis were shown to confer tolerance to HTS, hypersalinity and osmotic stress, albeit the response in hypersalinity was not as potent as HTS (Xu et al. 2013).

Osmotic and oxidative stresses are considered as secondary stress in plants. Song et al. (2009a, b) reported that suggested various types of Hsp90 in different compartments are essential for cellular homeostasis under such stress (Song et al. 2009a, b). Nishizawa-Yokoi et al. (2010) also reported that Hsp90 is responsible for induction of HsfA2 under oxidative stress (Nishizawa-Yokoi et al. 2010). Oxidative stress has previously been shown to induced the accumulation of ClpB-cyt/Hsp100, ClpC2 and ClpD1 in rice (Queitsch et al. 2000). Chankova et al. (2014) reported that Hsp70 functions as biomarker in oxidative stress tolerance. An earlier study reported in the involvement of cloroplastic sHSP in protection of photosynthetic electron transport from oxidative damage (Downs et al. 1999). Various forms of Hsp17 and Hsp16.4 were also found to be highly induced in moss (Ruibal et al. 2013), Arabidopsis (Scarpeci et al. 2008; Jiang et al. 2009) and carrot (Ahn and Song 2012). Furthermore, mitochondrial Hsp22 were found to be over-accumulated in response to oxidative stress in tomato (Banzet et al. 1998).

## 3.5 Hsp and Multistress Resistance: Connecting the Dots

Abiotic stress elicits multivarious responses in plants that encompass a sequence of physicochemical and molecular events. Multiple stress response mechanisms often function coordinately or synergistically to avoid cellular damage (Ahuja et al. 2010). While, the function of HSP in various abiotic stresses have been elucidated (Wang et al. 2004; Kotak et al. 2007; Al-Whaibi 2011), the precise mechanism is yet to be fully understood. The functions of HSP are not restricted to folding and maintenance of protein structures as some HSP and their co-chaperones have evidently been associated to signaling, besides protein targeting and degradation (Al-Whaibi 2011). Almost all the abiotic stresses cause to certain extent of oxidative stress, and there is a cross-talk between oxidative and abiotic stress signaling. The generation of $H_2O_2$ is a common phenomenon owing to the NADPH oxidase activity. The production of $H_2O_2$ has intimately been associated with the induction of stress-responsive genes in general and HTS-responsive genes in particular. This process is itself anticipated to be mediated by HSFs through direct sensing of $H_2O_2$ (Kotak et al. 2007). These facts suggest that the ROS production may influence *de novo* synthesis of HSP. Nonetheless, the information about the involvement of ROS in regulating abiotic stress-mediated HSP expression is still fragmentary (Kotak et al. 2007). Some of the HSP such as Hsp90 and Hsp70 class of chaperones and their co-chaperones interact with several components of signaling molecules viz., serine/threonine and/or tyrosine-kinase receptors, nuclear hormone receptors, and many more (Wang et al. 2004). Furthermore, existence of the redox status of thiol-containing molecules is key to the maintenance and balance of many crucial cellular functions. Some sHSP are implicated for the maintenance of the redox status per se in mammalian cells and likely to be playing a similar role in plants. The HSFs are involved not only for the basal tolerance against different abiotic stresses, but also initiate their acquisition. Several earlier reports addressed the possible role of $Ca^{2+}$/calmodulin-mediated signaling in abiotic stress-response. The HTS-induced cytosolic $Ca^{2+}$ flux has been well documented, and an association through calmodulin (CaM) has been proposed. These altogether help HSFs and other transcription factors to bind to heat shock elements or their respective response elements to activate transcription of HSP (Kotak et al. 2007; Wang et al. 2004). A schematic representation of how HSP work in association with other elements in acquiring stress tolerance is presented in Fig. 3.2.

## 3.6 Conclusions

The stress-response and the tolerance thereof are complex sequence of events, which are intimately interwoven in plants. Significant information is available about HSP and their roles in different stresses, their types and variety of functions. However, every class of HSP and their different members have precise task, yet their

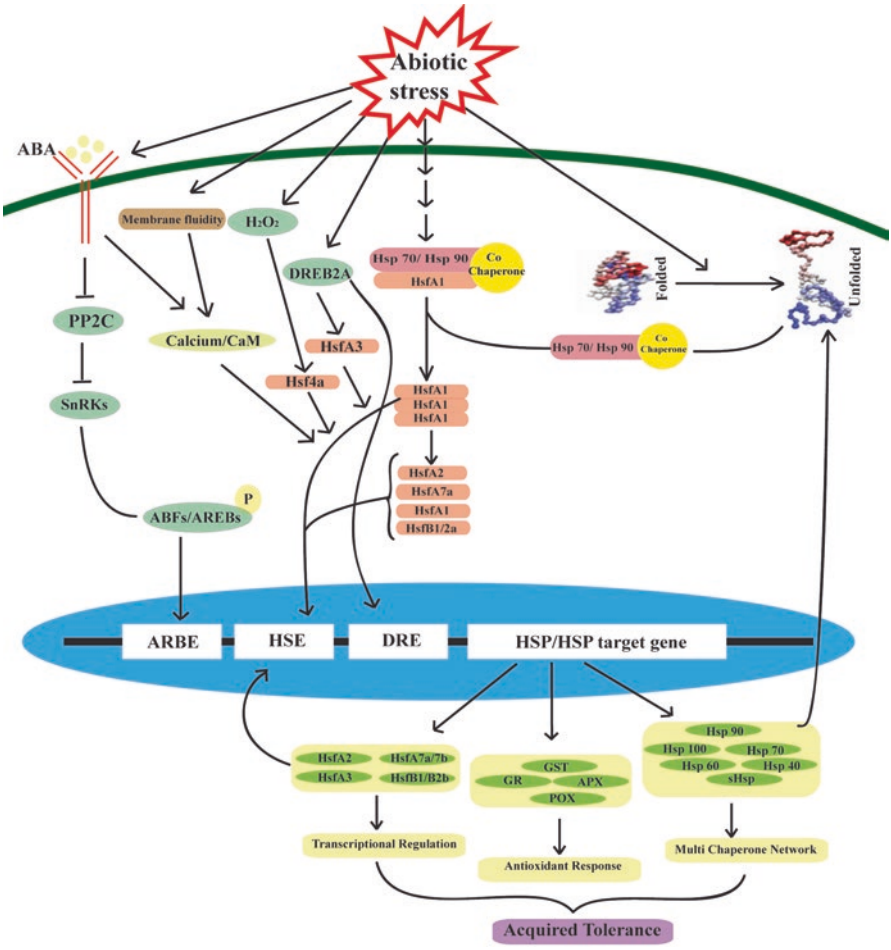


**Fig. 3.2** Schematic representation of the involvement of HSP in defense pathway for acquired tolerance. Schematic diagram shows the role of HSP/chaperone and their interaction with other pathway proteins to circumvent the harsh environmental stress. The protein folding process is greatly affected during stress, which in turn results in propagation of misfolded proteins. To alleviate the misfolded protein response, a number of signaling pathways are activated in cellular milieu. The HSP and co-chaperones bind to misfolded protein facilitating the release of HsfA1, which further form trimer and translocate into the nucleus and binds to heat shock element (HSE) to activate the target genes. Major abiotic stresses activate the expression of DREBs, which eventually induces the upregulation of HsfA3. Furthermore, secondary stresses also increase intracellular $H_2O_2$ and trigger Hsf4a. Consequently, the fluidity of plasma membrane is disturbed which stimulates the cytosolic calcium and calmodulin, and results the upregulation of many HSFs. Abiotic stress especially, dehydration and hypersalinity increases the abundance of ABA and binds to ABA receptor, and inhibits the PP2C. Hence active SnRKs activates the ABF/AREBs and binds to ABRE of promotor region. The Hsfs and other regulatory proteins (DREB, ABFs etc.) bind to different cis-regulatory region and initiate the transcription of HSP, HSFs and antioxidant proteins. Accumulation of these biomolecules contribute to acquisition of stress tolerance in plants

synergy across different networks seems to be a fundamental aspect for the assimilated function. Under natural growth and developmental conditions or during the stress, the HSP/chaperone machinery decides the consequence of the non-native or denatured protein/s. The questions remain to be answered as to how a non-native or denatured protein is recognized by a particular chaperone, and how and when it is stabilized or protected from aggregation, or refolded in native conformations or destined for degradation? The current knowledge about the role of HSP as regulatory module as to how they sense the signal and eventually induce the transcriptional activation of other stress-responsive genes is limited. Almost all recent studies in plants and other organisms have been focused on the changes in the expression of HSP or HSFs and/or their co-chaperones. In most cases, the role of HSP under stress have been attributed on the basis of in vitro studies, primarily due to the lack of suitable mutants wherein the expression studies can be carried out. Therefore, future studies should be focused on generation of HSP-mutants in plants, their interaction with pathway proteins and so forth to elucidate the underlying mechanism of stress tolerance. Studies about the structural changes in different HSP and co-chaperones vis-à-vis to single or collective stress-response mechanisms in plants are particularly significant. It can be anticipated that active research pertaining to the cross-talk between HSP and co-chaperones with that of other stress response mechanisms will provide a new insight into stress tolerance. In fact, genome sequence information alone is inadequate about gene function, their regulation, and the molecular switch to acclimatize under stress conditions. Future efforts must focus on unravelling the molecular association amongst the HSP and different abiotic stresses which may provide avenue for developing resilient crops through genetic engineering or molecular breeding.

**Acknowledgements** This work was supported by the National Institute of Plant Genome Research (NIPGR). We kindly acknowledge the University Grant Commission (UGC), Govt. of India for providing predoctoral fellowship to D.M, Department of Biotechnology (DBT), Govt. of India for providing predoctoral fellowship to D.S., and DST-SERB for providing postdoctoral fellowship to S.S.

## References

Adam, Z., & Clarke, A. K. (2002). Cutting edge of chloroplast proteolysis. *Trends in Plant Science, 7*, 451–456.

Adam, Z., Adamska, I., Nakabayashi, K., Ostersetzer, O., Haussuhl, K., Manuell, A., Zheng, B., Vallon, O., Rodermel, S. R., Shinozaki, K., & Clarke, A. K. (2001). Chloroplast and mitochondrial proteases in Arabidopsis. A proposed nomenclature. *Plant Physiology, 125*, 1912–1918.

Agrawal, L., Chakraborty, S., Jaiswal, D., Gupta, S., Datta, A., & Chakraborty, N. (2008). Comparative proteomics of tuber induction, development and maturation reveal the complexity of tuberization process in potato (*Solanum tuberosum* L.) *Journal of Proteome Research, 7*, 3803–3817.

Agrawal, L., Narula, K., Basu, S., Shekhar, S., Ghosh, S., Datta, A., Chakraborty, N., & Chakraborty, S. (2013). Comparative proteomics reveals a role for seed storage protein,

AmA1 in cellular growth, development and nutrient accumulation. *Journal of Proteome Research, 12*, 4904–4930.

Agrawal, L., Gupta, S., Mishra, S. K., Pandey, G., Kumar, S., Chauhan, P., Chakrabarty, D., & Nautiyal, C. (2016). Elucidation of complex nature of PEG induced drought-stress response in Rice root using comparative proteomics approach. *Frontiers in Plant Science, 7*, 1466.

Ahn, Y. J., & Song, N. H. (2012). A cytosolic heat shock protein expressed in carrot (*Daucus carota* L.) enhances cell viability under oxidative and osmotic stress conditions. *Hortscience, 47*, 143–148.

Ahuja, I., de Vos, R. C., Bones, A. M., & Hall, R. D. (2010). Plant molecular stress responses face climate change. *Trends in Plant Science, 15*, 664–674.

Alvim, F. C., Carolino, S. M., Cascardo, J. C., Nunes, C. C., Martinez, C. A., Otoni, W. C., & Fontes, E. P. (2001). Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiology, 126*, 1042–1054.

Al-Whaibi, M. H. (2011). Plant heat-shock proteins: A mini review. *Journal of King Saud University – Science, 23*, 139–150.

Bae, M. S., Cho, E. J., Choi, E.-Y., & Park, O. K. (2003). Analysis of the Arabidopsis nuclear proteome and its response to cold stress. *The Plant Journal, 36*, 652–663.

Balbuena, T. S., Salas, J. J., Martínez-Force, E., Garcés, R., & Thelen, J. J. (2011). Proteome analysis of cold acclimation in sunflower. *Journal of Proteome Research, 10*, 2330–2346.

Baniwal, S. K., Bharti, K., Chan, K. Y., Fauth, M., Ganguli, A., Kotak, S., Mishra, S. K., Nover, L., Port, M., Scharf, K. D., Tripp, J., Weber, C., Zielinski, D., & von Koskull-Döring, P. (2004). Heat stress response in plants: A complex game with chaperones and more than twenty heat stress transcription factors. *Journal of Biosciences, 29*, 471–487.

Banti, V., Mafessoni, F., Loreti, E., Alpi, A., & Perata, P. (2010). The heat-inducible transcription factor HsfA2 enhances anoxia tolerance in Arabidopsis. *Plant Physiology, 152*, 1471–1483.

Banzet, N., Richaud, C., Deveaux, Y., Kazmaier, M., Gagnon, J., & Triantaphylides, C. (1998). Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. *The Plant Journal, 13*, 519–527.

Benešová, M., Holá, D., Fischer, L., Jedelský, P. L., Hnilička, F., Wilhelmová, N., Rothová, O., Kočová, M., Procházková, D., Honnerová, J., Fridrichová, L., & Hniličková, H. (2012). The physiology and proteomics of drought tolerance in maize: Early stomatal closure as a cause of lower tolerance to short-term dehydration? *PLoS One, 7*, e38017.

Bharti, K., & Nover, L. (2002). Heat stress-induced signaling. In D. Scheel & C. Wasternack (Eds.), *Plant signal transduction: Frontiers in molecular biology* (pp. 74–115). Oxford., 2002: Oxford University Press.

Bhushan, D., Jaiswal, D. K., Ray, D., Basu, D., Data, A., Chakraborty, S., & Chakraborty, N. (2011). Dehydration-responsive reversible and irreversible changes in the extracellular matrix: Comparative proteomics of chickpea genotypes with contrasting tolerance. *Journal of Proteome Research, 10*, 2027–2046.

Bonhomme, L., Monclus, R., Vincent, D., Carpin, S., Lomenech, A. M., Plomion, C., Brignolas, F., & Morabito, D. (2009). Leaf proteome analysis of eight Populus ×euramericana genotypes: Genetic variation in drought response and in water-use efficiency involves photosynthesis-related proteins. *Proteomics, 9*, 41211–41242.

Boston, R. S., Viitanen, P. V., & Vierling, E. (1996). Molecular chaperones and protein folding in plants. *Plant Molecular Biology, 32*, 191–222.

Breiman, A. (2014). Plant Hsp90 and its co-chaperones. *Current Protein & Peptide Science, 15*, 232–244.

Burke, J. J., Hatfield, J. L., Klein, R. P., & Mullet, J. E. (1985). Accumulation of heat shock proteins in field-grown cotton. *Plant Physiology, 78*, 394–398.

Chandel, G., Dubey, M., & Meena, R. (2013). Differential expression of heat shock proteins and heat stress transcription factor genes in rice exposed to different levels of heat stress. *Journal of Plant Biochemistry and Biotechnology, 22*, 277–285.

Chankova, S. G., Dimova, E. G., Mitrovska, Z., Miteva, D., Mokerova, D. V., Yonova, P. A., & Yurina, N. P. (2014). Antioxidant and HSP70B responses in *Chlamydomonas reinhardtii* genotypes with different resistance to oxidative stress. *Ecotoxicology and Environmental Safety, 101*, 131–137.

Chen, Q., & Vierling, E. (1991). Analysis of conserved domains identifies a unique structural feature of a chloroplast heat shock protein. *Molecular & General Genetics, 226*, 425–431.

Chen, X., Lin, S., Liu, Q., Huang, J., Zhang, W., Lin, J., Wang, Y., Ke, Y., & He, H. (2014a). Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. *Biochimica et Biophysica Acta, 1844*, 818–828.

Chen, Y., Chen, X., Wang, H., Bao, Y., & Zhang, W. (2014b). Examination of the leaf proteome during flooding stress and the induction of programmed cell death in maize. *Proteome Science, 12*, 33.

Choudhary, M. K., Basu, D., Datta, A., Chakraborty, N., & Chakraborty, S. (2009). Dehydration-responsive nuclear proteome of rice (*Oryza sativa* L.) illustrates protein network, novel regulators of cellular adaptation, and evolutionary perspective. *Molecular & Cellular Proteomics, 8*, 1579–1598.

Cruz de carvalho, R., Bernardes DA Silva, A., Soares, R., Almeida, A. M., Coelho, A. V., Marques DA Silva, J., & Branquinho, C. (2014). Differential proteomics of dehydration and rehydration in bryophytes: Evidence towards a common desiccation tolerance mechanism. *Plant, Cell & Environment, 37*, 1499–1515.

Czarnecka, E., Nagao, R. T., Key, J. L., & Gurley, W. B. (1988). Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: Heavy-metal-induced inhibition of intron processing. *Molecular and Cellular Biology, 8*, 1113–1122.

Downs, C. A., Ryan, S. L., & Heckathorn, S. A. (1999). The chloroplast small heat-shock protein: Evidence for a general role in protecting photosystem II against oxidative stress and photoinhibition. *Journal of Plant Physiology, 155*, 488–496.

Duck, N. B., & Folk, W. R. (1994). Hsp70 heat shock protein cognate is expressed and stored in developing tomato pollen. *Plant Molecular Biology, 26*, 1031–1039.

Dumont, E., Bahrman, N., Goulas, E., Valot, B., Sellier, H., Hilbert, J. L., Vuylsteker, C., Lejeune-Hénaut, I., & Delbreil, B. (2011). A proteomic approach to decipher chilling response from cold acclimation in pea (*Pisum sativum* L.) *Plant Science, 180*, 86–98.

Dupuis, I., & Dumas, C. (1990). Influence of temperature stress on *in vitro* fertilization and heat shock protein synthesis in maize (*Zea mays* L.) reproductive tissues. *Plant Physiology, 94*, 665–670.

Duressa, D., Soliman, K., Taylor, R., & Senwo, Z. (2011). Proteomic analysis of soybean roots under aluminum stress. *International Journal of Plant Genomics, 2011*, 1–12.

Echevarría-Zomeño, S., Fernández-Calvino, L., Castro-Sanz, A. B., López, J. A., Vázquez, J., & Castellano, M. M. (2016). Dissecting the proteome dynamics of the early heat stress response leading to plant survival or death in Arabidopsis. *Plant, Cell & Environment, 39*, 1264–1278.

Fragkostefanakis, S., Röth, S., Schleiff, E., & Scharf, K. D. (2015). Prospects of engineering thermotolerance in crops through modulation of heat stress transcription factor and heat shock protein networks. *Plant, Cell & Environment, 38*, 1881–1895.

Giacomelli, L., Rudella, A., & van Wijk, K. J. (2006). High light response of the thylakoid proteome in Arabidopsis wild type and the ascorbate-deficient mutant *vtc2-2*. A comparative proteomics study. *Plant Physiology, 141*, 685–701.

Guo, S. J., Zhou, H. Y., Zhang, X. S., Li, X. G., & Meng, Q. W. (2007). Overexpression of *CaHSP26* in transgenic tobacco alleviates photoinhibition of PSII and PSI during chilling stress under low irradiance. *Journal of Plant Physiology, 164*, 126–136.

Guo, M., Liu, J. H., Lu, J. P., Zhai, Y. F., Wang, H., Gong, Z. H., Wang, S. B., & Lu, M. H. (2015). Genome-wide analysis of the *CaHsp20* gene family in pepper: Comprehensive sequence and expression profile analysis under heat stress. *Frontiers in Plant Science, 6*, 806.

Guo, M., Liu, J. H., Ma, X., Luo, D. X., Gong, Z. H., & Lu, M. H. (2016). The plant Heat Stress Transcription Factors (HSFs): Structure, regulation, and function in response to abiotic stresses. *Frontiers in Plant Science, 7*, 114.

Gurley, W. B. (2000). HSP101: A key component for the acquisition of thermotolerance in plants. *Plant Cell, 12*, 457–460.

Hahn, M., & Walbot, V. (1989). Effects of cold-treatment on protein synthesis and mRNA levels in rice leaves. *Plant Physiology, 91*, 930–938.

Hamilton, E. W., & Heckathorn, S. A. (2001). Mitochondrial adaptations to NaCl complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. *Plant Physiology, 126*, 1266–1274.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature, 381*, 571–580.

Helm, K. W., Lafayete, P. R., Nago, R. T., Key, J. L., & Vierling, E. (1993). Localization of small heat shock proteins to the higher plant endomembrane system. *Molecular and Cellular Biology, 13*, 238–247.

Hlaváčková, I., Vítámvás, P., Šantrůček, J., Kosová, K., Zelenková, S., Prášil, I. T., Ovesná, J., Hynek, R., & Kodíček, M. (2013). Proteins involved in distinct phases of cold hardening process in frost resistant winter barley (*Hordeum vulgare* L.) cv Luxor. *International Journal of Molecular Sciences, 14*, 8000–8024.

Hoang, T. M. L., Moghaddam, L., Williams, B., Khanna, H., Dale, J., & Mundree, S. G. (2015). Development of salinity tolerance in rice by constitutive-overexpression of genes involved in the regulation of programmed cell death. *Frontiers in Plant Science, 6*, 175.

Hu, W., Hu, G., & Han, B. (2009). Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. *Plant Science, 176*, 583–590.

Hu, X., Li, Y., Li, C., Yang, H., Wang, W., & Lu, M. (2010). Characterization of small heat shock proteins associated with maize tolerance to combined drought and heat stress. *Journal of Plant Growth Regulation, 29*, 455–464.

Huang, S., Ratliff, K. S., Schwartz, M. P., Spenner, J. M., & Matouschek, A. (1999). Mitochondrial unfold precursor proteins by unraveling them from their N-termini. *Nature Structural Biology, 6*, 1132–1138.

Hubert, D. A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K., & Dangl, J. L. (2003). Cytosolic HSP90 associates with and modulates the ARABIDOPSIS RPM1 disease resistance protein. *The EMBO Journal, 22*, 5679–5689.

Hüther, C. M., Martinazzo, E. G., Rombaldi, C. V., & Bacarin, M. A. (2017). Effects of flooding stress in 'Micro-Tom' tomato plants transformed with different levels of mitochondrial sHSP23.6. *Brazilian Journal of Biology, 77*, 43–51.

Jackson-Constan, D., Akita, M., & Keegstra, K. (2001). Molecular chaperones involved in chloroplast protein import. *Biochimica et Biophysica Acta, 1541*, 102–113.

Jaiswal, D. K., Ray, D., Choudhary, M. K., Subba, P., Kumar, A., Verma, J., Kumar, R., Datta, A., Chakraborty, S., & Chakraborty, N. (2013). Comparative proteomics of dehydration response in the rice nucleus: New insights into the molecular basis of genotype-specific adaptation. *Proteomics, 13*, 3478–3497.

Jiang, C., Xu, J., Zhang, H., , Zhang, X., Shi, J., Li, M. and Ming, F. (2009) A cytosolic class I small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana.* Plant, Cell & Environment 32, 1046–1059.

Jin, Y., Zhang, C., Yang, H., Yang, Y., Huang, C., Tian, Y., & Lu, X. (2011). Proteomic analysis of cold stress responses in tobacco seedlings. *African Journal of Biotechnology, 10*, 18991–19004.

Jung, Y. J., Nou, S. I., & Kang, K. K. (2014). Overexpression of *Oshsp16.9* gene encoding small heat shock protein enhances tolerance to abiotic stresses in rice. *Plant Breeding and Biotechnology, 2*, 370–379.

Jungkunz, I., Link, K., Vogel, F., Voll, L. M., Sonnewald, S., & Sonnewald, U. (2011). AtHsp70-15-deficient Arabidopsis plants are characterized by reduced growth, a constitutive cytosolic protein response and enhanced resistance to TuMV. *The Plant Journal, 66*, 983–995.

Kaufman, R. J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. *Genes & Development, 13*, 1211–1233.

Keeler, S., Boettger, C. M., Haynes, J. G., Kuches, K. A., Johnson, M. M., Thureen, D. L., Keeler, C. L., Jr., & Kitto, S. L. (2000). Acquired thermotolerance and expression of the HSP100/ClpB genes of Lima bean. *Plant Physiology, 123*, 1121–1132.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., & Harter, K. (2007). The AtGenExpress global stress expression data set: Protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *The Plant Journal, 50*, 347–363.

Kim, S. R., & An, G. (2013). Rice chloroplast-localized heat shock protein 70, OsHsp70CP1, is essential for chloroplast development under high-temperature conditions. *Journal of Plant Physiology, 170*, 854–863.

Kim, B. H., & Schöffl, F. (2002). Interaction between Arabidopsis heat shock transcription factor 1 and 70 kDa heat shock proteins. *Journal of Experimental Botany, 53*, 371–375.

Kollipara, K. P., Saab, I. N., Wych, R. D., Lauer, M. J., & Singletary, G. W. (2002). Expression profiling of reciprocal maize hybrids divergent for cold germination and desiccation tolerance. *Plant Physiology, 129*, 974–992.

Komatsu, S., Yamamoto, A., Nakamura, T., Nouri, M. Z., Nanjo, Y., Nishizawa, K., & Furukawa, K. (2011). Comprehensive analysis of mitochondria in roots and hypocotyls of soybean under flooding stress using proteomics and metabolomics techniques. *Journal of Proteome Research, 10*, 3993–4004.

Komatsu, S., Makino, T., & Yasue, H. (2013). Proteomic and biochemical analyses of the cotyledon and root of flooding-stressed soybean plants. *PLoS One, 8*, e65301.

Koo, H. J., Park, S. M., Kim, K. P., Suh, M. C., Lee, M. O., Lee, S. K., Xinli, X., & Hong, C. B. (2015). Small heat shock proteins can release light dependence of tobacco seed during germination. *Plant Physiology, 167*, 1030–1038.

Korotaeva, N. E., Antipina, A. I., Grabelnykh, O. I., Varakina, N. N., Borovskii, G. B., & Voinikov, V. K. (2001). Mitochondrial low-molecular-weight heat-shock proteins and the tolerance of cereal mitochondria to hyperthermia. *Russian Journal of Plant Physiology, 48*, 798–803.

Kosová, K., Vítámvás, P., Planchon, S., Renaut, J., Vanková, R., & Prášil, I. T. (2013). Proteome analysis of cold response in spring and winter wheat (Triticum aestivum) crowns reveals similarities in stress adaptation and differences in regulatory processes between the growth habits. *Journal of Proteome Research, 12*, 4830–4845.

Kotak, S., Larkindale, J., Lee, U., von Koskull-Doring, P., Vierling, E., & Scharf, K. D. (2007). Complexity of the heat stress response in plants. *Current Opinion in Plant Biology, 10*, 310–316.

Krishna, P., & Gloor, G. (2001). The Hsp90 family of proteins in *Arabidopsis thaliana*. *Cell Stress & Chaperones, 6*, 238–246.

Kropat, J., Oster, U., Rüdiger, W., & Beck, C. F. (1997). Chlorophyll precursors are signals of chloroplast origin involved in light induction of nuclear heat-shock genes. *Proceedings of the National Academy of Sciences, 94*, 14168–14172.

Kumar, M., Padula, M. P., Davey, P., Pernice, M., Jiang, Z., Sablok, G., Contreras-Porcia, L., & Ralph, P. J. (2017a). Proteome analysis reveals extensive light stress-response reprogramming in the seagrass *Zostera muelleri* (Alismatales, Zosteraceae) metabolism. *Frontiers in Plant Science, 17*, 2023.

Kumar, N., Suyal, D. C., Sharma, I. P., Verma, A., & Singh, H. (2017b). Elucidating stress proteins in rice (*Oryza sativa* L.) genotype under elevated temperature: A proteomic approach to understand heat stress response. *3 Biotech, 7*, 205.

Larkindale, J., Mishkind, M., & Vierling, E. (2005). Plant responses to high temperature. In M. A. Jenks & P. M. Hasegawa (Eds.), *Plant Abiotic Stress* (pp. 100–144). Oxford: Blackwell Publishing Ltd.

Lee, J., & Ahn, Y.-J. (2013). Heterologous expression of a carrot small heat shock protein increased Escherichia coli viability under lead and arsenic stresses. *Hortscience, 48*, 1323–1326.

Lee, U., Rioflorido, I., Hong, S.-W., Larkindale, J., Waters, E. R., & Vierling, E. (2007). The Arabidopsis ClpB/Hsp100 family of proteins: Chaperones for stress and chloroplast development. *The Plant Journal, 49*, 115–127.

Lee, D. G., Ahsan, N., Lee, S. H., Lee, J. J., Bahk, J. D., Kang, K. Y., & Lee, B. H. (2009). Chilling stress-induces proteomic changes in rice roots. *Journal of Plant Physiology, 166*, 1–11.

Lehesranta, S. J., Davies, H. V., Shepherd, L. V. T., Koistinen, K. M., Massat, N., Nunan, N., McNicol, J. W., & Kärenlampi, S. O. (2006). Proteomic analysis of the potato tuber life cycle. *Proteomics, 6*, 6042–6052.

Li, W., Wei, Z., Qiao, Z., Wu, Z., Cheng, L., & Wang, Y. (2013). Proteomics analysis of alfalfa response to heat stress. *PLoS One, 8*, e82725.

Liao, J. L., Zhou, H. W., Zhang, H. Y., Zhong, P. A., & Huang, Y. J. (2014). Comparative proteomic analysis of differentially expressed proteins in the early milky stage of rice grains during high temperature stress. *Journal of Experimental Botany, 65*, 655–671.

Lim, C. J., Yang, K. A., Hong, J. K., Choi, J. S., Yun, D. J., Hong, J. C., Chung, W. S., Lee, S. Y., Cho, M. J., & Lim, C. O. (2006). Gene expression profiles during heat acclimation in *Arabidopsis thaliana* suspension-culture cells. *Journal of Plant Research, 119*, 373–383.

Lin, S. K., Chang, M. C., Tsai, Y. G., & Lur, H. S. (2005). Proteomic analysis of the expression of proteins related to rice quality during caryopsis development and the effect of high temperature on expression. *Proteomics, 5*, 2140–2156.

Lin, C. J., Li, C. Y., Lin, S. K., Yang, F. H., Huang, J. J., Liu, Y. H., & Lur, H. S. (2010). Influence of high temperature during grain filling on the accumulation of storage proteins and grain quality in Rice (*Oryza sativa* L.) *Journal of Agricultural and Food Chemistry, 58*, 10545–10552.

Liu, Y., Burch-Smith, T., Schiff, M., Feng, S., & Dinesh-Kumar, S. P. (2004). Molecular chaperone hsp90 associates with resistance protein n and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. *The Journal of Biological Chemistry, 279*, 2101–2108.

Lopes-Caitar, V. S., de Carvalho, M. C. C. G., Darben, L. M., Kuwahara, M. K., Nepomuceno, A. L., Dias, W. P., Abdelnoor, R. V., & Marcelino-Guimarães, F. C. (2013). Genome-wide analysis of the Hsp20 gene family in soybean: Comprehensive sequence, genomic organization and expression profile analysis under abiotic and biotic stresses. *BMC Genomics, 14*, 577.

Low, D., Brandle, K., Nover, L., & Forreiter, C. (2000). Cytosolic heat stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones *in vivo*. *Planta, 211*, 575–582.

Lubben, T. H., Donaldson, G. K., Viitanen, P. V., & Gatenby, A. A. (1989). Severa1 proteins imported into chloroplasts form stable complexes with the GroEL-related chloroplast molecular chaperone. *Plant Cell, 1*, 1223–1230.

Maimbo, M., Ohnishi, K., Hikichi, Y., Yoshioka, H., & Kiba, A. (2007). Induction of a small heat shock protein and its functional roles in nicotiana plants in the defense response against *Ralstonia solanacearum*. *Plant Physiology, 145*, 1588–1599.

Majoul, T., Bancel, E., Triboï, E., Ben Hamida, J., & Branlard, G. (2004). Proteomic analysis of the effect of heat stress on hexaploid wheat grain: Characterization of heat-responsive proteins from non-prolamins fraction. *Proteomics, 4*, 505–513.

Malik, M. K., Slovin, J. P., Hwang, C. H., & Zimmerman, J. L. (1999). Modified expression of a carrot small heat shock protein gene, Hsp17.7, results in increased or decreased thermotolerance. *The Plant Journal, 20*, 89–99.

Manaa, A., Ben Ahmed, H., Valot, B., Bouchet, J. P., Aschi-Smiti, S., Causse, M., & Faurobert, M. (2011). Salt and genotype impact on plant physiology and root proteome variations in tomato. *Journal of Experimental Botany, 62*, 2797–2813.

Merret, R., Carpentier, M. C., Favory, J. J., Picart, C., Descombin, J., Bousquet-Antonelli, C., Tillard, P., Lejay, L., Deragon, J. M., & Charng, Y. Y. (2017). Heat shock protein HSP101 affects the release of ribosomal protein mRNAs for recovery after heat shock. *Plant Physiology, 174*, 1216–1225.

Mertz-Henning, L. M., Pegoraro, C., Maia, L. C., Venske, E., Rombaldi, C. V., & Costa de Oliveira, A. (2016). Expression profile of rice Hsp genes under anoxic stress. *Genetics and Molecular Research, 15*(2.) gmr.15027954.

Mishra, R. C., Richa, M. R. C., & Grover, A. (2016). Constitutive over-expression of rice ClpD1 protein enhances tolerance to salt and desiccation stresses in transgenic Arabidopsis plants. *Plant Science, 250*, 69–78.

Mishra, D., Shekhar, S., Agrawal, L., Chakraborty, S., & Chakraborty, N. (2017). Cultivar-specific high temperature stress responses in bread wheat (*Triticum aestivum* L.) associated with physicochemical traits and defense pathways. *Food Chemistry, 221*, 1077–1087.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends in Plant Science, 11*, 15–19.

Mu, C., Zhang, S., Yu, G., Chen, N., Li, X., & Liu, H. (2013). Overexpression of small heat shock protein LimHSP16.45 in Arabidopsis enhances tolerance to abiotic stresses. *PLoS One, 8*, e82264.

Murakami, T., Matsuba, S., Funatsuki, H., Kawaguchi, K., Saruyama, H., Tanida, M., & Sato, Y. (2004). Over-expression of a small heat shock protein, sHSP17.7, confers both heat tolerance and UV-B resistance to rice plants. *Molecular Breeding, 13*, 165–175.

Muthusamy, S. K., Dalal, M., Chinnusamy, V., & Bansal, K. C. (2016). Differential regulation of genes coding for organelle and cytosolic Clp ATPases under biotic and abiotic stresses in wheat. *Frontiers in Plant Science, 7*, 929.

Muthusamy, S. K., Dalala, M., Chinnusamy, V., & Bansal, K. C. (2017). Genome-wide identification and analysis of biotic and abiotic stress regulation of small heat shock protein (HSP20) family genes in bread wheat. *Journal of Plant Physiology, 211*, 100–113.

Nakamoto, H., & Vigh, L. (2007). The small heat shock proteins and their clients. *Cellular and Molecular Life Sciences, 64*, 294–306.

Navascués, J., Pérez-Rontomé, C., Sánchez, D. H., Staudinger, C., Wienkoop, S., Rellán-Álvarez, R., & Becana, M. (2012). Oxidative stress is a consequence, not a cause, of aluminum toxicity in the forage legume *Lotus corniculatus*. *The New Phytologist, 193*, 625–636.

Neumann, D., Lichtenberger, O., Günther, D., Tschiersch, K., & Nover, L. (1994). Heat-shock proteins induce heavy-metal tolerance in higher plants. *Planta, 194*, 360–370.

Nieto-Sotelo, J., Martínez, L. M., Ponce, G., Cassab, G. I., Alagón, A., Meeley, R. B., Ribau, J. M., & Yang, R. (2002). Maize HSP101 plays important roles in both induced and basal Thermotolerance and primary root growth. *Plant Cell, 14*, 1621–1633.

Nishizawa-Yokoi, A., Tainaka, H., Yoshida, E., Tamoi, M., Yabuta, Y., & Shigeoka, S. (2010). The 26S proteasome function and Hsp90 activity involved in the regulation of HsfA2 expression in response to oxidative stress. *Plant & Cell Physiology, 51*, 486–496.

Ogawa, I., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2009). Time course analysis of gene regulation under cadmium stress in rice. *Plant and Soil, 325*, 97.

Ono, K., Hibino, T., Kohinata, T., Suzuki, S., Tanaka, Y., Nakamura, T., Takabe, T., & Takabe, T. (2001). Overexpression of DnaK from a halotolerant cyanobacterium *Aphanothece halophytica* enhances the high-temperature tolerance of tobacco during germination and early growth. *Plant Science, 160*, 455–461.

Pandey, A., Chakraborty, S., Datta, A., & Chakraborty, N. (2008). Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (*Cicer arietinum* L.) *Molecular & Cellular Proteomics, 7*, 88–107.

Pandey, A., Rajamani, U., Verma, J., Subba, P., Chakraborty, N., Data, A., Chakraborty, S., & Chakraborty, N. (2010). Identification of extracellular matrix proteins of rice (*Oryza sativa* L.) involved in dehydration-responsive network: A proteomic approach. *Journal of Proteome Research, 9*, 3443–3464.

Pareek, A., Singla, S. L., & Grover, A. (1998). Plant Hsp90 family with special reference to rice. *Journal of Biosciences, 23*, 361–367.

Parsell, P. A., & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. *Annual Review of Genetics, 27*, 437–496.

Pi, E., Qu, L., Hu, J., Huang, Y., Qiu, L., Jiang, B., Liu, C., Peng, T., Zhao, Y., Wang, H., Tsai, S. T., Ngai, S., & Du, L. (2016). Mechanisms of soybean roots tolerances to salinity revealed by

proteomic and phosphoproteomic comparisons between two cultivars. *Molecular & Cellular Proteomics, 15*, 266–288.

Prasad, B. D., Goel, S., & Krishna, P. (2010). *In Silico* identification of carboxylate clamp type tetratricopeptide repeat proteins in Arabidopsis and Rice as putative co-chaperones of Hsp90/Hsp70. *PLoS One, 5*, e12761.

Pratt, W. B., & Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Experimental Biology and Medicine, 228*, 111–133.

Pratt, W. B., Galigniana, M. D., Harrell, J. M., & Deranco, D. B. (2004). Role of hsp90 and the hsp90-binding immunophilins in signaling protein movement. *Cellular Signalling, 16*, 857–872.

Pyatrikas, D. V., Rikhvanov, E. G., Fedoseeva, I. V., Varakina, N. N., Rusaleva, T. M., Tauson, E. L., Stepanov, A. V., Borovskii, G. B., & Voinikov, V. K. (2014). Mitochondrial retrograde regulation of HSP101 expression in *Arabidopsis thaliana* under heat stress and amiodarone action. *Russian Journal of Plant Physiology, 61*, 80–89.

Qi, Y., Wang, H., Zou, Y., Liu, C., Wang, Y., & Zhang, W. (2011). Over-expression of mitochondrial heat shock protein 70 suppresses programmed cell death in rice. *FEBS Letters, 585*, 231–239.

Queitsch, C., Hong, S. W., Vierling, E., & Lindquist, S. (2000). Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. *Plant Cell, 12*, 479–492.

Reddy, R., Chaudhary, S., Patil, P., & Krishna, P. (1998). The 90 kDa heat shock protein (Hsp90) is expressed throughout *Brassica napus* seed development and germination. *Plant Science, 131*, 131–137.

Reddy, P. S., Kavi Kishor, P. B., Seiler, C., Kuhlmann, M., Eschen-Lippold, L., Lee, J., Reddy, M. K., & Sreenivasulu, N. (2014). Unraveling regulation of the small heat shock proteins by the heat shock factor HvHsfB2c in barley: Its implications in drought stress response and seed development. *PLoS One, 9*, e89125.

Rensink, W. A., Lobst, S., Hart, A., Stegalkina, S., Liu, J., & Buell, C. R. (2005). Gene expression profiling of potato responses to cold, heat, and salt stress. *Functional & Integrative Genomics, 5*, 201–207.

Rinalducci, S., Egidi, M. G., Mahfoozi, S., Godehkahriz, S. J., & Zolla, L. (2011). The influence of temperature on plant development in a vernalization-requiring winter wheat: A 2-DE based proteomic investigation. *Journal of Proteomics, 74*, 643–659.

Ristic, Z., Gifford, D. J., & Cass, D. D. (1991). Heat shock proteins in two lines of *Zea mays* L. that differ in drought and heat resistance. *Plant Physiology, 97*, 1430–1434.

Rizhsky, L., Liang, H., & Mittler, R. (2002). The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiology, 130*, 1143–1151.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., & Mittler, R. (2004). When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. *Plant Physiology, 134*, 1683–1696.

Rollins, J. A., Habte, E., Templer, S. E., Colby, T., Schmidt, J. and von Korff M. (2013) Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.). Journal of Experimental Botany 64, 3201–3212.

Rossel, J. B., Wilson, I. W., & Pogson, B. J. (2002). Global changes in gene expression in response to high light in Arabidopsis. *Plant Physiology, 130*, 1109–1120.

Rozenzvieg, D., Elmaci, C., Samach, A., Lurie, S., & Porat, R. (2004). Isolation of four heat shock protein cDNAs from grapefruit peel tissue and characterization of their expression in response to heat and chilling temperature stresses. *Physiologia Plantarum, 121*, 421–428.

Ruibal, C., Castro, A., Carballo, V., Szabados, L., & Vidal, S. (2013). Recovery from heat, salt and osmotic stress in *Physcomitrella patens* requires a functional small heat shock protein PpHsp16.4. *BMC Plant Biology, 13*, 174.

Sabehat, A., Lurie, S., & Weiss, D. (1998). Expression of small heat-shock proteins at low temperatures: A possible role in protecting against chilling injuries. *Plant Physiology, 117*, 651–658.

Sanchez-Bel, P., Egea, I., Sanchez-Ballesta, M. T., Sevillano, L., del Carmen Bolarin, M., & Flores, F. B. (2012). Proteome changes in tomato fruits prior to visible symptoms of chilling

injury are linked to defensive mechanisms, uncoupling of photosynthetic processes and protein degradation machinery. *Plant & Cell Physiology, 53*, 470–484.

Sarkar, N. K., Kim, Y. K., & Grover, A. (2009). Rice sHsp genes: Genomic organization and expression profiling under stress and development. *BMC Genomics, 10*, 393.

Sarry, J. E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J. B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J., & Bourguignon, J. (2006). The early responses of Arabidopsis thaliana cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics, 6*, 2180–2198.

Sato, Y., & Yokoya, S. (2008). Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. *Plant Cell Reports, 27*, 329–334.

Scarpeci, T. E., Zanor, M. I., & Valle, E. M. (2008). Investigating the role of plant heat shock proteins during oxidative stress. *Plant Signaling & Behavior, 3*, 856–857.

Schöffl, F., Prändl, R., & Reindl, A. (1999). Molecular responses to heat stress. In *Molecular responses to cold, drought, heat and salt stress in higher plants* (Vol. 1). Texas: Biotechnology intelligence unit.

Schroda, M., Vallon, V., Wlollman, F., & Beck, C. F. (1999). A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant Cell, 11*, 11165–11178.

Shekhar, S., Mishra, D., Gayali, S., Buragohain, A. K., Chakraborty, S., & Chakraborty, N. (2016). Comparison of proteomic and metabolomic profiles of two contrasting ecotypes of sweetpotato (*Ipomoea batata* L.) *Journal of Proteomics, 143*, 306–317.

Siddique, M., Gernhard, S., von Koskull-Döring P, ., Vierling, E. and Scharf, K. D. (2008) The plant sHSP superfamily: Five new members in *Arabidopsis thaliana* with unexpected properties. Cell Stress & Chaperones 13, 183–197.

Singh, A., Singh, U., Mittal, D., & Grover, A. (2010). Genome-wide analysis of rice ClpB/HSP100, ClpC and ClpD genes. *BMC Genomics, 11*, 95.

Singh, R. K., Jaishankar, J., Muthamilarasan, M., Shweta, S., Dangi, A., & Prasad, M. (2016). Genome-wide analysis of heat shock proteins in $C_4$ model, foxtail millet identifies potential candidates for crop improvement under abiotic stress. *Scientific Reports, 6*, 32641.

Singla, S. L., Pareek, A., & Grover, A. (1997). Yeast HSP104 homologue rice HSP110 is developmentally- and stress-regulated. *Plant Science, 125*, 211–219.

Singla, S. L., Pareek, A., & Grover, A. (1998). Plant Hsp100 family with special reference to rice. *Journal of Biosciences, 23*, 337–345.

Soll, J. (2002). Protein import into chloroplasts. *Current Opinion in Plant Biology, 5*, 529–535.

Song, H., Fan, P., & Li, Y. (2009a). Overexpression of organellar and cytosolic *AtHSP90* in *Arabidopsis thaliana* impairs plant tolerance to oxidative stress. *Plant Molecular Biology Reporter, 27*, 342–349.

Song, H., Zhao, R., Fan, P., Wang, X., Chen, X., & Li, Y. (2009b). Overexpression of *AtHsp90.2*, *AtHsp90.5* and *AtHsp90.7* in *Arabidopsis thaliana* enhances plant sensitivity to salt and drought stresses. *Planta, 229*, 955–964.

Song, H. M., Wang, H. Z., & Xu, X. B. (2012). Overexpression of *AtHsp90.3* in *Arabidopsis thaliana* impairs plant tolerance to heavy metal stress. *Biologia Plantarum, 56*, 197–199.

Soto, A., Allona, I., Collada, C., Guevara, M., Casado, R., Emilio, R., Aragoncillo, C., & Gomez, L. (1999). Heterologous expression of a plant small heat-shock protein enhances *Escherichia coli* viability under heat and cold stress. *Plant Physiology, 120*, 521–528.

Su, P. H., & Li, H. M. (2008). Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiology, 146*, 1231–1241.

Subba, P., Barua, P., Kumar, R., Data, A., Soni, K. K., Chakraborty, S., & Chakraborty, N. (2013a). Phosphoproteomic dynamics of chickpea (*Cicer arietinum* L.) reveals shared and distinct components of dehydration response. *Journal of Proteome Research, 12*, 5025–5047.

Subba, P., Kumar, R., Gayali, S., Shekhar, S., Parveen, S., Pandey, A., Data, A., Chakraborty, S., & Chakraborty, N. (2013b). Characterisation of the nuclear proteome of a dehydration-sensitive

cultivar of chickpea and comparative proteomic analysis with a tolerant cultivar. *Proteomics, 13*, 1973–1992.

Sugino, M., Hibino, T., Tanaka, Y., Nii, N., & Takabe, T. (1999). Overexpression of DnaK from a halotolerant cyanobacterium *Aphanothece halophytic* acquires resistance to salt stress in transgenic tobacco plants. *Plant Science, 146*, 81–88.

Süle, A., Vanrobaeys, F., Hajós, G., Van Beeumen, J., & Devreese, B. (2004). Proteomic analysis of small heat shock protein isoforms in barley shoots. *Phytochemistry, 65*, 1853–1863.

Sun, W., Bernard, C., Van de Cotte, B., Van Montagu, M., & Verbruggen, N. (2001). At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. *The Plant Journal, 27*, 407–415.

Sun, J. H., Chen, J. Y., Kuang, J., Chen, W., & Lu, W. (2010). Expression of sHSP genes as affected by heat shock and cold acclimation in relation to chilling tolerance in plum fruit. *Postharvest Biology and Technology, 55*, 91–96.

Sung, D. Y., Vierling, E., & Guy, C. L. (2001). Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. *Plant Physiology, 126*, 789–800.

Swindell, W. R., Huebner, M., & Weber, A. P. (2007). Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics, 8*, 125.

Talamè, V., Ozturk, N. Z., Bohnert, H. J., & Tuberosa, R. (2007). Barley transcript profiles under dehydration shock and drought stress treatments: A comparative analysis. *Journal of Experimental Botany, 58*, 229–240.

Thao, N. P., Chen, L., Nakashima, A., Hara, S., Umemura, K., Takahashi, A., Shirasu, K., Kawasaki, T., & Shimamoto, K. (2007). RAR1 and HSP90 form a complex with Rac/Rop GTPase and function2 in innate-immune responses in rice. *Plant Cell, 19*, 4035–4045.

Vierling, E. (1991). The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology, 42*, 579–620.

Vinocur, B., & Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: Achievements and limitations. *Current Opinion in Biotechnology, 16*, 123–132.

Vítámvás, P., Prášil, I. T., Kosová, K., Planchon, S., & Renaut, J. (2012). Analysis of proteome and frost tolerance in chromosome 5A and 5B reciprocal substitution lines between two winter wheats during long-term cold acclimation. *Proteomics, 12*, 68–85.

Wang, W., Vinocur, B., Shoseyov, O., & Altman, A. (2004). Role of plant heatshock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science, 9*, 244–252.

Wang, A., Yu, X., Mao, Y., Liu, Y., Liu, G., Liu, Y., & Niu, X. (2015). Overexpression of a small heat-shock-protein gene enhances tolerance to abiotic stresses in rice. *Plant Breeding, 134*, 384–393.

Wang, J., Yu, Q., Xiong, H., Wang, J., Chen, S., Yang, Z., & Dai, S. (2016). Proteomic insight into the response of Arabidopsis chloroplasts to darkness. *PLoS One, 11*, e0154235.

Wang, M., Zou, Z., Li, Q., Sun, K., Chen, X., & Li, X. (2017). The CsHSP17.2 molecular chaperone is essential for thermotolerance in Camellia Sinensis. *Scientific Reports, 7*(1237).

Waters, E. R. (2013). The evolution, function, structure, and expression of the plant sHSPs. *Journal of Experimental Botany, 64*, 391–403.

Waters, E. R., Lee, G. J., & Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany, 47*, 325–338.

Waters, E. R., Lee, G. J., & Vierling, E. (2013). Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany, 47*, 325–338.

Xu, C., & Huang, B. (2010). Comparative analysis of drought responsive proteins in kentucky bluegrass cultivars contrasting in drought tolerance. *Crop Science, 50*, 2543–2552.

Xu, X. B., Song, H. M., Zhou, Z. H., Shi, N. N., Ying, Q. C., & Wang, H. Z. (2010). Functional characterization of AtHsp90.3 in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* under heat stress. *Biotechnology Letters, 32*, 979–987.

Xu, Y., Zhan, C., & Huang, B. (2011). Heat shock proteins in association with heat tolerance in grasses. *International Journal of Proteomics, 2011*(529648).

Xu, J., Xue, C., Xue, D., Zhao, J., Gai, J., Guo, N., & Xing, H. (2013). Overexpression of *GmHsp90s*, a heat shock protein 90 (Hsp90) gene family cloning from soybean, decrease damage of abiotic stresses in *Arabidopsis thaliana*. *PLoS One, 8*, e69810.

Yamada, K., Fukao, Y., Hayashi, M., Fukazawa, M., Suzuki, I., & Nishimura, M. (2007). Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in *Arabidopsis thaliana*. *The Journal of Biological Chemistry, 282*, 37794–37804.

Yang, Y., Li, X., Yang, S., Zhou, Y., Dong, C., Ren, J., Sun, X., & Yang, Y. (2015). Comparative physiological and proteomic analysis reveals the leaf response to cadmium-induced stress in poplar (*Populus yunnanensis*). *PLoS One, 10*, e0137396.

Young, T. E., Ling, J., Geisler-Lee, C. J., Tanguay, R. L., Caldwell, C., & Gallie, D. R. (2001). Developmental and thermal regulation of the maize heat shock protein, HSP101. *Plant Physiology, 127*, 777–791.

Young, L. W., Wilen, R. W., & Bonham-Smith, P. C. (2004). High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production. *Journal of Experimental Botany, 55*, 485–495.

Yu, A., Li, P., Tang, T., Wang, J., Chen, Y., & Liu, L. (2015). Roles of Hsp70s in stress responses of microorganisms, plants, and animals. *BioMed Research International, 2015*, 1–8.

Yu, J., Cheng, Y., Feng, K., Ruan, M., Ye, Q., Wang, R., Li, Z., Zhou, G., Yao, Z., Yang, Y., & Wan, H. (2016). Genome-wide identification and expression profiling of tomato Hsp20 gene family in response to biotic and abiotic stresses. *Frontiers in Plant Science, 7*, 1215.

Zhang, L., Yu, Z., Jiang, L., Jiang, J., Luo, H., & Fu, L. (2011). Effect of post-harvest heat treatment on proteome change of peach fruit during ripening. *Journal of Proteomics, 74*, 1135–1149.

Zhang, Y., Xu, L., Zhu, X., Gong, Y., Xiang, F., Sun, X., & Liu, L. (2013). Proteomic analysis of heat stress response in leaves of radish (*Raphanus sativus* L.) *Plant Molecular Biology Reporter, 31*, 195–203.

Zhang, L., Zhang, Q., Gao, Y., Pan, H., Shi, S., & Wang, Y. (2014a). Overexpression of heat shock protein gene *PfHSP21.4* in *Arabidopsis thaliana* enhances heat tolerance. *Acta Physiologiae Plantarum, 36*, 1555–1564.

Zhang, Y., Sun, M., & Zhang, Q. (2014b). Proteomic analysis of the heat stress response in leaves of two contrasting chrysanthemum varieties. *Plant OMICS, 7*, 229–232.

Zhang, Y., Pan, J., Huang, X., Guo, D., Lou, H., Hou, Z., Su, M., Liang, R., Xie, C., Mingshan You, M., & Li, B. (2017). Differential effects of a post-anthesis heat stress on wheat (*Triticum aestivum* L.) grain proteome determined by iTRAQ. *Scientific Reports, 7*, 3468.

Zou, J., Liu, C., Liu, A., Zou, D., & Chen, X. (2012). Overexpression of *OsHsp17.0* and *OsHsp23.7* enhances drought and salt tolerance in rice. *Journal of Plant Physiology, 169*, 628–635.

Zhou, Y., Chen, H., Chu, P., Li, Y., Tan, B., Ding, Y., Tsang, E. W. T., Jiang, L., Wu, K., & Huang, S. (2012). NnHSP17.5, a cytosolic class II small heat shock protein gene from Nelumbo nucifera, contributes to seed germination vigor and seedling thermotolerance in transgenic Arabidopsis. *Plant Cell Reports, 31*, 379–389.

# Chapter 4
# Heat Shock Proteins in Stress in Teleosts


**Bimal Prasanna Mohanty, Arabinda Mahanty, Tandrima Mitra, Subash Chandra Parija, and Sasmita Mohanty**

**Abstract** Heat shock proteins (HSP) play important role in maintenance of cellular homeostasis. These proteins constitute around 5–10% total proteins of all normal cells and mediate the correct assembly of proteins and intracellular localization. In unstressed cells, HSP play various constitutive functions; however, when cells face stressed condition, multifold increase in the synthesis of HSP is observed. Fish is an important animal in aquatic ecosystem and the health of fish reflects the health status of its environment. Moreover, fish is a health food and fisheries and aquaculture is one of the the fastest growing food production sectors. Fishes are poikilothermic animals and confront a wide range of biotic and abiotic stressors, and like other animals and plants, in fish also HSP play important role in combating and/or withstanding the stress. So the HSP have potential applications in monitoring and management of stress in fish. The present chapter discusses the different types of HSP that have been reported in fish and their potential applications in monitoring and management of fish health under biotic and abiotic stress; further, the knowledge from the lower vertebrates could be useful in health and disease management in higher vertebrates including humans.

**Keywords** Environmental pollutants · Fish · Heat shock proteins · Stress · Thermal stress

B. P. Mohanty (✉) · A. Mahanty · T. Mitra
ICAR-Central Inland Fisheries Research Institute, FREM Division-Biochemistry Laboratory, Barrackpore, Kolkata, India
e-mail: bimal.mohanty@icar.gov.in

S. C. Parija
Department of Pharmacology and Toxicology, College of Veterinary Sciences, Orissa University of Agriculture and Technology, Bhubaneswar, India

S. Mohanty
School of Biotechnology, KIIT University, Bhubaneswar, India



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_4

## Abbreviations

| | |
|---|---|
| CP | Chlorpyrifos |
| CYP1A | Cytochrome monooxygenase P450 1A |
| DC | Dendritic cell |
| EV | Esfenvalerate |
| Hsc | Heat shock cognates |
| HSP | Heat shock proteins |
| IL | Interleukin |
| IPCC | Intergovernmental Panel on Climate Change |
| MT | Metallothionine |
| MHC | Major histocompatibility complex |
| ppt | Parts per trillion |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| TNF | Tumor necrosis factor |

## 4.1 Introduction

Heat shock proteins (HSP) are the most important proteins that mediate the correct assembly of proteins and their intracellular localization. They constitute about 5–10% total proteins of all normal unstressed cells and perform many constitutive homeostatic functions (Pockley 2003). Such HSP are referred as "Constitutive chaperones" or "Heat shock cognates" (Roberts et al. 2010). However, when a cell is subjected to any stress, a rapid increase in the rate of synthesis of HSP occurs. The discovery of HSP is often attributed to the reporting of chromosomal puffs in the salivary gland cells of the fruit fly, *Drosophila busckii*, after a heat shock (Ritossa 1962; Iwama et al. 1999; Iwama 1998). However, the actual revelation of the fact that the transcription of the HSP is a response to heat shock occurred in the early 70s (Lindquist-McKenzie et al. 1975). Later, it was found that the synthesis oh HSP is not exclusive to *Drosohila* and these proteins were found in many other organisms including animals, plants and microbes. Reports on the teleosts HSP started coming in the early 80s. HSP have been classified into several families based on their molecular weight such as HSP110 (100–110 kDa), HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60, HSP47, and small HSP (12–43 kDa) (Park et al. 2007; Hallare et al. 2004). These HSP vary in their localization and functions (Jee 2016). The details on the different HSP families, their localization and functions are provided in Table 4.1.

**Table. 4.1** Different families of Heat shock proteins, their localization and functions

| Hsp Family | Localization | Role/Functions | References |
|---|---|---|---|
| HSP10 | Mitochondria Cell surface, cytosol, extracellular space | Plays important role in mitochondrial protein folding, functions related to pregnancy, cancer and autoimmune inhibition. | Jia et al. 2010 |
| HSP27 | Cytosol, nucleus and endoplasmatic reticulum | Inhibition of apoptosis, regulation of cell development, and cell differentiation | Gusev et al. 2002, Haslbeck 2002 |
| HSP40 | Cytosol | Chaperoning intermediate filament, plays important human diseases like neurodegenerative disorders, autoimmune diseases and cancer | Albani et al. 1995, Muchowski and Wacker 2005 |
| HSP47 | Endoplasmic reticulum | Maturation of collagen | Ishida and Nagata (2011) |
| HSP60 | Mitochondria | Synthesis and transportation of mitochondrial proteins, plays important immunological functions | Kaufman 2003 |
| HSP70 | Cytoplasm | Assist folding of large variety of proteins | Mayer and Bukau 2005 |
| HSP78 | Mitochondria, endoplasmic reticulum | Confers thermotolerance to the mitochondrial compartment but also participates in protein degradation by the matrix protease Pim1. | Leidhold et al. 2006 |
| HSP90 | Cytoplasm | Assists the folding, and maintenance of cytosolic proteins. | Picard 2006 |
| HSP110 | Nucleus and cytoplasm | Convert stable protein aggregates into Native proteins; helps in mounting immune response | Mattoo et al. 2013 |

## 4.2 Heat Shock Proteins (Hsp) in Fish

Fishes are poikilothermic animals and confront a wide range of biotic and abiotic stressors. The biotic stressors include predators and pathogenic infections of bacteria, virus, helminthes etc. The abiotic factors that influence the metabolism and survival of fish include pH, temperature, dissolved oxygen and salinity. For optimal growth and reproduction, fish needs an environment where these abiotc factors are within a range that supports the survival of fish. Alterations in any of these abiotc factors beyond the optimal ranges render the fish to be stressed and like other organisms, fish also elicit a generalized response in such stressed conditions. This stress response includes physiological alterations characterized by increase in stress hormones like catecholamines and a multitude of metabolites which helps in maintenance of homeostasis (Iwama 2004). Other than the stress hormones, elicitation of

**Table 4.2** Hsps expressed in different fish tissues/cell lines in response to various stressors

| Common name (Species) | Tissue/ Cell line | Stressor | Hsps | Reference |
|---|---|---|---|---|
| Catla *(Catla catla)* | Larvae | UV-B radiation | *hsp70* | Singh et al. 2015 |
| | Muscle | – | *hsp27*, *hsp47*, *hsp60*, *hsp70*, *hsp90*, *hsp110* | Mohanty et al. 2013 |
| Snakehead murrel *(Channa striatus)* | Liver, gill, muscle | Heat stress | *hsp27*, *hsp47*, *hsp60*, *hsp70*, *hsp78*, *hsp90*, *hsp110* | Purohit et al. 2014 |
| Mrigal *(Cirrhinus mrigala)* | Liver, gill, brain, kidney | Heat stress | *hsp70* | Das et al. 2005 |
| Zebrafish *(Danio rerio)* | Embryo | – | *h*spb1, *h*spb2, *h*spb3, *h*spb4, *h*spb5a, *h*spb5b, *h*spb6, *h*spb7, *h*spb8, *h*spb9, *h*spb11, *h*spb12,*h*spb15 | Elicker and Hutson 2007 |
| Rohu *(Labeo rohita)* | Liver | Arsenic | *hsp47*, *hsp60*, *hsp70*, *hsc71*, *hsp78*, *hsp90* | Mohanty et al. 2015, Banerjee et al. 2015 |
| | Liver | Starvation | *hsp70* | Yengkokpam et al. 2008 |
| | Liver, anterior kidney, Spleen | *Aeromonas hydrophila* Infection | *hsp30*, *hsp70*, *hsp90* | Das et al. 2015 |
| Punti *(Puntius sophore)* | Liver, gill, muscle | Heat stress | *hsp27*, *hsp47*, *hsp60*, *hsp70*, *hsp78*, *hsp90*, *hsp110* | Mahanty et al. 2016a, b, 2017 |
| Rita *(Rita rita)* | Liver, gill | Pollution | *hsp27*, *hsp47*, *hsp60*, *hsp70*, *hsp90*, *hsp110* | Mohanty et al. 2015, Mitra et al. 2017 |
| Salmon *(Salmo salar)* | Skeletal muscle | Fasting | *hsp90α1a*, *hsp90α1b*, *hsp90α2a*, *hsp90α2b*, *hsp90ß1a* | de la serrana et al. 2013 |

stress response is also accompanied by readjustments in metabolites like glucose, amino acids, fatty acids etc. It has been seen that the energy metabolism increases rapidly to compensate for the negative influences of the stressor (Iwama 2004; Mahanty 2016a). Another most important component of the stress response is the increased synthesis of heat shock proteins (HSP). Similar to other organisms, a number of families of HSP are also found in fishes. A list of HSP that have been found to be expressed in different fish tissues or cell lines are summarized in Table 4.2. These HSP are involved in varieties of functions; both constitutive and specialized functions during stress conditions. Functions and regulations of different HSP during various stressed conditions are discussed in this chapter.

## 4.3 Hsp and Thermal Stress

### *4.3.1 Heat Stress Response in Fish*

Temperature in an ecosystem is known to play an important role in modulating the physiology of biological organisms. Thus, increase or decrease in environmental temperature from its optimal value can alter various homeostatic mechanisms. The impact of high temperature is found to be severe in poikilothermic animals like fish (Allan et al. 2015) leading to retardation of their growth, reproduction and may even cause death: all such factors are likely to affect the overall loss of productivity in a scenario of pisciculture Fig. 4.1.

Thermal acclimation and adaptation are strategies adapted by organisms to survive in the changing environmental temperature climes. Thermal acclimation has been defined as the phenotypic response of organisms to changing environmental temperature that alters performance and plausibly changes fitness (Narum et al. 2013; Mahanty et al. 2017). Acclimatory responses are exhibited in organisms to maintain constancy in functional performance and energy production on a shorter time scale (Fangue 2007). In contrast, thermal adaptation refers to evolution of a population over a longer period of time (can be over generations) to an altered reaction norm for temperature (Narum et al. 2013; Portner and Knust 2007). Thermal adaptation enables species to inhabit over broad geographic ranges with highly variable climate regimes and the ability of fish species to adapt to the changing temperature climes determines the "Winners and Loosers" in an aquatic ecosystem (Narum et al. 2013; Somero 2010). This means that populations at extreme temperatures consist

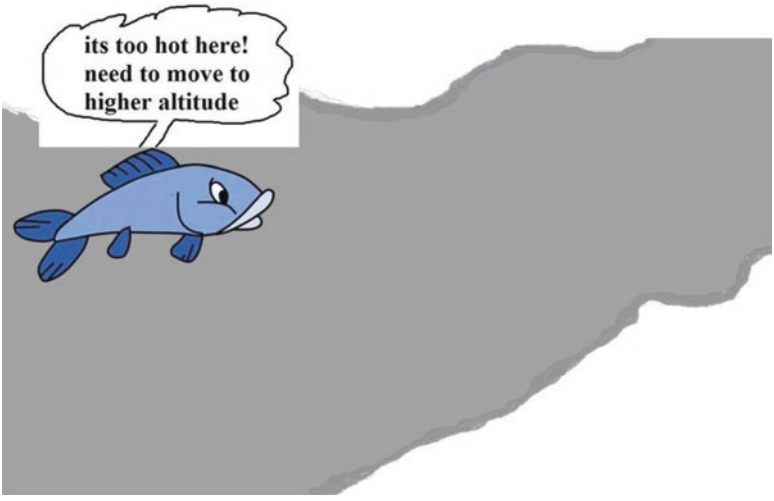


**Fig. 4.1** Migration of fish to higher altitudes or cooler habitats is one of the adaptive mechanisms to combat high temperature stress

of specialists, and populations elsewhere contain generalists (Narum et al. 2013). The acclimatory responses to changing environmental temperature generally start with behavioral changes like swimming. Fishes tend to move to places where the temperature is more ambient. Due to increase in the oceanic temperatures, high temperature driven pole ward movement of fish species have been evidenced in recent times (Goldfarb 2017; Poloczanska et al. 2016) (Fig. 4.1). Because of such migrations, fishes that were previously not available in higher altitudes are now available in these areas. For instance, black sea bass, which were the inhabitants of North Carolina, have shifted two degrees of latitude north, to New Jersey, over the last half-century (Goldfarb 2017; OceanAdapt http://oceanadapt.rutgers.edu/regional_data/). Similarly, in Portugal at least 20 new species have been found that belong to higher temperature climes (Goldfarb 2017). Other than that the energy demand increases which results in consumption of energy stores (Mahanty et al. 2016a, b).

### *4.3.2 HSP and the Heat Stress Response*

Heat stress has become one of the major environmental concerns because of the rise in climatic temperature in recent times. According to a report by Intergovernmental Panel on Climate Change (IPCC 2007) 20–30% of fish species assessed so far are at risk of extinction, if the climatic temperature increases by 1.5–2 °C relative to the pre-industrial era (IPCC 2007). The ability to adapt to the increased temperature climes would determine the winner and looser species i.e. species that would survive and species that would go extinct, at least locally (Somero 2010). Transient changes or precisely the increased synthesis of HSP is one of the integral components of heat stress tolerance in fish and other organisms. The species with higher ability to modulate this response would be able to better adapt to the changing temperature whereas those lacking this response will have reduced abilities to minimize the damage to their protein pool (Somero 2010; Podrabsky and Somero 2006). This ability of species to withstand different temperature climes has set a latitudinal thermal gradient of species distribution in aquatic ecosystems.

The family of HSP that get up-regulated in response to heat stress depends on many factors like the species, population, temperature, duration of exposure etc. Lack of certain types of heat-shock proteins leads to disruption of important cellular regulatory processes, including those that control the intrinsic (mitochondria-dependent) and extrinsic (death-receptor-mediated) pathways of programmed cell death (apoptosis), where heat-shock protein 70 (HSP70) is of critical regulatory significance (Creagh et al. 2000; Beere 2004). Upregulation of HSP70 and other molecular chaperones can block apoptosis through inhibition of the caspase proteins that are pivotal in the apoptotic destruction of the cell (Beere 2004). This regulatory reaction can be viewed as a means of providing molecular chaperones with adequate time to renature the damaged proteins. The ability of fish to withstand heat stress largely depend on two interdependent factors; the first one is the prior conditioning or hardening of the fish to higher temperature and the second one is the

ability to increase the synthesis of the HSP. It has been seen that population of a species which have been exposed to high temperature for generations develop a genetic makeup that help them to withstand temperature variations (Narum et al. 2013).

The increase in synthesis of different HSP in response high or low temperature is indicative of distinct cellular processes. Heat stress induces expression of a number of HSP gene families; HSP70 and HSP90 being the most prominent ones. Expression of induced form of HSP70 and HSP90 protein and genes in response to heat stress has been observed in many fish species like *Haliotis discus, Caretta caretta, Garra rufa, Clarias batrachus, Sparus aurata, Chromis viridis* (Park et al. 2007; Tedeschi et al. 2015; Oksala et al. 2014; Singh et al. 2013; Healy et al. 2010). Therefore, both HSP70 and HSP90 can be useful biomarkers for assessing heat stress in the fish (Tedeschi et al. 2015). Other than HSP70 and HSP90, up-regulation of other HSP families has also been reported in several fish species. Up-regulation of HSP60 in response to heat stress has been reported in fishes like *Caretta caretta*, *Garra rufa* (Tedeschi et al. 2015; Oksala et al. 2014). Similarly, up-regulation of other HSP like HSP47, HSP27 and HSP78 has also been reported in case of some fish species like *Garra rufa* (Oksala et al. 2014). A list of HSP genes those are up-regulated in response to heat shock in different fish species are summarized in Table 4.3. In one of the studies in murrel *Channa straitus* we have found the expression of *hsp90* and *hsp110* to be upregulated in the fishes that were stressed for shorter time period (4 days) which gradually decreased with increase in time period of temperature

**Table 4.3** Increased expression of Hsp genes in different fish/shellfish species in response to high temperature stress

| Common Name (species) | Stress condition | *Hsps* induced | Tissues examined | References |
|---|---|---|---|---|
| Doctor fish (*Garra rufa*) | Naturally living in a hot spring temp. (34.4 °C) | *hsp*70, *hsp*60, *hsp*90, *hsc*70, *grp*75 | Muscles | Oksala et al. 2014 |
| *Squalius* (*Squalius. torgalensis* and *Squalius carolitertii*) | 20, 25, 30 and 35 °C for 1 °C per day | *hsp70*, *hsc70* | Pectoral, pelvic, upper caudal fins, muscle | Jesus et al. 2013 |
| Large yellow croaker (*Larimichthys crocea*) | Low temp. (19 °C) and high temp. (27 °C and 31 °C) | *hsp*27 | Muscle, brain, liver, spleen, kidney, gill, and blood | Yang et al. 2012 |
| Atlantic cod (*Gadus morhua*) | Increased temp.,2 °C (2 °C/ hr) and control 10 °C | *hsp70* | Plasma | Perez-Casanova et al. 2008 |

(continued)

**Table 4.3** (continued)

| Common Name (species) | Stress condition | *Hsps* induced | Tissues examined | References |
|---|---|---|---|---|
| Killifish (*Fundulus heteroclitus*) | Thermal stress from 2–34 °C | *hsp70 and hsp90* | Whole-organism | Fangue et al. 2006 |
| Goldfish (*Carassius auratus*) | 4 hr. heat shock form 20 °C to 40 °C | *hsp*30, *hsp*70 mRNA | Cells derived from caudal fin | Kondo et al. 2004 |
| | 2 hr. heat shock from 22 °C to 32 °C | *hsp*72, *hsp*90 | Brain | Kagawa 2004 |
| Rainbow trout (*Oncorhynchus mykiss*) | 8 hr. heat shock from 10 °C to 30 °C | *hsp*70 mRNA | Red blood cells | Lund and Tufts (2003) |
| | 18 °C were exposed to an elevated temp. (25 °C) | *hsp*60 mRNA | Gill, liver, spleen, heart, and head kidney | Shi et al. 2015 |
| | 8 hr. heat shock from 13 °C to 25 °C with 18–24 hr. recovery | *hsp*70, *hsp*90 | Liver and heart tissues | Rendell et al. 2006 |
| Green sturgeon (*Acipense medtrostrs*) | 3 day heat shock from 17 °C to 26 °C at 1.5 °C/hr | *hsp*72, *hsp*78, *hsp*89 | Whole larvae | Werner et al. 2007 |
| Rohu (*Labeo rohita*) | 30 day heat shock at 31, 33 and 36 °C | *hsp*70 | Kidney, gill, liver and brain | Das et al. 2006 |
| Tiger prawn (*Penaeus monodon*) | 24 hr. heat shock from 29 °C to 35 °C | *hsp*70 | Tail muscle | de la Vega et al. 2006 |
| Channel catfish (*Ictalurus Punctatus*) | Exposure to low temp. From 25 °C to 10.5 °C for 14 and 28 day | *hsp*70 mRNA | Muscles | Weber and Bosworth 2005 |
| Monsoon river prawn (*Macrobrachium malcolmsonni*) | 3 hr. heat shock from 25 °C to 32–34 °C and 30 °C to 36 °C-38 °C with 1 hr. recovery. | *hsp*70 | Gill and heart | Selvakumar and Geraldine 2005 |
| Freshwater giant prawn (*Macrobrachium rosenbergii*) | 2 hr. heat shock form 25 °C to 30 °C and 35 °C | *hsp*70 mRNA | Hepatopancreas and thoracic glands | Liu et al. 2004 |
| Olympia oyster (*Ostrea conchaphila*) | 1 hr. heat shock from 12–15 °C to 33–38 °C | *hsp*70 | Gill | Brown et al. 2004 |
| Europeanflat oyster (*Ostreaedulis*) | 1 hr. heat shock from 18 °C to 34 °C with 24 hr. recovery at 18 °C | *hsp*70 | Gill | Piano et al. 2004 |
| *Snakehead murrel* (*Channa striatus*) | Heat shock treatment at 36 °C for 4/15/30 days | *hsp60, hsp70, hsp78* | Liver | Purohit et al. 2014 |

exposure. Unlike these *hsp,* expression of *hsp60, hsp70* and *hsp78* were found to be upregulated in the fishes heat stressed for both short term (4 days) and long term (15 days, 30 days and beyond) (Purohit et al. 2014).

### *4.3.3 HSP Expression in Fishes from Naturally Heat Stressed (Hot Spring) Environments*

Thermal stress has become a cause of concern due to the rapid increase in global atmospheric temperature. The ability of fish to survive in heat stressed condition depends on factors such as the temperature, the genetic potential of fish to modulate its metabolism, prior hardening of fish at elevated temperature etc. Organisms inhabiting the environment which are perennially heat stressed like the hot spring run-offs or thermal discharge sites are constantly exposed to high-temperature stress. Studying the physiology of such organisms has led to a understanding of thermotolerance mechanism. *Hsp* gene expression of a number of fish species from hot spring runoffs has been studied by different researchers. Purohit et al. (2014) and Mahanty et al. (2016a, b) have reported the *hsp* gene expression in *Channa striatus* and *Puntius sophore* collected from runoff water of a hot spring located at Atri (Odisha, India) whereas Okasala et al. (2014) have reported the *hsp* gene expression of doctor fish (*Garra rufa*) from Kangal hot spring place. There are commonalities in the expression of *hsp* in these fishes; however, there are species specific responses too. HSP90 was found to be a common *hsp* that was upregulated in all the three species. HSP60 and HSP70 were upregulated in *Channa striatus* and *Garra rufa* but not in *Puntius sophore.* Additionally, *hsp78* was upregulated in *Channa striatus* whereas *hsp47* was upregulated in *Puntius sophore* collected from the hot spring runoff (Figs. 4.2 and 4.3).

## 4.4 Hsp and Environmental Pollutatants

### *4.4.1 Aquatic Environmental Pollutants*

Aquatic pollution with biologically active chemicals and xenobiotics has become an issue of great public health concern and environmental challenge. Aquatic environment is being continuously loaded with contaminants, either due to direct discharge or due to hydrologic and atmospheric processes. Increasing number and amount of industries, urbanization, agriculture and aquaculture operations are the sources of the pollution. Joint toxicity is a major problem of open water ecosystems. Long term exposures to cocktail of pollutants like heavy metals, pesticides, insecticides, bacteria, organic pollutants, industrial waste and other contaminants into the aquatic environment have led to various deleterious effects on open water ecosystems as well as aquatic organisms (Padmini and Usha Rani et al. 2008).

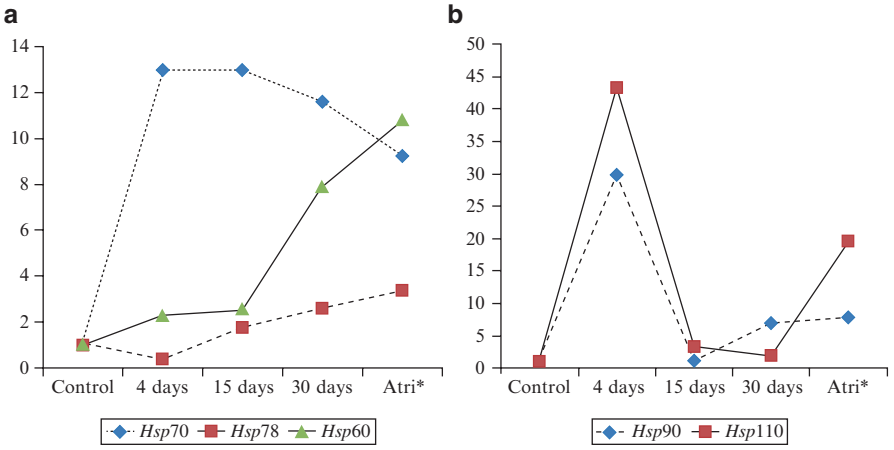


**Fig. 4.2** Trends in *hsp* gene expression in liver tissues of *Channa striatus* in response to heat stress. The *hsps* have been grouped into three clusters **(a)**, **(b)**, based on their similarity/near similarity in response to the heat stress; **(a)** *hsp70, hsp78,* and *hsp60*; **(b)** *hsp90* and *hsp110*; ∗Atri-fish collected from the Atri hot spring runoff. (Adapted from Purohit et al. 2014)

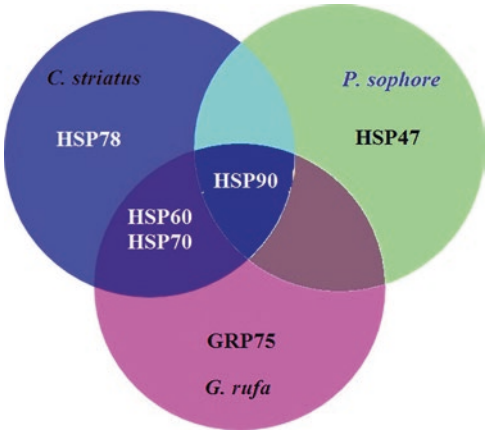


**Fig. 4.3** Increased expressions of Hsps in fishes collected from hot spring runoff environment; *Channa striatus* and *Puntius sophore* from Atri hot spring runoff (India) and *Garra rufa* from Kangal hot spring (Turkey). Hsp90 has been found to be the common Hsp in the three fishes (Purohit et al. 2014; Mahanty et al. 2016b; Oksala et al. 2014)

### *4.4.2 Impact of Environmental Pollutants on Aquatic Organisms*

Aquatic organisms, especially fish species encounter variety of stress during their continuous interaction with the environment from very early stages of their life and adverse habitat condition can lead to impairment of their health, growth, fitness (Padmini and Geetha 2007; Amaeze et al. 2015; Sueiro and Palacios, 2016). When large quantities of pollutants are released, there may be an immediate impact at molecular level followed by responses at the cellular (biochemical), tissue/organ

and whole-body levels. Responses that occur at individual, population and ecosystem levels are generally accepted to have ecological relevance and tend to be less reversible and more detrimental than effects at lower levels (Oost et al., 2003). The end results, which may occur long after the pollutants have passed through the environment, include immunosuppression, reduced metabolism, and damage to gills and epithelia.

### *4.4.3 Response of HSP in Fish against Pollution Stress*

The elevated concentration of contaminants can modify the physicochemical characteristics of water and have significant ecological impact on aquatic flora and fauna. Living systems encounter a variety of stresses during their continuous interaction with aquatic environment which affect the physiological and biochemical activities of fish (Padmini and Geetha 2007; Amaeze et al. 2015). Constant exposure to stressors induce oxidative stress in aquatic organisms by accelerating the endogenous production of reactive oxygen species (ROS). Earlier studies have demonstrated that high concentrations of metals, xenobiotics, pesticides, organic pollutants in water are mainly responsible for oxidative stress. Hence, reduced metabolic activities with enhanced cytoprotection, such as induction of chaperons as well as antioxidant defense to deal with oxidative stress occurs in organisms (Amaeze et al. 2015) (Fig. 4.4).

#### 4.4.3.1 Heavy Metals and HSP

The contamination of the aquatic environment by heavy metals is the consequence of urbanization and industrialization activities such as urban runoff, sewage treatment, and domestic garbage which expose the aquatic organisms to high level of metal contamination in its natural habitat. (Heath, 1987; Pinto et al. 2003; Sampaio et al. 2008). HSP are the important member of chaperone family and many studies have reported the induction HSP in response to heavy metals. Copper (Cu), cadmium (Cd) and Zinc (Zn) are the most common heavy metals that are available in aquatic environments on a global scale (Jiang et al. 2015). For this reason, these heavy metals are used in *in vivo* studies also, to assess their impact on aquatic organisms. Jiang et al. (2015) has reported the significant induction in mRNA levels of *hsp60, hsp70* and *hsp90* in common carp after 96 h of exposure to Cd and Cu. Similarly, significant increased abundance of HSP60 and HSP90 protein has been reported in response to combined Cd and Cu toxicity (Jiang et al. 2015). Different concentration of CuSO4 showed dose-dependent elevation in heat shock protein 70 (*hsp70*) expression at 24 and 48 h post-exposure in rainbow trout hepatocytes which indicates the crucial role of HSP70 in protecting cells from metal toxicity (Feng et al. 2003). Ali et al. (2003) has reported the induction of *hsp70* in common carp, *Cyprinus carpio* in response to Cd toxicity in a time and dose dependent manner

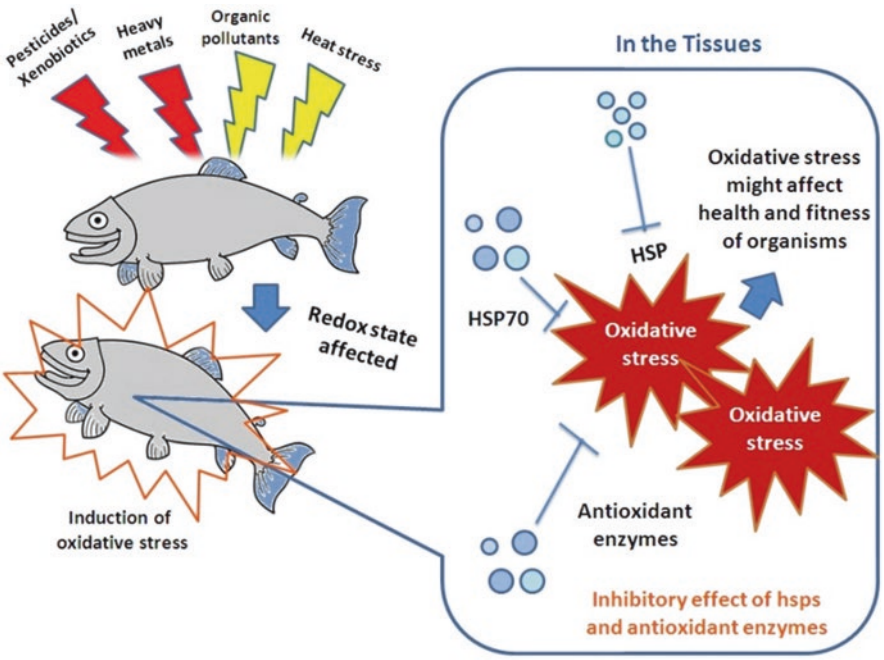


**Fig. 4.4** Induction of oxidative stress in fish and inhibitory effect of heat shock proteins

(Ali et al. 2003). Juvenile rainbow trout exposed to metals in the water or feed showed significantly higher HSP70 levels in the gill tissue (Williams et al. 1996). However, the fish samples collected from environmental water sources (Lake Hornträsket water) containing the same levels of Zn, Cu and Cd showed significant but a much lower induction of the *hsp* genes, which indicates that it is not sufficient to use the biological effects observed from laboratory condition to extrapolate the effects observed in complex environmental condition (Kumar et al. 2015).

#### 4.4.3.2 Arsenic

Arsenic is a major toxic environmental contaminant and a potential human carcinogen. Chronic arsenic toxicity (arsenicosis), due to intake of arsenic-contaminated drinking water and food, is a major environmental health hazard throughout the world (Nordstrom 2002; Banerjee et al. 2017). Expression of *hsp* transcript in response to arsenic toxicity has been extensively studied both in transcript and protein levels. In a study, Banerjee et al. (2015) reported increased expression of different *hsp* genes; *hsp90, hsp78, hsp60* in Indian major carp *Labeo rohita* (Fig. 4.5). The abundance of HSP70 and HSP90 proteins has also been found to be increasing with exposure to arsenic.

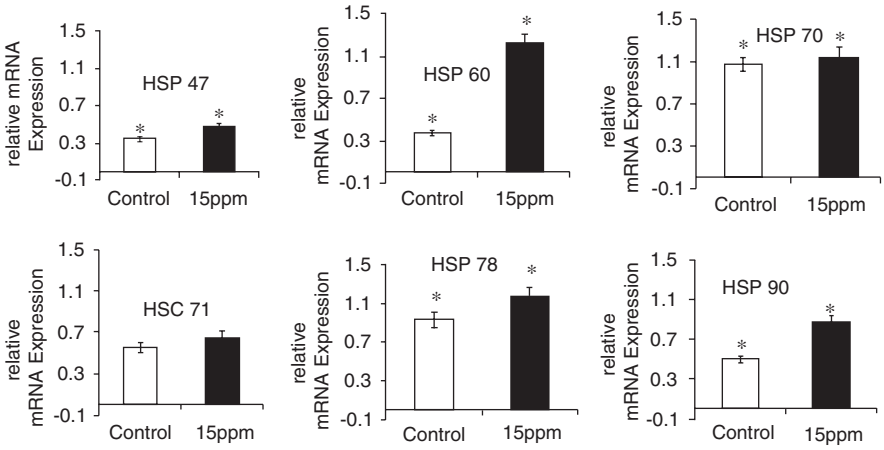


**Fig. 4.5** Gene expression of stress protein genes **(a)** HSP47, **(b)** HSP60, **(c)** HSP70, **(d)** HSC71, **(e)** HSP78 **(f)** HSP90 in liver of control and 15 ppm As-exposed *Labeo rohita*. (Banerjee et al. 2015)

#### 4.4.3.3 Pesticides, Insecticides and Xenobiotics

Pesticides, insecticides have been considered as the major anthropogenic contaminants in aquatic environments. Contamination from pesticides is now widespread in open water ecosystems such as rivers, lakes, streams and ground water from the agricultural runoff, urban runoff, and industrial effluents. Higher concentrations of pesticides and insecticides have deleterious effect in fish health. HSP induction is generally sensitive to aquatic contaminants and studies suggest that these could be used as monitoring tools in aquatic toxicology. A study on rainbow trout, *Oncorhynchus mykiss* has reported the induction of *hsp70* in exposure to 2, 4-dichloroaniline in skin epidermal cells (Kilemade and Mothersill 2001). Similarly, Porte et al. (2001) reported that HSP70 could be used as a biomarker in *Mytilus galloprovincialis* against PAH toxicity. Eder et al. 2004 reported the significant increase of *hsp70* and *hsp90* in exposure to Chlorpyrifos (CP) and induction of *hsp60, hsp70* and *hsp90* levels in response to Esfenvalerate (EV) in muscle tissues of juvenile Chinook salmon. Both the pesticides show no significant difference in expression of *hsp60* and decrease the abundance in gill tissues indicates the increased toxicity in gill (Eder et al. 2004). A study on striped bass (*Morone saxitalis*) showed the altered expression of *hsp70* and *hsp90* genes in liver and spleen but not in muscle, gill and kidney tissues in exposure for 24 h to EV (Geist et al. 2007). Another study on juvenile Chinook salmon demonstrated the induction of *hsp90* in liver and *hsp70*, *hsp60* in muscle tissues on exposure to Esfenvalerate (EV) and Chlorpyrifos (CP), respectively (Eder et al. 2009).

#### 4.4.3.4 Salinity

Deane et al. (2005) reported high induction of *hsp* in response to hypersaline and hypoosmotic salinities, compared to isosmotic salinities in an in vitro study in Black sea bream (*Myliomacro cephalus*) in exposure to different salinity concentration including 50 ppt (hypersaline), 33 ppt (seawater), 12 ppt (iso osmotic), and 6 ppt (hypoosmotic) for 8 months (Deane et al. 2005).

## 4.5 Hsp as Biomonitoring Tools for Aquatic Environmental Health Assessment

Under field conditions, interpretation of cause-and-effect relations of a given alteration in organisms is difficult, thus biomonitoring does allow a wider assessment of the natural conditions to which the organisms are subjected, and provide promising results for identifying environmental impacts (Ghisi et al. 2017). HSP play a critical role in the stress response of fish and it has been reported that expression pattern of HSP could be influenced by environmental factors such as heavy metals, pesticides, xenibiotics, organic pollutants, hypoxia. The role of HSP during stress is related to cytoprotective function as these proteins can act to pevent and repair protein damage (Beere 2004). Hence, several pollution biomonitoring studies use alteration of HSP expression to study the fish health in response to complex environmental condition. Among the HSP families, HSP70 has been the most comprehensively studied in fish and accumulation of these HSP has been linked to the intensity of stress (Porte et al. 2011). Vincze et al. (2015) reported the induction of HSP70 in protein level in the gill, liver and kidney of Brown trout collected from downstream of the Neckar River (Southern Germany) compared to the pristine zone. Similarly, expression of a battery of *hsp* genes (*hsp30*, *hsp47*, *hsp70*, and *hsp90*) have studied in liver tissues of goldfish (*Carassius auratus*) inhabiting Gaobeidian Lake in Beijing, China. Expression profile these hsp suggested the down-regulation of *hsp30* as a biomarker for complex environmental pollution (Wang et al. 2007). In contrast, other studies on wild crucian carp have reported the up-regulation liver *hsp70* and kidney *hsp30* as sensitive biomarkers for monitoring water quality in Hun River, China (An et al. 2014). A pollution monitoring study in river Ganga has reported the gene expression pattern of a battery of *hsp* genes (*hsp27*, *hsp47*, *hsp70*, *hsc70*, *hsp90*) in almost entire length of river by using riveine catfish *Rita rita* as experimental organism. The study reported that the increased expression of *hsp70* and *hsp47* in the fishes collected from down-stream and up-stream of the river, compared to the middle stretches (Fig. 4.6). An pollution induced isoform of *hsp70* (*hsp70b*) has been identified from fishes collected from lower streches of the river (Mitra et al. 2015). Similar appearance of *hsp70* isoform in protein level has also been reported in white muscle tissues of the same fish from the lower stretches of the river (Mohanty et al. 2010a, b). Previously, a study with marine mussels has reported two distinct HSP70 bands

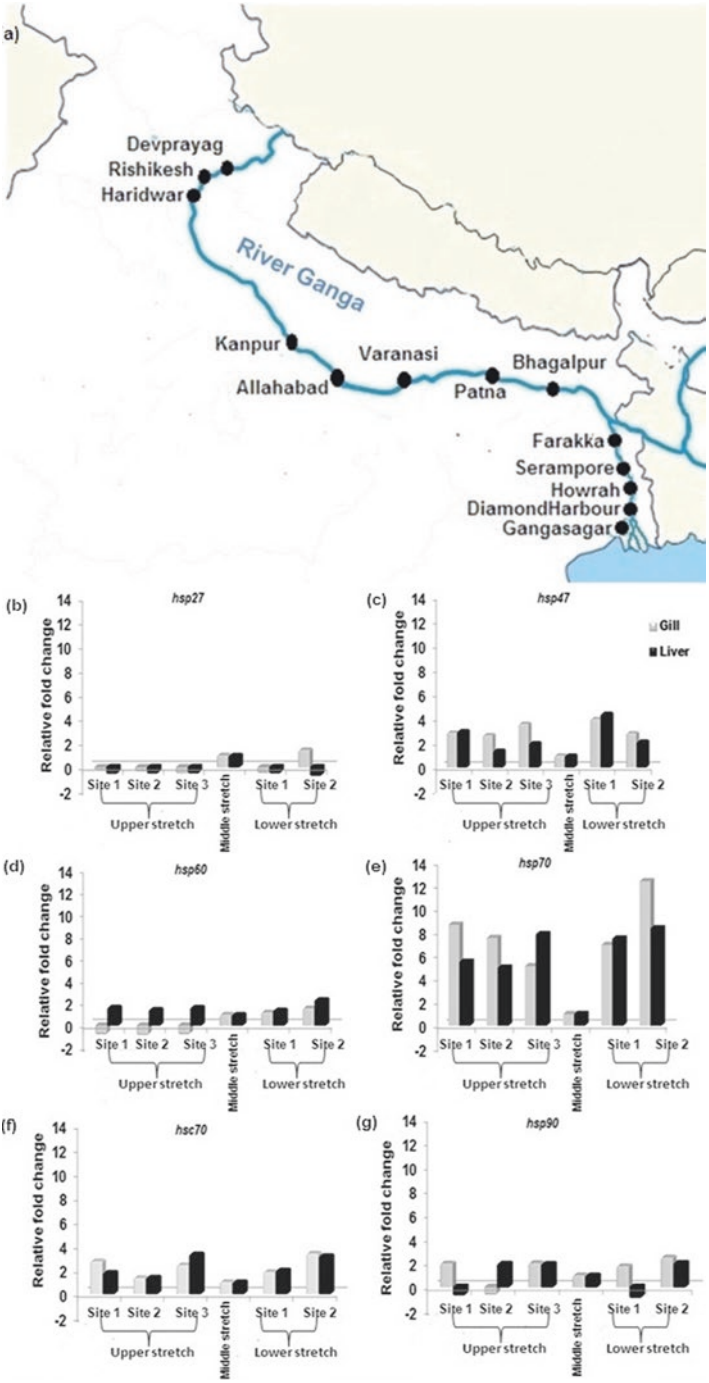


**Fig. 4.6** **(a)** Stretches of river Ganga. Relative gene expression pattern of heat shock proteins in different stretches of river Ganga. **(b)** *hsp27*, **(c)** *hsp47*, **(d)** *hsp60*, **(e)** *hsp70*, **(f)** *hsc70,* and **(g)** *hsp90*

in western blot analysis of gill protein extracts of *Mytilus galloprovinialis*; an intense band of 78 kDa and a thinner one of 72 kDa. Semi-quantitative analysis of these blots indicated a significant increase of the 72 kDa inducible band in samples collected from polluted zone indicating a correlation between levels of HSP70 and environmental pollution (Porte et al. 2001). A series of studies on estuarian ecosystem showed that complicated, changeable environment and external stressor can perturb mitochondrial function and cells evolve multiple signalling pathways to maintain cellular homeostasis (Fu et al. 2011). Padmini et al. (2009) reported the up-regulation of mitochondrial *hsp70* and decreased respiratory chain enzyme activities, ATP/ADP ratio, reduced mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase (Gpx) levels in *Mugil cephalas* in polluted (Ennore) estuary, compared to unpolluted estuaries (Kovalam) (Padmini et al. 2009). Another study from Jiulong river estuary, showed that significant down regulation of *hsp* in gills tissues of *Crassostrea hongkongensis* collected from a heavy metal contminated zone, which indicated the long term heavy metal exposure may significantly suppress the stress and immune response system of oysters (Luo et al. 2014). Not only freshwater and estruian ecosystem, induction of isofroms of *hsp70* (*hsp70a* and *hsp70b*), *hsp47* along with CYP1A and MT has been reported in *Epinephles guaza* collected from Gulf of Suez, a PAH contaminated zone (Abdel-Gawad and Khalil 2013).

## 4.6 Hsp and Immune Functions: Implications in Fish Health Management

Health management of fish is quiet challenging in both aquaculture and open water ecosystems due to their substantially higher exposure to pathogen than non-aquatic vertebrates with millions of bacteria and virus in per millilitres of water (Fuhrman 1999). In aquaculture operations, fishes rapidly grow under controlled or semi-controlled conditions. For commercial success, in order to reduce the production costs, the fishes can be adequately cultivated at densities that greatly exceed those normally present in nature (Aksungur et al. 2007; Baer et al. 2011). Previous studies have that high stocking density affects the physiological responses of fish (Jia et al. 2016). When these fishes are exposed to other environmental stressors such as hypoxia, bacterial or viral contamination, and the defense mechanism especially immune response of the organism may be weakened and reduce the ability of disease-resistance. Unlike aquaculture, open water ecosystems are highly contaminated with uncontrolled disposal of natural and anthropogenic pollutants, including industrial wastes, pesticides, insecticides, heavy metal, bacteria, organic pollutants, which wreaks adverse impacts on the health of aquatic organisms as well as health of aquatic environment (Hook et al. 2014). Immune system is fundamental for survival against invading pathogens. In contrast to mammals, in fish the innate immune response is highly developed and the adaptive immune response is rudimentary (Secombs et al. 2011).

Heat-shock proteins also play an important role in innate and adaptive immunity. Gaston 2002 has reported that *hsp* are up-regulated at sites of inflammation and capable stimulation of cross-reactive *hsp*-specific T cells which have the ability to induce autoimmune diseases (Van et al. 1988; Elias et al. 1995). The involvement of *hsp* in T cell-mediated immunity was demonstrated by the studies of Srivastava (Srivastava 2002; Srivastava 2008), who showed that the chaperone function of many HSP especially HSP70 and HSP90 (their ability to bind to and protect other polypeptides) allows them to deliver tumour antigens very effectively to antigen presenting cells (Anderton et al. 1995; Blachere et al. 1997). The third immunological role of HSP is their ability to stimulate cells of the innate immune system (Gaston 2002), which is more applicable for fish species because the innate immune system is the dominating immune component of fish.

For innate immunity, HSP are reported to mediate both humoral and cellular innate immune responses (Zanin-Zhorov et al. 2005). In fishes, activation of HSP served as a danger signal to activate cells innate such as dendritic cells (DCs) and macrophages (Quintana and Cohen 2005; Zanin-Zhorov et al. 2005), which helps maturation of MHC molecules. Similarly, several cytokines can be induced by HSP, such as tumor-necrosis-α (TNF-α), interleukin-1β (IL-1β), interleukin-12 (IL-12), nitric oxide and some chemokines (Lehner et al. 2000; Panjwani et al. 2002). For adaptive immune response, HSP act as membrane bound ligands such as toll like receptors, cluster of differentiation to stimulate immune system (Basu et al. 2001; Ohashi et al. 2000; Vabulas et al. 2001). Among the HSPs, *hsp60* has been found to be found to be highly immunogenic molecules in fish and activate both innate and adaptive immune response. It can activate macrophages as a signal or directly activate anti-inflammatory activities which can inhibit the pro-inflammatory response. As a part of adaptive immunity, it is capable of activating a large number of T cells and implicated in a variety of autoimmune and inflammatory conditions (Xu et al. 2011). Besides *hsp60*, *hsp70* and *hsp90* also play an important role in the development of inflammation and the specific and non-specific immune responses to bacterial and viral infections in both finfish and shrimp (Roberts et al. 2010).

## 4.7 Conclusions

A number of HSP families are found in fish which play important role in varieties of cellular functions both in normal and stressed cells. However, in stressed condition, the turnover of these proteins increases rapidly. But out of the HSP family not all respond to every stressor in similar manner; for instance, in response to bacterial contamination in aquatic ecosystem the expression of small HSP is more sensitive than the larger ones (Nicosia et al. 2014; Mitra et al. 2017). Similarly, there are numerous examples where the expression of a particular *hsp* can be correlated with particular stressor. Thus the different HSP families could be used as monitoring to detect the type and magnitude of stress in fish and could be helpful in stress management. However, a large varieties of fish species are available across the world and

there could identical responses across species along with species specific responses. For example, the in response to heat stress the expression *hsp90* and *hsp47* increased in *Puntius sophore* whereas the expression of *hsp60, hsp70, hsp78* and *hsp90* has been found to up-regulated in *Channa striatus* (Mahanty et al. 2016a, b; Purohit et al. 2014). Therefore, it is necessary to validate the suitability of the *hsp* across different fish species before their application as biomonitoring and management tools. Although many *hsp* have been found to be correlating well with different stressors and could be used as biomonitoring tools, field level applications have been scanty. Therefore, there is need for development of user friendly technology especially strip based detection kits that could use be used for monitoring and management of stress in fish. Along with being seen as potential stress monitoring tools, HSP have also found potential applications in immunotherapy and treatment of various human ailments like cancer (Srivastava 2000). Such studies on application of HSP in management of fish health are scanty and can be explored. Further, the knowledge gained from the lower vertebrates on role of HSP in stress response could provide the insights in managing biotic and abiotic stress in higher vertebrates, including human subjects.

**Acknowledgements** The authors are thankful to the Indian Council of Agricultural Research for funding support under the projects ICAR-CIFRI Core project FREM/ER/12/03/05, ICAR-NFBSFARA (AS-2001), World Bank funded NAIP (Component-4; Basic and Strategic Research).

## References

Abdel-Gawad, F. K., & Khalil, W. K. B. (2013). Modulation of stress protein genes in the bass (*Epinephelus sguaza*) caught from the Gulf of Suez, the Red Sea, Egypt. *Ecotoxicology and Environmental Safety, 96*, 175–181.

Aksungur, N., Aksungur, M., Akbulut, B., & Kutlu, I. (2007). Effects of stocking density on growth performance, survival and food conversion ratio of turbot (psetta maxima) in the net cages on the southeastern coast of the Black Sea, Turk. *Journal of Fisheries and Aquatic Science, 7*, 147–152.

Albani, S., et al. (1995). Positive selection in autoimmunity: Abnormal immune responses to a bacterial dnaJ antigenic determinant in patients with early rheumatoid arthritis. *Nature Medicine, 1*, 448–452.

Ali, K. S., Dorgai, L., Gazdag, A., Abraham, M., & Hermesz, E. (2003). Identification and induction of hsp 70 gene by heat shock and cadmium exposure in carp. *Acta Biologica Hungarica, 54*(3–4), 323–334.

Allan, B. J. M., Domenici, P., Munday, P. L., & McCormick, M. I. (2015). Feeling the heat: The effect of acute temperature changes on predator–prey interactions in coral reef fish. *Conservation Physiology, 3*(1), cov011.

Amaeze, N. H., Adeyemi, R. O., & Adebesin, A. O. (2015). Oxidative stress, heats shock protein and histopathological effects in the gills of African catfish, Clarias Gariepinus induced by bridge runoffs. *Environmental Monitoring and Assessment, 187*(4), 172.

An, H. L., Lei, K., & Bing-hui, Z. (2014). Use of heat shock protein mRNA expressions asbiomarkers in wild crucian carp for monitoringwater quality. *Environmental Toxicology and Pharmacology, 37*, 248–255.

Anderton, S. M., van der Zee, R., Prakken, B., Noordzij, A., & van Eden, W. (1995). Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *The Journal of Experimental Medicine, 181*, 943–952.

Baer, A., Schulz, C., Traulse, I., & Krieter, J. (2011). Analysing the growth of turbot (Psetta maxima) in a commercial recirculation system with the use of three different growth models. *Aquaculture International, 19*, 497–511.

Banerjee, S., Mahanty, A., Mohanty, S., Guhamazumder, D. N., Cash, P., & Mohanty, B. P. (2017). Identification of potential biomarkers of hepatotoxicity by plasma proteome analysis of arsenic-exposed carp *Labeo rohita*. *Journal of Hazardous Material, 336*, 71–80.

Banerjee, S., Mitra, T., Purohit, G. K., Mohanty, S., & Mohanty, B. P. (2015). Immunomodulatory effect of arsenic on cytokine and HSP gene expression in *Labeo rohita* fingerlings. *Fish & Shellfish Immunology, 44*, 43–49.

Basu, S., Binder, R. J., Ramalingam, T., & Srivastava, P. K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity, 14*, 303–313.

Beere, H. M. (2004). The stress of dying: The role of heat shock proteins in the regulation of apoptosis. *Journal of Cell Science, 117*(13), 2641–2651.

Blachere, N. E., Li, Z. H., & Chandawarkar, R. Y. (1997). Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *The Journal of Experimental Medicine, 186*, 1315–1322.

Creagh, E. M., Carmody, R. J., & Cotter, T. G. (2000). Heat shock protein 70 inhibits Caspase-dependent and -independent apoptosis in Jurkat T cells. *Experimental Cell Research, 257*(1), 58–66.

Das, P., Gupta, A., & Manna, S. K. (2005). Heat shock protein 70 expression in different tissues of Cirrhinus mrigala (Ham.) following heat stress. *Aquaculture Research, 36*, 525–529.

Das, S., Mohapatra, A., & Sahoo, P. K. (2015). Expression analysis of heat shock protein genes during *Aeromonas hydrophila* infection in rohu, *Labeo rohita*, with special reference to molecular characterization of Grp78. *Cell Stress & Chaperones, 20*(1), 73–84.

de la serrana, D. G., Johnston, I. A., & Picard, D. (2013). Expression of heat shock protein (Hsp90) Paralogues is regulated by amino acids in skeletal muscle of Atlantic salmon. *PLoS One, 8*(9), e74295.

Deane, E. E., Kelly, S. P., Luk, J. C., & Woo, N. Y. (2005). Chronic salinity adaptation modulates hepatic heat shock protein and insulin-like growth factor I expression in black sea bream. *Marine Biotechnology, 4*(2), 193–205.

Eder, K. J., Leutenegger, C. M., Wilson, B. W., & Werner, I. (2004). Molecular and cellular biomarker responses to pesticide exposure in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *Marine Environmental Research, 58*(2–5), 809–813.

Eder, K. J., Leutenegger, C. M., Kohler, H. R., & Werner, I. (2009). Effects of neurotoxic insecticides on heat-shock proteins and cytokine transcription in Chinook salmon (*Oncorhynchus tshawytscha*). *Ecotoxicology and Environmental Safety, 72*, 182–190.

Elias, D., Marcus, H., Reshef, T., Ablamunits, V., & Cohen, I. R. (1995). Induction of diabetes in standard mice by immunization with the p277 peptide of a 60-kDa heat shock protein. *European Journal of Immunology, 25*, 2851–2857.

Elicker, K. S., & Hutson, L. D. (2007). Genome-wide analysis and expression profiling of the small heat shock proteins in zebrafish. *Gene, 403*(1–2), 60–69.

Fangue, N. A. (2007). *Thermal acclimation and adaptation in the common killfish Fundulus heteoclitus: Thermal reaction norms and underlying mechanisms*. Ph.D. Thesis submitted to University of British Columbia.

Feng, Q., Boone, A. N., & Vijayan, M. M. (2003). Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. *Comparative Biochemistry and Physiology, 35 C*(3), 345–355.

Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature, 399*(6736), 541–548.

Fu, D., Chen, J., Zhang, Y., & Yu, Z. (2011). Cloning and expression of a heat shock protein (HSP) 90 gene in the haemocytes of Crassostrea hongkongensis under osmotic stress and bacterial challenge. *Fish & Shellfish Immunology, 31*(1), 118–125.

Gaston, J. S. H. (2002). Heat shock proteins and innate immunity. *Clinical and Experimental Immunology, 127*(1), 1–3.

Geist, J., Werner, I., Eder, K. J., & Leutenegger, C. M. (2007). Comparisons of tissue-specific transcription of stress response genes with whole animal endpoints of adverse effect in striped bass (*Morone saxatilis*) following treatment with copper and esfen valerate. *Aquatic Toxicology, 85*, 28–39.

Ghisi, N. C., Oliveira, E. C., Guiloski, I. C., Lima, S. B., Silva, A. H., Longhi, S. J., & Prioli, A. J. (2017). Multivariate and integrative approach to analyze multiple biomarkers in ecotoxicology: A field study in Neotropical region. *The Science of the Total Environment, 609*(31), 1208–1218.

Goldfarb, M. (2017). Feeling the heat: How fish are migrating from warming waters. https://e360.yale.edu/features/feeling-the-heat-warming-oceans-drive-fish-into-cooler-waters. Accessed on 25 Oct 2017.

Gusev, N. B., Bogatcheva, V., & Marston, S. B. (2002). Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. *Biochemistry, 67*(5), 511–519.

Haslbeck, M. (2002). sHsps and their role in the chaperone network. *Cellular and Molecular Life Sciences, 59*(10), 1649–1657.

Hallare, A. V., Köhler, H. R., & Triebskorn, R. (2004). Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. *Chemosphere, 56*(7), 659–666.

Healy, T. M., Tymchuk, W. E., Osborne, E. J., & Schulte, P. M. (2010). Heat shock response of killifish ( ): Candidate gene and heterologous microarray approaches. *Physiological Genomics, 41*(2), 171–184.

Heath, A. G. (1987). *Water pollution and fish Physiology*. Florida: CRC Press.

Hook, S. E., Gallagher, E. P., & Batley, G. E. (2014). The role of biomarkers in the assessment of aquatic ecosystem health. *Integrated Environmental Assessment and Management, 10*, 327–341.

IPCC. (2007). *Fourth assessment report - climate change 2007: Synthesis report*. IPCC, Geneva, Switzerland.

Ishida, Y., & Nagata, K. (2011). Hsp47 as a collagen-specific molecular chaperone. *Methods in Enzymology, 499*, 167–182.

Iwama, G. K. (1998). Stress in fish. *Annals of the New York Academy of Sciences, 851*(1 STRESS OF LIF), 304–310.

Iwama, G. K. (2004). Are hsps suitable for indicating stressed states in fish? *Journal of Experimental Biology, 207*(1), 15–19.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B., & Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. *American Zoologist, 39*(6), 901–909.

Jee, H. (2016). Size dependent classification of heat shock proteins: A mini-review. *Journal of Exercise Rehabilitation, 12*(4), 255–259.

Jia, H., Halilou, A. I., Hu, L., Cai, W., Liu, J., & Huang, B. (2010). Heat shock protein 10 (Hsp10) in immune-related diseases: One coin, two sides. *International Journal of Biochemistry and Molecular Biology, 2*(1), 47–57.

Jia, R., Liu, B., Feng, W., Han, C., & Huang, B. (2016). Stress and immune responses in skin of turbot (Scophthalmus maximus) under different stocking densities. *Fish & Shellfish Immunology, 55*, 131–139.

Jiang, X., Guan, X., Yao, Z., Jin, X., & Han, Y. (2015). Effects of single and joint subacute exposure of copper and cadmium on heat shock proteins in common carp (*Cyprinus carpio*). *Biological Trace Element Research, 169*(2), 374–381.

Kagawa, N. (2004). A drastic reduction in the basal level of heat-shock protein 90 in the brain of goldfish (Carassius auratus) after administration of geldanamycin. *Zoological Science, 21*(11), 1085–1089.

Kaufman, B. A. (2003). A function for the mitochondrial chaperonin Hsp60 in the structure and transmission of mitochondrial DNA nucleoids in Saccharomyces Cerevisiae. *The Journal of Cell Biology, 163*(3), 457–461.

Kilemade, M., & Mothersill, C. (2001). Heat shock protein 70 levels in rainbow trout primary epidermal cultures in response to 2,4-dichloroaniline exposure: A novelin vitro aquatic toxicity marker. *Environmental Toxicology, 16*(3), 253–259.

Kumar, R., Pradhan, A., Khan, F. A., Lindström, P., Ragnvaldsson, D., & Ivarsson, P. (2015). Comparative analysis of stress induced gene expression in Caenorhabditiselegans following exposure to environmental and lab reconstituted complex metal mixture. *PLoS One, 10*(7), e0132896.

Lehner, T., Bergmeier, L. A., Wang, Y., Tao, L., Sing, M., & Spallek, R. (2000). Heat shock proteins generate beta-chemokines which function as innate adjuvants enhancing adaptive immunity. *European Journal of Immunology, 30*, 594–603.

Leidhold, C., von Janowsky, B., Becker, D., Bender, T., & Voos, W. (2006). Structure and function of Hsp78, the mitochondrial ClpB homolog. *Journal of Structural Biology, 156*(1), 149–164.

Lindquist-McKenzie, S., Henikoff, S., & Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in Drosophila melanogaster. *Proceedings of the National Academy of Sciences, 72*(3), 1117–1121.

Lund, S. G., & Tufts, B. L. (2003). The physiological effects of heat stress and the role of heat shock proteins in rainbow trout (Oncorhynchus mykiss) red blood cells. *Fish Physiology and Biochemistry, 29*(1), 1–12.

Luo, L., Ke, C., Guo, X., Shi, B., & Huang, M. (2014). Metal accumulation and differentially expressed proteins in gill ofÂ oyster (Crassostrea hongkongensis) exposed to long-term heavy metal-contaminated estuary. *Fish & Shellfish Immunology, 38*(2), 318–329.

Mahanty, A., Purohit, G. K., Banerjee, S., Karunakaran, D., Mohanty, S., & Mohanty, B. P. (2016a). Proteomic changes in the liver of *Channa striatus* in response to high temperature stress. *Electrophoresis, 37*, 1704–1717.

Mahanty, A., Purohit, G. K., Yadav, R. P., Mohanty, S., & Mohanty, B. P. (2016b). *hsp90* and *hsp47* appear to play an important role in minnow Puntius sophore for surviving in the hot spring run-off aquatic ecosystem. *Fish Physiology and Biochemistry, 43*(1), 89–102.

Mahanty, A., Purohit, G. K., Nayak, N. R., Mohanty, S., & Mohanty, B. P. (2017). Suitable reference gene for quantitative real-time PCR analysis of gene expression in gonadal tissues of minnow Puntius Sophore under high-temperature stress. *BMC Genomics, 18*, 617. https://doi.org/10.1186/s12864-017-3974-1

Mattoo, R. U. H., Sharma, S. K., Smriti Priya, S., Finka, A., & Goloubinoff, P. (2013). Hsp110 is a bona fide chaperone using ATP to unfold stable misfolded polypeptides and reciprocally collaborate with Hsp70 to solubilize protein aggregates. *The Journal of Biological Chemistry, 288*, 21399. https://doi.org/10.1074/jbc.M113.479253

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*(6), 670–684.

Mitra, T., Mahanty, A., Ganguly, S., Banerjee, S., Bhowmick, S., Mahaver, L. R., Paul, S. K., Abdulla, T., Parida, P. K., & Mohanty, B. P. (2015). *Fish health assessment in inland openwater ecosystems-Evaluating stress protein transcript profiles in fish biomonitoring tools*. Abstract no. GHRN(P)-2. 2nd International symposium on genomics in aquaculture 28–30 January, 2016, ICAR-CIFA, Bhubaneswar, Odisha.

Mitra, T., Mahanty, A., Ganguly, S., Purohit, G. K., Mohanty, S., Das, B. K., & Mohanty, B. P. (2017). Gene expression patterns of heat shock proteins as biomarker response against environmental pollution in fish (*Rita rita*) from its natural riverine habitat. *Science of the Total Environment* (communicated).

Mohanty, B. P., Bhattacharjee, S., Mondal, K., & Das, M. K. (2010a). HSP70 expression profiles in white muscles of riverine catfish Rita rita show promise as biomarker for pollution monitoring in tropical rivers. *National Academy Science Letters, 33*, 177–182.

Mohanty, B. P., Mohanty, S., Sahoo, J. K., & Sharma, A. P. (2010b). Climate change: Impacts on fisheries and aquaculture. In S. Simard (Ed.), *Climate change and variability, InTech open* (pp. 119–138). https://doi.org/10.5772/9805.

Mohanty, B. P., Banerjee, S., Bhattacharjee, S., Mitra, T., Purohit, G. K., Sharma, A. P., Karunakaran, D., & Mohanty, S. (2013). Muscle proteomics of the Indian major carp catla (Catla catla, Hamilton). *Journal of Proteomics Bioinformatics, 6*, 252–263.

Mohanty, B. P., Banerjee, S., Bhattacharjee, S., Mitra, T., et al. (2015). Muscle proteomics of the Indian major carp Catla (*Catla catla,* Hamilton). *Journal of Proteomics and Bioinformatics, 6*, 11.

Muchowski, P. J., & Wacker, J. L. (2005). Modulation of neurodegeneration by molecular chaperones. *Nature Reviews. Neuroscience, 6*, 1–22.

Narum, S. R., Campbell, N. R., Meyer, K. A., Miller, M. R., & Hardy, R. W. (2013). Thermal adaptation and acclimation of ectotherms from differing aquatic climates. *Molecular Ecology, 22*(11), 3090–3097.

Nicosia, A., Maggio, T., Mazzola, S., Gianguzza, F., Cuttitta, A., Costa, S., & Ianora, A. (2014). Characterization of small HSPs from Anemonia viridis reveals insights into molecular evolution of alpha crystallin genes among cnidarians. *PLoS One, 9*(9), e105908.

Nordstrom, D. K. (2002). Public health. Worldwide occurrences of arsenic in groundwater. *Science, 296*, 2143–2145.

Ohashi, K., Burkart, V., Flohe, S., & Kolb, H. (2000). Cutting edge: Heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *Journal of Immunology, 164*, 558–561.

Oksala, N. K. J., GülerEkmekçi, F., Ozsoy, G., Kirankaya, S., Kokkola, T., Emecen, G., Lappalainen, J., Kaarniranta, K., & Atalay, M. (2014). Natural thermal adaptation increases heat shock protein levels and decreases oxidative stress. *Redox Biology, 3*, 25–28.

Oost, V. R., Beyer, J., & Vermeulen, N. P. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology, 13*(2), 57–149.

Padmini, E., & Geetha, B. (2007). A comparative seasonal pollution assessment study on Ennore Estuary with respect to metal accumulation in the grey mullet, Mugil cephalus. *Oceanological and Hydrobiological Studies, 36*(4), 91–103.

Padmini, E., & Usha Rani, M. (2008). Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 151*(3), 278–285.

Padmini, E., Lavanya, S., & Uthra, V. (2009). Preeclamptic placental stress and over expression of mitochondrial HSP70. *Clinical Chemistry and Laboratory Medicine, 47*(9), 1073–1080.

Panjwani, N. N., Popova, L., & Srivastava, P. K. (2002). Heat shock proteins grp96 and hsp70 activate the release of nitric oxide by APCs. *Journal of Immunology, 168*, 2997–3003.

Park, H., Ahn, I. H., & Lee, H. E. (2007). Expression of heat shock protein 70 in the thermally stressed Antarctic clam *Laternula elliptica*. *Cell Stress & Chaperones, 12*(3), 275–282.

Picard, D. (2006). Intracellular dynamics of the Hsp90 co-chaperone p23 is dictated by Hsp90. *Experimental Cell Research, 312*(2), 198–204.

Pinto, E., Sigaud-Kutner, T. C. S., Leitao, M. A. S., Okamoto, O. K., Morse, D., & Colepicolo, P. (2003). Heavy metal-induced oxidative stress in algae. *Journal of Phycology, 39*, 1008–1018.

Pockley, A. G. (2003). Heat shock proteins as regulators of the immune response. *Lancet, 362*, 469–476.

Podrabsky, J. E., & Somero, G. N. (2006). Inducible heat tolerance in Antarctic notothenioid fishes. *Polar Biology, 30*(1), 39–43.

Poloczanska, E. S., et al. (2016). Responses of marine organisms to climate change across oceans. *Frontiers in Marine Science, 3*. https://doi.org/10.3389/fmars.2016.00062

Porte, C., Biosca, X., Sole, M., & Albaiges, J. (2001). The integrated use of chemical analysis, cytochrome P450 and stress proteins in mussels to assess pollution along the Calician coast (NW Spain). *Environmental Pollution, 112*, 261–268.

Porte, C., Riboldi, E., & Sica, A. (2011). Mechanisms linking pathogens-associated inflammation and cancer. *Cancer Letters, 305*(2), 250–262.

Portner, H. O., & Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science, 315*(5808), 95–97.

Purohit, G. K., Mahanty, A., Suar, M., Sharma, A. P., Mohanty, B. P., & Mohanty, S. (2014). Investigating hsp gene expression in liver of *Channa striatus* under heat stress for understanding the upper thermal acclimation. *Biotechnology Research International*. https://doi.org/10.1155/2014/381719*, 2014*, 1.

Quintana, F. J., & Cohen, I. R. (2005). Heat shock proteins as endogenous adjuvants in sterile and septic inflammation. *Journal of Immunology, 175*, 2777–2782.

Ritossa, F. M. (1962). A new puffing pattern induced by a temperature shock and DNP in drosophila. *Experientia, 18*, 571–573.

Roberts, R. J., Agius, C., Saliba, C., Bossier, P., & Sung, Y. Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: A review. *Journal of Fish Diseases, 33*, 789–801.

Sampaio, F. G., Boijink, C. L., Oba, E. T., Santos, L. R. B., Kalinin, A. L., & Rantin, F. T. (2008). Antioxidant defenses and biochemical changes in pacu (Piaractus mesopotamicus) in response to single and combined copper and hypoxia exposure. *Comparative Biochemistry and Physiology, 147*, 43–51.

Secombes, C. J., Wang, T., & Bird, S. (2011). The interleukins of fish. *Developmental and Comparative Immunology, 35*, 1336–1345.

Selvakumar, S., & Geraldine, P. (2005). Heat shock protein induction in the freshwater prawn Macrobrachium malcolmsonii: Acclimation-influenced variations in the induction temperatures for Hsp70. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 140*(2), 209–215.

Singh, M. K., Sharma, J. G., & Chakrabarti, R. (2015). Simulation study of natural UV-B radiation on *Catla catla* and its impact on physiology, oxidative stress, Hsp 70 and DNA fragmentation. *Journal of Photochemistry and Photobiology. B, 149*, 156–163.

Somero, G. N. (2010). The physiology of climate change: How potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *Journal of Experimental Biology, 213*(6), 912–920.

Srivastava, P. K. (2000). Immunotherapy of human cancer: Lessons from mice. *Nature Immunology, 1*, 363–366.

Srivastava, P. K. (2002). Roles of heat shock proteins in innate and adaptive immunity. *Nature Reviews Immunology, 2*, 185–194.

Srivastava, P. K. (2008). New jobs for ancient chaperones. *Scientific American, 283*, 50–55.

Sueiro, S. C., & Palacios, M. G. (2016). Immunological and health-state parameters in the Patagonian rockfish Sebastes Oculatus. Their relation to chemical stressors and seasonal changes. *Fish & Shellfish Immunology, 48*, 71–78.

Tedeschi, J. N., Kennington, W. J., Berry, O., Whiting, S., Meekan, M., & Mitchell, N. J. (2015). Increased expression of Hsp70 and Hsp90 mRNA as biomarkers of thermal stress in loggerhead turtle embryos (*Caretta Caretta*). *Journal of Thermal Biology, 47*, 42–50.

Vabulas, R. M., Ahmad-Nejad, P., Costa, C., Miethke, T., Kirschning, C. J., & Hacker, H. (2001). Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *The Journal of Biological Chemistry, 276*, 31332–31339.

Van, E. W., Thole, J., & van der Zee, R. (1988). Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature, 331*, 171–173.

Vincze, K., Scheil, V., Kuch, B., Köhler, H. R., Triebskorn, R. (2015). Impact of wastewater on fish health: A case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. *Environmental Science and Pollution Research International, 22*(15), 11822–11839.

Wang, J., Wei, Y., Li, X., Cao, H., Xu, M., & Dai, J. (2007). The identification of heat shock protein genes in goldfish (*Carassius auratus*) and their expression in a complex environ-

ment in Gaobeidian Lake, Beijing, China. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 145*(3), 350–362.

Weber, T. E., & Bosworth, B. G. (2005). Effects of 28 day exposure to cold temperature or feed restriction on growth, body composition, and expression of genes related to muscle growth and metabolism in channel catfish. *Aquaculture, 246*(1–4), 483–492.

Williams, J. H., Farag, A. M., Stansbury, M. A., Young, P. A., Bergman, H. L., & Petersen, N. S. (1996). Accumulation of hsp70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and or diet. *Environmental Toxicology and Chemistry, 15*, 1324–1328.

Xu, X. Y., Shen, Y. B., Fu, J. J., Liu, F., Guo, S. Z., Yang, X. M., & Li, J. (2011). Molecular cloning, characterization and expression patterns of HSP60 in the grass carp (*Ctenopharyngodon idella*). *Fish & Shellfish Immunology, 31*(6), 864–870.

Yengkokpam, S., Pal, A. K., Sahu, N. P., Jain, K. K., Dalvi, R., Misra, S., & Debnath, D. (2008). Metabolic modulation in *Labeo rohita* fingerlings during starvation: Hsp70 expression and oxygen consumption. *Aquaculture, 285*, 234–237.

Zanin-Zhorov, A., Bruck, R., Tal, G., Oren, S., Aeed, H., & Hershkoviz, R. (2005). Heat shock protein 60 inhibits Th1-mediated hepatitis model via innate regulation of Th1/Th2 transcription factors and cytokines. *Journal of Immunology, 174*, 3227–3236.

# Chapter 5
# Life in Suspended Animation: Role of Chaperone Proteins in Vertebrate and Invertebrate Stress Adaptation

**Yichi Zhang and Kenneth B. Storey**

**Abstract** When confronted by environmental stress, organisms can employ physiological and biochemical adaptations for survival. These include hibernation, estivation, anhydrobiosis, anaerobiosis, and freeze tolerance. Underlying most of these is strong metabolic rate depression that suppresses rates of ATP-expensive processes like transcription, translation, and protein degradation to achieve major energy savings. Chaperone proteins are crucial to hypometabolism; helping to preserve cell viability and stabilize the proteome over extended periods of time. Two types of chaperones are important to the stress response: heat shock proteins (HSP) and glucose regulated proteins (GRP). Both act to fold new proteins or refold damaged and unfolded proteins, and can also have anti-apoptotic or other protective roles. This review summarizes recent knowledge on the involvement of HSP and GRP in animal responses to environmental stress, showing that chaperone upregulation is a consistent feature of hypometabolism. Hibernating mammals, cold-hardy insects, anoxic turtles, and dehydrated frogs and snails all show upregulation in HSP and GRP gene or protein expression during seasonal or stress-induced dormancies. This widespread chaperone response to environmental stress also provides insights into ways to improve strategies for medical applications such as organ preservation or combating diseases where oxidative and proteotoxic stresses contribute to the condition.

**Keywords** Anoxia · Dehydration · Estivation · Freeze tolerance · Hibernation · Metabolic rate depression

Y. Zhang
Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA

K. B. Storey (✉)
Department of Biology, Carleton University, Ottawa, ON, USA
e-mail: kenneth.storey@carleton.ca



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_5

## Abbreviations

| | |
|---|---|
| ATF4 | Activating transcription factor 4 |
| BAT | Brown adipose tissue |
| BCL-2 | B-cell lymphoma 2 |
| CCT | Chaperone containing TCP-1 |
| CREB1 | cAMP response element binding protein 1 |
| DMD | Duchenne muscular dystrophy |
| eEF2 | Eukaryotic elongation factor 2 |
| eIF2α | Eukaryotic initiation factor 2 alpha |
| ER | Endoplasmic reticulum |
| GRP | Glucose regulated protein |
| HSF1 | Heat shock factor 1 |
| HSP | Heat shock protein |
| miRNAs | microRNAs |
| ND | Non-diapause |
| PERK | Protein kinase RNA-like endoplasmic reticulum kinase |
| pO2 | Partial pressure of oxygen |
| SD | Summer diapause |
| SERCA | Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase |
| sHSP | small heat shock protein |
| SMA | Spinal muscular atrophy |
| SOD | Superoxide dismutase |
| Tb | Body temperature |
| TRIC | TCP-1 ring complex |
| UPR | Unfolded protein response |
| WD | Winter diapause |

## 5.1 Introduction

During their lifetimes, all organisms are bound to encounter environmental stresses that disrupt homeostasis and require them to make physiological and/or biochemical changes to ensure survival. Environmental stresses can include variations in the availability of oxygen, nutrients, or water, changes in atmospheric pressure, temperature, and salinity, or exposure to toxic chemicals or UV irradiation, among others. Every organism can tolerate at least minor changes in its environment, but many have developed extreme strategies for dealing with severe environmental stress. These organisms provide excellent models for investigating and understanding the biochemical mechanisms that confer cytoprotection and preserve viability under extreme stress. For example, several species of turtles can live underwater for three or more months during the winter without consuming oxygen, establishing

themselves as exemplary models for the study of the molecular adaptations that allow facultative anaerobiosis. This contrasts dramatically with the human condition, where we suffer brain damage in as little as 4 min when deprived of oxygen.

When exposed to severe environmental conditions, one method that many organisms employ to cope with stress is to retreat into a hypometabolic or dormant state. They do this by shutting down many energy-costly processes while retaining core vital processes, and as a result, they slow their rates of ATP production and ATP expenditure to the point where a minimum metabolic rate can be sustained for a long period of time without additional food consumption. These animals can sustain the hypometabolic state until environmental conditions are once again permissible for growth, development, and normal activities to resume. For example, 13-lined ground squirrels (*Ictidomys tridecemlineatus*) conserve energy by hibernating during winter months, where they undergo cycles of torpor and arousal. During torpor episodes lasting for a week or more, they suppress their metabolic rate and allow their core body temperature (Tb) to sink as low as about 4°C. This method of energy conservation allows squirrels to save as much as 88% of total ATP expenditure that would otherwise be needed to sustain normal physiological activities over the winter months (Wang and Lee 2011). Aside from hibernation, hypometabolism is a characteristic of many other situations including hypoxia/anoxia tolerance, freeze tolerance (survival of 50-70% of total body water converted to ice), estivation (dormancy triggered during a dry or hot period), and anhydrobiosis (dormancy induced by severe dehydration) (Hochachka and Lutz 2001; Clegg 2002; Storey and Storey 2004, 2010a).

There are many ways by which hypometabolism is induced in different organisms: some do so seasonally (e.g. obligate winter hibernation), others in response to environmental change (e.g. subzero temperatures in winter or arid conditions in summer), and some at particular life stages (e.g. diapause at a certain developmental stage). The molecular mechanisms activated during hypometabolism in these different organisms share many commonalities, and they have been the subject of study for many research groups. In general, studies of hypometabolism centered around 1) controlling the entry and exit from the dormant state, 2) regulatory mechanisms that adjust metabolism to the realities of the hypometabolic condition (e.g. changes in fuel use), and 3) strategies to ensure cell viability over long periods of hypometabolism. In order to suppress metabolism while ensuring cell viability, processes requiring both ATP synthesis and expenditure are targeted through various molecular mechanisms on a priority basis so that those critical for viability are preserved. As such, processes like transcription, translation, protein degradation, and cell cycle control are globally suppressed during hypometabolism in most, if not all, organisms (Storey and Storey 2004, 2007, 2010a). For example, estivating land snails (*Otala lactea*) showed an 80-90% reduction in the incorporation of the 3H-leucine into new proteins produced in the hepatopancreas compared to active snails. In addition, protein degradation measured by 20S proteasome activity decreased by 70-75% (Ramnanan et al. 2009).

The regulation of transcription, translation, protein degradation, and many other processes during hypometabolism are controlled efficiently by post-transcriptional and post-translational modifications. Reversible phosphorylation of various proteins such as ATPases, transcription factors, ribosomal initiation and elongation factors, and numerous metabolic enzymes is an important facet of metabolic rate depression (Storey and Storey 2004, 2007, 2010a). For instance, two ribosomal proteins that are important for regulating protein synthesis, the eukaryotic initiation factor 2 alpha (eIF2α) and the eukaryotic elongation factor 2 (eEF2), are both inactivated by protein phosphorylation. The levels of eIF2α phosphorylation at the Serine 51 (Ser51) residue and eEF2 phosphorylation at the Thr56 residue both increased strongly in the hepatopancreas of *O. lactea* during estivation (Ramnanan et al. 2009). A more recently discovered mechanism suggests that microRNAs (miRNAs) also play crucial roles in regulating the expression of genes that promote hypometabolism during environmental stress (Bansal et al. 2016; Hadj-Moussa et al. 2016; Luu et al. 2016; Wu et al. 2016). miRNAs can impart post-transcriptional control over most gene transcripts by binding to and inhibiting the translation of mRNA transcripts or targeting them for degradation. Hence, this is one important mechanism for the suppression of protein translation during hypometabolism. A recent study found that a group of 17 miRNAs that were differentially regulated during hibernation in *I. tridecemlineatus* regulated numerous genes which code for ion motive ATPases, as well as those involved in processes such as protein modification and cell signaling (Luu et al. 2016). This indicates that these miRNAs could be crucial to adaptation during hibernation by suppressing the expression of proteins involved in the above-mentioned processes.

One mechanism of cell preservation that is consistent across all organisms that are well-adapted to dealing environmental stress is the use of chaperone proteins to protect/stabilize the cellular proteome. This large class of proteins is involved in various cellular processes, but they are known best for participating in the folding or refolding of proteins to ensure they attain and retain their native and functional conformations (Jacob et al. 2017). Given that protein misfolding is a common occurrence under many stress conditions, the actions of molecular chaperones during environmental stress is an important subject of study. Therefore, the purpose of this review is to summarize the current literature on the role of molecular chaperones in stress adaptation for a variety of organisms subjected to different environmental stresses. The best known class of chaperone proteins are the heat shock proteins (HSP), and these proteins will be the main focus on this review. In addition to HSP, we will also assess the literature on another class of chaperones known as glucose-regulated proteins (GRP). Work from several groups have begun to identify the roles that GRP play in response to extreme environmental stress (Carey et al. 1999; Lee et al. 2002; Mamady and Storey 2006; Richards et al. 2008; Kesaraju et al. 2009; Zhang et al. 2011). We will end the review by pointing out some of the medical applications that could be derived from studying hypometabolism and dormant states in animals that undergo environmental stress, focusing particularly on chaperones.

## 5.2 Heat Shock Proteins and Stress

HSP were first discovered due to their induction under high temperature stress conditions. Since then, this group of proteins have also been associated with numerous other environmental stresses including cold, dehydration, osmotic, UV, oxidative, and light-induced stresses (Swindell et al. 2007). HSP have a main role in protein folding and are recruited in response to essentially any stressor that causes protein misfolding (Carey et al. 2006). However, other roles have been identified for HSP, including actions in the degradation of irreparable proteins, prevention of apoptosis or aging, cytoskeletal protection, regulation of cell signaling pathways and transcription, as well as promoting nitric oxide synthesis (Kültz 2004; Zilaee and Shirali 2016). Due to the abundance of unfolded proteins and the number of cellular roles played by HSP, copious amounts of these proteins are found in all cells. In fact, 1-2% of total eukaryotic protein content is comprised of HSP90 alone (Krukenberg et al. 2011).

HSPs are classified into families according to their molecular weights, which includes Hsp100, Hsp90, Hsp70, Hsp60, and HSP of a range of low molecular masses (40-10 kDa) that are called small HSPs (sHSP) (Gething and Sambrook 1992; Feder and Hofmann 1999; Kampinga et al. 2009; Carra et al. 2017). Of these families, the Hsp70 and Hsp90 families are most prominent. The molecular structure, function, and regulation of various HSP proteins have been reviewed in detail (Echtenkamp and Freeman 2012; Niforou et al. 2014; Radons 2016; Carra et al. 2017; Schopf et al. 2017), and therefore this review will only briefly discuss these aspects. The Hsp70 family contains a constitutive member known as Hsc70 and an inducible member known as Hsp70, each with various isoforms. For example, in the desiccation-resistant land snail (*Sphincterochila zonata*) and desiccation-sensitive land snail (*Sphincterochila cariosa*), two isoforms of Hsp70 were found at 72 kDa and 74 kDa (Mizrahi et al. 2010). Numerous isoforms of Hsp70 are found due to diversification of Hsp70 genes, and they can assume various cellular functions due to a large variety of co-chaperones such as Hsp40 and Hsp110 (Mayer and Bukau 2005). Hsp70 proteins are ATP-dependent chaperones that have an ATPase domain on their amino terminus and a substrate-binding domain on their carboxy-terminus that recognizes the hydrophobic amino acids of unfolded proteins (Rüdiger et al. 1997; Hartl et al. 2011). Hsp70 recognizes the hydrophobic amino acids to prevent proteins from aggregating since hydrophobic amino acid exposure is related to protein aggregation (Rousseau et al. 2006). Once ATP binds to Hsp70, its previous substrate is released and Hsp70 changes to an open confirmation so that it can bind another unfolded protein. The co-chaperone Hsp40 contributes to substrate binding by mediating the recruitment of substrates and by stimulating ATPase activity. On the other hand, Hsp110 works with Hsp70 to disaggregate large protein aggregates (Rousseau et al. 2006; Radons 2016) (Fig. 5.1a). As mentioned previously, the Hsp90 family of proteins are highly abundant in cells, and are ubiquitously expressed (Krukenberg et al. 2011). One reason for this is that they bind specific substrates that play roles in various cellular signaling cascades and in the cell cycle; Hsp90 specifically regulates these processes to ensure the survival of eukaryotic cells.

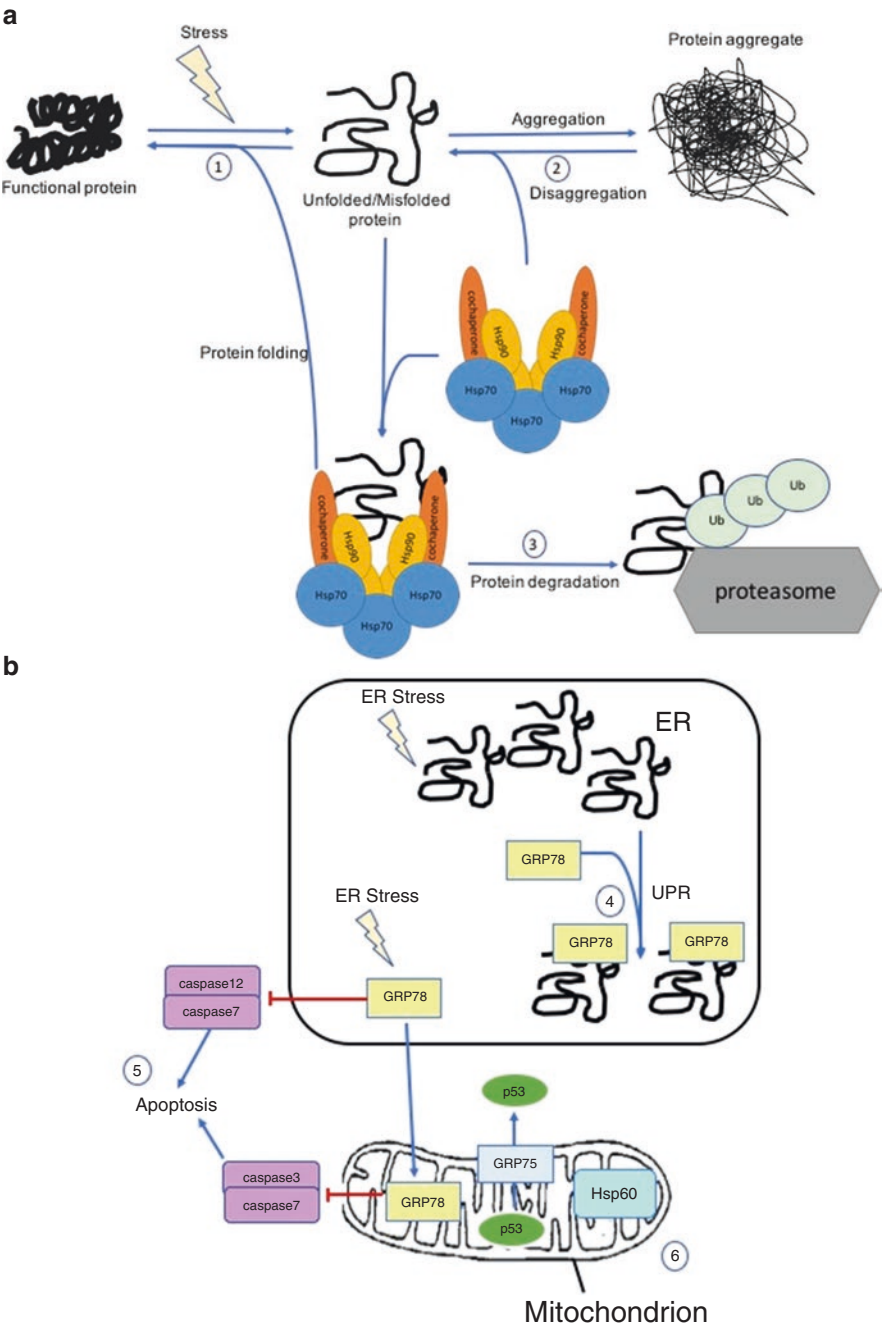


**Fig. 5.1** Schematic representation of the diverse roles played by chaperones in the cell. (a) (1) Under stress conditions (chemical, UV, heat, oxidative, etc.), native proteins can become misfolded. Complexes of heat shock proteins (HSP) and their co-chaperones (i.e. HSP110 and small HSP) can bind to misfolded proteins and refold them back to their native conformation.

Although Hsp90 mainly functions to prevent protein aggregation like many other chaperones, it does so through interactions with numerous co-regulators and co-chaperones, thus allowing it to affect many different processes in the cell (Brychzy et al. 2003; Saibil 2013) (Fig. 5.1a). The Hsp60 family is classified into two subgroups called group I and II, where group I is found in the mitochondria and group II is found in the cytosol (Ranford et al. 2000; Horwich et al. 2007). Group I Hsp60s act to ensure that mitochondrial proteins are folded correctly by binding unfolded proteins at their unbound hydrophobic amino acids to ensure that they cannot aggregate or misfold further (Motojima et al. 2004) (Fig. 5.1b). Group II Hsp60s include the TRIC (TCP-1 Ring Complex), otherwise known as CCT (Chaperone Containing TCP-1), and assist in protein folding of various cytoskeletal proteins such as α- and β-tubulins (Horwich et al. 2007). The sHSP family of proteins contains many members that have a diverse range of functions, although they all contribute to maintaining protein stability without the use of ATP (Bukau et al. 2006; Saibil 2013). In general, sHSP are involved in modulating the assembly of cytoskeletal components in addition to helping prevent protein aggregation. For example, αA-crystallin and αB-crystallin prevent actin depolymerization in the cytoskeleton (Wang and Spector 1996). In addition to modulating protein folding, HSPs also target unrepaired proteins and aggregates for degradation. For instance, αB-crystallin and Hsp27 target damaged proteins for degradation via the ubiquitin proteasome, whereas Hsp22 contributes to chaperone-mediated autophagy of protein aggregates (Carra et al. 2009; Ahner et al. 2013; Li et al. 2013). Aside from protein folding, stability, and degradation roles, certain HSPs also have other actions that would be beneficial for cell preservation during stress. For example, Hsp90, Hsp70, and Hsp27 have all been suggested to take part in inhibiting apoptosis as they can bind the apoptosis protease activating factor-1, which inhibits its activity, thus preventing its activation of caspases (Beere et al. 2000; Pandey et al. 2000; Gorman et al. 2005).

## 5.3 HSP and Hibernation

Hibernation is an essential process utilized by many mammals that live in cold climates to survive winter conditions (low temperature, lack of food). Although typical hibernators are small mammals such as bats and squirrels, there are larger hibernators as well (e.g. bears). During hibernation, these animals depress their metabolic

**Fig. 5.1** (continued) (2) Misfolded proteins tend to form larger protein aggregates, which HSPs can help to disaggregate. (3) Proteins that cannot be repaired are tagged by ubiquitin (Ub) ligases, and shunted to the ubiquitin proteasome system or the autophagy-lysosome pathway for degradation. (b) (4) In the endoplasmic reticulum, glucose regulated protein 78 (GRP78) binds unfolded proteins and under stress conditions activates the unfolded protein response (UPR). (5) As part of the UPR, GRP78 binds caspases to inhibit apoptosis and can translocate to the mitochondria to protect them from apoptosis. (6) GRP75 and HSP60 are mitochondrial chaperones. GRP75 transports p53 into the cytoplasm and HSP60 associates with co-chaperones to fold misfolded proteins

rate in a cyclic fashion; typically resulting in long periods of torpor (days or weeks) that are interspersed with short periods of arousal (hours to 1–2 days) back to 37°C (Frerichs and Hallenbeck 1998; Storey and Storey 2004; Storey 2010; Wang and Lee 2011). For some species, hibernation is a facultative response depending on winter conditions and available food stores whereas others show a circannual rhythm of obligate hibernation. Thirteen-lined ground squirrels are one of the most commonly studied models of obligate hibernation. These animals can drop their metabolic rate during torpor to just 2-4% of euthermic values, while allowing Tb to fall from 37°C to near 0°C. By doing so, hibernating squirrels can save up to 88% of energy expenditure that would otherwise be needed to sustain euthermic conditions over the winter (Wang and Lee 2011). To save energy, squirrels undergo a global reduction in gene expression, especially for genes that contribute to energy-expensive processes like transcription and translation. However, certain genes and proteins remain highly expressed during torpor to meet physiological demands (van Breukelen et al. 2004; Morin and Storey 2009). Among the proteins that are overexpressed during torpor are HSP, however there is still some debate about the role and involvement of HSP in hibernation. Table 5.1 summarizes the currently available information on HSP expression in hibernating mammals. Part of the difficulty in coming to a consensus regarding the involvement of HSP in hibernation is due to 1) species- and tissue-specific responses during hibernation, 2) variations in the methods used for quantitative analysis of mRNA and protein expression between studies, and 3) the experimental conditions or sampling points used in these studies. For example, a hibernation sampling point comprised of torpor conditions may be compared to a control that might be either a summer active animal (Lee et al. 2008) or a winter animal during interbout arousal. In other studies, specific time points across a torpor-arousal cycle are compared to one another (Carey et al. 2003; Eddy et al. 2005; Wu et al. 2015). In addition, the control time points used in these studies can also vary since some studies use controls collected at outdoor or room temperatures (Carey et al. 2003; Eddy et al. 2005; Lee et al. 2008) whereas others use euthermic animals in a cold environment (4-5°C) sampled either before or after a torpor bout (ie. in the interbout arousal stage) (Yan et al. 2007; Wu et al. 2015).

Aside from ground squirrels, other commonly studied hibernators are bats. For example, the expression of five HSP (Hsp90, Hsp70, Hsp60, Hsp27, and αB-crystallin) were evaluated in *Murina leucogaster* and showed that protein levels of Hsp70 were elevated in skeletal muscle during both hibernation and arousal compared to active summer controls (Lee et al. 2008). In another hibernating bat, *Myotis lucifugus*, total protein levels of Hsp27 were unchanged during torpor compared to euthermic controls, but phosphorylated Hsp27 levels were elevated by 3.2-fold during torpor in skeletal muscle (Table 5.1). In bat hearts, however, both total and phosphorylated Hsp27 levels were elevated during torpor (Eddy et al. 2005). It was suggested that phosphorylation of Hsp27 at Ser 78/82 leads to ubiquitination and degradation of the inhibitor protein IκB-α, which allows the transcription factor NF-κB to translocate to the nucleus and regulate the transcription of numerous genes important for torpor (Eddy et al. 2005). As mentioned previously, Hsp70 is a chaperone that plays many different roles in the cell, including assisting with

**Table 5.1** Heat shock protein expression during mammalian hibernation

| Reference | Heat shock proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Lee et al. 2008) | Hsp70 | *Myotis leucogaster* | Hibernation (torpor and arousal relative to active) | Muscle: Hsp70 ↑ during torpor and arousal | Immunoblotting (Protein) |
| (Eddy et al. 2005) | Hsp27, p-Hsp27 (Ser 78/82) | *Myotis lucifugus* | Hibernation (torpor relative to arousal) | Heart: Total Hsp27 and p-Hsp27 ↑<br>Skeletal muscle: p-Hsp27 ↑ | Immunoblotting (Protein) |
| (Carey et al. 1999) | Hsp70 | *Ictidomys tridecemlineatus* | Hibernation (torpor relative to active) | Brown adipose tissue (BAT): Hsp70 ↓ | Immunoblotting (Protein) |
| (Carey et al. 2003) | Hsp70 | *Ictidomys tridecemlineatus* | Hibernation | Hsp70 levels ↑ in the intestines of squirrels entering or in early torpor, and ↓ during arousal and interbout. | ELISA-based assay (Protein) |
| (Storey 2003) | Hsp40, Hsp60, Hsp70 | *Ictidomys tridecemlineatus* | Hibernation (torpor relative to active) | Liver: *hsp40, hsp60, hsp70* ↑ | Nylon microarray (mRNA) |
| (Epperson et al. 2010) | Hsp40 (DNAJC3), Hsp60 (HSPD1), Hsp70 (HSPA5), Hsp90 (HSP90B1) | *Ictidomys tridecemlineatus* | Hibernation (entrance into torpor relative to active) | Liver: Hsp90, Hsp70, Hsp40 ↑<br>Hsp60 ↓ | Liquid chromatography – mass spectrometry (protein) |
| (Yan et al. 2006) | Hsp10 (*Hspe1*), Hsp40 (*Dnajb9*), Hsp70 (*Hspa9a*), Hsp90 (*Hspca*) | *Spermophilus parryii* | Hibernation (torpor relative to active) | BAT: Hsp40 and Hsp70 families ↑, Hsp10 and Hsp90 families ↓ | Nylon microarray (mRNA) |
| (Yan et al. 2007) | Hsp10 (*Hspe1*), Hsp90 (*Hsp90ab1*) | *Spermophilus parryii* | Hibernation (torpor relative to active) or (arousal relative to torpor) | Muscle: Hsp90 ↓ in torpor, ↑ in arousal<br>Heart: Hsp90 ↓ in torpor, Hsp10 ↑ in torpor<br>Liver: Hsp90 ↑ in arousal, Hsp10 ↓ in torpor | Illumina gene expression array (mRNA) |
| (Wu et al. 2015) | Hsp90α, Hsp70, Hsp60 | *Microcebus murinus* | Hibernation (overnight torpor relative to active) | BAT: Hsp70 and Hsp90α ↑<br>Liver: Hsp60 ↑ | Luminex multiplex assay (protein) |

protein degradation (Radons 2016), and therefore the upregulation of Hsp70 in *M. leucogaster* and the phosphorylation of Hsp27 in *M. lucifugus* during torpor could indicate that they play similar roles in the skeletal muscle of hibernating bats. In addition, hibernators are known to shiver during arousal in order to restore $T_b$ to euthermic conditions, and the heat generated in skeletal muscles could also contribute to the upregulation of Hsp70 during arousal in *M. leucogaster* (Lee et al. 2008).

The chaperone response during hibernation has been well-studied in thirteen-lined ground squirrels (*I. tridecemlineatus*), where gene screening revealed the putative upregulation of various HSP transcripts (hsp40, hsp60, hsp70) in the liver of ground squirrels during torpor (Storey 2003) (Table 5.1). Since then, a proteomic approach was used to examine Hsp40 (DNAJC3), Hsp60 (HSPD1), Hsp70 (HSPA5), and Hsp90 (HSP90B1) protein levels in the liver upon entry into torpor. Findings suggested that Hsp40, Hsp70, and Hsp90 levels were elevated during entry into torpor but Hsp60 levels decreased by 1.3-fold (Epperson et al. 2010). Other studies used immunoblotting to show that Hsp70 was significantly reduced in brown adipose tissue (BAT) during torpor, but in the intestines its levels were highest during entry and early torpor, and lowest during arousal (Carey et al. 1999, 2003). These results illustrate the difficulty in reaching a consensus on the role of HSP in hibernation, since different methods were used to analyze HSP expression, different time points were examined in different studies, and tissue-specific differences can also occur. For example, Hsp70 appeared to be reduced only in BAT of *I. tridecemlineatus*, but it was elevated in liver and intestine during torpor (Table 5.1). When comparing gene expression and protein levels, it is expected that there would be differences in the findings, which was the case with decreased Hsp60 protein expression but increased hsp60 gene expression in *I. tridecemlineatus* during torpor (Storey 2003; Epperson et al. 2010). Disjuncts between gene and protein expression are well-documented, and could be caused during hypometabolism by a variety of factors such as the inhibition of mRNA translation via microRNA action or the suppression of the translational machinery (Ramnanan et al. 2009; Luu et al. 2016; Wu et al. 2016). In addition, BAT plays a key thermogenic role in hibernators, allowing them to rewarm their bodies during arousal from torpor by generating energy through uncoupled mitochondrial respiration (Frank and Storey 1995; Vertommen et al. 2017). Since BAT is not highly active during torpor itself, this could be a reason why Hsp70 levels were reduced. On the other hand, the liver is still a crucial organ during torpor, as its metabolic profile indicates that it regulates the switch from a primarily carbohydrate to a mainly lipid catabolism during hibernation (Serkova et al. 2007). This affects the fuel supplies to other organs since liver carries out crucial functions for the whole body such as providing key substrates to the brain in the form of ketone bodies and glucose. In addition, ground squirrel livers sampled during torpor or arousal are more resistant to cold storage in comparison with those from other mammals or summer squirrels (Lindell et al. 2005). Therefore, this predicts that the various HSP upregulated during torpor in ground squirrel liver play important cryoprotective roles.

HSP have also been studied in another hibernating ground squirrel, the arctic ground squirrel, *Spermophilus parryii*. Gene screening determined that hsp10

(hspe1), hsp40 (dnajb9), hsp70 (hspa9a), and hsp90 (hspca) were differentially expressed in BAT during torpor as compared to summer active controls. Hsp70 was upregulated by 3.62- or 1.76-fold during torpor, as assessed by microarrays or RT-PCR, respectively. Hsp40 was also overexpressed during torpor, but hsp90 and hsp10 were both downregulated (Yan et al. 2006). The same research group compared summer active *S. parryii* gene expression with that of torpid and aroused squirrels using a more advanced Illumina gene expression array. They found that only hsp10 (Hspe1) and hsp90 (Hsp90ab1) transcripts were differentially expressed during torpor or arousal in muscle, heart, and liver tissues. During torpor compared to summer active conditions, hsp90 decreased in muscle and heart, and hsp10 decreased in liver. In aroused squirrels compared to torpid ones, hsp90 was overexpressed in muscle and liver, and hsp10 was increased in heart. These findings for *I. tridecemlineatus* and *U. parryii* demonstrate that there are species-specific differences in chaperone involvement during hibernation, where hsp40, hsp60, and hsp70 were overexpressed in thirteen-lined but not arctic ground squirrels (Table 5.1). These differences could possibly be attributed to the physiological differences between the two squirrels during hibernation. For example, *U. parryii* can achieve a deeper state of torpor (e.g. Tb can fall to -2.9°C and metabolic rate can decrease to just 1-2% of basal metabolism during euthermia) compared with values found in I. tridecemlineatus during torpor (Barnes 1989; Buck and Barnes 2000). The expression of hsp40, hsp60, and hsp70 could be lower due to the greater degree of hypometabolism in *S. parryii* that may cause a greater reduction in gene expression compared to I. tridecemlineatus.

Unlike ground squirrels or bats, which inhabit cold regions, there are hibernators that enter torpor-arousal cycles in more temperate surroundings to cope with similar stressors (lack of food, dropping ambient temperature) (Schmid 2001). The grey mouse lemur (*Microcebus murinus*) is one such example, and it is a new model in the study of hibernation and primate adaptation to environmental stress. A recent study found that protein levels of Hsp70 and Hsp90α were upregulated in BAT, and Hsp60 was elevated in the liver during torpor (Table 5.1) (Wu et al. 2015). It is interesting that the expression of Hsp70 and Hsp90α were both increased during torpor in BAT, which is in contrast with the results for *I. tridecemlineatus* BAT (Carey et al. 1999). This indicates that there is considerable variability between studies that have examined HSP involvement in mammalian hibernation, although overexpression of HSP during torpor occurs in most cases. A large part of the variability could be attributed to the complexity of mammalian hibernation, as it involves not only metabolic rate depression but also major changes in body temperature that can range from as little as about 7°C reduction for *M. murinus* to over 30-35°C for ground squirrels (Biggar et al. 2015). The increased expression of Hsp70 and Hsp90α in *M. murinus* BAT during torpor could be due to the higher Tb during torpor since BAT would likely be more active during torpor in lemurs compared with ground squirrels. Various stresses are induced during hibernation including oxidative stress, which is known to induce protein misfolding, therefore HSP are needed to refold proteins and prevent protein aggregation (Swindell et al. 2007; Grune et al. 2011). In addition, Hsp90, Hsp70, Hsp27, and possibly other

HSP could play a large role in the inhibition of apoptosis during hibernation (Rouble et al. 2013; Logan et al. 2016a, b). What could be causing a downregulation in HSP expression in specific tissues of various hibernators is the dramatic decrease in body temperature, which may indicate that the constitutive complement of HSP is sufficient to deal with challenges to the proteome at low temperature. Therefore, future research needs to be conducted to determine the requirements for protein folding and repair following large changes in temperature, and the effects of long term Tb reduction on protein stability and chaperone function. Also, some mammals, like lemurs, are able to undergo both daily torpor and long-term/multi-day hibernation that takes place over several days (Schmid and Ganzhorn 2009). In the study by Wu et al. (2015), HSP levels were only evaluated during daily torpor and not over the long-term. As a result, the changes in HSP levels or lack thereof are particularly indicative of the effects of multi-day hibernation on HSP expression.

## 5.4 HSP and Anoxia

Oxidative phosphorylation is the primary method by which most organisms produce ATP due to the higher ATP yield of this pathway in comparison with anaerobic ATP production. This is why anoxia presents a severe challenge to most organisms due to decreased ATP yields from glucose/glycogen breakdown, the inability to use lipids as fuels, and the acidosis associated with high lactate accumulation. Certain animals that are normally aerobic can tolerate extended periods of anoxia, often by undergoing metabolic rate depression to reduce their ATP demand to match the reduced rate of ATP production. This means that many energy-expensive biosynthetic processes like transcription and translation are suppressed during anoxia. Therefore, to maintain cellular function and ensure survival, the "lifespan" of existing proteins needs to be extended. Hence, HSPs have been a relevant subject of study with several species that display well-developed anoxia tolerance. As shown in Table 5.2, the most commonly studied vertebrate facultative anaerobes are anoxia tolerant turtles, whereas a wide range of invertebrates (particularly many intertidal marine species) also have strong anoxia tolerance.

One example of an invertebrate facultative anaerobe is the Antarctic midge, *Belgica antarctica*, which is subjected to anoxia in its natural environment (Hermes-Lima and Zenteno-Savín 2002). Following 2 days of anoxia exposure, it was observed that sHSP and Hsp70 transcripts were both downregulated in the larvae, but were upregulated in adults. Hsp90 transcript levels were also reduced in larvae exposed to anoxia. It is believed that *B. antarctica* larvae encounter anoxia much more frequently than adults, therefore they may maintain constitutive expression of HSP at early stages in life (Lopez-Martinez et al. 2008). Two other invertebrates that have been studied in our laboratory are the goldenrod gall moth, *Epiblema scudderiana*, and the goldenrod gall fly, *Eurosta solidaginis*. The larvae of both insects are cold hardy: *E. solidaginis* is freeze tolerant, meaning that it can tolerate ice formation within its body, whereas *E. scudderiana* is freeze avoiding, meaning

**Table 5.2** Heat shock protein expression during anoxia stress

| Reference | Heat shock proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Chang et al. 2000) | Hsp60, Hsp70 family members (Hsp72/73) | *Chrysemys picta bellii, Trionys spinifer* | Anoxia (12h) | Cardiac muscle: Hsp72/73 ↑ in *C. p. bellii* Hsp72/73 ↓ in *T. spinifer* | Immunoblotting (protein) |
| (Prentice et al. 2004) | Hsp70 family members (Hsp72/73) | *Trachemys scripta* | Anoxia (4, 8, 12 h) | Brain: Hsp72 ↑ after 4–8 h, then ↓ to baseline by 12 h<br>Hsp73 ↑ from 4-12 h | Immunoblotting (protein) |
| (Ramaglia 2004) | Hsp70 family members (Hsp72/73), Hsp90 | *Chrysemys picta bellii* | Anoxia (2, 6, 12, 18, 24, and 30 h of forced diving) | Liver: Hsp72 ↓ at 12-24 h, ↑ at 30 h<br>Hsp73 ↑ at 30 h, Hsp90 ↑ at 18-30 h<br>Skeletal muscle: Hsp72 ↑ at 24-30 h<br>Hsp90 ↑ at 24-30 h<br>Heart: Hsp72 ↑ at 24–30 h<br>Brain: Hsp72 ↑ at 30 h<br>Hsp73 ↑ at 30 h, Hsp90 ↑ at 18-30 h | Immunoblotting (protein) |
| (Storey 2007) | Hsp70 (Hsp72), Hsp40, αB-crystallin | *Trachemys scripta elegans* | Anoxia (20 h) | Brain: Hsp70, Hsp40, αB-crystallin ↑ | Nylon microarray (mRNA) |
| (Kesaraju et al. 2009) | Hsp72, Hsp60, Hsp27 | *Trachemys scripta* | Anoxia (1, 4, 24 h) | Brain: Hsp72, Hsp27 ↑ at 1, 4, 24 h<br>Hsp60 ↑ at 1, 24 h | Immunoblotting (protein) |
| (Krivoruchko and Storey 2010) | Hsp25, Hsp40, Hsp60, Hsp90, Hsp70 family (Hsc70, Hsp70) | *Trachemys scripta elegans* | Anoxia (20 h) | Skeletal muscle: Hsp25, Hsp40, Hsp70, Hsc70, Hsp90 ↑<br>Liver: Hsp40, Hsp60, Hsp70, Hsc70 ↑<br>Kidney: Hsp40, Hsc70, Hsp90 ↑ | Immunoblotting (protein) |
| (Lopez-Martinez et al. 2008) | Hsp90, Hsp70, small Hsp | *Belgica Antarctica* | Anoxia (2 d) | Larvae: Hsp90, Hsp70, sHsp ↓<br>Adult: Hsp70, sHsp ↑ | Northern blot (mRNA) |
| (Zhang et al. 2011) | Hsp110, Hsp70, Hsp60, Hsp40, αA-crystallin, αB-crystallin | *Eurosta solidaginis* | Anoxia (4, 24 h) | September-collected last instar larvae: Hsp110, Hsp60 ↓<br>αA-crystallin, αB-crystallin, Hsp70 ↑ | Immunoblotting (protein) |
| (Zhang et al. 2017) | Hsp110, Hsp70, Hsp60, Hsp40 | *Epiblema scudderiana* | Anoxia (1, 4, 24 h) | September-collected last instar larvae: Hsp110 and Hsp60 ↓ | Immunoblotting (protein) |

that it supercools its body fluids to avoid freezing. These strategies are essential due to the cold climates that these insects inhabit, and *E. solidaginis* needs to tolerate anoxia as well during winter months. Following 24 h of anoxia exposure to the freeze tolerant *E. solidaginis*, Hsp70, as well as αA- and αB-crystallin proteins were overexpressed. Hsp110 and Hsp60 levels on the other hand, decreased by 58 and 62% during anoxia, respectively (Zhang et al. 2011). A similar study was conducted to assess HSP involvement during anoxia exposure in the freeze avoiding *E. scudderiana*. It was found that after 24 h of anoxia, Hsp110 and Hsp60 both decreased by 46 and 75%, respectively (Zhang et al. 2017). Therefore, in both cold hardy insects, the expression of Hsp110 and Hsp60 were decreased (Table 5.2), possibly indicating that at very low temperatures misfolded proteins do not accumulate abundantly in the mitochondrion, where Hsp60 is localized. Since both insects are more accustomed to dealing with cold stress than anoxia in isolation, it could be that chaperone recruitment is not as pronounced in response to anoxia alone. The interaction between temperature and oxygen deprivation will be discussed in a later section.

The best studied facultative anaerobes among vertebrate species are freshwater turtles such as the red-eared slider (*Trachemys scripta elegans*) and the painted turtle (*Chrysemys picta*). These turtles use extended periods of breath-hold diving to hunt for prey or evade predators, and in the winter months they submerge themselves for weeks or months at a time with little or no opportunity to breathe air or to take up oxygen by extrapulmonary mechanisms (Ultsch 1989). Instead, they have developed extreme anoxia tolerance, that is supported by huge reserves of anaerobic fuel (liver glycogen), a high capacity to buffer lactic acid or move lactate into their shells, and strong metabolic rate depression to just 10-20% of aerobic metabolic rates at the same temperature (Jackson 1968, 2002; Herbert and Jackson 1985). In this hypometabolic state, both the synthesis and degradation of proteins were reduced by more than 90% when turtle hepatocytes were incubated under anoxic conditions (Hochachka et al. 1996). The involvement of chaperones in maintaining/protecting the cellular proteome has been indicated as another crucial adaptation for long term anaerobiosis. For example, in the brains of *T. s. elegans*, Hsc73 protein levels progressively increased by 4 and 12 h of anoxia exposure as compared to normoxic controls. Hsp72 levels were also elevated at 4 h (2.64-fold) and 8 h (2.58-fold) of anoxia exposure, but then returned to normoxic levels at 12 h of anoxia (Prentice et al. 2004). In the brains of unstressed mice, Hsp72 is not detectable, so the induction of Hsp72 in anoxic turtle brains suggests that it has a key role to play in the protection of brain cells against oxygen deprivation. Another study of *T. s. elegans* brains showed that Hsp72 protein expression could be induced (by 2.07-fold) in the first hour of anoxia, and remained elevated at 24 h. This study also showed that Hsp60 and Hsp27 expression increased between 1 h and 24 h (Kesaraju et al. 2014). These studies on *T. s. elegans* suggest that the Hsp70 family plays an important role in the preservation and protection of turtle brains against anoxia. Interestingly, Hsp70 has been shown to have neuroprotective roles by reducing the

formation of pathological protein aggregates in various neurodegenerative diseases (Klucken et al. 2004; Brehme et al. 2014; Chen et al. 2016). A study on the red-eared slider in our laboratory initially identified the hsp70, hsp40, and αB-crystallin genes as putatively upregulated during anoxia through a cDNA microarray screen (Storey 2007). A follow-up study analyzed the protein expression of Hsp25, Hsp40, Hsp60, Hsp90, and two members of the Hsp70 family (Hsc70, Hsp70). Results for skeletal muscle showed that the expression of all HSPs analyzed except Hsp60 increased following 20 h of anoxia exposure. In the liver, Hsp40, Hsp60, Hsc70, and Hsp70 were all overexpressed under anoxia, and Hsp40, Hsc70, and Hsp90 levels were increased in the kidney during anoxia (Table 5.2) (Krivoruchko and Storey 2010). The same study also showed that most HSPs returned to baseline levels after 5 h of aerobic recovery in all organs, thus suggesting that the regulation of HSP expression is dynamic as the turtle transitions in or out of anoxic conditions. These findings were also supported by the activation the heat shock transcription factor (HSF1) under anoxic conditions: the amount of active HSF1 increased by about five-fold in heart and skeletal muscle along with strong increases in the nuclear localization of HSF1 in all four tissues examined (Krivoruchko and Storey 2010).

In another anoxia-tolerant turtle, *C. p. belli*, HSP expression was analyzed and compared to that of softshell turtles during anoxia and normoxia, as well as to responses by normoxic rats and rabbits. Even during normoxia, the painted turtle showed the highest myocardial Hsp60 expression of the four species, followed by softshell turtles, then rabbits and rats (Chang et al. 2000). Hsp60 is primarily found in the mitochondria and these findings suggest that more stress-tolerant organisms like *C. p. belli* have a greater intrinsic level of protection against protein misfolding that could be activated whenever stress is induced. The involvement of HSPs in anoxia tolerance was also supported by the finding that painted turtle myocardial Hsp72/73 protein levels were elevated after 12 h of anoxia, whereas Hsp72/73 levels decreased in softshell turtles (Chang et al. 2000). In another study, the time course of HSP expression in painted turtles was assessed by examining expression following various durations of diving (Table 5.2). The expression of all HSP proteins examined remained unchanged for at least the first 12 h of diving. Then, from 18 to 30 h, Hsp72 levels increased in all organs examined (liver, heart, brain, muscle), Hsp73 levels increased at 30 h in the brain and liver, and Hsp90 increased after 18-30 h of diving in the liver, skeletal muscle, and brain (Ramaglia 2004). The findings from this study are fascinating because 1) they illustrate that the chaperone response to anoxia exposure becomes elevated over time, and 2) there are tissue-specific responses in HSP expression. These patterns fit with a biphasic pattern of turtle response to anoxia that includes an initial transition into hypoxia where metabolic rate is maintained until blood oxygen levels fall below a critical pO2. From this point onwards turtles invoke hypometabolism to save energy and also initiate survival mechanisms including HSP overexpression.

## 5.5 HSP and Cold Tolerance

Many invertebrates are able to enter a period of suspended development and hypometabolism called diapause. Insects may enter this state for different reasons, one of which is exposure to environmental changes that present a challenge to the organism, such as extreme temperatures, or food and water shortages (Heldmaier et al. 2004; Dong et al. 2014). The goldenrod gall fly, discussed earlier, enters diapause during winter months. This insect, and many others such as the goldenrod gall moth, the Antarctic midge, and the Harlequin ladybird (*Harmonia axyridis*) are all able to withstand subzero temperatures during the winter through cold-hardiness and overwintering strategies (Sugg et al. 1983; Storey 1997; Wang et al. 2009). These insects have different overwintering measures that mainly revolve around behavioral or physiological changes during winter. They may move to a warmer, more sheltered location during the winter to reduce the chances of damage. More commonly used are physiological changes to the body's metabolism and the accumulation of cryoprotective molecules like trehalose, glycerol, sorbitol and other polyhydric alcohols that assist in the supercooling of body fluids (Watanabe 2002; Gagnon et al. 2013). In addition, some species of insects are freeze-tolerant and endure the accumulation of ice in extracellular spaces, which is mediated by the action of ice-nucleating proteins and modulated by the accumulation of the above-mentioned cryoprotectants (Storey 1997).

Due to the extreme variations in winter temperatures for different species of invertebrates (often from 0 to -50°C), well-developed chaperone responses may aid them in stabilizing protein conformation over wide ranges in temperature. Studies of the onion maggot, *Delia antiqua*, examined hsp70 gene expression in non-diapause (ND), summer diapause (SD), and winter diapause (WD) pupae subjected to cold-stress (10 °C or 15 °C). Hsp70 expression was shown to increase slightly in SD but rose by 3- and 2-fold for WD and ND insects, respectively (Chen et al. 2006) (Table 5.3). It appears that not only is Hsp70 involved in *D. antiqua* cold tolerance, there may also be an interaction between cold-stress and seasonal/environmental effects so that Hsp70 is more substantially upregulated by cold stress when environmental temperatures are lower. Moreover, non-hardy *D. antiqua* pupae showed actin depolymerization resulting in membrane damage after cold exposure, whereas cold-hardy (cold-acclimated and diapause) pupae did not. It is believed that this lack of actin depolymerization in cold-hardy pupae was due to elevated CCT expression (Kayukawa and Ishikawa 2009). Recall that CCT is a type II (cytoplasmic) Hsp60 family member, and actin as well as tubulin cytoskeletal proteins are substrates of CCT (Houry et al. 1999). This is one example showing that chaperones can play a variety of roles in stress response aside from folding damaged proteins.

The ladybird *H. axyridis* is another cold-hardy insect that has overwintering as well as summer populations, the overwintering population having much greater cold tolerance (Zhao et al. 2010). Specifically, overwintering beetles had much lower supercooling and freezing points than the summer population (Wang et al. 2017). Six sHSP were cloned from *H. axyridis* and their expression during

cold stress, low temperature storage, and development were assessed. Hsp36.77, Hsp16.25, and Hsp21.00 showed the lowest transcript levels at -5°C, and Hsp21.62 increased significantly by approximately 15-fold in comparison with the 25°C control (Table 5.3). These results may suggest that Hsp21.62 is one sHSP that is involved in cold tolerance in *H. axyridis*. Melanic (black) and non-melanic (yellow) forms of ladybirds were both studied to analyze possible color-specific mechanisms of cold-hardiness involving sHSP. In the overwintering black population, Hsp36.77 was highly expressed at day 10 of 5°C storage and Hsp16.25 and Hsp10.87 were highly expressed at day 5, whereas Hsp21.00 increased at day 20. In the overwintering yellow population on the other hand, Hsp36.77 was also highly expressed at day 10, but Hsp16.25 showed a delayed increase at day 15 and 20, and Hsp21.62 expression was elevated at day 15. Therefore, it appears that there is color-specificity in the expression of sHSP during the development of cold tolerance. In general, it appears that there is delayed sHSP expression in the yellow population compared to the black population, possibly suggesting that the yellow population can endure cold-stress for a longer duration before activating a chaperone response. Changes in the allele frequency in different populations of *H. axyridis* indeed suggest that melanism could be related to temperature changes and may confer protection during the winter (Michie et al. 2010).

As reviewed in the previous section, the Antarctic midge, *B. antarctica*, demonstrates anoxia tolerance. In addition, due to the frigid environment that it inhabits, *B. antarctica* is also tolerant of freezing as it spends much of its lifetime as a larva encased in ice (Sugg et al. 1983). In addition, it is known that these midges are susceptible to oxidative stress due to UV irradiation (Weatherhead and Andersen 2006). In *B. antarctica* larvae, it was found that the transcript levels of sHsp, Hsp70, and Hsp90 all decreased following 2 days of freezing exposure at -5°C (Table 5.3). In addition, transcript levels of the antioxidant enzymes, superoxide dismutase (SOD) and catalase, also decreased following freezing (Lopez-Martinez et al. 2008). These results are somewhat surprising considering that midges are susceptible to oxidative stress, which can lead to protein misfolding. However, these results are supported by a similar study on *B. antarctica* larvae that showed no changes in the transcript levels of the same three HSPs following 3 days of freezing treatment at -5°C instead of 2 days (Rinehart et al. 2006). Since HSP transcript levels were reduced or remained constant during freezing, it could be that there is minimal oxidative stress induced during freezing, since the UV irradiation in its environment is not replicated experimentally. Also, *B. antarctica* spends most of its life frozen, where sHsp, Hsp70, and Hsp90 all show constitutively strong expression throughout larval life (Rinehart et al. 2006). Most animals discussed so far elevate their chaperone expression as a stress response during hypometabolism. However, the high constitutive expression of defenses observed in *B. antarctica* may suggest that these larvae are continuously prepared to deal with environmental stresses when needed. Therefore, the chaperone response in the midge may be activated in preparation for freezing in combination with UV irradiation, but the response may actually diminish during freezing if there is no protein misfolding induced by oxidative stress.

**Table 5.3** Heat shock protein expression during cold stress

| Reference | Heat shock proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Chen et al. 2006) | Hsp70 | *Delia antiqua* | Cold [−10°C for winter diapause (WD) and non-diapause (ND), −15°C for summer diapause (SD)] | WD: Hsp70 ↑<br>ND: Hsp70 ↑<br>SD: Hsp70 ↑ | Q-PCR (mRNA) |
| (Lopez-Martinez et al. 2008) | Hsp90, Hsp70, small Hsp | *Belgica antarctica* | Cold (4°C to −5°C for 2 d) | Larvae: Hsp90, Hsp70, sHsp ↓ | Northern blot (mRNA) |
| (Zhang et al. 2011) | Hsp110, Hsp70, Hsp60, Hsp40, αA-crystallin, αB-crystallin | *Eurosta solidaginis* | Cold (late autumn and winter compared to September), (−16°C for 1 d) | Larvae: Hsp110, Hsp70, Hsp40, αB-crystallin ↑ and Hsp60 ↓ over late autumn and winter months compared to September<br>Hsp70 and Hsp40 ↑ at -16°C | Immunoblotting (protein) |
| (Wang et al. 2017) | Small Hsps (*Hsp36.77, Hsp16.25, Hsp21.00, Hsp21.62, Hsp10.87, Hsp21.56*) | *Harmonia axyridis* | Cold<br>(2 h cooling from 25°C to −5°C) | *Hsp36.77*: ↓ relative to 0°C<br>*Hsp16.25*: ↓ relative to 10°C<br>*Hsp21.00*: ↓ relative to 15°C<br>*Hsp21.62*: ↑ relative to 25°C, 15°C, 10°C, 5°C, 0°C<br>*Hsp21.56*: ↑ relative to 25°C, 5°C | Q-PCR (mRNA) |
| (Zhang et al. 2017) | Hsp110, Hsp70, Hsp60, Hsp40 | *Epiblema scudderiana* | Cold (late autumn and winter compared to September), (−4°C 1 d or 7 d, −20°C 7 d) | Larvae: Hsp110, Hsp70, Hsp60, and Hsp40 ↑ between October and March compared to September.<br>Hsp110, Hsp70 ↑ at −4°C, -20°C<br>Hsp60 ↓ at −20°C | Immunoblotting (protein) |

The two other anoxia tolerant and cold-hardy insects previously discussed were *E. solidaginis* and *E. scudderiana*. Last instar larvae of both species were collected in September. Some were held outdoors over the winter season and sampled every month from September to April, whereas others were acclimated in the lab to 15°C followed by experimental exposures to 3°C and then -16°C (Zhang et al. 2011). In freeze tolerant *E. solidaginis* larvae, Hsp70 and Hsp40 levels increased by 1.43- and 1.5-fold following freezing at 16°C for 24 h (Table 5.3). In addition, αB-crystallin, Hsp40, Hsp70, and Hsp110 protein expression all rose at various times by as much as 2-fold between October to April as compared with September values. However, Hsp60 protein levels decreased by ~50% in the months of December, February, and March (Zhang et al. 2011). Since Hsp60 is a predominantly mitochondrial HSP, this result correlates with the reduced activities of mitochondrial enzymes and mitochondrial DNA content also found in this species, indicating that substantial mitochondrial degradation occurs over the winter (Joanisse and Storey 1994; Levin et al. 2003; McMullen and Storey 2008). On the other hand, the freeze avoiding *E. scudderiana* larvae did not exhibit the same level of mitochondrial degradation during the winter. This correlates with the data for *E. scudderiana* HSP expression, which showed that the protein levels of Hsp60 were upregulated during the winter to the same extent as Hsp110, Hsp70 and Hsp40 (all rose by 2- to 3-fold from October to April). Following experimental exposures in the lab to -4°C for 1 week, Hsp110 and Hsp70 both increased relative to the 15°C control. When temperature was decreased further to -20°C for 1 week, Hsp110 and Hsp70 levels increased even further. However, Hsp60 expression decreased by approximately 50% when exposed to -20°C for 1 week (Zhang et al. 2017), similar to what was observed in *E. solidaginis.*

Increased HSP levels in various invertebrates during cold stress and/or the winter could contribute substantially to insect survival in the cold. For insects that freeze during the winter, extracellular ice formation also leads to anoxic conditions. As discussed in the previous section, chaperone action contributes to protein preservation during hypometabolism induced by the restriction of oxidative ATP production. When the two conditions are combined, there is potentially higher expression of HSPs that are involved in both processes. For instance, in the freeze-tolerant *E. solidaginis*, Hsp70 levels were upregulated in response to both anoxia and freezing, suggesting that HSPs contribute to protection against these stresses individually or in combination (Zhang et al. 2011). On the other hand, the freeze avoiding *E. scudderiana* may not be as well-adapted to dealing with anoxia as *E. solidaginis*, so this could be why Hsp70 remained unchanged during anoxia exposure (Zhang et al. 2017). In the study of stress adaptation, most research has been limited to analysis of individual stresses to isolate and identify stress-resistance traits. However, in nature, organisms may rarely encounter purely individual stresses and may often need to deal with more than one stressor at a time. In fact, transcriptomic studies have shown that when comparing dual stresses with single stresses, 61% of genes induced during dual stress were not induced by either of the singular stresses (Rasmussen et al. 2013). These studies of chaperone responses in the gall fly and gall moth highlight the need to study stress response mechanisms in animals that demonstrate natural stress tolerance.

## 5.6 HSP in Estivation and Dehydration

Estivation and dehydration tolerance/anhydrobiosis/cryptobiosis are mechanisms of stress-tolerance that are often induced by arid and/or hot environmental conditions. Studies of these strategies have centered around frogs/toads, snails, various species of insects, brine shrimp, and tardigrades (Storey and Storey 2010a). Although dehydration is a common feature shared by all these animals, very different levels of dehydration can accrue and different mechanisms of stress adaptation are activated. For example, during anhydrobiosis, otherwise known as cryptobiosis, organisms undergo an extreme level of metabolic rate depression where there is almost no metabolic activity detectable because there is no free water available (Clegg 2002). On the other hand, the African clawed frog (*Xenopus laevis*) can tolerate a loss of only about 30% of total body water, but studies indicate that there is little to no decrease in its metabolic rate during dehydration. It should be also noted that, many different physiological variables (i.e. heart rate, hematocrit content), and cellular signaling pathways (mitogen-activated protein kinases and antioxidant enzymes) are increased during dehydration in this animal (Hillman 1978; Malik and Storey 2009a, b, 2011).

In *X. laevis*, several HSP (Hsp90, Hsp70, Hsc70, Hsp60, Hsp40, and Hsp27) were studied under medium (16%) and high (30%) dehydration conditions in tissues (Luu et al. 2017). Findings showed that HSP expression in the clawed frog varied based on the tissue and the severity of dehydration. For example, liver Hsp40 levels increased progressively in response to 16% and 30% dehydration, whereas both Hsp70 proteins were upregulated only during 30% dehydration. In the kidney, Hsc70 levels also increased during both medium and high dehydration, and Hsp70 and Hsp27 levels increased during medium and high dehydration, respectively. In addition, both Hsp27 and Hsp70 were upregulated during medium dehydration in lungs (Table 5.4). Between the other two tissues evaluated (skeletal muscle, testes), HSP expression was also highly tissue-specific (Luu et al. 2017). It appeared that Hsp70, Hsc70, and Hsp27 were overexpressed in tissues that are more susceptible to dehydration stress, such as the kidneys that are the primary osmoregulatory organ and the lungs that are in direct contact with the air. Therefore, it is believed that these two HSP play particularly crucial roles in *X. laevis* osmoregulation during dehydration. For example, it has been shown that Hsp70 and Hsc70 play important roles in the expression and localization of sodium channels, where overexpression of Hsc70 decreases the expression and function of sodium channels unless accompanied by moderate Hsp70 expression (Goldfarb et al. 2006). In addition, it was suggested that increased Hsc70 levels in the kidney could contribute to osmoregulation by promoting the function of aquaporin-2, which is a water channel in the collecting ducts of the kidney that aids in the reabsorption of water that is filtered by the kidney (Brown 2003; Lu et al. 2007). Furthermore, during acute kidney injury and acute lung injury, phosphorylated Hsp27 was found to be highly expressed, and it is believed to contribute to injury through F-actin polymerization, resulting in the impairment of pulmonary endothelial function (Ma et al. 2015).

**Table 5.4** Heat shock protein expression during dehydration stress

| Reference | Heat shock proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Ramnanan et al. 2009) | Hsp10, Hsp40, Hsp60, Hsp90, Hsp110 | *Otala lactea* | Estivation for 14 d | Hepatopancreas:<br>Hsp10, Hsp60, Hsp90, and Hsp110 ↑ Hsp40 ↓ | Immunoblotting (Protein) |
| (Mizrahi et al. 2010) | Hsp90, Hsp70 family (Hsp72, Hsp74), Hsp25 family (Hsp15, Hsp17, Hsp25, Hsp30) | *Sphincterochila zonata* (desiccation-resistant species) | Desiccation (1, 3, 7, 10 d) | Foot muscle: Hsp90, Hsp74, Hsp72, Hsp25 ↑ at 10 d, Hsp72 ↑ at 7 d, Hsp25 ↑ at 3 d, Hsp90 ↓ 2 d<br>Hepatopancreas: Hsp90, Hsp30, Hsp17 ↑ at 7, 10 d<br>Kidney: Hsp30 ↓ at 7, 10 d, Hsp25 ↓ at 3 d | Immunoblotting (Protein) |
| (Mizrahi et al. 2010) | Hsp90, Hsp70 family (Hsp72, Hsp74), Hsp25 family (Hsp15, Hsp17, Hsp25, Hsp30) | *Sphincterochila cariosa* (desiccation-sensitive species) | Desiccation (1, 3, 7, 10 d) | Foot muscle: Hsp90 ↑ at 1, 2, 3, 10 d, Hsp74 ↑ from 1-7 d, Hsp72 ↑ at 3, 10 d, Hsp25 ↑ at 3 d<br>Hepatopancreas: Hsp90 ↑ at 7, 10 d, Hsp72 ↑ at 7 d, Hsp30 ↑ at 1, 7, 10 d, Hsp17 ↑ at 7, 10 d<br>Kidney: Hsp90 ↑ at 7d, Hsp30 ↓ at 3 d | Immunoblotting (Protein) |
| (Clark et al. 2009) | Hsp70, small Hsp | *Megaphorura arctica* | Dehydration (salt dehydrated to 0.9 or 0.2 g/g dry weight, cold dehydrated to −2°C, −7°C) | 0.9 salt: sHSP, HSP70 ↓<br>0.2 salt: sHSP ↓, HSP70 ↑<br>−2°C: sHSP, HSP70 ↑<br>−7°C: sHSP ↑, HSP70 ↓ | cDNA microarray (mRNA), Q-PCR (mRNA) |
| (Reuner et al. 2010) | Hsp90, Hsp70 isoforms (*hsp70-1, hsp70-2, hsp70-3*), Hsp60, Hsp10, α-crystallin family (*hsp17.2, hsp19.5*) | *Milnesium tardigradium* | Dehydration (6 h), Cryptobiosis (14 d) | Dehydration: *hsp19.5*, *hsp70-1* ↑ *hsp70-3* ↓<br>Cryptobiosis: Hsp90 ↑ | Q-PCR (mRNA) |

(continued)

**Table 5.4** (continued)

| Reference | Heat shock proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Gusev et al. 2011) | Hsp90, Hsp70 family (*hsp70, hsc70*), Hsp60, α-crystallin family (*hsp20, p23*) | *Polypedilum vanderplanki* | Dehydration (8, 16, 24, 48 h) | Larvae Hsp90: ↑ from 8–48 h<br>Larvae Hsc70: ↑ from 8–48 h<br>Larvae Hsp70: ↑ from 8–48 h<br>Larvae Hsp60: ↑ from 8–48 h<br>Larvae Hsp20: ↑ from 8–48 h<br>Larvae p23: ↑ at 48 h | Q-PCR (mRNA) |
| (Giraud-Billoud et al. 2013) | Hsp90, Hsp70 family (Hsp70, Hsc70) | *Pomacea canaliculata* | Estivation for 45 d, Arousal for 20 min, 24 h | Kidney: Hsp90 ↓ from control to estivation, Hsp90 ↑ from estivation to 20 min arousal<br>Foot: Hsp70 ↓ from estivation to 20 min and 24 h arousal, Hsp90 ↓ from estivation to 20 min and 24 h arousal | Immunoblotting (protein) |
| (Luu et al. 2017) | Hsp90, Hsp70 family (Hsp70, Hsc70), Hsp60, Hsp40, Hsp27 | *Xenopus laevis* | Dehydration (16%, 30% relative to control) | Liver: Hsp40 ↑ at 16%, 30%<br>Hsp70, Hsc70 ↑ at 30%<br>Skeletal muscle: Hsp40 ↑ at 30%<br>Hsc70 ↓ at 16%, 30%<br>Hsp90 ↓ at 30%<br>Kidney: Hsp27 ↑ at 30%<br>Hsc70 ↑ at 16%, 30%<br>Hsp60, Hsp90 ↓ at 30%<br>Hsp70 ↑ at 16%, ↓ at 30%<br>Lung: Hsp27, Hsp70 ↑ at 30%<br>Hsp60 ↓ at 16%, 30%<br>Hsp90 ↓ at 30%<br>Testes: Hsp90 ↑ at 30%<br>Hsp40, Hsp70 ↑ at 16%, 30% | Immunoblotting (protein), Luminex multiplex assay (protein) |

Future studies should be conducted to characterize the levels of dephosphorylated and phosphorylated Hsp27 levels in the lungs and kidneys of dehydrated *X. laevis* since a decrease or lack of change in phosphorylated levels would indicate that Hsp27 plays a protective role in the lungs and kidneys during dehydration.

Dehydration is also tolerated by many insects and invertebrate microfauna through a hypometabolic process called cryptobiosis or anhydrobiosis (Goyal et al. 2005; Alpert 2006; Watanabe 2006). Whereas the physiology behind cryptobiosis is generally well-understood, some of the molecular mechanisms underlying this process are relatively unknown. A study of chaperone responses in the African chironomid (*Polypedilum vanderplanki*) characterized the mRNA expression of Hsp90, the Hsp70 family (Hsp70, Hsc70), Hsp60, and the α-crystallin family (Hsp20, p23) (Table 5.4). Following desiccation for 8, 16, 24, and 48 h, transcript levels of all HSPs analyzed were overexpressed at all time points (Gusev et al. 2011). This suggests that chaperones are closely linked to anhydrobiosis. A well-studied cryptobiotic organism is the moss-dwelling tardigrade (*Milnesium tardigradum*). Tardigrades are commonly studied because they are well-known for their ability to tolerate extreme conditions during cryptobiosis, such as low (-196°C) and high temperatures, ionizing radiation and high pressure (Ramløv and Westh 1992; Seki and Toyoshima 1998; Horikawa et al. 2006; Hengherr et al. 2009). During the process of anhydrobiosis, various HSP transcripts were found to be expressed in tardigrades including hsp90, 3 isoforms of hsp70 (hsp70-1, hsp70-2, hsp70-3), hsp60, hsp10, and 2 isoforms of α-crystallins (hsp17.2, hsp19.5) (Reuner et al. 2010). However, on the 6th day of the anhydrobiotic transition, the expression of hsp19.5, hsp70-1, and hsp70-3 were all downregulated. When the anhydrobiotic state was fully established on the 12th day, the expression of hsp90 was upregulated. These results suggest that despite the extreme level of dehydration and hypometabolism tolerated by tardigrades, there is still a need to address problems of protein misfolding and aggregation. Hsp90 transcript levels may have been upregulated as full anhydrobiosis was reached because Hsp90 is known to be involved in several different cellular functions beyond protein folding. The encysted embryos of the brine shrimp, *Artemia salina*, also exhibit anhydrobiosis (MacRae 2016). In fact, one of the first examples of shock protein involvement during hypometabolism was the discovery of p26 accumulation in *A. salina* cysts (Willsie and Clegg 2001). P26 is a sHSP that has been shown to play key roles in transcriptional repression during stress (Clegg 2007). In addition, two other sHSP have also been shown to enhance bring shrimp survival, and they are called ArHsp21 and ArHsp22. These HSPs are restricted to diapause-destined embryos and both play roles in preventing stress-induced aggregation or denaturation of proteins in vitro (Qiu and Macrae 2008a; Qiu and MacRae 2008b).

The Arctic springtail (*Megaphorura arctica*) is an insect that uses a mechanism known as cryptobiotic dehydration, whereby these insects dehydrate when exposed to cold temperatures to such an extent that no freezable water remains and they are in a state of anhydrobiosis (Holmstrup and Westh 1994; Worland 1996). A cDNA microarray was conducted on springtails dehydrated through exposure to cold temperatures (-2°C and -7°C for 7 days) or salt-dehydrated to dry weights of

0.9 gram/gram and 0.2 g/g water content/dry weight post-dehydration. Following -2°C dehydration for 7 days, sHSP and Hsp70 were both putatively upregulated, but only sHSP levels were elevated at -7 °C, whereas Hsp70 levels decreased. Salt dehydration produced the opposite results, where both HSPs were underexpressed following dehydration to 0.9 g/g, and only Hsp70 was upregulated when dehydrated to 0.2 g/g whereas sHSP declined (Clark et al. 2009). These findings suggest that the two methods of dehydration may activate different stress adaptive mechanisms, where the cryptobiotic dehydration in response to low temperatures involves chaperones but salt-induced dehydration does not seem to induce chaperone expression to the same extent. Specifically, Hsp70 appears to be involved in cryptobiotic dehydration but not salt-induced dehydration. This finding coincides with those in the cold-hardy gall moth and gall fly, where Hsp70 levels were elevated during low temperature exposure in both insects (Zhang et al. 2011, 2017). Therefore, there may be a link between dehydration and cold-tolerance that results in a cumulative effect to activate a chaperone response. However, validation of the cDNA microarray screening of *M. arctica* needs to be conducted to substantiate the results, and analysis of protein level responses needs to be conducted for all three invertebrates discussed above in order to have a better understanding of functional HSP proteins involved in cryptobiosis.

Another survival strategy under dry conditions is estivation, and the land snail (*O. lactea*) is one organism that is known to estivate by globally suppressing both the synthesis and degradation of proteins during this process (Ramnanan et al. 2009). Therefore, a mechanism must exist which allows this animal to preserve the function of existing proteins. Hence, we studied the involvement of HSPs in this process and found that Hsp10, Hsp60, Hsp90, and Hsp110 were all upregulated in the foot muscle following 14 days of estivation (Table 5.4). Hsp40, however decreased during estivation (Ramnanan et al. 2009). In an early study in *O. lactea*, estivation-responsive proteins were labeled with 35S, and this resulted in the identification of four main proteins with molecular weights of 30, 50, 70, and 91 kDa that were strongly labeled during estivation (Brooks and Storey 1995). Therefore, considering how abundant Hsp90 and Hsp70 are in eukaryotic cells, it is likely that these two HSPs correspond to the two 35S-labelled proteins identified at 91 and 70 kDa in *O. lactea*. Several HSPs were also studied in two other land snails, *Sphincterochila cariosa*, which is sensitive to desiccation, and *Sphincterochila zonata*, which is tolerant of desiccation (Mizrahi et al. 2010). It was notable that sHSPs like Hsp30, Hsp25, and Hsp17 showed higher protein expression in the foot muscle and hepatopancreas following 1, 3, 7, and 10 days of desiccation in the desiccation-resistant species. The expression of larger HSPs like Hsp90, Hsp74, and Hsp72 showed elevated expression only after 7 or 10 days of dehydration. However, in the desiccation-sensitive species, larger HSPs like Hsp90, Hsp74, and Hsp72 showed earlier and higher expression in the foot muscle, hepatopancreas, and kidney (Mizrahi et al. 2010). These results suggest that the desiccation-resistant species involves the primary, larger chaperones at higher levels of dehydration because they are more stress-tolerant. In another land snail, the apple snail (*Pomacea canaliculata*), levels of various HSPs (Hsp90, Hsp70, Hsc70) were analyzed, and it was found that

Hsp90 levels decreased after estivation for 45 days (Giraud-Billoud et al. 2013). In contrast to *O. lactea* and *S. zonata*, which showed elevated HSP expression following estivation, expression was unchanged or decreased in *P. canaliculata*. However, no sHSPs were studied in *P. canaliculata*, and the authors suggested that the expression of large HSPs in the kidneys may resemble those of *S. cariosa* (Giraud-Billoud et al. 2013). This explanation is plausible given that both snails are known to be less tolerant of dehydration because their habitats are located near water. Therefore, it appears that the involvement of HSPs during estivation depends on the snail's level of tolerance for dehydration, where snails with higher tolerance may show higher expression of HSP. Further evidence is provided from a study involving the Mediterranean grunt snail (*Cantareus apertus*), which is also less tolerant of dehydration than desert species (Shoshana and Heller 1989). After 6 months of estivation, *hsp70* mRNA and Hsp70 protein expression were analyzed, and no changes in either was observed (Reuner et al. 2008). It is clear that chaperone responses during dehydration stress are complex given the different environmental factors that contribute to dehydration (cold temperature, salt, etc.) and the different strategies that organisms employ to cope with dehydration (estivation, cryptobiosis, etc.). Furthermore, it seems that the chaperone response to dehydration stress may be developed over time in organisms that need to adapt and become tolerant to dry conditions in their environment.

## 5.7 Glucose Regulated Proteins and Stress

Although HSPs are the most commonly studied group of chaperones, there are many other chaperones that play similar integral roles in cellular function. The family of proteins called the glucose regulated proteins (GRP) is another type of stress-inducible chaperone, and many of its members (Grp78, Grp94, Grp75, and Grp170) belong to the HSP family (Lee 2014). GRPs were given their name due to the strong expression of these proteins in cells cultured in glucose-free medium (Shiu et al. 1977). They can be found predominantly in either the endoplasmic reticulum (ER) or the mitochondria, and their expression is generally induced by ER stress. In the ER, GRPs play a major role in the folding of nascent proteins or refolding of misfolded proteins. Misfolded proteins that cannot be repaired must be targeted for protein degradation through proteasomes located proximal to the ER (Vashist and Ng 2004). However, GRPs can be transported to other parts of the cell and assume other functions related to cellular proliferation, apoptosis, signaling, inflammation, and immunity (Gonzalez–Gronow et al. 2009; Ni et al. 2011; Gray and Vale 2012).

Grp78 is possibly the most commonly studied of the GRP. It is predominantly an ER chaperone that binds to the hydrophobic residues in misfolded/unfolded proteins (Hendershot et al. 1996). When the ER is under stress, Grp78 is released from its substrate and it is free to activate a variety of downstream factors to regulate gene expression and translation (Prostko et al. 1992). This cumulative effect is known as

the unfolded protein response (UPR), and it serves to restore cellular stability (Wu and Kaufman 2006; Ron and Walter 2007; Hetz 2012) (Fig. 5.1b). One particularly important function of Grp78 is its regulation of apoptosis as it inhibits various caspases (Luo et al. 2006; Minamino et al. 2010; Zhu et al. 2013), which would be crucial for cell survival under environmentally stressful conditions. Grp78 can also localize to different parts of the cell; for example, it is transported to the mitochondrion during ER stress, where it protects mitochondria from apoptosis through the inhibition of caspases 3 and 7 (Wang et al. 2010; Wey et al. 2012). Grp78 can also regulate various signaling pathways such as the WNT-β-catenin proliferation pathway and the PI3K-Akt pathway (Verras et al. 2008; Liu et al. 2013; Tseng et al. 2013). Grp75 is a mitochondrial protein that acts as a channel to transport p53 from the mitochondrion to the cytosol (Mizukoshi et al. 1999). Furthermore, Grp94 and Grp170 are both regulators of ER calcium homeostasis (Reddy et al. 2006; Sanson et al. 2009).

As one could expect, the involvement of HSPs in environmental stress adaptation has been relatively well-studied in comparison with the involvement of GRPs. However, a variety of studies have been conducted on the role of GRPs in selected environmental stress response (hibernation, anoxia, cold tolerance). Among mammalian hibernators, the mRNA and protein expression of Grp75 and Grp78 were analyzed in thirteen-lined ground squirrels. In the intestines of torpid squirrels, there was approximately a 6-fold increase in Grp75 protein expression relative to active squirrels (Table 5.5). In the liver and muscle, Grp75 was also elevated (Carey et al. 1999). Grp78 showed a more tissue-specific response during torpor, where both *grp78* transcript and Grp78 protein levels were higher in BAT and brain during torpor. However, Grp78 protein levels were significantly decreased in the liver and heart of hibernating animals (Mamady and Storey 2006). It was suggested that perhaps Grp78 is more active in the brain and BAT because these tissues must be remain metabolically active during hibernation. The brain must regulate adjustments to Tb during cycles of torpor and arousal, and BAT is key for initiating thermogenesis during arousal. Hence, there would be a need for protein synthesis to remain relatively active in BAT and brain during hibernation, compared with other organs (Hittel and Storey 2002; Storey and Storey 2004). In addition, the heart rates of squirrels are strongly reduced from 350-400 beat per minute (bpm) during euthermia to only 5-10 bpm during torpor (Frerichs et al. 1994; Frerichs and Hallenbeck 1998), suggesting that this organ is less metabolically active during torpor. In *I. tridecemlineatus*, the regulation of GRP78 was also studied and the data suggested that it may be regulated by the protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathway. Under ER stress conditions, PERK phosphorylation of the ribosomal eukaryotic initiation factor α (eIF2α) has both a global inhibiting effect on protein translation as well as a specific effect because p-eIF2α stimulates activating transcription factor 4 (ATF4) and its cofactor, cAMP response element binding protein 1 (CREB1). ATF4 and CREB1 move to the nucleus to increase transcription of *grp78* and elevate GRP78 protein, thereby helping to relieve ER stress (Mamady and Storey 2008). In the hibernating bat (*Rhinolophus ferrumequinum*), results showed that Grp75, Grp78, and Grp94 protein levels were all lower before arousal

compared to during arousal (Lee et al. 2002), however it is difficult to make a comparison to the previous studies as it is not evident that the bats sampled before arousal were torpid.

The study of GRP responses in anoxia tolerant vertebrates has centered around red-eared slider turtles, with a particular focus on brain since GRPs have been shown to play neuroprotective roles against apoptosis and excitotoxicity via suppression of oxidative stress and the maintenance of calcium homeostasis by Grp94 and Grp170 (Hori et al. 1996; Yu et al. 1999). A cDNA array screen for anoxia-responsive genes in the brains of *T. s. elegans* showed that Grp75 was putatively upregulated (Storey 2007). Another study on Grp78 and Grp94 in T. s. elegans brains showed that the protein expression of Grp94 increased at 1, 4, and 24 h of anoxia exposure (Kesaraju et al. 2009) (Table 5.5). Both Grp78 and Grp94 are believed to protect cells from ER stress brought about by ischemia, which is often associated with anoxic conditions in mammalian studies (Kudo et al. 2008). However, only Grp94 was upregulated in *T. s. elegans*, possibly because Grp94 and Grp78 are regulated by different transcription factors and are highly expressed in different parts of the brain (Kim et al. 2003; Schröder and Kaufman 2005; Kudo et al. 2008). In addition, turtles undergoing anoxic submergence under water are not ischemic and, hence, the upregulation of Grp94 in turtles under anoxia may indicate a preferential function for Grp94 during anoxia not associated with ischemia.

GRP involvement in insect stress tolerance was also evaluated in *E. solidaginis* and *E. scudderiana* under anoxia or subzero temperatures as experimental conditions. When freeze-tolerant gall fly (*E. solidaginis*) larvae were exposed to 4 or 24 h of anoxia, Grp78 protein levels were elevated after 24 h of anoxia, and Grp75 levels increased after freezing at -16°C for 24 h (Zhang et al. 2011) (Table 5.5). When *E. solidaginis* and *E. scudderiana* were sampled from outdoor conditions over a time course from September to April, levels of Grp78 were consistently elevated from October to March as compared to September values (Zhang et al. 2011, 2017). In addition, Grp75 was also elevated in December. Therefore, both GRPs may play important roles in the winter survival of this insect. Hsp60 and Grp75 are both mitochondrial chaperones, but as discussed earlier, Hsp60 expression during both freezing and over the winter months was depressed in the freeze tolerant *E. solidaginis*. Therefore, Grp75 may be one of the primary mitochondrial chaperones utilized by *E. solidaginis* to endure subzero conditions during the winter. In addition, the high expression of Grp78 throughout winter months may be a response to ER stress induced by low temperatures and/or low oxygen availability. Grp78 is also able to localize to the mitochondrion and, therefore, it could also be part of the mitochondrial chaperone response, along with Grp75, to preserve stability and function of these organelles during freezing. In a similar study on freeze avoiding larvae of the gall moth *E. scudderiana*, Grp78 was overexpressed following 1 and 7 days of subzero temperature exposure at 4°C, and after 1 day at 20°C. In addition, Grp170 was also elevated by 1.8-fold at -20°C for 1 day (Zhang et al. 2017). Furthermore, Grp170 was elevated following 4 and 24 h of anoxia, but Grp78 was reduced after 1 h of anoxia. Over the late fall and winter months, Grp78 was elevated in December, and Grp94 and Grp170 were both

**Table 5.5** Glucose Regulated Protein expression during hibernation, anoxia, and cold stresses

| Reference | Glucose regulated proteins | Animal | Stress | Changes during stress | Method |
|---|---|---|---|---|---|
| (Carey et al. 1999) | GRP75 | *Ictidomys tridecemlineatus* | Hibernation (torpor relative to active) | Intestine: GRP75 ↑<br>Liver: GRP75 ↑<br>Muscle: GRP75 ↑ | Immunoblotting (Protein) |
| (Lee et al. 2002) | GRP75, GRP78, GRP94 | *Rhinolophus ferrumequinum* | Hibernation (torpor relative to arousal) | Brain: GRP75, GRP78, GRP94 ↓ | Immunoblotting (Protein) |
| (Mamady and Storey 2006) | GRP78 | *Ictidomys tridecemlineatus* | Hibernation (torpor relative to active) | Brain: GRP78 mRNA and protein ↑<br>Brown adipose tissue: GRP78 mRNA and protein ↑<br>Heart: GRP78 protein ↓<br>Liver: GRP78 protein ↓ | Semi-quantitative (RT-PCR), Immunoblotting (Protein) |
| (Storey 2007) | GRP75 | *Trachemys scripta elegans* | Anoxia (20 h) | Brain: GRP75 ↑ | Nylon microarray (mRNA) |
| (Kesaraju et al. 2009) | GRP78, GRP94 | *Trachemys scripta elegans* | Anoxia (1, 4, 24 h of anoxia) | Brain: GRP94 ↑ at 1, 4, 24 h | Immunoblotting (Protein) |
| (Zhang et al. 2011) | GRP75, GRP78 | *Eurosta solidaginis* | Anoxia (4, 24 h) | Larvae: GRP78 ↑ at 24 h | Immunoblotting (protein) |
| (Zhang et al. 2017) | GRP78, GRP94, GRP170 | *Epiblema scudderiana* | Anoxia (1, 4, 24 h) | September-collected larvae:<br>GRP78 ↓ at 1 h<br>GRP170 ↑ at 4, 24 h | Immunoblotting (protein) |
| (Richards et al. 2008) | GRP78 | *Osmerus mordax* | Cold (winter relative to fall) | Liver: GRP78 ↓ | Semi-quantitative (RT-PCR) |
| (Zhang et al. 2011) | GRP75, GRP78 | *Eurosta solidaginis* | Cold<br>(−16°C 1 d) | Larvae: GRP75 ↑ | Immunoblotting (protein) |
| (Zhang et al. 2017) | GRP78, GRP94, GRP170 | *Epiblema scudderiana* | Cold<br>(−4°C 1 d or<br>1 w, −20°C 1 w) | Larvae: GRP78 ↑ at −4°C 1 d and 1 w, −20°C<br>GRP170 ↑ at −20°C | Immunoblotting (protein) |

elevated from December to March (Zhang et al. 2017). This suggests that in both *E. scudderiana* and *E. solidaginis*, Grp78 may play a similar role as it was upregulated during the winter months in both species and was also responsive to anoxia and/or subzero temperature exposure in both. Overall, it is evident that the GRP response is activated in various organisms during hibernation, anoxia, and cold tolerance. Specifically, Grp78 appears to be consistently involved in stress adaptation, likely because protein folding and trafficking occurs to a large degree in the ER. As a result, the misfolding of proteins during ER stress caused by various factors leads to the activation of the UPR, thus requiring the involvement of Grp78.

## 5.8 Chaperones and Disease

Diseases like cancer are characterized by increased proteotoxic and oxidative stresses caused by factors such as dramatically increased cellular proliferation, cell metabolism, genetic lesions, acidosis, viral infection, as well as hypoxia and hypoglycemia (Ma and Hendershot 2004; Luo and Lee 2013; Trougakos et al. 2013). Therefore, high levels of chaperones, both HSPs and GRPs, are found in cancerous cells, and they contribute to tumor resistance against medical treatments because they play an important role in protecting the proteome of tumor cells (Trougakos et al. 2013). As we have shown through this review, environmental stress can induce a similar elevation of chaperones in stress-tolerant animals. Thus, by studying stress adaptation mechanisms initiated in stress-tolerant organisms as a response to oxidative, proteotoxic, and other combinations of stresses resembling those triggered in cancer cells, we can improve our understanding of the biological mechanisms that may be used by cancer cells. These studies could potentially identify novel biomarkers for tumor identification and cancer prognosis as well.

Although the relationships between the chaperone response and disease are most commonly studied in the context of cancer, there is also a link between chaperones and several neuromuscular and neurodegenerative diseases, including Duchenne muscular dystrophy (DMD) (Brinkmeier and Ohlendieck 2014). DMD is the most frequently inherited childhood neuromuscular disorder that results in the progressive wasting of muscle, and it is caused by a mutation in a gene coding for dystrophin (Muntoni et al. 2003). Protein level analysis suggested that increasing Hsp72 levels would be beneficial for treating DMD since an upregulation of muscular Hsp72 helps to restore the main SERCA pump of the sarcoplasmic reticulum. Since elevated intracellular calcium concentration in muscle is an initiator of degenerative pathways, rescue of the SERCA pump can restore cellular levels of calcium (Gehrig et al. 2012). In addition, a proteomics screen identified a decrease in Hsp27 in the muscles of a mouse model for DMD, so it was suspected that reduced Hsp27 levels may contribute to disease progression (Carberry et al. 2012). It is believed that since Hsp27 may have antioxidant and anti-apoptotic functions (Vidyasagar et al. 2012), its reduced concentrations in DMD muscles could contribute to lower resistance to oxidative stress and cell death (Carberry et al. 2012). As we have reviewed, studies

on the skeletal muscle of hibernating bats have shown that both Hsp70 and the active/phosphorylated form of Hsp27 increased during torpor (Eddy et al. 2005; Lee et al. 2008) (Table 5.1). Hibernators are excellent models for studying the mechanisms underlying resistance against disuse-induced muscle atrophy because many hibernators do not lose muscle mass despite being inactive for weeks at a time during hibernation (Gao et al. 2012; Xu et al. 2013; Cotton and Harlow 2015). In addition, ground squirrels are able to regulate their intracellular calcium concentrations to a greater extent than non-hibernating animals at the same temperature due to sharper and periodic influxes of intracellular calcium while maintaining the overall calcium concentration of the cell (Liu et al. 1990, 1993; Wang et al. 2000). The tight regulation of calcium homeostasis in hibernators could provide novel insights into the development of therapeutics that allow for correction of the calcium imbalances seen in DMD. The involvement of chaperones during hibernation and DMD seem to share similarities that suggest that studying the biochemistry and physiology of mammalian hibernators could provide clues to better understand and create therapeutics for DMD. Beyond DMD, altered HSP and GRP levels have been found in other neuromuscular diseases like dysferlinopathy, myofibrillar myopathies, spinal muscular atrophy (SMA), myasthenia gravis, X-linked muscular dystrophy, and many other neuromuscular diseases (De Palma et al. 2006; Lewis et al. 2009; Sela et al. 2011; Gomez et al. 2013; Mutsaers et al. 2013). Therefore, the chaperone response appears to play a large role in the pathogenesis of numerous neuromuscular diseases.

Anoxia-tolerant turtles are also used as models for the effects of oxygen deprivation on brain function and the adaptive strategies that are needed to mitigate the damaging consequences that anoxia/ischemia have for the brains of most mammals. Research has shown that increased levels of Hsp70 can provide greater tolerance to cerebral ischemia (Nishi et al. 1993; van der Weerd et al. 2005), and other chaperones like Hsp60, Hsp27, Grp94, and Grp78 have also been shown to be involved (Kato et al. 1994; Chen et al. 1996; Kirino 2002; Badin et al. 2006). In addition, cell death pathways also play a large role in cerebral ischemia and neurodegenerative diseases (Cao et al. 2001, 2002; Saito et al. 2005; Mehta et al. 2007). The turtle studies reviewed here show that Hsp70, Hsp27, Grp94, and numerous other chaperones are overexpressed in *T. s. elegans* and *C. p. belli* in response to anoxia (Table 5.2, 5). In addition, the anti-apoptotic protein Bcl-2 was also elevated during anoxia, suggesting that there is also a suppression of apoptosis in *T. s. elegans* (Kesaraju et al. 2009). Upregulation of the chaperone response in addition to anti-apoptosis suggest that anoxia-tolerant turtles may be excellent models for the continuing study of stroke and protection against neurodegenerative diseases.

Potentially, the most important applications that can be derived from studies of natural metabolic rate depression, cold hardiness and anoxia tolerance are to the fields of transplant medicine and tissue/organ cryopreservation and, indeed, various lessons learned from natural systems are finding application in these medical fields. There is currently a great shortage of organs for transplantation, and the World Health Organization estimated that only 10% of the global need for transplants are being met (Giwa et al. 2017). One of the main barriers limiting transplants is the

limited amount of time that viable organs can be preserved, given that they often need to be transported long distances. Although advancements have been made in machine perfusion, methods that improve organ hypothermic- or cryo-preservation, organ tolerance of ischemic injury, or that can induce metabolic rate depression could be extremely valuable for improving transplant medicine (Fahy et al. 2004; Storey and Storey 2004, 2007, 2010b; Wszola et al. 2013). For example, a real breakthrough in cryopreservation would allow for organ banking in a hypometabolic, suspended state at subzero temperatures – essentially freezing the organ while protecting it from ischemic injury. During ischemia and cold stresses, protein stability and function need to be maintained especially given that the organs are in hypometabolic states. Therefore, any method of organ preservation will likely involve chaperones. If this could be accomplished, organs could be stored for extended periods of time and, hence, the extraction, transportation, and transplantation of organs while running tests to ensure compatibility and acquiring consent would not need to take place under such tight time constraints. In addition, with the innovations in 3-D printing and xenotransplantation to create organs in the lab, organ banking would also make these approaches both practical and cost-effective since the organs could be better preserved for longer (Giwa et al. 2017). For organ banking to become possible, our knowledge of the fundamental biology behind freeze tolerance and freeze-avoidance, metabolic rate depression, ischemia/anoxia tolerance, and other methods of stress adaptation, must continue to be improved. Currently, methods to suppress the metabolism of organs through cold storage result in injury due to a lack of oxygen and macromolecules needed for organs to survive hypometabolism (Storey 2002). In this review, we have shown that activation of the chaperone response is essential for tissue and organ preservation during stress adaptation (Tables 5.1–5.5). Therefore, chaperones may be key factors for facilitating the preservation of organs not only through stabilizing protein folding, but also via other actions including anti-apoptosis actions and stabilization of the cytoskeleton. Hence, advancing our fundamental knowledge of natural stress adaptation and the chaperone response may aid in the discovery of novel cryoprotectants and help develop new methods of organ preservation.

## 5.9 Conclusions

This review summarizes literature on both HSPs and GRPs involved in stress adaptive processes including hibernation, anaerobiosis, cryptobiosis, estivation, and cold hardiness. Although these chaperones are best known for their actions in protein folding and preventing protein aggregation (thereby ensuring stability of the proteome), other functions have been identified. In response to environmental stress, the anti-apoptotic functions of chaperones could also be integral to the survival of these organisms. In addition to the upregulation of chaperones in response to stress, chaperones may also be overexpressed as a preparatory mechanism in anticipation of challenging seasonal conditions that can lead to extreme metabolic rate depression

in various organisms. During hypometabolism, chaperone involvement is essential since both protein synthesis and degradation are suppressed and therefore it is crucial for chaperones to ensure the stability and function of existing proteins. The binding of chaperones to existing proteins could also act as a protective mechanism against stress-mediated protein denaturation. Overall, the present review demonstrates the importance of chaperones as part of the adaptive mechanisms in animals subjected to challenging environmental conditions. The lessons that are learned from these studies of chaperones in nature can also increase our understanding of the biological dysfunctions underlying selected human diseases and point the way to improve therapeutic approaches that are derived from these mechanisms.

**Acknowledgements** We thank J.M. Storey for editorial review of the manuscript and her helpful comments. Research in the Storey lab was supported by a Discovery grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada (#6793) to Kenneth B. Storey. Kenneth B. Storey holds the Canada Research Chair in Molecular Physiology; Yichi Zhang held postgraduate Queen Elizabeth II Graduate Scholarships in Science and Technology.

## References

Ahner, A., Gong, X., Schmidt, B. Z., et al. (2013). Small heat shock proteins target mutant cystic fibrosis transmembrane conductance regulator for degradation via a small ubiquitin-like modifier-dependent pathway. *Molecular Biology of the Cell, 24*, 74–84. https://doi.org/10.1091/mbc.E12-09-0678

Alpert, P. (2006). Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? *The Journal of Experimental Biology, 209*, 1575–1584. https://doi.org/10.1242/jeb.02179

Badin, R. A., Lythgoe, M. F., van der Weerd, L., et al. (2006). Neuroprotective effects of virally delivered HSPs in experimental stroke. *Journal of Cerebral Blood Flow and Metabolism, 26*, 371–381. https://doi.org/10.1038/sj.jcbfm.9600190

Bansal, S., Luu, B. E., & Storey, K. B. (2016). MicroRNA regulation in heart and skeletal muscle over the freeze-thaw cycle in the freeze tolerant wood frog. *Journal of Comparative Physiology B, 186*, 229–241. https://doi.org/10.1007/s00360-015-0951-3

Barnes, B. M. (1989). Freeze avoidance in a mammal: Body temperatures below 0°C in an Arctic Hibernator. *Science, 244*, 1593–1595. https://doi.org/10.1126/science.2740905

Beere, H. M., Wolf, B. B., Cain, K., et al. (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nature Cell Biology, 2*, 469–475. https://doi.org/10.1038/35019501

Biggar, K. K., Wu, C. W., Tessier, S. N., et al. (2015). Primate torpor: Regulation of stress-activated protein kinases during daily torpor in the gray mouse lemur, *Microcebus murinus*. *Genomics Proteomics Bioinformatics, 13*, 81–90. https://doi.org/10.1016/j.gpb.2015.03.002

Brehme, M., Voisine, C., Rolland, T., et al. (2014). A chaperome subnetwork safeguards proteostasis in aging and neurodegenerative disease. *Cell Reports, 9*, 1135–1150. https://doi.org/10.1016/j.celrep.2014.09.042

van Breukelen, F., Sonenberg, N., & Martin, S. L. (2004). Seasonal and state-dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 287*, R349–R353. https://doi.org/10.1152/ajpregu.00728.2003

Brinkmeier, H., & Ohlendieck, K. (2014). Chaperoning heat shock proteins: Proteomic analysis and relevance for normal and dystrophin-deficient muscle. *Proteomics - Clinical Applications, 8*, 875–895. https://doi.org/10.1002/prca.201400015

Brooks, S. P. J., & Storey, K. B. (1995). Evidence for aestivation specific proteins in *Otala lactea*. *Molecular and Cellular Biochemistry, 143*, 15–20. https://doi.org/10.1007/BF00925922

Brown, D. (2003). The ins and outs of aquaporin-2 trafficking. *American Journal of Physiology. Renal Physiology, 284*, F893–F901. https://doi.org/10.1152/ajprenal.00387.2002

Brychzy, A., Rein, T., Winklhofer, K. F., et al. (2003). Cofactor Tpr2 combines two TPR domains and a J domain to regulate the Hsp70/Hsp90 chaperone system. *The EMBO Journal, 22*, 3613–3623. https://doi.org/10.1093/emboj/cdg362

Buck, C. L., & Barnes, B. M. (2000). Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 279*, R255–R262.

Bukau, B., Weissman, J., & Horwich, A. (2006). Molecular chaperones and protein quality control. *Cell, 125*, 443–451.

Cao, G., Pei, W., Lan, J., et al. (2001). Caspase-activated DNase/DNA fragmentation factor 40 mediates apoptotic DNA fragmentation in transient cerebral ischemia and in neuronal cultures. *The Journal of Neuroscience, 21*, 4678–4690.

Cao, G., Luo, Y., Nagayama, T., et al. (2002). Cloning and characterization of rat caspase-9: implications for a role in mediating caspase-3 activation and hippocampal cell death after transient cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism, 22*, 534–546. https://doi.org/10.1097/00004647-200205000-00005

Carberry, S., Zweyer, M., Swandulla, D., & Ohlendieck, K. (2012). Proteomics reveals drastic increase of extracellular matrix proteins collagen and dermatopontin in the aged mdx diaphragm model of Duchenne muscular dystrophy. *International Journal of Molecular Medicine, 30*, 229–234. https://doi.org/10.3892/ijmm.2012.1006

Carey, H. V., Sills, N. S., & Gorham, D. A. (1999). Stress proteins in mammalian hibernation. *American Zoologist, 39*, 825–835. https://doi.org/10.1093/icb/39.6.825

Carey, H. V., Rhoads, C. A., & Aw, T. Y. (2003). Hibernation induces glutathione redox imbalance in ground squirrel intestine. *Journal of Comparative Physiology B, 173*, 269–276. https://doi.org/10.1007/s00360-003-0330-3

Carey, C. C., Gorman, K. F., & Rutherford, S. (2006). Modularity and intrinsic evolvability of Hsp90-buffered change. *PLoS One, 20*, e76. https://doi.org/10.1371/journal.pone.0000076

Carra, S., Brunsting, J. F., Lambert, H., et al. (2009). HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2{alpha} phosphorylation. *The Journal of Biological Chemistry, 284*, 5523–5532. https://doi.org/10.1074/jbc.M807440200

Carra, S., Alberti, S., Arrigo, P. A., et al. (2017). The growing world of small heat shock proteins: from structure to functions. *Cell Stress & Chaperones, 22*, 601–611. https://doi.org/10.1007/s12192-017-0787-8

Chang, J., Knowlton, A. A., & Wasser, J. S. (2000). Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 278*, R209–R214.

Chen, J., Graham, S. H., Zhu, R. L., & Simon, R. P. (1996). Stress proteins and tolerance to focal cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism, 16*, 566–577. https://doi.org/10.1097/00004647-199607000-00006

Chen, B., Kayukawa, T., Monteiro, A., & Ishikawa, Y. (2006). Cloning and characterization of the HSP70 Gene, and its expression in response to diapauses and thermal stress in the onion maggot, *Delia antiqua*. *Journal of Biochemistry and Molecular Biology, 39*, 749–758. https://doi.org/10.5483/BMBRep.2006.39.6.749

Chen, H. J., Mitchell, J. C., Novoselov, S., et al. (2016). The heat shock response plays an important role in TDP-43 clearance: Evidence for dysfunction in amyotrophic lateral sclerosis. *Brain, 139*, 1417–1432. https://doi.org/10.1093/brain/aww028

Clark, M. S., Thorne, M. A., Purać, J., et al. (2009). Surviving the cold: molecular analyses of insect cryoprotective dehydration in the Arctic springtail *Megaphorura arctica* (Tullberg). *BMC Genomics, 10*, 328. https://doi.org/10.1186/1471-2164-10-328

Clegg, J. S. (2002). Cryptobiosis - A peculiar state of biological organization. *Tsitologiya, 44*, 1011–1014. https://doi.org/10.1016/S1096-4959(01)00300-1

Clegg, J. S. (2007). Protein stability in Artemia embryos during prolonged anoxia. *The Biological Bulletin, 212*, 74–81.

Cotton, C. J., & Harlow, H. J. (2015). Avoidance of skeletal muscle atrophy in spontaneous and facultative hibernators. *Physiological and Biochemical Zoology, 83*, 551–560. https://doi.org/10.1086/650471.

De Palma, S., Morandi, L., Mariani, E., et al. (2006). Proteomic investigation of the molecular pathophysiology of dysferlinopathy. *Proteomics, 6*, 379–385. https://doi.org/10.1002/pmic.200500098

Dong, Y., Desneux, N., Lei, C., & Niu, C. (2014). Transcriptome characterization analysis of *Bactrocera minax* and new insights into its pupal diapause development with gene expression analysis. *International Journal of Biological Sciences, 10*, 1051–1063. https://doi.org/10.7150/ijbs.9438

Echtenkamp, F. J., & Freeman, B. C. (2012). Expanding the cellular molecular chaperone network through the ubiquitous cochaperones. *Biochimica et Biophysica Acta-Molecular Cell Research, 1823*, 668–673. https://doi.org/10.1016/j.bbamcr.2011.08.011

Eddy, S. F., McNally, J. D., & Storey, K. B. (2005). Up-regulation of a thioredoxin peroxidase-like protein, proliferation-associated gene, in hibernating bats. *Archives of Biochemistry and Biophysics, 435*, 103–111. https://doi.org/10.1016/j.abb.2004.11.020

Epperson, L. E., Rose, J. C., Carey, H. V., & Martin, S. L. (2010). Seasonal proteomic changes reveal molecular adaptations to preserve and replenish liver proteins during ground squirrel hibernation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 298*, R329–R340. https://doi.org/10.1152/ajpregu.00416.2009

Fahy, G. M., Wowk, B., Wu, J., et al. (2004). Cryopreservation of organs by vitrification: Perspectives and recent advances. *Cryobiology, 48*, 157–178. https://doi.org/10.1016/j.cryobiol.2004.02.002

Feder, M. E., & Hofmann, G. E. (1999). Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology, 61*, 243–282.

Frank, C. L., & Storey, K. B. (1995). The optimal depot fat composition for hibernation by golden-mantled ground squirrels (*Spermophilus lateralis*). *Journal of Comparative Physiology B, 164*, 536–542. https://doi.org/10.1007/BF00261394

Frerichs, K. U., & Hallenbeck, J. M. (1998). Hibernation in ground squirrels induces state and species-specific tolerance to hypoxia and aglycemia: an in vitro study in hippocampal slices. *Journal of Cerebral Blood Flow and Metabolism, 18*, 168–175. https://doi.org/10.1097/00004647-199802000-00007

Frerichs, K. U., Kennedy, C., Sokoloff, L., & Hallenbeck, J. M. (1994). Local cerebral blood flow during hibernation, a model of natural tolerance to "cerebral ischemia". *Journal of Cerebral Blood Flow and Metabolism, 14*, 193–205. https://doi.org/10.1038/jcbfm.1994.26

Gagnon, D. D., Rintamäki, H., Gagnon, S. S., et al. (2013). Cold exposure enhances fat utilization but not non-esterified fatty acids, glycerol or catecholamines availability during submaximal walking and running. *Frontiers in Physiology, 4*. https://doi.org/10.3389/fphys.2013.00099

Gao, Y. F., Wang, J., Wang, H. P., et al. (2012). Skeletal muscle is protected from disuse in hibernating dauria ground squirrels. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 161*, 296–300. https://doi.org/10.1016/j.cbpa.2011.11.009

Gehrig, S. M., van der Poel, C., Sayer, T. A., et al. (2012). Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. *Nature, 484*, 394–398. https://doi.org/10.1038/nature10980

Gething, M.-J., & Sambrook, J. (1992). Protein folding in the cell. *Nature, 355*, 33–45. https://doi.org/10.1038/355033a0

Giraud-Billoud, M., Vega, I. A., Tosi, M. E. R., et al. (2013). Antioxidant and molecular chaperone defences during estivation and arousal in the South American apple snail *Pomacea canaliculata*. *The Journal of Experimental Biology, 216*, 614–622. https://doi.org/10.1242/jeb.075655

Giwa, S., Lewis, J. K., Alvarez, L., et al. (2017). The promise of organ and tissue preservation to transform medicine. *Nature Biotechnology, 35*, 530–542. https://doi.org/10.1038/nbt.3889

Goldfarb, S. B., Kashlan, O. B., Watkins, J. N., et al. (2006). Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 5817–5822. https://doi.org/10.1073/pnas.0507903103

Gomez, A. M., Vanheel, A., Losen, M., et al. (2013). Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis. *Journal of Neuroimmunology, 261*, 141–145. https://doi.org/10.1016/j.jneuroim.2013.05.008

Gonzalez–Gronow, M., Selim, M. A., Papalas, J., & Pizzo, S. V. (2009). GRP78: A Multifunctional receptor on the cell surface. *Antioxidants & Redox Signaling, 11*, 2299–2306. https://doi.org/10.1089/ars.2009.2568

Gorman, A. M., Szegezdi, E., Quigney, D. J., & Samali, A. (2005). Hsp27 inhibits 6-hydroxydopamine-induced cytochrome c release and apoptosis in PC12 cells. *Biochemical and Biophysical Research Communications, 327*, 801–810. https://doi.org/10.1016/j.bbrc.2004.12.066

Goyal, K., Walton, L. J., Browne, J. A., et al. (2005). Molecular anhydrobiology: Identifying molecules implicated in invertebrate anhydrobiosis. *Integrative and Comparative Biology, 45*, 702–709. https://doi.org/10.1093/icb/45.5.702

Gray, P. C., & Vale, W. (2012). Cripto/GRP78 modulation of the TGF-β pathway in development and oncogenesis. *FEBS Letters, 586*, 1836–1845.

Grune, T., Catalgol, B., Licht, A., et al. (2011). HSP70 mediates dissociation and reassociation of the 26S proteasome during adaptation to oxidative stress. *Free Radical Biology & Medicine, 51*, 1355–1364. https://doi.org/10.1016/j.freeradbiomed.2011.06.015

Gusev, O., Cornette, R., Kikawada, T., & Okuda, T. (2011). Expression of heat shock protein-coding genes associated with anhydrobiosis in an African chironomid *Polypedilum vanderplanki*. *Cell Stress & Chaperones, 16*, 81–90. https://doi.org/10.1007/s12192-010-0223-9

Hadj-Moussa, H., Moggridge, J. A., Luu, B. E., et al. (2016). The hibernating South American marsupial, *Dromiciops gliroides*, displays torpor-sensitive microRNA expression patterns. *Scientific Reports, 6*, 24627. https://doi.org/10.1038/srep24627

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*, 324–332. https://doi.org/10.1038/nature10317

Heldmaier, G., Ortmann, S., & Elvert, R. (2004). Natural hypometabolism during hibernation and daily torpor in mammals. *Respiratory Physiology & Neurobiology, 141*, 317–329. https://doi.org/10.1016/j.resp.2004.03.014

Hendershot, L., Wei, J., Gaut, J., et al. (1996). Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 5269–5274. https://doi.org/10.1073/pnas.93.11.5269

Hengherr, S., Worland, M. R., Reuner, A., et al. (2009). High-Temperature tolerance in anhydrobiotic tardigrades is limited by glass transition. *Physiological and Biochemical Zoology, 82*, 749–755. https://doi.org/10.1086/605954

Herbert, V., & Jackson, D. C. (1985). Temperature effects on the responses to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid-base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiological Zoology, 58*, 670–681. https://doi.org/10.1086/physzool.58.6.30156071

Hermes-Lima, M., & Zenteno-Savín, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 133*, 537–556. https://doi.org/10.1016/S1532-0456(02)00080-7

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nature Reviews. Molecular Cell Biology, 13*, 89–102. https://doi.org/10.1038/nrm3270

Hillman, S. S. (1978). The roles of oxygen delivery and electrolyte levels in the dehydrational death of *Xenopus laevis*. *Journal of Comparative Physiology B, 128*, 169–175. https://doi.org/10.1007/BF00689481

Hittel, D., & Storey, K. B. (2002). The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). *Archives of Biochemistry and Biophysics, 401*, 244–254. https://doi.org/10.1016/S0003-9861(02)00048-6

Hochachka, P. W., & Lutz, P. L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 130*, 435–459. https://doi.org/10.1016/S1096-4959(01)00408-0

Hochachka, P. W., Buck, L. T., Doll, C. J., & Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 9493–9498. https://doi.org/10.1073/pnas.93.18.9493

Holmstrup, M., & Westh, P. (1994). Dehydration of earthworm cocoons exposed to cold: a novel cold hardiness mechanism. *Journal of Comparative Physiology B, 164*, 312–315. https://doi.org/10.1007/BF00346448

Hori, O., Matsumoto, M., Kuwabara, K., et al. (1996). Exposure of astrocytes to hypoxia/reoxygenation enhances expression of glucose-regulated protein 78 facilitating astrocyte release of the neuroprotective cytokine interleukin 6. *Journal of Neurochemistry, 66*, 973–979.

Horikawa, D. D., Sakashita, T., Katagiri, C., et al. (2006). Radiation tolerance in the tardigrade Milnesium tardigradum. *International Journal of Radiation Biology, **82***, 843–848. https://doi.org/10.1080/09553000600972956

Horwich, A. L., Fenton, W. A., Chapman, E., & Farr, G. W. (2007). Two families of chaperonin: physiology and mechanism. *Annual Review of Cell and Developmental Biology, 23*, 115–145. https://doi.org/10.1146/annurev.cellbio.23.090506.123555

Houry, W. A., Frishman, D., Eckerskorn, C., et al. (1999). Identifcation of in vivo substrates of the chaperonin GroEL. *Nature, 402*, 147–154. https://doi.org/10.1038/45977

Jackson, D. C. (1968). Metabolic depression and oxygen depletion in the diving turtle. *Journal of Applied Physiology, 24*, 503–509.

Jackson, D. C. (2002). Hibernating without oxygen: Physiological adaptations of the painted turtle. *The Journal of Physiology, 543*, 731–737. https://doi.org/10.1113/jphysiol.2002.024729

Jacob, P., Hirt, H., & Bendahmane, A. (2017). The heat-shock protein/chaperone network and multiple stress resistance. *Plant Biotechnology Journal, 15*, 405–414. https://doi.org/10.1111/pbi.12659

Joanisse, D. R., & Storey, K. B. (1994). Mitochondrial enzymes during overwintering in two species of cold-hardy gall insects. *Insect Biochemistry and Molecular Biology, 24*, 145–150. https://doi.org/10.1016/0965-1748(94)90080-9

Kampinga, H. H., Hageman, J., Vos, M. J., et al. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress & Chaperones, 14*, 105–111. https://doi.org/10.1007/s12192-008-0068-7

Kato, H., Liu, Y., Kogure, K., & Kato, K. (1994). Induction of 27-kDa heat shock protein following cerebral ischemia in a rat model of ischemic tolerance. *Brain Research, 634*, 235–244.

Kayukawa, T., & Ishikawa, Y. (2009). Chaperonin contributes to cold hardiness of the onion maggot *Delia antiqua* through repression of depolymerization of actin at low temperatures. *PLoS One, 4*, e8277. https://doi.org/10.1371/journal.pone.0008277

Kesaraju, S., Schmidt-Kastner, R., Prentice, H. M., & Milton, S. L. (2009). Modulation of stress proteins and apoptotic regulators in the anoxia tolerant turtle brain. *Journal of Neurochemistry, 109*, 1413–1426. https://doi.org/10.1111/j.1471-4159.2009.06068.x

Kesaraju, S., Nayak, G., Prentice, H. M., & Milton, S. L. (2014). Upregulation of Hsp72 mediates anoxia/reoxygenation neuroprotection in the freshwater turtle via modulation of ROS. *Brain Research, 1582*, 247–256. https://doi.org/10.1016/j.brainres.2014.07.044

Kim, S. W., Park, S., You, K. H., & Kwon, O. Y. (2003). Expression of the endoplasmic reticulum chaperone GRP94 gene in ischemic gerbil brain. *Zeitschrift für Naturforschung. Section C, 58*, 736–739.

Kirino, T. (2002). Ischemic tolerance. *Journal of Cerebral Blood Flow and Metabolism, 22*, 1283–1296. https://doi.org/10.1097/00004647-200211000-00001.

Klucken, J., Shin, Y., Masliah, E., et al. (2004). Hsp70 reduces α-synuclein aggregation and toxicity. *The Journal of Biological Chemistry, 279*, 25497–25502. https://doi.org/10.1074/jbc.M400255200

Krivoruchko, A., & Storey, K. B. (2010). Regulation of the heat shock response under anoxia in the turtle, *Trachemys scripta elegans*. *Journal of Comparative Physiology B, 180*, 403–414. https://doi.org/10.1007/s00360-009-0414-9

Krukenberg, K. A., Street, T. O., Lavery, L. A., & Agard, D. A. (2011). Conformational dynamics of the molecular chaperone Hsp90. *Quarterly Reviews of Biophysics, 44*, 229–255. https://doi.org/10.1017/S0033583510000314

Kudo, T., Kanemoto, S., Hara, H., et al. (2008). A molecular chaperone inducer protects neurons from ER stress. *Cell Death and Differentiation, 15*, 364–375. https://doi.org/10.1038/sj.cdd.4402276

Kültz, D. (2004). Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology, 67*, 225–257. https://doi.org/10.1146/annurev.physiol.67.040403.103635.

Lee, A. S. (2014). Glucose-regulated proteins in cancer: Molecular mechanisms and therapeutic potential. *Nature Reviews Cancer, 14*, 263–276. https://doi.org/10.1038/nrc3701

Lee, M., Choi, I., & Park, K. (2002). Activation of stress signaling molecules in bat brain during arousal from hibernation. *Journal of Neurochemistry, 82*, 867–873. https://doi.org/10.1046/j.1471-4159.2002.01022.x

Lee, K., Joo, Y. P., Yoo, W., et al. (2008). Overcoming muscle atrophy in a hibernating mammal despite prolonged disuse in dormancy: Proteomic and molecular assessment. *Journal of Cellular Biochemistry, 104*, 642–656. https://doi.org/10.1002/jcb.21653

Levin, D. B., Danks, H. V., & Barber, S. A. (2003). Variations in mitochondrial DNA and gene transcription in freezing-tolerant larvae of *Eurosta solidaginis* (Diptera: Tephritidae) and *Gynaephora groenlandica* (Lepidoptera: Lymantriidae). *Insect Molecular Biology, 12*, 281–289. https://doi.org/10.1046/j.1365-2583.2003.00413.x

Lewis, C., Carberry, S., & Ohlendieck, K. (2009). Proteomic profiling of x-linked muscular dystrophy. *Journal of Muscle Research and Cell Motility, 30*, 267–279.

Li, M.-L., Defren, J., & Brewer, G. (2013). Hsp27 and F-box protein β-TrCP promote degradation of mRNA decay factor AUF1. *Molecular and Cellular Biology, 33*, 2315–2326. https://doi.org/10.1128/MCB.00931-12

Lindell, S. L., Klahn, S. L., Piazza, T. M., et al. (2005). Natural resistance to liver cold ischemia-reperfusion injury associated with the hibernation phenotype. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 288*, G473–G480. https://doi.org/10.1152/ajpgi.00223.2004

Liu, B., Wohlfart, B., & Johansson, B. W. (1990). Effects of low temperature on contraction in papillary muscles from rabbit, rat, and hedgehog. *Cryobiology, 27*, 539–546. https://doi.org/10.1016/0011-2240(90)90041-2

Liu, B., Wang, L. C. H., & Belke, D. D. (1993). Effects of temperature and pH on cardiac myofilament Ca-2+ sensitivity in rat and ground squirrel. *The American Journal of Physiology, 264*, R104–R108.

Liu, R., Li, X., Gao, W., et al. (2013). Monoclonal antibody against cell surface GRP78 as a novel agent in suppressing PI3K/AKT signaling, tumor growth, and metastasis. *Clinical Cancer Research, 19*, 6802–6811. https://doi.org/10.1158/1078-0432.CCR-13-1106

Logan, S. M., Luu, B. E., & Storey, K. B. (2016a). Turn down genes for WAT? Activation of anti-apoptosis pathways protects white adipose tissue in metabolically depressed thirteen-lined ground squirrels. *Molecular and Cellular Biochemistry, 416*, 47–62. https://doi.org/10.1007/s11010-016-2695-0

Logan, S. M., Tessier, S. N., Tye, J., & Storey, K. B. (2016b). Response of the JAK-STAT pathway to mammalian hibernation in 13-lined ground squirrel striated muscle. *Molecular and Cellular Biochemistry, 414*, 115–127. https://doi.org/10.1007/s11010-016-2665-6

Lopez-Martinez, G., Elnitsky, M. A., Benoit, J. B., et al. (2008). High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochemistry and Molecular Biology, 38*, 796–804. https://doi.org/10.1016/j.ibmb.2008.05.006

Lu, H. A. J., Sun, T. X., Matsuzaki, T., et al. (2007). Heat shock protein 70 interacts with aquaporin-2 and regulates its trafficking. *The Journal of Biological Chemistry, 282*, 28721–28732. https://doi.org/10.1074/jbc.M611101200

Luo, B., & Lee, A. S. (2013). The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene, 32*, 805–818. https://doi.org/10.1038/onc.2012.130

Luo, S., Mao, C., Lee, B., & Lee, A. S. (2006). GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Molecular and Cellular Biology, 26*, 5688–5697. https://doi.org/10.1128/MCB.00779-06

Luu, B. E., Biggar, K. K., Wu, C.-W., & Storey, K. B. (2016). Torpor-responsive expression of novel microRNA regulating metabolism and other cellular pathways in the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*. *FEBS Letters, 590*, 3574–3582. https://doi.org/10.1002/1873-3468.12435

Luu, B. E., Wijenayake, S., Malik, A. I., & Storey, K. B. (2017). The regulation of heat shock proteins in response to dehydration in *Xenopus laevis*. *Cell Stress & Chaperones, 23*(1), 45–53. https://doi.org/10.1007/s12192-017-0822-9

Ma, Y., & Hendershot, L. M. (2004). The role of the unfolded protein response in tumour development: friend or foe? *Nature Reviews. Cancer, 4*, 966–977. https://doi.org/10.1038/nrc1505

Ma, T., Liu, X., & Liu, Z. (2015). Role of asymmetric dimethylarginine in rat acute lung injury induced by acute ischemic kidney injury. *Molecular Medicine Reports, 12*, 1923–1928. https://doi.org/10.3892/mmr.2015.3619

MacRae, T. H. (2016). Stress tolerance during diapause and quiescence of the brine shrimp, *Artemia*. *Cell Stress & Chaperones, 21*, 9–18. https://doi.org/10.1007/s12192-015-0635-7

Malik, A. I., & Storey, K. B. (2009a). Activation of antioxidant defense during dehydration stress in the African clawed frog. *Gene, 442*, 99–107. https://doi.org/10.1016/j.gene.2009.04.007

Malik, A. I., & Storey, K. B. (2009b). Activation of extracellular signal-regulated kinases during dehydration in the African clawed frog, *Xenopus laevis*. *The Journal of Experimental Biology, 212*, 2595–2603. https://doi.org/10.1242/jeb.030627

Malik, A. I., & Storey, K. B. (2011). Transcriptional regulation of antioxidant enzymes by FoxO1 under dehydration stress. *Gene, 485*, 114–119. https://doi.org/10.1016/j.gene.2011.06.014

Mamady, H., & Storey, K. B. (2006). Up-regulation of the endoplasmic reticulum molecular chaperone GRP78 during hibernation in thirteen-lined ground squirrels. *Molecular and Cellular Biochemistry, 292*, 89–98. https://doi.org/10.1007/s11010-006-9221-8

Mamady, H., & Storey, K. B. (2008). Coping with the stress: Expression of ATF4, ATF6, and downstream targets in organs of hibernating ground squirrels. *Archives of Biochemistry and Biophysics, 477*, 77–85. https://doi.org/10.1016/j.abb.2008.05.006

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*, 670–684. https://doi.org/10.1007/s00018-004-4464-6

McMullen, D. C., & Storey, K. B. (2008). Mitochondria of cold hardy insects: Responses to cold and hypoxia assessed at enzymatic, mRNA and DNA levels. *Insect Biochemistry and Molecular Biology, 38*, 367–373. https://doi.org/10.1016/j.ibmb.2007.12.003

Mehta, S. L., Manhas, N., & Raghubir, R. (2007). Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Research Reviews, 54*, 34–66.

Michie, L. J., Mallard, F., Majerus, M. E. N., & Jiggins, F. M. (2010). Melanic through nature or nurture: Genetic polymorphism and phenotypic plasticity in *Harmonia axyridis*. *Journal of Evolutionary Biology, 23*, 1699–1707. https://doi.org/10.1111/j.1420-9101.2010.02043.x

Minamino, T., Komuro, I., & Kitakaze, M. (2010). Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. *Circulation Research, 107*, 1071–1082.

Mizrahi, T., Heller, J., Goldenberg, S., & Arad, Z. (2010). Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell Stress & Chaperones, 15*, 351–363. https://doi.org/10.1007/s12192-009-0150-9

Mizukoshi, E., Suzuki, M., Loupatov, A., et al. (1999). Fibroblast growth factor-1 interacts with the glucose-regulated protein GRP75/mortalin. *Biochemical Journal, 343*, 461. https://doi.org/10.1042/0264-6021:3430461

Morin, P., & Storey, K. B. (2009). Mammalian hibernation: Differential gene expression and novel application of epigenetic controls. *The International Journal of Developmental Biology, 53*, 433–442.

Motojima, F., Chaudhry, C., Fenton, W. A., et al. (2004). Substrate polypeptide presents a load on the apical domains of the chaperonin GroEL. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 15005–15012. https://doi.org/10.1073/pnas.0406132101

Muntoni, F., Torelli, S., & Ferlini, A. (2003). Dystrophin and mutations: One gene, several proteins, multiple phenotypes. *Lancet Neurology, 2*, 731–740.

Mutsaers, C. A., Lamont, D. J., Hunter, G., et al. (2013). Label-free proteomics identifies Calreticulin and GRP75/Mortalin as peripherally accessible protein biomarkers for spinal muscular atrophy. *Genome Medicine, 5*, 95. https://doi.org/10.1186/gm498

Ni, M., Zhang, Y., & Lee, A. S. (2011). Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signaling and therapeutic targeting. *Biochemical Journal, 434*, 181–188. https://doi.org/10.1042/BJ20101569

Niforou, K., Cheimonidou, C., & Trougakos, I. P. (2014). Molecular chaperones and proteostasis regulation during redox imbalance. *Redox Biology, 2*, 323–332. https://doi.org/10.1016/j.redox.2014.01.017

Nishi, S., Taki, W., Uemura, Y., et al. (1993). Ischemic tolerance due to the induction of HSP70 in a rat ischemic recirculation model. *Brain Research, 615*, 281–288.

Pandey, P., Saleh, A., Nakazawa, A., et al. (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *The EMBO Journal, 19*, 4310–4322. https://doi.org/10.1093/emboj/19.16.4310

Prentice, H. M., Milton, S. L., Scheurle, D., & Lutz, P. L. (2004). The upregulation of cognate and inducible heat shock proteins in the anoxic turtle brain. *Journal of Cerebral Blood Flow and Metabolism, 24*, 826–828. https://doi.org/10.1097/01.WCB.0000126565.27130.79

Prostko, C. R., Brostrom, M. A., Malara, E. M., & Brostrom, C. O. (1992). Phosphorylation of eukaryotic initiation factor (eIF) 2α and inhibition of eIF-2B in GH3 pituitary cells by perturbants of early protein processing that induce GRP78. *The Journal of Biological Chemistry, 267*, 16751–16754.

Qiu, Z., & Macrae, T. H. (2008a). ArHsp21, a developmentally regulated small heat-shock protein synthesized in diapausing embryos of *Artemia franciscana*. *Biochemical Journal, 411*, 605–611. https://doi.org/10.1042/BJ20071472

Qiu, Z., & MacRae, T. H. (2008b). ArHsp22, a developmentally regulated small heat shock protein produced in diapause-destined *Artemia* embryos, is stress inducible in adults. *The FEBS Journal, 275*, 3556–3566. https://doi.org/10.1111/j.1742-4658.2008.06501.x

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress & Chaperones, 21*, 379–404. https://doi.org/10.1007/s12192-016-0676-6

Ramaglia, V. (2004). Time-dependent expression of heat shock proteins 70 and 90 in tissues of the anoxic western painted turtle. *The Journal of Experimental Biology, 207*, 3775–3784. https://doi.org/10.1242/jeb.01211

Ramløv, H., & Westh, P. (1992). Survival of the cryptobiotic eutardigrade *Adorybiotus coronifer* during cooling to -196 °C: Effect of cooling rate, trehalose level, and short-term acclimation. *Cryobiology, 29*, 125–130. https://doi.org/10.1016/0011-2240(92)90012-Q

Ramnanan, C. J., Allan, M. E., Groom, A. G., & Storey, K. B. (2009). Regulation of global protein translation and protein degradation in aerobic dormancy. *Molecular and Cellular Biochemistry, 323*, 9–20. https://doi.org/10.1007/s11010-008-9959-2

Ranford, J. C., Coates, A. R., & Henderson, B. (2000). Chaperonins are cell-signalling proteins: The unfolding biology of molecular chaperones. *Expert Reviews in Molecular Medicine, **2***, 1–17. https://doi.org/10.1017/S1462399400002015

Rasmussen, S., Barah, P., Suarez-Rodriguez, M. C., et al. (2013). Transcriptome responses to combinations of stresses in arabidopsis. *Plant Physiology, 161*, 1783–1794. https://doi.org/10.1104/pp.112.210773

Reddy, G., Kumar, P., & Kumar, M. (2006). Chaperone-like activity and hydrophobicity of α-crystallin. *IUBMB Life, 58*, 632–641. https://doi.org/10.1080/15216540601010096.

Reuner, A., Brümmer, F., & Schill, R. O. (2008). Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (*Cantareus apertus*). *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology, 151*, 28–31. https://doi.org/10.1016/j.cbpb.2008.05.004

Reuner, A., Hengherr, S., Mali, B., et al. (2010). Stress response in tardigrades: Differential gene expression of molecular chaperones. *Cell Stress & Chaperones, 15*, 423–430. https://doi.org/10.1007/s12192-009-0158-1

Richards, R. C., Achenbach, J. C., Short, C. E., et al. (2008). Seasonal expressed sequence tags of rainbow smelt (*Osmerus mordax*) revealed by subtractive hybridization and the identification of two genes up-regulated during winter. *Gene, 424*, 56–62. https://doi.org/10.1016/j.gene.2008.08.002

Rinehart, J. P., Hayward, S. A. L., Elnitsky, M. A., et al. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 14223–14227. https://doi.org/10.1073/pnas.0606840103

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews. Molecular Cell Biology, 8*, 519–529. https://doi.org/10.1038/nrm2199

Rouble, A. N., Hefler, J., Mamady, H., et al. (2013). Anti-apoptotic signaling as a cytoprotective mechanism in mammalian hibernation. *PeerJ, **1***, e29. https://doi.org/10.7717/peerj.29

Rousseau, F., Serrano, L., & Schymkowitz, J. W. H. (2006). How evolutionary pressure against protein aggregation shaped chaperone specificity. *Journal of Molecular Biology, 355*, 1037–1047. https://doi.org/10.1016/j.jmb.2005.11.035

Rüdiger, S., Buchberger, A., & Bukau, B. (1997). Interaction of Hsp70 chaperones with substrates. *Nature Structural Biology, 4*, 342–349. https://doi.org/10.1038/nsb0597-342

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nature Reviews. Molecular Cell Biology, 14*, 630–642. https://doi.org/10.1038/nrm3658

Saito, A., Tominaga, T., & Chan, P. H. (2005). Neuroprotective role of neurotrophins: relationship between nerve growth factor and apoptotic cell survival pathway after cerebral ischemia. *Current Atherosclerosis Reports, 7*, 268–273.

Sanson, M., Augé, N., Vindis, C., et al. (2009). Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells: Prevention by oxygen-regulated protein 150 expression. *Circulation Research, 104*, 328–336. https://doi.org/10.1161/CIRCRESAHA.108.183749

Schmid, J. (2001). Daily torpor in free-ranging gray mouse lemurs (*Microcebus murinus*) in Madagascar. *International Journal of Primatology, 22*, 1021–1031. https://doi.org/10.1023/A:1012069706237.

Schmid, J., & Ganzhorn, J. U. (2009). Optional strategies for reduced metabolism in gray mouse lemurs. *Naturwissenschaften, 96*, 737–741. https://doi.org/10.1007/s00114-009-0523-z

Schopf, F., Biebl, M., & Buchner, J. (2017). The Hsp90 chaperone machinery. *Nature Reviews. Molecular Cell Biology, 18*, 345–360. https://doi.org/10.1038/nrm.2017.20

Schröder, M., & Kaufman, R. J. (2005). The Mammalian Unfolded Protein Response. *Annual Review of Biochemistry, 74*, 739–789. https://doi.org/10.1146/annurev.biochem.73.011303.074134

Seki, K., & Toyoshima, M. (1998). Preserving tardigrades under pressure. *Nature, 395*, 853–854. https://doi.org/10.1038/27576

Sela, I., Krentsis, I. M., Shlomai, Z., et al. (2011). The proteomic profile of hereditary inclusion body myopathy. *PLoS One, 6*, e16334. https://doi.org/10.1371/journal.pone.0016334

Serkova, N. N. J., Rose, J. J. C., Epperson, L. E., et al. (2007). Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. *Physiological Genomics, 31*, 15–24. https://doi.org/10.1152/physiolgenomics.00028.2007

Shiu, R. P., Pouyssegur, J., & Pastan, I. (1977). Glucose depletion accounts for the induction of two transformation-sensitive membrane proteinsin Rous sarcoma virus-transformed chick embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America, 74*, 3840–3844.

Shoshana, G. Z. A., & Heller, J. (1989). Resistance to desiccation and distribution patterns in the land snail *Sphincterochila*. *Journal of Zoology, 218*, 353–364. https://doi.org/10.1111/j.1469-7998.1989.tb02549.x

Storey, K. B. (1997). Organic Solutes in Freezing Tolerance. *Comparative Biochemistry and Physiology. Part A, Physiology, 117*, 319–326. https://doi.org/10.1016/S0300-9629(96)00270-8

Storey, K. B. (2002). Natural hypothermic preservation: The mammalian hibernator. *Cell Preservation and Technology, 1*, 3–16. https://doi.org/10.1089/15383440260073257

Storey, K. B. (2003). Mammalian hibernation. Transcriptional and translational controls. *Advances in Experimental Medicine and Biology, 543*, 21–38.

Storey, K. B. (2007). Anoxia tolerance in turtles: Metabolic regulation and gene expression. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 147*, 263–276. https://doi.org/10.1016/j.cbpa.2006.03.019

Storey, K. B. (2010). Out cold: Biochemical regulation of mammalian hibernation - A mini-review. *Gerontology, 56*, 220–230.

Storey, K. B., & Storey, J. M. (2004). Metabolic rate depression in animals: Transcriptional and translational controls. *Biological Reviews of the Cambridge Philosophical Society, 79*, 207–233. https://doi.org/10.1017/S1464793103006195

Storey, K. B., & Storey, J. M. (2007). Tribute to P. L. Lutz: putting life on 'pause' - molecular regulation of hypometabolism. *The Journal of Experimental Biology, 210*, 1700–1714. https://doi.org/10.1242/jeb.02716

Storey, K. B., & Storey, J. M. (2010a). Metabolic regulation and gene expression during aestivation. *Progress in Molecular and Subcellular Biology, 49*, 25–45.

Storey, K. B., & Storey, J. M. (2010b). Metabolic rate depression. The biochemistry of mammalian hibernation. *Advances in Clinical Chemistry, 52*, 77–108.

Sugg, P., Edwards, J. S., & Baust, J. (1983). Phenology and life history of an Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae). *Ecological Entomology, 8*, 105–113.

Swindell, W. R., Huebner, M., & Weber, A. P. (2007). Transcriptional profiling of *Arabidopsis* heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics, 8*, 125. https://doi.org/10.1186/1471-2164-8-125

Trougakos, I. P., Sesti, F., Tsakiri, E., & Gorgoulis, V. G. (2013). Non-enzymatic post-translational protein modifications and proteostasis network deregulation in carcinogenesis. *Journal of Proteomics, 92*, 274–298.

Tseng, Y. Z. C. C., Tsai, Y. L., Fu, X., et al. (2013). Cancer cells resistant to therapy promote cell surface relocalization of GRP78 which complexes with PI3K and enhances PI (3,4,5)P3 production. *PLoS One, 8*, e80071. https://doi.org/10.1371/journal.pone.0080071

Ultsch, G. R. (1989). Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. *Biological Reviews, 64*, 435–515. https://doi.org/10.1111/j.1469-185X.1989.tb00683.x

Vashist, S., & Ng, D. T. W. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *The Journal of Cell Biology,* ***165***, 41–52. https://doi.org/10.1083/jcb.200309132

Verras, M., Papandreou, I., Lim, A. L., & Denko, N. C. (2008). Tumor hypoxia blocks Wnt processing and secretion through the induction of endoplasmic reticulum stress. *Molecular and Cellular Biology, 28*, 7212–7224. https://doi.org/10.1128/MCB.00947-08

Vertommen, D., Herinckx, G., Hussain, N., et al. (2017). Changes in the phosphoproteome of brown adipose tissue during hibernation in the ground squirrel, *Ictidomys tridecemlineatus*. *Physiological Genomics, 49*, 462–472. https://doi.org/10.1152/physiolgenomics.00038.2017

Vidyasagar, A., Wilson, N. A., & Djamali, A. (2012). Heat shock protein 27 (HSP27): biomarker of disease and therapeutic target. *Fibrogenesis & Tissue Repair, 5*, 7. https://doi.org/10.1186/1755-1536-5-7

Wang, L. C. H., & Lee, T. F. (2011). Torpor and hibernation in mammals: Metabolic, physiological, and biochemical adaptations. *Comprehensive Physiology*, 507–532. https://doi.org/10.1002/cphy.cp040122

Wang, K., & Spector, A. (1996). Alpha-crystallin stabilizes actin filaments and prevents cytochalasin-induced depolymerization in a phosphorylation-dependent manner. *European Journal of Biochemistry, 242*, 56–66. https://doi.org/10.1111/j.1432-1033.1996.0056r.x

Wang, S., Zhou, Z., & Qian, H. (2000). Recording of calcium transient and analysis of calcium removal mechanisms in cardiac myocytes from rats and ground squirrels. *Science in China. Series C, Life Sciences, 43*, 191–199. https://doi.org/10.1007/BF02879128

Wang, S., Michaud, J. P., Zhang, R., et al. (2009). Seasonal cycles of assortative mating and reproductive behaviour in polymorphic populations of *Harmonia axyridis* in China. *Ecological Entomology, 34*, 483–494. https://doi.org/10.1111/j.1365-2311.2008.01075.x

Wang, M., Ye, R., Barron, E., et al. (2010). Essential role of the unfolded protein response regulator GRP78/BiP in protection from neuronal apoptosis. *Cell Death and Differentiation, 17*, 488–498. https://doi.org/10.1038/cdd.2009.144

Wang, H.-J., Shi, Z.-K., Shen, Q.-D., et al. (2017). Molecular cloning and induced expression of six small heat shock proteins mediating cold-hardiness in *Harmonia axyridis* (Coleoptera: Coccinellidae). *Frontiers in Physiology, 8*, 1–15. https://doi.org/10.3389/fphys.2017.00060

Watanabe, M. (2002). Cold tolerance and myo-inositol accumulation in overwintering adults of a lady beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). *European Journal of Entomology, 99*, 5–9.

Watanabe, M. (2006). Anhydrobiosis in invertebrates. *Applied Entomology and Zoology, 41*, 15–31. https://doi.org/10.1303/aez.2006.15

Weatherhead, E. C., & Andersen, S. B. (2006). The search for signs of recovery of the ozone layer. *Nature, 441*, 39–45. https://doi.org/10.1038/nature04746

van der Weerd, L., Lythgoe, M. F., Badin, R. A., et al. (2005). Neuroprotective effects of HSP70 overexpression after cerebral ischaemia–an MRI study. *Experimental Neurology, 195*, 257–266. https://doi.org/10.1016/j.expneurol.2005.05.002

Wey, S., Luo, B., & Lee, A. S. (2012). Acute inducible ablation of GRP78 reveals its role in hematopoietic stem cell survival, lymphogenesis and regulation of stress signaling. *PLoS One, 7*, e39047. https://doi.org/10.1371/journal.pone.0039047

Willsie, J. K., & Clegg, J. S. (2001). Nuclear p26, a small heat shock/alpha-crystallin protein, and its relationship to stress resistance in *Artemia franciscana* embryos. *The Journal of Experimental Biology, 204*, 2339–2350.

Worland, M. R. (1996). The relationship between water content and cold tolerance in the arctic collembolan *Onychiurus arcticus* (Collembola: Onychiuridae). *European Journal of Entomology, 93*, 341–348.

Wszola, M., Kwiatkowski, A., Diuwe, P., et al. (2013). One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation. *Transplant International, 26*, 1088–1096.

Wu, J., & Kaufman, R. J. (2006). From acute ER stress to physiological roles of the unfolded protein response. *Cell Death and Differentiation, 13*, 374–384. https://doi.org/10.1038/sj.cdd.4401840

Wu, C. W., Biggar, K. K., Zhang, J., et al. (2015). Induction of antioxidant and heat shock protein responses during torpor in the gray mouse lemur, *Microcebus murinus*. *Genomics, Proteomics & Bioinformatics, 13*, 119–126. https://doi.org/10.1016/j.gpb.2015.03.004

Wu, C. W., Biggar, K. K., Luu, B. E., et al. (2016). Analysis of microRNA expression during the torpor-arousal cycle of a mammalian hibernator, the 13-lined ground squirrel. *Physiological Genomics, 48*, 388–396. https://doi.org/10.1152/physiolgenomics.00005.2016

Xu, R., Andres-Mateos, E., Mejias, R., et al. (2013). Hibernating squirrel muscle activates the endurance exercise pathway despite prolonged immobilization. *Experimental Neurology, 247*, 392–401. https://doi.org/10.1016/j.expneurol.2013.01.005

Yan, J., Burman, A., Nichols, C., et al. (2006). Detection of differential gene expression in brown adipose tissue of hibernating arctic ground squirrels with mouse microarrays. *Physiological Genomics, 25*, 346–353. https://doi.org/10.1152/physiolgenomics.00260.2005

Yan, J., Barnes, B. M., Kohl, F., & Marr, T. G. (2007). Modulation of gene expression in hibernating arctic ground squirrels. *Physiological Genomics, 32*, 170–181. https://doi.org/10.1152/physiolgenomics.00075.2007.

Yu, Z., Luo, H., Fu, W., & Mattson, M. P. (1999). The Endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: Suppression of oxidative stress and stabilization of calcium homeostasis. *Experimental Neurology, 155*, 302–314. https://doi.org/10.1006/exnr.1998.7002

Zhang, G., Storey, J. M., & Storey, K. B. (2011). Chaperone proteins and winter survival by a freeze tolerant insect. *Journal of Insect Physiology, 57*, 1115–1122. https://doi.org/10.1016/j.jinsphys.2011.02.016

Zhang, G., Storey, J.M., & Storey, K.B. (2017). Elevated chaperone proteins are a feature of winter freeze avoidance by larvae of the goldenrod gall moth, *Epiblema scudderiana*. *Journal of Insect Physiology* (in press). doi: https://doi.org/10.1016/j.ymeth.2009.04.019.

Zhao, J., Chen, Z.-Z., Qu, J.-J., et al. (2010). Responses of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) adults to cold acclimation and the related changes of activities of several enzymes in their bodies. *Acta Entomologica Sinica, 53*, 147–153.

Zhu, G., Ye, R., Jung, D. Y., et al. (2013). GRP78 plays an essential role in adipogenesis and postnatal growth in mice. *The FASEB Journal, 27*, 955–964. https://doi.org/10.1096/fj.12-213330

Zilaee, M., & Shirali, S. (2016). Heat shock proteins and diabetes. *Canadian Journal of Diabetes, 40*, 594–602. https://doi.org/10.1016/j.jcjd.2016.05.016

# Chapter 6
# Heat Shock Proteins as Sensors for Mechanical Stress


**Daniel J. MacPhee**

**Abstract** Heat shock proteins (HSP) are highly responsive to stresses including temperature and oxidative stress. Some heat shock proteins bind to improperly or unfolded protein substrates and directly promote refolding in an ATP-dependent manner. Others can act as nucleotide-exchange factors while small HSP can hold unfolded proteins, prevent their aggregation, maintain them in a folding competent state, and pass them on to the ATP-dependent chaperone networks. It is now clear that HSP are also very responsive to mechanical stresses such as compression, shear, and tensile forces and new roles in modulating inflammation and cytoskeletal reorganization have been attributed to these proteins following such mechanical stress. This chapter will summarize findings within the stress protein field demonstrating HSP are sensors for mechanical forces in many tissues.

**Keywords** CRYAB · Heat shock proteins · HSP70 · HSPB1 · Mechanical stress

## Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BAG3 | bcl2-associated athanogene 3 |
| ERK | Extracellular signal-regulated kinase |
| FERMT | Fermitin family homology |
| HSC70 | Heat shock cognate 70 |
| HSF | Heat shock factor |
| HSP | Heat shock proteins |
| IkB | Inhibitor kappa B |
| IL-1β | Interleukin 1 beta |

D. J. MacPhee (✉)
Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

One Reproductive Health Research Group, University of Saskatchewan, Saskatoon, SK, Canada
e-mail: d.macphee@usask.ca



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_6

| | |
|---|---|
| MAPK | Mitogen activated protein kinase |
| mRNA | Messenger ribonucleic acid |
| NFkB | Nuclear factor kappa B |
| PDL | Periodontal ligament |
| PKC | Protein kinase C |
| sHSP | Small heat shock protein |

## 6.1 Introduction

Cells and tissues as a whole experience a wide variety of physical or mechanical stresses including compression forces, shear stress, tensile stretch, hydrostatic pressures and differential adhesion forces (Zhang and Labouesse 2012; Yusko and Asbury 2014; Yu et al. 2016). These forces can be considered forms of mechanical loading and cells sense and respond to these forces in a variety of ways including generating forces themselves. In fact, cells must respond to mechanical forces if they are to continue to perform important cellular processes including mitosis and differentiation (Zhang and Labouesse 2012; Yusko and Asbury 2014).

For cell signalling to occur, it must be transmitted, directed and transduced. Biochemical signals can cause changes in the active site of a target protein via conformational changes (i.e. allosteric changes) or via phosphorylation of a target (Yusko and Asbury 2014). The latter example can also lead to changes in active sites or conformational changes to a protein. Similarly, mechanical loads or physical forces can also change the conformation of a protein(s) and direct and transmit signals (Ingber 1997; Yusko and Asbury 2014; Ingber et al. 2014). With respect to mechanical forces, the cytoskeleton has a key responsibility in transmission, direction and ultimately conversion of the force(s) into biochemical signals, or mechanotransduction, via force-sensitive proteins. Considerable evidence now indicates that heat shock proteins (HSP) also play important roles as force sensitive proteins in assisting the cytoskeleton with mechanotransduction. Thus, this chapter will review examples of HSP as force sensitive proteins in the promotion of signalling from mechanical stresses within different cells and tissues. The author apologizes in advance for the inevitable omission of some published works in the field.

### *6.1.1 Heat Shock Proteins*

The discovery of the heat shock response occurred over 50 years ago during research on nucleic acid synthesis in Drosophila salivary glands inadvertently exposed to higher than normal temperature (Ritossa 1962). Heat shock proteins were subsequently discovered (Tissieres et al. 1974) and they are now known to comprise large families of very conserved proteins with important roles in cellular homeostasis and

cytoprotection from chronic or acute stressors including mechanical forces. The HSP are now grouped under HUGO Gene Nomenclature Committee guidelines (former names in parentheses) as: HSPA (HSP70), HSPB (small HSP), HSPC (HSP90), HSPH (HSP110), DNAJ (HSP40), and the human chaperonin (HSP60 and CCT) families (Kampinga et al. 2009), but use of the older nomenclature may still be used here for consistency with the original papers reviewed. As molecular chaperones HSP can participate in physiological processes and protect proteins from aggregation, promote their proper folding, and help mediate protein degradation under physiological or stressful conditions (Lanneau et al. 2010; Hartl et al. 2011; Mymrikov et al. 2011). If the protein substrates cannot be refolded then the HSP will direct them to the degradation machinery.

The larger molecular weight HSPA, HSPC and chaperonin families in particular are complex molecular machines that directly promote protein folding in an ATP-regulated manner (Hartl et al. 2011). Co-chaperones also bind with these HSP to modulate chaperone function (Lanneau et al. 2010). The HSPB family is comprised of eleven small molecular weight proteins (HSPB1–11; 15–40 kDa) that are also important for cellular homeostasis and induced by physiological stressors (reviewed by Laskowksa et al. 2010; Kampinga and Garrido 2012; Mymrikov et al. 2011). Canonically, HSPB members act as ATP-independent molecular chaperones by binding unfolded proteins post-stress and preventing them from irreversible aggregation until they are passed on to the ATP-dependent chaperone networks (e.g. HSP70/HSPA family) for refolding (Laskowska et al. 2010; Mymrikov et al. 2017). The HSPB proteins also assist in other processes such as cytoskeletal rearrangements, regulation of cell death, and immune system activation (Acunzo et al. 2012; van Noort et al. 2012; Wettstein et al. 2012). Many HSPB members can heteroligomerize with one another, thus adding greater functional complexity (Zantema et al. 1992; Fontaine et al. 2005). Overall, this chapter will largely focus on the HSPA, B and C families.

### *6.1.2 Sensing Mechanical Stress in the Heart, Vascular Wall and Visceral Smooth Muscle*

Mechanical overload of the heart has been known for quite some time to induce the expression of HSP70 (Izumo et al. 1988; Delcayre et al. 1988). Knowlton et al. (1991) used isolated perfused rabbit hearts, apparently free of hormonal and neural factors, and elongated the heart by inserting a cannula through the mitral valve then pushing against the endocardial surface until elongation was observed. They showed myocardial fibre stretch significantly increased HSP70 mRNA and protein expression. From a signalling standpoint, HSP70 induction was not influenced by inhibition of Protein Kinase C (PKC) activity with the specific inhibitor H7.

Heat shock factor (HSF) expression and activation occurs rapidly in response to stresses and as transcription factors can regulate HSP expression (Silver and Noble

2012). Since HSF1 activation and HSP70 expression had been reported in rat aorta (Udelsman et al. 1993; Xu et al. 1995, 1996), Chang et al. (2001) examined HSF1 activation and the effect on HSP70 expression in the perfused rabbit heart. When hearts were subjected to stretch, HSF1 was activated and it led to a simultaneous induction of HSP70 mRNA expression. Notably, the effect of stretch on HSF1 and HSP70 expression was found to be mediated by stretch-activated channels in these hearts, but not L-type calcium channels.

Mechanical stresses in the vascular wall include stretch by muscular action, cyclic pulsatile stretch due to pressure and shear stress from blood flow (Luo et al. 2007). All of these mechanical stresses are potent inducers of HSP expression. Wang et al. (2007) conducted a proteome analysis of bovine vascular endothelial cells exposed to laminar shear stress. HSP70 protein expression increased dramatically 12 fold after 6 h of shear flow stress. This correlated well with work by Brooks et al. (2002) where they reported a significant increase in HSP70 mRNA expression after prolonged steady laminar flow. When endothelial cells are exposed to high shear stress loading or stretch they respond by forming stress fibres (Franke et al. 1984; Langille et al. 1991). These fibres are largely made up of actin fibres and connected to integrins at the plasma membrane where they are important in maintaining cell integrity (Burridge et al. 1988). Luo et al. (2007) showed that rat arterial endothelial cells exposed to mechanical stretch increased expression of HSP70 and HSP27 (HSPB1), but not HSP90. Strikingly, exposure of the cells to quercetin, an inhibitor of HSP70, inhibited both the expression of HSP70 and stress fibre formation in the cells in response to stretch suggesting a role for HSP70 in stress fibre formation.

The response of HSP to mechanical forces can vary depending on the force applied, its duration, or even in a tissue-dependent manner. For example, unlike Luo et al. (2007) where HSP90α was not induced by stretch in endothelial cells, HSP90α expression and secretion was markedly induced in three-dimensionally cultured fibroblasts exposed to sustained compressive loading (Kanazawa et al. 2014). Expression of HSF1 and HSF2 as well as HSP including HSP40, HSP60 and HSC70 were also induced by the compressive loading stress.

Smooth muscle is a tissue that is particularly responsive to mechanical forces, particularly since it must quickly adapt to its local environment (Salinthone et al. 2008). For example, Xu et al. (2000) demonstrated that cyclic strain in vascular smooth muscle activated HSF1 and induced HSP70 expression. Although utilizing an overexpression strategy, which should be interpreted carefully, the authors showed that the response observed was regulated by Rac and Ras GTP-binding proteins. Inhibition of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) with specific inhibitors did not affect HSF1 activation. Clearly, the literature suggests great complexity in HSP responses and regulation.

The small heat shock protein B (HSPB) family has been intensely studied in smooth muscle (Salinthone et al. 2008). McGregor et al. (2004) examined the response of saphenous vein smooth muscle to hemodynamic stress. The human saphenous vein is a common choice for bypass grafting, but in the scenario of the

arterial bypass the grafted vessel is subsequently exposed to high pressure, pulsatile arterial circulation. This is in marked contrast to the normal environment and leads to hemodynamic stress. Using 2-D gel electrophoresis, the authors reported increased abundance of HSPB1 (~2 fold) in saphenous vein smooth muscle following exposure to simulated arterial flow compared to simulated venous flow.

Chaudhuri and Smith (2008) also examined cyclic strain effects in airway smooth muscle. When these cells were cultured and exposed to cyclic strain with a Flexcell tension system (Flexcell International, Burlington, NC, USA), HSPB1 was rapidly phosphorylated. As a means to understand mechanism, the authors investigated potential upstream mediators with inhibitor studies and found HSPB1 phosphorylation was abolished following cell incubation with p38 MAPK inhibitors. The inhibition of ERK, PKC, or Rho kinase had no effect on HSPB1 phosphorylation upon cyclic strain. With transfection of various isoforms of HSPB1, the authors demonstrated that HSPB1 activation was necessary for actin microfilament stability in these cells upon cyclic strain.

Lately, the uterine smooth muscle or myometrium has been a focus of research on HSPB family member expression and response to stretch. The myometrium undergoes structural, physiological, and biochemical changes throughout pregnancy over four periods: proliferative, synthetic, contractile and labour phases (MacPhee and Miskiewicz 2017). There is clearly a transitional period prior to the synthetic phase marked by activation of an intrinsic apoptotic pathway in the myometrium, yet no indication of significant apoptosis (Shynlova et al. 2009, 2010). The transition could be a result, in part, of uterine conversion (Reynolds 1949; Shynlova et al. 2009). At this time the fetus appears to reach a maximal spherical radius resulting in a locally stretched and stressed uterus marked by ischemia (Shynlova et al. 2010). The embryo then undergoes a change to a more ellipsoid shape, presumably releasing local tension stress and aiding re-establishment of uterine blood flow. Subsequently, one of the key biophysical stressors during the synthetic phase that then continues through to the labour phase is increasing uterine distension due to ever increasing fetal growth inducing myometrial hypertrophy.

White et al. (2005) demonstrated that HSPB1 mRNA expression and protein levels were significantly increased during late pregnancy and labour in the rat myometrium corresponding to late synthetic, contractile and labour phases. Despite this knowledge, it was not known how expression was regulated in this tissue. White and MacPhee (2011) examined the effect of uterine distension on myometrial HSPB1 expression using a unilaterally pregnant rat model. In this strategy, female rats (bicornuate uteri) prior to mating underwent a surgical ligation of one of their oviducts so that fertilization could not take place and the respective uterine horn would contain no fetuses (i.e. unstretched uterine horn). Fertilization could still take place in the contralateral oviduct and the respective uterine horn would become gravid and exposed to distension. HSPB1 mRNA and pSer15-HSPB1 protein expression were significantly elevated in distended gravid uterine horns at both late pregnancy (Day (D) 19) and labour (D23) of gestation compared to non-gravid horns. Similarly, pSer15-HSPB1 protein detection in situ was only observed in the distended horns compared to the non-gravid horns at both timepoints. HSPB1 mRNA and

pSer15-HSPB1 protein expression were also markedly increased in myometrium from ovariectomized, non-pregnant rat uteri distended for 24 h with laminaria tents compared to empty horns (White and MacPhee 2011). This ruled out the necessity, but not necessarily the ability, of ovarian hormones to contribute to the increased expression observed during pregnancy. It was postulated that the response of HSPB1 to uterine stretch could regulate actin cytoskeleton dynamics at focal adhesion sites and support uterine distension-induced hypertrophy and subsequent focal adhesion reorganization during late pregnancy (White and MacPhee 2011).

CRYAB or HSPB5, previously known as αB-crystallin, was discovered as a highly abundant eye lens protein that helped maintain the transparency of the structure (Bloemendal 1982; Clark et al. 2012). Nicoletti et al. (2016) also discovered that uterine stretch was a major player in regulation of CRYAB expression. Both total and pSer59-CRYAB protein expression were significantly elevated in gravid uterine horns at both D19 and D23 of gestation compared to contralateral non-gravid horns of unilaterally pregnant rats. The authors also reported that pSer59-CRYAB co-localized with the focal adhesion protein FERMT2 at the ends of actin filaments in myometrial cells. Thus, Nicoletti et al. (2016) postulated that pSer59-CRYAB may be part of a mechano-adaptive response, perhaps in partnership with pSer15-HSPB1, to modulate actin polymerization dynamics at focal adhesions in the myometrium during late pregnancy and to facilitate subsequent phasic labour contractions.

The small HSP (sHSP) HSPB8 is also highly expressed in smooth muscle and it is an important facilitator of protein quality control such as in protecting cells from the accumulation of aggregated proteins (Mymrikov et al. 2011). HSPB8 was found to interact with the adapter protein Bcl2-associated athanogene 3 (BAG3), which is known to serve as a stimulator of a protein quality control pathway known as macroautophagy (Carra et al. 2008; Fuchs et al. 2010; Hishiya et al. 2011). Like some sHSP, BAG3 gene expression is stress inducible (Rosati et al. 2011). Both HSPB8 and BAG3 protein expression were significantly elevated during the early synthetic phase of myometrial differentiation marked by initiation of uterine distension and myometrial hypertrophy (Marsh et al. 2015). Analysis of HSPB8 and BAG3 protein expression in myometrium from unilateral pregnancies also revealed that expression of both proteins was significantly increased on D15 of gestation by uterine distension. Since muscle tissue relies on an effective proteostasis network (Arndt et al. 2010), it was postulated that HSPB8 and BAG3 were important to help maintain protein homeostasis, particularly during the early synthetic phase of myometrial differentiation where they could prevent aggregation or promote degradation of misfolded proteins during the initiation of myometrial hypertrophy.

As already demonstrated, sHSP are highly expressed in smooth muscle and some like HSPB1 and HSPB6 have some role(s) in regulating the contractile machinery (Salinthone et al. 2008) although the exact mechanism(s) utilized is far from clear. Recently, Wang et al. (2015) stretched bladder smooth muscle cells (14.8% elongation) and reported that this markedly disrupted the actin cytoskeleton and decreased the filamentous/globular (F/G) actin ratio in these cells. However, actin filament structures recovered and the F/G ratio significantly increased at 12 h post-stretch in cells

overexpressing HSPB1 compared to control stretched cells with normal endogenous HSPB1 levels. This indicated HSPB1 promoted cytoskeletal recovery following stretch. The authors also provided evidence that HSPB1 overexpressing bladder smooth muscle cells exhibited enhanced contractile force and this was reduced by HSPB1 knockdown. Such information continues to add to the growing body of evidence (e.g. Miron et al. 1991; Lavoie et al. 1993a, b; Benndorf et al. 1994; Ibitayo et al. 1999; Bitar 2002) that HSPB1 has a role(s) in smooth muscle contraction.

HSPB family members also respond to mechanical forces in other types of muscle. Functional overload of skeletal muscle is a potent inducer of hypertrophy and mechanical stress. Several studies have shown that exposure of skeletal muscle to decreases or increases in muscle loading leads to changes in HSPB1 expression (Inaguma et al. 1993; Huey et al. 2004). For example, Huey et al. (2006) showed that functional overload of soleus and plantaris muscles in rats led to the induction of HSPB1 mRNA expression and increased levels of total HSPB1 and phosphorylated HSPB1 (pHSPB1) within insoluble fractions of muscle consistent with a possible role in stabilizing the actin cytoskeleton. Other roles for HSPB members occur in muscle following mechanic stress. In cardiomyocytes, CRYAB expression is also induced in cells under stretch (Goncalves et al. 2016). CRYAB can regulate signal transduction networks including the focal adhesion protein focal adhesion kinase (FAK) and the tyrosine phosphatase Shp2 (Pereira et al. 2014; Goncalves et al. 2016). Pereira et al. (2014) showed that CRYAB interacted with FAK in cardiomyocytes and protected FAK from degradation and cells from apoptosis during stretch.

### *6.1.3 Sensing Mechanical Forces in The Lung*

It had been previously reported that HSP70 expression was responsive to volutrauma in the lung (Ribeiro et al. 2001). In the lung, mechanical ventilation or stretch can change lung cell activity which can lead to inflammation. To better understand the mechanotransduction pathway in the lung in vivo, Copland et al. (2003) studied alterations in gene expression within adult rat lungs exposed to high tidal volume ventilation, without causing injury, using gene arrays. HSP70 mRNA expression was significantly increased in lungs exposed to a 30 min ventilation. Temporally, HSP70 expression followed the earlier induction of c-Jun expression and HSP70 expression was particularly noted in bronchiolar and alveolar epithelium (Copland et al. 2003). Furthermore, interleukin-1 beta (IL-1β) mRNA expression followed a temporal pattern of expression similar to HSP70 suggesting a link to inflammation as IL-1β has been associated with stretch-induced lung injury (Copland et al. 2004).

Using a Flexcell in vitro cyclic stretch system, Copland and colleagues (2007) then confirmed the stretch-induced expression of HSP70 among early response genes in fetal rat lung epithelial cells. They also examined the potential role of various signaling proteins in eliciting the expression of the early response genes. Nuclear factor kappa B (NFkB) mediated the sustained and gradual expression of HSP70 following the stretch, which had also been reported in

cardiomyocytes and vascular endothelium (Hamilton et al. 2004 a, b). Furthermore, upstream inhibition of calcium mobilization in the fetal lung epithelial cells prevented stretch-induced NFkB activation and HSP70 expression. Since the small GTPase Ras is induced by calcium influx (Ghosh and Greenberg 1995), Copland and colleagues inhibited Ras in the fetal rat lung epithelial cells and found that this also reduced HSP70 expression. The authors postulated that NFkB-mediated the stretch-induced expression of HSP70 in the lung, perhaps as a negative feedback mechanism that would regulate the duration of NFkB activation since HSP70 could bind and stabilize Inhibitor kappa B (IkB), thereby maintaining a complex with NFkB, and prevent NFkB activation (Wong and Wispe 1997).

### *6.1.4 Sensing Mechanical Stress in Supporting Tissues and Cells*

Supporting tissues and cells that comprise them, such as fibroblasts, are important sensors and responders to mechanical forces. The periodontal ligament (PDL) is an example of these characteristics. It is comprised of vascular and cellular tissue and located between the tooth and alveolar bone where it acts as a shock absorber that anchors the tooth root to the jaw bone (Takano-Yamamoto et al. 1994; Lekic and McCulloch 1996). Fibroblasts are a major cellular component of the PDL. The PDL participates in tissue remodelling under the influence of mechanical stresses such as occlusal and orthodontic forces (Araujo et al. 2007; Arai et al. 2010). The latter is important for inducing early stages of tooth movement.

Arai et al. (2010) examined HSP expression on the pressure side of the PDL during early stages of orthodontic tooth movement with an in vivo rat model, laser microdissection, real time PCR and immunohistochemistry. They reported structural changes in the pressure zone of the PDL and within this zone mRNA expression of inducible HSP70 (HSPA1A) was significantly increased after 6 h of orthodontic stress. Immunohistochemical analysis also showed increased detection of HSPA1A in this zone after 6 h, specifically in fibroblasts and osteoblasts. Furthermore, HSPA1A was detected extracellularly, consistent with other reports and a possible role as a pro-inflammatory mediator when expressed in this manner (DeMaio 2011).

Muraoka et al. (2010) also examined HSP expression in mouse periodontal tissue following orthodontic mechanical stress. As an example of how HSP can respond differently to mechanical forces, unlike results of Arai et al. (2010) where expression of HSPA1A was in the pressure zone, Muraoka et al. reported that HSPB1 and pSer15-HSPB1 expression was largely in the tension side of the PDL following mechanical stress. HSPB1 expression was specific to fibroblasts and cementoblasts of the tension side after 3 h of stress and also became expressed in osteoblasts after 9 h of stress. The expression pattern of HSPB1 may reflect the need for tissue remodelling, especially cytoskeletal reorganization, on the tension side of the PDL. Both of these papers utilized more in vivo approaches and extended early

in vitro work by Maeda et al. (1997) and Okazaki et al. (2000). The former reported that cultured human PDL-derived fibroblasts had increased HSP60, HSP70 and HSP90 expression following continuous compression while the latter reported increased expression of HSP47, HSP60 and HSP70 following cyclic stretching of human PDL fibroblasts.

## 6.2 Conclusions

Despite the data reviewed above, a significant deficit in our understanding of mechanical forces and the induction of HSP expression in cells and tissues is: what signalling pathways are involved in such situations? While an NFkB signalling pathway may play a role, especially in relation to induction of HSP70 expression and inflammation (see above), recent work on fibroblasts has shed some light on another pathway that pertains to HSPB members and the cytoskeleton, the latter being critical for mechanotransduction. Hoffman et al. (2017) cultured mouse fibroblasts and then exposed them to uniaxial cyclic stretch. This led to activation of p38 MAPK and phosphorylation of HSPB1 on Serine 86 (analogous to human serine 82) in a p38 MAPK-dependent manner. Actin stress fibres were thickened or reinforced in these cells following stretch and pHSPB1 became localized prominently to actin comet tails, which are sites of actin polymerization at focal adhesions (Guo and Wang, 2007). When HSPB1 phosphorylation was inhibited, its accumulation at the cytoskeleton was reduced following stretch. HSPB1 null fibroblasts also displayed reduced actin reinforcement after stretch and an increase in cell migration. Furthermore, it appeared that HSPB1 localized to free F-actin barbed ends, as accumulation of pHSPB1 at comet tails was reduced with Cytochalasin D treatment. These findings now add to our understanding of the role of HSPB1 and p38 MAPK in actin remodelling following mechanical stress, and provide information that could be relevant to specific tissues. For example, p38 MAPK expression in the uterine myometrium is also responsive to uterine distension during late pregnancy (Oldenhof et al. 2002) and could be responsible, in part, for modulating HSPB1 phosphorylation and function in the uterine musculature. In total, more research is needed to understand the signalling pathways underlying HSP expression and function during mechanical stresses.

Many HSP are now identified (Kampinga et al. 2009) and yet it is unclear how many can act as sensors for mechanical stress. Continued exploration and documentation of mechanical stress sensitive HSP are needed including defining whether or not certain HSP are required to respond to specific mechanical forces. There are also large numbers of co-chaperones and co-regulators that modulate HSP function (Gamerdinger et al. 2011). As already reviewed earlier, the adapter BAG3 is stretch inducible (Marsh et al. 2015). How many other proteins of this nature are mechanical sensors and how do they specifically modulate mechanical stress responses in concert with HSP? This is a major area for future research.

In conclusion, HSP are highly responsive to stresses including temperature, ischaemia, hypoxia and endotoxins. It is now clear that different mechanical stresses

also induce HSP expression in a variety of cells and tissues including cardiac, skeletal and smooth muscle, the vascular wall, lung and supporting tissues. Furthermore, signalling pathways such as the NFkB and p38MAPK pathways can modulate the increased expression and post-translational modification of HSP, but given that HSP are proteins with many talents it is likely regulation of HSP expression and function under mechanical stress will be even more complex than currently appreciated.

**Acknowledgements** This work was supported in part by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (RGPIN-2017-04951) and a Canada Foundation for Innovation John R Evans Leaders Fund (Project # 32512).

## References

Acunzo, J., Katsogiannou, M., & Rocchi, P. (2012). Small heat shock proteins HSP27 (HSPB1), alphaB-crystallin (HSPB5) and HSP22 (HSPB8) as regulators of cell death. *The International Journal of Biochemistry & Cell Biology, 44*, 1622–1631.

Arai, C., Nomura, Y., Ishikawa, M., Noda, K., Choi, J. W., Yashiro, Y., Hanada, N., & Nakamura, Y. (2010). HSPA1A is upregulated in periodontal ligament at early stage of tooth movement in rats. *Histochemistry and Cell Biology, 134*, 337–343.

Araujo, R. M. S., Oba, Y., & Moriyama, K. (2007). Identification of genes related to mechanical stress in human periodontal ligament cells using microarray analysis. *Journal of Periodontal Research, 42*, 15–22.

Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., Furst, D. O., Saftig, P., Saint, R., Fleischmann, B. K., Hoch, M., & Hohfeld, J. (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. *Current Biology, 20*, 143–148.

Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J., & Lutsch, G. (1994). Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization inhibiting activity. *The Journal of Biological Chemistry, 269*, 20780–20784.

Bitar, K. N. (2002). HSP27 phosphorylation and interaction with actin and myosin in smooth muscle contraction. *The American Journal of Physiology, 282*, G894–G903.

Bloemendal, H. (1982). Lens protein. *Critical Reviews in Biochemistry and Molecular Biology, 12*, 1–38.

Brooks, A. R., Lelkes, P. I., & Rubanyi, G. M. (2002). Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. *Physiological Genomics, 9*, 27–41.

Burridge, K., Fath, K., Kelly, T., Nuckollos, G. C., & Turner, C. (1988). Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annual Review of Cell Biology, 4*, 487–525.

Carra, S., Seguin, S. J., & Landry, J. (2008). HSPB8 and Bag3: A new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy, 4*, 237–239.

Chang, J., Wasser, J. S., Cornelussen, R. N. M., & Knowlton, A. A. (2001). Activation of heat-shock factor by stretch-activated channels in rat hearts. *Circulation, 104*, 209–214.

Chaudhuri, S., & Smith, P. G. (2008). Cyclic strain-induced hsp27 phosphorylation modulates actin filaments in airway smooth muscle cells. *American Journal of Respiratory Cell and Molecular Biology, 39*, 270–278.

Clark, A. R., Lubsen, N. H., & Slingsby, C. (2012). sHSP in the eye lens: Crystallin mutations, cataract and proteostasis. *The International Journal of Biochemistry & Cell Biology, 44*, 1687–1697.

Copland, I. B., & Post, M. (2007). Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. *Journal of Cellular Physiology, 210*, 133–143.

Copland, I. B., Kavanagh, B. P., Engelberts, D., McKerlie, C., Belik, J., & Post, M. (2003). Early changes in lung gene expression due to high tidal volume. *American Journal of Respiratory and Critical Care Medicine, 168*, 1051–1059.

Copland, I. B., Martinez, F., Kavanagh, B. P., Engelberts, D., McKerlie, C., Belik, J., & Post, M. (2004). High tidal volume ventilation causes different inflammatory responses in newborn versus adult lung. *American Journal of Respiratory and Critical Care Medicine, 169*, 739–748.

De Maio, A. (2011). Extracellular heat shock proteins, cellular export vesicles, and the stress observation system: A form of communication during injury, infection, and cell damage. *Cell Stress & Chaperones, 16*, 235–249.

Delcayre, C., Samuel, J.-L., Marotte, F., Best-Belpomme, M., Mercadier, J. J., & Rappaport, L. (1988). Synthesis of stress proteins in rat cardiac myocytes 2–4 days after imposition of hemodynamic overload. *The Journal of Clinical Investigation, 82*, 460–468.

Fontaine, J. M., Sun, X., Benndorf, R., & Welsh, M. J. (2005). Interactions of HSP22 (HSPB8) with HSP20, alphaB-crystallin, and HSPB3. *Biochemical and Biophysical Research Communications, 337*, 1006–1011.

Franke, R.-P., Grafe, M., Schnittler, H., Seiffge, D., Mitter-Mayer, C., & Drenckhahan, D. (1984). Induction of human vascular endothelial stress fibers by fluid shear stress. *Nature, 307*, 648–649.

Fuchs, M., Poirier, D. J., Seguin, S. J., Lambert, H., Carra, S., Charette, S. J., & Landry, J. (2010). Identification of the key structural motifs involved in HSPB8/HSPB6-Bag3 interaction. *The Biochemical Journal, 425*, 245–255.

Gamerdinger, M., Carra, S., & Behl, C. (2011). Emerging roles of molecular chaperones and co-chaperones in selective autophagy: Focus on BAG proteins. *Journal of Molecular Medicine, 89*, 1175–1182.

Ghosh, A., & Greenberg, M. E. (1995). Calcium signaling in neurons: Molecular mechanisms and cellular consequences. *Science, 268*, 239–247.

Goncalves, D. C., Marin, T. M., Pereira, M. B. M., Santos, A. M., Leme, A. F. P., & Franchini, K. G. (2016). αB-Crystallin interacts and attenuates the tyrosine phosphatase activity of Shp2 in cardiomyocytes under mechanical stress. *FEBS Letters, 590*, 2232–2240.

Guo, W. H., & Wang, Y. L. (2007). Retrograde fluxes of focal adhesion proteins in response to cell migration and mechanical signals. *Molecular Biology of the Cell, 18*, 4519–4527.

Hamilton, K. L., Gupta, S., & Knowlton, A. A. (2004a). Estrogen and regulation of heat shock protein expression in female cardiomyocytes: Cross-talk with NF kappa B signaling. *Journal of Molecular and Cellular Cardiology, 36*, 577–584.

Hamilton, K. L., Mbai, F. N., Gupta, S., & Knowlton, A. A. (2004b). Estrogen, heat shock proteins, and NFkappaB in human vascular endothelium. *Arteriosclerosis, Thrombosis, and Vascular Biology, 24*, 1628–1633.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*, 324–332.

Hishiya, A., Salman, M. N., Carra, S., Kampinga, H. H., & Takayama, S. (2011). BAG3 directly interacts with mutated alphaB-crystallin to suppress its aggregation and toxicity. *PLoS One, 6*, e16828.

Hoffman, L., Jensen, C. C., Yoshigi, M., & Beckerle, M. (2017). Mechanical signals activate p38 MAPK pathway-dependent reinforcement of actin via mechanosensitive HspB1. *Molecular Biology of the Cell, 28*, 2661–2675.

Huey, K. A. (2006). Regulation of HSP25 expression and phosphorylation in functionally overloaded rat plantaris and soleus muscles. *Journal of Applied Physiology, 100*, 451–456.

Huey, K. A., Thresher, J. S., Brophy, C. M., & Roy, R. R. (2004). Inactivity-induced modulation of Hsp20 and Hsp25 content in rat hindlimb muscles. *Muscle & Nerve, 30*, 95–101.

Ibitayo, A. I., Sladick, J., Tuteja, S., Louis-Jacques, O., Yamada, H., Groblewski, G., Welsh, M., & Bitar, K. N. (1999). Hsp27 in signal transduction and association with contractile proteins in smooth muscle cells. *The American Journal of Physiology, 277*, G445–G454.

Inaguma, Y., Goto, S., Shinohara, H., Hasegawa, K., Ohshima, K., & Kato, K. (1993). Physiological and pathological changes in levels of the two small stress proteins, HSP27 and alpha B crystallin, in rat hindlimb muscles. *Journal of Biochemistry, 114*, 378–384.

Ingber, D. E. (1997). Tensegrity: The architectural basis of cellular mechanotransduction. *Annual Review of Physiology, 59*, 575–599.

Ingber, D. E., Wang, N., & Stamenovic, D. (2014). Tensegrity, cellular biophysics, and the mechanics of living systems. *Reports on Progress in Physics, 77*, 046603.

Izumo, S., Nadal-Ginard, B., & Mahdavi, V. (1988). Protooncogene induction and reprogramming of cardiac gene expression produced by pressure over-load. *Proceedings of the National Academy of Sciences of the United States of America, 85*, 339–343.

Kampinga, H. H., & Garrido, C. (2012). HSPBs: Small proteins with big implications in human disease. *The International Journal of Biochemistry & Cell Biology, 44*, 1706–1710.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B., & Hightower, L. E. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress & Chaperones, 14*, 105–111.

Kanazawa, T., Nakagami, G., Minematsu, T., Yamane, T., Huang, L., Mugita, Y., Noguchi, H., Mori, T., & Sanada, H. (2014). Biological responses of three-dimensional cultured fibroblasts by sustained compressive loading include apoptosis and survival activity. *PLoS One, 9*, e104676.

Knowlton, A. A., Eberli, F. R., Brecher, P., Romo, G. M., Owen, A., & Apstein, C. S. (1991). A single myocardial stretch or decreased systolic fiber shortening stimulates the expression of heat shock protein 70 in the isolated, erythrocyte-perfused rabbit heart. *The Journal of Clinical Investigation, 88*, 2018–2025.

Langille, B. L., Graham, J. J., Kim, D. W., & Gotlieb, A. I. (1991). Dynamics of shear-induced redistribution of F-actin in endothelial cells in vivo. *Arteriosclerosis Thrombosis, 11*, 1814–1820.

Lanneau, D., Wettstein, G., Bonniaud, P., & Garrido, C. (2010). Heat shock proteins: Cell protection through protein triage. *Scientific World Journal, 10*, 1543–1552.

Laskowska, E., Matuszewska, E., & Kuczynska-Wisnik, D. (2010). Small heat shock proteins and protein-misfolding diseases. *Current Pharmaceutical Biotechnology, 11*, 146–157.

Lavoie, J. N., Gingras-Breton, G., Tanguay, R. M., & Landry, J. (1993a). Induction of Chinese hamster Hsp27 gene expression in mouse cells confers resistance to heat shock. Hsp27 stabilization of the microfilament organization. *The Journal of Biological Chemistry, 268*, 3420–3429.

Lavoie, J. N., Hickey, E., Weber, L. A., & Landry, J. (1993b). Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. *The Journal of Biological Chemistry, 268*, 24210–24214.

Lekic, P., & McCulloch, C. A. G. (1996). Periodontal ligament cell populations: The central role of fibroblasts in creating a unique tissue. *The Anatomical Record, 245*, 327–341.

Luo, S.-S., Sugimoto, K., Fujii, S., Takemasa, T., Fu, S.-B., & Yamashita, K. (2007). Role of heat shock protein 70 in induction of stress fiber formation in rat arterial endothelial cells in response to stretch stress. *Acta Histochemica et Cytochemica, 40*, 9–17.

MacPhee, D. J., & Miskiewicz, E. I. (2017). The potential functions of small heat shock proteins in the uterine musculature during pregnancy. *Advances in Anatomy, Embryology, and Cell Biology, 222*, 95–116.

Maeda, T., Kameda, T., & Kameda, A. (1997). Loading of continuously applied compressive force enhances production of heat shock protein 60, 70 and 90 in human periodontal ligament-derived fibroblast-like cells. *The Journal of Japan Orthodontic Society, 56*, 296–302.

Marsh, N. M., Wareham, A., White, B. G., Miskiewicz, E. I., Landry, J., & MacPhee, D. J. (2015). HSPB8 and the cochaperone BAG3 are highly expressed during the synthetic phase of rat myometrium programming during pregnancy. *Biology of Reproduction, 92*, 131.

McGregor, E., Kempster, L., Wait, R., Gosling, M., Dunn, M. J., & Powell, J. T. (2004). F-actin capping (CapZ) and other contractile saphenous vein smooth muscle proteins are altered by hemodynamic stress. *Molecular & Cellular Proteomics, 3*, 115–124.

Miron, T., Vancompernolle, K., Vanderkerckhove, J., Wilchek, M., & Geiger, B. (1991). A 25 kDa inhibitor of actin polymerization is a low molecular mass heat shock protein. *The Journal of Cell Biology, 114*, 255–261.

Muraoka, R., Nakano, K., Kurihara, S., Yamada, K., & Kawakami, T. (2010). Immunohistochemical expression of heat shock proteins in the mouse periodontal tissues due to orthodontic mechanical stress. *European Journal of Medical Research, 15*, 475–482.

Mymrikov, E. V., Seit-Nebi, A. S., & Gusev, N. B. (2011). Large potentials of small heat shock proteins. *Physiological Reviews, 91*, 1123–1159.

Mymrikov, E. V., Daake, M., Richter, B., Haslbeck, M., & Buchner, J. (2017). The chaperone activity and substrate spectrum of human small heat shock proteins. *The Journal of Biological Chemistry, 292*, 672–684.

Nicoletti, J. G., White, B. G., Miskiewicz, E. I., & MacPhee, D. J. (2016). Induction of expression and phosphorylation of heat shock protein B5 (CRYAB) in rat myometrium during pregnancy and labour. *Reproduction, 152*, 69–79.

Okazaki, M., Shimizu, Y., Chiba, M., & Mitani, H. (2000). Expression of heat shock proteins induced by cyclical stretching stress in human periodontal ligament fibroblasts. *Tohoku University Dental Journal, 19*, 108–115.

Oldenhof, A. D., Shynlova, O., Liu, M., Langille, B. L., & Lye, S. J. (2002). Mitogen-activated protein kinases mediate stretch-induced c-fos mRNA expression in myometrial smooth muscle cells. *American Journal of Physiology. Cell Physiology, 283*, C1530–C1539.

Pereira, M. B. M., Santos, A. M., Goncalves, D. C., Cardoso, A. C., Consonni, S. R., Gozzo, F. C., Oliveira, P. S., Pereira, A. H. M., Figueiredo, A. R., Tiroli-Cepeda, A. O., Ramos, C. H. I., de Thomaz, A. A., Cesar, C. L., & Franchini, K. G. (2014). αB-crystallin interacts with and prevents stress-activated proteolysis of focal adhesion kinase by calpain in cardiomyocytes. *Nature Communications, 5*, 5159.

Reynolds, S.R.M. (1949) Gestational mechanisms. In Physiology of the uterus, 2nd Ed. Hoeber, New York, p. 218–234.

Ribeiro, S. P., Rhee, K., Tremblay, L., Veldhuizen, R., Lewis, J. F., & Slutsky, A. S. (2001). Heat stress attenuates ventilator-induced lung dysfunction in an ex vivo rat lung model. *American Journal of Respiratory and Critical Care Medicine, 163*, 1451–1456.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia, 18*, 571–573.

Rosati, A., Graziano, V., DeLaurenzi, V., Pascale, M., & Turio, M. C. (2011). BAG3: A multifaceted protein that regulates major cell pathways. *Cell Death & Disease, 2*, e141.

Salinthone, S., Tyagi, M., & Gerthoffer, W. T. (2008). Small heat shock proteins in smooth muscle. *Pharmacology & Therapeutics, 119*, 44–54.

Shynlova, O., Tsui, P., Jaffer, S., & Lye, S. J. (2009). Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 144*, S2–S10.

Shynlova, O., Kwong, R., & Lye, S. J. (2010). Mechanical stretch regulates hypertrophic phenotype of the myometrium during pregnancy. *Reproduction, 139*, 247–253.

Silver, J. T., & Noble, E. G. (2012). Regulation of survival gene hsp70. *Cell Stress & Chaperones, 17*, 1–9.

Takano-Yamamoto, T., Takemura, T., Kitamura, Y., & Nomura, S. (1994). Site-specific expression of mRNAs for osteonectin, osteocalcin, and osteopontin revealed by in situ hybridization in rat periodontal ligament during physiological tooth movement. *The Journal of Histochemistry and Cytochemistry, 42*, 885–896.

Tissieres, A., Mitchell, M. K., & Tracy, U. (1974). Protein synthesis in salivary glands of Drosophila melanogaster. Relation to chromosome puffs. *Journal of Molecular Biology, 84*, 389–398.

Udelsman, R., Blake, M. J., Stagg, C. A., Li, D. G., Putney, D. J., & Holbrook, N. J. (1993). Vascular heat shock protein expression in response to stress. *The Journal of Clinical Investigation, 91*, 465–473.

van Noort, J. M., Bsibsi, M., Nacken, P., Gerritsen, W. H., & Amor, S. (2012). The link between small heat shock proteins and the immune system. *The International Journal of Biochemistry & Cell Biology, 44*, 1670–1679.

Wang, X.-L., Fu, A., Raghavakaimal, S., & Lee, H.-C. (2007). Proteomic analysis of vascular endothelial cells in response to laminar shear stress. *Proteomics, 7*, 588–596.

Wang, Y., Xiong, Z., Gong, W., Zhou, P., Xie, Q., Zhou, Z., & Lu, G. (2015). Expression of heat shock protein 27 correlates with actin cytoskeletal dynamics and contractility of cultured human bladder smooth muscle cells. *Experimental Cell Research, 338*, 39–44.

Wettstein, G., Bellaye, P. S., Micheau, O., & Bonniaud, P. (2012). Small heat shock proteins and the cytoskeleton: An essential interplay for cell integrity? *The International Journal of Biochemistry & Cell Biology, 44*, 1680–1686.

White, B. G., & MacPhee, D. J. (2011). Distension of the uterus induces HspB1 expression in rat uterine smooth muscle. *The American Journal of Physiology, 301*, R1418–R1426.

White, B. G., Williams, S. J., Highmore, K., & MacPhee, D. J. (2005). Small heat shock protein 27 (Hsp27) expression is highly induced in rat myometrium during late pregnancy and labour. *Reproduction, 129*, 115–126.

Wong, H. R., & Wispe, J. R. (1997). The stress response and the lung. *The American Journal of Physiology, 273*, L1–L9.

Xu, Q., Li, D., Holbrook, N. J., & Udelsman, R. (1995). Acute hypertension induces heat shock protein 70 gene expression in rat aorta. *Circulation, 92*, 1223–1229.

Xu, Q., Fawcett, T. W., Udelsman, F. R., & Holbrook, N. J. (1996). Activation of heat shock transcription factor 1 in rat aorta in response to high blood pressure. *Hypertension, 28*, 53–57.

Xu, Q., Schett, G., Li, C., Hu, Y., & Wick, G. (2000). Mechanical stress–induced heat shock protein 70 expression in vascular smooth muscle cells is regulated by rac and ras small G proteins but not mitogen-activated protein kinases. *Circulation Research, 86*, 1122–1128.

Yu, H.-S., Kim, J.-J., Kim, H.-W., Lewis, M. P., & Wall, I. (2016). Impact of mechanical stretch on the cell behaviors of bone and surrounding tissues. *Journal of Tissue Engineering, 7*, 1–24.

Yusko, E. C., & Asbury, C. L. (2014). Force is a signal that cells cannot ignore. *Molecular Biology of the Cell, 25*, 3717–3725.

Zantema, A., Vries, M. V.-D., Maasdam, D., Bol, S., & Avd, E. (1992). Heat shock protein 27 and αB-crystallin can form a complex, which dissociates by heat shock. *The Journal of Biological Chemistry, 267*, 12936–12941.

Zhang, H., & Labouesse, M. (2012). Signalling through mechanical inputs – A coordinated process. *Journal of Cell Science, 125*, 3039–3049.

# Part II
# Chaperone Function of HSP

# Chapter 7
# The 70 KDA Heat Shock Protein Hsp70 as Part of a Protein Disaggregase System

**Maria Luiza Caldas Nogueira, Juliana Crotti Franco, Gabriela de Mello Gandelini, and Carlos Henrique Inacio Ramos**

**Abstract** Proteins participate in a wide variety of cellular functions, which enable many activities in our body. However, proteins need to reach their correct state of folding to function properly. In the cell, the folding of many nascent proteins is aided by the 70 kDa heat shock protein (Hsp70). When folding is not favorable, misfolded species accumulate leading to the formation of aggregates, which leads to loss of function and is the basis of several diseases. In addition to its function of aiding folding, Hsp70 is also an important agent in disaggregation, sometimes acting in a bichaperone system together with Hsp100 in several organisms, such as bacteria, fungi and plants. Surprisingly, animals lack a bonafide Hsp100 orthologue. To overcome this limitation, animals evolved a Hsp70-based disaggregation system, in which Hsp70 cooperates with Hsp40 and Hsp110 co-chaperones to reactivate aggregated substrates. This chapter revises the most recent models for the mechanism of interaction between these proteins and how they cooperate to solubilize protein aggregates.

**Keywords** Disaggregase · Hsp40 · Hsp70 · Hsp100 · Hsp110 · Misfolding

## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| Hsc | Heat shock cognate protein |
| Hsp | Heat shock protein |
| NBD | Nucleotide binding domain |
| NEF | Nucleotide exchange factor |
| PQC | Protein quality control |
| SBD | Substrate binding domain |

M. L. C. Nogueira · J. C. Franco · G. de Mello Gandelini · C. H. I. Ramos (✉)
Department of Organic Chemistry, Institute of Chemistry,
University of Campinas – UNICAMP, Campinas, SP, Brazil
e-mail: cramos@iqm.unicamp.br



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_7

## 7.1 Introduction

Proteins must assume their correct folding state to function properly. On the other hand, protein misfolding, aggregation and deposition (Fig. 7.1a) result in many human disorders from neurodegeneration (Alzheimer's and Parkinson's diseases) to diabetes and cancers (Anderson et al. 2011; Castellano and Shorter 2012; Chiti and Dobson 2006, 2017; De Felice et al. 2014; Douglas and Dillin 2010; Foguel and Silva 2004; Holmes et al. 2014; Johnson et al. 2012; Knowles et al. 2014; Macario and Conway de Macario 2000; Prahlad and Morimoto 2009; Ramos and Ferreira 2005; Silva et al. 2014; Stewart and Radford 2017; Valastyan and Lindquist 2014; Vicente Miranda and Outeiro 2010; Wolfe and Cyr 2011) (Table 7.1).

One of the most well-known and well-studied neurodegenerative diseases, Alzheimer's disease, is caused by the deposition of amyloid β-peptide (Aβ) in brain tissue (Hardy and Selkoe 2002), and it is estimated that it will afflict more than 70 million individuals during the next 40 years (Alzheimer's Association 2016). Other examples are amyotrophic lateral sclerosis (ALS) and Type II diabetes that affects more than 300 million of people (Ebrahimi-Fakhari et al. 2012; Knowles et al. 2014) and is known for increasing the risk of a heart attack

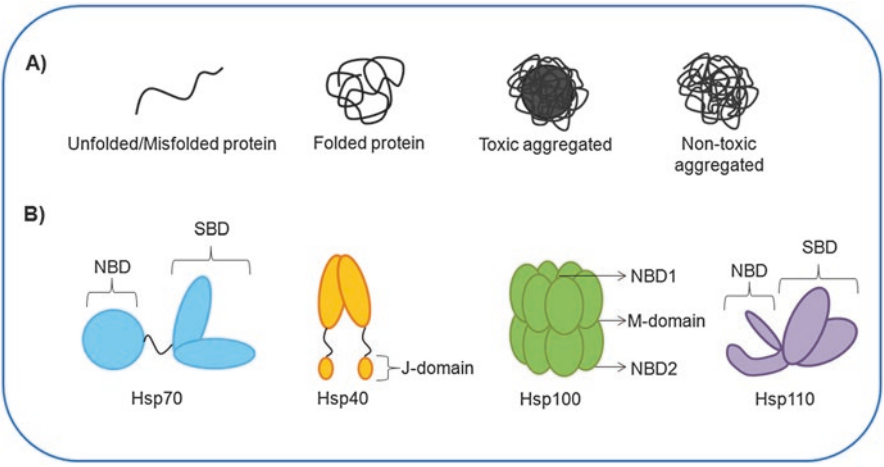


**Fig. 7.1** **(a)** Proteins in the cell. Proteins are produced in an unfolded state by the ribosome and have to fold to reach the native state. In some cases, proteins remain disordered (not shown) to accomplish their function. Misfolding leads to the loss of function and the formation of aggregates, which in many cases are toxic to the cell. **(b)** Hsp70, Hsp40, Hsp100 and Hsp110. Cartoon representing the two chaperone families, Hsp70 and Hsp100, and two co-chaperone families, Hsp40 and Hsp110, that are discussed in this chapter. Hsp70 family is monomeric and has two domains, the nucleotide binding domain (NBD) and the substrate binding domain (SBD). Hsp40 family is dimeric and is characterized by the presence of one J-domain in each monomer. Hsp100 family forms a hexameric structure and each monomer is formed by two NBDs, one at the N-terminus and the other at the C-terminus, which are connected by a middle domain (M). Hsp110 family is monomeric and similar to Hsp70 having both a NBD and a SBD

**Table 7.1 Examples of human diseases caused by protein misfolding and aggregates** (For reviews see (Abu-Hamad et al. 2017; Akiyama et al. 2004; Chaudhuri and Paul 2006, Cheng et al. 1990, Chiti and Dobson 2006; Collier et al. 2017; Fan and Ishii 2007; Knowles et al. 2014; Lv et al. 2016; Quinlan et al. 2007; Rinaldi and Fischbeck 2015; Satyal et al. 2000; Silverman et al. 2013; Takahashi et al. 2010; Tamarappoo et al. 1999; Valastyan and Lindquist 2014))

| Disease | Aggregating protein | Main protein characteristics | Localization |
|---|---|---|---|
| **α1 Antitrypsin deficiency** | α1 Antitrypsin Z | Mutants | Endoplasmic Reticulum |
| **Alexander disease** | GFAP | Mutants (Rosenthal fibres) | Cytosol |
| **Alzheimer disease** | Amyloid beta, Tau | Abeta peptide 1-42, 1-43 | Cytosol |
| **Amyotrophyic lateral sclerosis** | Superoxide dismutase | Mutants | Cytosol |
| **Familial british dementia** | Bri/Abri | Full-length extended | Cytosol |
| **Cancer** | p53 | Mutants | Cytosol |
| **Cataract** | Crystallin | Covalent protein damage | Cytosol |
| **Cystic fibrosis** | Cystic fibrosis membrane transporter | Mutants | Endoplasmic Reticulum |
| **Fabry disease** | α galactosidase A | Mutants | Lysosome |
| **Familial amyloidotic polyneuropathy** | Transthyretin | Mutants | Endoplasmic Reticulum |
| **Hemodialysis-related amyloidosis** | $\beta_2$-microglobulin | Full-length or truncated protein | Extracellular |
| **Huntington's** | Huntingtin | Mutants Poli(Q) | Nucleus and cytosol |
| **Insulin-related amyloid** | Insulin | Full-length | Extracellular |
| **Lysozyme amyloidosis** | Lysozyme | Full length, Mutants | Endoplasmic Reticulum, extracelular |
| **nephrogenic diabetes insipidus** | Aquaporin-2 / V2asopressin | Mutants | Endoplasmic Reticulum |
| **Parkinson disease** | Alpha synuclein | Mutants | Cytosol |
| **Polyglutamine diseases** | Polyglutamine | Mutants (poli Q) | Nucleus or cytosol |
| **Sickle cell anemia** | Hemoglobin | Mutants | Cytosol |
| **Spongiform encephalopathies** | Prion | Full-length | Endoplasmic Reticulum |
| **Type II diabetes** | Amylin | Full-length | Extracellular |
| **Wilson disease** | ATP7B | Mutants | Endoplasmic Reticulum |

(Olshansky et al. 2005) (Table 7.1). For all of these disorders, the current therapeutic approaches are, thus far, palliative and inefficient, since they are not able to reverse protein aggregates or misfolded proteins and then recover the proteostasis in affected cells (Shorter 2017).

Among the several lines of research towards the development of therapeutic strategies for misfolding maladies and even aging, the study of how the maintenance of proteostasis affects the development of the disease has been fruitful. A plethora of reviews has been prepared on this topic (for recent reviews see for example: (Balch et al. 2008; Calamini and Morimoto 2012; Chiti and Dobson 2017; Cuanalo-Contreras et al. 2013; Dubnikov et al. 2017; Goloubinoff 2016; Hipp et al. 2014; Hoogstra-Berends et al. 2012; Jeng et al. 2015; Kaushik and Cuervo 2015; Labbadia and Morimoto 2015; Mack and Shorter 2016; Vashist et al. 2010; Young 2014)). Molecular chaperones, also known as heat shock proteins (Hsp), although the terms are not always synonymous (Tiroli-Cepeda and Ramos 2011), allow for protection against misfolded aggregation-prone proteins and are among the most potent suppressors of neurodegeneration in human diseases ((Muchowski and Wacker 2005); also see the reviews above). Briefly these chaperones, together with the proteasome that includes the proteases, form a system termed protein quality control (PQC) that is responsible for the correct folding of client proteins and the degradation of misfolded proteins not repaired by the chaperones, helping to maintain protein homeostasis or proteostasis (Borges and Ramos 2005; Tiroli-Cepeda and Ramos 2011). Molecular chaperones are organized based on the molecular mass of their monomers, despite the fact that most of them exist as oligomers. Two of the most important chaperone families are Hsp70 and Hsp90, each being at the center of sub-systems that combine proper co-chaperones and specific client proteins, which are major players in the maintenance of proteostasis. Therefore, several reviews have been produced about these machines, and some recent reviews on the Hsp70 machinery are (Alderson et al. 2016; Bhandari and Houry 2015; Clerico et al. 2015; Cyr and Ramos 2015; da Silva and Borges 2011; De Maio 2014; Duncan et al. 2015; Li et al. 2016; Shrestha and Young 2016; Mayer 2013; Mayer and Kityk 2015; Przyborski et al. 2015; Young 2010; Zuiderweg et al. 2013; Zuiderweg et al. 2017).

The subject of disaggregase systems mainly deals with two chaperone families, Hsp70 and Hsp100, and two co-chaperone families, Hsp40 and Hsp110 (Fig. 7.1b and Table 7.2). The Hsp100 family is the classical disaggregase per se, and the other three, together or in collaboration with Hsp100, form a system with disaggregase function (Aguado et al. 2015; Mokry et al. 2015; Nillegoda and Bukau 2015; Sousa 2014; Sweeny and Shorter 2016). Therefore, it is possible to classify chaperones taking in account their function when interacting with substrates as holders, foldases, and disaggregases (Mayer 2010; Tiroli-Cepeda and Ramos 2011) (Fig. 7.2). Foldases (Hsp70, Hsp90, etc.) help the substrate adopt its native state in an ATP-dependent manner and are responsible for the major refolding activities in the chaperone system. Holders (Hsp40, small Hsp, etc.) are able to recognize, bind and stabilize unfolded or partially folded polypeptides to avoid aggregation and deliver

**Table 7.2 Some exemples for Hsp70, Hsp40, Hsp110 and Hsp100 genes in *Escherichia coli*, yeast, plants and humans.** The two chaperone families Hsp70 and Hsp100 as well as Hsp40 and Hsp110, two co-chaperone families, vary among the organisms and are the main subjects of this chapter

| | Hsp70 | | Hsp40 | | | |
|---|---|---|---|---|---|---|
| ORGANISM | iHsp70 | Hsc70 | TYPE I | TYPE II | Hsp100 | Hsp110 |
| ***E. coli*** | DnaK, HscA e HscC | – | DnaJ | CbpA | ClpB | – |
| **Yeast** | Ssa3p, Ssa4p | Ssa1p, Ssa2p | Ydj1, Xdj1, Apj1, Mdj1, Scj1 | Sis1, Djp1, Caj1, Hlj1 | Hsp104 | Sse1p, Sse2p |
| **Plant (*A. thaliana*)** | AtHsp70-1, AtHsp70-2, AtHsp70-3, AtHsp70-4, AtHsp70-5 | Hsc70-1, Hsc70-2, Hsc70-3, mtHsc70-1 and mtHsc70-2, cpHsc70-1, cpHsc70-2 | atDjA, | atDjB | atHsp101 | atHsp70-14 |
| **Human** | Hsp1A, Hsp1B, HspA6, HspA7, HspA14 | HspA1L, HspA2, HspA5, HspA8, HspA9, HspA12A, HspA12B, HspA13 | DnajA1, DnajA2, DnajA3, DnajA4 | DnajB1, DnajB2, DnajB3, DnajB4, DnajB5, DnajB6, DnajB7, DnajB8, DnajB9, DnajB10, DnajB11, DnajB12, DnajB13, DnajB14 | – | Hsp110 (or Hsp105), Apg1, Apg2 |

them to foldases in an ATP-independent manner. Disaggregases (Hsp100 and some other chaperone complexes; see below) use ATP hydrolysis to promote the reactivation of aggregated proteins inside the cell.

## 7.2 The HSP70/HSP40 Complex

The 70 kDa heat shock protein Hsp70 is the most important and well-studied family of chaperones because it plays a key role in the cellular network of the cellular PQC (protein quality control) system (Alderson et al. 2016; Clerico et al. 2015; da Silva and Borges 2011; Duncan et al. 2015; Li et al. 2016; Shrestha and Young 2016;

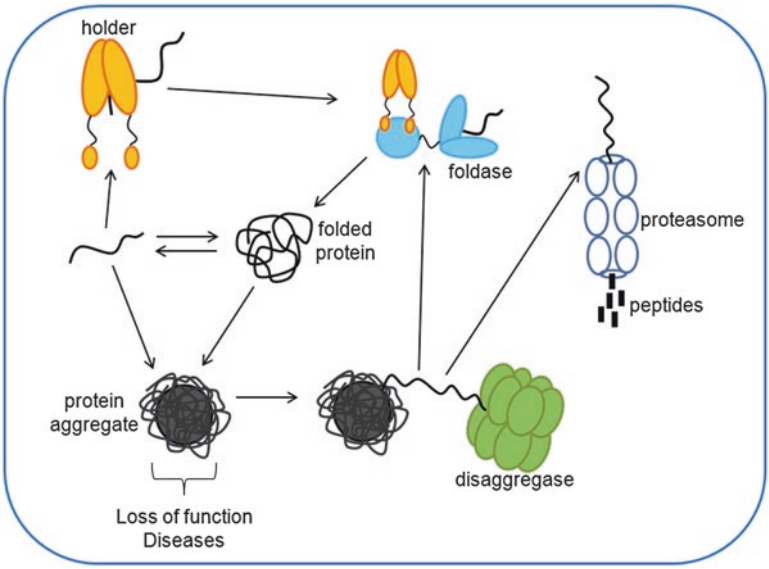


**Fig. 7.2** Holders, foldases, and disaggregases promote proteostasis. Chaperones can be classified by taking into account their function when interacting with substrates as holders, foldases or disaggregases. Foldases help the substrate to adopt its native state in an ATP-dependent manner and are responsible for the major refolding activities in the chaperone system. Holders bind client proteins in an ATP-independent manner, protecting against misfolding or aggregation and delivering them to further action by other chaperones, commonly a foldase. Disaggregases promote the reactivation of aggregated proteins inside the cell. Together they promote proteostasis, the precise balance between folding/refolding of nascent or disaggregated/reactivated proteins. The proteasome, which is involved in the degradation of misfolded proteins, is also involved in the maintenance of proteostasis. Adapted from (Tiroli-Cepeda and Ramos 2011)

Mayer 2013; Mayer and Kityk 2015; Przyborski et al. 2015; Tiroli-Cepeda and Ramos 2011; Young 2010). Hsp70 functions as a foldase, facilitates protein damage repair, which increases cell feasibility, and is involved in the folding process of nascent proteins, working along with other chaperones (Radons 2016; Tiroli-Cepeda and Ramos 2011). Hsp70 is found intracellularly (cytosol or organelles such as the mitochondria and endoplasmic reticulum), but recent works show that it can also be found in the cell membrane and in the extracellular environment (Pockley et al. 2014; Radons 2016). Hsc70, the 70-kDa heat shock cognate protein, is the constitutively expressed cytosolic Hsp70 protein in mammals (Table 7.2). The Hsp70 proteins are abundant proteins in human cells: cytosolic Hsc70 (HSPA8) and mitochondrial HSP70 (HSPA9) may constitute approximately 3% of the total protein mass of unstressed human cells (Finka et al. 2015).

The Hsp70 monomeric chaperones are characterized by the presence of two functional domains: the N-terminal with a nucleotide binding domain, NBD, and the C-terminal with a substrate binding domain, SBD (Tiroli-Cepeda and Ramos 2011) (Fig. 7.1b ). The NBD domain is responsible for ATPase activity, binding ATP

or ADP. On the other hand, the SBD domain, formed by a flexible lid α subdomain composed of a long α-helix and a rigid β-sheet-rich subdomain, switches from closed to open conformations and is able to bind to hydrophobic amino acid residues of the substrate (Finka et al. 2015; Borges and Ramos 2006; Sarbeng et al. 2015). On its extreme C-terminal, cytoplasmic Hsp70 harbors a variable region, composed by G/P-rich residues and the EEVD motif, which is involved in binding to TPR co-chaperones (Radons 2016). Meanwhile, the Hsp70 from mitochondria and the ER has a N-terminal localization signal, and some ER HSP70 contain a KDEL retention signal (Munro and Pelham 1987; Radons 2016).

The Hsp70 chaperone family has many physiological functions in proteostasis, such as protein assembly/disassembly, protein activation/inactivation, polypeptide translocation, degradation and involvement in protecting against protein aggregation (Ebrahimi-Fakhari et al. 2012). Hsp70s are also cancer biomarkers since they are overexpressed in most cancer cells and are related to tumorigenicity (Radons 2016), have an anti-apoptotic activity and also have an important role in the modulation of the immunological system. Additionally, Hsp70s bind to antigenic peptides, arousing intracellular antigen processing, are secreted and exposed (as a complex with antigen peptide) on the extracellular surface, and bind to APCs's surface receptors, thus stimulating MHC and NK cells. Chaperokine and Hsp-antigen complexes stimulate the innate immune response by releasing cytokines and triggering pro-inflammatory molecules (Radons 2016; Srivastava 2005; Tytell 2005).

The most important co-chaperones of Hsp70 belong to the Hsp40 family (Cheetham and Caplan 1998; Cyr et al. 1994; Cyr and Ramos 2015; Dekker et al. 2015; Qiu et al. 2006; Walsh et al. 2004). They help Hsp70 in many cellular functions, are essential for maintaining cell survival (Borges et al. 2005) and work as holders, binding unfolded or misfolded polypeptides and delivering them to Hsp70 (Sarbeng et al. 2015). Hsp40s are formed by the highly conserved α-helical N-terminal domain, termed the J-domain (Fig. 7.1b), which is responsible for the interaction with the NBD of Hsp70, inducing ATPase activity. The position of the J-domain determines the classes of Hsp40s, as it is located at the N-terminus in type I and II classes and elsewhere in the type III class. Adjacent to the J-domain, there is a disordered and flexible glycine/phenylalanine-rich region. The central region consists of a cysteine-rich domain that folds in a zinc-dependent manner. The C-terminal domain is formed by a β-sheet structure related to the dimerization of Hsp40, and together with the Cys-rich and C-terminal domains, it is responsible for substrate binding and presentation (Borges et al. 2005). However, the mechanism regarding the substrate transfer from Hsp40 to Hsp70 is still unknown (Borges et al. 2005; Summers et al. 2009).

The Hsp70/Hsp40 complex involves consecutive cycles of binding, folding and release of client proteins in an ATP-dependent manner (Fig. 7.3). When Hsp70 is in the ATP-bound state (bound to the NBD), it has an open conformation, and there is a low affinity for substrate (throughout the SBD). Hsp40s deliver the client protein to Hsp70, and the interaction through the Hsp70 NBD and Hsp40 J-domain stimulates Hsp70 ATPase activity (ATP hydrolysis), resulting in the predominance of the ADP-bound state when Hsp70 has a closed conformation, thus increasing the affinity

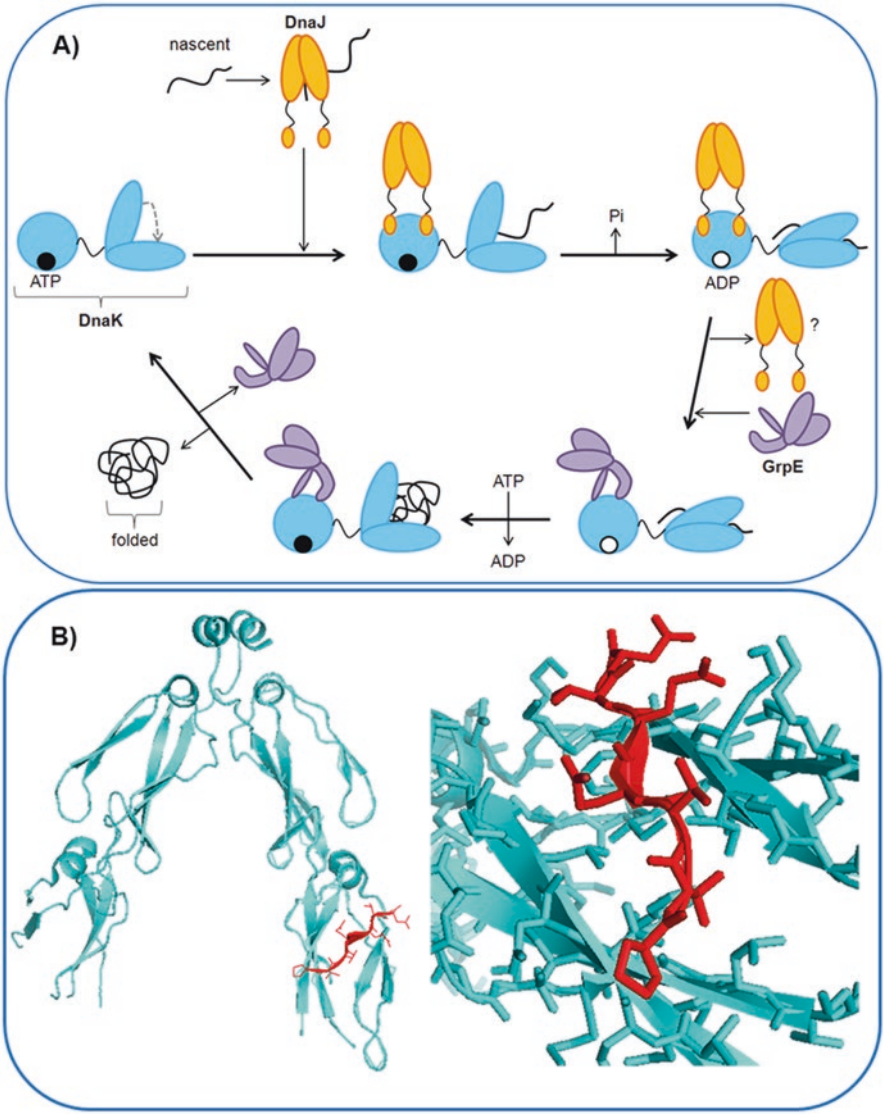


**Fig. 7.3** Hsp70/Hsp40 complex. **(a)** Hsp70/Hsp40 chaperone cycle. The figure represents the Hsp70/Hsp40 chaperone cycle in *Escherichia coli*. Nascent/client proteins are delivered to ATP-bound Hsp70/DnaK (open; low substrate affinity) by Hsp40/DnaJ. ATP hydrolysis is stimulated by Hsp40/DnaJ binding and Hsp70/DnaK is converted into its ADP-bound state (closed; high substrate affinity). Hsp40 is released and subsequently a nucleotide exchange factor (NEF/GrpE) stimulates the ATP/ADP exchange and substrate release. If the substrate is not released folded, it can reenter for another cycle. **(b)** Interaction between Hsp70 and type II Hsp40: Crystal structure of the complex formed by the extreme eight C-terminal residues G634PTVEEVD641 of the Ssa1 (Hsp70; red) lid domain and the dimeric Sis1 (Hsp40; cyan) peptide-binding fragment (residues 171–352) from yeast. PDB accession number 2B26 from reference (Li et al. 2006). A closer view of the interaction is shown on the right

for the substrate. It is noteworthy that there are many types of Hsp40 proteins, which is consistent with the diversity of substrates that can be delivered to Hsp70 (Cyr and Ramos 2015; Gonçalves and Ramos 2016) (Table 7.2). Subsequently, Hsp40 is released, and a nucleotide exchange factor (NEF) stimulates the ATP/ADP exchange and substrate release. If a substrate is not released folded, it can reenter for another cycle.

## 7.3 HSP100, the Classical Disaggregase

The ClpB/Hsp104 chaperone family, or simply Hsp100, is a subset of the AAA+ (ATPases associated with various cellular activities) superfamily of ATPases and is present in all non-metazoan eukaryotes, all eubacteria and some archaebacterial (Lee et al. 2003; Li et al. 2006; Nyquist and Martin 2014; Olivares et al. 2016; Snider and Houry 2008; Sweeny and Shorter 2016; Yedidi et al. 2017) ((Figs. 7.1b and 7.4). Hsp104 is a yeast protein that brings over the ATP hydrolysis energy to dissolve disordered aggregates, prions, stress-induced misfolded proteins, pre-amyloids and even remodel extremely stable amyloids fibers (Lindquist 2006; Lo Bianco et al. 2008; Shorter 2011; Shorter and Lindquist 2004, 2008; Wallace et al. 2015). Hexameric Hsp100s assembled into ring-shaped structures undergo conformational changes triggered by ATP binding and hydrolysis (Weibezahn et al. 2004; Zolkiewski 2006) (Fig. 7.1b). The Hsp100 family is characterized by a N-terminal domain (NTD) that contributes to the high-binding affinities that Hsp100s have for aggregates and contains canonical Walker A and B motifs as well as sensor-1 and sensor-2 motifs, which are important components in ATP binding and hydrolysis

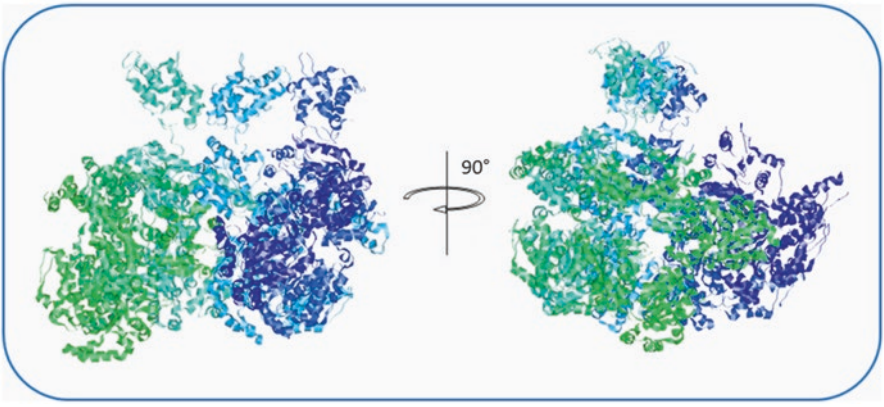


**Fig. 7.4** Hsp100, the classical disaggregase. CryoEM reconstruction of Hsp104. Two rotating views from the Hsp104 hexamer structure reconstructed from CryoEM (electron microscopy) from PDB number 5KNE (Yokom et al. 2016). The structure on the right is a 90° rotation from that on the left

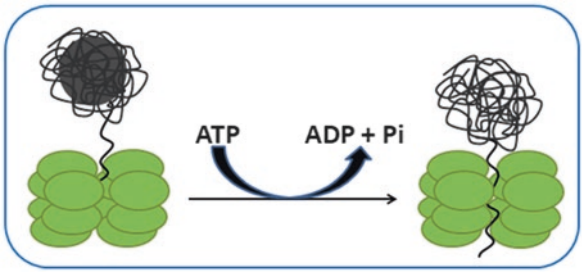


**Fig. 7.5** The disaggregation mechanism of Hsp100s by a process called "threading". The pulling force resulting in substrate unfolding and threading is triggered by ATP hydrolysis. Adapted from reference (Mogk et al. 2015)

(Beinker et al. 2002; Mogk et al. 2003a, ; Schlieker et al. 2004). Following the NTDs is a coiled-coil middle (M) domain, which is not involved in ATPase and protein translocation but is essential for reactivating protein aggregates (Desantis and Shorter 2012; Jackrel et al. 2014; Lee et al. 2013; Oguchi et al. 2012; Sielaff and Tsai 2010). Studies with Hsp104 have shown that the C-terminal region located after the second NBD is involved in oligomerization and co-chaperone interaction (Mackay et al. 2008). For full ATPase activity, Hsp104 requires both NBD1 and NBD2 ATP binding domains, both A and B conserved Walker motifs, the "sensor residues" from one protomer, and the Arg finger from the adjacent protomer (Erzberger and Berger 2006; Gates et al. 2017; Sweeny and Shorter 2016).

The disaggregation mechanism of Hsp100 involves continuous extraction of unfolded proteins from the aggregate that are solubilized into the central channel in a process called "threading" (Mogk et al. 2015; Schlieker et al. 2004) (Fig. 7.5). The structure of ClpB from *Thermus thermophilus* shows that its hexameric conformation is crucial for activity because it provides multiple sites for polypeptide substrate binding, which are translocated through its central channel during aggregate reactivation (Barends et al. 2010; Doyle et al. 2013; Lee et al. 2003; Mayer 2010; Schirmer et al. 1996; Schlieker et al. 2004; Weibezahn et al. 2004; Zolkiewski 2006; Zolkiewski et al. 2012). Recently, studies with cryo-EM have demonstrated the mechanism by which Hsp104 binds and mechanically translocates its substrate (Gates et al. 2017). The hexamer in the closed (ATPγS + substrate bind) and extended (AMPPNP-bind, an ADP analogue) conformations differs in the arrangement of P1 and P6 protomers as well as the Tyr pore loop strands in the SBD, which contacts the substrate and becomes ordered in a right hand spiral package (Carroni et al. 2014; Lee et al. 2003). The conserved amino acid residues Tyr257, Tyr662 (P1-P5 protomers) and V663 (NBD2) hydrophobically bind the substrate into the channel, the lower loops from NBD 1 and 2 seemingly make contact with the substrate and the protomer P6 is disconnected from the substrate (Gates et al. 2017). In summary, ATP binding and hydrolysis result in changes in the positions of bound substrates relative to each other followed by the translocation of the polypeptide through the central channel during aggregate reactivation (Weibezahn et al. 2004; Zolkiewski 2006).

## 7.4 The Role of HSP70 in Disaggregase Systems

The Hsp70 protein family and its respective co-chaperones Hsp40 and Hsp110 (Fig. 7.3) form a complex system that is utilized by cells in many ways, not only in folding but also in disaggregation (see below) (Mayer and Bukau 2005). Actually, the Hsp70/Hsp40/Hsp110 system is the most important system that can unfold toxic protein conformers in neurons (Hinault et al. 2010). It also has crucial functions in protein metabolism due to the fact that it plays key roles, receiving and handing out unfolded proteins to other molecular chaperones. Hence, studying the role of Hsp70 in disaggregase systems is important because they may play a role in disease progression by providing ways of treating neurodegenerative diseases associated with incorrect protein conformation and toxicity (Warrick et al. 1999). Protein disaggregase systems can mitigate the traumatic variation in the function of misfolded proteins. Therefore, it might be a future therapeutic option to recover the quality of life for patients with these diseases, which have incidence rates that are increasing annually.

The 110 kDa heat shock protein (Hsp110) is a cytosolic protein present exclusively in eukaryotic organisms, as it is a co-chaperone belonging to the Hsp70 family (Raviol et al. 2006a) (Fig. 7.1b). Structurally, Hsp110s have expressive amino acid sequence homology in the N-terminal ATPase domain compared with the Hsp70 chaperones (34% of identity according to (Oh et al. 1999), while its C-terminal domain is notably larger than that of Hsp70, presenting differences principally due to the insertion of multiple dimensions of negatively charged residues (Dragovic et al. 2006; Raviol et al. 2006a). There are also differences regarding substrate specificity since Hsp70s bind to aliphatic-rich peptides whereas Hsp110s usually bind aromatic residue-rich peptides. The negatively charged residues at the C-terminus are characteristic of Hsp110s (Oh et al. 1999). The co-chaperone Hsp110 cooperates with Hsp70 (Fig. 7.6) to aid correct protein folding (Dragovic

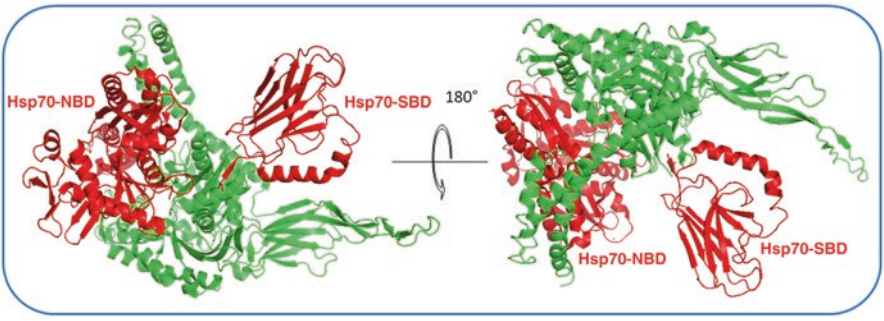


**Fig. 7.6** Interaction between Hsp70 and Hsp110. Two rotating views of the crystal structure of the complex formed by the nucleotide binding domain (NBD) of bovine Hsp70 *(red)* and yeast Hsp110 *(green)*. PDB accession number 3C7N from reference (Schuermann et al. 2008). The structure on the left side is a 180° rotation from that on the right side

et al. 2006), in addition to avoiding aggregation, acting as a holder (Oh et al. 1997) and helping in the correct refolding of model proteins after heat induced denaturation when in the presence of Hsp70s and some co-chaperones (Raviol et al. 2006a).

The Hsp110 co-chaperone also acts as an effective nucleotide exchange factor (NEF) for Hsp70, promoting the release of ADP as well as the replacement of ATP, allowing the cycling of Hsp70 to the ATP-bound state (Borges and Ramos 2005; Tiroli-Cepeda and Ramos 2011; Young 2010). This role was already demonstrated by the yeast Hsp110 homologue Sse1. It was shown that there is bonding between Sse1 and the nucleotide that generates necessary conformational changes for the interaction of Hsp110 with the yeast Hsp70 Ssa1. This interaction promotes ADP release from Hsp70, and then, the consequent rebinding of ATP causes the dissolution of the complex (Andreasson et al. 2008b). It was also observed that the protein Sse1p can accelerate the dissociation of ADP from Hsp70 in vitro (Raviol et al. 2006b). However, whether the action of Hsp110 as a foldase is capable of being modulated by Hsp40s as is Hsp70 is still a matter of intense debate (Andreasson et al. 2008a; Dragovic et al. 2006; Goeckeler et al. 2008; Nillegoda and Bukau 2015; Raviol et al. 2006a).

Metazoans, despite of the absence of a bona fide Hsp100, have evolved a disaggregase system and thus are able to resolubilize aggregated proteins (for reviews and discussion on this subject see for example: (Gao et al. 2015; Abrahão et al. 2017; Mokry et al. 2015; Nillegoda and Bukau 2015; Nillegoda et al. 2015; Rampelt et al. 2012; Shorter 2011; Torrente and Shorter 2013). In this system, Hsp110 co-chaperone cooperates with Hsp70 and Hsp40 to form a complex with disaggregase function in mammals (Shorter 2011). To date, Hsp70/Hsp40/NEF has been the only known chaperone machinery in the cytoplasm of mammalian cells that is able to use the ATP hydrolysis energy to unfold toxic misfolded protein conformers into non-toxic natively refoldable or protease-degradable species (Hinault et al. 2010). Using a cell-free system (Shorter 2011) showed that cytosolic preparations of either rat liver or HeLa cells were able to reactivate model proteins that have been aggregated by urea. The disaggregase activity was lower compared to that of Hsp104 alone and was dependent on ATP-hydrolysis. Depletion of either of the two Hsp110 variants, Hsp105 (heat-inducible) or Apg-2 (constitutive), reduced the disaggregase activity, with the reduction being greater when Apg-2 was depleted. As a confirmation of that result, the addition of Apg-2 restored the activity. Thus, the addition of Hsp100 to the mammalian disaggregase system enhances its activity(DeSantis et al. 2012; Duennwald et al. 2012).

One of the first models for the combined action between Hsp70 and Hsp110 (Mattoo et al. 2013) was based on the information about the structural interaction between the two chaperones (shown in Fig. 7.6). This model considers that the two SBDs and NBDs of the chaperone heterodimers are in different states of binding. For example, the NBD of Hsp70 is binding ADP, and its SBD is bound to an unfolded substrate, while the NBD of Hsp110 is binding ATP and the SBD is opened. When the ATP bound to Hsp110-NBD is hydrolyzed, its SBD can reach a new misfolded structure and bind to it. Alternate cycles of ADP release and ATP binding and hydrolysis along with open and closed states in the domains of the two

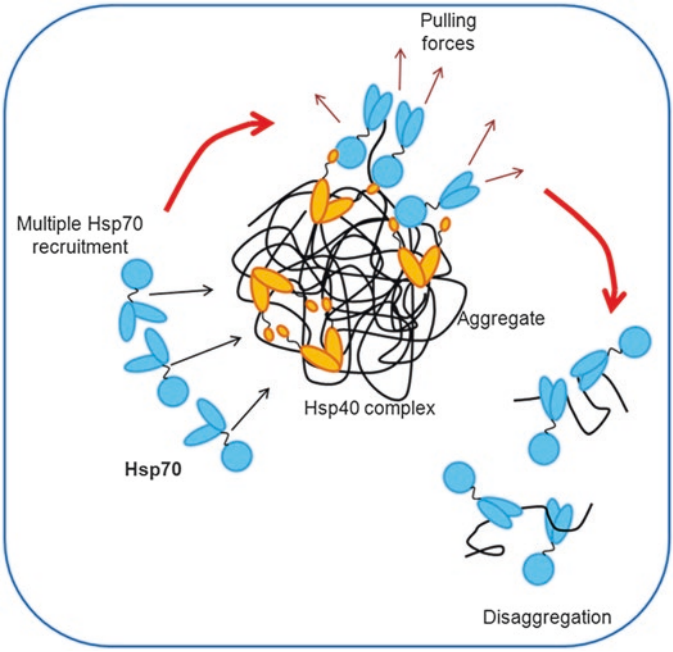


**Fig. 7.7** A model for the cooperation between Hsp70 and Hsp110 for protein disaggregation. In this model, the Hsp40 co-chaperone associates with the surface of the protein aggregates and recruits Hsp70. Hsp110 associates with Hsp70 forming a heterodimer. Alternate cycles of ADP release and ATP binding and hydrolysis along with open and closed states in the domains of the two chaperones will unfold the misfolded structure at several points (see text). Adapted from reference (Nillegoda and Bukau 2015)

chaperones will unfold the misfolded structure at several points. Such action, when successful, results in disaggregation followed by the refolding of individual polypeptides along the way. It is noteworthy that the authors (Mattoo et al. 2013) found an optimal stoichiometry of 1:1 for the combined action of Hsp70 and Hsp110. This model was discussed in reference (Nillegoda and Bukau 2015) and is shown in Fig. 7.7.

The activity of Hsp110 was also shown to be conserved in yeast (Shorter 2011). However, Hsc70 and Hsp40 (Hdj1) from humans seem to exhibit higher intrinsic disaggregation capacities than their yeast homologs Hsp70 Ssa1 and Hsp40s Ydj1 and Sis1 (Rampelt et al. 2012). Human cytosolic Hsp110s (HSPH1 and HSPH2) are bona fide chaperones by themselves, and together with Hsp40 (DNAJA1 and DNAJB1), they can hydrolyze ATP and convert stable misfolded polypeptides into natively refolded proteins. Besides, equimolar amounts of Hsp70 (HSPA1A) and Hsp110 (HSPH1) form a powerful molecular machinery that is able to reactivate stable luciferase aggregates in an ATP- and DNAJA1-dependent manner in a disaggregation mechanism (Mattoo et al. 2013). Furthermore, selective NEF requirements were demonstrated because Apg2 and HSP105α are more efficient than a Bag

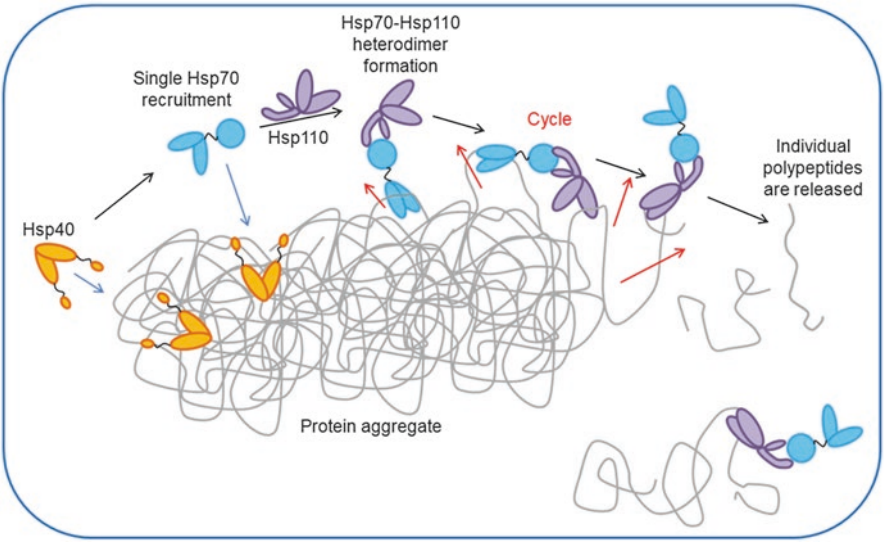


**Fig. 7.8** A model for the synergistic interaction of types I and II Hsp40s for protein disaggregation. In this model, Hsp40 type I and II complexes interact with the aggregated substrate and Hsp70, which interacts with Hsp110. Hsp70 binding to the aggregates generates forces that pull individual chains helping to accelerate disaggregation. In this model, the transient nature of the Hsp40 interactions allows for multiple kinds of responses as each type could also act alone. Adapted from reference (Nillegoda et al. 2015)

family-type NEF (Bag1) in performing this function (Gao et al. 2015). It is noteworthy that the proportions of the components in the disaggregase system can have contrary effects, enhancement or inhibition of the activity with different substrates (Gao et al. 2015; Mattoo et al. 2013; Rampelt et al. 2012; Shorter 2011).

The Hsp40 J domain is able to stimulate the ATPase activity of Hsp70 and Hsp110, and it is necessary for the disaggregase activity (Shorter 2011). More recently, it was shown, using proteins from *Caenorhabditis elegans*, that the presence of Hsp40 for both types I and II enhanced the recovery of aggregated luciferase (Nillegoda et al. 2015) (Fig. 7.8). This work showed a synergistic effect between the two types and that increasing concentrations of one of the Hsp40 types, when in the absence of the other, had surprisingly inhibitory effects. Additionally, the interaction between the two types was transient and efficient for various sizes of aggregates. Finally, the authors conclude that disaggregation, different from refolding, requires both type I and type II Hsp40s. Fig. 7.7 shows a model suggested by these authors, in which the Hsp40 complex interacts with the aggregated substrate and Hsp70, and then, Hsp70 binding to the aggregates generates forces that pull individual chains helping to accelerate disaggregation. These forces originate from the entropy decrease caused by Hsp70 binding to aggregated polypeptides and then extracting them for folding or degradation.

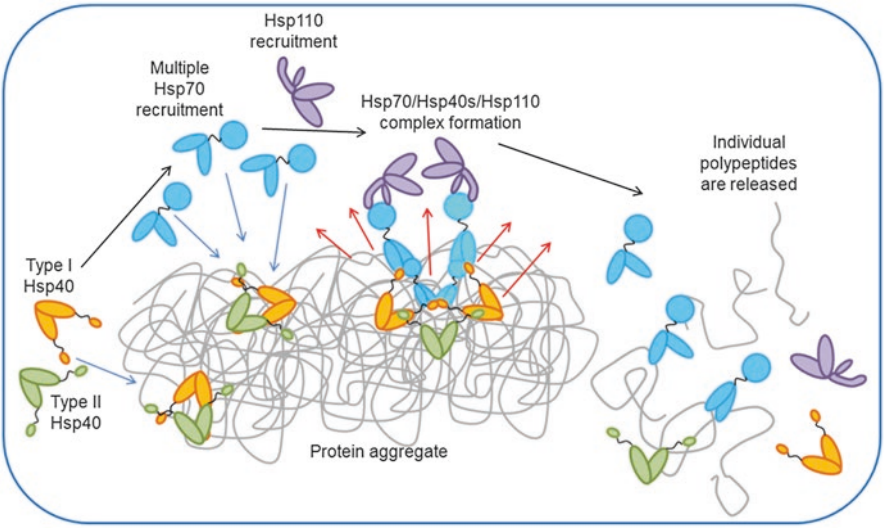


**Fig. 7.9** A more detailed mechanism for the action of the Hsp70/Hsp40/Hsp110 disaggregase complex. In this model, heterocomplexes formed by Hsp40s from types I and II bind to the aggregate complexes and potentiate the action of Hsp70 proteins. Hsp110 is recruited forming a Hsp70/Hsp40s/Hsp110 complex that binds to the aggregates generating forces that pull individual chains, thus helping to accelerate disaggregation. Adapted from reference (Nillegoda and Bukau 2015)

A more detailed mechanism for the action of the Hsp70/Hsp40/Hsp110 disaggregase complex has been developed (Nillegoda and Bukau 2015) (Fig. 7.9). This model considers extensive data contrary to Hsp110 being a bona fide foldase to place its function in the disaggregase system as a nucleotide exchange factor that has a holder activity (see above and further discussion in reference (Nillegoda and Bukau 2015)). Additionally, this model points to the essential Hsp40 function in the process as a specific Hsp40 works equally well with either Hsp110 or Bag1 in the disaggregase system (Rampelt et al. 2012). Thus, the action of Hsp110 will vary depending on the type of Hsp40 present and will also be substrate-specific. Mechanistically, this new model (Fig. 7.8) considers that the multiple Hsp70 chaperones will be interacting with aggregated substrate in a manner similar to that shown in Fig. 7.7, thus generating forces that pull individual chains. As mentioned above, a critical step in this mechanism is the coordinated action of Hsp40s. Heterocomplexes formed by Hsp40s from types I and II bind to the aggregate complexes and potentiate the action of Hsp70 proteins. Since Hsp40 are dimers, each heterocomplex presents four J-domains, and each of them could bind one Hsp70 to potentiate their effect on the aggregate substrate. The nucleotide exchange function of Hsp110 will then contribute to the efficacy of the entire system.

Besides giving support to the hypothesis that metazoans do have a disaggregase system based on Hsp70, new findings show that this system has a fundamental role in maintaining proteostasis and therefore is a potential therapeutic target for several diseases (Deane and Brown 2017; Gao et al. 2015). One example shows that a

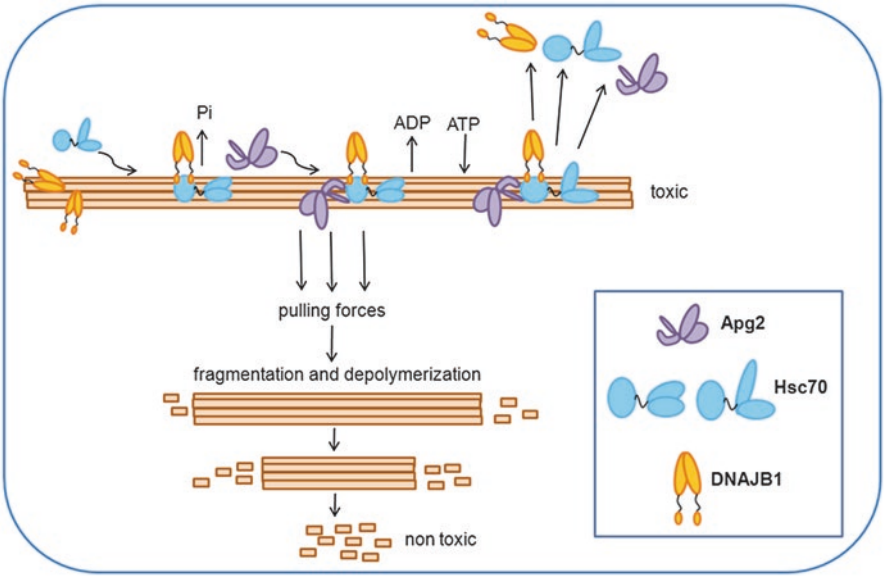


**Fig. 7.10** Predicted model for the disassembly of alpha-synuclein fibrils mediated by the complex formed by the proteins Hsc70/DNAJB1/Apg2. Hsp40s interact with alpha-synuclein fibrils and recruit ATP-bound Hsp70 stimulating hydrolysis. Subsequently, the Hsp70/Hsp40 complex recruits Hsp110 stimulating nucleotide exchange and then releases the disaggregase system from the fibril surface. This release involves structural changes that liberate individual alpha-synuclein polypeptides from fibril ends. As the cycle repeats itself, fibrils disintegrate liberating free alpha-synuclein and exposing free fibril ends for further action of the disaggregation system. Adapted from reference (Gao et al. 2015)

Hsp70-based disaggregation system is involved in the resolubilization of alpha-synuclein, a protein involved in Parkinon's disease (Gao et al. 2015). In this study, the combination (but not individualized) of Hsc70, Hsp70 (HSPA1A), Hsp40s types I and II (DNAJA1 and DNAJB1) and Apg2 or Hsp105α increases the solubilization of alpha-synuclein in an ATP-dependent manner as monitored by electron microscopy and immunoblotting. The authors proposed a mechanistic model (Fig. 7.10) in which Hsp40s interact with alpha-synuclein fibrils and recruit ATP-bound Hsp70 stimulating hydrolysis. In the following step, the Hsp70/Hsp40 complex recruits Hsp110 stimulating nucleotide exchange and the subsequent release of the disaggregase system from the fibril surface. This release involves structural changes that liberate individual alpha-synuclein polypeptides from fibril ends. As the cycles repeat, fibrils disintegrate liberating free alpha-synuclein and exposing free fibril ends for further action of the disaggregation system.

Hsp100 requires a synergistic interaction with Hsp70 and Hsp40 molecular chaperones to completely recover functional proteins from the majority of aggregates (Goloubinoff et al. 1999; Glover and Lindquist 1998; Mogk et al. 1999). As a matter of fact, ClpB/Hsp104 and Hsp70 form a bichaperone system (Figs. 7.11a, b) that is species-specific (Glover and Lindquist 1998; Miot et al. 2011; Olzscha et al.

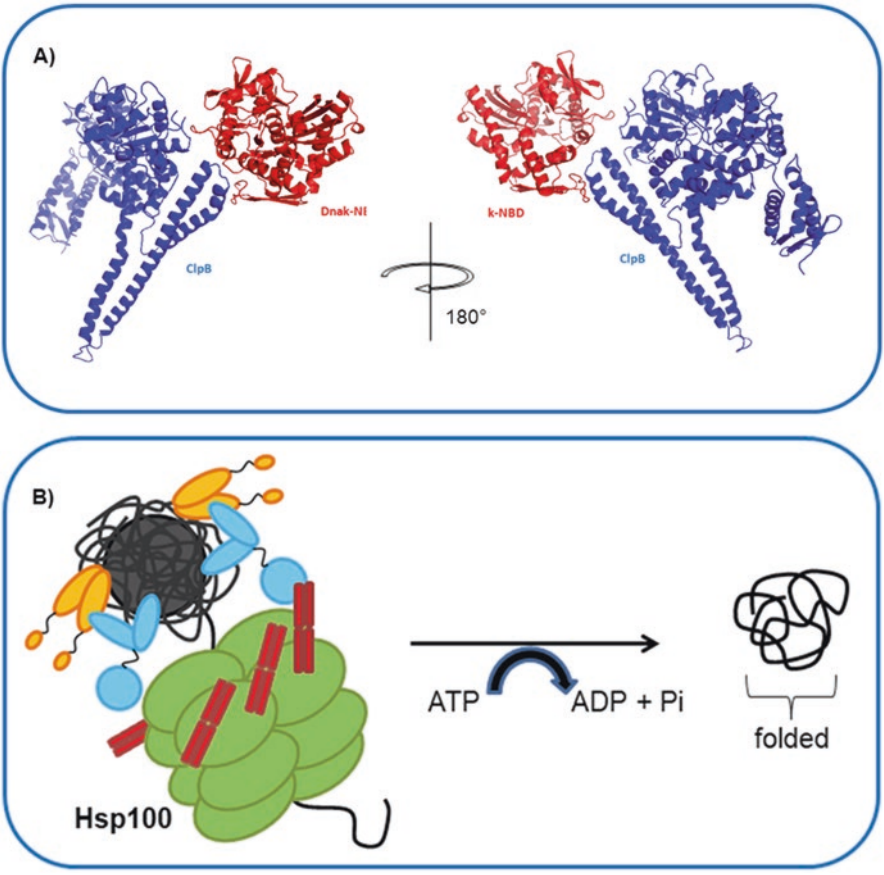


**Fig. 7.11** Interaction between Hsp70 and Hsp100. **(a)** Structure. Structure from NMR (nuclear magnetic resonance) of the complex formed by the nucleotide binding domain of the DnaK (Hsp70; red) and ClpB (Hsp100; blue) portions comprising the N-terminal domain (NTD), nucleotide binding domain 1 (NBD1), and coil-coil domain (CCD) from the bacteria *Escherichia coli*. Structure from reference (Rosenzweig et al. 2013). The structure in the bottom is a 180° rotation from that on the top. **(b)** Model for the interaction of Hsp70/Hsp40/Hsp100 for protein disaggregation. This model is from reference (Mogk et al. 2015). Yellow, Hsp40; blue, Hsp70; green, Hsp100 (M-domain is in brown); black, client protein. In this model, the complex formed by client protein/Hsp40/Hsp70 binds to Hsp100. The later is activated by Hsp70, which interacts only with detached M-domains derepressing the activated states of Hsp100. In the activated state, Hsp100 is capable of deaggregating the client protein favoring the folded state

2011; Schlee et al. 2004; Zietkiewicz et al. 2004; Zietkiewicz et al. 2006) as shown, for example, by experiments showing that the replacement of the M-domain of yeast Hsp104 with that of bacterial ClpB is sufficient to exchange the species specificity, allowing the yeast Hsp104 chimera to cooperate with the bacterial Hsp70 system in protein disaggregation and vise-versa (Sielaff and Tsai 2010). The authors further demonstrated that the M-domain controls Hsp104 function through direct

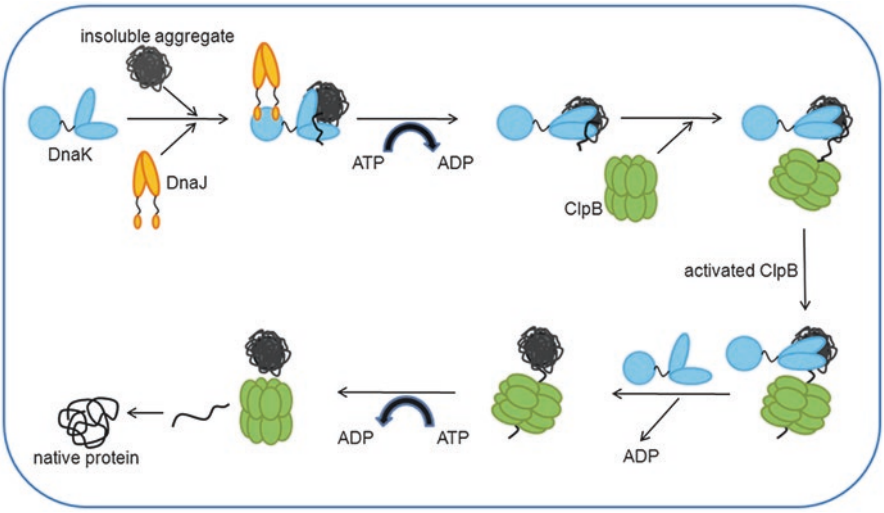


**Fig. 7.12** DnaK(Hsp70)/DnaJ(Hsp40)/ClpB)Hsp100) disaggregation model. In this model, the interaction between DnaK/DnaJ and aggregated proteins initiates the exposition of extended polypeptides that are the starting point for disaggregation. A direct interaction between the NBD of DnaK and the CCD of ClpB will concomitantly deliver the exposed polypeptide segment to the central pore of ClpB, thus activating the later. In this situation, there is an enhancement of nucleotide turnover resulting in the pulling and unfolding of the exposed segments through the axial channel of ClpB. An individual polypeptide will then emerge at the other side being ready for folding. Adapted from reference (Rosenzweig et al. 2013)

interaction with the cognate Hsp70 system that is required to unleash the Hsp104 protein remodeling activity. Additionally, Hsp70/Hsp40 has little disaggregase activity in the absence of Hsp100 (Doyle et al. 2007; Goloubinoff et al. 1999) and both ClpB and Hsp104 require their Hsp70 counterparts for binding to some heat-denaturated aggregates and prion fibrils (Winkler et al. 2012).

Although Hsp100 can bind and reactivate aggregates even in the absence of Hsp70/40, the presence of a Hsp70/40 system greatly enhances this capability, suggesting that the two systems may physically interact. From the structure presented in Fig. 7.7, the authors proposed a model for the Hsp70/Hsp40/Hsp100 interaction (Rosenzweig et al. 2013) (Fig. 7.12). In this model, the interaction between DnaK(Hsp70)/DnaJ(Hsp40) and aggregated proteins initiates the exposition of extended polypeptides that are the starting point for disaggregation. A direct interaction between the NBD of DnaK(Hsp70) and the CCD of ClpB(Hsp100) will concomitantly deliver the exposed polypeptide segment to the central pore of the ClpB, thus activating the later. In this situation, there is an enhancement of nucleotide turnover resulting in the pulling and unfolding of the exposed segments through the axial channel of ClpB. An individual polypeptide will then emerge at the other side being ready for folding.

## 7.5 Conclusions

One of the most important families of chaperones is the Hsp70 system formed by Hsp70 and its co-chaperone Hsp40. This system assists many cellular processes involving proteins, including folding, transportation through membranes, degradation and escape from aggregation. Hsp70 functions as a pivot point that receives and distributes unfolded proteins from and to other molecular chaperones. Cytosolic Hsp70 (Hsc70) transports proteins from ribosomes to the Hsp60 system for folding, participates in a system with Hsp100 that recovers protein aggregates and reactivates heat-denatured proteins. This chapter described Hsp70-based disaggregase systems formed by Hsp70, Hsp40 and Hsp110 that also can cooperate with the classical disaggregase Hsp100. The fact that metazoans lack a bona fide Hsp100 has been puzzling researchers in this area due to the high importance of the disaggregase function to protein homeostasis in the cell. A Hsp70-based disaggregase system being established in metazoans has only happened recently and is now being exploited as a potential target for therapeutic strategies against diseases or conditions that are caused or worsened by misfolded proteins.

**Acknowledgments** FAPESP (2012/50161-8), CNPq and CAPES for grants and fellowships.

## References

Abrahão, J., Mokry, D. Z., & Ramos, C. H. (2017). Hsp78 (78 kDa heat shock protein), a representative AAA family member found in the mitochondrial matrix of Saccharomyces Cerevisiae. Mini review article. *Frontiers in Molecular Biosciences, 4*, 60.

Abu-Hamad, S., Kahn, J., Leyton-Jaimes, M. F., Rosenblatt, J., & Israelson, A. (2017). Misfolded SOD1 accumulation and mitochondrial association contribute to the selective vulnerability of motor neurons in familial ALS: Correlation to human disease. *ACS Chemical Neuroscience, 8*(10), 2225–2234.

Aguado, A., Fernandez-Higuero, J. A., Moro, F., & Muga, A. (2015). Chaperone-assisted protein aggregate reactivation: Different solutions for the same problem. *Archives of Biochemistry and Biophysics, 580*, 121–134.

Akiyama, H., Kondo, H., Arai, T., Ikeda, K., Kato, M., Iseki, E., Schwab, C., & McGeer, P. L. (2004). Expression of BRI, the normal precursor of the amyloid protein of familial British dementia, in human brain. *Acta Neuropathologica, 107*, 53–58.

Alderson, T. R., Kim, J. H., & Markley, J. L. (2016). Dynamical structures of Hsp70 and Hsp70-Hsp40 complexes. *Structure (London, England), 24*, 1014–1030.

Alzheimer's Association. (2016). 2016 Alzheimer's disease facts and figures. *Alzheimer's & Dementia, 12*, 459–509.

Anderson, J. F., Siller, E., & Barral, J. M. (2011). Disorders of protein biogenesis and stability. *Protein and Peptide Letters, 18*, 110–121.

Andreasson, C., Fiaux, J., Rampelt, H., Druffel-Augustin, S., & Bukau, B. (2008a). Insights into the structural dynamics of the Hsp110-Hsp70 interaction reveal the mechanism for nucleotide exchange activity. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 16519–16524.

Andreasson, C., Fiaux, J., Rampelt, H., Mayer, M. P., & Bukau, B. (2008b). Hsp110 is a nucleotide-activated exchange factor for Hsp70. *The Journal of Biological Chemistry, 283*, 8877–8884.

Finka, A., Sharma, S. K., & Goloubinoff, P. (2015). Multi-layered molecular mechanisms of polypeptide holding, unfolding and disaggregation by HSP70/HSP110 chaperones. *Frontiers in Molecular Biosciences, 2*, 29.

Balch, W. E., Morimoto, R. I., Dillin, A., & Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science (New York, N.Y.), 319*, 916–919.

Barends, T. R. M., Werbeck, N. D., & Reinstein, J. (2010). Disaggregases in 4 dimensions. *Current Opinion in Structural Biology, 20*, 46–53.

Beinker, P., Schlee, S., Groemping, Y., Seidel, R., & Reinstein, J. (2002). The N terminus of ClpB from Thermus thermophilus is not essential for the chaperone activity. *The Journal of Biological Chemistry, 277*, 47160–47166.

Bhandari, V., & Houry, W. A. (2015). Substrate interaction networks of the Escherichia coli chaperones: Trigger factor, DnaK and GroEL. *Advances in Experimental Medicine and Biology, 883*, 271–294.

Borges, J. C., Fischer, H., Craievich, A. F., & Ramos, C. H. I. (2005). Low resolution structural study of two human HSP40 chaperones in solution. DJA1 from subfamily a and DJB4 from subfamily B have different quaternary structures. *The Journal of Biological Chemistry, 280*, 13671–13681.

Borges, J. C., & Ramos, C. H. I. (2005). Protein folding assisted by chaperones. *Protein and Peptide Letters, 12*, 257–261.

Borges, J. C., & Ramos, C. H. I. (2006). Spectroscopic and thermodynamic measurements of nucleotide-induced changes in the human 70-kDa heat shock cognate protein. *Archives of Biochemistry and Biophysics, 452*, 46–54.

Calamini, B., & Morimoto, R. I. (2012). Protein homeostasis as a therapeutic target for diseases of protein conformation. *Current Topics in Medicinal Chemistry, 12*, 2623–2640.

Carroni, M., Kummer, E., Oguchi, Y., Wendler, P., Clare, D. K., Sinning, I., Kopp, J., Mogk, A., Bukau, B., & Saibil, H. R. (2014). Head-to-tail interactions of the coiled-coil domains regulate ClpB activity and cooperation with Hsp70 in protein disaggregation. *eLife, 3*, e02481.

Castellano, L. M., & Shorter, J. (2012). The surprising role of amyloid fibrils in HIV infection. *Biology, 1*, 58–80.

Chaudhuri, T. K., & Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. *The FEBS Journal, 273*, 1331–1349.

Cheetham, M. E., & Caplan, A. J. (1998). Structure, function and evolution of DnaJ: Conservation and adaptation of chaperone function. *Cell Stress & Chaperones, 3*, 28–36.

Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R., & Smith, A. E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell, 63*, 827–834.

Chiti, F., & Dobson, C. M. (2006). Protein misfolding, functional amyloid, and human disease. *Annual Review of Biochemistry, 75*, 333–366.

Chiti, F., & Dobson, C. M. (2017). Protein Misfolding, amyloid formation, and human disease: A summary of progress over the last decade. *Annual Review of Biochemistry, 86*, 27–68.

Clerico, E. M., Tilitsky, J. M., Meng, W., & Gierasch, L. M. (2015). How hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology, 427*, 1575–1588.

Collier, T. J., Srivastava, K. R., Justman, C., Grammatopoulous, T., Hutter-Paier, B., Prokesch, M., Havas, D., Rochet, J. C., Lui, F., Jock, K., de Oliveira, P., Stirtz, G. L., Dettermer, U., Sortweel, C. E., Feany, M. B., Lansbury, P., Lapidus, L., & Paumier, K. L. (2017). Nortriptyline inhibits aggregation and neurotoxicity of alpha-synuclein by enhancing reconfiguration of the monomeric form. *Neurobiology of Disease, 106*, 191–204.

Cuanalo-Contreras, K., Mukherjee, A., & Soto, C. (2013). Role of protein misfolding and proteostasis deficiency in protein misfolding diseases and aging. *International Journal of Cell Biology, 2013*, 638083.

Cyr, D. M., Langer, T., & Douglas, M. G. (1994). DnaJ-like proteins: Molecular chaperones and specific regulators of Hsp70. *Trends in Biochemical Sciences, 19*, 176–181.

Cyr, D. M., & Ramos, C. H. I. (2015). Specification of Hsp70 function by Type I and Type II Hsp40. In G. Blatch & A. L. Edkins (Eds.), *The networking of chaperones by co-chaperones, subcellular biochemistry* (Vol. 78, pp. 91–102). *Cham*: *Springer International Publishing*.

da Silva, K. P., & Borges, J. C. (2011). The molecular chaperone Hsp70 family members function by a bidirectional heterotrophic allosteric mechanism. *Protein and Peptide Letters, 18*, 132–142.

De Felice, F. G., Lourenco, M. V., & Ferreira, S. T. (2014). How does brain insulin resistance develop in Alzheimer's disease? *Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 10*, S26–S32.

De Maio, A. (2014). Extracellular Hsp70: Export and function. *Current Protein & Peptide Science, 15*, 225–231.

Deane, C. A. S., & Brown, I. R. (2017). Differential targeting of Hsp70 heat shock proteins HSPA6 and HSPA1A with components of a protein disaggregation/refolding machine in differentiated human neuronal cells following thermal stress. *Frontiers in Neuroscience, 11*, 227.

Dekker, S. L., Kampinga, H. H., & Bergink, S. (2015). DNAJs: More than substrate delivery to HSPA. *Frontiers in Molecular Biosciences, 2*(35).

DeSantis, M. E., Leung, E. H., Sweeny, E. A., Jackrel, M. E., Cushman-Nick, M., Neuhaus-Follini, A., Vashist, S., Sochor, M. A., Knight, M. N., & Shorter, J. (2012). Operational plasticity enables hsp104 to disaggregate diverse amyloid and nonamyloid clients. *Cell, 151*, 778–793.

Desantis, M. E., & Shorter, J. (2012). The elusive middle domain of Hsp104 and ClpB: Location and function. *Biochimica et Biophysica Acta, 1823*, 29–39.

Douglas, P. M., & Dillin, A. (2010). Protein homeostasis and aging in neurodegeneration. *The Journal of Cell Biology, 190*, 719–729.

Doyle, S. M., Genest, O., & Wickner, S. (2013). Protein rescue from aggregates by powerful molecular chaperone machines. *Nature Reviews Molecular Cell Biology, 14*, 617–629.

Doyle, S. M., Hoskins, J. R., & Wickner, S. (2007). Collaboration between the ClpB AAA+ remodeling protein and the DnaK chaperone system. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 11138–11144.

Dragovic, Z., Broadley, S. A., Shomura, Y., Bracher, A., & Hartl, F. U. (2006). Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s. *The EMBO Journal, 25*, 2519–2528.

Dubnikov, T., Ben-Gedalya, T., & Cohen, E. (2017). Protein quality control in health and disease. *Cold Spring Harbor Perspectives in Biology, 2017*(9), a023523.

Duennwald, M. L., Echeverria, A., & Shorter, J. (2012). Small heat shock proteins potentiate amyloid dissolution by protein disaggregases from yeast and humans. *PLoS Biology, 10*, e1001346.

Duncan, E. J., Cheetham, M. E., Chapple, J. P., & van der Spuy, J. (2015). The role of HSP70 and its co-chaperones in protein misfolding, aggregation and disease. *Sub-Cellular Biochemistry, 78*, 243–273.

Ebrahimi-Fakhari, D., Wahlster, L., & McLean, P. J. (2012). Protein degradation pathways in Parkinson's disease – Curse or blessing. *Acta Neuropathologica, 124*, 153–172.

Erzberger, J. P., & Berger, J. M. (2006). Evolutionary relationships and structural mechanisms of AAA+ proteins. *Annual Review of Biophysics and Biomolecular Structure, 35*, 93–114.

Fan, J. Q., & Ishii, S. (2007). Active-site-specific chaperone therapy for Fabry disease. Yin and Yang of enzyme inhibitors. *FEBS Journal, 274*, 4962–4971.

Foguel, D., & Silva, J. L. (2004). New insights into the mechanisms of protein misfolding and aggregation in amyloidogenic diseases derived from pressure studies. *Biochemistry, 43*, 11361–11370.

Gao, X., Carroni, M., Nussbaum-Krammer, C., Mogk, A., Nillegoda, N. B., Szlachcic, A., Guilbride, D. L., Saibil, H. R., Mayer, M. P., & Bukau, B. (2015). Human Hsp70 Disaggregase reverses Parkinson's-linked alpha-Synuclein amyloid fibrils. *Molecular Cell, 59*, 781–793.

Gates, S. N., Yokom, A. L., Lin, J., Jackrel, M. E., Rizo, A. N., Kendsersky, N. M., Buell, C. E., Sweeny, E. A., Mack, K. L., Chuang, E., Torrente, M. P., Su, M., Shorter, J., & Southworth, D. R. (2017). Ratchet-like polypeptide translocation mechanism of the AAA+ disaggregase Hsp104. *Science (New York, NY), 357*, 273–279.

Glover, J. R., & Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell, 94*, 73–82.

Goeckeler, J. L., Petruso, A. P., Aguirre, J., Clement, C. C., Chiosis, G., & Brodsky, J. L. (2008). The yeast Hsp110, Sse1p, exhibits high-affinity peptide binding. *FEBS Letters, 582*, 2393–2396.

Goloubinoff, P. (2016). Mechanisms of protein homeostasis in health, aging and disease. *Swiss Medical Weekly, 146*, w14306.

Goloubinoff, P., Mogk, A., Zvi, A. P., Tomoyasu, T., & Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 13732–13737.

Gonçalves, C. C., & Ramos, C. H. I. (2016). Molecular Chaperones and HSPs in Sugarcane and Eucalyptus. In A. Asea, P. Kaur, & S. Calderwood (Eds.), *Heat Shock Proteins and Plants* (Vol. 10, pp. 245–283). *Cham*: *Springer International Publishing*.

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science (New York, N.Y.), 297*, 353–356.

Hinault, M. P., Cuendet, A. F., Mattoo, R. U., Mensi, M., Dietler, G., Lashuel, H. A., & Goloubinoff, P. (2010). Stable alpha-synuclein oligomers strongly inhibit chaperone activity of the Hsp70 system by weak interactions with J-domain co-chaperones. *The Journal of Biological Chemistry, 285*, 38173–38182.

Hipp, M. S., Park, S. H., & Hartl, F. U. (2014). Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends in Cell Biology, 24*, 506–514.

Holmes, W. M., Klaips, C. L., & Serio, T. R. (2014). Defining the limits: Protein aggregation and toxicity in vivo. *Critical Reviews in Biochemistry and Molecular Biology, 49*, 294–303.

Hoogstra-Berends, F., Meijering, R. A., Zhang, D., Heeres, A., Loen, L., Seerden, J. P., Kuipers, I., Kampinga, H. H., Henning, R. H., & Brundel, B. J. (2012). Heat shock protein-inducing compounds as therapeutics to restore proteostasis in atrial fibrillation. *Trends in Cardiovascular Medicine, 22*, 62–68.

Jackrel, M. E., DeSantis, M. E., Martinez, B. A., Castellano, L. M., Stewart, R. M., Caldwell, K. A., Caldwell, G. A., & Shorter, J. (2014). Potentiated Hsp104 variants antagonize diverse proteotoxic misfolding events. *Cell, 156*, 170–182.

Jeng, W., Lee, S., Sung, N., Lee, J., & Tsai, F. T. (2015). Molecular chaperones: Guardians of the proteome in normal and disease states. *F1000Research, 4*, 1448.

Johnson, S. M., Connelly, S., Fearns, C., Powers, E. T., & Kelly, J. W. (2012). The transthyretin amyloidoses: From delineating the molecular mechanism of aggregation linked to pathology to a regulatory-agency-approved drug. *Journal of Molecular Biology, 421*, 185–203.

Kaushik, S., & Cuervo, A. M. (2015). Proteostasis and aging. *Nature Medicine, 21*, 1406–1415.

Knowles, T. P., Vendruscolo, M., & Dobson, C. M. (2014). The amyloid state and its association with protein misfolding diseases. *Nature Reviews Molecular Cell Biology, 15*, 384–396.

Labbadia, J., & Morimoto, R. I. (2015). The biology of proteostasis in aging and disease. *Annual Review of Biochemistry, 84*, 435–464.

Lee, J., Kim, J. H., Biter, A. B., Sielaff, B., Lee, S., & Tsai, F. T. (2013). Heat shock protein (Hsp) 70 is an activator of the Hsp104 motor. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 8513–8518.

Lee, S., Sowa, M. E., Watanabe, Y. H., Sigler, P. B., Chiu, W., Yoshida, M., & Tsai, F. T. (2003). The structure of ClpB: A molecular chaperone that rescues proteins from an aggregated state. *Cell, 115*, 229–240.

Li, J., Wu, Y., Qian, X., & Sha, B. (2006). Crystal structure of yeast Sis1 peptide-binding fragment and Hsp70 Ssa1 C-terminal complex. *The Biochemical Journal, 398*, 353–360.

Li, X., Shao, H., Taylor, I. R., & Gestwicki, J. E. (2016). Targeting allosteric control mechanisms in heat shock protein 70 (Hsp70). *Current Topics in Medicinal Chemistry, 16*, 2729–2740.

Lo Bianco, C., Shorter, J., Regulier, E., Lashuel, H., Iwatsubo, T., Lindquist, S., & Aebischer, P. (2008). Hsp104 antagonizes alpha-synuclein aggregation and reduces dopaminergic degeneration in a rat model of Parkinson disease. *The Journal of Clinical Investigation, 118*, 3087–3097.

Lv, T., Li, X., Zhang, W., Zhao, X., Ou, X., & Huang, J. (2016). Recent advance in the molecular genetics of Wilson disease and hereditary hemochromatosis. *European Journal of Medical Genetics, 59*, 532–539.

Macario, A. J., & Conway de Macario, E. (2000). Stress and molecular chaperones in disease. *International Journal of Clinical & Laboratory Research, 30*, 49–66.

Mack, K. L., & Shorter, J. (2016). Engineering and evolution of molecular chaperones and protein Disaggregases with enhanced activity. *Frontiers in Molecular Biosciences, 3*, 8.

Mackay, R. G., Helsen, C. W., Tkach, J. M., & Glover, J. R. (2008). The C-terminal extension of Saccharomyces Cerevisiae Hsp104 plays a role in oligomer assembly. *Biochemistry, 47*, 1918–1927.

Mattoo, R. U., Sharma, S. K., Priya, S., Finka, A., & Goloubinoff, P. (2013). Hsp110 is a bona fide chaperone using ATP to unfold stable misfolded polypeptides and reciprocally collaborate with Hsp70 to solubilize protein aggregates. *The Journal of Biological Chemistry, 288*, 21399–21411.

Mayer, M. P. (2010). Gymnastics of molecular chaperones. *Molecular Cell, 39*, 321–331.

Mayer, M. P. (2013). Hsp70 chaperone dynamics and molecular mechanism. *Trends in Biochemical Sciences, 38*, 507–514.

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences: CMLS, 62*, 670–684.

Mayer, M. P., & Kityk, R. (2015). Insights into the molecular mechanism of allostery in Hsp70s. *Frontiers in Molecular Biosciences, 2*, 58.

Miot, M., Reidy, M., Doyle, S. M., Hoskins, J. R., Johnston, D. M., Genest, O., Vitery, M. C., Masison, D. C., & Wickner, S. (2011). Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 6915–6920.

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., & Bukau, B. (2003a). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Molecular Microbiology, 50*, 585–595.

Mogk, A., Kummer, E., & Bukau, B. (2015). Cooperation of Hsp70 and Hsp100 chaperone machines in protein disaggregation. *Frontiers in Molecular Biosciences, 2*, 22.

Mogk, A., Schlieker, C., Strub, C., Rist, W., Weibezahn, J., & Bukau, B. (2003b). Roles of individual domains and conserved motifs of the AAA+ chaperone ClpB in oligomerization, ATP hydrolysis, and chaperone activity. *The Journal of Biological Chemistry, 278*, 17615–17624.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rudiger, S., Roder, D., Langen, H., & Bukau, B. (1999). Identification of thermolabile Escherichia coli proteins: Prevention and reversion of aggregation by DnaK and ClpB. *The EMBO Journal, 18*, 6934–6949.

Mokry, D. Z., Abrahao, J., & Ramos, C. H. (2015). Disaggregases, molecular chaperones that resolubilize protein aggregates. *Anais da Academia Brasileira de Ciências, 87*, 1273–1292.

Muchowski, P. J., & Wacker, J. L. (2005). Modulation of neurodegeneration by molecular chaperones. *Nature Reviews Neuroscience, 6*, 11–22.

Munro, S., & Pelham, H. R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell, 48*, 899–907.

Nillegoda, N. B., & Bukau, B. (2015). Metazoan Hsp70-based protein disaggregases: Emergence and mechanisms. *Frontiers in Molecular Biosciences, 2*, 57.

Nillegoda, N. B., Kirstein, J., Szlachcic, A., Berynskyy, M., Stank, A., Stengel, F., Arnsburg, K., Gao, X., Scior, A., Aebersold, R., Guilbride, D. L., Wade, R. C., Morimoto, R. I., Mayer, M. P., & Bukau, B. (2015). Crucial HSP70 co-chaperone complex unlocks metazoan protein disaggregation. *Nature, 524*, 247–251.

Nyquist, K., & Martin, A. (2014). Marching to the beat of the ring: Polypeptide translocation by AAA+ proteases. *Trends in Biochemical Sciences, 39*, 53–60.

Oguchi, Y., Kummer, E., Seyffer, F., Berynskyy, M., Anstett, B., Zahn, R., Wade, R. C., Mogk, A., & Bukau, B. (2012). A tightly regulated molecular toggle controls AAA+ disaggregase. *Nature Structural & Molecular Biology, 19*, 1338–1346.

Oh, H. J., Chen, X., & Subjeck, J. R. (1997). Hsp110 protects heat-denatured proteins and confers cellular thermoresistance. *The Journal of Biological Chemistry, 272*, 31636–31640.

Oh, H. J., Easton, D., Murawski, M., Kaneko, Y., & Subjeck, J. R. (1999). The chaperoning activity of hsp110. Identification of functional domains by use of targeted deletions. *The Journal of Biological Chemistry, 274*, 15712–15718.

Olivares, A. O., Baker, T. A., & Sauer, R. T. (2016). Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines. *Nature Reviews Microbiology, 14*, 33–44.

Olshansky, S. J., Passaro, D. J., Hershow, R. C., Layden, J., Carnes, B. A., Brody, J., Hayflick, L., Butler, R. N., Allison, B. D., & Ludwing, D. S. (2005). A potential decline in life expectancy in the United States in the 21st century. *The New England Journal of Medicine, 352*, 1138–1145.

Olzscha, H., Schermann, S. M., Woerner, A. C., Pinkert, S., Hecht, M. H., Tartaglia, G. G., Vendruscolo, M., Hayer-Hartl, M., Hartl, F. U., & Vabulas, R. M. (2011). Amyloid-like aggregates sequester numerous metastable proteins with essential cellular functions. *Cell, 144*, 67–78.

Pockley, A. G., Henderson, B., & Multhoff, G. (2014). Extracellular cell stress proteins as biomarkers of human disease. *Biochemical Society Transactions, 42*, 1744–1751.

Prahlad, V., & Morimoto, R. I. (2009). Integrating the stress response: Lessons for neurodegenerative diseases from C. elegans. *Trends in Cell Biology, 19*, 52–61.

Przyborski, J. M., Diehl, M., & Blatch, G. L. (2015). Plasmodial HSP70s are functionally adapted to the malaria parasite life cycle. *Frontiers in Molecular Biosciences, 2*, 34.

Qiu, X. B., Shao, Y. M., Miao, S., & Wang, L. (2006). The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences: CMLS, 63*, 2560–2570.

Quinlan, R. A., Brenner, M., Goldman, J. E., & Messing, A. (2007). GFAP and its role in Alexander disease. *Experimental Cell Research, 313*, 2077–2087.

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress & Chaperones, 21*, 379–404.

Ramos, C. H., & Ferreira, S. T. (2005). Protein folding, misfolding and aggregation: Evolving concepts and conformational diseases. *Protein and Peptide Letters, 12*, 213–222.

Rampelt, H., Kirstein-Miles, J., Nillegoda, N. B., Chi, K., Scholz, S. R., Morimoto, R. I., & Bukau, B. (2012). Metazoan Hsp70 machines use Hsp110 to power protein disaggregation. *The EMBO Journal, 31*, 4221–4235.

Raviol, H., Bukau, B., & Mayer, M. P. (2006a). Human and yeast Hsp110 chaperones exhibit functional differences. *FEBS Letters, 580*, 168–174.

Raviol, H., Sadlish, H., Rodriguez, F., Mayer, M. P., & Bukau, B. (2006b). Chaperone network in the yeast cytosol: Hsp110 is revealed as an Hsp70 nucleotide exchange factor. *The EMBO Journal, 25*, 2510–2518.

Rinaldi, C., & Fischbeck, K. H. (2015). Pathological mechanisms of Polyglutamine diseases. *Nature Education, 8*, 5.

Rosenzweig, R., Moradi, S., Zarrine-Afsar, A., Glover, J. R., & Kay, L. E. (2013). Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. *Science (New York, N.Y.), 339*, 1080–1083.

Sarbeng, E. B., Liu, Q., Tian, X., Yang, J., Li, H., Wong, J. L., Zhou, L., & Liu, Q. (2015). A functional DnaK dimer is essential for the efficient interaction with Hsp40 heat shock protein. *The Journal of Biological Chemistry, 290*, 8849–8862.

Satyal, S. H., Schmidt, E., Kitagawa, K., Sondheimer, N., Lindquist, S., Kramer, J. M., & Morimoto, R. I. (2000). Polyglutamine aggregates alter protein folding homeostasis in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 5750–5755.

Schirmer, E. C., Glover, J. R., Singer, M. A., & Lindquist, S. (1996). HSP100/Clp proteins: A common mechanism explains diverse functions. *Trends in Biochemical Sciences, 21*, 289–296.

Schlee, S., Beinker, P., Akhrymuk, A., & Reinstein, J. (2004). A chaperone network for the resolubilization of protein aggregates: Direct interaction of ClpB and DnaK. *Journal of Molecular Biology, 336*, 275–285.

Schlieker, C., Tews, I., Bukau, B., & Mogk, A. (2004). Solubilization of aggregated proteins by ClpB/DnaK relies on the continuous extraction of unfolded polypeptides. *FEBS Letters, 578*, 351–356.

Schuermann, J. P., Jiang, J., Cuellar, J., Llorca, O., Wang, L., Gimenez, L. E., Jin, S., Taylor, A. B., Demeler, B., Morano, K. A., Hart, P. J., Valpuesta, J. M., Lafer, E. M., & Sousa, R. (2008). Structure of the Hsp110: Hsc70 nucleotide exchange machine. *Molecular Cell, 31*, 232–243.

Shorter, J. (2011). The mammalian disaggregase machinery: Hsp110 synergizes with Hsp70 and Hsp40 to catalyze protein disaggregation and reactivation in a cell-free system. *PLoS One, 6*, e26319.

Shorter, J. (2017). Designer protein disaggregases to counter neurodegenerative disease. *Current Opinion in Genetics & Development, 44*, 1–8.

Shorter, J., & Lindquist, S. (2004). Hsp104 catalyzes formation and elimination of self-replicating Sup35 prion conformers. *Science (New York, NY), 304*, 1793–1797.

Shorter, J., & Lindquist, S. (2006). Destruction or potentiation of different prions catalyzed by similar Hsp104 remodeling activities. *Molecular Cell, 23*, 425–438.

Shorter, J., & Lindquist, S. (2008). Hsp104, Hsp70 and Hsp40 interplay regulates formation, growth and elimination of Sup35 prions. *The EMBO Journal, 27*, 2712–2724.

Shrestha, L., & Young, J. (2016). Function and Chemotypes of human Hsp70 chaperones. *Current Topics in Medicinal Chemistry, 16*, 2812–2828.

Sielaff, B., & Tsai, F. T. (2010). The M-domain controls Hsp104 protein remodeling activity in an Hsp70/Hsp40-dependent manner. *Journal of Molecular Biology, 402*, 30–37.

Silva, J. L., De Moura Gallo, C. V., Costa, D. C., & Rangel, L. P. (2014). Prion-like aggregation of mutant p53 in cancer. *Trends in Biochemical Sciences, 39*, 260–267.

Silverman, G. A., Pak, S. C., & Perlmutter, D. H. (2013). Disorders of protein misfolding: Alpha-1-antitrypsin deficiency as prototype. *The Journal of Pediatrics, 163*, 320–326.

Snider, J., & Houry, W. A. (2008). AAA+ proteins: Diversity in function, similarity in structure. *Biochemical Society Transactions, 36*, 72–77.

Sousa, R. (2014). Structural mechanisms of chaperone mediated protein disaggregation. *Frontiers in Molecular Biosciences, 1*, 12.

Srivastava, P. K. (2005). Immunotherapy for human cancer using heat shock protein-peptide complexes. *Current Oncology Reports, 7*, 104–108.

Stewart, K. L., & Radford, S. E. (2017). Amyloid plaques beyond αβ: A survey of the diverse modulators of amyloid aggregation. *Biophysical Reviews, 4*, 405–419.

Summers, D. W., Douglas, P. M., Ramos, C. H., & Cyr, D. M. (2009). Polypeptide transfer from Hsp40 to Hsp70 molecular chaperones. *Trends in Biochemical Sciences, 34*, 230–233.

Sweeny, E. A., & Shorter, J. (2016). Mechanistic and structural insights into the Prion-Disaggregase activity of Hsp104. *Journal of Molecular Biology, 428*, 1870–1885.

Takahashi, T., Katada, S., & Onodera, O. (2010). Polyglutamine diseases: Where does toxicity come from? What is toxicity? Where are we going? *Journal of Molecular Cell Biology, 2*, 180–191.

Tamarappoo, B. K., Yang, B., & Verkman, A. S. (1999). Misfolding of mutant aquaporin-2 water channels in nephrogenic diabetes insipidus. *The Journal of Biological Chemistry, 274*, 34825–34831.

Tiroli-Cepeda, A. O., & Ramos, C. H. (2011). An overview of the role of molecular chaperones in protein homeostasis. *Protein and Peptide Letters, 18*, 101–109.

Torrente, M. P., & Shorter, J. (2013). The metazoan protein disaggregase and amyloid depolymerase system: Hsp110, Hsp70, Hsp40, and small heat shock proteins. *Prion, 7*, 457–463.

Tytell, M. (2005). Release of heat shock proteins (Hsps) and the effects of extracellular Hsps on neural cells and tissues. *International Journal of Hyperthermia: the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group, 21*, 445–455.

Valastyan, J. S., & Lindquist, S. (2014). Mechanisms of protein-folding diseases at a glance. *Disease Models & Mechanisms, 7*, 9–14.

Vashist, S., Cushman, M., & Shorter, J. (2010). Applying Hsp104 to protein-misfolding disorders. *Biochemistry and Cell Biology = Biochimie et biologie cellulaire, 88*, 1–13.

Vicente Miranda, H., & Outeiro, T. F. (2010). The sour side of neurodegenerative disorders: The effects of protein glycation. *The Journal of Pathology, 221*, 13–25.

Wallace, E. W., Kear-Scott, J. L., Pilipenko, E. V., Schwartz, M. H., Laskowski, P. R., Rojek, A. E., Katanski, C. D., Riback, J. A., Dion, M. F., Franks, A. M., Airoldi, E. M., Pan, T., Budnik, B. A., & Drummond, D. A. (2015). Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress. *Cell, 162*, 1286–1298.

Walsh, P., Bursac, D., Law, Y. C., Cyr, D., & Lithgow, T. (2004). The J-protein family: Modulating protein assembly, disassembly and translocation. *EMBO Reports, 5*, 567–571.

Warrick, J. M., Chan, H. Y., Gray-Board, G. L., Chai, Y., Paulson, H. L., & Bonini, N. M. (1999). Suppression of polyglutamine-mediated neurodegeneration in drosophila by the molecular chaperone HSP70. *Nature Genetics, 23*, 425–428.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E. U., Dougan, D. A., Tsai, F. T., Mogk, A., & Bukau, B. (2004). Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell, 119*, 653–665.

Winkler, J., Tyedmers, J., Bukau, B., & Mogk, A. (2012). Chaperone networks in protein disaggregation and prion propagation. *Journal of Structural Biology, 179*, 152–160.

Wolfe, K. J., & Cyr, D. M. (2011). Amyloid in neurodegenerative diseases: Friend or foe? *Seminars in Cell & Developmental Biology, 22*, 476–481.

Yedidi, R. S., Wendler, P., & Enenkel, C. (2017). AAA-ATPases in protein degradation. *Frontiers in Molecular Biosciences, 4*, 42.

Yokom, A. L., Gates, S. N., Jackrel, M. E., Mack, K. L., Su, M., Shorter, J., & Southworth, D. R. (2016). Spiral architecture of the Hsp104 disaggregase reveals the basis for polypeptide translocation. *Nature Structural & Molecular Biology, 23*, 830–837.

Young, J. C. (2010). Mechanisms of the Hsp70 chaperone system. *Biochemistry and Cell Biology = Biochimie et biologie cellulaire, 88*, 291–300.

Young, J. C. (2014). The role of the cytosolic HSP70 chaperone system in diseases caused by misfolding and aberrant trafficking of ion channels. *Disease Models & Mechanisms, 7*, 319–329.

Zietkiewicz, S., Krzewska, J., & Liberek, K. (2004). Successive and synergistic action of the Hsp70 and Hsp100 chaperones in protein disaggregation. *The Journal of Biological Chemistry, 279*, 44376–44383.

Zietkiewicz, S., Lewandowska, A., Stocki, P., & Liberek, K. (2006). Hsp70 chaperone machine remodels protein aggregates at the initial step of Hsp70-Hsp100-dependent disaggregation. *The Journal of Biological Chemistry, 281*, 7022–7029.

Zolkiewski, M. (2006). A camel passes through the eye of a needle: Protein unfolding activity of Clp ATPases. *Molecular Microbiology, 61*, 1094–1100.

Zolkiewski, M., Zhang, T., & Nagy, M. (2012). Aggregate reactivation mediated by the Hsp100 chaperones. *Archives of Biochemistry and Biophysics, 520*, 1–6.

Zuiderweg, E. R., Bertelsen, E. B., Rousaki, A., Mayer, M. P., Gestwicki, J. E., & Ahmad, A. (2013). Allostery in the Hsp70 chaperone proteins. *Topics in Current Chemistry, 328*, 99–153.

Zuiderweg, E. R., Hightower, L. E., & Gestwicki, J. E. (2017). The remarkable multivalency of the Hsp70 chaperones. *Cell Stress & Chaperones, 22*, 173–189.

# Chapter 8
# Molecular Chaperones: Structure-Function Relationship and their Role in Protein Folding

**Bhaskar K. Chatterjee, Sarita Puri, Ashima Sharma, Ashutosh Pastor, and Tapan K. Chaudhuri**

**Abstract** During heat shock conditions a plethora of proteins are found to play a role in maintaining cellular homeostasis. They play diverse roles from folding of non-native proteins to the proteasomal degradation of harmful aggregates. A few out of these heat shock proteins (Hsp) help in the folding of non-native substrate proteins and are termed as molecular chaperones. Various structural and functional adaptations make them work efficiently under both normal and stress conditions. These adaptations involve transitions to oligomeric structures, thermal stability, efficient binding affinity for substrates and co-chaperones, elevated synthesis during shock conditions, switching between 'holding' and 'folding' functions etc. Their ability to function under various kinds of stress conditions like heat shock, cancers, neurodegenerative diseases, and in burdened cells due to recombinant protein production makes them therapeutically and industrially important biomolecules.

**Keywords** Chaperone assisted folding · Heat shock · Molecular chaperones · Protein folding · Structure-function of chaperones

## Abbreviations

| | |
|---|---|
| ACD | α-crystallin domain |
| ADP | Adenosine di-phosphate |
| ATP | Adenosine tri-phosphate |
| CCT | Chaperonin containing TCP-1 |
| CIRCE | Controlling inverted repeat of chaperone expression |

Bhaskar K. Chatterjee, Sarita Puri, Ashima Sharma, and Ashutosh Pastor authors are equally contributed.

B. K. Chatterjee · S. Puri · A. Sharma · A. Pastor · T. K. Chaudhuri (✉)
Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, HauzKhas, New Delhi, India
e-mail: tkchaudhuri@bioschool.iitd.ac.in



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_8

| | |
|---|---|
| CNX | Calnexin |
| CRT | Calreticulin |
| CS | Citrate synthase |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum associated degradation |
| FRET | Fluorescence energy resonance transfer |
| HOP | HSP90/HSP70 organizing protein |
| HSC70 | Heat shock cognate |
| HSEs | Heat shock elements |
| HSFs | Heat shock response and specific transcription factors |
| Hsp | Heat shock proteins |
| HSP | Heat shock protein family |
| HSR | Heat shock response |
| MalZ | Maltodextrin glucosidase |
| NAC | Nascent chain associated complex |
| NEF | Nucleotide-exchange factors |
| NTD | n-terminal domain |
| PBD | Peptide binding domain |
| PPIase | Peptidyl-prolyl isomerases |
| PTP | Permeability transition pore complex |
| RAC | Ribosome associated complex |
| RuBisCO | Ribulose-1,5-bisphosphate oxygenase-carboxylase |
| SHR | Steroid hormone receptors |
| sHsp | Small heat shock proteins |
| sHSP | Small heat shock protein family |
| TF | Trigger factor |
| TPR | Tetratricopeptide |
| TRiC | TCP-1 ring complex |
| UPR | Unfolded protein response pathway |

## 8.1 Introduction

Living systems respond to threatening conditions at multiple levels in their quest for survival. It may in the form of a fight or flight response, which is a result of any imminent physical threat either to an organism or their inner homeostasis. For example the temperature, ionic and sugar balance are regulated within a fixed range in our bodies and are probably optimized by evolutionary mechanisms. Similarly, homeostasis is also maintained at the cellular level and maintaining such a balance is imperative for the survival and efficient functioning of the cell. One of the major homeostasis mechanisms operating at the cellular level is the protein homeostasis, commonly referred to as proteostasis (Balch et al. 2008). Starting with maintaining the structural organization of a cell to catalysing various metabolic reactions; from the transport of macromolecules within and across cells to various recognition and

immune functions, proteins play vital roles in our bodies and are regarded as the actual workhorses of the cells. Proteins undergo various post-translational modifications and move through trafficking pathways before they are ready to take up their function. However, acquiring their specific three-dimensional structure supersedes all this because only in their specific structural forms can they undertake the function they are meant to carry out. In this chapter, we shall discuss the cellular machinery responsible for maintaining proteins in their functional state during stress conditions; specifically focusing on the Hsp that assist in the folding and refolding of misfolded and aggregation prone proteins.

## 8.2 What Is Stress Response?

The stress response can be defined as our involuntary defense reaction to threatening conditions. This may occur at multiple levels as a response to a different range of conditions. At a cellular level, the response to any alarming condition, like a chronic change in the environment away from normal conditions (which may interfere with the physiological functioning of the cells and cause damage to nucleic acids and proteins) can be identified as a stress response (Kültz 2004). While at the organism level our adaptive responses are driven by hormonal changes (Charmandari et al. 2005), the cellular response to damages occurring at a molecular level involves a cascade of pathways, and molecules that work in cohort to bring the cells back to their normal functional state. Various kinds of stress at the cellular level include oxidative, heat, radiation and nutrient deprivation. The major consequences of these stress are DNA damage, loss of cellular signalling, protein unfolding, misfolding, aggregation, proteolysis, cellular necrosis and apoptosis. The cellular stress response may be of a protective nature where the cell can defend and restore its normal functioning, or of a destructive nature where the conditions are beyond the cell's ability to repair. The type, level, and duration of stressful conditions may ultimately determine the fate a cell. During stress conditions, proteins are misfolded due to changes in the overall energy landscape. This causes loss of protein function and the accumulation of misfolded proteins in the form of toxic aggregates. The protective stress response for proteins includes pathways of the heat shock response and the unfolded protein response (UPR); the destructive response pathways include apoptosis, necrosis or autophagy (Fulda et al. 2010). The DNA damage response consists of multiple, complex pathways that restore genomic integrity. These include the base excision repair, nucleotide excision repair, and non-homologous end joining (Kourtis and Tavernarakis 2011). The oxidative stress response helps the cell cope with the reactive oxygen species, maintain redox homeostasis; and a number of enzymes like superoxide dismutase and non-enzymatic antioxidants are involved (Trachootham et al. 2008). In this chapter, we shall mainly focus on the diverse mechanisms governing heat shock response and the various factors that are involved in mediating such a response.

## 8.3 Heat Shock Response

The heat shock response was one of the earliest explored stress response mechanisms that was initially observed in Drosophila as changes in puffing patterns of salivary gland chromosomes (Ritossa 1996), followed by changes in gene expression patterns after heat treatment (Tissiéres et al. 1974; Hightower 1991). The heat shock response imparts thermo-tolerance to the cells and protects them when they are stressed due to prolonged exposure to heat. This response is activated by the rise of a few degrees in temperature from the normal dwelling temperature of the organism. The regular transcription and translation processes in the cells are halted during heat shock response, and specific transcription factors (HSFs) which selectively enhance expression of a set of proteins having protective functions are activated. However, even during normal conditions these HSFs play important roles in differentiation and development of the organisms (Morimoto et al. 1996). The HSF1 predominantly regulates the heat shock responses, and is itself regulated by its interactions with heat shock proteins HSP70 and HSP90 (Pirkkala et al. 2001). The ability to activate transcription and bind to DNA are uncoupled in HSF1 imparting a higher degree of regulation. The HSF1 exists as a monomer or in complex with Hsp under normal conditions. During heat shock, HSF1 homotrimerizes and undergoes hyperphosphorylation which leads to its activation (Cotto et al. 1996). These HSFs facilitate the overexpression of Hsp by binding to *cis* acting sequences on the genome known as heat shock elements (HSEs). HSP are commonly known as molecular chaperones for their role in assisting proteins to acquire their native structures. Hsp prevent heat induced denaturation and aggregation of proteins, facilitate the proteins to fold, and assist in the refolding of already denatured proteins (Lindquist 1986). The Hsp play an active role in facilitating the degradation of proteins that are unable to fold in order to maintain protein homeostasis and thus promote cell survival.

## 8.4 Cellular Components Providing HS Response

Heat shock response in cells is mediated by concerted actions of heat shock factors (HSF), heat shock elements (HSE) and Hsp. The heat shock factors as described above are transcription factors activated during a heat shock. Gram negative bacteria *E.coli* has a specific sigma factor 32 (σ32), coded by the rpoH gene, which is a heat shock promoter specific subunit of RNA polymerase. The σ32 is a positive regulator and is suppressed by DnaJ during normal conditions (Bukau 1993). The gram-positive bacteria *B. subtilis* has a negative regulator HrcA which binds to negatively acting CIRCE elements (controlling inverted repeat of chaperone expression). Folding of HrcA is mediated by GroE chaperones. During heat shock response, HrcA doesn't fold due to the unavailability of free GroE chaperones and hence the negative regulation of Hsp is switched off (Hietakangas and Sistonen 2006).

A majority of the eukaryotes have either one or multiple of the four heat shock factors HSF1-HSF4; the condition being different in plants. *Arabidopsis* has 21 genes encoding various heat shock factors, divided among three classes and 14 different groups. The plant HSFs are induced and expressed during heat shock (Hietakangas and Sistonen 2006). Unlike plants, the transcription factors in animals are like HSF1. They are constitutively expressed but functionally repressed during normal conditions by HSP70 and HSP90 (Shi et al. 1998). The DNA binding domain of HSF recognizes the HSE in a major grove of the DNA double helix. HSEs are highly conserved and contain inverted repeats of nGAAn (e.g. nTTCnnGAAnnTTCn) (Amin et al. 1988; Akerfelt et al. 2010). There might be multiple HSEs in the promoter region of a heat shock gene and hence multiple HSFs can bind simultaneously in a cooperative manner.

Expression of Hsp is invariably upregulated during heat shock irrespective of the difference in their mechanism of action, as discussed above. Overall, Hsp or molecular chaperones, are one of the seven different classes of proteins to be overexpressed during stress response and were the earliest discovered components of the heat shock response. The second class comprises components of the proteolytic system which helps in the clearance of misfolded and aggregated proteins. The proteolytic machinery of the proteasome has a similar structural organization amongst different organisms differing only in their subunit compositions. The protein degradation machinery requires the translocation of misfolded or partially unstructured intermediates between cytosol, ER and Golgi in eukaryotes and multiple HSP members coordinating protein folding and degradation in different cellular compartments. The third class of proteins help in DNA and RNA repair, like counteracting the heat induced methylation of RNA. The fourth category comprises enzymes of the metabolic pathways which reorganize the energy supply of the cell. The fifth category includes kinases and transcription factors that further initiate or inhibit expression cascades to support the stress response. The sixth class of proteins maintain the integrity of the cytoskeleton. Transport proteins and membrane-modulating proteins make up the seventh class. All these proteins function together to respond to the heat stress conditions (Richter et al. 2010). However, we will be discussing the molecular chaperones in detail in the following sections.

The Hsp are divided into multiple families based primarily on their size. These different classes of molecular chaperones and their localization in different cellular compartments provide a great degree of organizational control and distribution of roles to execute particular functions within the overall cascade of stress response. HSP60, HSP70, HSP90, HSP100, and small HSP are found across all organisms and are known to have high similarities within their classes among different organisms. One similarity among all chaperones is that they recognize the hydrophobic surfaces of proteins which are increasingly exposed among misfolded and unfolded proteins, and function to either fold the protein (foldases) or mask the hydrophobic regions to prevent aggregation (holdases). Despite the name suggesting a particular function, Hsp play significant roles in multiple stress response pathways in all organisms, including oxidative stress in all organisms and drought and osmotic stress in plants. The roles of molecular chaperones have been observed in the correct

folding of a newly translated protein, refolding of misfolded proteins, disaggregation, translocation, and in the degradation of proteins. HSP60, HSP70, and HSP90 are ATP dependent chaperones which actively support protein folding. The small HSP and other chaperones simply prevent the misfolding of proteins. The heat shock factors are regulated by molecular chaperones like HSP70 and HSP90, thus proving their importance in the overall heat shock response of the cell. HSP70 helps in the translocation of proteins to cellular compartments like ER and also facilitates retrotranslocation (The reverse movement of the protein from ER to the cytosol, where it can be degraded by the proteasomal machinery) during ER associated degradation. HSP90 clients include many kinases and steroid receptors, which help in regulating a multitude of functions through signaling pathways. HSP104 is known to disaggregate proteins and redirect them to correct refolding pathways. The widespread presence of chaperones in almost all cellular components where protein folding occurs, proves that they are key players in the heat shock response machinery. Almost all cellular proteins might have to interact with molecular chaperones at least once in their lifetimes, be it during synthesis, folding, targeting, or degradation.

## 8.5 Role of Chaperones in Mediating Cellular Heat Shock Response

Cells grow optimally within a narrow range of temperature, pH, and other physiological conditions, but adapt to moderate deviation from such conditions. One of the most well studied cellular adaptations is the heat shock response (HSR) (Guisbert et al. 2004). During heat shock conditions, many cellular proteins work to either rescue the cells from dying, or trigger apoptosis when the damage incurred is irreversible. These proteins are referred to as heat shock proteins (Hsp) (Herman and Gross 2000). Few out of several such Hsp protect proteins from undergoing aggregation, unfold aggregated proteins to make them folding compatible and refold damaged proteins. These proteins are termed as chaperones (Morimoto et al. 1994). Molecular chaperone is a major class of protein found at all levels of cellular organizations ranging from bacteria to humans. They have variable organization and function depending on the cellular location and complexity of the organism. Bacterial chaperone proteins are found only in the cytosol as they are not compartmentalized, but in case of higher organisms, these are also localized in mitochondria, endoplasmic reticulum, and chloroplasts. Structural and functional organizations of chaperones are evolutionally conserved within the same kingdom but vary between them. On the basis of gross molecular weight, the major chaperone are classified as: HSP60 (GroEL/GroES, Cpn60/Cpn10, HSP60/HSP10, Tric/CCT, Thermosome), HSP70 (DnaK, DnaJ, GrpE, HSP70), HSP90 (HSP90, TRAP, HtpG), HSP100 (ClpA, ClpB, ClpP), sHSP and Trigger Factor (Georgopoulous et al. 1994; Gross 1996). The other important chaperone proteins playing a role

in heat shock are Prefoldin, Calnexin / Calreticulin**,** GRP94, GRP170, AAA ATPasesPPIases, PDIases, NAC (Nascent polypeptide Associated Complex), CasA and HtpX (Rani et al. 2016).

### *8.5.1 Small Heat Shock Proteins (sHsp)*

Most organisms have a well-developed sHSP system, which help in their protection from the thermal, osmotic and salt stresses (Jakob et al. 1993). sHsp have subunit molecular masses of 12–43 kDa. The common feature of all sHSP is the presence of a highly conserved stretch of 80–100 amino acids in their C-terminus termed as the "α-crystallin domain" (ACD). It is flanked on both side by less conserved N-terminal domain (NTD) and a C-terminal extension (Kappé et al. 2003; Franck et al. 2004; Kriehuber et al. 2010). In *E.coli* major sHsp are IbpA and IbpB. Under normal cellular conditions they help in aggregation prevention and folding of substrate proteins in an ATP independent manner. During stress conditions, along with an increased expression, these proteins undergo drastic conformational rearrangements in order to bind to the misfolded proteins and prevent cellular aggregation (Mani et al. 2016). HSP31 of *E.coli* functions as a holding chaperone. It cooperates with the DnaK-DnaJ-GrpE system in managing protein misfolding during stress conditions (Mujacic et al. 2004). In the pathogenic *Halobacterium sp.*, sHSP1, HSP-5 and sHSP2 impart protection from thermal stress, solar radiation and high salt concentration (Vanghele and Ganea 2010). HSP20 of *Mycobacterium tuberculosis* protects them from macrophage induced stress response and helps in solubilization of heat induced aggregates (Vanghele and Ganea 2010).

In yeast, sHsp HSP26 and HSP42 together, add an additional layer of protection against a cellular assault like heat shock. Both HSP26 and HSP42 are poorly expressed during exponential growth, but their expression increases 10-fold under heat stress suggesting the dominant role they play in a thermally stressed cell (Haslbeck et al. 2004a). HSP26 exists as a 24-mer under normal conditions, acts like a holdase for damaged or misfolded proteins and transfers client proteins to the HSP70 chaperone machinery during heat shock (Haslbeck et al. 2004a, b; 1999). During heat shock, the 24-mer of HSP26 gets reversibly dissociated into dimers. This dimeric form then interacts with the unfolded polypeptides and eventually forms a larger complex, to be presented to chaperones capable of folding the substrate (Stromer et al. 2004). HSP26 also interacts with aggregated proteins, making them accessible to the HSP104 chaperone (Glover and Lindquist 1998). HSP42 oligomer is a symmetric assembly of dimers organized into two hexameric rings. HSP42 binds with 30% of total yeast cytosolic proteins (Haslbeck et al. 2004a). It is a more effective chaperone than HSP26, as a higher HSP26 to substrate ratio is needed to prevent aggregation (Haslbeck et al. 2004a). HSP12 exhibits low sequence homology to the sHSP superfamily and is structurally and functionally distinct, as it exists exclusively as a monomer (Welker et al. 2010). Like the other sHsp, HSP12

is weakly expressed in exponentially growing cells but overexpressed (100-fold) during heat shock (Welker et al. 2010).

In plants, there are distinct gene families for sHSP found in different organelles and a total of 6 families have been classified. The HSP17.6 and HSP17.9 reside in the cytoplasm, HSP21 in the chloroplast, HSP22 in the ER, HSP23 in the mitochondria and HSP22.3 in the cell membrane (Wang et al. 2004). They have been suggested to be involved either in maintaining the structure of a heat stressed cell (Lindquist and Craig 1988) or to protect the photosynthesis machinery during heat shock (Schuster et al. 1988; Chen et al. 1990). Genetically modified plants with higher thermo-tolerance have been designed by constitutive upregulation of sHSP. For example, the HSF3 gene of *Arabidopsis thaliana* was modified to express it in non-heat shock conditions and was shown to increase the basal thermotolerance of the plant (Prändl et al. 1998). Overexpression of HSP17.6 results in a higher tolerance to drought and salinity in *Arabidopsis thaliana*, while the natural elevated gene expression is observed in case of heat shock (Sun et al. 2001).

In humans, Group I sHsp consists of HSP27, HSP20, HSP22, and αB-crystallin. They are found in various tissues and are heat inducible. Group II sHsp consists of HSPB9, HSPB10, HSPB3, HSPB2 and α-crystallin, and they are involved in cell differentiation and are restricted to certain tissues (Taylor and Benjamin 2005). HSP27 forms oligomeric species when subjected to higher temperatures and this results in increased chaperone activity (Bakthisaran et al. 2015). They predominantly function as 'holdases', keeping the substrates in a folding competent globular state that can later be presented to 'foldases' like HSP60/10 or HSP70 to make them functional (Eyles and Gierasch 2010). Under conditions of heat stress, they also prevent aggregation by binding to late unfolding intermediates and keep them in a stable, soluble complex. sHSP may also be involved in the transient/reversible reactivation of early unfolded intermediates and this process may be ATP dependent, although no ATP hydrolysis has been observed (Rajaraman et al. 2001). ATP binding is thought to trigger a conformational change that aides Hsp to release their refolding-competent substrates (Muchowski et al. 1999).

### *8.5.2 The Chaperonin System (HSP60)*

Chaperonins are double ring complexes of 800–900 kDa which help in the folding of many cellular proteins under normal and stress conditions (Spiess et al. 2004; Hemmingsen et al. 1988; Vabulas et al. 2010). These are further classified as group I and group II chaperonins (Horwich et al. 2007).

In bacteria and symbiotic organelles like mitochondria and chloroplasts, Group I chaperonins (cpn60) are found. These chaperonins are termed as GroEL in *E.coli*, mtHsp60 in mitochondria and Rubisco binding protein in chloroplast (Figueiredo et al. 2004). These require co-chaperonin GroES or HSP10 to function in prokaryotic and eukaryotic cells respectively. Under normal cellular conditions, GroEL/ES is constitutively expressed in bacterial cytoplasm and helps in the folding of

substrate proteins in an ATP dependent fashion. Under environmental stress conditions, the expression of these proteins increase by 15–20% (Georgopoulos et al. 1994; Gross 1996) and also leads to a few structural and functional modifications of the chaperone system, which enable them to fold or hold the aggregation prone proteins. One such modification is the phosphorylation of the double ring which mediates substrate folding in an ATP independent manner (Sherman and Goldberg 1994). GroEL also acts as a holding chamber for substrate proteins during thermal stress and helps them regain their folding function once normality is restored (Carrascosa et al. 1998). Mitochondrial Hsp60, with the help of its co-chaperonin HSP10 helps in the folding and assembly of imported proteins inside the matrix of mitochondria. In HSP60 conditional mutants, aggregates accumulate and are unable to assemble into functional complexes. The plastid HSP60 chaperonin found in chloroplasts consists of two different subunits which make them different from other members of the HSP60 family (Levy-Rimler et al. 2002). These two distinct subunits known as CPN60α and CPN60β share about 50% sequence identity (Boston et al. 1996). The tetradecameric structure consists of α7β7oligomers where the assembly of α7 is dependent on the β7 homo-oligomer, thus forming two homogeneous rings, and the oligomers having a seven fold symmetry (Waldmann et al. 1995). The chloroplast co-chaperonin can bind to both GroEL and ch-CPN60 and assists in the folding of proteins in both cases. Its structure however, is markedly different from GroES and contains two GroES like domains connected by a short linker region. Some reports have confirmed the presence of 10 kDa CPN10 and 20 kDa dimer like CPN20 existing simultaneously in the chloroplasts (Hill and Hemmingsen 2001; Levy-Rimler et al. 2002).

Group II chaperonins are found in archaea (Thermosome) and the cytoplasm of eukaryotes (TCP/CCT) (Ditzel et al. 1998; Leitner et al. 2012). They do not require the co-factor HSP10 as they have specialized α-helical extensions in their apical domain that function as a built-in lid (Vabulaset al. 2010). Archaeal Group II chaperonins (Thermosome) consists of two stacked octameric rings with two different kinds of subunits α & β (Klumpp et al. 1997). The mechanism of folding of non-native protein substrates is similar to GroEL and involves binding of the substrate at the apical domain, followed by ATP hydrolysis and release of the folded substrate from the cavity (Lopez et al. 2016). During stress conditions, Group II chaperonins form a large octadecameric β complex which is more efficient in substrate binding. It also helps in membrane stabilization of archaeal cells during stress conditions (Chaston et al. 2016). The group II chaperonins in the eukaryotic cytosol are known as TRiC (TCP-1 ring complex) or CCT (chaperonin containing TCP-1) and like their archaeal counterparts have eight or nine rings, each containing eight paralogous subunits (Frydman 2001). The general domain structure of the group II chaperonins is akin to GroEL (Ditzel et al. 1998). The closing and opening of these segments to encapsulate the substrate in the TriC/CCT cavity is ATP dependent (Meyer et al. 2003a). TriCs interact functionally with the co-chaperone prefoldin (Vainberg et al. 1998; Siegert et al. 2000) and HSP70 (Langer et al. 1992), which serve to transfer substrates to this chaperonin.

### *8.5.3 HSP70*

HSP70 family is involved in a multitude of functions in all organisms and are found in various cellular compartments. HSP70 facilitates translocation, protein import, and signal transduction along with assisting in the refolding of substrate proteins and preventing their aggregation (Frydman 2001; Miemyk 2017). HSP70 consists of two domains, a highly conserved 44 kDa N-terminal ATP binding domain, and a 15 kDa C-terminal peptide binding domain (PBD) which consists of a β-sandwich motif and an α-helical lid segment (Vabulas et al. 2010; Yu et al. 2015). In eukaryotes, under normal conditions, HSP70 exist as complexes with either HSP90 or co-chaperones like HSP40 and others, and is known as Heat Shock Cognate 70 (HSC70). The constitutively expressed HSC70 assist in the folding and translocation of newly synthesized proteins during normal conditions (Hartl et al. 2011). Under heat stress conditions, the stress-inducible HSP70 isoforms are overexpressed. This is achieved by its interaction with HSF, which transcriptionally activates several heat shock genes including that of HSP70. HSP70s function is generally conserved in all organisms (Akerfelt et al. 2010). Nascent polypeptides form the bulk of its clients and undergo chaperoning via the ATP-dependent reaction cycle of HSP70 which is regulated by HSP40 and nucleotide-exchange factors (NEF) (Kampinga and Craig 2010; Mayer 2010). The β-sandwich motif of the PBD recognizes an extended, seven-residue hydrophobic patch of an aggregation prone protein, especially when they are locally surrounded by positively charged residues (Rudiger et al. 1997). The binding of the peptide is ATP-dependent and is regulated by a conformational change in the β-sandwich motif (Mayer 2010). In the ATP-bound state, the lid adopts an open conformation resulting in a high binding affinity for the peptide. Hydrolysis of ATP facilitates lid closure and is accelerated by HSP40, leading to the peptide getting locked inside. Following the hydrolysis of ATP, NEF binds to the ATPase domain and catalyses ADP-ATP exchange that result in the opening of the lid and release of the substrate, presumably in their non-native conformation. These intermediates might then undergo multiple rounds of binding and release till they acquire their native conformation (Hartl et al. 2011). Under stress conditions (apart from folding nascent polypeptides) HSP70 prevents aggregation of non-native substrates by transiently shielding exposed hydrophobic segments and keeping them in a folding competent state, which may subsequently be transferred to the chaperonin cage for completion of the folding process (Vabulas et al. 2010). BiP, the HSP70 paralog in the ER, binds to unfolded regions of the protein manifesting exposed hydrophobic residues and has an ATP dependent mechanism of substrate binding and release, similar to its cytosolic counterpart. BiP plays an active role in the Unfolded Protein Response pathway (UPR) and in ER Associated Degradation (ERAD), both of which occur when misfolded proteins start accumulating in ER. It does so by interacting with several co-chaperones that assist in protein folding and quality control. One of them is Erdj3, an HSP40 paralog in the ER, which interacts with partially folded intermediates and presents them to BiP. Another cohort is BAP, a NEF that plays a similar role as its cytosolic

counterparts, promoting ADP release and facilitating BiPs transition to the open state (Ma and Hendershot 2004).

In yeast, SSA and SSB are subclasses of cytosolic Hsp70 that are constitutively expressed, while SSA3 and SSA4 are known to be induced upon exposure to heat shock (Santoro et al. 1998). Interaction with the chaperone Ydj1 (Hsp40), a homologue of the bacterial DnaJ protein, accelerates the ATPase activity of Hsp70. KAR2 (ER Hsp70) is a yeast homolog of BiP protein which gets induced within 10 minutes of heat shock (Normington et al. 1989). Kar2 is responsible for the translocation of proteins across the ER membrane through co-translational as well as post-translational pathways (Brodsky et al. 1995). It is also involved in the retrograde transport of defective non-functional substrates from the ER to the cytosol for proteosomal degradation via ERAD, and thereby contributes to ER proteostasis (McCracken and Brodsky 1996). The promoter region of the Kar2 gene contain unfolded protein response elements (UPREs), and it gets induced when unfolded polypeptides start accumulating in the ER lumen (Cox and Walter 1996). As is the case for human cytosolic Hsp70, Kar 2 interacts with co-chaperones Hsp40/J proteins (Sec63, Scj1, and Jem1) and nucleotide exchange factors (Sil1) (Nishikawa and Endo 1997; Sadler et al. 1989; Schlenstedt et al. 1995). Sec63 acts as a Kar2 ATPase activator (Lyman and Schekman 1995), while Scj1 functions to counter the misfolding of proteins occurring due to lack of a carbohydrate modification (Silberstein et al. 1998). SSC1 and SSC 3 are the two mitochondrial Hsp70 chaperones which are involved in the heat shock response (Wagner et al. 1994). Binding of the SSC1 to the unfolded protein is critical for post-stress (Baumann et al. 2000). Mdj1 (J protein), the yeast mitochondrial HSP70 cofactor, helps in preventing heat induced protein aggregation in mitochondria (Rowley et al. 1994). It is also involved in protein folding. Furthermore, in addition to folding, its interaction with SSC1 also facilitates the clearance of misfolded mitochondrial proteins. MGE1 is the only mitochondrial NEF known to interact with, and therefore assist the Ssc1 chaperone action by promoting the release of bound nucleotide (Wagner et al. 1994).

The HSP70 family in plants has been subdivided into 4 subgroups based on C-terminal sequence motifs and their cellular localization. The cytosolic HSP70 contains the EEVD motif; the ER HSP70 contains the HDEL motif, the HSP70 molecules found in plastids have the PEGDVIDADFTDSK motif and those in mitochondria have a conserved PEAEYEEAKK motif (Guy and Li 1998). These motifs, known as anchors, are identified by co-chaperones and are involved in substrate binding through the assistance of co-chaperones (Freeman et al. 1995). Plant cells are different in that they contain multiple (2-5) HSP70 family members within the ER (Ray et al. 2016). In *Arabidopsis,* the total HSP70 family members are encoded by about 18 genes, (Lin et al. 2001) and about 12 genes of the HSP70 family have been identified in the spinach genome (Guy and Li 1998). This gives an idea about the functional diversity of this chaperone family (Wang et al. 2004). Mechanisms by which HSP70 mediates its cellular function have been difficult to study in plants due to the poor survival of deletion mutants and the unavailability of efficient inhibitors (Sarkar et al. 2013). In prokaryotes, the DnaK/DnaJ system belongs to HSP70/HSP40 family of heat shock proteins. They function with the prokaryotic

NEF GrpE. DnaK is an ATP-dependent chaperone which requires DnaJ and GrpE for substrate binding through its ATPase cycle. During stress conditions, DnaJ undergoes certain functional modifications which stimulates the ATPase activity of DnaK (~500 fold). During stress conditions, DnaK/DnaJproteins also interact with ClpB and reactivate the inactivated proteins (Liberek et al. 1992). DnaK/DnaJ system is absent in archaeal species except for a few halophiles, hence may not have any particular role in alleviating archaeal stress. The functional analogs of DnaK/DnaJ system in archaea are prefoldin and GimC protein (Rani et al. 2016).

### *8.5.4 HSP90*

HSP90 is another major player that functions downstream of HSP70 in the conformational maturation and functional activation of several important classes of proteins that actively take part in various cell signaling pathways. While the basal HSP90 content is about 1% of the total cellular protein content, it may increase to 4–6% during the stress response (Young et al. 2001; Wegele et al. 2004). HSP90 is usually present in the cytosol, but they may also be found in the ER, mitochondria and plastids (Krishna and Gloor 2001). HSP90 functions as a dimer; the monomer units covalently joined at their C-terminal domains via the dimerization domain. The N-terminal domain binds and hydrolyzes ATP and is joined to the C-terminal domain by a middle domain, which participates in substrate and co-chaperone binding. In all organisms HSP90 acts as a scaffold for several if not all protein folding pathways and components. HSP90 dimer undergoes an ATP driven reaction cycle of substrate binding and release achieved by the transition from an open nucleotide-free to a closed ATP-bound state ‘committed to hydrolysis. HSP90 works in cohort with multiple co-chaperones like HSP70, HOP, the J-domain proteins (HSP40) and p23. These co-chaperones mediate the interactions between HSP90 and the substrates in most of the cases (Li et al. 2012).

In humans, clients of this ~ 90Kda chaperone number over 200, which includes kinases, nuclear receptors, transcription factors, telomerase and many other proteins (Zhao et al. 2005; McClellan et al. 2007). Under stress conditions HSP90α is over-expressed and shows marked increase in interactions with certain clients. HSP90α has a higher potential for oligomerization and undergoes temperature-dependent oligomerization above 45 °C, and denatured substrates like DHFR bind specifically with these higher order oligomers (4-mer, 6-mer, and 8-mer). Oligomeric forms of HSP90 display manifold higher affinity for denatured substrates as they themselves undergo ‘unfolding', where their hydrophobic patches are exposed (Csermely et al. 1998). Like HSP70, HSP90 prevents irreversible denaturation of substrates like luciferase and can act as ‘holdases’. When cells go back to the resting phase, these substrates can be captured by ‘foldases’ and reactivated (Minami and Minami 1999). Tumour Necrosis Factor Receptor-Associated protein 1 is the mitochondrial homologue of Hsp90 (Felts et al. 2000). It is a ~75 kDa protein (Chen et al. 1996) which is structurally similar to its cytosolic counterpart, except the absence of an

MEEVD motif in the C-terminus that binds to co-chaperones like p23, indicating that the regulation of TRAP-1 might be orchestrated by a different set of co-chaperones in a yet unknown fashion (Felts et al. 2000). TRAP-1 functionally differs from its ER counterpart, GRP94 in that it adopts a closed conformation upon ATP binding, but this alone is insufficient for commitment towards ATP hydrolysis as the propensity to adopt an open confirmation even before ATP hydrolysis is greater than its ATP hydrolysis rate. This kinetic partitioning essentially reduces the turnover number of ATP and signifies that the dissociation of ATP is favoured to its ATPase activity (Leskovar et al. 2008). Under heat shock conditions, its ATPase activity increases by ~ 200 fold (Altieri et al. 2012). Although downstream targets involved in this process is not fully understood, it is probably the chaperone Cyclophilin D (a mitochondrial matrix PPIase), a key component of the Permeability Transition Pore complex (PTP). Opening of the PTP is known to induce mitochondrial apoptosis, and by refolding Cyclophilin D in a closed PTP configuration through its ATPase activity, TRAP-1 is perhaps able to mediate cell survival (Green and Kroemer 2004; Kang et al. 2007). The proteins classified in HSP90 family range from 80–94 kDa in size and about 70% sequence identity has been observed between the plant and other eukaryotic counterparts of HSP90 (Lindquist and Craig 1988). The HSP90 family in *Arabidopsis* has seven members where AtHSP90–1 to AtHSP90–4 are present in the cytoplasm, AtHSP90–5 is present in the plastids, AtHSP90–6 in the mitochondria and AtHSP90–7 in the ER (Krishna and Gloor 2001; Wang et al. 2004).It has been observed in *Arabidopsis* that HSP90 is involved in mediating the stress response pathways through its interactions with the heat shock factor (HSF) (Yamada et al. 2007). Overall, studies carried out in plants suggest important roles of HSP90 in many aspects of plant development and stress response. HtpG is the *Escherichia coli* homolog of HSP90. It normally acts as a holder chaperone that transiently maintains the nascent protein in a conformation accessible to DnaK. During stress conditions, its expression increases 5–10-fold and it binds to aggregated proteins with the help of ClpB and re-presents them to the DnaK/DnaJ system (Thomas and Baneyx 2000). In yeast, HSP90 chaperone is encoded by the *Hsp82* gene, which is overexpressed under heat shock conditions (Smith et al. 1991). Yeast HSP90 chaperone system is also involved in overcoming the deleterious impact of heat shock on the cell surface (Imahi & Yahara 2000).

### *8.5.5 HSP100/Clp*

The HSP100 family is primarily known for its unique function of remodeling protein complexes and disassembling protein aggregates, facilitating either refolding or degradation of the aggregated proteins (Goloubinoff et al. 1999). The HSP100 are hexameric ring structures belonging to AAA+ ATPase family (Burton and Baker 2005) with two defined classes based on distinct N and C-domains. The class I proteins are ClpA, B, C, D, E having two AAA modules, whereas the class II proteins ClpM, N, X, and Y have only one AAA module (Lee et al. 2007). In *E.coli* the major

HSP100 are ClpA, ClpB, ClpC, ClpX and ClpY. They help in the disassembly of oligomers and aggregates during stress (Smith et al. 1999). HSP104 is the yeast homolog of ClpB. It recognizes misfolded proteins within an aggregate, unfolds them and ultimately delivers the substrates into various refolding pathways (Schirmer et al. 1996). Together with HSP70 and HSP40, it resolubilizes and refolds the substrates. HSP78 (a yeast mitochondrial aggregase), plays a role in the reactivation of proteins damaged due to stress, thus imaprting thermotolerance (Janowsky et al. 2006). It binds to misfolded polypeptides in the matrix and stabilizes them, thereby preventing their aggregation (Schmitt et al. 1995). One of the major roles of HSP78 is resolubilization of the Ssc1 (mtHsp70) chaperone which itself tends to misfold during stress (Sichting et al. 2005). HEP1 (mtHsp70 escort protein) and Pim1 (involved in mitochondrial proteolysis) are some of the other proteins in mitochondria which are involved in HSR (Sichting et al. 2005; Wagner et al. 1994). HEP1 plays a complementary role to Hsp78 in maintaining functional SSC1 (Sichting et al. 2005). It assists Ssc1 to maintain its solubility and function during stress (Sichting et al. 2005). Multiple HSP100 members exist in *Arabidopsis;* four ClpB, two ClpC, and one ClpD. They are present at basal levels in cells under normal conditions and their expression is observed to increase under heat stress. The cytosolic ClpB1 of *Arabidopsis*, known as AtHSP101 is present in high levels during heat stress and imparts thermotolerance to plants (Glover and Lindquist 1998; Lee et al. 2007).

### *8.5.6 Other Chaperones*

Many other chaperones also play a role in stress tolerance; e.g., PPIases, AAA ATPases and so on. PPIase proteins include cyclophilins, FKBPs and parvulins (Maruyama et al. 2004). They help in cis-trans proline isomerization in a polypeptide chain and help in the fast folding of kinetically trapped proteins. AAA ATPases are mainly found in archaea and eukaryotes. The major AAA ATPase of archaea is CDC48 and AMA which function as major proteasomal ATPases. These proteins regulate proteasomal protein degradation in archaea (Forouzan et al. 2012). In yeast and humans, most proteins that are translocated into the ER are N-glycosylated with a branched glucose-3-mannose-9-N-acetylglucosamine-2 (Glc3Man9) glycan chain (Helenius and Aebi 2004). This oligosaccharide moiety serves as a recognition signal for lectin-like chaperones calnexin (CNX) and calreticulin (CRT) (Daniels et al. 2003). CRT prevents thermal aggregation and promotes recovery of nonglycosylated substrates. This happens due to enhanced polypeptide binding property of CRT under heat stress. CRT also forms oligomers under heat stress via certain conformational changes that occur in its C-terminal acidic domain. Oligomerization of CRT and enhanced polypeptide binding are concomitant and explain how CRT acts as a chaperone. CNX may act to recruit other chaperones like the PDI family member Erp57 that catalyses disulphide bond formation in a highly oxidized ER lumen or it may even act as a chaperone, binding to exposed polypeptide stretches of misfolded

**Table 8.1** Major Chaperones described herein and their subcellular localization (Modified from (Graner et al. 2014))

| | Subcellular localization | | | | |
|---|---|---|---|---|---|
| Major chaperones | ER | Mitochondria | Cytosol | Nucleus | Cell Surface[a] |
| HSP27 | | | ✓ | ✓ | ✓ |
| HSP60 | | ✓ | ✓ | | ✓ |
| HSP70/HSC70 | | | ✓ | ✓ | ✓ |
| GRP78 (BiP) | ✓ | ✓ | ✓ | ✓ | ✓ |
| HSP90 | | | ✓ | ✓ | ✓ |
| HSP110 | | | ✓ | ✓ | ✓ |
| GRP94 (gp96) | ✓ | | | ✓ | ✓ |
| CNX/CALR[b] | ✓ | | ✓ | | ✓ |
| PDI[c] | ✓ | | | | ✓ |

[a]*Cell surface localization is mostly associated with tumour cell surfaces*
[b]*Calnexin/Calreticulin*
[c]*Prolyl Disulphide Isomerase*

glycoproteins. Both these functions of CNX are enhanced under heat stress conditions. However, these chaperones may also 'generally' bind to glycoproteins independent of their folding state, suggesting that there is no specific recognition of the attached polypeptide chain (Buchberger et al. 2010). Other chaperones like GRP94, Peptidyl-Prolyl Isomerases (PPIase) and GRP170 along with the ones mentioned above form a large ER-localized multi-protein complex that functions as a network and bind to unfolded proteins in the ER rather than existing as free pools that get individually assembled onto protein clients. Grp94 most likely acts as a scaffold like its cytosolic HSP90 counterpart and forms an important part of this multi-chaperone complex (Ma and Hendershot 2004). Table 8.1 lists the major chaperones involved in attenuating the adverse effects of heat shock and their subcellular localization.

## 8.6 Detailed Mechanism of Chaperone Assisted Protein Folding

Out of the many chaperone systems described briefly in the previous section, our group has mainly focussed on a couple of chaperone systems namely GroEL/ES (*E. coli*) and HSP90 (human). Based on the various structural and functional studies carried out by our group since the last decade on the chaperonin GroEL/ES system, we have better understood the importance of this system in aggregation prevention of denatured substrates, refolding of substrates and survival of *E. coli*. While we have only recently started working on the human HSP90 system, we have obtained novel information regarding the contribution of HSP90s structure in modulating its chaperone properties using certain HSP90 inhibitors. The following section describes in detail the structure and function of GroEL/ES and HSP90 during normal and under stress conditions.

## 8.7 GroEL/ES Mediated Protein Folding

### *8.7.1 GroEL/ES Structure*

GroEL in its normal state is a porous cylindrical protein made of 14 subunits arranged in nearly 7- fold rotationally symmetrical rings stacked back to back, and forms a cage like structure with a central cavity (Braig et al. 1994). Each GroEL subunit further folds into three domains (Braig et al. 1994; 1995). The apical domain (residues 190–345) which is rich in hydrophobic residues and acts as a binding site for the non-native substrates and co-chaperonin GroES (Fenton et al. 1994). The intermediate domain (residues 134–190, 377–408) acts like a hinge between the apical and equatorial domains. This also helps in allosteric communication between the two domains. The equatorial domain consists of sub-domains E1 (residues 4–133) and E2 (residues 409–523) that form all the intra and inter-subunit interactions required for the proper folding of the monomeric subunit and their assembly into the tetra-decameric form (Braig et al. 1994, Hayer-Hartl et al. 2016). It also houses the nucleotide binding pocket since GroEL works in ATP-dependent manner (Braig et al. 1994). GroES is a single seven membered ring with identical subunits of 10 kDa that binds to one or both ends of the GroEL cylinder in presence of a nucleotide. Each subunit consists of a β-barrel body with an extended hydrophobic mobile loop that interacts with the apical domain of GroEL and provides an enclosed cavity for the folding of substrate proteins (Braig et al. 1994, Fenton et al. 1994; Hendrick and Hartl 1993). Binding of GroES to the GroEL cylinder doubles the volume of central cavity to provide sufficient space for the folding of substrate proteins. Under normal conditions, GroEL interacts with non-native substrates post-translationally (Fenton et al. 1997), while native proteins under stress are subjected to unfolding that leads to the formation of intermediate 'aggregation prone' states that remain bound to GroEL. These can then be refolded back to their native form, presented to other chaperones, or taken up by the proteolysis machinery (Hartl et al. 2011).

### *8.7.2 GroEL Mechanism of Substrate Folding under Normal Conditions*

GroEL helps in the folding of about 5–15% of total cellular protein under normal conditions (Ewalt et al. 1997). Depending upon the size of the substrate protein, it assists in folding in two different ways: (I) Cis- mechanism of folding and (II) Trans- mechanism of folding. The GroEL cavity can encapsulate substrate proteins ranging between 54–57 kDa (Sakikawa et al. 1999) and help in their folding by providing an Anfinsen cage inside the cavity. This is termed as the cis-mechanism of substrate folding. GroEL is not able to encapsulate large proteins (> 60 kDa) inside its cavity and they can undergo multiple rounds of binding and release at the apical

domain of GroEL before reaching their final folded or 'committed to folding' state. Both GroES and ATP are required to release the folded/partially folded substrate from the cis-ring (Fenton and Horwich 1997; Dahiya and Chaudhuri 2014).

### *8.7.3 Cis- Mechanism of GroEL Action*

The asymmetric complex of GroEL-GroES is the most common form found in the cellular milieu, along with a few symmetric complexes of GroEL (Weissman et al. 1995). A few other reports however, show that both types of complexes can exist in equimolar concentration in cellular milieu and that the ratio of symmetric to the asymmetric complex is an ADP dependent phenomenon (Yang et al. 2013; Lizuka and Funatsu 2016). The asymmetric complex has one ring of GroEL occupied by GroES, while another ring remains ready to accept a non-native substrate protein. Due to the presence of hydrophobic residues at the apical domain, newly synthesised polypeptides or partially folded substrates bind to the empty GroEL ring. The binding of substrate protein leads to a conformational change in the same ring which promotes subsequent binding of GroES and ATP. This leads to the formation of an isolated cage for the folding of the substrate, known as the cis-ring. The conformational changes that occur during the binding of GroES and ATP make the GroEL cavity hydrophilic, which in turn provides a proper environment for the folding of substrate protein. ATP hydrolysis is a slow process with one molecule of ATP hydrolysed in 8–10 seconds. The hydrolysis of ATP lowers the binding affinity of GroES to the apical domain. This releases GroES and the folded substrate from the GroEL cis ring; simultaneously ATP binds to the opposite ring along with another substrate and a new cycle is initiated. (Fenton and Horwich 1997; Weissman et al. 1995). Recent reports show that both rings of GroEL act in a concomitant fashion during the folding process (Yang et al. 2013). The following schematic explains the mechanism of cis- folding (Fig. 8.1).

### *8.7.4 Trans Mechanism of GroEL*

Large proteins with molecular weight (>60 kDa) are too big to fit inside the GroEL cavity. This does not involve cis-encapsulation, but requires GroES binding to the trans ring to release either folded or partially folded substrates (Chaudhuri et al. 2001). As the cis-ring binds with the substrate protein, the trans ring acts as a binding site for GroES, so this mechanism is termed as folding in-trans. There are many substrate proteins, e.g., 70 kDa tail spike protein of phage p22, 86 kDa α/β heterodimer, 82 kDa mitochondrial aconitase, dimeric citrate synthase, etc. that fold via this mechanism (Weissman et al. 1995, Chaudhuri et al. 2001). In this process, the non-native substrate binds to the apical domain of the asymmetric GroEL-GroES complex, and undergoes folding with domain rearrangements or prevents aggregation

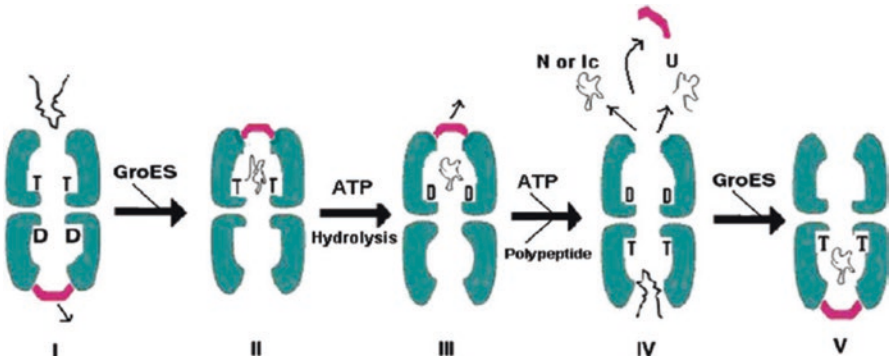


**Fig. 8.1 Proposed model for GroEL/ES assisted folding of small proteins via cis-mechanism**): (**I**) Open ring of GroEL–GroES–ADP complex acts as an acceptor state for the non-native polypeptide. (**II**) Binding of GroES to the GroEL–ATP complex leads to conformational changes in the apical domain of GroEL; consequently, polypeptide enters in the central cavity. (**III**) Folding occurs in the cis cavity before the ATP gets hydrolysed, this weakens the interaction between GroEL and GroES. (**IV**) Binding of ATP to the trans ring promotes the release of (N – Native, Ic – partially folded, U – misfolded) from the cis ring. (**V**) At the same time, binding of GroES to GroEL allows GroEL to alternate its rings between binding-active and folding-active states. (Copyright clearance order number 4177600370026

(Chaudhuri et al. 2001). The apical domain of GroEL can also bind with the 'burst phase intermediate' state of substrate proteins. To prove this hypothesis, experiments were carried out using slow folding Malate Synthase G (MSG), a 89 kDa multi-domain monomeric protein. Our observations suggest that binding of MSG to GroEL accelerates the slowest kinetic phase of the spontaneous protein folding pathway. Due to the large size of the substrate, GroES is not able to bind to this ring, but ATP hydrolysis continues, which helps in the release of the folded substrate from the ring. Sometimes the release of the substrate depends on the binding of both GroES and ATP to the opposite ring. The binding of GroES and ATP to the opposite ring also accelerates the release of folded/partially folded substrate from the GroEL ring (Paul et al. 2007; Dahiya and Chaudhuri 2014). The trans mechanism of substrate folding occurs under normal cellular conditions and also during thermal stress. It helps in preventing the irreversible aggregation of thermally denatured proteins. Study of the thermal unfolding pathway of citrate synthase (CS) show that CS unfolds via an inactive dimeric intermediate. Further unfolding of these intermediates led to their irreversible aggregation. GroEL interacts with this dimeric unfolding intermediate, dissociating them into monomers which stably associate with GroEL (Grallert et al. 1998). The following schematic explains the mechanism of Trans-folding (Fig. 8.2).

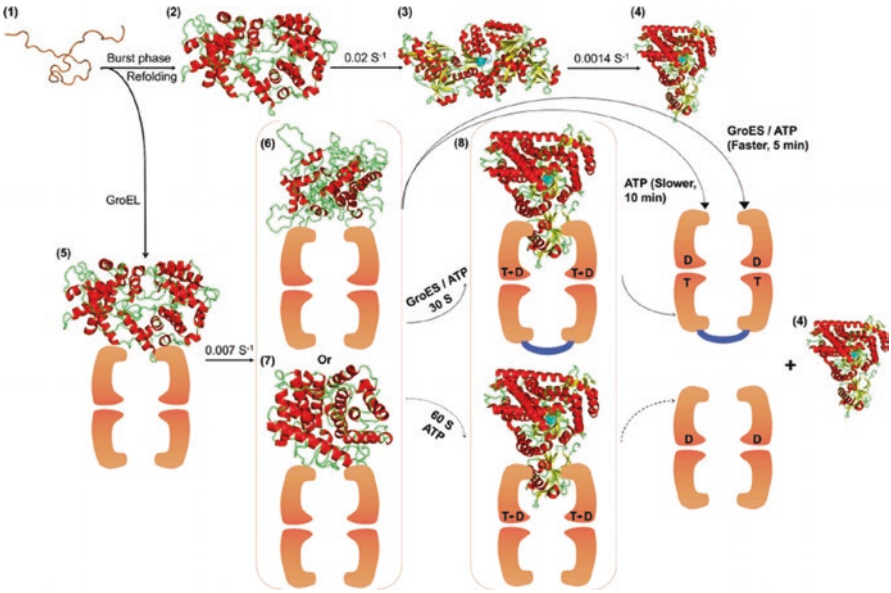


**Fig. 8.2 Proposed model for GroEL/ES assisted folding of large proteins via trans-mechanism:** The burst phase intermediate of MSG is captured by GroEL (orange colored) to form GroEL-MSG complex. This binding induces minor structural rearrangements to give rise to a more folding-compatible state. Further addition of GroES/ATP or ATP releases the GroEL-bound form of MSG, which folds to the native state via formation of a compact intermediate, that is structurally quite close to the native MSG. GroES (shown in blue) binds in Trans to the folding polypeptide and doubles the ATP-dependent reactivation rate

### *8.7.5 Passive Models of GroEL-Mediated Folding*

GroEL passively suppresses protein aggregation (Pelham 1986; Ellis and van der Vies 1991; Agard 1993) by binding to the exposed hydrophobic regions of aggregation prone proteins. GroEL specifically recognises and binds with the on-pathway intermediate states, which shifts the overall folding equilibrium away from aggregation and provides a pool of folding competent monomers that could reach their native state by further folding or assembly. In a purely passive folding model, high concentrations of an aggregating molecule cause non-linear increase in the rate of aggregation (Van den Berg 1999; Ellis 2001). GroEL prevents the formation of unfavourable intermediates that could lead to aggregation and hence aid in the proper folding of its substrates.

## 8.8 GroEL in Stress

### *8.8.1 Synthesis of GroEL during Stress*

During heat shock, activation of σ32 transcription factor leads to an increased production of Hsp inside *E.coli.* GroEL population increases from a basal level of 1–2% to 10–15% (of the total cellular protein content). Non-native substrates binding to GroEL results in a 2-fold increase (30% of the total cytoplasmic protein content) in the clientele of GroEL, as compared to normal conditions (10–15%) (Bukau 1993).

### *8.8.2 Structural and Functional Modifications during Heat Shock*

**Foldase** During heat shock, GroEL undergoes phosphorylation allowing it to function without GroES. Enhanced ATPase activity in the phosphorylated form ensures relatively fast release of the folded substrate from GroEL. The folding efficiency of phosphorylated GroEL is 50–100 fold higher than its native counterpart. Phosphorylation is a reversible process and once the cell is relieved of stress, GroEL undergoes de-phosphorylation and resumes its normal function inside the cell (Sherman and Goldberg 1994).

**Holdase** Our studies on monomeric GroEL show that the apical domain is the most stable region in the GroEL subunit as it requires 4.0 M urea and 70°C to undergo complete unfolding (Golbik et al. 1998; Puri and Chaudhuri 2017). This is a kind of adaptation in the GroEL structure that can possibly hold aggregating substrates under stress conditions. It is also observed that during thermal stress, GroELs protein folding activity is reduced and it starts acting as holdase by binding to the aggregation prone proteins, thus behaving as a storehouse of proteins. The molecular basis for such a functional transition is the loss of inter-ring signalling and negative cooperativity, which slows down the release of GroES and the unfolded proteins from the GroEL cavity. This phenomenon is also reversible with GroEL reverting to its normal function after heat shock (Llorca et al. 1998).

**Unfoldase** Strong binding of substrate proteins at the GroEL apical domain can be the main cause of unfolding or ‘stretching’ of bound substrates. The unfolding activity occurs through inter-domain movement where stretching of the apical domain helps in ‘opening up’ of the aggregating substrates. The unfolded substrate remains bound to the GroEL apical domain during stress conditions, but once cellular conditions become normal, GroEL is able to perform its folding function (Grallert et al. 1998).

### 8.8.3 ATP Independent Aggregation Prevention by GroEL Protein

Aggregation prevention tendency of GroEL is an ATP independent process. This proves useful during stress conditions, because of increasing load of aggregating proteins and relatively more ATP consumption to maintain cellular homeostasis (Soini, et al. 2005). In vivo studies in bacteria and yeast demonstrated that depletion of either GroEL or mitochondrial Hsp60 resulted in aggregation of a large number of newly translated proteins (Cheng et al. 1989; Horwich et al. 1993). Similarly, many in vitro studies demonstrated that GroEL can rapidly and efficiently bind to non-native states of several proteins and arrest their aggregation e.g., malate dehydrogenase (MDH; Ranson et al. 1995), ribulose-1,5-bisphosphate oxygenase-carboxylase (RuBisCO; Goloubinoff et al. 1989), etc. Our current study on aggregation prevention of Maltodextrin glucosidase (MalZ) demonstrate that GroEL can prevent aggregation of this protein without the involvement of GroES and ATP (Puri and Chaudhuri 2017). The passive binding of non-native intermediates to GroEL can prevent their aggregation by disallowing random hydrophobic interactions. Once captured proteins reach the folding competent state, they must be released back into the free solution, to undergo completion of the final steps of folding or oligomer assembly without ATP (Lin and Rye 2006).

## 8.9 HSP90 Mediated Protein Folding

**Structure**

HSP90 functions as a flexible dimer inside the cell; the dimers consist of two monomers joined at their C-terminus via the dimerization domain. The N-terminus consists of a conserved Bergerat ATP/ADP binding fold that also binds to Geldanamycin and Radicicol, competitive inhibitors of ATP binding (Pearl 2016; Bergerat et al. 1997; Stebbins et al. 1997; Roe et al. 1999). An N-terminus ‘lid’ segment that responds to ATP binding has been found, consisting of two highly conserved glycine clusters (Prodromou et al. 2000). The N-terminus is connected to the Middle (M) domain by a flexible linker that has been shown to convey allosteric modulations from the M-domain and the C-terminus to the N-terminus, resulting in global conformational changes. The M-domain binds to co-chaperones that “present” client proteins to HSP90. The C-terminus contains a unique, conserved MEEVD domain that binds to tetratricopeptide (TPR) domain containing co-chaperones. The nucleotide binding site lies in a deep pocket on the helical face of the N-domain. The adenine base, sugar, and the α- phosphate group make extensive contacts within this pocket, whereas the β- and the γ- phosphate groups display weak and no contacts respectively. A hydrogen bond connects the adenine base with Asp79, while all other contacts observed are polar in nature with water molecules interacting with the

ribose sugar moiety. The α- and the β- phosphate groups are bound to an octahedrally co-ordinated $Mg^{2+}$ ion, making an indirect coupling with the protein (Pearl 2016; Panaretou et al. 1998).

**ATPase Cycle**

HSP90s function as a chaperone is dependent on its inherent ATPase activity. The molecule undergoes a series of conformational changes upon ATP and client protein binding that culminates in ATP hydrolysis and release of the partially folded client protein (Li et al. 2012). Understanding the mechanism of this ATPase coupled conformational cycle has progressively advanced with several research groups using sophisticated biophysical techniques like FRET and Analytical Ultracentrifugation to identify the kinetic states in this cycle and the switch that occurs between these states (Hessling et al. 2009; Mickler et al. 2009). The defining mechanism is the 'molecular clamp mechanism', (Ali et al. 2006) where ATP binding to the open V-shaped constitutive HSP90 dimer (apo-state) induces structural changes via intermediates, the formation of each of which is regulated by co-chaperones that have specific roles to play in the cycle. The rate limiting step is the formation of a closed 'tensed' state, where the N-domains are dimerized and associated with the M-domains. This closed conformation is committed to ATP hydrolysis with subsequent release of the substrate and ADP occurring concomitantly as the N-domains dissociate and HSP90 returns to its open conformation, ready for another round of the ATPase cycle (Pearl 2016; Li et al. 2012). The rate limiting step involves major structural changes occurring in the N-domain in two distinct 'switch' regions: (a) the β-strand in the N-domain of one monomer hydrogen bonds with the main β-sheet in the N-domain of the other monomer. Simultaneous movement of the α-helix exposes a large hydrophobic patch that dimerizes with the equivalent patch of the other monomer; and (b) the 'lid' segment, which flips over ~180° from its apo conformation to fold over the bound nucleotide in the pocket and 'cradle' the γ-phosphate of ATP in a series of main-chain hydrogen bonds (Pearl 2016). Additionally, the flexible loop from the M-domain associates with the lid segment of the N-domain via hydrophobic residues. This intra-molecular docking of the N and M-domains facilitates the interaction between the γ-phosphate and the R380 residue, resulting in the assembly of the two- halves of a split active site for ATP hydrolysis (Pearl 2016; Meyer et al. 2003a, b; Cunningham et al. 2012). The stable HSP90 C-terminal dimer model was challenged by Ratzke and his team (Ratzke et al. 2010) who observed through FRET experiments that apart from the transient dimerization observed at the N-domains, the C-terminus can also open and close with fast kinetics, even when ATP/ADP is bound to the N-domain. They proposed a unique mechanism where HSP90 may undergo multiple transitions from being a dimer at the C-domain and open at the N-domains, to being open at the C-terminus while N-domains are dimerized, thereby associating a higher degree of dynamic flexibility that may influence substrate release (Ratzke et al. 2010). The following schematic depicts the conformational change from the open to the closed state of HSP90 via several transition intermediates (Fig. 8.3).

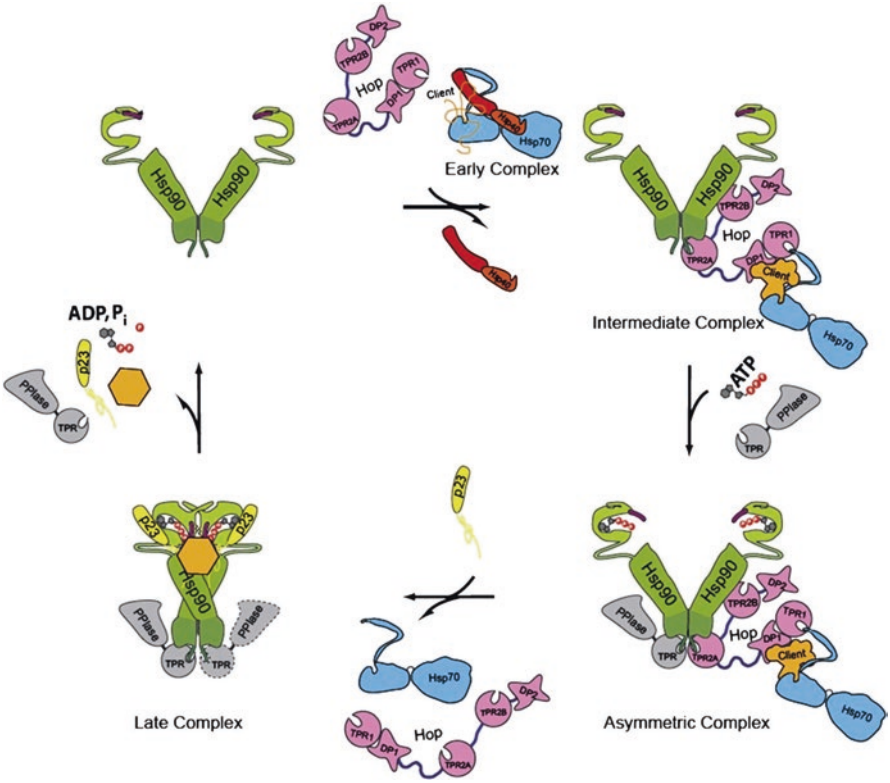


**Fig. 8.3 An overview of the conformational cycle of HSP90:** Several co-chaperones and ATP mediate the transition between the early, intermediate and late stages of substrate-bound HSP90 (Copyright clearance order number- 4177600710200)

## HSP90 co-Chaperones

HSP90 can only keep its substrates in a folding competent state and prevent them from undergoing aggregation, but cannot refold any of its clients by itself (Freeman and Morimoto 1996). It requires the assistance of other proteins to carry out its biological function. These proteins are also known as co-chaperones. Around 20 co-chaperones have been identified in eukaryotes and while some of them have been well-characterized, the mechanism by which some of the other co-chaperones function is unclear. These co-chaperones mainly regulate the ATPase activity and binding of client proteins to HSP90 (Prodromou et al. 1999; 2002; Richter et al. 2004; Roe et al. 2004; Chen and Smith 1998). They associate and dissociate dynamically and help in the transition from one intermediate conformation to another, or stabilize a certain conformation in the protein folding cycle. Some of these co-chaperones like HSP90/HSP70 Organizing Protein (HOP) (Johnson et al. 1998), protein phosphatase PP5 (Silverstein et al. 1997) and PPIase family members FKBP51

**Table 8.2** List of some well-studied co-chaperones of HSP90 and their role in the ATPase cycle (Modified from (Li et al. 2012))

| Protein Name | Function |
|---|---|
| *TPR containing co-chaperones* | |
| HOP | Stabilizes the open conformation of HSP90; inhibits ATP hydrolysis; simultaneously binds HSP90 and HSP70 and aids in transfer of nascent polypeptides recognized by HSP70. |
| FKBP51/52 | Maturation of steroid hormone receptors (SHR); chaperone. |
| CYP40 | Maturation of Estrogen receptors specifically; chaperone (Chen et al. 1998; Pirkl and Buchner 2001). |
| PP5 | Post translational modification of HSP90; dephosphorylates HSP90 and CDC37; plays a role in processing of client proteins. |
| TPR2 | Recognizes both HSP90 and HSP70 through its TPR domain; may act in the client transfer from HSP70 to HSP90 (Brychzy et al. 2003). |
| *Non-TPR co-chaperones* | |
| AHA1 | Stimulates ATPase activity; induces conformational change (Retzlaff et al. 2010; Sato et al. 2000). |
| P23 | Binds and stabilizes closed client bound HSP90 heterocomplex; maturation of client proteins; inhibits ATP hydrolysis; chaperone (Johnson and Toft 1994; Obermann et al. 1998; Bose et al. 1996; Freeman et al. 1996). |
| CDC37 | Binds specifically to client kinases and 'presents' them to HSP90 by binding to HSP90s N-terminal domain via its C-terminal; inhibits ATP binding and ATPase activity of HSP90; chaperone (MacLean and Picard 2003; Gaiser et al. 2010; Siligardi et al. 2002; Ali et al. 2006). |

(Nair et al. 1997) and FKBP52 (Cox et al. 2007; Johnson and Toft 1994) have a TPR domain that binds to the MEEVD sequence located in the C-terminus of the chaperone (Scheufler et al. 2000; Das et al. 1998). Table 8.2 (Modified from Li et al. 2012) lists some of the well-studied co-chaperones that have been identified and their role in the ATPase cycle of Hsp90.

**HSP90 co-chaperone Cycle**

The most recent understanding of the chaperone cycle is that there are three different complexes formed in a chronological fashion with different co-chaperone composition (Smith 1993). The first complex, called the early complex consists of HSP70, HSP40 and a client protein (Smith et al. 1992; Patricia Hernández et al. 2002; Cintron and Toft 2006), which presumably is a misfolded or nascent polypeptide. This complex docks to HSP90 via HOP which acts as a scaffold. One TPR domain of HOP binds to one MEEVD motif of the HSP90 dimer while another HOP domain binds to the early complex (Li et al. 2011). This HOP bound HSP90 complex can be called the intermediate complex. Addition of ATP and a PPIase results in the formation of an asymmetric complex, where the TPR domain of the PPIase binds with the unoccupied MEEVD motif of the other monomer, and

concomitantly ATP binds to the N-terminus (Smith 1993). The chaperone is still in its open confirmation. HSP90 adopts the closed 'committed to ATP hydrolysis' conformation after p23 binds to the intermediate complex (Johnson et al. 1994; Johnson and Toft 1995; McLaughlin et al. 2006; Freeman et al. 2000). This is called the late complex where HOP and HSP70/40 dissociate from HSP90 as their binding affinity is weakened due to the conformational change. After ATP hydrolysis, p23 and PPIase is released along with the partially folded client protein. Not much is known about the ADP bound conformation that re-positions the relative orientations of the N-domains just prior to the release of the client (Pearl 2016). Dynamic X-ray scattering data has revealed that HSP90 can exist in a highly flexible conformational ensemble (Rice et al. 2008; Zhang et al. 2004), even in the nucleotide free state and that the ADP bound state is a partially closed one with the N-domains close to each other, but different to the ATP bound closed complex where the N-domains dimerize (Scherrer et al. 1990).

**Expression and Regulation of HSP90s Function under Thermal Stress**

The upregulation of chaperones under heat shock conditions is mediated by the Heat Shock Transcription Factor 1 (HSF-1) (Fiorenza et al. 1995). HSF-1 under normal conditions remains in its inactive monomeric state bound to HSP90. Upon heat shock, HSP90 dissociates from the complex and HSF-1 undergoes trimerization (Baler et al. 1993; Sarge et al. 1993). This trimer can bind to DNA regulatory elements called the Heat Shock Elements (HSEs) and upregulate the transcription of HSP genes like HSP90 and HSP70 (Akerfelt et al. 2010). Elevated levels of HSP lead to inactivation of HSF-1; HSP90 and its cohorts FKBP2 and p23 bind to the trimeric state and attenuates its DNA binding affinity (Zou et al. 1998; Bharadwaj et al. 1999; Guo et al. 2001) while HSP70/HSP40 bind to HSF-1 and prevent its transactivation (Shi et al. 1998; Abravaya et al. 1992; Baler et al. 1992). This negative feedback loop regulates the chaperones at the transcription level, and thereby modulates the levels of misfolded nascent polypeptides inside the cell. Additionally, certain post translational modifications affect the chaperone functions of HSP90 (Scroggins and Neckers 2007). Under normal circumstances, HSP90 remains extensively phosphorylated, with as many as four phosphorylated residues in each isoform (Sefton et al. 1978; Kelley and Schlesinger 1982). Under heat shock conditions, the general understanding is that HSP90 is rapidly dephosphorylated, leading to the loss of HSP90s ability to stimulate the activity of its client proteins (Lees-Miller and Anderson 1989; Morange and Bensaude 1991). Dephosphorylation is mediated by Protein Phosphatase 1 (PP1), while phosphorylation is mediated by several kinases like CKII, DNA-PK, and Akt (Wandinger et al. 2006; Dougherty et al. 1987; Walker et al. 1985; Lees-Miller and Anderson 1989; Chalovich and Eisenberg 2012). After clients that are bound to HSP90 under normal temperature get released due to dephosphorylation, one of HSP90s client proteins, heme-regulated Inhibitor Kinase (HRI), is activated by the chaperone upon rapid phosphorylation and this in-turn down regulates protein synthesis by inactivating eukaryotic initiation Factor-2α subunit (Scroggins and Neckers 2007). This cycle of dephosphorylation and phosphorylation of HSP90 reduces the load of misfolded proteins inside the cell.

## 8.10 Combinatorial Assistance of Various Chaperones

There exists various checkpoints to ensure correct folding from the beginning of protein synthesis till it attains its native, biologically active conformation. Molecular chaperones act as a crucial buffer providing conditions conducive for the partially unfolded intermediate to fold. The chaperone machinery helps newly synthesized protein to navigate the complex energy landscape in order to achieve their native form. The following paragraph describes the presence of various chaperones at different spatial and temporal points, coordinating with each other to regulate protein folding.

The chaperones are present at different levels: The first chaperonic tier consists of ribosome associated chaperones. These chaperones stabilize the nascent polypeptides which are being synthesized on the ribosome and initiate their folding process. The second tier of components act subsequently and aid in the complete folding of the proteins. Both systems cooperate to form one single folding pathway. Chaperones involved in the first tier include prokaryotic chaperones Trigger Factor (TF) and eukaryotic Ribosome Associated Complex (RAC) (Kramer et al. 2009). These are present on the large ribosome near the exit tunnel of a polypeptide chain. This RAC complex comprises HSP70 homologs Ssb1, Ssb2 and Ssz1, zuotin (in yeast) and the corresponding homologs in higher eukaryotes (Hundley et al. 2005; Otto et al. 2005). These chaperones primarily bind to the exposed hydrophobic regions of the proteins. TF works in an ATP independent manner to fold a newly synthesized polypeptide. The bound polypeptide tries to bury its hydrophobic regions which facilitate the folding process. Interestingly most of the nascent chains (~70%) seek the assistance of TF and are successfully folded without any further assistance. Small proteins also fold spontaneously after their synthesis without any further assistance (Ferbitz et al. 2004; Merz et al. 2008).

Another class of proteins (~ 20%) possess strong hydrophobic elements (Teter et al. 1999; Thulasiraman et al. 1999) and thus TF and other ribosome associated chaperones do not provide enough assistance for their folding. It has been reported that TF dissociates from the ribosome while bound to the polypeptide chain (Agashe et al. 2004), thereby presenting the unfolded polypeptides to the downstream chaperones like DnaJ/DnaK (Martinez-Hackert and Hendrickson 2009). The DnaJ/DnaK system further interacts with the longer polypeptide chains and helps in their folding in an ATP dependent manner. In eukaryotes the second tier also consists of NAC (Nascent chain associated complex) and like TF, it interacts with the newly synthesized polypeptides and helps them attain their folded conformation with the assistance of RAC, HSP70, and other cofactors.

HSP70s along with other cofactors like HSP40, J-proteins and various NEFs aid in the folding of substrates in an ATP dependent manner (Zhu et al. 1996; Mayer et al. 2000; Pellecchia et al. 2000). HSP40 also binds with the misfolded polypeptides and recruits HSP70 to assist in the proper folding of substrates (Young et al. 2003). In eukaryotes, subsequent to the HSP70 system, HSP90 with the help of numerous regulators and co-chaperones acts as a finisher, helping the polypeptide

attain its biologically active structure (Pearl and Prodromou 2006; Zhao and Houry 2007; Scheufler et al. 2000). The remaining 10% of substrates that remain in a non-functional, yet non-aggregated state, are shifted to the chaperonin cage for their folding (Hartl 1996; Horwich et al. 2007). The environment in the nano cage of chaperonin facilitates the misfolded proteins to attain a native-like structure (Gromiha and Selvaraj 2004). In bacteria, GroEL/ES help in the folding of substrate proteins. In eukaryotes, substrate proteins are presented to the chaperonin TRiC. The reaction is mediated by HSP70 and Prefoldin which interact directly with the TRiC system resulting in the release of folded, functional proteins (Hartl and Hayer-Hartl 2002).

## 8.11 Conclusions

Compilation of studies both past and recent, on chaperone mediated protein folding unequivocally show that chaperones play a basal role in maintaining protein homeostasis inside the cell. While some of them function as 'foldases', hydrolysing ATP to carry out the folding and release of various substrates, a few others function independent of ATP, primarily as 'holdases'. Under stress conditions however, chaperones undergo multiple levels of modification; from the enhanced rates of ATP hydrolysis and more efficient substrate binding to various post-translational modifications that aid in the transcriptional upregulation of several hsp genes. Chaperones can also prevent aggregation of misfolded proteins that tend to accumulate under heat shock conditions. Chaperones play a dual role inside the cell; either rescuing misfolded or unfolded polypeptides and preventing aggregation or triggering degradation of substrates when cell damage is irreversible, both of which needs to be tightly regulated to maintain proteostasis. Such properties of chaperones are currently being utilized in the industry as well as in designing therapy for certain debilitating diseases like cancer and neurodegenerative disorders. Overexpression of certain proteins can be a major problem, especially when they are being expressed in a different host. Several studies including some of our own have shown that a chaperone or a combination of chaperones have been proven effective in increasing the yield as well as the active fraction of total protein expressed. Some of those proteins are considered therapeutically important; hence, large-scale productions of such proteins are regularly uptaken by the pharmaceutical industry. Chaperones thus ablate a significant clog in this giant wheel of industrial-scale protein production and such strategies have come to the rescue over the years. HSP90 has been used as a target to design inhibitors that have been successful in the amelioration of tumor progression and development, and is considered a hot prospect for drug-based therapy for the treatment of certain types of cancer. Although a promising approach, only a few compounds have reached the clinical trials and none of them have made it to the market. On the other end of the spectrum, the ability to prevent aggregation of misfolded proteins and even refold partially folded 'aggregation-prone' intermediates can be used to treat neurodegenerative disorders. We are

currently working on one such compound that stimulates the chaperone functions of HSP90 *in-vitro*, and plan to carry out several studies to investigate its potential in preventing certain neurodegenerative conditions. Overall, chaperones make an interesting topic of study, not only because they play such important roles in regulating protein function, stability and degradation but also because they possess tremendous value both industrially and therapeutically.

**Acknowledgments** The authors acknowledge the financial assistance from IIT Delhi and infrastructural facility from IIT Delhi, India. AS and AP acknowledge financial assistance from CSIR, Government of India for providing fellowships in their doctoral course programme. SP acknowledge financial assistance from UGC, Government of India for providing fellowships in their doctoral course programme. BKC acknowledges IIT Delhi for providing fellowship in the doctoral course program.

## References

Abravaya, K., et al. (1992). The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes and Development, 6*(7), 1153–1164.

Agard, D. (1993). To fold or not to fold. *Science, 260*(5116), 1903–1904.

Agashe, V. R., et al. (2004). Function of trigger factor and DnaK in multidomain protein folding. *Cell, 117*(2), 199–209.

Akerfelt, M., Morimoto, R. I., & Sistonen, L. (2010). Heat shock factors: Integrators of cell stress, development and lifespan. *Nature Reviews. Molecular Cell Biology, 11*(8), 545–555.

Ali, M. M. U., et al. (2006). Crystal structure of an Hsp90–nucleotide–p23/Sba1 closed chaperone complex. *Nature, 440*(7087), 1013–1017.

Altieri, D. C., et al. (2012). TRAP-1, the mitochondrial Hsp90. *Biochimica et Biophysica Acta-Molecular Cell Research, 1823*(3), 767–773.

Amin, J., Ananthan, J., & Voellmy, R. (1988). Key features of heat shock regulatory elements. *Molecular and Cellular Biology, 8*(9), 3761–3769.

Bakthisaran, R., Tangirala, R., & Rao, C. M. (2015). Small heat shock proteins: Role in cellular functions and pathology. *Biochimica et Biophysica Acta - Proteins & Proteomics, 1854*(4), 291–319.

Balch, W. E., et al. (2008). Adapting proteostasis for disease intervention. *Science, 319*(5865), 916–919.

Baler, R., Welch, W. J., & Voellmy, R. (1992). Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *The Journal of Cell Biology, 117*(6), 1151–1159.

Baler, R., Dahl, G., & Voellmy, R. (1993). Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Molecular and Cellular Biology, 13*(4), 2486–2496.

Baumann, F., Milisav, I., Neupert, W., & Herrmann, J. M. (2000). Ecm10, a novel hsp70 homolog in the mitochondrial matrix of the yeast Saccharomyces Cerevisiae. *FEBS Letters, 487*(2), 307–312.

van den Berg, B. (1999). Effects of macromolecular crowding on protein folding and aggregation. *The EMBO Journal, 18*(24), 6927–6933.

Bergerat, A., et al. (1997). An atypical topoisomerase II from archaea with implications for meiotic recombination. *Nature, 386*, 414–417.

Bharadwaj, S., Ali, A., & Ovsenek, N. (1999). Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. *Molecular and Cellular Biology, 19*(12), 8033–8041.

Bose, S., et al. (1996). Chaperone function of Hsp90-associated proteins. *Science, 274*(5293), 1715–1717.

Boston, R. S., Viitanen, P. V., & Vierling, E. (1996). Molecular chaperones and protein folding in plants. *Plant Molecular Biology, 32*(1), 191–222.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., & and-Sigler, P. B. (1994). The crystal structure of bacterial chaperonin GroEL at 2.8A$^{o}$. *Nature, 371*(6498), 578–586.

Braig, K., Adams, P. D., & Brunger, A. T. (1995). Conformational variability in the refined structure of the chaperonin GroEL at 2.8A$^{o}$ resolution. *Nature Structural and Molecular Biology, 2*(12), 1083–1094.

Brodsky, J. L., Goeckeler, J., & Schekman, R. (1995). BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. *Proceedings of the National Academy of Sciences, 92*(21), 9643–9646.

Brychzy, A., et al. (2003). Cofactor Tpr2 combines two TPR domains and a J domain to regulate the Hsp70/Hsp90 chaperone system. *The EMBO Journal, 22*(14), 3613–3623.

Buchberger, A., Bukau, B., & Sommer, T. (2010). Protein quality control in the cytosol and the endoplasmic reticulum: Brothers in arms. *Molecular Cell, 40*(2), 238–252.

Bukau, B. (1993). Regulation of the Escherichia Coli heat-shock response. *Molecular Microbiology, 9*(4), 671–680.

Burton, B. M., & Baker, T. A. (2005). Remodeling protein complexes: Insights from the AAA+ unfoldase ClpX and mu transposase. *Protein Science, 14*(8), 1945–1954.

Carrascosa, L., Muga, A., & Valpuesta, M. (1998). GroEL under Heat-Shock. *The Journal of Biological Chemistry, 273*(49), 32587–32594.

Chalovich, J. M., & Eisenberg, E. (2012). A proteomic screen identified stress-induced chaperone proteins as targets of Akt phosphorylation in Mesangial cells. *Biophysical Chemistry, 257*(5), 2432–2437.

Charmandari, E., Tsigos, C., & Chrousos, G. (2005). Endocrinology of the stress response. *Annual Review of Physiology, 67*, 259–284.

Chaston, J. J., et al. (2016). Structural and functional insights into the evolution and stress adaptation of type II Chaperonins. *Structure, 24*(3), 364–374.

Chaudhuri, T. K., Farr, G. W., Fenton, W. A., Rospert, S., & Horwich, A. L. (2001). GroEL/GroES-mediated folding of a protein too large to be encapsulated. *Cell, 107*(2), 235–246.

Chen, S., & Smith, D. F. (1998). Hop as an adaptor in the heat shock protein 70 (Hsp70) and Hsp90 chaperone machinery. *The Journal of Biological Chemistry, 273*(52), 35194–35200.

Chen, Q., et al. (1990). Accumulation, stability, and localization of a major chloroplast heat-shock protein. *The Journal of Cell Biology, 110*(6), 1873–1883.

Chen, C. F., et al. (1996). A new member of the hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat shock. *Molecular and Cellular Biology, 16*(9), 4691–4699.

Chen, S., et al. (1998). Differential interactions of p23 and the TPR-containing proteins hop, Cyp40, FKBP52 and FKBP51 with Hsp90 mutants. *Cell Stress & Chaperones, 3*(2), 118–129.

Cheng, M. Y., et al. (1989). Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature, 337*(6208), 620–625.

Cintron, N. S., & Toft, D. (2006). Defining the requirements for Hsp40 and Hsp70 in the Hsp90 chaperone pathway. *The Journal of Biological Chemistry, 281*(36), 26235–26244.

Cotto, J., Kline, M., & Morimoto, R. I. (1996). Activation of heat shock factor 1 DNA binding precedes stress- induced serine phosphorylation. *The Journal of Biological Chemistry, 271*(7), 3355–3358.

Cox, J. S., & Walter, P. (1996). A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell, 87*(3), 391–404.

Cox, M. B., et al. (2007). FK506-binding protein 52 phosphorylation: A potential mechanism for regulating steroid hormone receptor activity. *Molecular Endocrinology, 21*(12), 2956–2967.

Csermely, P., et al. (1998). The 90-kDa molecular chaperone family: Structure, function, and clinical applications. A comprehensive review. *Pharmacology & Therapeutics, 79*(2), 129–168.

Cunningham, C. N., et al. (2012). The conserved arginine 380 of Hsp90 is not a catalytic residue, but stabilizes the closed conformation required for ATP hydrolysis. *Protein Science, 21*, 1162–1171.

Dahiya, V., & Chaudhuri, T. K. (2014). GroEL/ES accelerates the refolding of a multi-domain protein through modulating on pathway intermediates. *The Journal of Biological Chemistry, 289*(1), 286–298.

Daniels, R., et al. (2003). N-linked glycans direct the cotranslational folding pathway of influenza hemagglutinin. *Molecular Cell, 11*(1), 79–90.

Das, A. K., et al. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: Implications for TPR-mediated protein-protein interactions. *The EMBO Journal, 17*(5), 1192–1199.

Ditzel, L., Lowe, J., Stock, D., Stetter, K. O., Huber, H., Huber, R., & Steinbacher, S. (1998). Crystal structure of the thermosome, the archaeal chaperonin and homolog of CCT. *Cell, 93*(1), 125–138.

Dougherty, J., et al. (1987). Identification of the 90Kda substrate of rat liver type II casein kinase with the heat shock protein which binds steroid receptors. *Biochimica et Biophysica Acta, 927*, 74–80.

Ellis, R. J. (2001). Macromolecular crowding: Obvious but underappreciated. *Trends in Biochemical Sciences, 26*(10), 597–604.

Ellis, R. J., & van der Vies, S. M. (1991). Molecular Chaperones. Annual Review of Biochemistry, 60(1), 321–347.

Ewalt, K. L., Hendrick, J. P., Houry, W. A., & Hartl, F. U. (1997). In Vivo observation of polypeptide flux through the bacterial chaperonin system. *Cell, 90*(3), 491–500.

Eyles, S. J., & Gierasch, L. M. (2010). Nature's molecular sponges: Small heat shock proteins grow into their chaperone roles. *Proceedings of the National Academy of Sciences, 107*(7), 2727–2728.

Felts, S. J., et al. (2000). Protein structure and folding: The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties the hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. *The Journal of Biological Chemistry, 275*(5), 3305–3312.

Ferbitz, L., et al. (2004). Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins. *Nature, 431*(7008), 590–596.

Fenton, W. A., & Horwich, A. L. (1997). Review, GroEL mediated protein folding. *Protein Science, 6*, 743–760.

Fenton, W. A., Kashi, Y., Furtak, K., & Horwich, A. L. (1994). Residues in chaperonin GroEL required for polypeptide binding and release. *Nature, 371*(6498), 614–619.

Figueiredo, L., Klunker, D., Ang, D., Naylor, D. J., Kerner, M. J., Georgopoulos, C., Hartl, F. U., & Hayer-Hartl, M. (2004). Functional characterization of an archaeal GroEL/GroES chaperonin system: Significance of substrate encapsulation. *The Journal of Biological Chemistry, 279*(2), 1090–1099.

Fiorenza, M. T., et al. (1995). Complex expression of murine heat shock transcription factors. *Nucleic Acids Research, 23*(3), 467–474.

Forouzan, D., Ammelburg, M., Hobel, C. F., Ströh, L. J., Sessler, N., Martin, J., & Lupas, A. N. (2012). The archaeal proteasome is regulated by a network of AAA ATPases. *The Journal of Biological Chemistry, 287*(46), 39254–39262.

Franck, E., et al. (2004). Evolutionary diversity of vertebrate small heat shock proteins. *Journal of Molecular Evolution, 59*(6), 792–805.

Freeman, B. C., & Morimoto, R. I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *The EMBO Journal, 15*(12), 2969–2979.

Freeman, B. C., et al. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *The EMBO Journal, 14*(10), 2281–2292.
Freeman, B. C., Toft, D. O., & Morimoto, R. I. (1996). Molecular chaperone machines: Chaperone activities of the cyclophilin Cyp-40 and the steroid aporeceptor-associated protein p23. *Science, 274*, 1718–1720.
Freeman, B. C., et al. (2000). The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. *Genes and Development, 14*(4), 422–434.
Frydman, J. (2001). Folding of newly translated proteins in vivo: The role of molecular chaperones. *Annual Review of Biochemistry, 70*, 603–647.
Fulda, S., et al. (2010). Cellular stress responses: Cell survival and cell death. *Int J Cell Biol, 2010*(2010), 1–23.
Gaiser, A. M., Kretzschmar, A., & Richter, K. (2010). Cdc37-Hsp90 complexes are responsive to nucleotide-induced conformational changes and binding of further cofactors. *The Journal of Biological Chemistry, 285*(52), 40921–40932.
Georgopoulos, C., Liberek, K., Zylicz, M., & Ang, D. (1994). Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. *Biol Heat Shock Prot Mol Chaperones*, 209–249.
Glover, J. R., & Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell, 94*(1), 73–82.
Golbik, R., Zahn, R., Harding, S. E., & Alan, F. R. (1998). Thermodynamic stability and folding of GroEL minichaperones. *Journal of Molecular Biology, 276*, 505–515.
Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989). GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in Escherichia coli. *Nature, 337*(6202), 44–47.
Goloubinoff, P., et al. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proceedings of the National Academy of Sciences, 96*(24), 13732–13737.
Grallert, H., Rutkat, K., & Buchner, J. (1998). GroEL traps dimeric and monomeric unfolding intermediates of citrate synthase. *The Journal of Biological Chemistry, 273*(50), 33305–33310.
Graner, M. W., Lillehei, K. O., & Katsanis, E. (2014). Endoplasmic reticulum chaperones and their roles in the immunogenicity of cancer vaccines. *Frontiers in Oncology, 4*(379), 1–12.
Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626–629.
Gromiha, M. M., & Selvaraj, S. (2004). Inter-residue interactions in protein folding and stability. *Progress in Biophysics and Molecular Biology, 86*(2), 235–277.
Gross, C. A. (1996). Function and regulation of the heat shock proteins in *Escherichia coli* and *Salmonella* cellular and molecular biology. *American Society for Microbiology*, 1382–1399.
Guisbert, E., Herman, C., Lu, C. Z., & Gross, C. A. (2004). A chaperone network controls the heat shock response in E. Coli. *Genes & Development, 18*(22), 2812–2821.
Guo, Y., et al. (2001). Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex. *The Journal of Biological Chemistry, 276*(49), 45791–45799.
Guy, C. L., & Li, Q.-B. (1998). The organization and evolution of the spinach stress 70 molecular chaperone gene family. *Plant Cell, 10*(4), 539–556.
Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature, 381*(6583), 571–580.
Hartl, F. U., Bracher, A., & Hartl, M. H. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*(7356), 325–332.
Hartl, F. U., & Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: From nascent chain to folded protein. *Science, 295*(5561), 1852–1858.
Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S., Saibil, H. R., & Buchner, J. (1999). Hsp26: A temperature-regulated chaperone. *The EMBO Journal, 18*(23), 6744–6751.

Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauf, S., & Buchner, J. (2004a). Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. *The EMBO Journal, 23*(3), 638–649.

Haslbeck, M., Ignatiou, A., Saibil, H., Helmich, S., Frenzl, E., Stromer, T., & Buchner, J. (2004b). A domain in the N-terminal part of Hsp26 is essential for chaperone function and oligomerization. *Journal of Molecular Biology, 343*(2), 445–455.

Hayer-Hartl, M., Bracher, A., & Hartl, F. U. (2016). The GroEL-GroES chaperonin machine: A nano-cage for protein folding. *Trends in Biochemical Sciences, 41*(1), 62–76.

Helenius, A., & Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. *Annual Review of Biochemistry, 73*, 1019–1049.

Hemmingsen, S. M., et al. (1988). Homologous plant and bacterial protein chaperone oligomeric protein assembly. *Nature, 333*, 330–334.

Hendrick, J. P., & Hartl, F. U. (1993). Molecular chaperone functions of heat-shock proteins. *Annual Review of Biochemistry, 62*(1), 349–384.

Herman, C., & Gross, C. A. (2000). Heat stress. In J. Lederberg (Ed.), *Encyclopedia of microbiology* (pp. 598–606). Academic Press.

Hessling, M., Richter, K., & Buchner, J. (2009). Dissection of the ATP-induced conformational cycle of the molecular chaperone Hsp90. *Nature Structural & Molecular Biology, 16*(3), 287–293.

Hietakangas, V., & Sistonen, L. (2006). Regulation of the heat shock response by heat shock transcription factors. *Chaperones*, 1–34.

Hightower, L. E. (1991). Heat shock, stress proteins, chaperones, and Proteotoxicity. *Cell, 66*(2), 191–197.

Hill, J. E., & Hemmingsen, S. M. (2001). Arabidopsis Thaliana type I and II chaperonins. *Cell Stress and Chaperones, 6*(3), 190–200.

Horwich, A. L., Fenton, W. A., Chapman, E., & Farr, G. W. (2007). Two families of chaperonin: Physiology and mechanism. *Annual Review of Cell and Developmental Biology, 23*, 115–145.

Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N., & Furtak, K. (1993). Folding in vivo of bacterial cytoplasmic proteins: Role of GroEL. *Cell, 74*(5), 909–917.

Hundley, H. A. (2005). Human Mpp11 J protein: Ribosome-tethered molecular chaperones are ubiquitous. *Science, 308*(5724), 1032–1034.

Imai, J., & Yahara, I. (2000). Role of HSP90 in salt stress tolerance via stabilization and regulation of calcineurin. *Molecular and Cellular Biology, 20*(24), 9262–9270.

Jakob, U., Gaestel, M., Engel, K., & Buchner, J. (1993). Small heat shock proteins are molecular chaperones. *The Journal of Biological Chemistry, 268*(3), 1517–1520.

Janowsky, B., Major, T., Knapp, K., & Voos, W. (2006). The disaggregation activity of the mitochondrial ClpB homolog Hsp78 maintains Hsp70 function during heat stress. *Journal of Molecular Biology, 357*(3), 793–807.

Johnson, J. L., & Toft, D. O. (1994). A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. *The Journal of Biological Chemistry, 269*(40), 24989–24993.

Johnson, J. L., & Toft, D. O. (1995). Binding of p23 and hsp90 during assembly with the progesterone receptor. *Molecular Endocrinology, 9*(6), 670–678.

Johnson, J. L., et al. (1994). Characterization of a novel 23-kilodalton protein of unactive progesterone receptor complexes. *Molecular and Cellular Biology, 14*(3), 1956–1963.

Johnson, B. D., et al. (1998). Hop modulates hsp70 / hsp90 interactions in protein folding. *The Journal of Biological Chemistry, 273*(6), 3679–3686.

Kampinga, H. H., & Craig, E. A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews. Molecular Cell Biology, 11*(8), 579–592.

Kang, B. H., et al. (2007). Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell, 131*(2), 257–270.

Kappé, G., et al. (2003). The human genome encodes 10 alpha-crystallin-related small heat shock proteins: HspB1-10. *Cell Stress and Chaperones, 8*(1), 53–61.

Kelley, P. M., & Schlesinger, M. J. (1982). Antibodies to two major chicken heat shock proteins cross-react with similar proteins in widely divergent species. *Molecular and Cellular Biology, 2*(3), 267–274.

Klumpp, M., Baumeister, W., & Essen, L. O. (1997). Structure of the substrate binding domain of the thermosome, an archaeal group II chaperonin. *Cell, 91*(2), 263–270.

Kourtis, N., & Tavernarakis, N. (2011). Cellular stress response pathways and ageing: Intricate molecular relationships. *The EMBO Journal, 30*(13), 2520–2531.

Kramer, G., Boehringer, D., Ban, N., & Bukau, B. (2009). The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nature Structural & Molecular Biology, 16*(6), 589–597.

Kriehuber, T., et al. (2010). Independent evolution of the core domain and its flanking sequences in small heat shock proteins. *The FASEB Journal, 24*(10), 3633–3642.

Krishna, P., & Gloor, G. (2001). The Hsp90 family of proteins in Arabidopsis Thaliana. *Cell Stress and Chaperones, 6*(3), 238–246.

Kültz, D. (2004). Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology, 67*(1), 225–257.

Langer, T., et al. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature, 356*(6371), 683–689.

Lee, U., et al. (2007). The Arabidopsis ClpB/Hsp100 family of proteins: Chaperones for stress and chloroplast development. *The Plant Journal, 49*(1), 115–127.

Lees-Miller, S. P., & Anderson, C. W. (1989). Two human 90-kDa heat shock proteins are phosphorylated in vivo at conserved serines that are phosphorylated in vitro by casein kinase II. *The Journal of Biological Chemistry, 264*(5), 2431–2437.

Leitner, A., Joachimiak, L. A., Bracher, A., Mönkemeyer, L., Walzthoeni, T., Chen, B., Pechmann, S., Holmes, S., Cong, Y., & Ma, B. (2012). The molecular architecture of the eukaryotic chaperonin TRiC/CCT. *Structure, 20*(5), 814–825.

Leskovar, A., et al. (2008). The ATPase cycle of the mitochondrial Hsp90 analog trap1. *The Journal of Biological Chemistry, 283*(17), 11677–11688.

Levy-Rimler, G., et al. (2002). Type I chaperonins: Not all are created equal. *FEBS Letters, 529*(1), 1–5.

Li, J., Richter, K., & Buchner, J. (2011). Mixed Hsp90-cochaperone complexes are important for the progression of the reaction cycle. *Nature Structural and Molecular Biology, 18*(1), 61–66.

Li, J., Soroka, J., & Buchner, J. (2012). The Hsp90 chaperone machinery: Conformational dynamics and regulation by co-chaperones. *Biochimica et Biophysica Acta – Molecular Cell Research, 1823*(3), 624–635.

Liberek, K., Galitski, T. P., Zylicz, M., & Georgopoulos, C. (1992). The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the $\sigma^{32}$ transcription factor. *Proceedings of the National Academy of Sciences, 89*, 3516–3520.

Lin, Z., & Rye, H. S. (2006). GroEL mediated protein folding: Making the impossible, possible. *Critical Reviews in Biochemistry and Molecular Biology, 41*(4), 211–239.

Lin, B. L., et al. (2001). Genomic analysis of the Hsp70 superfamily in Arabidopsis Thaliana. *Cell Stress and Chaperones, 6*(3), 201–208.

Lindquist, S. (1986). The heat-shock response. *Annual Review of Biochemistry, 55*(1), 1151–1191.

Lindquist, S., & Craig, E. A. (1988). The heat-shock proteins. *Annual Review of Genetics, 22*(1), 631–677.

Lizuka, R., & Funatsu, T. (2016). Chaperonin GroEL uses asymmetric and symmetric reaction cycles in response to the concentration of non-native substrate proteins. *Biophy and Physicobiol, 13*, 63–69.

Llorca, O., Galán, A., Carrascosa, J. L., Muga, A., & Valpuesta, J. M. (1998). GroEL under heat-shock. *Journal of Biological Chemistry, 273*(49), 32587–32594.

Lopez, T., Dalton, K., & Frydman, J. (2016). The mechanism and function of group II chaperonins. *Journal of Molecular Biology, 427*(18), 2919–2930.

Lyman, S. K., & Schekman, R. (1995). Interaction between BiP and Sec63p is required for the completion of protein translocation into the ER of Saccharomyces Cerevisiae. *The Journal of Cell Biology, 131*(5), 1163–1171.

Ma, Y., & Hendershot, L. M. (2004). ER chaperone functions during normal and stress conditions. *Journal of Chemical Neuroanatomy, 28*(1–2), 51–65.

MacLean, M., & Picard, D. (2003). Cdc37 goes beyond Hsp90 and kinases. *Cell Stress & Chaperones, 8*(2), 114–119.

Mani, N., et al. (2016). Multiple oligomeric structures of a bacterial small heat shock protein. *Scientific Reports, 6*, 1–12.

Martinez-Hackert, E., & Hendrickson, W. A. (2009). Promiscuous substrate recognition in folding and assembly activities of the trigger factor chaperone. *Cell, 138*(5), 923–934.

Maruyama, T., Suzuki, R., & Furutani, M. (2004). Archaeal peptidylprolyl cis-trans isomerases (PPIases) update. *Frontiers in Bioscience, 9*, 1680–1700.

Mayer, M. P. (2010). Gymnastics of molecular chaperones. *Molecular Cell, 39*(3), 321–331.

Mayer, M. P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T., & Bukau, B. (2000). Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Structural Biology, 7*, 586–593.

McClellan, A. J., et al. (2007). Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. *Cell, 131*(1), 121–135.

McCracken, A. A., & Brodsky, J. L. (1996). Assembly of ER-associated protein degradation in vitro: Dependence on cytosol, calnexin, and ATP. *The Journal of Cell Biology, 132*(3), 291–298.

McLaughlin, S. H., et al. (2006). The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. *Journal of Molecular Biology, 356*(3), 746–758.

Merz, F., et al. (2008). Molecular mechanism and structure of trigger factor bound to the translating ribosome. *The EMBO Journal, 27*(11), 1622–1632.

Meyer, A. S., et al. (2003a). Closing the folding chamber of the eukaryotic chaperonin requires the transition state of ATP hydrolysis. *Cell, 113*(3), 369–381.

Meyer, P., et al. (2003b). Structural and functional analysis of the middle segment of Hsp90: Implications for ATP hydrolysis and client protein and Cochaperone interactions. *Molecular Cell, 11*, 647–658.

Mickler, M., et al. (2009). The large conformational changes of Hsp90 are only weakly coupled to ATP hydrolysis. *Nature Structural and Molecular Biology, 16*(3), 281–286.

Miemyk, J. (2017). The 70 kDa stress-related proteins as molecular chaperones. *Trends in Plant Science, 2*(5), 180–187.

Minami, Y., & Minami, M. (1999). Hsc70/Hsp40 chaperone system mediates the Hsp90-dependent refolding of firefly luciferase. *Genes to Cells, 4*(12), 721–729.

Morange, M., & Bensaude, O. (1991). Heat shock increases turnover of 90 groups in HeLa cells. *Intl J Biol Stress, l*(2), 359–362.

Morimoto, R. I., Tissieres, A., & Georgopoulos, C. (1994). Progress and perspectives on the biology of heat shock proteins and molecular chaperones. *Biol Heat Shock Prot Mol Chaperones*, 1–30.

Morimoto, R. I., Kroeger, P. E., & Cotto, J. J. (1996). The transcriptional regulation of heat shock genes: A plethora of heat shock factors and regulatory conditions. In U. Feige et al. (Eds.), *Stress-Inducible Cellular Responses* (Vol. 77, pp. 139–163). Basel: Birkhäuser Basel EXS.

Muchowski, P. J., et al. (1999). ATP and the core "alpha-Crystallin" domain of the small heat-shock protein alphaB-crystallin. *The Journal of Biological Chemistry, 274*(42), 30190–30195.

Mujacic, M., Bader, M. W., & Baneyx, F. (2004). *Escherichia coli* Hsp31 functions as a holding chaperone that cooperates with the DnaK-DnaJ-GrpE system in the management of protein misfolding under severe stress conditions. *Molecular Microbiology, 51*, 849–859.

Nair, S. C., et al. (1997). Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. *Molecular and Cellular Biology, 17*(2), 594–603.

Nishikawa, S., & Endo, T. (1997). The yeast Jem1p is a DnaJ-like protein of the endoplasmic reticulum membrane required for nuclear fusion. *The Journal of Biological Chemistry, 272*(20), 12889–12892.

Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J., & Sambrook, J. (1989). S. Cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell, 57*(7), 1223–1236.

Obermann, W. M. J., et al. (1998). In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *The Journal of Cell Biology, 143*(4), 901–910.

Otto, H., et al. (2005). The chaperones MPP11 and Hsp70L1 form the mammalian ribosome-associated complex. *Proceedings of the National Academy of Sciences, 102*(29), 10064–10069.

Panaretou, B., et al. (1998). ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *The EMBO Journal, 17*(16), 4829–4836.

Panaretou, B., et al. (2002). Activation of the ATPase activity of Hsp90 by the stress-regulated cochaperone Aha1. *Molecular Cell, 10*(6), 1307–1318.

Patricia Hernández, M., Chadli, A., & Toft, D. O. (2002). HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. *The Journal of Biological Chemistry, 277*(14), 11873–11881.

Paul, S., Singh, C., Mishra, S., & Chaudhuri, T. K. (2007). The 69-kDa Escherichia Coli Maltodextrin Glucosidase does not get encapsulated underneath GroES and folds through trans mechanism during GroEL/GroES assisted folding. *The FASEB Journal, 21*(11), 2874–2885.

Pearl, L. H. (2016). Review: The HSP90 molecular chaperone - an enigmatic ATPase. *Biopolymers, 105*(8), 594–607.

Pearl, L. H., & Prodromou, C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annual Review of Biochemistry, 75*(1), 271–294.

Pelham, H. R. B. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell, 46*(7), 959–961.

Pellecchia, M., Montgomery, D. L., Stevens, S. Y., Vander Kooi, C. W., Feng, H., Gierasch, L. M., & Zuiderweg, E. R. P. (2000). Structural insights into substrate binding by the molecular chaperone DnaK. *Nature Structural Biology, 7*, 298–303.

Pirkkala, L., Nykänen, P., & Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *The FASEB Journal, 15*(7), 1118–1131.

Pirkl, F., & Buchner, J. (2001). Functional analysis of the Hsp90-associated human peptidylprolyl cis/trans isomerases FKBP51, FKBP52 and Cyp40. *Journal of Molecular Biology, 308*(4), 795–806.

Prändl, R., et al. (1998). HSF3, a new heat shock factor from Arabidopsis Thaliana, derepresses the heat shock response and confers thermotolerance when overexpressed in transgenic plants. *Molecular & General Genetics, 258*(3), 269–278.

Prodromou, C., et al. (1999). Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. *The EMBO Journal, 18*(3), 754–762.

Prodromou, C., et al. (2000). The ATPase cycle of Hsp90 drives a molecular ` clamp via transient dimerization of the N-terminal domains. *The EMBO Journal, 19*(16), 4383–4392.

Puri, S., & Chaudhuri, T. K. (2017). Folding and unfolding pathway of chaperonin GroEL monomer and elucidation of thermodynamic parameters. *International Journal of Biological Macromolecules, 96*, 713–726.

Rajaraman, K., et al. (2001). Interaction of human recombinant alphaA- and alphaB-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. *FEBS Letters, 497*(2–3), 118–123.

Rani, S., Srivastava, A., Kumar, M., & Goel, M. (2016). CrAgDb - a database of annotated chaperone repertoire in archaeal genomes. *FEMS Microbiology Letters, 363*(6), 1–6.

Ranson, N. A., Dunster, N. J., Burston, S. G., & Clarke, A. R. (1995). Chaperonins can catalyse the reversal of early aggregation steps when a protein misfolds. *Journal of Molecular Biology, 250*(5), 581–586.

Ratzke, C., et al. (2010). Dynamics of heat shock protein 90 C-terminal dimerization is an important part of its conformational cycle. *Proceedings of the National Academy of Sciences, 107*(37), 16101–16106.

Ray, D., et al. (2016). Plant stress response: Hsp70 in the spotlight. In A. A. A. Asea, P. Kaur, & S. K. Calderwood (Eds.), *Heat shock Prot plants, Heat shock proteins* (Vol. 10, pp. 123–147). Cham: Springer.

Retzlaff, M., et al. (2010). Asymmetric activation of the Hsp90 dimer by its Cochaperone Aha1. *Molecular Cell, 37*(3), 344–354.

Rice, L. M., et al. (2008). Article multiple conformations of *E .coli* Hsp90 in solution : Insights into the conformational dynamics of Hsp90. *Structure, 16*(5), 755–765.

Richter, K., Walter, S., & Buchner, J. (2004). The co-chaperone Sba1 connects the ATPase reaction of Hsp90 to the progression of the chaperone cycle. *Journal of Molecular Biology, 342*(5), 1403–1413.

Richter, K., Haslbeck, M., & Buchner, J. (2010). The heat shock response: Life on the verge of death. *Molecular Cell, 40*(2), 253–266.

Ritossa, F. (1996). Discovery of the heat shock response. *Cell Stress and Chaperones, 1*(2), 97–98.

Roe, S. M., et al. (1999). Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics Radicicol and Geldanamycin. *Journal of Medicinal Chemistry, 42*(2), 260–266.

Roe, S. M., et al. (2004). The mechanism of Hsp90 regulation by the protein kinase-specific Cochaperone $p50^{cdc37}$. *Cell, 116*(1), 87–98.

Rowley, N., Prip-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barreil, B., & Neupertt, W. (1994). Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. *Cell, 77*(2), 249–259.

Rudiger, S., Buchberger, A., & Bukau, B. (1997). Interaction of Hsp70 chaperones with substrates. *Nature, 4*(5), 686–689.

Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J., & Silver, P. (1989). A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *The Journal of Cell Biology, 109*(6), 2665–2675.

Sakikawa, C., Taguchi, H., Makino, Y., & Yoshida, M. (1999). On the maximum size of proteins to stay and fold in the cavity of GroEL underneath GroES. *Journal of Biological Chemistry, 274*(30), 21251–21256.

Santoro, N., Johansson, N., & Thiele, D. J. (1998). Heat shock element architecture is an important determinant in the temperature and transactivation domain requirements for heats hock transcription factor. *Molecular and Cellular Biology, 18*(11), 6340–6352.

Sarge, K. D., Murphy, S. P., & Morimoto, R. I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and Cellular Biology, 13*(3), 1392–1407.

Sarkar, N. K., Kundnani, P. and Grover, A. (2013). Functional analysis of Hsp70 superfamily proteins of rice (Oryza Sativa). *Cell Stress Chaperones*. Dordrecht: Springer Netherlands 18 (4), pp. 427–437.

Sato, S., Fujita, N., & Tsuruo, T. (2000). Modulation of Akt kinase activity by binding to Hsp90. *Proceedings of the National Academy of Sciences, 97*(20), 10832–10837.

Scherrer, C., et al. (1990). Structural and functional reconstitution of the glucorcorticoid receptor-Hsp90 complex. *The Journal of Biological Chemistry, 265*(35), 21397–21400.

Scheufler, C., et al. (2000). Structure of TPR domain-peptide complexes: Critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell, 101*(2), 199–210.

Schirmer, E. C., Glover, J. R., Singer, M. A., & Lindquist, S. (1996). HSP100/Clp proteins: A common mechanism explains diverse functions. *Trends in Biochemical Sciences, 21*(8), 289–296.

Schlenstedt, G., Harris, S., Risse, B., Lill, R., & Silver, P. A. (1995). A yeast DnaJ homologue, Scj1p, can function in the endoplasmic reticulum with BiP/ Kar2p via a conserved domain that specifies interactions with Hsp70s. *The Journal of Cell Biology, 129*(4), 979–988.

Schmitt, M., Neupert, W., & Langer, T. (1995). Hsp78, a Clp homologue within mitochondria, can substitute for chaperone functions of mt-hsp70. *The EMBO Journal, 14*(14), 3434–3444.
Schuster, G., et al. (1988). Evidence for protection by heat-shock proteins against photoinhibition during heat-shock. *The EMBO Journal, 7*(1), 1–6.
Scroggins, B. T., & Neckers, L. (2007). Post-translational modification of heat-shock protein 90: Impact on chaperone function. *Expert Opinion on Drug Discovery, 2*(10), 1403–1414.
Sefton, B. M., Beemon, K., & Hunter, T. (1978). Comparison of the expression of the src gene of Rous sarcoma virus in vitro and in vivo. *Journal of Virology, 28*(3), 957–971.
Sherman, M., & Goldberg, A. L. (1994). Heat shock induced phosphorylation of GroEL alters its binding and dissociation from unfolded protein. *The Journal of Biological Chemistry, 269*(50), 31479–31483.
Shi, Y., Mosser, D. D., & Morimoto, R. I. (1998). Molecular chaperones as HSF1-specific transcriptional repressors. *Genes and Development, 12*(5), 654–666.
Sichting, M., Mokranjac, D., Azem, A., Neupert, W., & Hell, K. (2005). Maintenance of structure and function of mitochondrial Hsp70 chaperones requires the chaperone Hep1. *The EMBO Journal, 24*(5), 1046–1056.
Siegert, R., et al. (2000). Structure of the molecular chaperone Prefoldin. *Cell, 103*(4), 621–632.
Silberstein, S., Schlenstedt, G., Silver, P. A., & Gilmore, R. (1998). A role for the DnaJ homologue Scj1p in protein folding in the yeast endoplasmic reticulum. *The Journal of Cell Biology, 143*(4), 921–933.
Siligardi, G., et al. (2002). Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50 cdc37. *The Journal of Biological Chemistry, 277*(23), 20151–20159.
Silverstein, A. M., et al. (1997). Protein phosphatase 5 is a major component of glucocorticoid receptor°hsp90 complexes with properties of an FK506-binding immunophilin. *The Journal of Biological Chemistry, 272*(26), 16224–16230.
Smith, D. F. (1993). Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Molecular Endocrinology, 7*(11), 1418–1429.
Smith, et al. (1999). Lon and Clp family proteases and chaperones share homologous substrate-recognition domains. *Proceedings of the National Academy of Sciences, 96*, 6678–6682.
Smith, B. J., & Yaffe, M. P. (1991). A mutation in the yeast heat-shock factor gene causes temperature sensitive defects in both mitochondrial protein import and the cell cycle. *Molecular and Cellular Biology, 11*(5), 2647–2655.
Smith, D. F., et al. (1992). Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. *The Journal of Biological Chemistry, 267*(2), 1350–1356.
Spiess, C., et al. (2004). Mechanism of the eukaryotic chaperonin: Protein folding in the chamber of secrets. *Trends in Cell Biology, 14*(11), 598–604.
Stebbins, C. E., et al. (1997). Crystal structure of an Hsp90 – Geldanamycin complex: Targeting of a protein chaperone by an antitumor agent. *Cell, 89*(2), 239–250.
Stromer, T., Fischer, E., Richter, K., Haslbeck, M., & Buchner, J. (2004). Analysis of the regulation of the molecular chaperone Hsp26 by temperature- induced dissociation: The N-terminal domain is important for oligomer assembly and the binding of unfolding proteins. *The Journal of Biological Chemistry, 279*(12), 11222–11228.
Sun, W., et al. (2001). At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. *The Plant Journal, 27*(5), 407–415.
Taylor, R. P., & Benjamin, I. J. (2005). Small heat shock proteins: A new classification scheme in mammals. *Journal of Molecular and Cellular Cardiology, 38*(3), 433–444.
Teter, S. A., et al. (1999). Polypeptide flux through bacterial Hsp70. *Cell, 97*(6), 755–765.
Thomas, J. G., & Baneyx, F. (2000). ClpB and HtpG facilitate de novo protein folding in stressed Escherichia Coli cells. *Molecular Microbiology, 36*(6), 1360–1370.
Thulasiraman, V., Yang, C., & Frydman, J. (1999). In vivo newly translated polypeptides are sequestered in a protected folding environment. *The EMBO Journal, 18*(1), 85–95.
Tissiéres, A., Mitchell, H. K., & Tracy, U. M. (1974). Protein synthesis in salivary glands of Drosophila Melanogaster: Relation to chromosome puffs. *Journal of Molecular Biology, 84*(3), 389–398.

Trachootham, D., et al. (2008). Redox regulation of cell survival. *Antioxidants and Redox Signaling, 10*(8), 1343–1374.

Vabulas, R. M., et al. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harbor Perspectives in Biology*, 1–18.

Vainberg, I. E., et al. (1998). Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. *Cell, 93*(5), 863–873.

Vanghele, M., & Ganea, E. (2010). The role of bacterial molecular chaperones in pathogen survival within the host. Rom. *Journal of Biochemistry, 47*(1), 87–100.

Wagner, I., Arlt, H., van Dyck, L., Langer, T., & Neupert, W. (1994). Molecular chaperones cooperate with PIM1 protease in the degradation of mis- folded proteins in mitochondria. *The EMBO Journal, 13*(21), 5135–5145.

Waldmann, T., et al. (1995). The Thermosome of Thermoplasma acidophilum and its relationship to the eukaryotic Chaperonin TRiC. *European Journal of Biochemistry, 227*(3), 848–856.

Walker, A. I., et al. (1985). Double-stranded DNA induces the phosphorylation of several proteins including the 90000 mol. Wt. heat-shock protein in animal cell extracts. *The EMBO Journal, 4*(1), 139–145.

Wandinger, S. K., et al. (2006). The phosphatase Ppt1 is a dedicated regulator of the molecular chaperone Hsp90. *The EMBO Journal, 25*(2), 367–376.

Wang, W., et al. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science, 9*(5), 244–252.

Wegele, H., Müller, L. and Buchner, J. (2004). Hsp70 and Hsp90—a relay team for protein folding. *Reviews of Physiology, Biochemistry and Pharmacology*, Springer, Berlin, Heidelberg 151, pp. 1–44.

Weissman, J. S., et al. (1995). Mechanism of GroEL action: Productive release of polypeptide from a sequestered position under GroES. *Cell, 83*(4), 577–587.

Welker, S., Rudolph, B., Frenzel, E., Hagn, F., Liebisch, G., Schmitz, G., Scheuring, J., Kerth, A., Blume, A., Weinkauf, S., Haslbeck, M., Kessler, H., & Buchner, J. (2010). Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function. *Molecular Cell, 39*(4), 507–520.

Yamada, K., et al. (2007). Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in Arabidopsis Thaliana. *The Journal of Biological Chemistry, 282*(52), 37794–37804.

Yang, D., Yea, X., & Lorimer, G. H. (2013). Symmetric GroEL: $GroES_2$ complexes are the protein-folding functional form of the chaperonin nanomachine. *Proceedings of the National Academy of Sciences, 110*(46), 4298–4305.

Young, J. C., Moarefi, I., & Hartl, F. U. (2001). Hsp90. *The Journal of Cell Biology, 154*(2), 267–274.

Young, J. C., Barral, J. M., & Hartl, F. U. (2003). More than folding: Localized functions of cytosolic chaperones. *Trends in Biochemical Sciences, 28*(10), 541–547.

Yu, A., et al. (2015). Roles of Hsp70s in stress responses of microorganisms, plants, and animals. *BioMed Res Intl, 2015*(2015), 1–8.

Zhang, W., et al. (2004). Biochemical and structural studies of the interaction of Cdc37 with Hsp90. *Journal of Molecular Biology, 340*(4), 891–907.

Zhao, R., et al. (2005). Navigating the chaperone network: An integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell, 120*(5), 715–727.

Zhao, R., & Houry, W. A. (2007). Molecular interaction network of the Hsp90 chaperone system, *BT - molecular aspects of the stress response: Chaperones, membranes and networks*. In P. Csermely & L. Vígh (Eds.), (pp. 27–36). NY: Springer.

Zhu, X., et al. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science, 272*(5268), 1606–1614.

Zou, J., et al. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell, 94*(4), 471–480.

# Chapter 9
# ER Stress, Human Health and Role of $Ca^{2+}$-Binding Chaperones


**Sasirekha Narayanasamy and Gopala Krishna Aradhyam**

**Abstract** The Endoplasmic Reticulum (ER) is a dynamic and versatile organelle involved in many critical functions of the cell. It is the major $Ca^{2+}$ storehouse of the cell and the intra luminal [$Ca^{2+}$] influences several cellular processes including synthesis of protein, lipids and sterols. Therefore, the concentration of $Ca^{2+}$ is tightly controlled and buffered by several $Ca^{2+}$-binding proteins in the ER. Several physiological and pathological disturbances can also perturb ER homeostasis, leading to the accumulation of misfolded or unfolded proteins, a condition termed as ER stress. The ER responds well to the stress by activating a series of signaling cascades known as unfolded protein response (UPR) in order to rescue ER functions. The adaptive response of the UPR pathway activates the transcription of several genes including molecular chaperones which aid in the folding of misfolded proteins. In addition to $Ca^{2+}$-binding (that regulates their function), these ER-resident calcium binding proteins play a major role in folding, post translational processing and quality control of other nascent polypeptide chains and hence can be classified as calcium-binding chaperones (CaBC). The role of CaBC in the UPR pathway is quite indispensable and will be discussed in this chapter.

**Keywords** $Ca^{2+}$-Binding proteins · $Ca^{2+}$ Signaling · Chaperones · Endoplasmic reticulum · ER stress · Unfolded protein response

## Abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| ALS | Amyotrophic lateral sclerosis |
| APP | Amyloid precursor protein |
| ASK1 | Apoptosis signal regulated kinase 1 |

S. Narayanasamy · G. K. Aradhyam (✉)
Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India
e-mail: agk@iitm.ac.in



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_9

| | |
|---|---|
| ATF4 | Activating transcription factor 4 |
| ATF6 | Activating transcription factor 6 |
| BiP | Binding immunoglobulin protein |
| $Ca^{2+}$ | Calcium |
| CABC | Calcium binding chaperones |
| CALR | Calreticulin |
| Calu | Calumenin |
| CaN | Calcineurin |
| CHOP | C/EBP homologous protein |
| CLABPs | Chaperone-like amyloid binding proteins |
| CNX | Calnexin |
| CSP | Cysteine string protein |
| CSQ | Calsequestrin |
| eIF2α | Eukaryotic translation initiation factor 2 |
| ER | Endoplasmic reticulum |
| ERAD | ER-associated degradation |
| ERS | ER stress |
| ERp29 | Endoplasmic protein of 29 kDa |
| ERp72 | Endoplasmic protein of 72 kDa |
| GRP57 | Glucose regulated protein 57 |
| GRP78 | Glucose regulated protein 78 |
| GRP94 | Glucose regulated protein 94 |
| HD | Huntington's disease |
| HRC | Histidine rich calcium-binding protein |
| HSP60 | Heat shock protein 60 |
| HSP70 | Heat shock protein 70 |
| IP3 | Inositol trisphosphate |
| IRE1 | Inositol-requiring enzyme |
| JNK | c-Jun N-terminal protein kinase |
| NCS | Neuronal calcium sensor |
| NucB1 | Nucleobindin-1 (Calnuc) |
| PD | Parkinson's disease |
| PDI | Protein disulfide isomerase |
| PERK | Protein kinase (RNA)-like endoplasmic reticulum kinase |
| PPIB | Peptidyl prolyl isomerase B |
| PVALB | Parvalbumin |
| SCGN | Secretagogin |
| SER | Sarco endoplasmic reticulum |
| TRAF2 | Tumor necrosis factor receptor associated factor 2 |
| UGGT | UDP-Glucose:Glycoprotein glycosyltransferase |
| UPR | Unfolded protein response |

## 9.1 Introduction

The Endoplasmic reticulum (ER) is an extensive, interconnected branching network of tubular membranes found in all eukaryotes and plays many vital functions in the life of the cell (Baumann and Walz 2001; Schroder 2008). Studded with ribosomes, ER is the main site for the synthesis of proteins, lipids and sterols. Besides, it is also involved in $Ca^{2+}$ homeostasis by $Ca^{2+}$ storage/release, aided by transporters and pumps (Baumann and Walz 2001). The protein folding machineries in the ER require ATP, $Ca^{2+}$ and oxidizing condition which makes the luminal environment highly sensitive to several internal and external insults (Braakman et al. 1992; Dorner et al. 1990; Gaut and Hendershot 1993). As a consequences of perturbation of ER homeostasis, the protein folding capacity of the ER is impaired, resulting in the accumulation of aggregated proteins, a condition termed as ER Stress (ERS) (Szegezdi et al. 2006). The accumulated, misfolded or unfolded proteins can irrevocably damage cellular functions, pose a cytotoxic threat and contribute to severe disease progression including neurodegenerative disorders, diabetes mellitus, obesity, cardiovascular diseases, liver diseases, cancer, and ischemia (Kaufman 2002; Kim et al. 2008; Minamino and Kitakaze 2010; Ron and Walter 2007). In order to combat the stress and restore ER homeostasis, a protective mechanism has evolved called Unfolded Protein Response (UPR). The key actions executed by the active machineries of unfolded protein response include global translational arrest and induction of the transcription of molecular chaperones and folding enzymes which are involved in protein folding and quality control (Ashby and Tepikin 2001; Coe and Michalak 2009; Walter and Ron 2011). If all of these measures fail to rescue the ER function, the unfolded protein response switches from pro-survival to the pro-apoptotic pathway (Szegezdi et al. 2006).

$Ca^{2+}$ is an essential signaling molecule and affects nearly all the functions of the cell (Berridge et al. 2003). A specialized compartment in the ER termed as SER (Sarco Endoplasmic Reticulum) is the major $Ca^{2+}$ storage organelle and functions to release and uptake huge amounts of $Ca^{2+}$ with the aid of transporters and pump (Lipskaia et al. 2009; Rhodes and Sanderson 2009; Taylor and Laude 2002). Although ER stores abundant $Ca^{2+}$, most of the $Ca^{2+}$ is buffered by binding to several $Ca^{2+}$-binding proteins (CaBPs) that reside in the ER. Many of these CaBPs involved in maintaining $Ca^{2+}$ homeostasis have moonlighting functions and are also molecular chaperones (Fig. 9.1). The molecular functioning of these CaBCs are tightly regulated by $Ca^{2+}$. Hence, fluctuations in the ER free $Ca^{2+}$ concentration have several impacts on the structure of CaBPs and also the assembly of folding complexes as well (Corbett et al. 2000; Corbett and Michalak 2000). In this chapter, we provide a basic framework on the structure-function relationship of the ER resident calcium binding chaperones, their potential role in $Ca^{2+}$-mediated protein folding and quality control processes.

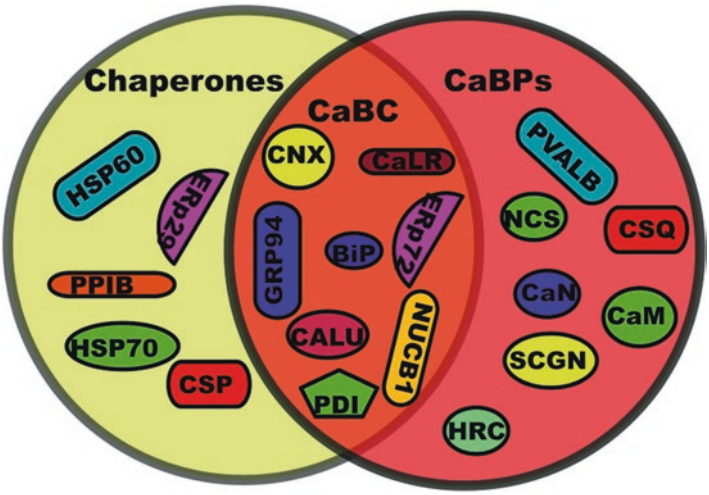


**Fig. 9.1** Dual molecular functions of calcium binding chaperones (CaBC) in the ER. $Ca^{2+}$-binding plays a regulatory role in chaperone function. These proteins have a variety of affinities for the metal ion

## 9.2 Protein Biogenesis in the Endoplasmic Reticulum

ER is a highly dynamic organelle, extending from nucleus to plasma membrane that harbours a large network of proteins which carry out many functions of ER including $Ca^{2+}$ homeostasis, lipid synthesis, sterol synthesis and folding of nascent polypeptides. Being the protein folding factory of the cell, the ER is the site for post-translational modification, folding and maturation, all necessary for their transport through the secretory pathway. Although, protein folding is a complex and error-prone process, >95% of newly synthesized polypeptide chains are properly folded with the aid of molecular chaperones and folding catalyst (Hammond and Helenius 1995). While the molecular chaperones prevent the aggregation of the nascent polypeptides by transient interaction, folding catalyst provides the correct disulfide bond formation in the protein. These properly folded proteins are then "enrouted" to Golgi apparatus and later destined for secretion or displayed on the plasma membrane surfaces (Walter and Ron 2011). Several factors such as the availability of oxidising conditions, ATP and $Ca^{2+}$ are required for the correct folding of the protein which eventuate the organelle susceptible to a variety of cellular stress (Gaut and Hendershot 1993).

## 9.3 Causes of ER Stress

A Variety of disturbances can perturb ER homeostasis and elicit an adaptive protective mechanism known as unfolded protein response (Malhotra and Kaufman 2007a; Ron and Walter 2007). Since most of the chaperone functions are $Ca^{2+}$ dependent, any alterations in the free calcium level can elicit ER stress and is the major cause for many unfolded protein complications (Ma and Hendershot 2004). Of note, small drugs such as thapsigargin and ionomycin interfere with the ER $Ca^{2+}$ store, resulting in either depletion /filling up of excess $Ca^{2+}$ and elicit UPR (Coe and Michalak 2009). Certain conditions such as hypoxia, exposure to oxidising or reducing agents can disturb the redox state of the lumen and interferes with disulfide bond formation in the proteins leading to the accumulation of misfolded proteins (Frand et al. 2000; Kim et al. 2008). Also, hypoglycaemic condition (deprivation of glucose) can elicit ER stress in the cell by intervening with the N-linked glycosylation of proteins (Kim et al. 2008; Schonthal 2012). Additionally, molecules such as tunicamycin and brefeldin A can disrupt protein glycosylation, and induce retrograde transport of proteins from Golgi to ER respectively (Navid and Colbert 2017). Furthermore, exposure to free radicals, acidosis, hyperthermia, inflammatory cytokines and even an elevated protein demand in the ER can cause stress (Oslowski and Urano 2011). The UPR can also be triggered in a cell upon viral infection, to be able to evade the viral encoded proteins from the system and prevent spread of the infection (Kim et al. 2008; Ron and Walter 2007). Even a mutation in a single gene and the resultant aberrant expression of the misfolded protein can generate ER stress (Oyadomari et al. 2002; Ron 2002). Furthermore, formation of protein-inclusion-bodies, typical of most chronic neurodegenerative diseases, as well as disorders such as inclusion-body myositis indirectly cause accumulation of unfolded proteins in the ER and induce ER stress (Cooper et al. 2006; Delaunay et al. 2008). Thus, the accumulation of misfolded proteins and aggregates pose a major threat to the cell which leads to various pathophysiological conditions.

## 9.4 Cellular Response to the ER Stress and Misfolded/ Unfolded Proteins

In order to maintain the fidelity of ER functions, a cascade of adaptive responses are activated as a result of ER stress to restrict the accumulation of unfolded/misfolded proteins (Cao and Kaufman 2012; Hetz 2012; Kozutsumi et al. 1988; Normington et al. 1989). The surveillance mechanism, as a part of quality control, together with the signaling network combats the stress and promotes cellular repair and survival. This, in turn, allows only the matured proteins to enter the secretory pathway (Malhotra and Kaufman 2007b).

The key actions in the UPR signaling can be majorly divided into three branches:

1. Attenuation of protein translation machineries in order to prevent the load of unfolded proteins and to disallow the entry of newly synthesized polypeptides into the ER.
2. Transcriptional induction of genes encoding for ER chaperones, folding enzymes and other essential functional components to cope up with the accumulated misfolded proteins.
3. Initiation of ER-associated degradation process (ERAD) in order to evacuate the load of mis- and unfolded proteins from the ER upon prolonged period of stress.

## 9.5 Regulation of the ER Stress by UPR Signaling

The UPR mediated protective response transduces the signal from the ER to nucleus and also to plasma membrane for transcriptional activation of genes encoding for ER resident chaperones and folding catalyst. The enriched molecular chaperone in the ER in turn inhibits the process of protein synthesis globally and promotes folding of proteins. However, the UPR also signals for apoptotic pathway under persisting stress condition and targets the misfolded proteins for degradation (Groenendyk et al. 2013; Michalak et al. 2009; Rutkowski and Kaufman 2004; Wang et al. 2015). The adaptive protective response mechanisms evoked by the UPR is well conserved among eukaryotes despite differences in the signal transduction mechanism (Walter and Ron 2011).

### *9.5.1 Activation of the Protective Response through UPR (Pro-Survival Pathway)*

There are three branches of ER stress sensors/transducers of the UPR pathway namely PERK, IRE1, and ATF6 which get activated in order to rescue the ER functions. These sensors are hibernated under normal conditions due to their association with the molecular chaperone, BiP/GRP78. Under stressed condition, the accumulation of misfolded proteins promotes the dissociation of BiP/GRP78 from these sensors leading to their active state, initiating the downstream signaling events (Fig. 9.2). While PERK is the first savior to be activated to salvage the ER-stressed cell, IRE1 is believed to be the last member to be dissociated from the BiP/GRP78 to initiate the protective response pathway (Szegezdi et al. 2006). Additionally, the stress response and the signaling mechanism evoked by these sensors vary and fade with time, irrespective of the persistence of stress. For instance, PERK has longer responsive time than ATF6 while the stress response

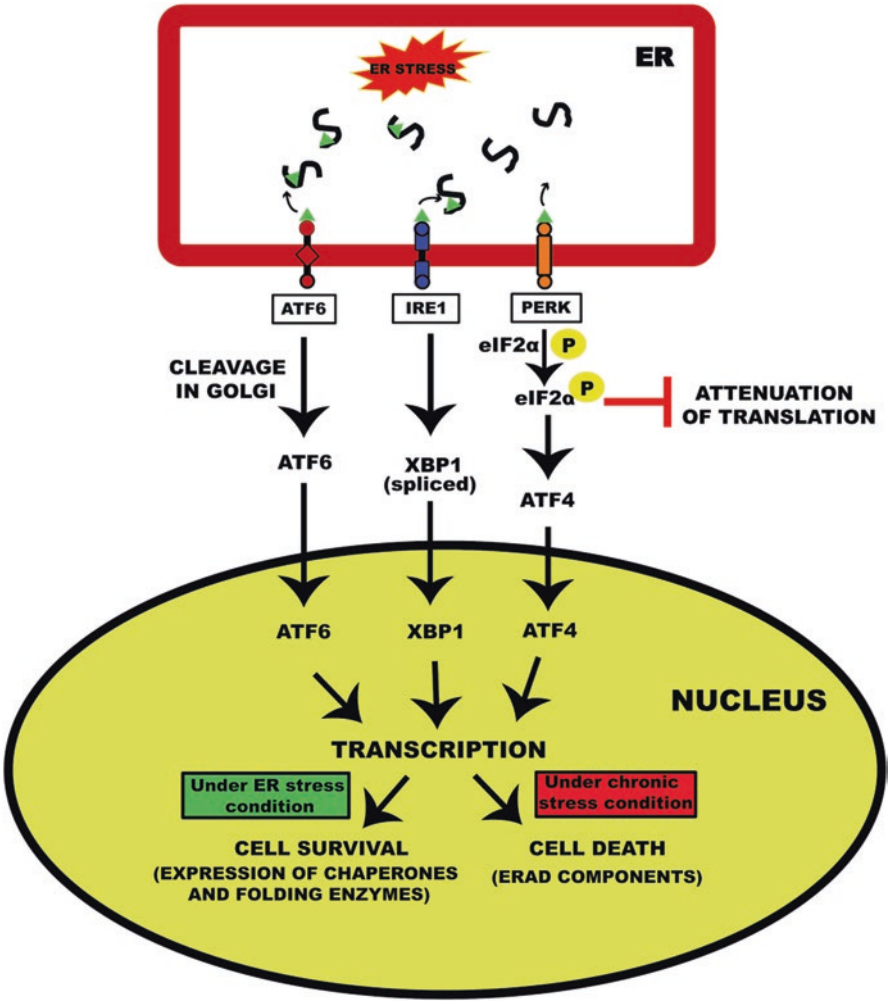


**Fig. 9.2** Schematic representation of unfolded protein response elicited during ER stress. The sensor proteins (ATF6, IRE1 and PERK) activate signals to the cell upon dissociation from BiP. Downstream effect could lead to either survival or death of cells

time for IRE1 is the shortest (Lin et al. 2007a). Intriguingly, the mode of signaling mechanism of ATF6 appears to be predominantly directed towards the survival of the cell (Logue and Gorman 2013). Whereas IRE1 and PERK, signal for both pro-apoptotic and pro-survival pathway (Szegezdi et al. 2006). The role of each of these UPR promoting ER stress sensors and the mechanisms are elucidated below.

### 9.5.2 IRE1 Signalling

IRE1 (Inositol Requiring Enzyme 1) is a type I ER transmembrane receptor protein which elicits the most conserved signaling branch among eukaryotic organisms (Chen and Brandizzi 2012, 2013; Hetz and Glimcher 2009; Hetz et al. 2011; Nagashima et al. 2011). The first isoform of IRE1 (IRE1α) is structurally organised into an N-terminal domain oriented towards the ER lumen followed by a single pass transmembrane domain and a C-terminal cytosolic domain. The cytoplasmic domain functions as a dual enzyme viz. Ser/Thr kinase and an endonuclease (RNase). Upon activation, as a result of dissociation of BiP/GRP78 from IRE1α during ER stress condition, IRE1α dimerizes and undergo conformational changes. The structural changes bring two IRE1α molecules in close proximity and trans-autophosphorylate each other through the kinase domain, leading to the activation of the endonuclease enzyme present in the cytosolic C-terminal domain (Ali et al. 2011; Aragon et al. 2009; Credle et al. 2005; Korennykh et al. 2009; Shamu and Walter 1996; Tirasophon et al. 1998). The IRE1α initiates the pro-survival pathway by tranducing the signal for the transcriptional activation of molecular chaperones through the endonuclease (RNAse) action on XBP1 (i.e., excision of nucleotides from an intron of XBP1 (X-box Binding Protein 1)) and renders the translation of XBP1 (Ron and Hubbard 2008). XBP1 is a transcription factor belonging to leucine zipper family which upregulates the expression of several protein folding genes such as protein disulfide isomerase (PDI) for the folding of misfolded proteins. However, XBP1 also activates the ERAD pathway upon high levels of persisting ER stress conditions (Lee et al. 2003). Simultaneously IRE1 also switches the protective response signaling to the pro-apoptotic pathway by recruiting TRAF2 (Tumor Necrosis factor receptor Associated Factor 2) which in turn activates ASK1 (Apoptosis Signal regulated Kinase 1) and JNK (c-Jun N-terminal protein Kinase) for the degradation of misfolded proteins under chronic stress conditions (Nishitoh et al. 2002; Nishitoh et al. 1998; Urano et al. 2000).

### 9.5.3 PERK Signalling

PERK (Protein kinase (RNA)-like Endoplasmic Reticulum Kinase) is a type I ER transmembrane kinase and has a significant role in protection of cell during unfolded protein response (Yang et al. 2015). Under ER stress conditions, the dissociation of BiP/GRP78 facilitates PERK dimerization and in turn becomes active through autophosphorylation of the cytoplasmic domain which contains Ser/Thr kinase activity. Thus, enabling the active cytoplasmic domain to inhibit the eukaryotic translation initiation factor 2 α (eIF2α) by phosphorylation via Ser/Thr protein kinase (Shi et al. 1998). Hence, the event of phosphorylation leads to the global translational arrest by preventing the complexation of the inactive eIF2α ribosomes. As a consequence, the heavy load of nascent proteins entering into the ER is

prevented, ensuring that the cell is protected from the cytotoxic effects of the accumulation of misfolded proteins (Harding et al. 2000; Kim et al. 2008; Oslowski and Urano 2011). Furthermore, the protective response of PERK signaling is augmented by the inactive eIF2α which preferentially translate ATF4 (Activating Transcription Factor 4) genes. ATF4 belongs to the leucine zipper family of transcription factors that regulate the transcription of several genes which either fold the proteins or target them to apoptosis (Fels and Koumenis 2006). However, under prolonged stress condition, PERK activates the CHOP signaling which is a vital switching element leading from pro-survival to the pro-apoptotic pathway during unfolded protein response (Szegezdi et al. 2006).

### *9.5.4 ATF6 Signalling*

ATF6 (Activating Transcription Factor 6), is a type II ER transmembrane protein and the associated signaling predominantly plays a role in the survival of the cell. Under normal condition, BiP/GRP78 is bound to the ATF6 molecule keeping it in an inactive state. Under ER-stress condition, the accumulation of mis- and un-folded proteins promotes the dissociation of BiP/GRP78 from ATF6 (Shen et al. 2002). The released ATF6 molecule migrates to the Golgi complex and gets converted into its active form by the action of site-1 (S1P) and site-2 (S2P) proteases (Chen et al. 2002; Ye et al. 2000). Thus, the activated ATF6 initiates the downstream signaling by entering into the nucleus and transcriptional activation of several genes coding for protein folding including GRP78, GRP94, PDI and also the transcription factors CHOP (C/EBP Homologous Protein) and XBP1 (X box-binding protein 1) (Schroder and Kaufman 2005b). Thus, the activity of ATF6 is unique among other sensors, since it always signals for the pro-survival pathway during unfolded protein responses.

## 9.6 Regulation of the ER Stress by Calcium Binding Chaperones (CaBC)

Chaperones belong to a class of proteins with versatile functions. Under normal conditions, they are designed to promote the folding process during post translational modification of proteins. However, under ER-stressed condition, the signal transducers such as IRE1α, PERK and ATF6 increase the expression level of these chaperones thereby improving the correct folding of the accumulated misfolded proteins. The primary role of these molecular chaperones is to bind the misfolded proteins and delay the folding process, thereby, increases the quality of the properly folded proteins and prevents the aggregation processes. However, on the flip side, molecular chaperones also guide the proteins that fail to fold to the ERAD pathway

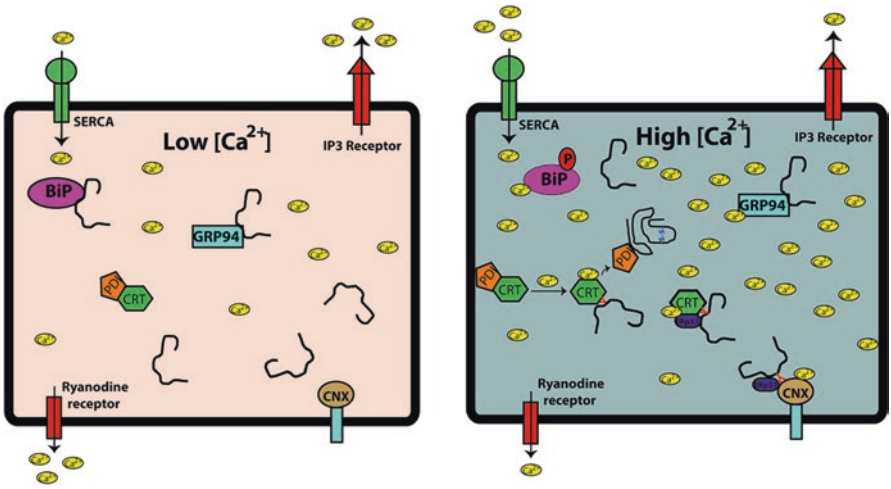


**Fig. 9.3** $Ca^{2+}$-dependent chaperone activity of calcium-binding chaperones

(Schroder and Kaufman 2005a; Xu et al. 2005). An additional physiological property of some class of molecular chaperones is that they are also $Ca^{2+}$-binding proteins and contributes to the overall $Ca^{2+}$ storage capacity of the ER (Coe and Michalak 2009; Michalak et al. 2002). Therefore, any alterations in the $Ca^{2+}$ concentration is expected to play a significant role in the regulation of chaperoning functions of these proteins attenuating their capacity to form folding complexes (Fig. 9.3). Since, these CaBC are regulated by $Ca^{2+}$, any differences in the free $Ca^{2+}$ in the ER as result $Ca^{2+}$ depletion/filling up the $Ca^{2+}$ stores might facilitate the passage of the unfolded 'client' substrate protein from one CaBC to another for proper folding. Except for calnuc and calumenin, most CaBCs lack well-defined $Ca^{2+}$-binding motifs (such as EF-hands), but ion binding is enabled by discrete stretches of acidic residues, widely distributed throughout the protein.

In general, ER consists of three groups of $Ca^{2+}$-binding molecular chaperones and folding catalyst contributing to the overall post translational modification of proteins coupled to native folding:-

- Classical molecular chaperones such as BiP (Hsp70) and its Co-chaperone (Hsp40) belonging to the heat shock protein family (HSP) which promote protein folding of non-glycosylated proteins with an intrinsic ATP-hydrolysing activity (Morito and Nagata 2015; Otero et al. 2010). These proteins need metal ion such as $Ca^{2+}$ for ATP binding and hydrolysis activity.
- The second group consists of oxidoreductases including protein disulfide isomerase (PDI), ERp57, and ERp72 of the PDI family which catalyse the oxidation of the cysteine residues and enable the formation and rearrangement of the disulfide bonds in the substrate protein (Ellgaard and Ruddock 2005).

- The third group comprises of lectins such as calnexin (CNX) and calreticulin (CALR) which recognize oligosaccharide chains attached on the nascent polypeptides and promote protein folding of glycoproteins (Helenius and Aebi 2004; Nishikawa et al. 2005). The lectin-like activity of these chaperones plays a crucial role in the quality control process during synthesis and folding of secretory proteins.

Each group of chaperones show difference in their substrate specificity. While the folding enzymes interact with the peptide moiety of their substrates and fasten the disulfide bond formation and rearrangements (disulfide interchange), lectins can recognize only the sugar moieties irrespective of the protein conformation (Rodan et al. 1996). Additionally, numerous factors regulate the chaperone activity in each group. For instances, the chaperoning activity of classical chaperones is an ATP driven processes and the chaperones cycles between ATP- and ADP bound form. During the ADP bound form, parts of the bound substrate peptide are transiently released to undergo protein folding (Bergeron et al. 1994; Hurtley and Helenius 1989). Interestingly, in lectin group of chaperones, the substrate glycoprotein undergoes continuous deglucosylation and reglucosylation cycles until the secretory proteins are correctly folded. Of note, these chaperones coordinate and assemble together and orchestrate the protein folding process (Xu and Ng 2015). The structure-function relationship of these CaBCs and their involvement in several diseases are summarised in Table 9.1.

## 9.7 Heat Shock Proteins

### *9.7.1 BiP*

BiP (The immunoglobulin (Ig) heavy chain (H) Binding Protein) also known as glucose regulated protein-78 (GRP78) is an ubiquitous and well-established chaperone belonging to the HSP70 family of stress-responsive proteins (Haas and Wabl 1983). It plays a critical role in regulating the UPR signaling pathway. Under normal conditions, it binds to the stress transducers such as ATF6, IRE1, and PERK and arrests them in a quiescent state. However, upon the condition of ER-stress, the accumulation of misfolded proteins in the ER leads to the dissociation of BiP from these sensors, thereby promoting the unfolded protein response. It is the major $Ca^{2+}$ storage protein of the ER and binds $Ca^{2+}$ as much as 25% of the total ER storage capacity with a relatively low capacity (Lievremont et al. 1997). Crystal structure of BiP demonstrates two $Ca^{2+}$-binding sites and lacks well-defined $Ca^{2+}$-binding motifs (Sriram et al. 1997). The many functions of BiP include protein synthesis, post-translational protein assembly, folding, translocation, uncoating of clathrin coated vesicles, protein degradation and modulation of protein expression (Beckmann et al. 1990; Sriram et al. 1997). Importantly, BiP plays a significant role in the

**Table 9.1** Involvement of Calcium binding chaperones in diseases

| S. No. | CaBC | Co-factors involved in chaperone function | Substrate proteins | Clinical features/ Disease | Causative element |
|---|---|---|---|---|---|
| 1. | BiP/GRP78 | ATP hydrolysis | Non-glycosylated proteins | Bipolar disorder | Deficiency of BiP gene Kakiuchi et al. (2005), Luo et al. (2006) |
| | | | | Cerebellar atrophy and degeneration of Purkinje cells | Loss of expression of GRP78 gene in purkinje cells Wang et al. (2009) |
| | | | | Neurodegeneration | Mutant BiP protein Jin et al. (2014) |
| 2. | GRP94 | ATP hydrolysis | Non-glycosylated proteins | Bipolar disorder | Deficiency of GRP94 gene Kakiuchi et al. (2007), Mao et al. (2010) |
| 3. | PDI | $Ca^{2+}$ | Disulfide bond containing proteins | Neurodegenerative diseases | S-Nitrosylation of PDI Uehara et al. (2006) |
| 4. | PDIA6 | ND | Disulfide bond containing proteins | Apoptosis | Partial loss of PDIA6 gene levels Vekich et al. (2012) |
| 5. | ERp44 | ND | Disulfide bond containing proteins | Defect in cardiac development and function | Deficiency of ERp44 gene Wang et al. (2014) |
| 6. | ERp57 | $Ca^{2+}$-binding increases activity | Disulfide bond containing proteins | Motor dysfunction and premature death; alterations of neuromuscular junctions | Abberant expression of ERp57 Woehlbier et al. (2016) |
| | | | | Amyotrophic Lateral Sclerosis (ALS) | Missense mutation in ERp57 gene Gonzalez-Perez et al. (2015) |
| 7. | ERp72 | $Ca^{2+}$-binding increases activity | Disulfide bond containing proteins | N.D | N.D |

(continued)

**Table 9.1** (continued)

| S. No. | CaBC | Co-factors involved in chaperone function | Substrate proteins | Clinical features/ Disease | Causative element |
|---|---|---|---|---|---|
| 8. | Calreticulin | $Ca^{2+}$ | Glycoproteins | Schizophrenia; | Point mutation in promoter of Calreticulin gene Aghajani et al. (2006) |
| | | | | Impairment of $Ca^{2+}$ release from IP3 receptor | Deficiency of Calreticulin gene Nakamura et al. (2001) |
| | | | | Compromised cardiac development | Aberrant expression of CRT Mesaeli et al. (1999) |
| | | | | Motoneuron degeneration | Decrease in calreticulin expression Bernard-Marissal et al. (2015) |
| 9. | Calnexin | $Ca^{2+}$ | Glycoproteins | Motor disorder | Deficiency in Calnexin gene Denzel et al. (2002) |
| | | | | Demyelination and decreased nerve conduction velocity | Deficiency in Calnexin gene Kraus et al. (2010) |
| 10. | Calnuc | $Ca^{2+}$-binding decreases activity | ND | Increase in the level of Amyloid Precursor Protein (APP) | Silencing of Calnuc gene Lin et al. (2007b) |
| 11. | Calumenin | $Ca^{2+}$-binding increases activity | ND | Apoptosis | Silencing of Calumenin gene Wang et al. (2017) |

N.D. Not determined

folding of non-glycosylated proteins (Hendershot 2004) or for glycoproteins where the attachment of N-terminal glycans occur later in the polypeptide after protein folding (Molinari and Helenius 2000). The overall structure of BiP can be divided into two distinct functional domains: - the N-terminal nucleotide binding domain (NBD) and a substrate binding domain (SBD) at the C-terminus (Bukau and Horwich 1998; Mayer and Bukau 2005). The NBD domain binds ATP and hydrolyses it to ADP whereas SBD of BiP recognizes the hydrophobic regions of the substrate protein and transiently binds to them. Binding of ATP and its consequent hydrolysis (to ADP bound form) induces conformational changes in the SBD

allosterically promoting closure of the C-terminal domain, thereby facilitating the attachment of the substrate polypeptide chain (Mayer and Bukau 2005). On nucleotide exchange of ADP for ATP, the interaction between BiP and the substrate protein is disrupted leading to the release of folded proteins. Thus, the chaperoning function of BiP appears to be an ATP dependent process and the nascent protein cycles between the BiP -bound and -free state until the protein has reached its native stable conformation (Jiang et al. 2005). The chaperoning activity of BiP is tightly controlled by phosphorylation which arrests the molecule in an inactive state. Additionally, phosphorylation of BiP takes place only in the presence of $Ca^{2+}$ indicating that the $Ca^{2+}$-bound structure of BiP demonstrate a state in which phosphorylation can occur (Sriram et al. 1997). Therefore, the event of phosphorylation in the presence of $Ca^{2+}$ and the associated inactive state of BiP suggest that $Ca^{2+}$ act as a negative regulator of BiP (Leustek et al. 1991).

### *9.7.2 GRP94*

GRP94 (Glucose regulated protein-94) is the most abundant chaperone of the ER belonging to the HSP90 family. Similar to other ER $Ca^{2+}$ storage proteins, GRP94 also binds $Ca^{2+}$ with low-affinity and high capacity, coordinated through the clusters of acidic residues (Argon and Simen 1999). Although, GRP94 is highly expressed and can be used as a bio-marker for detecting ER stress response system (Eletto et al. 2010), only a few proteins have been found to be its clients (Ostrovsky et al. 2009). The structure of GRP94 is organised into four domains: an N-terminal nucleotide binding domain (NTD), followed by an acidic linker domain, a middle domain and a C-terminal domain (CTD). The N-terminal domain of GRP94 contains an ATP and a peptide binding site. Similar to BiP, the chaperoning activity of GRP94 is coupled to ATP hydrolysis and is modulated by $Ca^{2+}$-binding. $Ca^{2+}$ binds to the charged residues in the acidic linker domain and induces major conformational changes in the N-terminal domain, which in turn enhances the peptide binding activity. Thus, $Ca^{2+}$-binding to GRP94 plays a regulatory role in the structure of GRP94 as it promotes binding of the unfolded protein in the presence of ATP (Nigam et al. 1994).

### *9.7.3 Folding Enzymes*

#### 9.7.3.1 PDI

Protein Disulfide Isomerase (PDI)/ERp58 is an efficient folding catalyst of the ER belonging to the thioredoxin superfamily which mediates the redox dependent disulfide-bond formation of proteins (Naidoo 2009). It is the major catalyst in the ER, the expression levels of PDI is almost equivalent to or in excess of nascent

proteins (Hartl et al. 1992). Under normal and stress conditions, the role of PDI assumes catalyzing the formation and rearrangement of disulfide bonds in the substrate proteins leading to subsequent protein folding processes. However, under stress condition, it prevents the cell from the cytotoxic effect of the accumulated misfolded proteins and clears the load of aberrant proteins by exporting them to the ERAD pathway (Nishikawa et al. 2005; Tu and Weissman 2004). Structurally, PDI enzyme contains four conserved thioredoxin-like domain (a-b-b'-a') which are necessary for the disulfide bond formation in the client proteins (Maattanen et al. 2006). PDI shows very high substrate specificity and high affinity towards unfolded proteins compared to the natively folded proteins (Klappa et al. 1998). PDI can discriminate completely unfolded proteins from the folded forms by recognising the hydrophobic regions in the unfolded protein. The b and b' domain are non-catalytic and are involved in the recognition of the hydrophobic regions in the substrate protein mediating the interaction of PDI with the unfolded client protein (Denisov et al. 2009; Klappa et al. 1998). Special feature, such as inter-domain mobility and flexibility of the PDI enables it to accommodate and catalyse folding of proteins with different size and shape (Irvine et al. 2014). Besides catalytic activity, PDI also binds $Ca^{2+}$ with low-affinity and high capacity (Lebeche et al. 1994; Macer and Koch 1988). However, the chaperoning activity of PDI is unaffected by $Ca^{2+}$-binding (Primm et al. 1996). For example, PDI shows chaperone activity in the absence of $Ca^{2+}$, but is augmented in its presence in case of refolding RNASe, insulin and BPTI demonstrating the modulation of PDI activity by $Ca^{2+}$ binding (Lucero and Kaminer 1999).

PDI interacts with several chaperones in the ER and any fluctuations in the free $Ca^{2+}$ concentration play a very significant role in the formation of coordination complexes. At very less concentration of $Ca^{2+}$ in the ER (below 100 μM; mimicking the ER empty $Ca^{2+}$ store), PDI binds to the N- and P-domain of calreticulin and decreases the concentration of the free calreticulin in the ER. This $Ca^{2+}$-independent interaction disrupts the chaperoning function of PDI as it fails to refold scrambled RNase A suggesting that calreticulin binding interface is at near the active sites of the PDI (Baksh et al. 1995). On the other hand, the interaction is short-lived and upon an increase in free $Ca^{2+}$ concentration in the ER, in the event of filling up the ER stores (>400 μM), calreticulin undergoes conformational changes upon binding $Ca^{2+}$ and dissociates from PDI. Thus, PDI and Calreticulin become active and resume its chaperoning function**.**

#### 9.7.3.2 ERP57

ERP57 is a thiol dependent oxidoreductase belongs to the PDI family of folding enzymes and shares high structural homology to PDI. Similar to PDI, ERp57 contains four thioredoxin-like domains and exhibits thiol-disulfide oxidoreductase activity thereby promoting disulfide bond formation in substrate proteins (Bourdi et al. 1995). ERp57 interacts with calnexin/calreticulin through the non-catalytic domain which has positively charged amino acids and in the presence of these

chaperones, the disulfide isomerase activity of ERp57 is enhanced several fold (Zapun et al. 1998; Pollock et al. 2004). This indicates that ERp57 functions as a part of the glycoprotein-specific quality control machinery in concert with calnexin/calreticulin (Kozlov et al. 2006; Maattanen et al. 2006; Russell et al. 2004; Oliver et al. 1997). Besides, it modulates the $Ca^{2+}$ homeostasis in the ER in a $Ca^{2+}$ dependent manner (Halperin et al. 2014; Li and Camacho 2004; Prins et al. 2011).

#### 9.7.3.3 ERp72/CaBP2/PDIA4

ERp72/CaBP2/PDIA4 belongs to the PDI family of folding catalyst and shares high sequence similarity to ERp57. It contains a protein disulfide isomerase activity and takes part in oxido-reduction reactions of secretory/membrane proteins. ERp72 also forms multi-protein complexes of chaperones which efficiently bind newly synthesized membrane and secretory proteins (Meunier et al. 2002). Structurally, ERp72 contains five thioredoxin-like domains. The N-terminal region contains a consecutive stretches of negatively charged residues which are disordered and unstructured by nature. These residues bind $Ca^{2+}$ and facilitate interaction with basic charged ER proteins/substrates. Despite sharing high homology, unlike ERp57, ERp72 does not interact with calnexin owing to the difference in the charged residues on the surface of ERp72 (Kozlov et al. 2009). Among the members of PDI family, ERp72 has an unique role of retaining misfolded proteins such as cholera toxin and mutant thyroglobulin without targeting to proteasome degradation (Forster et al. 2006; Kozlov et al. 2009). Although, $Ca^{2+}$-binding to the N-terminal domain facilitates protein-protein interaction of ERp72 (Chen et al. 2008), the exact role of $Ca^{2+}$ regulation of this protein is currently unknown.

### *9.7.4 Lectins*

#### 9.7.4.1 Calreticulin

Calreticulin (CALR) is the major $Ca^{2+}$ storage/chaperone protein of the ER (Michalak et al. 2009). It is an abundant protein and well-conserved in diverse organisms except yeast (Michalak et al. 1999). Calreticulin has been reported to play a variety of cellular processes and most of these functions in the ER owing to its ability to acts as the $Ca^{2+}$ buffer (Michalak et al. 2009). The structure of calreticulin can be divided into three distinct major domains (Fliegel et al. 1989; Nakamura et al. 2001; Ostwald and MacLennan 1974) namely an N-terminal globular domain and a C-terminal acidic domain which are connected through a central long flexible P-domain arm (Michalak et al. 2009). Secretory proteins undergo post translational glycosylation modification and trimming and are presented to the chaperones for proper folding. Lectins such as calreticulin recognise the mono-glycosylated oligosaccharides in the newly synthesized secretory proteins

via the N-domain (Kozlov et al. 2010). However, the central proline rich P-domain contains a weaker oligosaccharide recognition site and also contributes to the lectin-like functioning of calreticulin (Leach et al. 2002). This acidic P-domain also possess a $Ca^{2+}$-binding site with relatively high affinity and low capacity (Coe and Michalak 2009). That the lectin binding site overlaps with the $Ca^{2+}$-binding in the P-domain suggests that the $Ca^{2+}$-bound form of calreticulin is essential for the folding of lectins. It has been known that calreticulin binds over 50% of total $Ca^{2+}$ in the ER through the high capacity and low-affinity $Ca^{2+}$-binding site in the C-domain and is a major $Ca^{2+}$ reservoir of the ER (Baksh and Michalak 1991; Nakamura et al. 2001). Calreticulin also interacts with PDI and ERp57 through the $Ca^{2+}$-binding C-domain suggesting that the free luminal $Ca^{2+}$ concentration is mandatory for the regulation of these interactions. Thus, the molecular function of $Ca^{2+}$-binding to calreticulin secures dual advantages (Corbett et al. 2000; Martin et al. 2006); it regulates the free $Ca^{2+}$ concentration and acts as $Ca^{2+}$ store (Krause and Michalak 1997) as well as chaperoning the glycoproteins together with folding catalyst in the presence of $Ca^{2+}$. Intriguingly, the chaperoning activity of calreticulin is inhibited at very low concentration of $Ca^{2+}$ as it facilitates dissociation of $Ca^{2+}$ from calreticulin. The $Ca^{2+}$ free form of calreticulin evades the proteolytic tryptic digestion by its association with PDI. On the other hand, upon increase in $Ca^{2+}$ concentration, $Ca^{2+}$-binding leads to conformational change evident by its dissociation from the PDI and resistance to tryptic digestion by forming an N-terminal protease resistant core. This functional $Ca^{2+}$-bound form of the calreticulin recognises the mono-glycosylated proteins for folding. Thus recognition and folding of glycoproteins by calreticulin is a $Ca^{2+}$ dependent process.

#### 9.7.4.2 Calnexin

Calnexin (CNX) is an integral membrane protein of the ER which binds to the mono-glycosylated oligosaccharides of the un- and mis-folded glycoproteins and promotes their folding (Hebert and Molinari 2007). The crystal structure of the N-terminal construct, without the C-terminal region shows that it has two functional domains, namely P-domain and the globular carbohydrate binding domain (Schrag et al. 2001; Schrag et al. 2003). Since, calnexin shows high structural homology to P-domain of calreticulin, both the proteins share similar substrate specificity (Schrag et al. 2003; Williams 2006). The highly conserved P-domain of calnexin contains an ERp57-binding site indicating that the nascent glycoproteins are initially recognised and folded by calnexin/calreticulin and presented to the ERp57 for disulfide bond formation and rearrangement in order to achieve its native conformation (Pollock et al. 2004). Together with UGGT (UDP-Glucose:Glycoprotein glycosyltransferase) and glucosidases, these CaBC (calreticulin, calnexin and ERp57) play a significant role in the folding and quality control of nascent glycoproteins (Hebert and Molinari 2007). Because there is a direct relationship between high-affinity $Ca^{2+}$-binding and the lectin-like chaperone function of calreticulin and calnexin, it is likely that the folding of newly synthesized glycoproteins is highly sensitive to

changes in the free $Ca^{2+}$ ER. The intraluminal $Ca^{2+}$ plays a critical role in the chaperoning function of these proteins as the $Ca^{2+}$ chelators abrogates the formation of folding complexes (Bergeron et al. 1994). It has been well studied that these interactions are hampered in the absence of $Ca^{2+}$ resulting in the accumulation of misfolded proteins and also induces ER stress leading to cell death.

## 9.8 Chaperones of the New Kind

Recently, a new group of proteins have been characterised to have a promising chaperone activity in the cell. Unlike lectins and folding enzymes, these have well-defined $Ca^{2+}$-binding motifs such as EF-hands and are known to prevent aggregation of proteins induced by various stress. These are also known to inhibit the fibrillation of amyloid proteins leading to the identification of novel therapeutic approaches. Unlike other proteins, the recognition centre for the prevention of aggregation needs to be studied.

### *9.8.1 Calnuc*

Calnuc (NUCB1) also known as Nucleobindin-1 is an ubiquitously expressed and a well conserved protein of the Golgi apparatus (Kawano et al. 2000; Lin et al. 1998; Lin et al. 1999; Tsukumo et al. 2009). Overexpression of calnuc led to an increase in $Ca^{2+}$ storage, suggesting that calnuc is directly involved in $Ca^{2+}$ homeostasis in Golgi (Lin et al. 1999). Although calnuc constitutes the major $Ca^{2+}$-binding and storage protein of Golgi complex (Lin et al. 1998; Lin et al. 1999), it is initially targeted to ER via the ER signal peptide. The ER specific localisation signal gets proteolytically cleaved from the calnuc and it is translocated to the cis-Golgi network. Calnuc undergoes post-translational modifications such as O-linked glycosylation, sulfation and sialyation inside the Golgi complex and can be secreted from the cell (Lavoie et al. 2002; Wendel et al. 1995). The structural organisation of calnuc can be divided into DNA-binding motif, two EF-hand $Ca^{2+}$-binding motifs and a leucine zipper motif (Kanuru et al. 2009). Calnuc has been found to have an important role in ER-stress and is upregulated during unfolded protein response, a property similar to other chaperones. However, the molecular functioning of calnuc in the UPR pathway is still elusive. Conversely, calnuc has an inhibitory effect on the proteolytic cleavage of pro-survival ER-stress transducer, ATF6 (Tsukumo et al. 2007). During ER-stress condition, the processing of ATF6 is transiently increased and gradually declined through an unknown regulatory mechanism indicating that calnuc probably acting as a negative regulator of ATF6 (Okada et al. 2003; Tsukumo et al. 2007; Yoshida et al. 1998). One of the outcomes of ER stress is the upregulation

and induction of amyloid β-peptide production. Liu et al., (2014) have shown that APP (amyloid precursor protein) has a negative impact on cell survival and mediates ER-stress induced apoptosis through CHOP pathway (Takahashi et al. 2009). Calnuc has been found to bind APP, a major pathophysiological hallmark of several neurodegenerative diseases such as Alzheimer's Disease (AD) and prevents its biosynthesis (Lin et al. 2007b). This accounts for the chaperoning activity of calnuc during ER-induced stress condition. More recently, calnuc has been reported as a novel CLABP (Chaperone-Like Amyloid Binding Proteins) inhibiting fibrillation processes by binding to pre-fibrillar amyloids (Bonito-Oliva et al. 2017). Prevention of amyloid formation may propose the involvement of calnuc in controlling certain neurodegenerative disease through its effect on the pre-fibrillar formation. Unlike other CaBC, $Ca^{2+}$ acts as a negative regulator of the chaperone activity of Calnuc. The interaction between calnuc and APP is $Ca^{2+}$ sensitive (Gupta et al. 2012; Lin et al. 2007b) and the prevention of aggregation of thermally stressed proteins is disrupted in the presence of $Ca^{2+}$. On the other hand, $Zn^{2+}$-binding has no effect in calnuc's chaperoning activity (Kanuru and Aradhyam 2017).

### *9.8.2 Calumenin*

Calumenin (Calu) is a multiple EF-hand containing $Ca^{2+}$-binding protein belonging to the CREC family. It interacts with various binding partners and has been found to have a profounding role in $Ca^{2+}$ homeostasis (Honore 2009). Despite being an ER resident, it is also found in the secretory pathway and can be secreted into the extracellular space (Wang et al. 2012). However, the physiological function of this protein remains unknown and still emerging. Similar to other ER-resident $Ca^{2+}$-binding chaperones, calumenin also responds well to ER stress and is upregulated to protect the cells (Lee et al. 2013). Additionally, overexpression of calumenin downregulates the expression of stress responding elements such as GRP78, p-PERK and p-eIF2α thereby prevents the accumulation of misfolded proteins in the ER. Interestingly, it also prevents the cells succeeding to apoptosis by reducing the expression levels of apoptotic inducers such as CHOP and p-JNK proteins probably playing a survival role (Lee et al. 2013). Recently, calumenin has been identified as a G551D-CFTR chaperone and also the F508del-CFTR folding modulator protein (Tripathi et al. 2014). Furthermore, calumenin has known to strongly associate with G551D-CFTR than the wild type CFTR protein probably involved in the maturation of G551D-CFTR through an altered pathway. The anti-aggregation property of calumenin on F508del-CFTR mutant protein has been investigated. It has been shown that calumenin in the absence of $Ca^{2+}$ prevents the aggregation of F508-del mutant more strongly than in the presence of $Ca^{2+}$ (Tripathi et al. 2014).

## 9.9 Activation of the ERAD (ER-Associated Degradation) Pathway of UPR Signaling (Pro-Apoptotic Pathway)

The UPR diverts the signaling pathway to become pro-apoptotic only under the condition that, all the protective responses evoked by the signaling molecules have been unsuccessful and ineffective, and when the ER is experiencing stress for a prolonged period of time. The activated maladaptive response in turn expel out all the accumulated misfolded/aberrant proteins from the ER, transport back into cytoplasm and degrade with the aid of ubiquitin proteasome system known as ER-associated degradation (ERAD) pathway (Brodsky and McCracken 1997; McCracken and Brodsky 2003; Szegezdi et al. 2006; Tsai et al. 2002). Thus, the protective response evoked by IRE1 and PERK signalling also plays an indirect role in inducing the maladaptive response through the expression of the pro-apoptotic signaling molecules (Ferri and Kroemer 2001; Xu et al. 2005). However, the switching mechanism leading from survival to cell death remains unclear and is still emerging. Although, the unfolded protein responses can initiate pro-survival or pro-apoptotic pathway, depending on the severity of the stress, it is still remain to be answered as to how the ER differentiates these opposing functions i.e., folding of nascent proteins and activation of ERAD in the same compartment. It has been proposed that different regions of the ER might be populated by different groups of chaperones which contribute to establish these contrasting functions.

The key actions involved in maladaptive response signaling are as follows:

- Induction of the pro-apoptotic CHOP pathway through the downstream signaling of PERK/eIF2α.
- Activation of ASK1 and JNK through IRE1.
- $Ca^{2+}$ release from the ER through Bax/Bcl2 (Sano and Reed 2013).

### *9.9.1 Pro-Apoptotic CHOP Signalling*

CHOP (C/EBP Homologous Protein) also known as DDIT3/GADD153 belongs to the C/EP family of b-ZIP transcription factors (Oyadomari and Mori 2004). The levels of CHOP in the ER are tightly regulated and kept under low concentration in normal cells. However, during mild ER stress condition, the expression of CHOP protein is restored via the UPR signaling elements (IRE1 and PERK mediated signaling branch) to a level of significance. The full effect of CHOP signaling emerges only on chronic stress conditions when there is a steady increase in the accumulation of misfolded proteins in the ER (Schonthal 2012). The elevated levels of CHOP can act at different molecules leading the cell to apoptosis. Primarily, the activated CHOP reversibly dephosphorylates eIF2α which in turn recovers the protein translation processes and induce cell death by initiating the synthesis of many pro-apoptotic proteins. CHOP also lead the cell to death by inducing and

repressing respectively the expression of apoptotic Bim protein and antiapoptotic Bcl-2 protein (McCullough et al. 2001; Puthalakath et al. 2007). The severity of the ER stress is furthermore enhanced by CHOP by transcribing the expression of ERO1α which in turn hyperoxidize and perturb the redox state of the ER leading to apoptosis (Marciniak et al. 2004; Song et al. 2008). Additionally, the ERO1α can also aggravate ER stress and promote cell death by depleting the $Ca^{2+}$ stores in the ER through the action of releasing $Ca^{2+}$ from the ER to mitochondria through the IP3 receptor (Li et al. 2009).

## 9.10 ER Stress in Human Health and Diseases

Several pathological stimuli can disrupt protein folding in the ER resulting in the accumulation of misfolded proteins. The consequent cell injury leads to disease progression and contribute to pathology (Table 9.1). ER stress has been linked with several diseases such as cancer, diabetes, cardiovascular diseases, neurodegenerative disorders, immune disorders and several others. The detail information on the ER-stress associated disease is given in Schonthal 2012.

## 9.11 Drug Targeting for ER Stress Linked Diseases

The UPR initiated pro-survival and pro-apoptotic pathways have been implicated in various diseases associated with ER stress. The intruders in ER stress and the modulators of the stress sensors of the UPR pathway, therefore, would provide promising therapeutics for the treatment of ER stress induced diseases (Kim et al. 2008). There are primarily two main modalities to target the UPR for the development of therapeutic approaches against ER linked diseases. The first method involves ameliorating the protective pathway of the UPR signaling molecules which cope up with the stress (Park and Ozcan 2013). The second method is to suppress the components which are involved in the pro-apoptotic arm of the UPR pathway (Ma and Klann 2014; Tufanli et al. 2017). Although, targeting/altering the stress sensors of the UPR pathway seems convincing as a potential therapeutic goal, there are several limitations in this approach. Two out of the three ER inducible stress transducers elicit signal for both cell survival as well as cell death. Furthermore, the mechanism which switches from pro-survival to pro-apoptotic pathway is less understood (Urra et al. 2013). Therefore, at the present moment, targeting the UPR pathway for treatment of ER-stress related disease is still at the early stage for therapeutic use.

The increasing number of pathogenic role of the protofibrils which lead to amyloidosis attracts our attention for the need of therapeutic approaches in order to prevent the formation of protofibrils (Graham et al. 2017; Winblad et al. 2016). Recently, CLABPs (Chaperone-like amyloid binding proteins) have been identified

and known to bind the intermediate filaments and prevents the formation of amyloid deposits which are the hallmark of several neurodegenerative disorders (Arosio et al. 2016; Bonito-Oliva et al. 2017; Kakuyama et al. 2005; Mansson et al. 2014; Muchowski et al. 2000). Therefore, upregulation of these molecular chaperones and CLABPs warrants a novel drug discovery in preventing neuronal disorders (Muchowski and Wacker 2005). More research on ER stress will provide therapeutic opportunities in diseases linked to ER stress.

## 9.12 Conclusions

A wide range of pathological conditions can curb the biological folding of proteins in the ER and leads to ER stress. The UPR potentiates the protective signaling mechanism to restore ER homeostasis with the aid of molecular chaperones. However, under persisting stress conditions, it can switch to pro-apoptotic pathway of the UPR branch to destroy the cells. The signaling components in the UPR pathways play the major role in rescuing ER functions and the role of CaBC are indispensable. Because of its abundance, diverse functions, interactions with other chaperones, the role of CaBC are inevitable. Finally, developing specific therapeutics by targeting the individual UPR components would be beneficial for the treatment of various metabolic disorders linked to ER stress.

**Acknowledgements** The work of the authors presented herein was funded by DST, ICMR, CSIR and DBT. The authors also gratefully thank IIT Madras for the facility and financial support.

## References

Aghajani, A., Rahimi, A., Fadai, F., Ebrahimi, A., Najmabadi, H., & Ohadi, M. (2006). A point mutation at the calreticulin gene core promoter conserved sequence in a case of schizophrenia. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 141B*, 294–295.

Ali, M. M., Bagratuni, T., Davenport, E. L., et al. (2011). Structure of the Ire1 autophosphorylation complex and implications for the unfolded protein response. *The EMBO Journal, 30*, 894–905.

Aragon, T., van Anken, E., Pincus, D., et al. (2009). Messenger RNA targeting to endoplasmic reticulum stress signalling sites. *Nature, 457*, 736–740.

Argon, Y., & Simen, B. B. (1999). GRP94, an ER chaperone with protein and peptide binding properties. *Seminars in Cell & Developmental Biology, 10*, 495–505.

Arosio, P., Michaels, T. C., Linse, S., et al. (2016). Kinetic analysis reveals the diversity of microscopic mechanisms through which molecular chaperones suppress amyloid formation. *Nature Communications, 7*, 10948.

Ashby, M. C., & Tepikin, A. V. (2001). ER calcium and the functions of intracellular organelles. *Seminars in Cell & Developmental Biology, 12*, 11–17.

Baksh, S., & Michalak, M. (1991). Expression of calreticulin in Escherichia coli and identification of its $Ca^{2+}$ binding domains. *The Journal of Biological Chemistry, 266*, 21458–21465.

Baksh, S., Burns, K., Andrin, C., & Michalak, M. (1995). Interaction of calreticulin with protein disulfide isomerase. *The Journal of Biological Chemistry, 270*, 31338–31344.

Baumann, O., & Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. *International Review of Cytology, 205*, 149–214.

Beckmann, R. P., Mizzen, L. E., & Welch, W. J. (1990). Interaction of Hsp 70 with newly synthesized proteins: Implications for protein folding and assembly. *Science, 248*, 850–854.

Bergeron, J. J., Brenner, M. B., Thomas, D. Y., & Williams, D. B. (1994). Calnexin: A membrane-bound chaperone of the endoplasmic reticulum. *Trends in Biochemical Sciences, 19*, 124–128.

Bernard-Marissal, N., Sunyach, C., Marissal, T., Raoul, C., & Pettmann, B. (2015). Calreticulin levels determine onset of early muscle denervation by fast motoneurons of ALS model mice. *Neurobiology of Disease, 73*, 130–136.

Berridge, M. J., Bootman, M. D., & Roderick, H. L. (2003). Calcium signalling: Dynamics, homeostasis and remodelling. *Nature Reviews. Molecular Cell Biology, 4*, 517–529.

Bonito-Oliva, A., Barbash, S., Sakmar, T. P., & Graham, W. V. (2017). Nucleobindin 1 binds to multiple types of pre-fibrillar amyloid and inhibits fibrillization. *Scientific Reports, 7*, 42880.

Bourdi, M., Demady, D., Martin, J. L., et al. (1995). cDNA cloning and baculovirus expression of the human liver endoplasmic reticulum P58: Characterization as a protein disulfide isomerase isoform, but not as a protease or a carnitine acyltransferase. *Archives of Biochemistry and Biophysics, 323*, 397–403.

Braakman, I., Helenius, J., & Helenius, A. (1992). Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum. *Nature, 356*, 260–262.

Brodsky, J. L., & McCracken, A. A. (1997). ER-associated and proteasome mediated protein degradation: How two topologically restricted events came together. *Trends in Cell Biology, 7*, 151–156.

Bukau, B., & Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell, 92*, 351–366.

Cao, S. S., & Kaufman, R. J. (2012). Unfolded protein response. *Current Biology, 22*, R622–R626.

Chen, Y., & Brandizzi, F. (2012). AtIRE1A/AtIRE1B and AGB1 independently control two essential unfolded protein response pathways in Arabidopsis. *The Plant Journal, 69*, 266–277.

Chen, Y., & Brandizzi, F. (2013). IRE1: ER stress sensor and cell fate executor. *Trends in Cell Biology, 23*, 547–555.

Chen, X., Shen, J., & Prywes, R. (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *The Journal of Biological Chemistry, 277*, 13045–13052.

Chen, W., Shang, W. H., Adachi, Y., Hirose, K., Ferrari, D. M., & Kamata, T. (2008). A possible biochemical link between NADPH oxidase (Nox) 1 redox-signalling and ERp72. *The Biochemical Journal, 416*, 55–63.

Coe, H., & Michalak, M. (2009). Calcium binding chaperones of the endoplasmic reticulum. *General Physiology and Biophysics, 28*, F96–F103.

Cooper, A. A., Gitler, A. D., Cashikar, A., et al. (2006). Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. *Science, 313*, 324–328.

Corbett, E. F., & Michalak, M. (2000). Calcium, a signaling molecule in the endoplasmic reticulum? *Trends in Biochemical Sciences, 25*, 307–311.

Corbett, E. F., Michalak, K. M., Oikawa, K., et al. (2000). The conformation of calreticulin is influenced by the endoplasmic reticulum luminal environment. *The Journal of Biological Chemistry, 275*, 27177–27185.

Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M., & Walter, P. (2005). On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 18773–18784.

Delaunay, A., Bromberg, K. D., Hayashi, Y., et al. (2008). The ER-bound RING finger protein 5 (RNF5/RMA1) causes degenerative myopathy in transgenic mice and is deregulated in inclusion body myositis. *PLoS One, 3*, e1609.

Denisov, A. Y., Maattanen, P., Dabrowski, C., Kozlov, G., Thomas, D. Y., & Gehring, K. (2009). Solution structure of the bb' domains of human protein disulfide isomerase. *The FEBS Journal, 276*, 1440–1449.

Denzel, A., Molinari, M., Trigueros, C., et al. (2002). Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. *Molecular and Cellular Biology, 22*, 7398–7404.

Dorner, A. J., Wasley, L. C., & Kaufman, R. J. (1990). Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proceedings of the National Academy of Sciences of the United States of America, 87*, 7429–7432.

Eletto, D., Dersh, D., & Argon, Y. (2010). GRP94 in ER quality control and stress responses. *Seminars in Cell & Developmental Biology, 21*, 479–485.

Ellgaard, L., & Ruddock, L. W. (2005). The human protein disulphide isomerase family: Substrate interactions and functional properties. *EMBO Reports, 6*, 28–32.

Fels, D. R., & Koumenis, C. (2006). The PERK/eIF2alpha/ATF4 module of the UPR in hypoxia resistance and tumor growth. *Cancer Biology & Therapy, 5*, 723–728.

Ferri, K. F., & Kroemer, G. (2001). Organelle-specific initiation of cell death pathways. *Nature Cell Biology, 3*, E255–E263.

Fliegel, L., Burns, K., MacLennan, D. H., Reithmeier, R. A., & Michalak, M. (1989). Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. *The Journal of Biological Chemistry, 264*, 21522–21528.

Forster, M. L., Sivick, K., Park, Y. N., Arvan, P., Lencer, W. I., & Tsai, B. (2006). Protein disulfide isomerase-like proteins play opposing roles during retrotranslocation. *The Journal of Cell Biology, 173*, 853–859.

Frand, A. R., Cuozzo, J. W., & Kaiser, C. A. (2000). Pathways for protein disulphide bond formation. *Trends in Cell Biology, 10*, 203–210.

Gaut, J. R., & Hendershot, L. M. (1993). The modification and assembly of proteins in the endoplasmic reticulum. *Current Opinion in Cell Biology, 5*, 589–595.

Gonzalez-Perez, P., Woehlbier, U., Chian, R. J., et al. (2015). Identification of rare protein disulfide isomerase gene variants in amyotrophic lateral sclerosis patients. *Gene, 566*, 158–165.

Graham, W. V., Bonito-Oliva, A., & Sakmar, T. P. (2017). Update on Alzheimer's disease therapy and prevention strategies. *Annual Review of Medicine, 68*, 413–430.

Groenendyk, J., Agellon, L. B., & Michalak, M. (2013). Coping with endoplasmic reticulum stress in the cardiovascular system. *Annual Review of Physiology, 75*, 49–67.

Gupta, R., Kapoor, N., Raleigh, D. P., & Sakmar, T. P. (2012). Nucleobindin 1 caps human islet amyloid polypeptide protofibrils to prevent amyloid fibril formation. *Journal of Molecular Biology, 421*, 378–389.

Haas, I. G., & Wabl, M. (1983). Immunoglobulin heavy chain binding protein. *Nature, 306*, 387–389.

Halperin, L., Jung, J., & Michalak, M. (2014). The many functions of the endoplasmic reticulum chaperones and folding enzymes. *IUBMB Life, 66*, 318–326.

Hammond, C., & Helenius, A. (1995). Quality control in the secretory pathway. *Current Opinion in Cell Biology, 7*, 523–529.

Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., & Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. *Molecular Cell, 5*, 897–904.

Hartl, F. U., Martin, J., & Neupert, W. (1992). Protein folding in the cell: The role of molecular chaperones Hsp70 and Hsp60. *Annual Review of Biophysics and Biomolecular Structure, 21*, 293–322.

Hebert, D. N., & Molinari, M. (2007). In and out of the ER: Protein folding, quality control, degradation, and related human diseases. *Physiological Reviews, 87*, 1377–1408.

Helenius, A., & Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. *Annual Review of Biochemistry, 73*, 1019–1049.

Hendershot, L. M. (2004). The ER function BiP is a master regulator of ER function. *Mount Sinai Journal of Medicine, 71*, 289–297.

Hetz, C. (2012). The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews. Molecular Cell Biology, 13*, 89–102.

Hetz, C., & Glimcher, L. H. (2009). Fine-tuning of the unfolded protein response: Assembling the IRE1alpha interactome. *Molecular Cell, 35*, 551–561.

Hetz, C., Martinon, F., Rodriguez, D., & Glimcher, L. H. (2011). The unfolded protein response: Integrating stress signals through the stress sensor IRE1alpha. *Physiological Reviews, 91*, 1219–1243.

Honore, B. (2009). The rapidly expanding CREC protein family: Members, localization, function, and role in disease. *BioEssays, 31*, 262–277.

Hurtley, S. M., & Helenius, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annual Review of Cell Biology, 5*, 277–307.

Irvine, A. G., Wallis, A. K., Sanghera, N., et al. (2014). Protein disulfide-isomerase interacts with a substrate protein at all stages along its folding pathway. *PLoS One, 9*, e82511.

Jiang, J., Prasad, K., Lafer, E. M., & Sousa, R. (2005). Structural basis of interdomain communication in the Hsc70 chaperone. *Molecular Cell, 20*, 513–524.

Jin, H., Mimura, N., Kashio, M., Koseki, H., & Aoe, T. (2014). Late-onset of spinal neurodegeneration in knock-in mice expressing a mutant BiP. *PLoS One, 9*, e112837.

Kakiuchi, C., Ishiwata, M., Nanko, S., et al. (2005). Functional polymorphisms of HSPA5: Possible association with bipolar disorder. *Biochemical and Biophysical Research Communications, 336*, 1136–1143.

Kakiuchi, C., Ishiwata, M., Nanko, S., et al. (2007). Association analysis of HSP90B1 with bipolar disorder. *Journal of Human Genetics, 52*, 794–803.

Kakuyama, H., Soderberg, L., Horigome, K., et al. (2005). CLAC binds to aggregated Abeta and Abeta fragments, and attenuates fibril elongation. *Biochemistry, 44*, 15602–15609.

Kanuru, M., & Aradhyam, G. K. (2017). Chaperone-like activity of Calnuc prevents amyloid aggregation. *Biochemistry, 56*, 149–159.

Kanuru, M., Samuel, J. J., Balivada, L. M., & Aradhyam, G. K. (2009). Ion-binding properties of Calnuc, $Ca^{2+}$ versus $Mg^{2+}$-Calnuc adopts additional and unusual $Ca^{2+}$-binding sites upon interaction with G-protein. *The FEBS Journal, 276*, 2529–2546.

Kaufman, R. J. (2002). Orchestrating the unfolded protein response in health and disease. *The Journal of Clinical Investigation, 110*, 1389–1398.

Kawano, J., Kotani, T., Ogata, Y., et al. (2000). CALNUC (nucleobindin) is localized in the Golgi apparatus in insect cells. *European Journal of Cell Biology, 79*, 208–217.

Kim, I., Xu, W., & Reed, J. C. (2008). Cell death and endoplasmic reticulum stress: Disease relevance and therapeutic opportunities. *Nature Reviews Drug Discovery, 7*, 1013–1030.

Klappa, P., Ruddock, L. W., Darby, N. J., & Freedman, R. B. (1998). The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. *The EMBO Journal, 17*, 927–935.

Korennykh, A. V., Egea, P. F., Korostelev, A. A., et al. (2009). The unfolded protein response signals through high-order assembly of Ire1. *Nature, 457*, 687–693.

Kozlov, G., Maattanen, P., Schrag, J. D., et al. (2006). Crystal structure of the bb' domains of the protein disulfide isomerase ERp57. *Structure, 14*, 1331–1339.

Kozlov, G., Määttänen, P., Schrag, J. D., et al. (2009). Structure of the noncatalytic domains and global fold of the protein disulfide isomerase ERp72. *Structure, 17*, 651–659.

Kozlov, G., Bastos-Aristizabal, S., Maattanen, P., et al. (2010). Structural basis of cyclophilin B binding by the calnexin/calreticulin P-domain. *The Journal of Biological Chemistry, 285*, 35551–35557.

Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., & Sambrook, J. (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature, 332*, 462–464.

Kraus, A., Groenendyk, J., Bedard, K., et al. (2010). Calnexin deficiency leads to dysmyelination. *The Journal of Biological Chemistry, 285*, 18928–18938.

Krause, K. H., & Michalak, M. (1997). Calreticulin. *Cell, 88*, 439–443.

Lavoie, C., Meerloo, T., Lin, P., & Farquhar, M. G. (2002). Calnuc, an EF-hand $Ca^{2+}$-binding protein, is stored and processed in the Golgi and secreted by the constitutive-like pathway in AtT20 cells. *Molecular Endocrinology, 16*, 2462–2474.

Leach, M. R., Cohen-Doyle, M. F., Thomas, D. Y., & Williams, D. B. (2002). Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. *The Journal of Biological Chemistry, 277*, 29686–29697.

Lebeche, D., Lucero, H. A., & Kaminer, B. (1994). Calcium binding properties of rabbit liver protein disulfide isomerase. *Biochemical and Biophysical Research Communications, 202*, 556–561.

Lee, A. H., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Molecular and Cellular Biology, 23*, 7448–7459.

Lee, J. H., Kwon, E. J., & Kim, D. H. (2013). Calumenin has a role in the alleviation of ER stress in neonatal rat cardiomyocytes. *Biochemical and Biophysical Research Communications, 439*, 327–332.

Leustek, T., Toledo, H., Brot, N., & Weissbach, H. (1991). Calcium-dependent autophosphorylation of the glucose-regulated protein, Grp78. *Archives of Biochemistry and Biophysics, 289*, 256–261.

Li, Y., & Camacho, P. (2004). $Ca^{2+}$-dependent redox modulation of SERCA2b by ERp57. *The Journal of Cell Biology, 164*, 35–46.

Li, G., Mongillo, M., Chin, K. T., et al. (2009). Role of ERO1-alpha-mediated stimulation of inositol 1,4,5-triphosphate receptor activity in endoplasmic reticulum stress-induced apoptosis. *The Journal of Cell Biology, 186*, 783–792.

Lievremont, J. P., Rizzuto, R., Hendershot, L., & Meldolesi, J. (1997). BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of $Ca^{2+}$. *The Journal of Biological Chemistry, 272*, 30873–30879.

Lin, P., Le-Niculescu, H., Hofmeister, R., et al. (1998). The mammalian calcium-binding protein, nucleobindin (CALNUC), is a Golgi resident protein. *The Journal of Cell Biology, 141*, 1515–1527.

Lin, P., Yao, Y., Hofmeister, R., Tsien, R. Y., & Farquhar, M. G. (1999). Overexpression of CALNUC (nucleobindin) increases agonist and thapsigargin releasable $Ca^{2+}$ storage in the Golgi. *The Journal of Cell Biology, 145*, 279–289.

Lin, J. H., Li, H., Yasumura, D., et al. (2007a). IRE1 signaling affects cell fate during the unfolded protein response. *Science, 318*, 944–949.

Lin, P., Li, F., Zhang, Y. W., et al. (2007b). Calnuc binds to Alzheimer's beta-amyloid precursor protein and affects its biogenesis. *Journal of Neurochemistry, 100*, 1505–1514.

Lipskaia, L., Hulot, J. S., & Lompre, A. M. (2009). Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation. *Pflügers Archiv, 457*, 673–685.

Logue, S. E., & Gorman, A. M. (2013). Current Concepts in ER Stress-Induced Apoptosis. *Journal of Carcinogenesis & Mutagenesis*, s6.

Lucero, H. A., & Kaminer, B. (1999). The role of calcium on the activity of ER calcistorin/protein-disulfide isomerase and the significance of the Cterminal and its calcium binding. A comparison with mammalian protein-disulfide isomerase. *The Journal of Biological Chemistry, 274*, 3243–3251.

Luo, S., Mao, C., Lee, B., & Lee, A. S. (2006). GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Molecular and Cellular Biology, 26*, 5688–5697.

Ma, Y., & Hendershot, L. M. (2004). ER chaperone functions during normal and stress conditions. *Journal of Chemical Neuroanatomy, 28*, 51–65.

Ma, T., & Klann, E. (2014). PERK: A novel therapeutic target for neurodegenerative diseases? *Alzheimer's Research & Therapy, 6*, 30.

Maattanen, P., Kozlov, G., Gehring, K., & Thomas, D. Y. (2006). ERp57 and PDI: Multifunctional protein disulfide isomerases with similar domain architectures but differing substrate-partner associations. *Biochemistry and Cell Biology, 84*, 881–889.

Macer, D. R., & Koch, G. L. (1988). Identification of a set of calcium-binding proteins in reticuloplasm, the luminal content of the endoplasmic reticulum. *Journal of Cell Science, 91*, 61–70.
Malhotra, J. D., & Kaufman, R. J. (2007a). The endoplasmic reticulum and the unfolded protein response. *Seminars in Cell & Developmental Biology, 18*, 716–731.
Malhotra, J. D., & Kaufman, R. J. (2007b). Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? *Antioxidants & Redox Signaling, 9*, 2277–2293.
Mansson, C., Kakkar, V., Monsellier, E., et al. (2014). DNAJB6 is a peptide-binding chaperone which can suppress amyloid fibrillation of polyglutamine peptides at substoichiometric molar ratios. *Cell Stress & Chaperones, 19*, 227–239.
Mao, C., Wang, M., Luo, B., et al. (2010). Targeted mutation of the mouse Grp94 gene disrupts development and perturbs endoplasmic reticulum stress signaling. *PLoS One, 5*, e10852.
Marciniak, S. J., Yun, C. Y., Oyadomari, S., et al. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & Development, 18*, 3066–3077.
Martin, V., Groenendyk, J., Steiner, S. S., et al. (2006). Identification by mutational analysis of amino acid residues essential in the chaperone function of calreticulin. *The Journal of Biological Chemistry, 281*, 2338–2346.
Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*, 670–684.
McCracken, A. A., & Brodsky, J. L. (2003). Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *BioEssays, 25*, 868–877.
McCullough, K. D., Martindale, J. L., Klotz, L. O., Aw, T. Y., & Holbrook, N. J. (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Molecular and Cellular Biology, 21*, 1249–1259.
Mesaeli, N., Nakamura, K., Zvaritch, E., et al. (1999). Calreticulin is essential for cardiac development. *The Journal of Cell Biology, 144*, 857–868.
Meunier, L., Usherwood, Y. K., Chung, K. T., & Hendershot, L. M. (2002). A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. *Molecular Biology of the Cell, 13*, 4456–4469.
Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., & Opas, M. (1999). Calreticulin: One protein, one gene, many functions. *The Biochemical Journal, 344*, 281–292.
Michalak, M., Robert Parker, J. M., & Opas, M. (2002). $Ca^{2+}$ signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium, 32*, 269–278.
Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I., & Opas, M. (2009). Calreticulin, a multiprocess calcium-buffering chaperone of the endoplasmic reticulum. *The Biochemical Journal, 417*, 651–666.
Minamino, T., & Kitakaze, M. (2010). ER stress in cardiovascular disease. *Journal of Molecular and Cellular Cardiology, 48*, 1105–1110.
Molinari, M., & Helenius, A. (2000). Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science, 288*, 331–333.
Morito, D., & Nagata, K. (2015). Pathogenic hijacking of ER-associated degradation: Is ERAD flexible? *Molecular Cell, 59*, 335–344.
Muchowski, P. J., & Wacker, J. L. (2005). Modulation of neurodegeneration by molecular chaperones. *Nature Reviews. Neuroscience, 6*, 11–22.
Muchowski, P. J., Schaffar, G., Sittler, A., Wanker, E. E., Hayer-Hartl, M. K., & Hartl, F. U. (2000). Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 7841–7846.
Nagashima, Y., Mishiba, K., Suzuki, E., Shimada, Y., Iwata, Y., & Koizumi, N. (2011). Arabidopsis IRE1 catalyses unconventional splicing of bZIP60 mRNA to produce the active transcription factor. *Scientific Reports, 1*(29). https://doi.org/10.1038/srep00029.
Naidoo, N. (2009). ER and aging-protein folding and the ER stress response. *Ageing Research Reviews, 8*, 150–159.

Nakamura, K., Zuppini, A., Arnaudeau, S., et al. (2001). Functional specialization of calreticulin domains. *The Journal of Cell Biology, 154*, 961–972.

Navid, F., & Colbert, R. A. (2017). Causes and consequences of endoplasmic reticulum stress in rheumatic disease. *Nature Reviews Rheumatology, 13*, 25–40.

Nigam, S. K., Goldberg, A. L., Ho, S., Rohde, M. F., Bush, K. T., & Sherman, M. (1994). A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes $Ca^{(2+)}$-binding proteins and members of the thioredoxin superfamily. *The Journal of Biological Chemistry, 269*, 1744–1749.

Nishikawa, S., Brodsky, J. L., & Nakatsukasa, K. (2005). Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). *Journal of Biochemistry, 137*(5), 551.

Nishitoh, H., Saitoh, M., Mochida, Y., et al. (1998). ASK1 is essential for JNK/SAPK activation by TRAF2. *Molecular Cell, 2*, 389–395.

Nishitoh, H., Matsuzawa, A., Tobiume, K., et al. (2002). ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes & Development, 16*, 1345–1355.

Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J., & Sambrook, J. (1989). S. cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell, 57*, 1223–1236.

Okada, T., Haze, K., Nadanaka, S., et al. (2003). A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. *The Journal of Biological Chemistry, 278*, 31024–31032.

Oliver, J. D., van der Wal, F. J., Bulleid, N. J., & High, S. (1997). Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science, 275*, 86–88.

Oslowski, C. M., & Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods in Enzymology, 490*, 71–92.

Ostrovsky, O., Makarewich, C. A., Snapp, E. L., & Argon, Y. (2009). An essential role for ATP binding and hydrolysis in the chaperone activity of GRP94 in cells. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 11600–11605.

Ostwald, T. J., & MacLennan, D. H. (1974). Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *The Journal of Biological Chemistry, 249*, 974–979.

Otero, J. H., Lizak, B., & Hendershot, L. M. (2010). Life and death of a BiP substrate. *Seminars in Cell & Developmental Biology, 21*, 472–478.

Oyadomari, S., & Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death and Differentiation, 11*, 381–389.

Oyadomari, S., Koizumi, A., Takeda, K., et al. (2002). Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *The Journal of Clinical Investigation, 109*, 525–532.

Park, S. W., & Ozcan, U. (2013). Potential for therapeutic manipulation of the UPR in disease. *Seminars in Immunopathology, 35*, 351–373.

Pollock, S., Kozlov, G., Pelletier, M. F., et al. (2004). Specific interaction of ERp57 and calnexin determined by NMR spectroscopy and an ER two-hybrid system. *The EMBO Journal, 23*, 1020–1029.

Primm, T. P., Walker, K. W., & Gilbert, H. F. (1996). Facilitated protein aggregation. Effects of calcium on the chaperone and anti-chaperone activity of protein disulfide-isomerase. *The Journal of Biological Chemistry, 271*, 33664–33669.

Prins, D., Groenendyk, J., Touret, N., & Michalak, M. (2011). Modulation of STIM1 and capacitative $Ca^{2+}$ entry by the endoplasmic reticulum luminal oxidoreductase ERp57. *EMBO Reports, 12*, 1182–1188.

Puthalakath, H., O'Reilly, L. A., Gunn, P., et al. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell, 129*, 1337–1349.

Rhodes, J. D., & Sanderson, J. (2009). The mechanisms of calcium homeostasis and signalling in the lens. *Experimental Eye Research, 88*, 226–234.

Rodan, A. R., Simons, J. F., Trombetta, E. S., & Helenius, A. (1996). N-linked oligosaccharides are necessary and sufficient for association of glycosylated forms of bovine RNase with calnexin and calreticulin. *The EMBO Journal, 15*, 6921–6930.

Ron, D. (2002). Proteotoxicity in the endoplasmic reticulum: Lessons from the Akita diabetic mouse. *The Journal of Clinical Investigation, 109*, 443–445.

Ron, D., & Hubbard, S. R. (2008). How IRE1 reacts to ER stress. *Cell, 132*, 24–26.

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews. Molecular Cell Biology, 8*, 519–529.

Russell, S. J., Ruddock, L. W., Salo, K. E., et al. (2004). The primary substrate binding site in the b' domain of ERp57 is adapted for endoplasmic reticulum lectin association. *The Journal of Biological Chemistry, 279*, 18861–18869.

Rutkowski, D. T., & Kaufman, R. J. (2004). A trip to the ER: Coping with stress. *Trends in Cell Biology, 14*, 20–28.

Sano, R., & Reed, J. C. (2013). ER stress-induced cell death mechanisms. *Biochimica et Biophysica Acta, 1833*, 3460–3470.

Schonthal, A. H. (2012). Endoplasmic reticulum stress: Its role in disease and novel prospects for therapy. *Scientifica, 2012*(857516), 1–26.

Schrag, J. D., Bergeron, J. J., Li, Y., et al. (2001). The structure of calnexin, an ER chaperone involved in quality control of protein folding. *Molecular Cell, 8*, 633–644.

Schrag, J. D., Procopio, D. O., Cygler, M., Thomas, D. Y., & Bergeron, J. J. (2003). Lectin control of protein folding and sorting in the secretory pathway. *Trends in Biochemical Sciences, 28*, 49–57.

Schroder, M. (2008). Endoplasmic reticulum stress responses. *Cellular and Molecular Life Sciences, 65*, 862–894.

Schroder, M., & Kaufman, R. J. (2005a). ER stress and the unfolded protein response. *Mutation Research, 569*, 29–63.

Schroder, M., & Kaufman, R. J. (2005b). The mammalian unfolded protein response. *Annual Review of Biochemistry, 74*, 739–789.

Shamu, C. E., & Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *The EMBO Journal, 15*, 3028–3039.

Shen, J., Chen, X., Hendershot, L., & Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Developmental Cell, 3*, 99–111.

Shi, Y., Vattem, K. M., Sood, R., et al. (1998). Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Molecular and Cellular Biology, 18*, 7499–7509.

Song, B., Scheuner, D., Ron, D., Pennathur, S., & Kaufman, R. J. (2008). Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *The Journal of Clinical Investigation, 118*, 3378–3389.

Sriram, M., Osipiuk, J., Freeman, B., Morimoto, R., & Joachimiak, A. (1997). Human Hsp70 molecular chaperone binds two calcium ions within the ATPase domain. *Structure, 5*, 403–414.

Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Reports, 7*, 880–885.

Takahashi, K., Niidome, T., Akaike, A., Kihara, T., & Sugimoto, H. (2009). Amyloid precursor protein promotes endoplasmic reticulum stress-induced cell death via C/EBP homologous protein-mediated pathway. *Journal of Neurochemistry, 109*, 1324–1337.

Taylor, C. W., & Laude, A. J. (2002). IP3 receptors and their regulation by calmodulin and cytosolic $Ca^{2+}$. *Cell Calcium, 32*, 321–334.

Tirasophon, W., Welihinda, A. A., & Kaufman, R. J. (1998). A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes & Development, 12*, 1812–1824.

Tripathi, R., Benz, N., Culleton, B., Trouve, P., & Ferec, C. (2014). Biophysical characterisation of calumenin as a charged F508del-CFTR folding modulator. *PLoS One, 9*, e104970.

Tsai, B., Ye, Y., & Rapoport, T. A. (2002). Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nature Reviews. Molecular Cell Biology, 3*, 246–255.

Tsukumo, Y., Tomida, A., Kitahara, O., et al. (2007). Nucleobindin 1 controls the unfolded protein response by inhibiting ATF6 activation. *The Journal of Biological Chemistry, 282*, 29264–29272.

Tsukumo, Y., Tsukahara, S., Saito, S., Tsuruo, T., & Tomida, A. (2009). A novel endoplasmic reticulum export signal: Proline at the +2-position from the signal peptide cleavage site. *The Journal of Biological Chemistry, 284*, 27500–27510.

Tu, B. P., & Weissman, J. S. (2004). Oxidative protein folding in eukaryotes: Mechanisms and consequences. *The Journal of Cell Biology, 164*, 341–346.

Tufanli, O., Telkoparan Akillilar, P., Acosta-Alvear, D., et al. (2017). Targeting IRE1 with small molecules counteracts progression of atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America, 114*, E1395–EE404.

Uehara, T., Nakamura, T., Yao, D., et al. (2006). S-Nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature, 441*, 513.

Urano, F., Wang, X., Bertolotti, A., et al. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science, 287*, 664–666.

Urra, H., Dufey, E., Lisbona, F., Rojas-Rivera, D., & Hetz, C. (2013). When ER stress reaches a dead end. *Biochimica et Biophysica Acta, 1833*, 3507–3517.

Vekich, J. A., Belmont, P. J., Thuerauf, D. J., & Glembotski, C. C. (2012). Protein disulfide isomerase-associated 6 is an ATF6-inducible ER stress response protein that protects cardiac myocytes from ischemia/reperfusion-mediated cell death. *Journal of Molecular and Cellular Cardiology, 53*, 259–267.

Walter, P., & Ron, D. (2011). The unfolded protein response: From stress pathway to homeostatic regulation. *Science, 334*, 1081–1086.

Wang, M., Ye, R., Barron, E., et al. (2009). Essential role of the unfolded protein response regulator GRP78/BiP in protection from neuronal apoptosis. *Cell Death and Differentiation, 17*, 488.

Wang, Q., Feng, H., Zheng, P., et al. (2012). The intracellular transport and secretion of calumenin-1/2 in living cells. *PLoS One, 7*, e35344.

Wang, D. Y., Abbasi, C., El-Rass, S., et al. (2014). Endoplasmic reticulum resident protein 44 (ERp44) deficiency in mice and zebrafish leads to cardiac developmental and functional defects. *Journal of the American Heart Association, 3*, e001018.

Wang, Q., Groenendyk, J., & Michalak, M. (2015). Glycoprotein quality control and endoplasmic reticulum stress. *Molecules, 20*, 13689–13704.

Wang, Y., Sun, Y., Fu, Y., et al. (2017). Calumenin relieves cardiac injury by inhibiting ERS-initiated apoptosis during viral myocarditis. *International Journal of Clinical and Experimental Pathology, 10*, 7277–7284.

Wendel, M., Sommarin, Y., Bergman, T., & Heinegard, D. (1995). Isolation, characterization, and primary structure of a calcium-binding 63-kDa bone protein. *The Journal of Biological Chemistry, 270*, 6125–6133.

Williams, D. B. (2006). Beyond lectins: The calnexin/calreticulin chaperone system of the endoplasmic reticulum. *Journal of Cell Science, 119*, 615–623.

Winblad, B., Amouyel, P., Andrieu, S., et al. (2016). Defeating Alzheimer's disease and other dementias: A priority for European science and society. *Lancet Neurology, 15*, 455–532.

Woehlbier, U., Colombo, A., Saaranen, M. J., et al. (2016). ALS-linked protein disulfide isomerase variants cause motor dysfunction. *The EMBO Journal, 35*, 845–865.

Xu, C., & Ng, D. T. (2015). Glycosylation-directed quality control of protein folding. *Nature Reviews. Molecular Cell Biology, 16*, 742–752.

Xu, C., Bailly-Maitre, B., & Reed, J. C. (2005). Endoplasmic reticulum stress: Cell life and death decisions. *The Journal of Clinical Investigation, 115*, 2656–2664.

Yang, L., Zhao, D., Ren, J., & Yang, J. (2015). Endoplasmic reticulum stress and protein quality control in diabetic cardiomyopathy. *Biochimica et Biophysica Acta, 1852*, 209–218.

Ye, J., Rawson, R. B., Komuro, R., et al. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Molecular Cell, 6*, 1355–1364.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., & Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *The Journal of Biological Chemistry, 273*, 33741–33749.

Zapun, A., Darby, N. J., Tessier, D. C., Michalak, M., Bergeron, J. J., & Thomas, D. Y. (1998). Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *The Journal of Biological Chemistry, 273*, 6009–6012.

# Chapter 10
# Hsp70: A Multi-Tasking Chaperone at the Crossroad of Cellular Proteostasis


**Vignesh Kumar and Koyeli Mapa**

**Abstract** Molecular chaperones are key components of protein quality control machineries in all biological systems. Members of Hsp70 group of molecular chaperones are one of the most commonly found chaperones that accomplish multitude of cellular activities in concert with its co-chaperones. Hsp70s are involved in almost all aspects of protein quality control starting from de novo protein folding, prevention of misfolded or aggregated protein formation to membrane translocation and degradation of terminally misfolded proteins. Barring few exceptions, all known Hsp70s accomplish cellular activities by consuming energies from ATP-hydrolysis by their ATPase activity. The ATP-hydrolysis-driven chaperoning activities of Hsp70s are always assisted and regulated by two groups of co-chaperones; J-domain proteins (JDPs) or Hsp40s and nucleotide exchange factors (NEFs), to accomplish cellular functions in physiological time frames. As the co-chaperones especially the JDPs outnumber the Hsp70s, it is thought that different co-chaperone networks actually bestow the multi-tasking ability to particular Hsp70. In this chapter, an overview of recent understanding of various cellular activities of Hsp70s assisted by its co-chaperones have been discussed to highlight the extent of diversity of cellular functions achieved by this group of molecular chaperones.

**Keywords** Heat shock proteins · Hsp70 · Molecular chaperones · Protein folding · Proteostasis

V. Kumar
Hepatitis Division, National Institute of Virology, Pashan, Pune, India

K. Mapa (✉)
Department of Life Sciences, School of Natural Sciences, Shiv Nadar University, Greater Noida, UP, India
e-mail: koyeli.mapa@snu.edu.in



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_10

## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| ERAD | Endoplasmic reticulum associated degradation |
| FRET | Forster resonance energy transfer |
| Hsp | Heat shock protein |
| IMS | Inter-membrane space |
| JDP | J-domain protein |
| mtHSP70 | Mitochondrial heat shock protein 70 |
| NAC | Nascent polypeptide-associated complex |
| NBD | Nucleotide binding domain |
| NEF | Nucleotide exchange factors |
| PBD | Peptide binding domain |
| RAC | Ribosome associated complex |
| RNC | Ribosome nascent chain complex |
| SBD | Substrate binding domain |
| sm-FRET | Single molecule Forster resonance energy transfer |

## 10.1 Introduction

Hsp70s are one of the most ubiquitous molecular chaperones present in almost all forms of life and accomplish a wide variety of cellular functions (Clerico et al. 2015; Hartl et al. 2011; Hartl and Hayer-Hartl 2009; Mayer 2013; Mayer and Bukau 2005; Zuiderweg et al. 2013). Although the primary sequences of Hsp70 chaperones have diverged significantly over evolution, the two-domain structure of the members belonging to Hsp70 group, remained conserved. All members of Hsp70s are known to harbour two functional domains, the N-terminal nucleotide binding domain (NBD) or ATPase domain and the C-terminal substrate or peptide binding domain (SBD/PBD), connected by a short (8–10 amino acids) linker sequence (Mayer 2013; Mayer and Bukau 2005; Zuiderweg et al. 2013). The nomenclature of domains is in corroboration with the functional roles they fulfil. The two domains allosterically regulate each other's activity achieving extremely diverse functions in the cells in collaboration with co-chaperones. The NBD dictates the substrate binding affinity of the SBD which in turn accelerates the inherent ATPase activity of the Hsp70-NBD and help in rapid hydrolysis of bound ATP. All crucial cellular activities of Hsp70s are all driven by allosteric communication between its domains leading to successful binding and timely release of substrates by the chaperone (Mayer 2013; Zuiderweg et al. 2013). Co-ordinated substrate binding and release is vital for Hsp70-driven cellular activities; this is possible due to extremely stringent regulation of the Hsp70 activity by the co-chaperones. Two groups of co-chaperones complete the Hsp70 chaperone activity, the J-domain proteins, in short JDPs and the

nucleotide exchange factors (NEF) (Bracher and Verghese 2015; Fan et al. 2003; Hartl et al. 2011; Kampinga and Craig 2010). JDPs constitute a wide variety of proteins and as the initially discovered members of this group of co-chaperones belonged to the molecular weight range of ~40 kDa, JDPs are also known as Hsp40s although many members especially the ones belonging to type III JDPs are much smaller in size (Fan et al. 2003; Kampinga and Craig 2010). The other group of co-chaperones are nucleotide exchange factors, in short NEFs, which facilitate exchange of ADP from the NBDs of Hsp70s, thereby initiating a new chaperone cycle (Bracher and Verghese 2015). In contrast to JDPs where all members harbour at least a J-domain consisting of helix-loop-helix structure with conserved tripeptide motif (HPD), NEFs vary starkly in their sequence as well as in structure. There are plenty of examples of a singleHsp70 being assisted by structurally different NEFs, although the specificity of NEFs towards particular functions of Hsp70s, remain largely enigmatic.

## 10.2 Domain Organization and Allosteric Communication in Hsp70 Chaperones

Hsp70 chaperones harbour an N-terminal ~45 kDa nucleotide binding domain (NBD) and a C-terminal ~25 kDa substrate binding domain (SBD). The two domains remain connected by a hydrophobic linker. The NBD is horse-shoe shaped and each lobe is subdivided into two sub-domains (altogether 4 sub-domains, IA, IB, IIA and IIB). The SBD has two prominent parts, the ß-sheet base and an α-helical lid like structure constituted of five α-helices (Mayer 2013; Mayer and Bukau 2005; Mayer and Kityk 2015; Zuiderweg et al. 2013). Helical lid is followed by a conserved intrinsically unstructured region with a hitherto unknown function. Hsp70 chaperones are known to exert their chaperoning activity by inter-domain allostery. The substrate binding affinity of the C-terminal SBD is dictated by the nucleotide bound status of the N-terminal NBD. Hsp70s bind substrates with a weak affinity in the ATP-bound state and the substrate-binding affinity increases drastically following the hydrolysis of ATP to ADP. On the other hand, substrate binding by SBD accelerates the ATPase activity of the NBD (Mayer 2013; Mayer and Bukau 2005; Mayer and Kityk 2015; Zuiderweg et al. 2013). This allosteric communication is the basis of successful chaperoning activity in all biological functions of the chaperone. An understanding of the mechanism of inter-domain allostery at the molecular details remained obscure due to unavailability of two-domain structures of the Hsp70 proteins for years. Ironically, the structure of isolated domains (NBD and SBD) of prototype Hsp70, DnaK (bacterial Hsp70), was solved long ago but structure of isolated domains could not explain the inter-domain communication that forms the basis of allosteric regulation. Simultaneously, many groups have shown that the nucleotide binding activity and the substrate binding activity of NBD and SBD respectively, remain intact by the isolated -domains although domains are either deficient or significantly less efficient in chaperoning

activity in respect to the full length chaperones. This indicated the importance of inter-domain allosteric regulation for functionality of the chaperones. Due to dynamic nature of Hsp70 molecules, the X-ray crystal structures of the full length proteins remained unsolved for decades and came to light, fairly recently (Chang et al. 2008; Qi et al. 2013; Swain et al. 2007; Zhuravleva et al. 2012). In parallel, to capture this dynamic nature of Hsp70s, various Hsp70 proteins were subjected to different techniques in solution phase and indeed, the existence of conformational ensemble or co-existence of multiple conformational states in single allosteric states of Hsp70 family members came into light (Banerjee et al. 2016; Lai et al. 2017; Mapa et al. 2010; Marcinowski et al. 2011; Sikor et al. 2013). Many groups including ours have tried to understand the dynamic nature of Hsp70 molecules using state of the art techniques like single molecule FRET (FÖrster Resonance Energy Transfer) spectroscopy. Along with other techniques, Single molecule FRET experiments successfully revealed the structural heterogeneity in different biological states of various members of Hsp70 group of chaperones which remained so far masked in ensemble studies or in high resolution structural snapshots (Fig. 10.1).

Using biochemical and biophysical studies on purified Hsp70 chaperones, it was indicated that two types of prominent conformational changes take place during each allosteric chaperone cycle. The distance between domains changes drastically with ATP binding and hydrolysis and the α-helical lid of SBD opens or closes on top of the peptide binding pocket situated at ß-sheet base of SBD. Furthermore, the crystal structure of isolated peptide-bound SBD of DnaK (Zhu et al. 1996) demonstrated that the alpha helical lid closes on top of the substrate binding pocket of ß-sheet base. The state of lid in ATP-bound states were thought to be open from several other experimental evidences although the conformational heterogeneity, if any, remained unclear. Almost simultaneous to the discovery of two-domain structures of some members of Hsp70s, application of single molecule FRET on biologically relevant molecules became common. Some members of Hsp70 chaperones like bacterial Hsp70, DnaK, yeast mitochondrial Hsp70, Ssc1, yeast ER Hsp70, BiP were subjected to sm-FRET experiments to capture the conformational ensembles in various allosteric states (Banerjee et al. 2016; Mapa et al. 2010; Marcinowski et al. 2011; Sikor et al. 2013). From such single molecule FRET efficiency/ratio distribution plots, it became clear that Hsp70s are fairly dynamic molecules and contain inherent conformational heterogeneity in most of the functional states. Previous to this, the substrate-bound, lid-closed structure of isolated DnaK or in two-domain structure along in its ADP bound state hinted that the lid gets closed upon substrate binding (Bertelsen et al. 2009; Chang et al. 2008; Zhu et al. 1996). The lid-open structure of structural analogue of Hsp70s, Hsp110s showed the open state of lid in ATP state of the chaperone (Liu and Hendrickson 2007; Polier et al. 2008). It was expected from previous biochemical or biophysical studies that Hsp70 lids remain open in ATP-bound states. Although, the high resolution structures of full-length (without the C-terminal disordered region) Hsp70s in ATP state came after few years, sm-FRET studies had revealed the conformational ensemble in regard to the lid conformation. It was demonstrated that lid remains in open state in most of the molecules in ATP-bound state while the conformational states of lid in

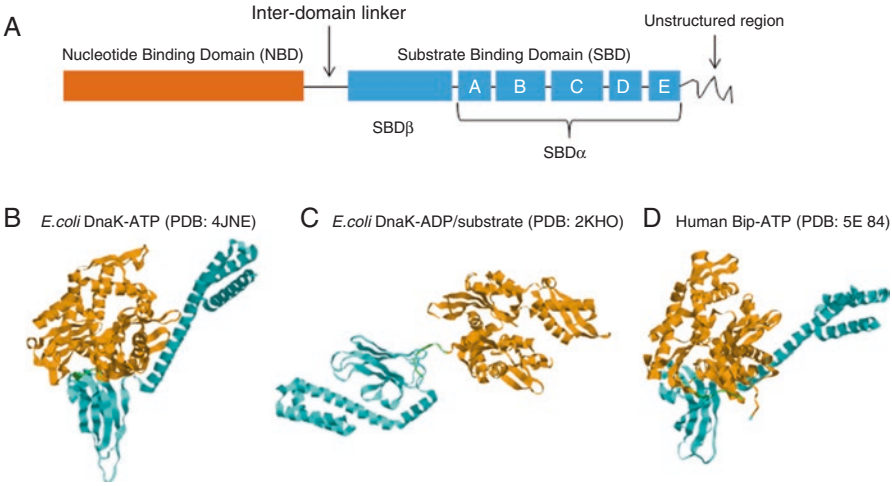


**Fig. 10.1** Domain organization and structure of Hsp70 molecular chaperones: Panel A: Almost all known Hsp70 chaperones consist of two domains, the N-terminal nucleotide binding domain (NBD) and the C-terminal substrate binding domain (SBD). The SBD is divided into the beta-sheet base (SBDß) followed by alpha helical lid like structure (SBDα). The lid consists of 5 helices, A to E. The SBDα is followed by a disordered region. Panel B-C: Ribbon diagram of crystal and NMR structure of E. coli Hsp70, DnaK in the ATP and ADP/substrate bound states respectively. Panel D: Crystal structure of human BiP protein in the ATP-bound state. The NBDs have been coloured in orange, the inter-domain linkers in green and the SBDs have been coloured in cyan in all three structures

the ADP-bound conformation is quite heterogeneous (Banerjee et al. 2016; Mapa et al. 2010; Marcinowski et al. 2011). Additionally, Sm-FRET experiments also revealed that exclusively upon binding the substrates, lid of SBD closes significantly. This finding was a significant advancement from the previous knowledge of mere ATP-hydrolysis driven lid closure of Hsp70 molecules. Capturing the heterogeneity by sm-FRET proved that just ADP state is not sufficient to close the SBD lid. A recent study from our group had shown that the lid closure is not mandatory for substrate capture and the degree of lid closure depends on the nature of substrate bound (Banerjee et al. 2016).

Furthermore, the high resolution structures of full length (except the C-terminal unstructured domains) Hsp70s in ADP and ATP-bound states revealed the stark difference in domain interaction in the ATP state and the ATP-hydrolysed state. In the ATP-bound states NBD and SBD comes into close contact (domain-docked state) and the linker becomes buried, whereas in the ADP-bound states, the two domains are uncoupled exposing the linker (Bertelsen et al. 2009; Kityk et al. 2012; Qi et al. 2013). While probing the conformational ensemble for inter-domain interaction, we and others observed that in the ADP-bound state, an appreciable population of molecules remain in the domain-docked state which remain hidden in the crystal structures (Mapa et al. 2010; Marcinowski et al. 2011) In the ATP-bound state the interaction of two domains (or docking of SBD to NBD) was found for

most of the molecules (Mapa et al. 2010; Marcinowski et al. 2011). Upon substrate binding the two domains get uncoupled. Thus, by employing parallel approaches of solving of high resolution structures and subjecting the Hsp70 proteins for state of the art methodologies like single molecule FRET, our knowledge about the allosteric communication of these molecular machines has significantly enriched.

## 10.3 Role of Hsp70 in Quality Control of Nascent Chains

In eukaryotes, members of Hsp70 group of molecular chaperones are present in multiple intracellular compartments. In unicellular eukaryote model, yeast, there are 9 members (including two of the Hsp110s, Sse1 and Sse2) of Hsp70 family (Ssa1–4, Ssb1–2, Sse1–2 and Ssz1) that exist only in cytoplasm of the cell. While Ssa1 and its paralog Ssa2 are mainly engaged in various aspects of protein quality control in the cytosol under physiological scenario, Ssa3 and its paralog Ssa4 are thought be expressed under stress. Ssb1 and Ssb2, together known as SSBs are ribosome associated chaperones and their role have been described in details in the next section. Sse1 and Sse2 belong to Hsp110s and have been shown to act as nucleotide exchange factors for cytosolic Hsp70s, although various other roles in protein quality control have been ascribed to Sse1.

The role of Hsp70s in maintenance of protein quality control in eukaryotes starts at a reasonably early stage of a protein's life. Ribosome-bound Hsp70 (SSBs in yeast) with help of co-chaperones (RAC or Ribosome associated complex and NAC or Nascent polypeptide-associated complex) bind the newly emerging nascent chains as soon as they emerge through the ribosome exit tunnel (Gautschi et al. 2001; Hubscher et al. 2017; Preissler and Deuerling 2012; Rakwalska and Rospert 2004). Although the discovery of interaction of some eukaryotic Hsp70s with ribosome is rather old, the structural intricacies of Hsp70-ribosome interaction and its functional relevance has been unravelled only recently. In yeast, SSBs encompass two extremely identical ribosome-bound Hsp70 chaperones, Ssb1 and Ssb2, which differ only by 4 amino acids. In a recent study, it was shown that alpha helical regions in the lid domain of SSB's PBD contain a conserved positively charged surface which is essential for binding to ribosome and rRNAs. SSBs interact with two ribosomal proteins Rpl35 and Rpl39 that are situated near the ribosome exit tunnel explaining its capacity of binding nascent polypeptide chains just after emergence from the ribosome exit tunnel (Gumiero et al. 2016). Like other members of Hsp70 chaperone family, SSB follows the same trend regarding their dependence on the co-chaperones. For SSBs, a heterodimeric complex known as RAC (Ribosome Associated Complex) acts as a co-chaperone. Yeast RAC consists of a JDP called Zuo1 (a homologue of mammalian Zuotin) and an atypical Hsp70, Ssz1 (Gautschi et al. 2001; Gautschi et al. 2002; Leidig et al. 2013; Peisker et al. 2008). A subunit of RAC (J-domain protein Zuo1) helps to accelerate the ATPase rate of SSBs and thereby ensures tight binding with the nascent polypeptides (Fig. 10.2). RAC is essential not only for the acceleration of ATPase activity of SSB but also for efficient binding of SSB to ribosome (Gumiero et al. 2016). Absence of RAC (Δzuo Δssz1) or presence

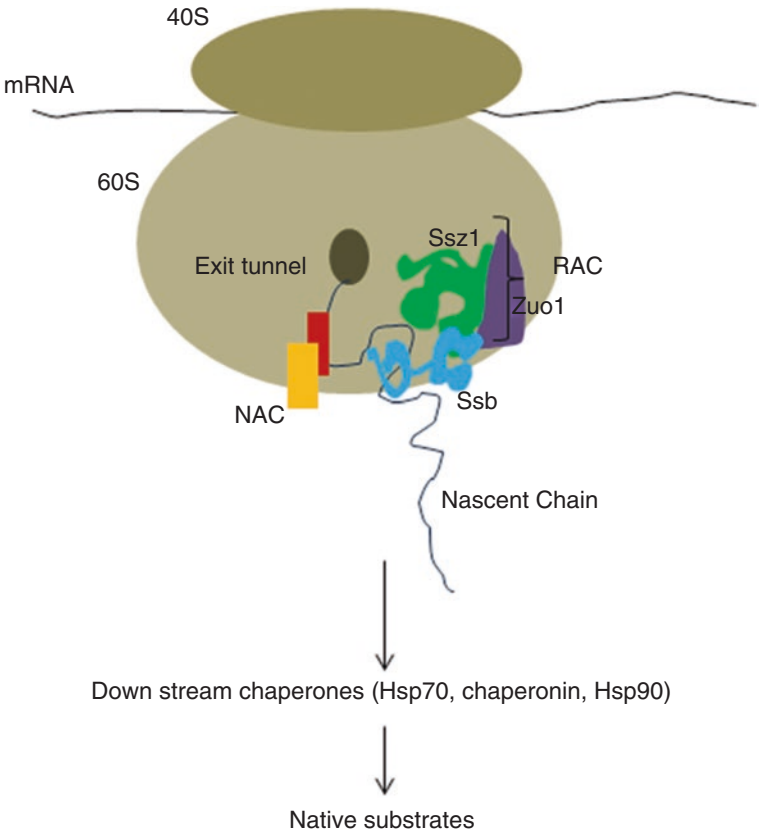


**Fig. 10.2** Ribosome bound Hsp70 and its co-chaperones: Ribosome-bound Hsp70, Ssb interacts with nascent polypeptide chains soon after its emergence from ribosome exit-tunnel. RAC or ribosome associated complex consists of atypical Hsp70 protein Ssz1 and J-domain protein Zuo1. RAC acts as co-chaperone for Ssb and helps it in tight binding to the nascent chains. NAC or nascent chain associated complex also binds the nascent chain near the ribosome exit tunnel. The Ssb-RAC-NAC hands over the nascent chains to downstream chaperones to fold into native conformation

of a non-functional RAC (Zuo1 H128Q) leads to severe abrogation of SSB-ribosome interaction. The importance of SSB and its co-chaperones in chaperoning the nascent chains and in overall cellular protein homeostasis is further highlighted by the evidences showing significant aggregation of ribosomal proteins and ribosome biogenesis factors in cells deleted of SSBs and its co-chaperones (Koplin et al. 2010).

Ssz1, the other component of yeast RAC, is a member of Hsp70 family although it has diverged significantly from typical Hsp70 features, both structurally and functionally (Conz et al. 2007; Huang et al. 2005). Ssz1 lacks the ATPase activity in vitro and the ATP binding activity has been shown to be dispensable for this unique Hsp70 (Conz et al. 2007; Huang et al. 2005). The substrate binding domain (SBD) of this protein is smaller in size compared to other Hsp70-PBDs and the

intrinsically unstructured region present at the extreme C-terminal part of most of the Hsp70s, is absent in Ssz1. It was shown that SBD of Ssz1 is essential for its interaction with its partner in RAC, Zuo1 and also play important role for its interaction with ribosome (Conz et al. 2007).

The functional significance of Hsp70-co-chaperone interaction with ribosome and nascent chains came pretty recently. In 2013, Judith Frydman's group had shown that ~70% of the nascent polypeptides interact with SSBs in yeast in a RAC dependent manner (Willmund et al. 2013). They had reported that the SSB-interacting nascent chains mainly belong to cytoplasmic and nuclear proteins, although nascent chains of some SRP (signal recognition particle)-independent ER targeted proteins were also found to interact with SSBs (Willmund et al. 2013). Very recently, Bernd Bukau's group has elegantly shown that not only the nascent chains of cytosolic and nuclear proteins but also a significant percentage of newly synthesized mitochondrial pre-proteins (~80%) interact with SSB (Doring et al. 2017). This finding is quite unexpected and worth exploring as absence of SSB will have significant impact on the biogenesis and functionality of this crucial organelle. Indeed, in this work, the authors have shown that the cells lacking SSB exhibit altered mitochondria dynamics and mass. Additionally, this study has also revealed that an appreciable percentage (~46%) of nascent chains of ER targeted proteins especially those which are translocated to ER without docking of RNC (Ribosome Nascent Chain) complex to ER-translocons, preferentially bind to SSB (Doring et al. 2017). Whether SSB-RNC complex works in collaboration with SRP for ER targeting of proteins or it serves as a parallel targeting pathway, remains to be explored. In summary, both canonical and non-canonical members of Hsp70 group of molecular chaperones play significant role in chaperoning of nascent chains and protein homeostasis as a whole.

## 10.4 Hsp70 in Mitochondria: A Multitasking Chaperone in Action

The multi-tasking nature of Hsp70 chaperones are extremely obvious in certain sub-cellular compartments like mitochondria where Hsp70 chaperones are involved in maintaining different aspects of protein homeostasis like membrane translocation, folding and assembly of newly imported unfolded precursor proteins (Neupert and Brunner 2002; 2007).

### *10.4.1 Role of Mitochondrial Hsp70 in Protein Translocation into Mitochondrial Matrix*

Majority of biochemical activities carried out by mitochondria is located in its matrix. Most of the matrix proteins except those that are encoded by mitochondrial genome, are translocated across the outer membrane without expending energy but

has to cross the tightly gated inner membrane by an energy consuming process (Bolender et al. 2008; Kutik et al. 2009; Neupert and Herrmann 2007; Wiedemann and Pfanner 2017). Translocation across the inner mitochondrial membrane needs active participation of a molecular motor, also called the "import motor". The energy-driven translocase of inner mitochondrial membrane, also called TIM23 translocase, is operationally divided into membrane-embedded translocation channel and the channel associated import motor. The import motor consists of mitochondrial Hsp70 (mtHsp70) and its co-chaperones (Neupert and Herrmann 2007; Wiedemann and Pfanner 2017). In the import motor, through an ATP-dependent cycle of consecutive binding and release from the incoming precursor protein, mtHsp70 leads to vectorial movements of precursors into the matrix. In unicellular eukaryote, yeast, a J-domain like protein called Tim44 assists Hsp70 in import of precursor proteins across the tightly gated inner mitochondrial membrane. Tim44 is considered merely as a "J-domain like protein" because of absence of the classical J-domain with conserved HPD motif found in all designated JDPs essential for acceleration of ATPase activity of Hsp70s. Tim44 is a peripheral inner mitochondrial membrane protein and remains attached to the matrix side of the TIM23 translocase. It is thought to act as a membrane anchor for mtHsp70 to position the chaperone in the vicinity of the incoming precursor proteins through the channel of the TIM23 translocase (Blom et al. 1993). Several studies have demonstrated that interaction of Tim44 with mtHsp70 is highly dependent on the nucleotide-bound status of mtHsp70 (Kronidou et al. 1994; Rassow et al. 1994; Schneider et al. 1994). It was shown that Tim44-mtHsp70 forms a stable complex in the ADP state of the chaperone while the complex dissociates in presence of ATP (Rassow et al. 1994; Schneider et al. 1994; von Ahsen et al. 1995). As the Tim44-mtHsp70 interaction is similar in behaviour to substrate-mtHsp70 interaction regarding the nucleotide dependence, it is still enigmatic whether a ternary complex of Tim44-mtHsp70-substrate is formed at some point of translocation process or binding of Tim44 and substrates (incoming precursor proteins) to mtHsp70 are mutually exclusive in nature (Fig. 10.3).

There are two contrasting hypothesis regarding the working mechanism of mtHsp70 as a motor protein during translocation; the power-stroke model and the Brownian ratchet model (Neupert and Brunner 2002). According to Brownian ratchet model the pre-proteins undergo spontaneous Brownian motion within the translocase pore of the TIM23 translocase (Ungermann et al. 1994) and mtHsp70 binds and traps the segments of precursor proteins preventing its retrograde movement or backsliding into intermembrane space (IMS) or the cytosol (Neupert and Brunner 2002; Schneider et al. 1994). As has been shown for bacterial homolog DnaK, mtHsp70 binds sequence motifs constituted of ~7–10 amino acids in length which are enriched in hydrophobic residues in the core and are flanked by positively charged residues within substrates (Rudiger et al. 1997). Our previous data have shown that portions of precursors proteins which do not get recognised by mtHsp70, are preferentially bound by Tim44 thereby helping in unidirectional import towards the matrix. Thus, according to this model, no pulling force is exerted by the chaperone during the translocation process. Previous experiments also pointed towards more in favour of Brownian ratchet model. In an elegant experiment, by introducing Hsp70 disfavoured regions like polygycine or polyglutamine stretches

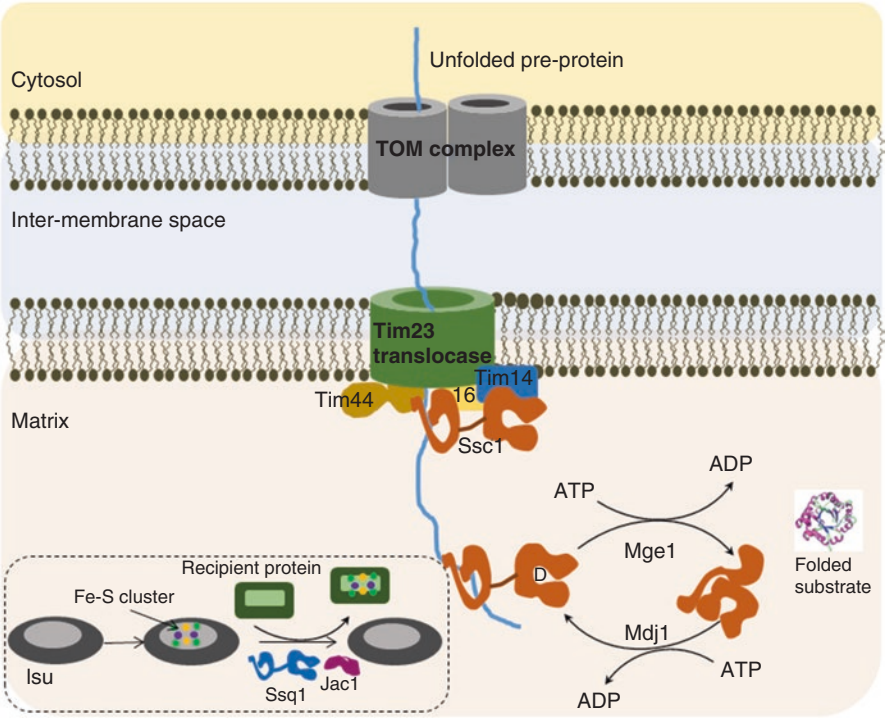


**Fig. 10.3** Multifaceted role of mitochondrial Hsp70: Mitochondrial Hsp70 is engaged in variety of functions within the mitochondrial matrix. In yeast, S. cerevisiae, the main mitochondrial Hsp70 also known as Ssc1 is involved in protein translocation across the tightly-gated inner mitochondrial membrane and also in folding of unfolded precursors after their translocation into matrix. Several co-chaperones of Ssc1; Tim44, Tim14, tim16 which are also components of import motor of the TIM23 complex, are essential for protein import across the inner membrane. For folding function, separate group of co-chaperones are there, Mdj1 (J-domain protein) and Mge1 (NEF). Another mitochondrial Hsp70, Ssq1 is involved in Fe-S cluster biogenesis in mitochondrial matrix, Jac1 acts as a J-domain co-chaperone for Ssq1. T: ATP-state of Ssc1, D: ADP-state of Ssc1

in the pre-proteins, it was shown that the import of these proteins remain unaffected once it is initiated by the membrane potential across the inner mitochondrial membrane ($\Delta\psi$)(Okamoto et al. 2002). On the contrary, according to the power-stroke model mtHsp70 should actively pull the incoming polypeptide chain and the force required should be generated mostly by nucleotide induced conformational changes of the chaperone (Glick 1995; Matouschek et al. 2000). As there are ample contrasting results in favour of both the mechanism of mitochondrial protein translocation by import motor, there is a possibility that multiple modes of action of mtHsp70 exist for efficient translocation of mitochondrial precursor proteins towards matrix.

Following the discovery of Tim14 (Pam18) (Mokranjac et al. 2003; Truscott et al. 2003) as the typical J-domain co-chaperone of mtHsp70 and another structurally similar, closely associated protein Tim16 (Pam16) (Frazier et al. 2004; Kozany

et al. 2004; Li et al. 2004), the understanding of the sequence of events of import motor during protein import across inner mitochondrial membrane has become highly convoluted. From the high resolution structure of Tim14/Tim16 complex, it is clearly evident that the two proteins form a closely associated structure and this structure indicates the possibility of Tim16 as a controller of undue J-domain activity by Tim14 on mtHsp70, thereby preventing the formation of a 'stuck' translocase (Mokranjac et al. 2006). The human Tim14/Tim16 complex have also shown to be of similar three-dimensional structure indicating a conserved mechanism of mt-Hsp70-co-chaperone interaction even in higher eukaryotes (Elsner et al. 2009). It is important to note here that all high resolution structures of Tim14/16 complexes so far have given glimpses of the structural arrangements of soluble domains of these proteins although the membrane association of the co-chaperone complex or the sequence of events of interaction of Tim14/16 with Ssc1 and Tim44, remains elusive. It is important to emphasize that not only mtHsp70 but also all of its co-chaperones involved in import motor, are essential for viability of cells which clearly indicate the importance of such an orchestra of co-chaperones for mtHsp70 in fulfilling the protein translocation across inner mitochondrial membrane.

### *10.4.2 Mitochondrial Hsp70 in Protein Folding and Assembly of Proteins in Mitochondria*

Following the translocation of newly imported precursor proteins, Hsp70 along with a new set of co-chaperones engage in folding of these unfolded precursors into their native structures. To assist in the folding activity of mtHsp70, Mdj1 acts as the JDP co-chaperone in yeast mitochondria (Prip-Buus et al. 1996; Rowley et al. 1994; Voos and Rottgers 2002; Westermann et al. 1996). Although it is thought that mtHsp70 carry out the folding of newly imported precursor proteins in the soluble phase of mitochondrial matrix, significant association of Mdj1 with the inner mitochondrial membrane raises some interesting possibilities (Duchniewicz et al. 1999; Mapa et al. 2010). Whether Mdj1 remain close to the import channel and helps mtHsp70 in folding of newly imported precursors without much delay, remains a question. Interestingly, despite the engagement of a plethora of JDPs and J-like proteins as co-chaperones, the nucleotide exchange activity on mtHsp70 is solely executed by Mge1 (Deloche et al. 1997; Laloraya et al. 1994; Miao et al. 1997; Sakuragi et al. 1999; Schmidt et al. 2001). It has been shown that Mge1 actually completes the chaperone cycle by dissociating the mt-Hsp70-JDP-substrate complex with an extremely fast reaction kinetics in presence of ATP (Mapa et al. 2010). Although the cytosolic Hsp70s interact with structurally diverse NEFs, mitochondrial Hsp70 is exclusively dependent on Mge1, the homologue of bacterial NEF, GrpE.

It is worth mentioning here that mtHsp70 (both Ssc1 and Ssq1 in *S. cerevisiae*), although harbour high homology to its bacterial counterpart DnaK, is more aggregation prone than DnaK. Interestingly, to chaperone this aggregation prone

Hsp70, a dedicated protein called Hep1 exists in mitochondrial matrix (Hsp70 escorting protein, also known as Zim17 or Tim15) (Sanjuan Szklarz et al. 2005; Sichting et al. 2005; Yamamoto et al. 2005). Hep1 interacts with the ATPase domain and linker region of yeast mtHsp70 (Ssc1) and prevents its aggregation (Blamowska et al. 2010). Recently, in mammalian cells, an orthologue of Hep1 has been discovered and was found to play critical role in solubility and maintenance of human mtHsp70 (Goswami et al. 2010). This example of chaperones for chaperones, especially for Hsp70s is quite unique and worth exploring for other aggregation prone chaperones.

Apart from the translocation and folding function in mitochondria, mtHsp70 is also engaged in assembly of protein complexes like the Fe-S clusters (Delewski et al. 2016; Dutkiewicz et al. 2003; Schilke et al. 2006). For the Fe-S cluster formation, interaction of Hsp70 with the scaffold protein (Isu1, in yeast) aided by a specialized JDP co-chaperone is required (Fig. 10.3). Even among yeasts, *S. cerevisiae* and *S. pombe*, there is significant diversification of mtHsp70s. In case of *S. cerevisiae*, due to genome duplication, mtHsp70 or Ssc1 has a given rise to a specialized Hsp70 called Ssq1 in the mitochondrial matrix (Delewski et al. 2016; Dutkiewicz et al. 2003; Schilke et al. 2006). Ssq1 is specifically engaged in Fe-S cluster biogenesis in collaboration with JDP, Jac1 and has not been found to participate in protein translocation or folding in mitochondrial matrix (Kim et al. 2001; Schilke et al. 2006; Voisine et al. 2001). Interestingly, in *S. cerevisiae*, it has been shown that even after alanine substitution in HPD motif of Jac1, mutated Jac1 retains the capability to interact with Ssq1 and the cells remain viable. On the other hand, in *S. pombe* Ssc1 is the sole mtHsp70 and is involved in Fe-S cluster formation apart from membrane translocation and folding activities. In *S. pombe,* the alanine substitution of HPD motif of Jac1 is not at all tolerated and cells become inviable with such mutations in Jac1 (Delewski et al. 2016). Thus, it is interesting that either by increasing the co-chaperone repertoire or by increasing the chaperone number itself (e.g. by genome duplication), the multi-tasking ability of Hsp70 chaperones has been achieved in different organisms as well as in different sub-cellular compartments.

## 10.5 Hsp70 in ER Homeostasis

The glucose regulated protein 78 (GRP78) is a ER resident Hsp70 (Gardner et al. 2013; Gething 1999). Grp78 is also known as BiP (Binding immunoglobulin protein) and Kar2 in yeast. BiP or Grp78 protein is essential for protein folding and unfolded protein response (UPR) signalling in ER (Gardner et al. 2013; Lewy et al. 2017), additionally BiP plays crucial role in protein translocation into ER lumen(Dudek et al. 2015; Hassdenteufel et al. 2014; Zimmermann et al. 2011), $Ca^{2+}$ homeostasis (Gething 1999; Hendershot 2004; Kania et al. 2015; Schauble et al. 2012) and protein degradation by ER-associated degradation (ERAD) (Casas 2017; Foufelle and Ferre 2007; Maattanen et al. 2010; McCaffrey and Braakman 2016;

Sano and Reed 2013; Wang et al. 2017). ER, being a folding and modification hub of membrane and secretory proteins, chaperones residing in ER play crucial role in maintenance of ER protein homeostasis in concert with ERAD machineries. BiP, being a member of Hsp70 family, consists of the typical nucleotide binding domain and the peptide/substrate binding domain. Additionally, for its ER localization, an ER retention signal (KDEL) is harboured by the protein at its extreme C-terminus. A recent crystal structure of human BiP in the ATP-bound state demonstrates the overall structural similarity with prokaryotic Hsp70 DnaK, although exhibiting sufficient differences in structural features, especially in the NBD-SBDα interface. The authors claimed that this NBD-SBDα interface is unique for eukaryotic cytosolic/ER resident Hsp70s and are in contrast to prokaryotic or mitochondrial Hsp70 (Yang et al. 2015). Although the domain-interface is important, it remains to be seen, if the evolution of the interface has indeed been functionally relevant in imparting specialized functions to the cytosolic and the ER counterparts.

Like other Hsp70s, BiP is assisted by members of two groups of co-chaperones, JDPs (Erdj1–7 in mammals) and NEFs like BAP (Sil1 in yeast), Grp170 (Lhs1 in yeast) etc. (Behnke et al. 2015) (Fig. 10.4). The partitioning of BiP between different activities in ER is thought to be guided by recruitment of specific JDPs during different activities. Although the role of all JDPs in folding and degradation are still not clear, JDPs involved in translocation are pretty specific, JDP like Sec63 is the one assisting BiP in protein translocation into ER lumen in yeast (Dudek et al. 2015; Hassdenteufel et al. 2014; Zimmermann et al. 2011). It is interesting to note here that, during Hsp70-motor mediated membrane translocation, presence of a co-chaperone like Tim44 (in case of inner mitochondrial membrane) or Sec63 (in ER membrane) as a membrane anchor for Hsp70s seems mandatory. Whether such membrane anchors actively participate in membrane translocation by holding or pulling the translocating chain, remains unanswered so far. Few more JDP co-chaperones of BiP have been shown to be involved in exclusive activities of the chaperone like Erdj3 and Erdj5 in mammals have been shown to be specifically involved in protein folding and degradation, respectively (Dong et al. 2008; Jin et al. 2008; Shen and Hendershot 2005).

BiP is highly expressed during ER stress and has been extensively used as ER stress marker for long time. It is also overexpressed in certain tumours associated with deregulation of glucose metabolism, glucose deficiency and hypoxia in tumour micro-environment. ER, as already mentioned is the main hub of folding of secretory and membrane proteins and as a result always experience presence of massive amount of unfolded or misfolded proteins inside the lumen. In physiological scenario, ER protein homeostasis machineries consisting of several chaperones like BiP, Grp94, ERp72, PDI, Grp170 etc. and the components of ERAD machinery are extremely efficient to prevent overloading of ER with misfolded or aggregated proteins. In situations where the ER proteostasis machinery is overwhelmed with misfolded or aggregated proteins, ER becomes stressed and a response pathway called Unfolded protein response (UPR) is elicited to restore the homeostasis.

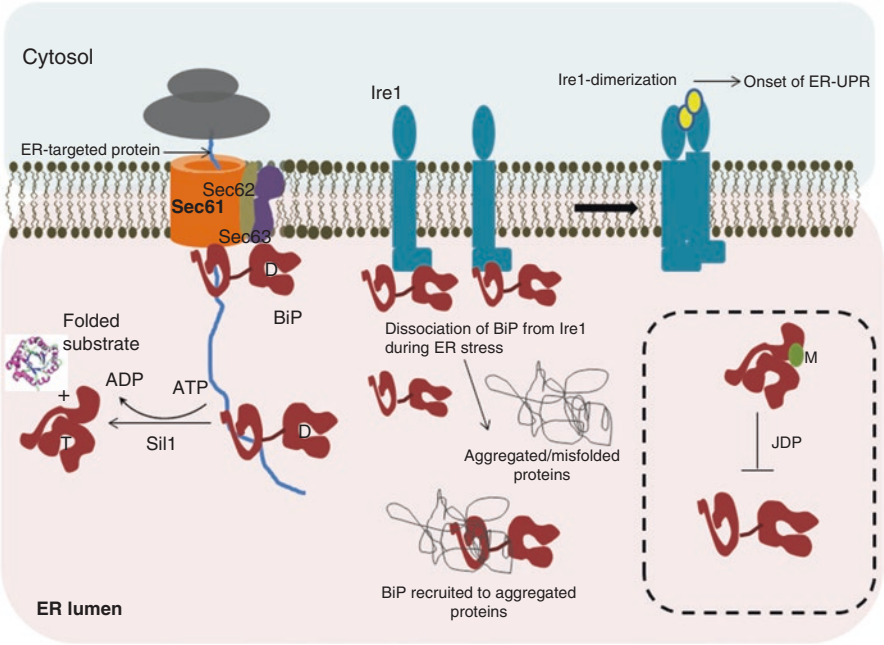


**Fig. 10.4** BiP, a multi-tasking Hsp70 of ER: ER resident Hsp70, also known as BiP is involved in protein translocation into ER-lumen through Sec61 complex. It acts a motor protein for protein import through Sec61 complex. It is also involved in protein folding in ER and a major component to initiate the signalling cascade of ER-UPR (unfolded protein response). Here, the role of BiP in yeast ER-UPR has been shown. Upon accumulation of misfolded or aggregated proteins in ER lumen, BiP dissociates from ER-membrane protein Ire1 which is the sole sensory molecule for ER-UPR in yeast. Upon dissociation of BiP from Ire1, Ire1 auto-phosphorylates (shown in yellow circles) and dimerizes leading to its activation and initiation of ER-UPR signalling cascade. In mammals, a special modification called AMPylation (shown as green circle) takes place on BiP which makes it refractory to J-domain co-chaperone induced ATP-hydolysis. T: ATP-state of BiP, D: ADP-state of BiP, M: AMPylated site

### *10.5.1 Role of BiP in ER-Unfolded Protein Response*

BiP plays a major role in initiating the signalling of ER-UPR (Foufelle and Ferre 2007; Gardner et al. 2013; Hendershot 2004; Lewy et al. 2017). In case of higher eukaryotes, there are three ER membrane embedded sensory molecules that sense the misfolding stress and initiate UPR signal. These proteins are Inositol requiring protein 1 (IRE1), PKR like ER kinase (PERK) and Activating transcription factor 6 (ATF6) (Gardner et al. 2013; Korennykh and Walter 2012; Walter and Ron 2011). All three UPR sensors have a cytosolic domain and an ER luminal domain. The luminal domains of these sensory molecules interact with ER-resident Hsp70, BiP. During the onset of ER-UPR, BiP dissociates from the luminal domains of UPR sensors leading to their activation and initiation of UPR signalling. In case of

IRE1 and PERK, BiP dissociation leads to the oligomerization of these proteins within ER membrane followed by phosphorylation of specific sites on the cytosolic domains of IRE1 and PERK leading to their activation (Fig. 10.4). Activated IRE1 exerts its endonuclease activity and splices the Xbp1 or Hac1 (yeast) mRNA and the protein products of spliced RNA act as a potent transcription factor to upregulate ER chaperones including BiP and components of ERAD machinery. PERK activation leads to a different response from IRE1 activation. Activated PERK leads to phosphorylation of EIF2α thereby abrogating the translation of new proteins and in turn decreases further protein load on ER. During generalized translation block, few genes are preferentially translated like ATF4 which acts as transcription factor to upregulate the genes for restoration of ER homeostasis. Similarly, displacement of BiP from the third UPR sensor ATF6, leads to its ER to Golgi transport and subsequent cleavage by Golgi specific proteases, SP1 and SP2. The cleaved cytosolic domain of ATF6 further acts as a transcription factor to bring back the ER homeostasis (Gardner et al. 2013; Korennykh and Walter 2012; Walter and Ron 2011). Thus, BiP not only binds to unfolded nascent chains, but also plays a crucial role in initiation of UPR signalling which is key in timely restoration of ER protein homeostasis.

### *10.5.2 BiP in $Ca^{2+}$ Homeostasis in ER*

Besides being a hub for post translational modification and folding of secretory and membrane proteins, ER also serves as the reservoir for cellular $Ca^{2+}$ ions. The role of ER resident Hsp70 chaperone, BiP, in ER $Ca^{2+}$ storage was discovered long ago (Lievremont et al. 1997). It was shown that increased BiP level leads to higher $Ca^{2+}$ storage capacity of ER although the mechanism remained unclear. Rather recently, a novel interaction between BiP and sec61α was uncovered as the regulatory interaction for the BiP-mediated $Ca^{2+}$ storage in ER (Schauble et al. 2012). The protein translocation channel or SEC complex situated in the ER membrane for translocation of ER targeted proteins also acts as a $Ca^{2+}$ leak channel. Cytosolic protein Calmodulin prevents undue $Ca^{2+}$ leakage by interaction with the IQ motif of the cytosolic domain of Sec61α protein of this complex. BiP was shown to play significant role in stopping of $Ca^{2+}$ leakage from the ER luminal side. It was demonstrated that BiP interacts with a ER-luminal loop of Sec61α between its two transmembrane helices and closes the Sec61α channel from the open state. The conclusion came from the experiment where amount of BiP in ER lumen was artificially reduced either by genetic manipulation or by increasing the misfolded protein load in ER leading to increased $Ca^{2+}$ release from ER. Furthermore, a mutation at the BiP-binding site of Sec61α (Y344H, originally found in diabetic animals) led to increased $Ca^{2+}$ leakage from ER leading to apoptosis. This increased $Ca^{2+}$ leakage by mutated Sec61α is independent of ER BiP levels and additional decrease in BiP level did not have added impact on the mutated SEC61 complex mediated Ca2+

leakage (Schauble et al. 2012). Altogether, BiP plays a crucial role in $Ca^{2+}$ homeostasis in ER by preventing its undue leakage from ER lumen.

### *10.5.3 Novel Regulatory Mechanism of BiP*

Extensive research on BiP has revealed a number of post-translational modifications of the protein which have been demonstrtaed to play crucial role in regulation of its activity. A recent discovery has shown that BiP is AMPylated at a specific amino acid (Thr 518) by an enzyme called FICD in mammalian cells (Ham et al. 2014; Preissler et al. 2015a; Sanyal et al. 2015). This modification has profound effect on BiP activity. AMPylated BiP was shown to be completely resistant to stimulation by J-domain co-chaperones. On the other hand, the substrate binding affinity of AMPylated BiP remains unchanged although substrates are dissociated with a much faster off rate from the AMPylated BiP in comparison to the unmodified version. Interestingly, AMPylation of BiP mimics an ATP-bound state in the ADP-bound BiP leading to low affinity for substrates. This feature of AMPylated BiP is in compelling contrast to the high affinity states of ADP-bound unmodified BiP. On the other hand, AMPylation of the ATP- bound BiP renders it resistant to JDP co-chaperone-induced stimulation of ATPase activity (Fig. 10.4). Altogether, AMPylation of BiP leads to an inactive state of the chaperone which is probably a unique regulatory mechanism to wean BiP activity when the load of misfolded proteins decline in ER (Preissler et al. 2015b). There are reports that show multiple other post translational modifications (PTMs) of BiP influence the activity of the protein by several ways. For example, acetylation of BiP on lysine 585 is commonly found in various cancer cell lines when treated with HDAC inhibitor, vorinostat (Kahali et al. 2010). This PTM on BiP has a tremendous implication as the K-585 acetylated BiP is unable to interact with PERK, leading to induction of UPR and apoptosis (Kahali et al. 2010). Thus, such modifications of BiP if explored in details may have potential therapeutic application in future.

## 10.6 Role of Hsp70 in Protein Degradation

Apart from Hsp70's roles in various constructive activities related to protein homeostasis in different sub-cellular locations as mentioned in the previous sections, the chaperone is also involved in another extremely important aspect of protein homeostasis, the degradation of damaged, aggregated or terminally misfolded proteins from cellular milieu. Eukaryotic cells have several parallel pathways to eliminate unwanted or harmful proteins: Ubiquitin-proteasome system (UPS), autophagy (microautophagy and macroautophagy) and Chaperone mediated autophagy (CMA). In many of these vital pathways, some members of Hsp70 family have been shown to be involved in association with co-chaperones.

### *10.6.1 Hsp70 in UPS Mediated Protein Degradation*

Ubiquin-proteasome system or UPS is a highly effective mechanism of degradation of mainly cytosolic proteins. UPS, as the name suggests consists of ubiquitin, a small protein that tags the proteins to be degraded whereas proteasome is a barrel shaped multicomponent protein complex harboring a cavity that acts as the site of degradation of ubiquitin-tagged proteins. The process of ubiquitination of proteins is quite elaborate and is achieved by an enzymatic cascade consisting of three groups of enzymes which work sequentially to tag a protein with ubiquitin (Amm et al. 2014; Ji and Kwon 2017). Three groups of proteins work in concert to achieve the ubiquitination of substrates; ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) (Amm et al. 2014; Ji and Kwon 2017). To assist Hsp70s in the UPS mediated degradation process, a protein called CHIP (carboxy terminus of Hsc70 interacting protein) acts as co-chaperone. CHIP is a dimeric protein and harbours three domains, the N-terminal TPR domain which interacts with the conserved EEVD motif of Hsp70s and Hsp90s, the C-terminal domain U-box binding domain harbouring E3 ubiquitin ligase activity and an intervening charged domain. CHIP hinders the chaperoning activity of Hsp70 and Hsp90 and the substrates hold by these chaperones are positioned near the E2 enzymes by CHIP for successful ubiquitin tagging which is completed by its own E3 ligase activity (Edkins 2015; McDonough and Patterson 2003; Paul and Ghosh 2014). Another group of co-chaperone of Hsp70s called BAG proteins also play crucial role in UPS mediated protein degradation by Hsp70s (Doong et al. 2003; Elliott et al. 2007). BAG1 has been shown to acts as NEF for Hsp70 but also harbours an ubiquitin-like domain that interacts with proteasome and can deliver the Hsp70-bound substrate proteins for degradation by proteasome (Doong et al. 2003; Elliott et al. 2007).

### *10.6.2 Hsp70 in Chaperone Mediated Autophagy (CMA)*

Chaperone mediated autophagy is a type of autophagy for efficient removal of misfolded or aggregated proteins from cytosol where the degradation prone proteins are translocated across the lysosomal membranes with the help of chaperones, mainly Hsp70s for degradation (Ciechanover and Kwon 2017; Jacob et al. 2017; Kettern et al. 2010; Li et al. 2011; Massey et al. 2006). In contrast to classical autophagy (macro or micro-autophagy) where the cargo is sequestered within autophagic vacuoles or autophagosomes followed by fusion of autophagosomes with lysosomes, CMA substrates with the help of cytosolic Hsp70s (Hsc70, HSPA8), interact with the lysosomal membranes protein (LAMP-2A) followed by translocation inside the lysosomal cavity. Presence of a specific sequence motif KFERQ is absolutely essential in CMA substrates to be degraded by this process. The cytosolic Hsc70 recognizes this motif on substrates and delivers the substrates to LAMP-2A (Rout et al.

2014). The interaction of Hsc70 is immensely important for CMA and is considered as one of the hallmarks of CMA substrates. Various proteins like transcription factors, lipid binding and calcium binding proteins, various enzymes and aggregation prone proteins like poly-Q-Huntingtin (are removed by this efficient degradation system (Arias and Cuervo 2011; Li et al. 2011; Qi and Zhang 2014). Once LAMP-2A binds substrates, it oligomerizes into ~700kDA complex for membrane translocation. Soon after the translocation of the protein substrate within the lysosomal cavity, LAMP-2A needs to revert to monomeric state for next round of binding and translocation of substrates. Lys-Hsc70 play a critical role in the rapid conversion of oligomeric LAMP-2A to its monomers and helps in next round of CMA-substrate binding and translocation within lysosomes (Arias and Cuervo 2011; Kaushik and Cuervo 2012).

## 10.7 Conclusions

In this chapter, we have summarized the roles of various members of Hsp70 chaperone family in diverse aspects of protein quality control. It is astonishing that despite similar structures, the members of this group can carry out such a varied function. It is quite evident from literature that the involvement of two groups of co-chaperones, especially the J-domain proteins impart the multi-tasking ability to Hsp70s. The mechanism of spatial segregation of co-chaperones within same subcellular location remains unanswered so far and needs to be explored in details. Apart from co-chaperones, modifications of Hsp70s and interaction with a number of other interacting partners, also help in achieving such diverse function.

**Acknowledgements** We sincerely acknowledge Dr. Kausik Chakraborty, Asmita Ghsoh and Joshua Jebakumar Peter for their critical comments on the chapter.

## References

Amm, I., Sommer, T., & Wolf, D. H. (2014). Protein quality control and elimination of protein waste: The role of the ubiquitin-proteasome system. *Biochimica et Biophysica Acta, 1843*, 182–196.

Arias, E., & Cuervo, A. M. (2011). Chaperone-mediated autophagy in protein quality control. *Current Opinion in Cell Biology, 23*, 184–189.

Banerjee, R., Jayaraj, G. G., Peter, J. J., Kumar, V., & Mapa, K. (2016). Monitoring conformational heterogeneity of the lid of DnaK substrate-binding domain during its chaperone cycle. *The FEBS Journal, 283*, 2853–2868.

Behnke, J., Feige, M. J., & Hendershot, L. M. (2015). BiP and its nucleotide exchange factors Grp170 and Sil1: Mechanisms of action and biological functions. *Journal of Molecular Biology, 427*, 1589–1608.

Bertelsen, E.B., Chang, L., Gestwicki, J.E., Zuiderweg, E.R. (2009). Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate. Proceedings of the National Academy of Sciences of the United States of America 106**,** 8471–8476.

Blamowska, M., Sichting, M., Mapa, K., Mokranjac, D., Neupert, W., & Hell, K. (2010). ATPase domain and interdomain linker play a key role in aggregation of mitochondrial Hsp70 chaperone Ssc1. *The Journal of Biological Chemistry, 285*, 4423–4431.

Blom, J., Kubrich, M., Rassow, J., et al. (1993). The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane. *Molecular and Cellular Biology, 13*, 7364–7371.

Bolender, N., Sickmann, A., Wagner, R., Meisinger, C., & Pfanner, N. (2008). Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Reports, 9*, 42–49.

Bracher, A., & Verghese, J. (2015). GrpE, Hsp110/Grp170, HspBP1/Sil1 and BAG domain proteins: Nucleotide exchange factors for Hsp70 molecular chaperones. *Sub-Cellular Biochemistry, 78*, 1–33.

Casas, C. (2017). GRP78 at the Centre of the Stage in cancer and neuroprotection. *Frontiers in Neuroscience, 11*, 177.

Chang, Y. W., Sun, Y. J., Wang, C., & Hsiao, C. D. (2008). Crystal structures of the 70-kDa heat shock proteins in domain disjoining conformation. *The Journal of Biological Chemistry, 283*, 15502–15511.

Ciechanover, A., & Kwon, Y. T. (2017). Protein quality control by molecular chaperones in neurodegeneration. *Frontiers in Neuroscience, 11*, 185.

Clerico, E. M., Tilitsky, J. M., Meng, W., & Gierasch, L. M. (2015). How hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology, 427*, 1575–1588.

Conz, C., Otto, H., Peisker, K., et al. (2007). Functional characterization of the atypical Hsp70 subunit of yeast ribosome-associated complex. *The Journal of Biological Chemistry, 282*, 33977–33984.

Delewski, W., Paterkiewicz, B., Manicki, M., et al. (2016). Iron-sulfur cluster biogenesis chaperones: Evidence for emergence of mutational robustness of a highly specific protein-protein interaction. *Molecular Biology and Evolution, 33*, 643–656.

Deloche, O., Kelley, W. L., & Georgopoulos, C. (1997). Structure-function analyses of the Ssc1p, Mdj1p, and Mge1p Saccharomyces Cerevisiae mitochondrial proteins in Escherichia Coli. *Journal of Bacteriology, 179*, 6066–6075.

Dong, M., Bridges, J. P., Apsley, K., Xu, Y., & Weaver, T. E. (2008). ERdj4 and ERdj5 are required for endoplasmic reticulum-associated protein degradation of misfolded surfactant protein C. *Molecular Biology of the Cell, 19*, 2620–2630.

Doong, H., Rizzo, K., Fang, S., Kulpa, V., Weissman, A. M., & Kohn, E. C. (2003). CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: Accumulation of poly-ubiquitinated Hsp90 client proteins. *The Journal of Biological Chemistry, 278*, 28490–28500.

Doring, K., Ahmed, N., Riemer, T., et al. (2017). Profiling Ssb-nascent chain interactions reveals principles of Hsp70-assisted folding. *Cell, 170*(298–311), e20.

Duchniewicz, M., Germaniuk, A., Westermann, B., Neupert, W., Schwarz, E., & Marszalek, J. (1999). Dual role of the mitochondrial chaperone Mdj1p in inheritance of mitochondrial DNA in yeast. *Molecular and Cellular Biology, 19*, 8201–8210.

Dudek, J., Pfeffer, S., Lee, P. H., et al. (2015). Protein transport into the human endoplasmic reticulum. *Journal of Molecular Biology, 427*, 1159–1175.

Dutkiewicz, R., Schilke, B., Knieszner, H., Walter, W., Craig, E. A., & Marszalek, J. (2003). Ssq1, a mitochondrial Hsp70 involved in iron-sulfur (Fe/S) center biogenesis. Similarities to and differences from its bacterial counterpart. *The Journal of Biological Chemistry, 278*, 29719–29727.

Edkins, A. L. (2015). CHIP: A co-chaperone for degradation by the proteasome. *Sub-Cellular Biochemistry, 78*, 219–242.

Elliott, E., Tsvetkov, P., & Ginzburg, I. (2007). BAG-1 associates with Hsc70.Tau complex and regulates the proteasomal degradation of tau protein. *The Journal of Biological Chemistry, 282*, 37276–37284.

Elsner, S., Simian, D., Iosefson, O., Marom, M., & Azem, A. (2009). The mitochondrial protein translocation motor: Structural conservation between the human and yeast Tim14/Pam18-Tim16/Pam16 co-chaperones. *International Journal of Molecular Sciences, 10*, 2041–2053.

Fan, C. Y., Lee, S., & Cyr, D. M. (2003). Mechanisms for regulation of Hsp70 function by Hsp40. *Cell Stress & Chaperones, 8*, 309–316.

Foufelle, F., & Ferre, P. (2007). Unfolded protein response: Its role in physiology and physiopathology. *Medical Science (Paris), 23*, 291–296.

Frazier, A. E., Dudek, J., Guiard, B., et al. (2004). Pam16 has an essential role in the mitochondrial protein import motor. *Nature Structural & Molecular Biology, 11*, 226–233.

Gardner, B. M., Pincus, D., Gotthardt, K., Gallagher, C. M., & Walter, P. (2013). Endoplasmic reticulum stress sensing in the unfolded protein response. *Cold Spring Harbor Perspectives in Biology, 5*, a013169.

Gautschi, M., Lilie, H., Funfschilling, U. et al. (2001). RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. Proceedings of the National Academy of Sciences of the United States of America 98**,** 3762–3767.

Gautschi, M., Mun, A., Ross, S., Rospert, S. (2002). A functional chaperone triad on the yeast ribosome. Proceedings of the National Academy of Sciences of the United States of America 99**,** 4209–4214.

Gething, M. J. (1999). Role and regulation of the ER chaperone BiP. *Seminars in Cell & Developmental Biology, 10*, 465–472.

Glick, B. S. (1995). Pathways and energetics of mitochondrial protein import in Saccharomyces Cerevisiae. *Methods in Enzymology, 260*, 224–231.

Goswami, A. V., Chittoor, B., & D'Silva, P. (2010). Understanding the functional interplay between mammalian mitochondrial Hsp70 chaperone machine components. *The Journal of Biological Chemistry, 285*, 19472–19482.

Gumiero, A., Conz, C., Gese, G. V., et al. (2016). Interaction of the cotranslational Hsp70 Ssb with ribosomal proteins and rRNA depends on its lid domain. *Nature Communications, 7*, 13563.

Ham, H., Woolery, A. R., Tracy, C., Stenesen, D., Kramer, H., & Orth, K. (2014). Unfolded protein response-regulated drosophila fic (dFic) protein reversibly AMPylates BiP chaperone during endoplasmic reticulum homeostasis. *The Journal of Biological Chemistry, 289*, 36059–36069.

Hartl, F. U., & Hayer-Hartl, M. (2009). Converging concepts of protein folding in vitro and in vivo. *Nature Structural & Molecular Biology, 16*, 574–581.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*, 324–332.

Hassdenteufel, S., Klein, M. C., Melnyk, A., & Zimmermann, R. (2014). Protein transport into the human ER and related diseases, Sec61-channelopathies. *Biochemistry and Cell Biology, 92*, 499–509.

Hendershot, L. M. (2004). The ER function BiP is a master regulator of ER function. *Mount Sinai Journal of Medicine, 71*, 289–297.

Huang, P., Gautschi, M., Walter, W., Rospert, S., & Craig, E. A. (2005). The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1. *Nature Structural & Molecular Biology, 12*, 497–504.

Hubscher, V., Mudholkar, K., & Rospert, S. (2017). The yeast Hsp70 homolog Ssb: A chaperone for general de novo protein folding and a nanny for specific intrinsically disordered protein domains. *Current Genetics, 63*, 9–13.

Jacob, J. A., Salmani, J. M. M., Jiang, Z., et al. (2017). Autophagy: An overview and its roles in cancer and obesity. *Clinica Chimica Acta, 468*, 85–89.

Ji, C. H., & Kwon, Y. T. (2017). Crosstalk and interplay between the ubiquitin-proteasome system and autophagy. *Molecules and Cells, 40*, 441–449.

Jin, Y., Awad, W., Petrova, K., & Hendershot, L. M. (2008). Regulated release of ERdj3 from unfolded proteins by BiP. *The EMBO Journal, 27*, 2873–2882.

Kahali, S., Sarcar, B., Fang, B., et al. (2010). Activation of the unfolded protein response contributes toward the antitumor activity of vorinostat. *Neoplasia, 12*, 80–86.

Kampinga, H. H., & Craig, E. A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews. Molecular Cell Biology, 11*, 579–592.

Kania, E., Pajak, B., & Orzechowski, A. (2015). Calcium homeostasis and ER stress in control of autophagy in cancer cells. *BioMed Research International*, 352794.

Kaushik, S., & Cuervo, A. M. (2012). Chaperone-mediated autophagy: A unique way to enter the lysosome world. *Trends in Cell Biology, 22*, 407–417.

Kettern, N., Dreiseidler, M., Tawo, R., & Hohfeld, J. (2010). Chaperone-assisted degradation: Multiple paths to destruction. *Biological Chemistry, 391*, 481–489.

Kim, R., Saxena, S., Gordon, D. M., Pain, D., & Dancis, A. (2001). J-domain protein, Jac1p, of yeast mitochondria required for iron homeostasis and activity of Fe-S cluster proteins. *The Journal of Biological Chemistry, 276*, 17524–17532.

Kityk, R., Kopp, J., Sinning, I., & Mayer, M. P. (2012). Structure and dynamics of the ATP-bound open conformation of Hsp70 chaperones. *Molecular Cell, 48*, 863–874.

Koplin, A., Preissler, S., Ilina, Y., et al. (2010). A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes. *The Journal of Cell Biology, 189*, 57–68.

Korennykh, A., & Walter, P. (2012). Structural basis of the unfolded protein response. *Annual Review of Cell and Developmental Biology, 28*, 251–277.

Kozany, C., Mokranjac, D., Sichting, M., Neupert, W., & Hell, K. (2004). The J domain-related cochaperone Tim16 is a constituent of the mitochondrial TIM23 preprotein translocase. *Nature Structural & Molecular Biology, 11*, 234–241.

Kronidou, N.G., Oppliger, W., Bolliger, L. et al. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. Proceedings of the National Academy of Sciences of the United States of America. 91**,** 12818–12822.

Kutik, S., Stroud, D. A., Wiedemann, N., & Pfanner, N. (2009). Evolution of mitochondrial protein biogenesis. *Biochimica et Biophysica Acta, 1790*, 409–415.

Lai, A. L., Clerico, E. M., Blackburn, M. E., et al. (2017). Key features of an Hsp70 chaperone allosteric landscape revealed by ion-mobility native mass spectrometry and double electron-electron resonance. *The Journal of Biological Chemistry, 292*, 8773–8785.

Laloraya, S., Gambill, B.D., Craig, E.A. (1994). A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. Proceedings of the National Academy of Sciences of the United States of America 91**,** 6481–6485.

Leidig, C., Bange, G., Kopp, J., et al. (2013). Structural characterization of a eukaryotic chaperone--the ribosome-associated complex. *Nature Structural & Molecular Biology, 20*, 23–28.

Lewy, T. G., Grabowski, J. M., & Bloom, M. E. (2017). BiP: Master regulator of the unfolded protein response and crucial factor in Flavivirus biology. *The Yale Journal of Biology and Medicine, 90*, 291–300.

Li, Y., Dudek, J., Guiard, B., Pfanner, N., Rehling, P., & Voos, W. (2004). The presequence translocase-associated protein import motor of mitochondria. Pam16 functions in an antagonistic manner to Pam18. *The Journal of Biological Chemistry, 279*, 38047–38054.

Li, W., Yang, Q., & Mao, Z. (2011). Chaperone-mediated autophagy: Machinery, regulation and biological consequences. *Cellular and Molecular Life Sciences, 68*, 749–763.

Lievremont, J.P., Rizzuto, R., Hendershot,.L., Meldolesi, J. (1997) BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. The Journal of Biological Chemistry 272**,** 30873–30879.

Liu, Q., & Hendrickson, W. A. (2007). Insights into Hsp70 chaperone activity from a crystal structure of the yeast Hsp110 Sse1. *Cell, 131*, 106–120.

Maattanen, P., Gehring, K., Bergeron, J. J., & Thomas, D. Y. (2010). Protein quality control in the ER: The recognition of misfolded proteins. *Seminars in Cell & Developmental Biology, 21*, 500–511.

Mapa, K., Sikor, M., Kudryavtsev, V., et al. (2010). The conformational dynamics of the mitochondrial Hsp70 chaperone. *Molecular Cell, 38*, 89–100.

Marcinowski, M., Holler, M., Feige, M. J., Baerend, D., Lamb, D. C., & Buchner, J. (2011). Substrate discrimination of the chaperone BiP by autonomous and cochaperone-regulated conformational transitions. *Nature Structural & Molecular Biology, 18*, 150–158.

Massey, A. C., Zhang, C., & Cuervo, A. M. (2006). Chaperone-mediated autophagy in aging and disease. *Current Topics in Developmental Biology, 73*, 205–235.

Matouschek, A., Pfanner, N., & Voos, W. (2000). Protein unfolding by mitochondria. The Hsp70 import motor. *EMBO Reports, 1*, 404–410.

Mayer, M. P. (2013). Hsp70 chaperone dynamics and molecular mechanism. *Trends in Biochemical Sciences, 38*, 507–514.

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*, 670–684.

Mayer, M. P., & Kityk, R. (2015). Insights into the molecular mechanism of allostery in Hsp70s. *Frontiers in Molecular Biosciences, 2*, 58.

McCaffrey, K., & Braakman, I. (2016). Protein quality control at the endoplasmic reticulum. *Essays in Biochemistry, 60*, 227–235.

McDonough, H., & Patterson, C. (2003). CHIP: A link between the chaperone and proteasome systems. *Cell Stress & Chaperones, 8*, 303–308.

Miao, B., Davis, J. E., & Craig, E. A. (1997). Mge1 functions as a nucleotide release factor for Ssc1, a mitochondrial Hsp70 of Saccharomyces Cerevisiae. *Journal of Molecular Biology, 265*, 541–552.

Mokranjac, D., Sichting, M., Neupert, W., & Hell, K. (2003). Tim14, a novel key component of the import motor of the TIM23 protein translocase of mitochondria. *The EMBO Journal, 22*, 4945–4956.

Mokranjac, D., Bourenkov, G., Hell, K., Neupert, W., & Groll, M. (2006). Structure and function of Tim14 and Tim16, the J and J-like components of the mitochondrial protein import motor. *The EMBO Journal, 25*, 4675–4685.

Neupert, W., & Brunner, M. (2002). The protein import motor of mitochondria. *Nature Reviews. Molecular Cell Biology, 3*, 555–565.

Neupert, W., & Herrmann, J. M. (2007). Translocation of proteins into mitochondria. *Annual Review of Biochemistry, 76*, 723–749.

Okamoto, K., Brinker, A., Paschen, S. A., et al. (2002). The protein import motor of mitochondria: A targeted molecular ratchet driving unfolding and translocation. *The EMBO Journal, 21*, 3659–3671.

Paul, I., & Ghosh, M. K. (2014). The E3 ligase CHIP: Insights into its structure and regulation. *BioMed Research International*, 918183.

Peisker, K., Braun, D., Wolfle, T., et al. (2008). Ribosome-associated complex binds to ribosomes in close proximity of Rpl31 at the exit of the polypeptide tunnel in yeast. *Molecular Biology of the Cell, 19*, 5279–5288.

Polier, S., Dragovic, Z., Hartl, F. U., & Bracher, A. (2008). Structural basis for the cooperation of Hsp70 and Hsp110 chaperones in protein folding. *Cell, 133*, 1068–1079.

Preissler, S., & Deuerling, E. (2012). Ribosome-associated chaperones as key players in proteostasis. *Trends in Biochemical Sciences, 37*, 274–283.

Preissler, S., Chambers, J. E., Crespillo-Casado, A., et al. (2015a). Physiological modulation of BiP activity by trans-protomer engagement of the interdomain linker. *eLife, 4*, e08961.

Preissler, S., Rato, C., Chen, R., et al. (2015b). AMPylation matches BiP activity to client protein load in the endoplasmic reticulum. *eLife, 4*, e12621.

Prip-Buus, C., Westerman, B., Schmitt, M., Langer, T., Neupert, W., & Schwarz, E. (1996). Role of the mitochondrial DnaJ homologue, Mdj1p, in the prevention of heat-induced protein aggregation. *FEBS Letters, 380*, 142–146.

Qi, L., & Zhang, X. D. (2014). Role of chaperone-mediated autophagy in degrading Huntington's disease-associated huntingtin protein. *Acta Biochimica et Biophysica Sinica Shanghai, 46*, 83–91.

Qi, R., Sarbeng, E. B., Liu, Q., et al. (2013). Allosteric opening of the polypeptide-binding site when an Hsp70 binds ATP. *Nature Structural & Molecular Biology, 20*(7), 900.

Rakwalska, M., & Rospert, S. (2004). The ribosome-bound chaperones RAC and Ssb1/2p are required for accurate translation in Saccharomyces Cerevisiae. *Molecular and Cellular Biology, 24*, 9186–9197.

Rassow, J., Maarse, A. C., Krainer, E., et al. (1994). Mitochondrial protein import: Biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *The Journal of Cell Biology, 127*, 1547–1556.

Rout, A. K., Strub, M. P., Piszczek, G., & Tjandra, N. (2014). Structure of transmembrane domain of lysosome-associated membrane protein type 2a (LAMP-2A) reveals key features for substrate specificity in chaperone-mediated autophagy. *The Journal of Biological Chemistry, 289*, 35111–35123.

Rowley, N., Prip-Buus, C., Westermann, B., et al. (1994). Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding. *Cell, 77*, 249–259.

Rudiger, S., Germeroth, L., Schneider-Mergener, J., & Bukau, B. (1997). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *The EMBO Journal, 16*(7), 1501.

Sakuragi, S., Liu, Q., & Craig, E. (1999). Interaction between the nucleotide exchange factor Mge1 and the mitochondrial Hsp70 Ssc1. *The Journal of Biological Chemistry, 274*, 11275–11282.

Sanjuan Szklarz, L. K., Guiard, B., Rissler, M., et al. (2005). Inactivation of the mitochondrial heat shock protein zim17 leads to aggregation of matrix hsp70s followed by pleiotropic effects on morphology and protein biogenesis. *Journal of Molecular Biology, 351*, 206–218.

Sano, R., & Reed, J. C. (2013). ER stress-induced cell death mechanisms. *Biochimica et Biophysica Acta, 1833*, 3460–3470.

Sanyal, A., Chen, A. J., Nakayasu, E. S., et al. (2015). A novel link between fic (filamentation induced by cAMP)-mediated adenylylation/AMPylation and the unfolded protein response. *The Journal of Biological Chemistry, 290*, 8482–8499.

Schauble, N., Lang, S., Jung, M., et al. (2012). BiP-mediated closing of the Sec61 channel limits Ca2+ leakage from the ER. *The EMBO Journal, 31*, 3282–3296.

Schilke, B., Williams, B., Knieszner, H., et al. (2006). Evolution of mitochondrial chaperones utilized in Fe-S cluster biogenesis. *Current Biology, 16*, 1660–1665.

Schmidt, S., Strub, A., Rottgers, K., Zufall, N., & Voos, W. (2001). The two mitochondrial heat shock proteins 70, Ssc1 and Ssq1, compete for the cochaperone Mge1. *Journal of Molecular Biology, 313*, 13–26.

Schneider, H. C., Berthold, J., Bauer, M. F., et al. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature, 371*, 768–774.

Shen, Y., & Hendershot, L. M. (2005). ERdj3, a stress-inducible endoplasmic reticulum DnaJ homologue, serves as a cofactor for BiP's interactions with unfolded substrates. *Molecular Biology of the Cell, 16*, 40–50.

Sichting, M., Mokranjac, D., Azem, A., Neupert, W., & Hell, K. (2005). Maintenance of structure and function of mitochondrial Hsp70 chaperones requires the chaperone Hep1. *The EMBO Journal, 24*, 1046–1056.

Sikor, M., Mapa, K., von Voithenberg, L. V., Mokranjac, D., & Lamb, D. C. (2013). Real-time observation of the conformational dynamics of mitochondrial Hsp70 by spFRET. *The EMBO Journal, 32*, 1639–1649.

Swain, J. F., Dinler, G., Sivendran, R., Montgomery, D. L., Stotz, M., & Gierasch, L. M. (2007). Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker. *Molecular Cell, 26*, 27–39.

Truscott, K. N., Voos, W., Frazier, A. E., et al. (2003). A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *The Journal of Cell Biology, 163*, 707–713.

Ungermann, C., Neupert, W., & Cyr, D. M. (1994). The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. *Science, 266*, 1250–1253.

Voisine, C., Cheng, Y.C., Ohlson, M. et al. (2001). Jac1, a mitochondrial J-type chaperone, is involved in the biogenesis of Fe/S clusters in Saccharomyces Cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 98**,** 1483–1488.

von Ahsen, O., Voos, W., Henninger, H., & Pfanner, N. (1995). The mitochondrial protein import machinery. Role of ATP in dissociation of the Hsp70.Mim44 complex. *The Journal of Biological Chemistry, 270*, 29848–29853.

Voos, W., & Rottgers, K. (2002). Molecular chaperones as essential mediators of mitochondrial biogenesis. *Biochimica et Biophysica Acta, 1592*, 51–62.

Walter, P., & Ron, D. (2011). The unfolded protein response: From stress pathway to homeostatic regulation. *Science, 334*, 1081–1086.

Wang, J., Lee, J., Liem, D., & Ping, P. (2017). HSPA5 gene encoding Hsp70 chaperone BiP in the endoplasmic reticulum. *Gene, 618*, 14–23.

Westermann, B., Gaume, B., Herrmann, J. M., Neupert, W., & Schwarz, E. (1996). Role of the mitochondrial DnaJ homolog Mdj1p as a chaperone for mitochondrially synthesized and imported proteins. *Molecular and Cellular Biology, 16*, 7063–7071.

Wiedemann, N., & Pfanner, N. (2017). Mitochondrial machineries for protein import and assembly. *Annual Review of Biochemistry, 86*, 685–714.

Willmund, F., del Alamo, M., Pechmann, S., et al. (2013). The cotranslational function of ribosome-associated Hsp70 in eukaryotic protein homeostasis. *Cell, 152*, 196–209.

Yamamoto, H., Momose, T., Yatsukawa, Y., et al. (2005). Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane. *FEBS Letters, 579*, 507–511.

Yang, J., Nune, M., Zong, Y., Zhou, L., & Liu, Q. (2015). Close and allosteric opening of the polypeptide-binding site in a human Hsp70 chaperone BiP. *Structure, 23*, 2191–2203.

Zhu, X., Zhao, X., Burkholder, W. F., et al. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science, 272*, 1606–1614.

Zhuravleva, A., Clerico, E. M., & Gierasch, L. M. (2012). An interdomain energetic tug-of-war creates the allosterically active state in Hsp70 molecular chaperones. *Cell, 151*, 1296–1307.

Zimmermann, R., Eyrisch, S., Ahmad, M., & Helms, V. (2011). Protein translocation across the ER membrane. *Biochimica et Biophysica Acta, 1808*, 912–924.

Zuiderweg, E. R., Bertelsen, E. B., Rousaki, A., Mayer, M. P., Gestwicki, J. E., & Ahmad, A. (2013). Allostery in the Hsp70 chaperone proteins. *Topics in Current Chemistry, 328*, 99–153.

# Part III
# HSP in Human Disease

# Chapter 11
# Heat Shock Protein Response to Overuse Injuries


**Mary F. Barbe, Danielle M. Salvadeo, and Brendan A. Hilliard**

**Abstract** The heat shock protein (Hsp) response is understudied with non-exercise overuse injuries. We focused on the Hsp response in muscles and tendons undergoing such injury or cyclical loading. Hsp25 mRNA and protein levels increase in muscles undergoing functional overload, and show greater increases in fast type muscles. In an operant rat model of reaching and grasping, the inducible form of Hsp70 increased in muscles and tendons showing injury, with the greatest increase in rats performing a high repetition high force for 12 weeks, compared to easier repetition/loading paradigms. These increases were paralleled by increases in several repair-associated proteins (osteoactivin, MMPs, and TGFB1). Trapezius biopsies from patients with myalgia show increased mRNA levels of Hsp72 and decreased levels of growth and metabolism regulators. Prolonged exercise interventions in general, when provided to subjects with trapezius myalgia, decreased Hsp72 mRNA levels, while specific strength training of shoulder and neck muscles increased mRNA levels of analytes related to carbohydrate oxidation. In a rat model of supraspinatus injury, the Hsp response appeared related to the cascade of stress-related programmed cell death in torn tendons. A mild mechanical stimulation of cultured tendon fibroblasts reduced apoptosis and increased cell proliferation and may be helpful for tissue regeneration.

**Keywords** High intensity exercise · Muscle · Repetitive strain injury · Tendon · Work-related musculoskeletal disorders · Work-related overuse

## Abbreviations

| | |
|---|---|
| APAF1 | Apoptotic protease activating factor 1 |
| cFLIP | Cellular FLICE-inhibitory protein |
| ERK | Extracellular signal regulated kinases |
| GPNMB | Glycoprotein nonmelanoma protein B |

M. F. Barbe (✉) · D. M. Salvadeo · B. A. Hilliard
Department of Anatomy and Cell Biology, Temple University, Philadelphia, PA, USA
e-mail: Mary.barbe@temple.edu



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_11

| | |
|---|---|
| HSF-1 | Heat shock factor-1 |
| Hsp | Heat shock protein |
| IL-1beta | Interleukin 1 beta |
| JNK | c-Jun N-terminal kinases |
| MMP | Matrix metalloproteinase |
| TGFB-1 | Transforming growth factor beta 1 |
| TNFalpha | Tumor necrosis factor alpha |

## 11.1 Introduction

The response of heat shock proteins to exercise has been studied intently for over 30 years. With prolonged intense exercise, heat shock protein 25/27, 70 and 90 have been shown to increase in muscles (McArdle et al. 2004; Paulsen et al. 2007). However, although several studies have shown that repetitive or cyclical movements can lead to tissue injury, the heat shock protein response is understudied with non-exercise-induced overuse injuries. Such injuries include work-related overuse activities, which are also termed work-related musculoskeletal disorders and repetitive strain injuries. Overuse injuries, whatever their cause, are now considered a leading cause of long-term pain and physical disability world-wide (Woolf and Pfleger 2003; World Health Organization 2007), with diagnoses including muscle disorders (myositis, myalgia, over-reaching syndrome) and tendinopathies (tendonitis, tendinosis, tendinopathy, lateral epicondylitis and rotator cuff tears) (Piligian et al. 2000; Rempel and Diao 2004; Rempel et al. 1992; van Rijn et al. 2009). According to the U.S. Bureau of Labor Statistics, musculoskeletal disorders in general accounted for 34% of lost workday injuries/illnesses in 2012, and upper extremity work-related musculoskeletal disorders comprised 24% and were among the most severe, with work absences ranging from 17 days (hand and wrist) to 24 days (shoulder) (Bureau of Labor Statistics 2012, 2014). Studies have identified repetition, force and duration as three key risk factors for upper extremity overuse injuries (Gallagher and Heberger 2013; Silverstein et al. 1986). Many overuse injuries commonly occur as a result of prolonged repetitive loading of the muscle-tendon unit (Barr and Barbe 2002; Kannus et al. 1997). Therefore, we will focus on the response of heat shock proteins in muscles and tendons to non-exercise-induced overuse injuries. We will also include studies reporting the response of heat shock proteins to cyclical/repetitive strain in cultured tendon fibroblasts.

Briefly, as an overview, heat shock proteins (Hsp) are primarily chaperones induced in tissues in response to tissue and cellular stress and that play important roles in facilitating protein synthesis, folding and assembly, and environmental adaptation (Craig et al. 1993; Hartl 1996). Hsp are thought to be provoked by infiltrating inflammatory cells, yet are anti-inflammatory in nature, and provide an anti-inflammatory influence (De et al. 2000; Senf et al. 2013; Wieten et al. 2007). Heat shock proteins are typically classified by their molecular weight and are upregulated in response to heat stress in order to confer protection against damage (Barbe et al. 1988; Tytell et al.

1989). However, they are also known to increase in response to tissue injury and mechanical stress (Banfi et al. 2004; Escobedo et al. 2004; Jagodzinski et al. 2006). Hsp25 (the rodent form) and Hsp27 (the human form) are thought to protect skeletal and cardiac muscles against oxidative damage (Escobedo et al. 2004) and mechanical damage (Koh 2002; Koh and Escobedo 2004). In mammalian cells, members of the Hsp70 molecular weight family are the most highly induced of the cellular stress response, and include Hsc70 (the constitutive form) and the inducible form of Hsp70 (also commonly known as Hsp72). Hsp70 increases after most types of tissue injury and is thought to play key roles in skeletal muscle repair, regeneration or adaptation (McArdle et al. 2004; Senf et al. 2008; Senf et al. 2013).

## 11.2 HSP Response in Muscles to Overuse Injuries

### *11.2.1 Response in Muscles to Functional Overload*

Functional overload of plantaris and soleus muscles was induced in female rats by bilateral removal of their major synergistic muscle, the gastrocnemius (Huey et al. 2007). These two muscles were chosen since the plantaris is a fast type muscle and the soleus is a slow type. Hsp25 and phosphorylation changes were then examined in each muscle at 0.5, 1, 2, 3 and 7 days after onset of functional overload and in controls. Hsp25 mRNA expression levels were greatest at the earlier time points after functional overload and then decreased thereafter, with more in the plantaris than in the soleus muscle, compared to control values. Consistently higher Hsp25 mRNA levels in functionally overloaded muscles were accompanied by increased total Hsp25 protein levels (soluble and insoluble fractions and phosphorylated Hsp25), although protein levels were higher at 2, 3 and 7 days after functional overload than earlier, and greater in the plantaris muscle than in the soleus at 3 and 7 days. The soluble Hsp25 response to functional overload was greater in the plantaris (i.e., the fast muscle) than in the soleus at all time points, and at 0.5 days in the insoluble fraction. There were also greater levels of phosphorylated Hsp25 in functionally overloaded plantaris muscles, which the authors postulated were due to the higher observed levels of TNFalpha in this muscle, compared to the soleus, and thus a greater need to protect against the cytotoxic effects of this pro-inflammatory cytokine (Huey et al. 2007). It has been proposed that one of the earliest responses to stress is phosphorylation of Hsp25, a change that disrupts the typically large aggregates of Hsp25 in unstressed cells. Such disruption allows Hsp25 to interact directly or indirectly with F-actin and protect it from damage and facilitate cytoskeleton reorganization and stabilization (Mounier and Arrigo 2002; Welsh and Gaestel 1998). Results of this study indicate that the initial responses of total Hsp25, phosphorylated Hsp25 and TNFalpha to mechanical stress and inflammation induced by functional overload are greater in fast type than in slow type muscles (Huey et al. 2007).

### *11.2.2 Response in Muscles to Repetitive Work Activities*

An operant rat model of reaching and grasping was developed in which reach rates and force can be varied (Barbe et al. 2003; Barbe et al. 2013). The parameters of the various work tasks are defined in Table 11.1. The target reach rate for high repetition (4 reaches/min) and high force pulling (50–70% of maximum voluntary pulling force) were chosen from epidemiological studies reporting thresholds of exposure above which there is a high risk for developing carpal tunnel syndrome in industrial populations; the low repetition rate and force targets used were below these threshold limits (Barr and Barbe 2004; Fine et al. 1986; Silverstein et al. 1986). Female rats perform these tasks chronically for 2 h per day, in four 30-min sessions per day, and 3 days per week for 6 to 24 weeks. This model has been used to examine the effects of performance of low and high demand work tasks on musculoskeletal tissues. In one study using this model, the temporal and spatial characteristics of the inducible form of Hsp70, osteoactivin and matrix metalloproteinases (MMPs) were studied in forelimb flexor digitorum muscles of rats performing a high repetition negligible force food retrieval task for 3 to 6 weeks (Frara et al. 2016). Osteoactivin, also known as glycoprotein nonmelanoma protein B (GPNMB), is an anabolic growth factor involved in skeletal muscle regeneration via upregulation of MMPs (Furochi et al. 2007a, b; Ogawa et al. 2005). MMPs are zinc-dependent proteases that participate in tissue repair and remodeling processes (Kherif et al. 1998). Levels of Hsp70 protein were progressively increased by weeks 3 and 6 within muscles of high repetition negligible force rats, compared to controls (Frara et al. 2016). The increased Hsp70 was concomitant with progressive increases of MMP-1, -2 and -3 (active forms only), and osteoactivin gene and protein expression. Immunohistochemistry showed co-localization of Hsp70 with osteoactivin in the sarcolemma surrounding myofibers of 6-week task rats, suggestive of an interaction between the two proteins. Modest myofiber injury was present in rats performing this particular task by week 6 (Barbe et al. 2003). It was also observed that IL-1beta, a pro-inflammatory cytokine, increased transiently by week 3, followed by declines to control levels by week 6 (Frara et al. 2016). The latter finding is consistent with studies indicating that Hsp may inhibit the release of pro-inflammatory cytokines, including IL-1beta (Cahill et al. 1996; Wieten et al. 2007). These results combined indicate that a repair response is present in muscles of 6-week high repetition negligible force rats, with Hsp70, osteoactivin and MMPs mediating this response, likely in a shared manner.

Exposure-dependent changes on Hsp and indices of tissue pathology were also examined using a force-repetition interaction design in which female rats performed a repetitive lever-pulling task for 12 weeks at one of four repetition and force levels: 1) low repetition with low force, 2) low repetition with high force, 3) high repetition with low force, and 4) high repetition with high force (See Table 11.1 for details of the task parameters) (Barbe et al. 2013). By week 12 of task performance, flexor digitorum muscles showed significant force x repetition interactions in muscle levels of inducible Hsp70 and the presence of macrophage infiltration into myofibers

**Table 11.1** Task parameters: Target versus actual (Mean ± SEM reported) this task was performed for 2 hours/day, in four 30 min session per day, for 3 days per week

| | LRNF/LRLF | | | HRNF/HRLF | | | LRHF | | | HRHF | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Successful Reps/min (n=12) | All Reps/min (n=12) | Grasp Force % MPF (n = 12) | Successful Reps/min (n = 24) | All Reps/min (n = 24) | Grasp Force % MPF (n = 15) | Successful Reps/min (n = 12) | All Reps/min (n = 12) | Grasp Force % MPF (n = 12) | Successful Reps/min (n = 24) | All Reps/min (n = 24) | Grasp Force % MPF (n = 24) |
| Target values | 2 | 2 | 5 or 15 ± 5%[a] MPF (0.23 N) | 4 | 4 | 5 or 15 ± 5%[a] MPF (0.23 N) | 2 | 2 | 53 ± 5 % MPF (1.02 N) | 4 | 4 | 53 ± 5 % MPF (1.02 N) |
| Week 1 Actual Values | 2.01±0.33 | 3.03±0.12 | 15.78±4.54 | 2.28±0.34 | 6.48±0.47 | 20.07±1.61 | 1.31±0.38 | 9.0±0.68 | 41.90±2.61 | 2.31±0.52 | 10.8 ± 0.80 | 39.88±2.22 |
| Week 12 Actual Values | 1.39±0.06 | 3.38±0.31 | 13.68±0.89 | 2.06±0.39 | 5.04±0.45 | 14.95±1.03 | 1.46±0.25 | 5.79±0.97 | 46.22±6.24 | 2.89±0.46 | 9.27 ± 0.64 | 48.64±0.90 |

[a]Negligible force required retrieval of a 45 mg pellet of food estimated to be 5% force, while low force required pulling on a lever bar at 15% of their maximum pulling force, as explained in [32]. *All Reps* all repetitions per minute, both successful and unsuccessful, *g* grams, *Grasp Force %* maximum isometric pulling force on lever bar, *MPF* average maximum isometric pulling force for these young adult rats, which was determined to be 1.93 Newtons on the last day of training by a subset of rats, *n* number of rats analyzed, *N* Newtons

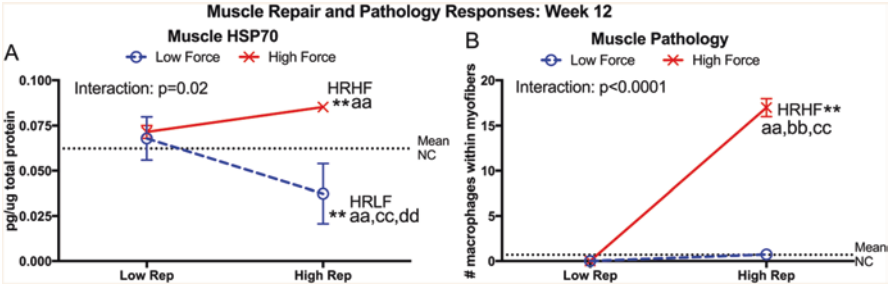


**Fig. 11.1** Histopathology and production of repair proteins in flexor digitorum muscles after performance of repetitive tasks for 12 weeks at one of four combinations of repetitive rates and force levels: low repetition low force (LRLF), high repetition low force (HRLF), low repetition high force (LRHF) or high repetition high force (HRHF). Repetitive rate is indicated on X-axis (Low Rep vs High Rep); low force is indicated by blue lines; high force is indicated by red lines. Results were compared in two-way ANOVAs to examine the factors repetition and force, and their interaction, and are indicated in individual panels. **(a)** Levels of inducible Hsp70 in muscles, assayed using ELISA. **(b)** Number of activated macrophages (CD-68 immunopositive) within myofibers, per area examined using a 40x objective. Symbols: aa: $p < 0.01$, compared to LRLF rats; bb: $p < 0.01$, compared to HRLF rats; cc: $p < 0.01$, compared to LRHF rats; **:$p < 0.01$, compared to normal control rats (NC) (indicated by horizontal dashed line). Mean and SEM are shown. Figure modified from (Barbe et al. 2013)

(Fig. 11.1a, b). Specifically, muscle levels of Hsp70 and evidence of muscle microdamage were present only in rats that had performed the high repetition high force task for 12 weeks, and the macrophage infiltration was only into atrophying myofibers (latter data not shown), matching hypothesized outcomes for fatigue failure in which only the highest demand tasks or loads result in tissue pathology (Barbe et al. 2013; Gallagher and Heberger 2013). Rats performing either low repetition task showed no changes in any of these attributes, compared to control rats (Fig. 11.1a, b). In contrast, muscles in rats performing a high repetition low force task for 12 weeks showed the lowest muscle levels of Hsp70, compared to the other groups (Fig. 11.1a), suggesting an apparent acclimation to the stress of the task. Furthermore, there were significant increases in several inflammatory cytokines (IL-1alpha and IL-1beta) in rats that had performed the high repetition high force task for 12 weeks, compared to the other groups (Barbe et al. 2013). This may seem in contrast to the earlier discussed results in which increased Hsp70 levels were associated with decreasing IL-1beta levels (Frara et al. 2016). However, a subsequent study examining cytokines longitudinally in high repetition high force rats showed that increases in most pro-inflammatory cytokines had abated in muscles by week 12, compared to earlier weeks (Fisher et al. 2015). There are several studies suggesting that such cytokines may be involved in inducing production of Hsp70 and that the Hsp70 response is then anti-inflammatory in nature (Cahill et al. 1996; Wieten et al. 2007). The 12-week time point also marked the peak protein production of a key repair cytokine, transforming growth factor (TGFB1), in the muscles (Fisher et al. 2015). Thus, indices of muscle injury were present in rats that had performed a high repetition high force for 12 weeks. These injury responses were accompanied by enhanced

repair responses, such as increased production of Hsp70 and TGFB1. Such injury and repair responses were lower or absent in rats performing easier tasks.

### *11.2.3 Response in Muscles to Repetitive Stressful Work and then Prolonged Exercise Training*

The above results from animal studies are consistent with findings by Sjogaard and colleagues showing that repetitive stressful work increases inducible Hsp70 levels in myalgic trapezius muscles of female humans (Sjogaard et al. 2013). Twenty-eight females with trapezius myalgia and 16 healthy females were included in the study, with all subjects employed in jobs with monotonous and repetitive work tasks (e.g., assembly line or office work). The diagnosis of myalgia was fulfilled when subjects had: 1) pain or discomfort for more than 30 days during the last year in the neck and shoulder region; 2) more than 30 days of pain or discomfort in maximally three of eight major body regions; and 3) intense and frequent pain, assayed using three different scales (Sjogaard et al. 2013), and 4) palpable tenderness and tightness in the trapezius muscle (Sjogaard et al. 2010). At baseline, all participants (Controls and Myalgic patients) had biopsies collected from the trapezius muscle. Eight of the Myalgic patients went on to participate in a repetitive stressful task and had a second sample collected approximately 7 h later: 2 h rest, performance of a 40-min repetitive-low-force unilateral pegboard task, two hours semi-rest (participants filled out a questionnaire on the computer, watched a movie or did some reading), performance of a stressful color-word-conflict test for 10 min (for details, see (Sjogaard et al. 2010)), and then another 1 h rest period before the second biopsy. No differences were observed in Control versus Myalgic patients samples at baseline. However, when examining the eight Myalgic patient muscles pre- versus post-task, mRNA content of Heme oxygenase-1 (HO-1) and Hsp72 was increased after the 7 h of repetitive/rest/stressful work, indicating that the trapezius muscle may have indeed been exposed to oxidative stress during the repetitive work task (HO-1 function) and was in need of protection against ischemia and/or preservation of cellular structure (Hsp72 function). This is of interest as two growth-related proteins and one metabolic regulation protein were significantly decreased in trapezius biopsies of these Myalgia subjects after the repetitive stress work day: insulin-like growth factor -1Ea, myostatin, and peroxisome proliferative activated receptor gamma coactivator 1alpha, respectively. No changes were observed for constitutive hsp70 (Hsc70).

Also in this Sjogaard study, all 28 Myalgic participants were then randomized into one of three different intervention groups: a specific strength training group (specifically, high intensity-specific neck and shoulder training regimens), a general fitness training group (leg bicycling), and a health counseling only group. The participants participated in these training or counseling activities for 20 min, 3 times per week for 10 weeks. Prolonged exercise training led to decreased levels of

Hsp70 in muscles. Both tenderness scores and Hsp72 mRNA levels decreased in the myalgic patients after both 10 week exercise programs, compared to the counseling only group. Glycogen synthase and pyruvate dehydrogenase E1alpha, a key enzyme in glycogenesis and a metabolic factor, respectively, increased significantly in the specific strength training group, compared to the other groups. Furthermore, levels of constitutive Hsp70 (Hsc70) also decreased in biopsies collected from the specific strength training group. Findings of this study combined indicate that in the trapezius biopsies from patients with trapezius myalgia, that repetitive stressful work increases mRNA content for Hsp72 and decreases levels of key regulators of growth and metabolism, that prolonged exercise interventions in general decreases Hsp72 mRNA content, while specific strength training of these same muscles increased mRNA levels of analytes related to carbohydrate oxidation.

## 11.3 HSP Response in Tendons to Overuse Injuries

### *11.3.1 Response in Tendons to Repetitive Work Activities*

In the same high repetitive negligible task rats as discussed above, Hsp70, osteoactivin, MMP-1 and the active form of MMP-3 were increased in flexor digitorum tendons with task performance to 6 weeks, compared to 3-week task and control female rat tendons (Frara et al. 2016). Both Hsp70 and osteoactivin were increased in tenocytes located in the endotendon. While IL-1beta levels were unchanged by task performance, IL-1alpha level increased transiently in week 3, but fell to control levels by week 6. Thus, as in muscle, tendons showed a peak of inflammatory responses in week 3 followed by the onset of tendon repair responses in week 6 including increases in Hsp70. Exposure-dependent changes of Hsp70, other repair proteins and tissue pathology were examined in flexor digitorum tendons using the force-repetition interaction design described above, again using female rats. Flexor digitorum tendons of 12-week high repetition high force rats showed the highest levels of Hsp70 protein simultaneously with increased histological indices of tendon pathology such as collagen disorganization (Fig. 11.2a, b). These changes were accompanied by increases in tendon levels of several other repair-related proteins (TGFB-1, MMP2, and platelet derived growth factor ab/bb) (Fig. 11.2c–f). The Hsp70 increases indicate that cell and tissue stresses were sufficient in tendons of high repetition high force rats to elicit this potentially mitigating response to tissue stress and injury (Noble et al. 1999; Noble and Shen 2012). In contrast, decreased tendon levels of Hsp70, an absence of tendon pathology, and no increases in any repair cytokine were observed in rats that had performed a low repetition low force task for 12 weeks, compared to control rats, suggesting that their tendon tissues had adapted to the task (Fig. 11.2a–f). These findings combined support a fatigue failure theory in which only the highest demand tasks or loads result in tissue pathology

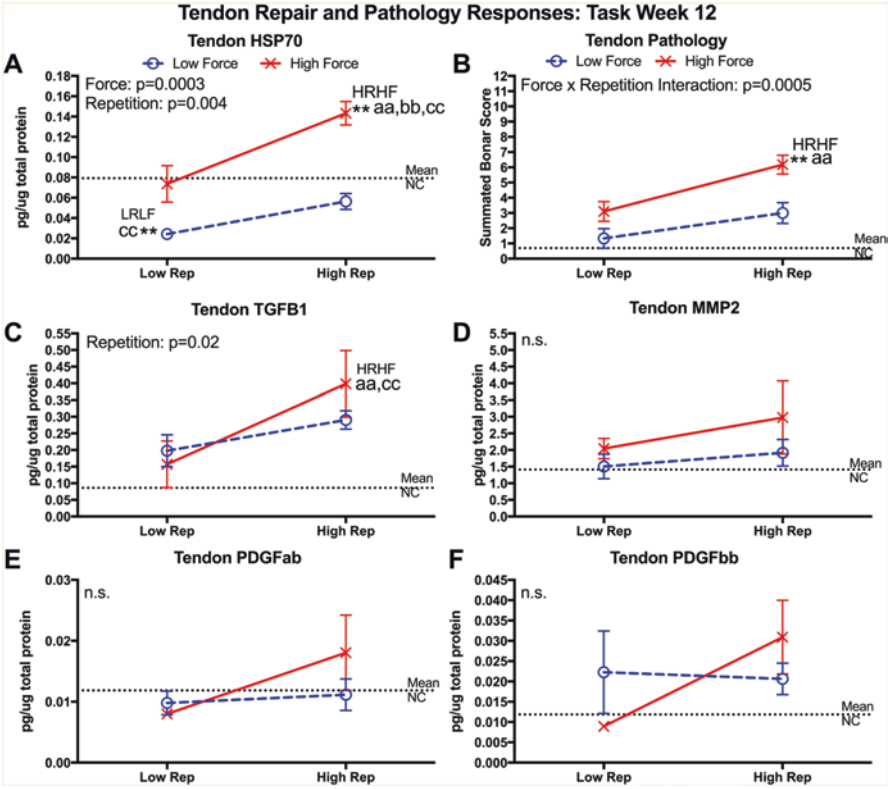


**Fig. 11.2** Histopathology and production of repair proteins in flexor digitorum tendons after performance of repetitive tasks for 12 weeks at one of four combinations of repetitive rates and force levels as defined in Fig. 11.1. Results were compared in two-way ANOVAs to examine the factors repetition and force, and their interaction, and are indicated in individual panels. **(a)** Levels of inducible Hsp70 in tendons, assayed using ELISA. **(b)** Combined tendon pathology scores for cellularity, cell shape and collagen organization using the semi-quantitative Bonar Scoring System in which 12 is the total score. **(c-f)** Levels of transforming growth factor beta 1 (TGFB-1), matrix metalloproteinase 2 (MMP2), and platelet derived growth factor ab and bb, assayed using ELISA. Symbols: aa: $p < 0.01$, compared to LRLF rats; bb: $p < 0.01$, compared to HRLF rats; cc: $p < 0.01$, compared to LRHF rats; **:$p < 0.01$, compared to normal control rats (NC). Mean and SEM are shown. Figure modified and extended from (Barbe et al. 2013)

(Fung et al. 2010; Fung et al. 2009; Gallagher and Heberger 2013; Neviaser et al. 2012) followed by enhanced repair processes, and that prolonged activity at low force parameters may activate metabolic changes that allow tendon tissues to handle more efficiently any potentially damaging changes occurring with task performance (Sjogaard et al. 2013).

### 11.3.2 Response in Tendons to Supraspinatus Tendinopathy

A rodent model of tendinopathy has been used to examine whether heat shock proteins play a role in the cascade of stress-related programmed cell death and degeneration in rotator cuff tears (Millar and Murrell 2012; Millar et al. 2008). Male rats were underwent daily training on a treadmill for two weeks, at increasing intensity, to acclimate them to treadmill exercise. They were subjected to running on a 10° decline at 17 m/min for 1 h per day, 5 days per week (which equals approximately 7500 strides per day), before collection of the supraspinatus tendon from these rats and from non-exercised controls. In this same study, samples of torn supraspinatus tendons were collected from patients undergoing arthroscopic shoulder surgery for rotator cuff tears. Samples of uninjured subscapularis tendons were also harvested from these same patients. A separate and independent set of subscapularis tendon samples were collected from patients undergoing arthroscopic surgery for shoulder stabilization in which no signs of rotator cuff tears were observed (termed control tendons). Gene microarray analysis showed that differential expression of apoptosis-related genes represented 6% (5 genes) of the significantly upregulated genes in degenerative supraspinatus tendons from rats subjected to daily downhill treadmill running: *Hsp27, Hsp70, caspase 3 and cFLIP* (cellular FLICE-inhibitory protein; a protease-dead, procaspase-8-like regulator of death ligand-induced apoptosis). These same genes were upregulated in the human samples of torn supraspinatus tendon, compared to both matched subscapularis and control subscapularis tendons. The increase in each apoptosis-related gene was verified in all torn supraspinatus tendon sample (rodent and human) as increased at the protein levels using immunohistochemical methods. This data confirms the presence of heat shock proteins in both animal and human models of tendinopathy, and their co-expression with other regulators of apoptosis. The authors hypothesize that heat shock proteins play a role in the cascade of degeneration in tendinopathy, perhaps to interfere with the caspase-driven apoptotic pathways in an attempt to limit tendon cell apoptosis (Millar and Murrell 2012; Millar et al. 2008). Their hypothesis is consistent with studies showing that Hsp27 inhibits the cytochrome C and ATP-triggered activity of caspase 9 on the apoptotic pathway (Martin-Ventura et al. 2006), and that Hsp70 interacts with Apaf-1 (apoptotic peptidase activating factor 1) to prevent its interactions with caspases (Saleh et al. 2000), that likewise can limit or prevent apoptosis.

### 11.3.3 Response of Tendon Fibroblasts to Cyclical Repetitive Strain

The influence of cyclical mechanical strain on Hsp72 in cultured human tendon fibroblasts (i.e., tenocytes (Frara et al. 2017)) has been examined in two separate studies (Barkhausen et al. 2003; Jagodzinski et al. 2006). In one, the time dependent increases in Hsp72 protein levels were examined using patellar tendon cells

collected from humans undergoing operative treatment for knee joint instability (Jagodzinski et al. 2006). The cells were plated on flexible silicone dishes for three days before reduction of serum concentration to 1% to align most cells to the $G_0$ phase of the cell cycle (Zeichen 2000). Cells were then stretched using an apparatus that stretched the silicone dishes longitudinally in biaxial directions for 15 min (group 1) or 60 min (group 2). The strain magnitude was 5% at a 1 Hz frequency in continuous cycles. After the stretching period, the fibroblasts were cultured for another 2, 4 or 8 h without further stretching. Hsp72 expression after 15 min of cyclical stretching was only slightly elevated above control levels at all endpoints; however, Hsp72 was significantly increased at 2 and 8 h after 60 min of cyclical stretching (but not at 4 h), a response that also involved nuclear translocation of the protein.

In the second study, tendon fibroblasts were collected and treated similarly, although three different patterns of stretching were used (Barkhausen et al. 2003). In Group 1, the first pattern, cyclical longitudinal stretching was applied twice for 30 min with a non-stress interval of 4 h between stress intervals. In Group 2, the second pattern, cyclical longitudinal stretching involved applying the stress in four blocks with durations of 15 min each, a non-stress interval of 4 h between these daily stress intervals, and with the stress distributed across two days. Group 3, the third pattern, had the same constellation as Group 1 but with stress durations of 15 and 30 min. The experiments were terminated after 0, 6, 12 or 24 h for the detection of proliferation and apoptosis rates, and after 0, 2, 4 and 8 h for the determination of Hsp72 levels using Western blot analysis. They found proliferation rates of the tendon fibroblasts increased similarly in Groups 1 and 3 at 24 hafter the repetitive stress, yet decreased in Group 2. A biphasic kinetic curve was observed for apoptosis in Groups 1 and 3, with the maximum increase after 24 h, but no change from basal levels in Group 2. These were interesting findings since Hsp70 was most increased in Group 2 cells at 24 h (its expression in Groups 1 and 3 were slight only at 2 h after stretching). These authors postulate that the Hsp72 increases inhibited tendon fibroblasts proliferation and apoptosis rate in Group 2 (Barkhausen et al. 2003). Past findings that Hsp72 (the inducible form of Hsp70) modulates activity of JNK (Skutek et al. 2003) may explain the low apoptosis rate in Group 2. Abundant Hsp72 may also suppress ERK (extracellular signal – regulated kinases) and p38 (Gabai et al. 1997; Song et al. 2001). These results combined indicate that repetitive stress can modulate several physiological parameters in human tendon fibroblasts, such as proliferation, apoptosis and Hsp70 expression (Barkhausen et al. 2003; Gabai et al. 1997; Song et al. 2001). A mild mechanical stimulation increased cell proliferation and may be helpful for tissue regeneration. However, only moderate increases in Hsp72 were observed in the latter study. A more pronounced level of mechanical stimulation may be needed to inhibit both proliferation and apoptotic responses in this cell type.

## 11.4 Conclusions

Hsp72 is known to mediate tissue repair after injury and to protect skeletal muscle from atrophy and damage (Gehrig et al. 2012; Miyabara et al. 2006; Miyabara et al. 2012; Senf et al. 2013). Hsp70 is known to play roles in skeletal muscle repair or regeneration and adaptation after high-force eccentric exercise (Escobedo et al. 2004; Koh 2002; McArdle et al. 2004; Paulsen et al. 2007) and increases concomitantly with MMP-2 in skeletal muscle following high intensity training [30], apparently acting together to promote muscle matrix remodeling (Carmeli et al. 2010; Hirunsai et al. 2015). We review here that Hsp70 is also involved in skeletal muscle and tendon repair/regeneration/adaptation processes after a variety of non-exercise related overloading/repetitive strain activities. The investigation of the response of heat shock proteins to overuse injuries needs to be extended to other family members, such as more examination of the roles of Hsp25/27, or examination of Hsp90 as it has been shown to have interesting protective roles in muscles and tendons post exercise or heat exposure (Fujii et al. 2017; Tuttle et al. 2017). Other future directions include the effect of sex, as studies have mostly focused on females. Although there is a propensity for females to show higher risk for work-related overuse-induced musculoskeletal injuries, males are certainly not immune to these disorders (Bae and Min 2016; Cote 2012; Millar et al. 2008). The precise biological significance of the mechanical- versus injury-induced induction of Hsp still needs elucidating, particularly in light of multiple means of "cross talk" between signaling pathways for heat shock proteins and other repair, growth and metabolic factors that are altered in parallel with the stress response proteins (Morton et al. 2009; Noble and Shen 2012). Upregulation of heat shock proteins in a non-pharmacological method may also serve as an interesting intervention for pain or discomfort occurring with repetitive strain injuries, as suggested in a recent study (cupping) (Subadi et al. 2017), or at least to limit muscle dysfunction occurring with both aging and overuse injuries (Morton et al. 2009; Xin et al. 2011). The tendon fibroblast studies also indicate that mechanical stress upregulated Hsp70 expression and reduced apoptosis. Different levels of mechanical stimulation, and the effects on both heat shock, apoptotic and repair proteins need to be examined to fully develop this idea into a potential tissue regeneration method.

**Acknowledgements** Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR056019 to MFB. This study utilized services offered by core facilities of the Center for Substance Abuse Research (Grant Number DA013429). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Competing Interests** None of the authors have any competing interests to declare.

## References

Bae, Y. H., & Min, K. S. (2016). Associations between work-related musculoskeletal disorders, quality of life, and workplace stress in physical therapists. *Industrial Health, 54*, 347–353.

Banfi, G., et al. (2004). Exercise raises serum heat-shock protein 70 (Hsp70) levels. *Clinical Chemistry and Laboratory Medicine: CCLM/FESCC, 42*, 1445–1446.

Barbe, M. F., et al. (1988). Hyperthermia protects against light damage in the rat retina. *Science, 241*, 1817–1820.

Barbe, M. F., et al. (2003). Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *Journal of Orthopaedic Research, 21*, 167–176.

Barbe, M. F., et al. (2013). The interaction of force and repetition on musculoskeletal and neural tissue responses and sensorimotor behavior in a rat model of work-related musculoskeletal disorders. *BMC Musculoskeletal Disorders, 14*, 303.

Barkhausen, T., et al. (2003). Modulation of cell functions of human tendon fibroblasts by different repetitive cyclic mechanical stress patterns. *Experimental and Toxicologic Pathology, 55*, 153–158.

Barr, A. E., & Barbe, M. F. (2002). Pathophysiological tissue changes associated with repetitive movement: A review of the evidence. *Physical Therapy, 82*, 173–187.

Barr, A. E., & Barbe, M. F. (2004). Inflammation reduces physiological tissue tolerance in the development of work-related musculoskeletal disorders. *Journal of Electromyography and Kinesiology, 14*, 77–85.

Bureau of Labor Statistics. (2012). Nonfatal occupational injuries and illnesses requiring days away from work, 2011. http://www.bls.gov/news.release/osh2.nr0.htm *News Release USDL-12-2204,* November 8, 2012.

Bureau of Labor Statistics (2014). Prevention of work-related musculoskeletal disorders. http://www.bls.gov/news.release/pdf/osh2.pdf.

Cahill, C. M., et al. (1996). Transcriptional repression of the prointerleukin 1beta gene by heat shock factor 1. *The Journal of Biological Chemistry, 271*, 24874–24879.

Carmeli, E., et al. (2010). Increased iNOS, MMP-2, and HSP-72 in skeletal muscle following high-intensity exercise training. *Journal of Basic and Clinical Physiology and Pharmacology, 21*, 127–146.

Cote, J. N. (2012). A critical review on physical factors and functional characteristics that may explain a sex/gender difference in work-related neck/shoulder disorders. *Ergonomics, 55*, 173–182.

Craig, E. A., et al. (1993). Heat shock proteins: Molecular chaperones of protein biogenesis. *Microbiological Reviews, 57*, 402–414.

De, A. K., et al. (2000). Exaggerated human monocyte IL-10 concomitant to minimal TNF-alpha induction by heat-shock protein 27 (Hsp27) suggests Hsp27 is primarily an antiinflammatory stimulus. *Journal of Immunology, 165*, 3951–3958.

Escobedo, J., et al. (2004). hsp25 protects skeletal muscle cells against oxidative stress. *Free Radical Biology & Medicine, 37*, 1455–1462.

Fine, L. J., et al. (1986). Detection of cumulative trauma disorders of upper extremities in the workplace. *Journal of Occupational Medicine, 28*(8), 674.

Fisher, P. W., et al. (2015). Increased CCN2, substance P and tissue fibrosis are associated with sensorimotor declines in a rat model of repetitive overuse injury. *Journal of Cell Communication and Signaling, 9*(1), 37–54.

Frara, N., et al. (2016). Growth and repair factors, osteoactivin, matrix metalloproteinase and heat shock protein 72, increase with resolution of inflammation in musculotendinous tissues in a rat model of repetitive grasping. *BMC Musculoskeletal Disorders, 17*, 34.

Frara, N., et al. (2017). Substance P increases CCN2 dependent on TGF-beta yet Collagen Type I via TGF-beta1 dependent and independent pathways in tenocytes. *Connective Tissue Research, 1*, 1–15.

Fujii, N., et al. (2017). Heat shock protein 90 contributes to cutaneous vasodilation through activating nitric oxide synthase in young male adults exercising in the heat. *Journal of Applied Physiology*, (*1985*), jap 00446 2017.

Fung, D. T., et al. (2009). Subrupture tendon fatigue damage. *Journal of Orthopaedic Research, 27*, 264–273.

Fung, D. T., et al. (2010). Early response to tendon fatigue damage accumulation in a novel in vivo model. *Journal of Biomechanics, 43*, 274–279.

Furochi, H., et al. (2007a). Osteoactivin fragments produced by ectodomain shedding induce MMP-3 expression via ERK pathway in mouse NIH-3T3 fibroblasts. *FEBS Letters, 581*, 5743–5750.

Furochi, H., et al. (2007b). Overexpression of osteoactivin protects skeletal muscle from severe degeneration caused by long-term denervation in mice. *The Journal of Medical Investigation: JMI, 54*, 248–254.

Gabai, V. L., et al. (1997). Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *The Journal of Biological Chemistry, 272*, 18033–18037.

Gallagher, S., & Heberger, J. R. (2013). Examining the interaction of force and repetition on musculoskeletal disorder risk: A systematic literature review. *Human Factors, 55*, 108–124.

Gehrig, S. M., et al. (2012). Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. *Nature, 484*, 394–398.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature, 381*, 571–579.

Hirunsai, M., et al. (2015). Heat stress promotes extracellular matrix remodelling via TGF-beta1 and MMP-2/TIMP-2 modulation in tenotomised soleus and plantaris muscles. *International Journal of Hyperthermia, 31*(4), 336–348.

Huey, K. A., et al. (2007). Modulation of HSP25 and TNF-alpha during the early stages of functional overload of a rat slow and fast muscle. *Journal of Applied Physiology*. (1985)*, 102*, 2307–2314.

Jagodzinski, M., et al. (2006). Influence of cyclic mechanical strain and heat of human tendon fibroblasts on HSP-72. *European Journal of Applied Physiology, 96*, 249–256.

Kannus, P., et al. (1997). Effects of training, immobilization and remobilization on tendons. *Scandinavian Journal of Medicine & Science in Sports, 7*, 67–71.

Kherif, S., et al. (1998). Matrix metalloproteinases MMP-2 and MMP-9 in denervated muscle and injured nerve. *Neuropathology and Applied Neurobiology, 24*, 309–319.

Koh, T. J. (2002). Do small heat shock proteins protect skeletal muscle from injury? *Exercise and Sport Sciences Reviews, 30*, 117–121.

Koh, T. J., & Escobedo, J. (2004). Cytoskeletal disruption and small heat shock protein translocation immediately after lengthening contractions. *American Journal of Physiology. Cell Physiology, 286*, C713–C722.

Martin-Ventura, J. L., et al. (2006). Biological significance of decreased HSP27 in human atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology, 26*, 1337–1343.

McArdle, A., et al. (2004). Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. *The FASEB Journal, 18*, 355–357.

Millar, N. L., & Murrell, G. A. (2012). Heat shock proteins in tendinopathy: Novel molecular regulators. *Mediators of Inflammation, 436203*.

Millar, N. L., et al. (2008). Heat shock protein and apoptosis in supraspinatus tendinopathy. *Clinical Orthopaedics and Related Research, 466*, 1569–1576.

Miyabara, E. H., et al. (2006). Overexpression of inducible 70-kDa heat shock protein in mouse attenuates skeletal muscle damage induced by cryolesioning. *American Journal of Physiology. Cell Physiology, 290*, C1128–C1138.

Miyabara, E. H., et al. (2012). Overexpression of inducible 70-kDa heat shock protein in mouse improves structural and functional recovery of skeletal muscles from atrophy. *Pflügers Archiv, 463*, 733–741.

Morton, J. P., et al. (2009). The exercise-induced stress response of skeletal muscle, with specific emphasis on humans. *Sports Medicine, 39*, 643–662.

Mounier, N., & Arrigo, A. P. (2002). Actin cytoskeleton and small heat shock proteins: How do they interact? *Cell Stress & Chaperones, 7*, 167–176.

Neviaser, A., et al. (2012). Basic mechanisms of tendon fatigue damage. *Journal of Shoulder and Elbow Surgery, 21*, 158–163.

Noble, E. G., & Shen, G. X. (2012). Impact of exercise and metabolic disorders on heat shock proteins and vascular inflammation. *Autoimmune Diseases, 836519*.

Noble, E. G., et al. (1999). Differential expression of stress proteins in rat myocardium after free wheel or treadmill run training. *Journal of Applied Physiology* .1985*, 86*, 1696–1701.

Ogawa, T., et al. (2005). Osteoactivin upregulates expression of MMP-3 and MMP-9 in fibroblasts infiltrated into denervated skeletal muscle in mice. *American Journal of Physiology. Cell Physiology, 289*, C697–C707.

Paulsen, G., et al. (2007). Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 293*, R844–R853.

Piligian, G., et al. (2000). Evaluation and management of chronic work-related musculoskeletal disorders of the distal upper extremity. *American Journal of Industrial Medicine, 37*, 75–93.

Rempel, D. M., & Diao, E. (2004). Entrapment neuropathies: Pathophysiology and pathogenesis. *Journal of Electromyography and Kinesiology, 14*, 71–75.

Rempel, D. M., et al. (1992). Work-related cumulative trauma disorders of the upper extremity. *JAMA, 267*, 838–842.

Saleh, A., et al. (2000). Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nature Cell Biology, 2*, 476–483.

Senf, S. M., et al. (2008). Hsp70 overexpression inhibits NF-kappaB and Foxo3a transcriptional activities and prevents skeletal muscle atrophy. *The FASEB Journal, 22*, 3836–3845.

Senf, S. M., et al. (2013). Loss of the inducible hsp70 delays the inflammatory response to skeletal muscle injury and severely impairs muscle regeneration. *PLoS One, 8*, e62687.

Silverstein, B. A., et al. (1986). Hand wrist cumulative trauma disorders in industry. *British Journal of Industrial Medicine, 43*, 779–784.

Sjogaard, G., et al. (2010). Muscle oxygenation and glycolysis in females with trapezius myalgia during stress and repetitive work using microdialysis and NIRS. *European Journal of Applied Physiology, 108*, 657–669.

Sjogaard, G., et al. (2013). Exercise training and work task induced metabolic and stress-related mRNA and protein responses in myalgic muscles. *BioMed Research International, 2013*, 984523.

Skutek, M., et al. (2003). Cyclic mechanical stretching of human patellar tendon fibroblasts: Activation of JNK and modulation of apoptosis. *Knee Surgery, Sports Traumatology, Arthroscopy, 11*, 122–129.

Song, J., et al. (2001). Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nature Cell Biology, 3*, 276–282.

Subadi, I., et al. (2017). Pain relief with wet cupping therapy in rats is mediated by heat shock protein 70 and ss-endorphin. *The Iran Journal of Medical Sciences, 42*, 384–391.

Tuttle, J. A., et al. (2017). The Hsp72 and Hsp90alpha mRNA responses to hot downhill running are reduced following a prior bout of hot downhill running, and occur concurrently within leukocytes and the vastus Lateralis. *Frontiers in Physiology, 8*, 473.

Tytell, M., et al. (1989). Photoreceptor protection from light damage by hyperthermia. *Progress in Clinical and Biological Research, 314*, 523–538.

van Rijn, R.M., et al. (2009) Associations between work-related factors and specific disorders at the elbow: A systematic literature review. Rheumatology (Oxford) 48**,** 528–536.

Welsh, M. J., & Gaestel, M. (1998). Small heat-shock protein family: Function in health and disease. *Annals of the New York Academy of Sciences, 851*, 28–35.

Wieten, L., et al. (2007). Cell stress induced HSP are targets of regulatory T cells: A role for HSP inducing compounds as anti-inflammatory immuno-modulators? *FEBS Letters, 581*, 3716–3722.

Woolf, A. D., & Pfleger, B. (2003). Burden of major musculoskeletal conditions. *Bulletin of the World Health Organization, 81*, 646–656.

World Health Organization (2007) Workers' health: Global plan of action Geneva: *World Health Organization,* http://www.who.int/occupational_health/publications/global_plan/en/

Xin, D. L., et al. (2011). Aging enhances serum cytokine response but not task-induced grip strength declines in a rat model of work-related musculoskeletal disorders. *BMC Musculoskeletal Disorders, 12*, 63.

Zeichen, J. (2000). The proliferative response of isolated human tendon fibroblasts to cyclic biaxial mechanical strain. *The American Journal of Sports Medicine, 28*, 888–892.

# Chapter 12
# Heat Shock Protein 90: Truly Moonlighting!


**Eusebio S. Pires**

**Abstract** Hsp90 is an essential and abundantly expressed molecular chaperone in any living cell. The multiplicity of Hsp90 cellular functions is driven by its interaction with a broad range of partner proteins and thereby establishing itself as a moonlighting molecule. There are newer insights emerging to ascertain the cellular and physiological roles of Hsp90, such as (and not limited to) chromatin remodeling, gene regulation and developmental pathways. Hsp90 has been recognized as an important therapeutic target and has been linked to an increasing number of diseases, including cancer. Development of Hsp90 therapeutic reagents would be valuable research tools towards the maintenance of the proteome in health and disease. This review revisits the expression, structure-function, and clinical significance of the Hsp90 and its forms and reinforces its impact as a disease target.

**Keywords** Hsp90 · Hsp90 diversity · HSPC · Moonlighting roles · Therapeutic candidate

## Abbreviations

| | |
|---|---|
| AOA | Anti-ovarian antibodies |
| GVBD | Eggs, germinal vesicle breakdown oocyte |
| Hsp | Heat shock proteins |
| IHC | Immunohistochemistry |
| IVF-ET | In vitro fertilization- embryo transfer |
| LC-MS | Liquid chromatography/mass spectrometry |
| MALDI-TOF/TOF | Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight |
| POF | Premature ovarian failure |
| POI | Primary ovarian insufficiency |

E. S. Pires (✉)
Department of Obstetrics & Gynecology at the School of Medicine, University of Virginia, Virginia, USA
e-mail: ep7d@virginia.edu



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_12

## 12.1 Introduction

Heat shock proteins (Hsp) or chaperonins, as they were previously called, are group of evolutionary conserved proteins that show high sequence homology between different species, from bacteria to humans (Morimoto 1993). These molecular chaperones are proteins that interact with and help other client proteins to acquire a functionally active form, and they then dissociate from the client once the final active structure is formed. They are classified based on molecular size, sequence similarities, location within the cell and function. Despite the significant degree of evolutionary conservation, HSP's are highly immunogenic and it has been postulated that they could activate antigen presenting cells, serving as a danger signal to the immune system (Gallucci and Matzinger 2001).

Of the several Hsp, Hsp90 (with an approximate molecular weight of 90 kilo Daltons) is an abundant, constitutively expressed chaperone constituting around 1–2% of total cellular protein under non-stress conditions (Falsone et al. 2005). In eukaryotes, Hsp90 is found in the cytosol, the nucleus and in organelles such as the endoplasmic reticulum. The nuclear localized Hsp90 represents a small fraction of cytosolic Hsp90 under physiological conditions. Studies have shown that Hsp90 is not only present inside the cell, but also on the cell surface of various cell types and secreted into the extracellular space suggesting distinct extracellular chaperoning activity (Li et al. 2012). Hsp90 has a crucial role in cellular signaling, as it participates in the folding of steroid hormone receptors, protein kinases and other signaling components. However, the well determined function of Hsp90 proteins is to suppress the aggregation of unfolded proteins. The full functional activity of Hsp90 is gained in concert with other co-chaperones, thereby playing an important role in the folding of newly synthesized proteins and stabilization and refolding of denatured proteins after stress. Apart from its co-chaperones, Hsp90 binds to an array of client proteins, where the co-chaperone requirement varies and depends on the actual client (Sreedhar et al. 2004). A current updated list of Hsp90 and its partner proteins is maintained by Dr. Didier Picard (http://www.picard.ch/downloads/downloads.htm). Hsp90 clients were shown to be functionally and structurally diverse thereby making it a central modulator of important processes that range from stress regulation and protein folding to DNA repair, development, the immune response, neuronal signaling and many other processes (Schopf et al. 2017). Very recently, studies by the late Susan Lindquist' team demonstrated that Hsp90 can modify the consequences of genetic variation in human, something long hypothesized but never proven. The study provided insights into the mechanisms by which Hsp90 buffering can alter the course of human diseases, broadly protective in nature and mitigating the deleterious effects of missense mutations. The study suggested that by buffering these kinds of human genetic variation, Hsp90 enables gene-environment interactions which are capable of shaping disease trajectories (Karras et al. 2017) thus indicating clinical significance.

## 12.2 HSP90 Nomenclature, Isoforms and Structure

This HSP90 family encodes five members as seen in the Table 12.1 namely *HSPC1, HSPC2, HSPC3, HSPC4 and HSPC5*. Their chromosomal location all varies and are known by various nick names in literature (Kampinga et al. 2009, Website: HUGO gene nomenclature committee). They can be found in different cell compartment such as cytosol, endoplasmic reticulum and the mitochondria (Csermely et al. 1998). Most studied members of this group are the cytoplasmic isoforms and an ER specific HSPC4. This protein from even the most distantly related eukaryotes has 50% amino acid identity and all have more than 40% identity with the Escherichia coli protein (Bardwell and Craig 1987) and has sequence similarities with other mammalian species as shown in Fig. (12.1a, b). There are two major cytoplasmic forms of Hsp90: Hsp90α [inducible form] and Hsp90β [constitutive form] which possibly arose by gene duplication roughly 500 million years ago (Gupta 1995). In contrast to bacteria, which typically contain only a single Hsp90 gene, budding yeast and humans contain two HSP90 genes that encode cytosolic proteins: Hsc82 and Hsp82 in *S. cerevisiae*, and Hsp90α and Hsp90β in *H. sapiens* (Schopf et al. 2017). A minimum of two additional gene duplication events, which took place at a later time, are required to explain the presence of two different forms of HSP90 that are found in these fungi and vertebrate species. In humans, sequence similarities between the alpha and the beta form are around 93.4% using the EBI tool: EMBOSS pairwise alignment algorithm. Hsp90α is a 732-amino acid long protein while Hsp90β has 724 amino acids and 8 amino acids (7–11 and 237–239) present in Hsp90α are missing in Hsp90β as seen in Fig. (12.1c, d). Although both are ubiquitously expressed, Hsp90α is heat-inducible in specific tissues, while Hsp90β typically has a more constitutive pattern of expression (Sreedhar et al. 2004).

**Table 12.1 Nomenclature of the HSP90 genes.** The table illustrates the HSP90 genes contained within the family. Five forms of the protein are encoded by genes *HSPC1-5*. Among these HSP90α and HSP90β are the two widely studied and described isoforms

| Gene name | Genes within HSP90 family | Aliases | Locus | Scientific name | Gene ID |
|---|---|---|---|---|---|
| *HSPC1* | HSP90AA1 | Hsp89, Hsp90, FLJ31884, HSP90N, HspC1 | 14q32.31 | Heat shock protein 90 alpha family class A member 1 | 3320 |
| *HSPC2* | HSP90AA3P | HSP90Alpha, HSPCA | 1q23.1 | Heat shock protein 90 alpha family class A member 3, pseudogene | 3324 |
| *HSPC3* | HSP90AB1 | HspC3 | 6p21.1 | Heat shock protein 90 alpha family class B member 1 | 3326 |
| *HSPC4* | HSP90B1 | GP96, GRP94, | 12q23.3 | Heat shock protein 90 beta family member 1 | 7184 |
| *HSPC5* | TRAP1 | HSP75, HSP90L | 16p13.3 | TNF receptor associated protein 1 | 10,131 |

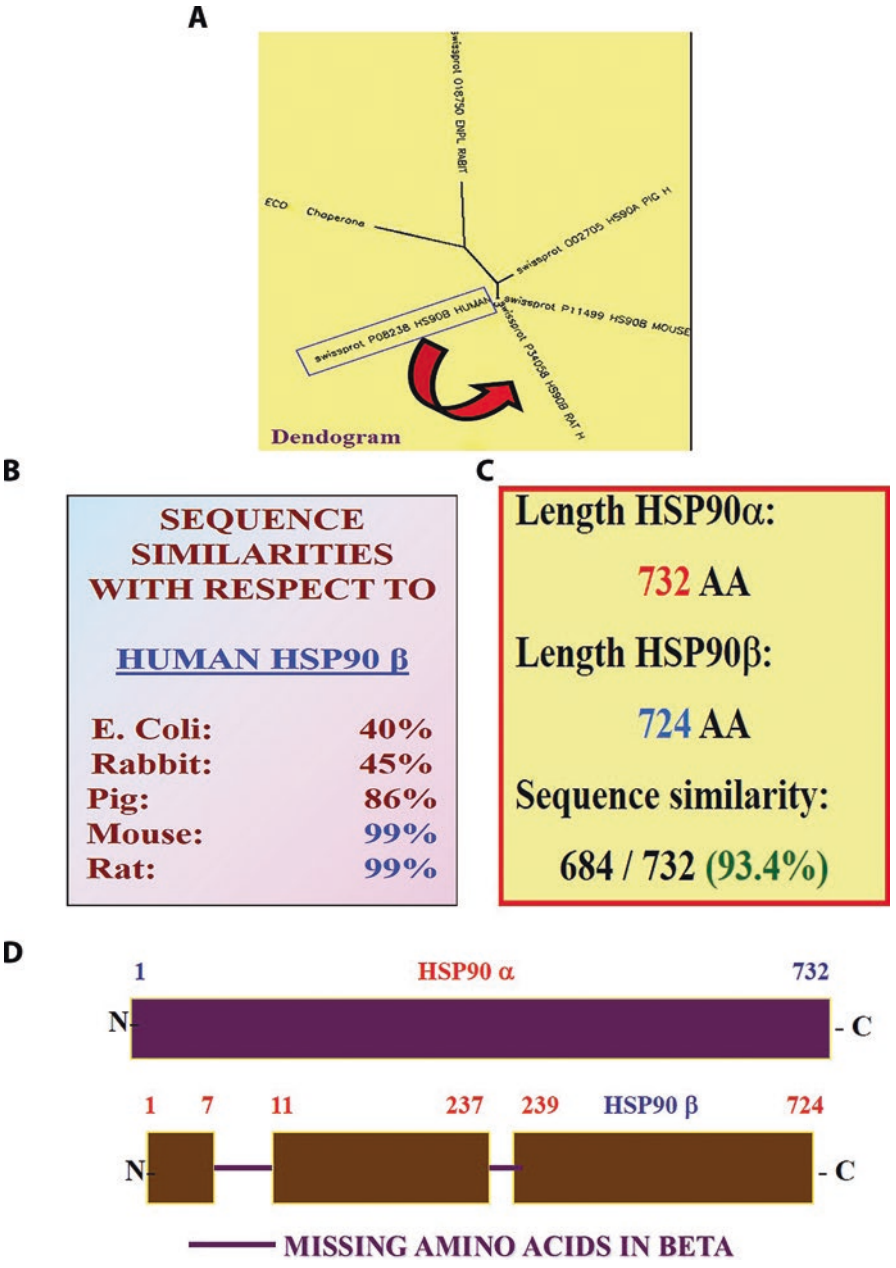


**Fig. 12.1 Distance relation of Hsp90β across species and Hsp90α versus Hsp90β structure.** (**a**) Hsp90 protein shows high sequence homology between different species, from bacteria to humans as seen in the dendogram which shows the distance relation between the species with respect to human Hsp90β. (**b**) Percentage sequence similarity with respect to human Hsp90β and other species. The rodents show the highest sequence similarity with respect to human Hsp90β protein. (**c**) Hsp90α is a 732-amino acid long protein while Hsp90β has 724 amino acids. (**d**) It can be clearly seen in the stick diagram that 8 amino acids (7–11 and 237–239) present in Hsp90α are missing in Hsp90β

These regulatory differences translate into varied responses to extracellular signals and stress events, with Hsp90α adopting a cytoprotective role by mediating a rapid response, whereas Hsp90β is associated with long-term cellular adaptation. Owing to its higher levels of expression, Hsp90β is the predominant Hsp90 isoform involved in normal cellular function, including the maintenance of cytoskeletal architecture, cellular transformation, and signal transduction. An important difference is that the α-form readily dimerizes, whereas the β-form does so with much less efficiency. Hsp90 is mainly a constitutive dimer [αα or ββ], however, monomers [α or β], heterodimers [αβ] and higher oligomers of both forms also exist (Sreedhar et al. 2004). Hsp90 contains three conserved domains: a N-terminal ATP-binding domain, a middle domain and a carboxy-terminal domain. All examined forms of Hsp90 bind and hydrolyze ATP (Zuehlke and Johnson 2010). The N-terminal of Hsp90 and the highly charged central region are responsible for the binding of different target proteins. A binding site for ATP/ ADP also can be found in the N-terminal region, while the C-terminal domain contains a dimerization site (Csermely et al. 1998). Some of the biological differences between the Hsp90α and Hsp90β isoforms are likely to be determined by client proteins and by cochaperones associated with Hsp90 chaperone machinery (Rohl et al. 2013; Millson et al. 2007). It is intriguing therefore that the cochaperone, GCUNC45, previously implicated in the Hsp90 chaperoning of the progesterone receptor, was found to bind preferentially to Hsp90β over Hsp90α, resulting in an efficient blockade of progesterone receptor chaperoning *in vitro* (Chadli et al. 2006, 2008).

## 12.3 Hsp90 Expression Profile

The isoform specificity is not restricted only to the biochemical level but extends to the functional role of Hsp90 in cell differentiation and development. On one hand Hsp90α has been shown to play a regulatory role in muscle cell differentiation of zebra fish while on the other hand it is shown to inhibit cellular differentiation of embryonal carcinoma cells to trophectoderm (Lele et al. 1999; Sreedhar et al. 2004). Hsp90β has been shown to play a major role in trophoblast differentiation, and Hsp90β-deficient homozygous mice with normal expression of Hsp90α failed to differentiate to form placental labyrinths (Voss et al. 2000). Hsp90β overexpression is observed throughout the germ cell lineage from very early stages of development to adult oocytes and spermatocytes (Hilscher et al. 1974) thereby indicating expression in different stages of development, and suggestive of the fact that Hsp90β is required for early embryonic development. Thus, there are several differences between Hsp90 isoforms in cell differentiation and embryonic development in various organisms. Hsp90α expression is lower compared to Hsp90β in most cells. Hsp90α is highly inducible in contrast to Hsp90β whose expression is thought to be constitutive (Hilscher et al. 1974; Gruppi et al. 1991).

Earlier studies from my group (Pires and Khole 2009a; Pires et al. 2011a) using isoform specific antibodies showed that Hsp90β is the predominant form in the

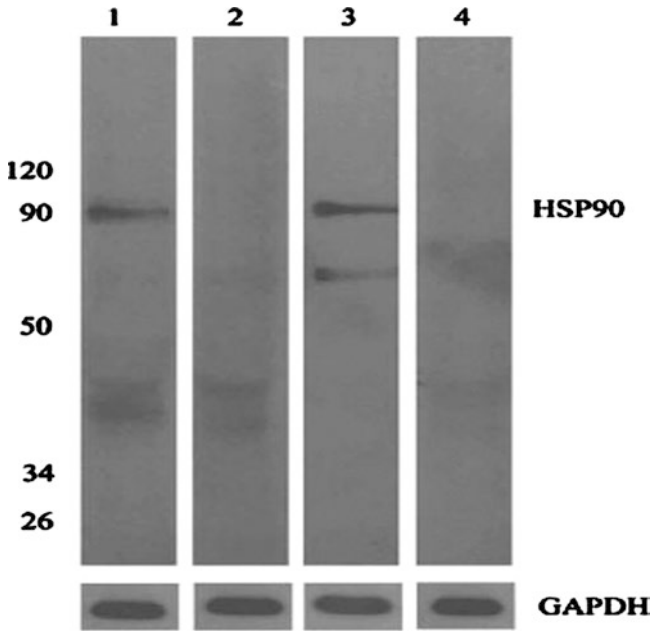


**Fig. 12.2 Hsp90α and Hsp90β gamete specific expression.** Hsp90β is the predominant isoform present in the ovary. Western blot using commercially available antibodies demonstrates the predominance of the beta isoform of Hsp90 in ovarian protein extracts (*lane 1*). No immunoreactivity to the 90 kDa locus was seen when the strip was probed with Hsp90α commercially available polyclonal antibody (*lane 2*), thereby suggesting Hsp90β to be the predominant isoform in ovary. Mouse testes extract was used as a positive control for Hsp90α (*lane 3*). A 'no primary' control showed no immunoreactivity to any of the ovarian proteins (*lane 4*). GAPDH served as an equal loading control

ovary, while the testes express Hsp90α form. This data was supported by a Western blot as shown in Fig. 12.2, which clearly demonstrates the predominance of the beta isoform of Hsp90 in ovarian protein extracts (lane 1). No immunoreactivity to the 90 kDa locus was seen when the blot was probed with commercially available Hsp90α polyclonal antibody (lane 2), thereby suggesting Hsp90β to be the predominant isoform in ovary. Mouse testes extract was used as a positive control for Hsp90α (lane 3). Data from mass spectrometry analysis of the immunoreactive dominant EP90 protein demonstrated dominance of Hsp90β peptides where 15 of the 18 LC/MS generated peptides and 9 of the 12 MS/MS generated peptides, other 2 peptides in each MS run belong to Hsp90α (Pires and Khole 2009a). Immunohistochemical (IHC) analysis using immunoreactive patient serum containing Hsp90 AOAs against a panel of multiple rodent tissue sections as seen in Fig. (12.3) shows strong immunoreactivity not only to oocyte ooplasm (panel O) but also to spermatogonial and spermatocytes of testes (panel N) and cilia of principal cells in epididymis (panel M). Since the mass spectrometry data revealed major beta peptides and minor alpha peptides, antibody cross reactivity to the alpha form in the

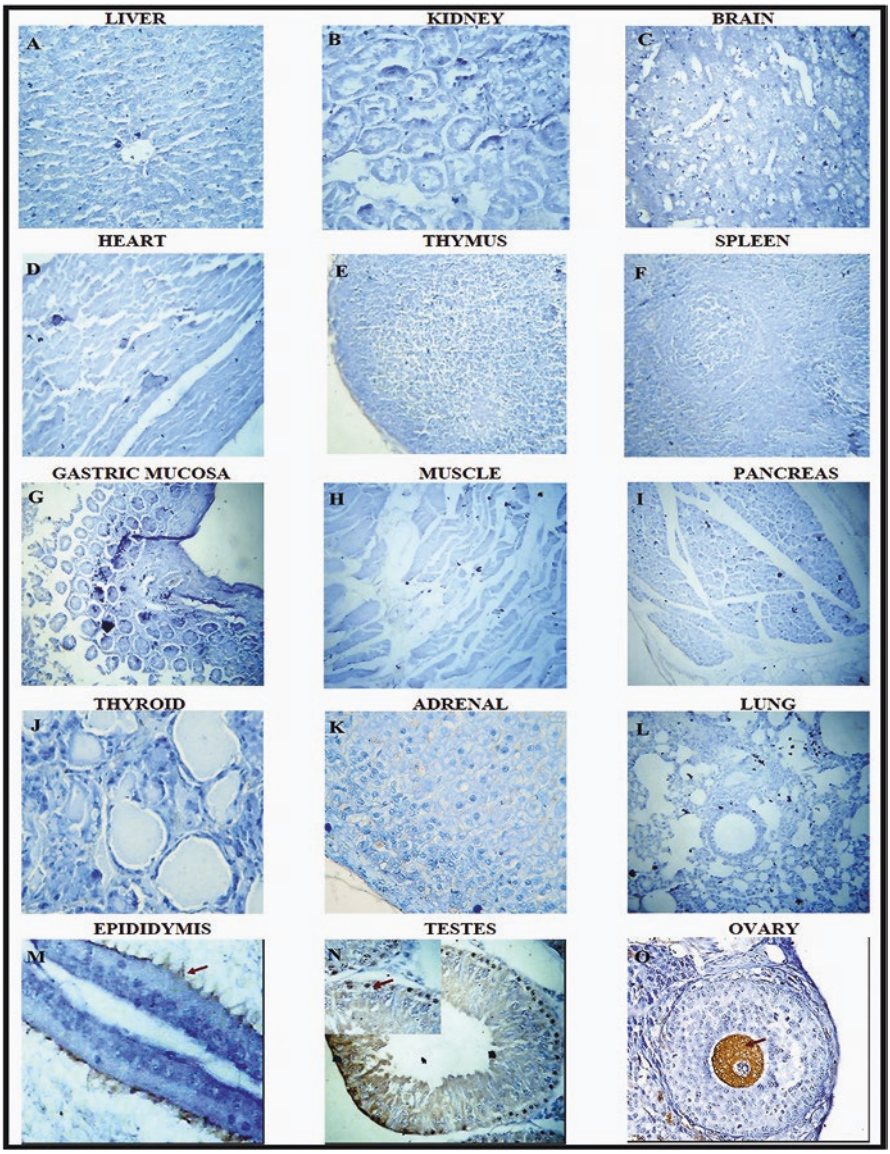


**Fig. 12.3 Multiple tissue immunohistochemistry with Hsp90 specific immunoreactive patient serum antibodies.** IHC using a Hsp90β AOA positive patient serum against a panel of multiple rodent tissue sections shows immunoreactivity oocyte ooplasm (*panel O*), to spermatocytes of testes (*panel N*) and cilia of principal cells in epididymis (*panel M*) indicating that the protein could be a reproductive specific protein

male reproductive system is of a high probability keeping in mind the polyclonal nature of human serum antibodies.

## 12.4 Clinical Significance of Hsp90

### *12.4.1 Hsp90 Expression in Diseases*

Tumor cells seem to be in a 'stressed' state, which puts additional pressure on controlling proteostasis. Hsp90 plays an important part in the survival of cancer cells, also because of their extensive dependence on Hsp90-assisted signaling pathways (Schopf et al. 2017). In breast cancer, Hsp90 up-regulation has been found as being linked to the expression of the estrogen receptor and Her-2/Erb-B2 associated with bad prognosis and decreased survival (Pick et al. 2007). A study with 52 epithelial ovarian carcinomas revealed an association of the Hsp90 expression with higher stages, but not with prognosis indicating that it might be a reliable indicator of aggressiveness (Elpek et al. 2003).

Studies on the predictive role of Hsp90 in prostate cancer are shown to be inconsistent. On the one hand, abnormal up-regulated levels of Hsp90 have been observed in human prostatic carcinoma cells with a stage-dependent and malignancy-dependent expression (Cardillo and Ippoliti 2006; Cornford et al. 2000). On the other hand, no significant association between Hsp90 expression and conventional diagnostic factors could be detected in 193 patients with clinically organ-confined prostate cancer who underwent radical prostatectomy without any neoadjuvant therapies (Miyake et al. 2010).

In a study on Hsp90α-deficient male mice (Grad et al. 2010), testicular atrophy and infertility was observed which was mediated likely due to apoptosis of spermatocytes, indicating that the Hsp90α isoform is required for spermatogenesis. A conditional deletion of Hsp90α showed that the chaperone is essential for germ cell development in mature adult testes (Kajiwara et al. 2012). Hsp90 was shown to be localized in the neck, midpiece, and tail regions of human sperm, and its expression increased during capacitation thus suggesting roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-stimulated sperm function (Li et al. 2014). Hsp90 (pro-apoptotic in nature) was shown to play an important role in apoptosis in mitochondrially-mediated aging and male infertility (Purandhar et al. 2014). This study clearly indicated a lucid expression and functional role of the Hsp90 during spermatogenesis and/or the process of aging.

### *12.4.2 Hsp90 in Ovarian Autoimmunity*

With a continuous interest in ovarian biology (Pires et al. 2013), female reproductive tract diseases (Pires et al. 2015) and the role Hsp90 plays in reproductive immunology (Pires 2010; Pires 2017), our laboratory focused on identifying targets that

played a role in female reproduction and infertility. Efforts were spent on establishing a diagnostic test to detect serum anti-ovarian antibodies (AOA) which could have a pathological role in this disease thereby leading to premature ovarian failure (POF) or insufficiencies (POI) and we were keen in identifying the proteins under target (Pires et al. 2006). The specificity of existing immunoassays detecting these AOAs were questioned. We reported earlier on the presence of naturally occurring anti-albumin antibodies as the likely factor for non-specificity (Pires et al. 2006). Having developed a novel blocking recipe, we show substantial elimination of this non-specificity. Subsequently using patient sera we reported multiple targets at the protein and histological levels. Our study demonstrated that 15 of 50 (30%) patients with POF/POI and 13 of 50 (26%) in vitro fertilization- embryo transfer (IVF-ET) patients showed the presence of these AOAs (Pires et al. 2011b). Western blotting showed a large number of patients making AOAs to a 90-kDa protein, followed by 97-kDa and 120-kDa proteins (Pires et al. 2007; Pires and Khole 2009a, b). The immunodominant 90-kDa protein was found to be conserved across species, was serine–threonine phosphorylated, and was expressed from the primordial stage to the Graafian-stage ooplasm of the oocytes during follicular development (Pires and Khole 2009a).

Using high-throughput proteomic technologies like liquid chromatography/mass spectrometry (LC-MS), matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF), and tandem mass spectrometry analysis revealed the identity of this protein to be Hsp90β (Pires and Khole 2009a). Using an immunoreactive Hsp90β patient sera to immunostain isolated mouse germinal vesicle breakdown oocyte (GVBD eggs) and growing blastocyst, expression of this protein was seen in the cytosol of the oocytes (ooplasm) as well as cells of the inner cell mass/trophectoderm as seen in Fig. (12.4a, b) respectively. Commercially available recombinant protein immunoreacted with the sera from patients with AOAs against the 90-kd antigen. In parallel, using monoclonal antibody to human Hsp90, we found that it reacts with the eluted protein from a crude ovarian extract (Pires and Khole 2009a). With an aim to identify potential immunoreactive Hsp90 epitopes using patient sera containing AOA to Hsp90, experimentally and statistically peptide EP6 (amino acids 380–389) seems to be the major antigenic epitope followed by EP1 (amino acids 1–12) and EP8 (amino acids 488–498). Predicted 3D structures of these peptides demonstrated that they exist in the loop conformation which is the most mobile part of the protein. Also, analysis of the sequences of Hsp90 beta across several species reveals that EP6 peptide forms a part of a well conserved motif. The polyclonal antibody generated to the immunodominant epitope-EP6 confirmed similar biochemical and cellular immunoreactivity as seen with the patients' sera having anti-Hsp90 autoantibodies (Pires et al. 2011a). The study proposed options to generate new tools for the detection of disease-inducing epitopes and a possible therapeutic intervention. Since our initial evidence suggested the involvement of Hsp90β in human ovarian autoimmunity, identifying the protein was a significant causative factor in early ovarian failure. These observations were supported by results from a mouse model in which fertilization and embryo development were highly disrupted in animals immunized with *in vitro* generated Hsp90

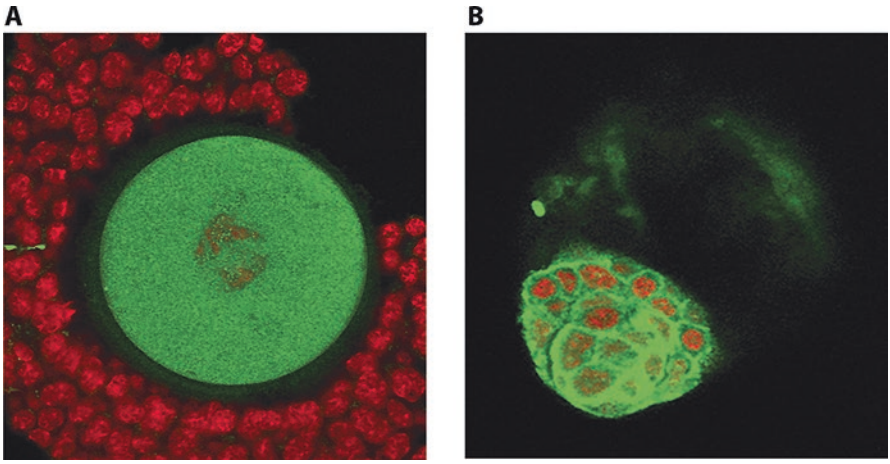


**Fig. 12.4 Hsp90β protein expression in mouse oocytes and developing embryos.** (**a**) Indirect immunofluorescence using an immunoreactive patient sera to Hsp90β protein shows strong ooplasm reactivity (green stain for Hsp90β, red is counterstain for DNA) to the cytoplasm of the germinal vesicle breakdown oocyte. (**b**) As the embryo develops and transforms to the blastocyst stage, it was seen that, apart from a strong signal (green stain for Hsp90β) in the inner cell mass, slight immunostaining also was seen in the cells of the trophectoderm. Neither the control sera nor the secondary alone control showed immunoreactivity to any of the stages in embryogenesis or to the cumulus cells (data not shown here)

antibodies. The study showed that there was a significant drop in the fertility index due to an increase in pre- and post-implantation loss, associated with an increased incidence of degenerated eggs and embryos. The ovaries showed an increase in the number of empty and degenerated follicles and extensive granulosa cell deaths, which was reflected by the decrease in the levels of Nobox and Gja1 gene expression. (Choudhary and Khole 2013).

In view of this, we proposed that as a result of a prolonged or repeated asymptomatic chronic infection early in the life of these infertile women they could have anti-Hsp90 antibodies in circulation. In the course of their reproductive life, these antibodies could then target the ovarian antigens (exposed to the immune system due to reasons such as accidents or trauma, also immune system has memory) leading to early ovarian failure (Pires 2017). This may be relevant to human reproduction, since many couples with fertility problems have had a previously undetected genital tract infection (Witkin et al. 1994a). In general, HSP are among the first proteins produced during embryogenesis (Bensaude and Morange 1983). Constitutive form of Hsp90 is known to be expressed at high levels during preimplantation mouse embryo development (Loones et al. 1997). Therefore presence of anti Hsp90 antibodies in women aiming for pregnancy is likely to have detrimental consequences. Women who have antibodies to Hsp90 in their circulation, the possible interactions between Hsp90 and the cytoskeletal proteins (such as actin and tubulin) may be disturbed and destroyed. Thus, there could likely be a collapse of the ovarian cytoarchitecture as the main role of Hsp90 as a chaperone is to maintain this cytoskeletal framework (Pires 2017).

### *12.4.3 Hsp90 and Immunotherapy*

Immunotherapies involving patients own T-cells have shown immense potential as new treatment strategies. Although inhibition of Hsp90 has received attention for therapeutic purposes in solid tumors and hematologic malignancies, Hsp90 inhibition has shown limited responses as single agents in cancer patients (Pacey et al. 2012; Solit et al. 2008). Although success rates vary, there is a need for improvement with the combinatorial approach. Hwu' group at MD Anderson recently discussed enhancement of cancer immunotherapy utilizing Hsp90 inhibition through an upregulation of interferon response genes (Mbofung et al. 2017). The highlights of this study include: [a] Hsp90 inhibition shows enhanced T-cell killing of tumors, [b] This mechanism is mediated via IFN-induced protein with tetratricopeptide repeats [IFIT genes], [c] Thereby potentiating immune checkpoint blockade therapy, [d] Combining with anti-CTLA4 enhances CD8 T-cell function. All in all, evidence that Hsp90 inhibition can potentiate T-cell-mediated anti-tumor immune responses and supports exploration of the combination of immunotherapy and Hsp90 inhibitors in the clinic was established.

An interesting paper was presented at the Society for immunotherapy of cancer (SITC; Fecek et al. 2015) where the group reported treatment of melanoma cell lines *in vitro* or *in vivo* with Hsp90 inhibitor, lead to the rapid proteasome-dependent degradation of Hsp90 client proteins, with the resulting peptides used to load MHC class I complexes on the tumor cell surface, thereby conditionally-enhancing specific CD8+ T cell recognition. These results suggested that a polyepitope vaccine based on BRAFi-resistance associated Hsp90 client proteins could define a novel immunotherapeutic strategy for the co-treatment of patients with advanced-stage melanomas. Thus, there is a growing wealth of evidence indicating the focal role of Hsp90 in tumorigenesis.

## 12.5 Conclusions

Hsp90 is one of the oldest proteins found in all organisms ranging from the simplest prokaryotic bacteria to the highly complexed eukaryotic human beings. The amino acid compositions have not changed dramatically as HSP of highly divergent species are similar to each other and thus is one of the classical evolutionary proteins studied till date. It's solely ascertained functions viz., molecular chaperoning and stress-elicited responses still holds good but studies have described newer functional predictions and thus Hsp90 rightly fits under "moonlighting" category. Of interest to many is to be able to differentiate Hsp90 in its "housekeeping" role in comparison to its "pathobiochemical role". Exploiting its structure-function towards human health and disease is of immense importance and attempts are being made by the Hsp90 pioneers towards this effect.

**Acknowledgements** The author would also like to place on record his gratitude to his Ph.D. mentor, the late Dr. Vrinda V Khole from the National Institute for Research in Reproductive Health (ICMR), Mumbai, India who mentored him during the course of his graduate degree. The author thanks the journal Fertility & Sterility for permitting reproduction of one figure from his previously published work [Fig. 12.4. Panel B2 and B5 of Pires ES and Khole VV. 2009: A 'block' in the road to fertility: autoantibodies to an immunodominant heat shock protein 90-beta in human ovarian autoimmunity. Fertility & Sterility 92:1395–1409]. Thank you to the journal of Reproductive Biology and Endocrinology for allowing him to reproduce one figure from his earlier work [Fig. 12.1a of Pires ES, Choudhury AK, Idicula-Thomas S, and Khole VV. Anti-Hsp90 autoantibodies in sera of infertile women identify a dominant, conserved epitope EP6 (380–389) of Hsp90 beta protein. Reprod Biol Endocrinol. 2011; 9: 16].

## References

Bardwell, J. C. A., & Craig, E. A. (1987). Eukaryotic Mr 83,000 heat shock protein has a homologue in Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America, 84*, 5177–5181.

Bensaude, O., & Morange, M. (1983). Spontaneous high expression of heat-shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryo. *The EMBO Journal, 2*, 173–177.

Cardillo, M. R., & Ippoliti, F. (2006). IL-6, IL-10 and HSP-90 expression in tissue microarrays from human prostate cancer assessed by computer-assisted image analysis. *Anticancer Research, 26*, 3409–3416.

Chadli, A., Graham, J. D., Abel, M. G., et al. (2006). GCUNC45 is a novel regulator for the progesterone receptor/Hsp90 chaperoning pathway. *Molecular and Cellular Biology, 26*(5), 1722–1730.

Chadli, A., Felts, S. J., & Toft, D. O. (2008). GCUNC45 is the first Hsp90 co-chaperone to show α/β isoform specificity. *Journal of Biological Chemistry, 283*(15), 9509–9512.

Choudhury, A., & Khole, V. V. (2013). Hsp90 antibodies: a detrimental factor responsible for ovarian dysfunction. *American Journal of Reproductive Immunology, 70*(5), 372–385.

Cornford, P. A., et al. (2000). Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Research, 60*, 7099–7105.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., & Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacology & Therapeutics, 79*, 129–168.

Elpek, G. O., Karaveli, S., Simsek, T., Keles, N., & Aksoy, N. H. (2003). Expression of heat-shock proteins Hsp27, Hsp70 and Hsp90 in malignant epithelial tumour of the ovaries. *APMIS, 111*, 523–530.

Falsone, F. S., Bernd, G., Florian, T., Anna-Maria, P., Andreas, J., & Kung, l. (2005). A proteomic snapshot of the human heat shock protein 90 interactome. *FEBS Letters, 579*, 6350–6354.

Fecek, R. J., Simeng, W., & Walter, J. S. (2015). Immunotherapeutic targeting of Hsp90 client proteins in BRAF-inhibitor resistant melanoma. *Journal for ImmunoTherapy of Cancer, 3*(Suppl2), 432.

Gallucci, S., & Matzinger, P. (2001). Danger signals: SOS to the immune system. *Current Opinion in Immunology, 13*, 114–119.

Grad, I., Cederroth, C. R., Walicki, J., et al. (2010). The molecular chaperone Hsp90α is required for meiotic progression of spermatocytes beyond pachytene in the mouse. *PLoS One, 5*(12), e15770.

Gruppi, C. M., Zakeri, Z. F., & Wolgemuth, D. J. (1991). Stage and lineage-regulated expression of two hsp90 transcripts during mouse germ cell differentiation and embryogenesis. *Molecular Reproduction and Development, 28*(3), 209–217.

Gupta, R. S. (1995). Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. *Molecular Biology and Evolution, 12*, 1063–1073.

Hilscher, B., Hilscher, W., Bulthoff-Ohnolz, B., Kramer, U., Birke, A., Pelzer, H., & Gauss, G. (1974). Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. *Cell and Tissue Research, 154*, 443–470.

Kajiwara, C., Kondo, S., Uda, S., et al. (2012). Spermatogenesis arrest caused by conditional deletion of Hsp90α in adult mice. *Biology Open, 1*(10), 977–982.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., et al. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress & Chaperones, 14*(1), 105–111.

Karras, G. I., Song, Y., Sahni, N., Máté, F., Jenny, X., Marc, V., Alan, D., Luke, W., & Lindquist, S. (2017). Hsp90 shapes the consequences of human genetic variation. *Cell, 168*(5), 856–866.

Lele, Z., Hartson, S. D., Martin, C. C., Whitesell, L., Matts, R. L., & Krone, P. H. (1999). *Developmental Biology, 210*, 56–70.

Li, J., Soroka, J., & Buchner, J. (2012). The Hsp90 chaperone machinery: Conformational dynamics and regulation by co-chaperones. *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research, 1823*(3), 624–635.

Li, K., Xue, Y., Chen, A., Jiang, Y., Xie, H., Shi, Q., et al. (2014). Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. *PLoS One, 9*(12), e115841.

Loones, M. T., Rallu, M., Mezger, V., & Morange, M. (1997). HSP gene expression and HSF2 in mouse development. *Cellular and Molecular Life Sciences, 53*, 179–190.

Mbofung, R. M., McKenzie, J. A., Malu, S., et al. (2017). Hsp90 inhibition enhances cancer immunotherapy by upregulating interferon response genes. *Nature Communications, 8*, 451.

Millson, S. H., Truman, A. W., Racz, A., et al. (2007). Expressed as the sole Hsp90 of yeast, the α and β isoforms of human Hsp90 differ with regard to their capacities for activation of certain client proteins, whereas only Hsp90β generates sensitivity to the Hsp90 inhibitor radicicol. *The FEBS Journal, 274*(7), 4453–4463.

Miyake, H., Muramaki, M., Kurahashi, T., Takenaka, A., & Fujisawa, M. (2010). Expression of potential molecular markers in prostate cancer: Correlation with clinicopathological outcomes in patients undergoing radical prostatectomy. *Urologic Oncology, 28*, 145–151.

Morimoto, R. I. (1993). Cells in stress: Transcriptional activation of heat shock genes. *Science, 259*(5100), 1409–1410.

Pacey, S., et al. (2012). A phase II trial of 17-allylamino, 17-demethoxygeldanamycin [17-AAG, tanespimycin] in patients with metastatic melanoma. *Investigational New Drugs, 30*, 341–349.

Pick, E., et al. (2007). High Hsp90 expression is associated with decreased survival in breast cancer. *Cancer Research, 67*, 2932–2937.

Pires, E. S. (2010). Multiplicity of molecular and cellular targets in human ovarian autoimmunity an update. *Journal of Assisted Reproduction and Genetics, 27*, 519–524.

Pires, E. S. (2017). The Unmysterious roles of Hsp90: Ovarian pathology and autoantibodies. In D. MacPhee (Ed.), *The Role of Heat Shock Proteins in Reproductive System Development and Function*, *Advances in Anatomy, Embryology and Cell Biology* (Vol. 222, pp. 29–44). Springer.

Pires, E. S., & Khole, V. V. (2009a). A 'block' in the road to fertility: Autoantibodies to an immunodominant heat shock protein 90-beta in human ovarian autoimmunity. *Fertility and Sterility, 92*, 1395–1409.

Pires, E. S., & Khole, V. V. (2009b). Anti- ovarian antibodies: Specificity, prevalence, multipleantigenicity and significance in human ovarian autoimmunity. In *Current Paradigm of Reprod Immunol* (pp. 159–190). Trivandrum: Research signpost Trivandrum. ISBN: 978-81-308-0373-9.

Pires, E. S., Parte, P. P., Meherji, P. K., Khan, S. A., & Khole, V. V. (2006). Naturally occurring anti-albumin antibodies are responsible for false positivity in diagnosis of autoimmune premature ovarian failure. *The Journal of Histochemistry and Cytochemistry, 54*(4), 397–405.

Pires, E. S., Meherji, P. K., Vaidya, R. R., Parikh, F. R., Ghosalkar, M. N., & Khole, V. V. (2007). Specific and sensitive immunoassays detect multiple anti-ovarian antibodies in women with infertility. *The Journal of Histochemistry and Cytochemistry, 55*(12), 1181–1190.

Pires, E. S., Choudhury, A. K., Idicula-Thomas, S., & Khole, V. V. (2011a). Anti-Hsp90 autoantibodies in sera of infertile women identify a dominant, conserved epitope EP6 (380–389) of Hsp90 beta protein. *Reproductive Biology and Endocrinology, 9*, 16.

Pires, E. S., Parikh, F. R., Mande, P. V., Uttamchandani, S. A., Savkar, S., & Khole, V. V. (2011b). Can anti-ovarian antibody testing be useful in an IVF-ET clinic? *Journal of Assisted Reproduction and Genetics, 28*(1), 55–64.

Pires, E. S., Hlavin, C., Macnamara, E., Ishola-Gbenla, K., Doerwaldt, C., Chamberlain, C., Klotz, K., Herr, A. K., Khole, A., Chertihin, O., Curnow, E., Feldman, S. H., Mandal, A., Shetty, J., Flickinger, C., & Herr, J. C. (2013). SAS1B protein [Ovastacin] shows temporal and spatial restriction to oocytes in several eutherian orders and initiates translation at the primary to secondary follicle transition. *Developmental Dynamics, 242*, 1405–1426.

Pires, E. S., D'Souza, R., Needham, M., Herr, A., Jazaeri, A., Li, H., Stoler, M., Anderson-Knapp, K., Thomas, T., Mandal, A., Gougeon, A., Flickinger, C., Bruns, D., Pollok, B., & Herr, J. C. (2015). Membrane associated cancer-oocyte neoantigen SAS1B/ovastacin is a candidate immunotherapeutic target for uterine tumors. *Oncotarget, 6*(30), 30194–30211.

Purandhar, K., Jena, P. K., Prajapati, B., Rajput, P., & Seshadri, S. (2014). Understanding the role of heat shock protein Isoforms in male fertility, Aging and Apoptosis. *The World Journal of Men's Health, 32*(3), 123–132.

Röhl, A., Rohrberg, J., & Buchner, J. (2013). The chaperone Hsp90: Changing partners for demanding clients. *Trends in Biochemical Sciences, 38*(5), 253–262.

Schopf, H. F., Maximilian, M. B., & Johannes, B. (2017). The Hsp90 chaperone machinery. *Nature Reviews Molecular Cell Biology, 18*, 345–360.

Solit, D. B., et al. (2008). Phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with metastatic melanoma. *Clinical Cancer Research, 14*, 8302–8307.

Sreedhar, S. A., Kalmar, E., Csermely, P., & Shen, Y. F. (2004). Hsp90 isoforms: functions, expression and clinical importance. *FEBS Letters, 562*, 11–15.

Website: http://www.picard.ch/downloads/downloads.htm

Website: https://www.genenames.org/cgi-bin/genefamilies/set/586

Voss, A. K., Thomas, T., & Gruss, P. (2000). *Development, 127*, 1–11.

Witkin, S. S., Sultan, K. M., Neal, G. S., et al. (1994). Unsuspected Chlamydia trachomatis infections in the female genital tract and in vitro fertilization outcome. *American Journal of Obstetrics and Gynecology, 171*, 1208–1214.

Zuehlke, A., & Johnson, J. L. (2010). Hsp90 and co-chaperones twist the functions of diverse client proteins. *Biopolymers, 93*(3), 211–217.

# Chapter 13
# Geroscience From Cell-body Dynamics and Proteostasis Cooperation Supported by αB-crystallin and Human will ~ A Proposal of "Body-Mind Integrative Science"

**Yoriko Atomi, Miho Shimizu, Eri Ohto-Fujita, Aya Atomi, Saaya Hayasaki, Yoshikazu Higashi, and Tomoaki Atomi**

**Abstract** The importance of small heat shock protein HSP in geroscience is increasing. We believe that research progress from life science should contribute to well-being. Molecular chaperone studies are considered superior when performed on model substrates and model animals. However, by itself, concrete measures for extending the healthy life of human beings are not provided. It is important to analyze the cell-animal-human results, interpret the relationship, and promote comprehensively studied HSP research. αB-crystallin (CRYAB) was identified for key molecule to explain the mechanism of exercise adaptation of slow-twitch muscle for a long ago. It is only human beings that stand up against gravity and move all day in their standing position. With CRYAB and tubulin/microtubule as a key word, we will introduce the principle of clarifying not only cells but also how to control the human body. Mechanistically fiber structure produced after protein assembly has not only multifunction but also is available as materials to make "body" and can sustain body weight by tension development at both micro- and macro-levels. This links cell's autonomous regulating ability and human will to keep standing. This manuscript may contribute to develop a new direction of HSP Geroscience research proposing real program to healthy aging and mature human world.

Y. Atomi (✉) · M. Shimizu · E. Ohto-Fujita · A. Atomi · S. Hayasaki · Y. Higashi
Material Health Science Laboratory, Tokyo University of Agriculture and Technology, Tokyo, Japan
e-mail: yatomi@cc.tuat.ac.jp

T. Atomi
Faculty of Health Sciences, Department of Physical Therapy, Kyorin University, Tokyo, Japan



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_13

**Keywords** αB-Crystallin (CRYAB) · Cytoskeleton · Dynamic Instability · Heat Shock Protein · Mechanical Stress · Bipedal standing

## Abbreviations

| | |
|---|---|
| CRYAB | αB-crystallin |
| ECM | Extracellular matrix |
| FRAP | Fluorescence recovery after photobleaching |
| FRET | Fluorescence resonance energy transfer |
| Hsp | Heat shock protein |
| HSP | Heat shock protein family |
| MAP | Microtubule-associated protein |
| MT | Microtubule |

## 13.1 Contents

**Geroscience from Proteostasis for Healthy Life**

- Inactivity is the third cause of death and 10 years bed-ridden life.
- Molecular chaperone supporting and physical exercise / physical activity.
- Moving shapes of cell and human and small HSP / CRYAB.
- Importance of mild stress: PGC1α coactivator inducing conversion to slow muscle at 39 °C is induced.

**Learning from Experimental Model in which Small HSP/CRYAB Disapperas: Meaning to Take on Weight**

- Reason why current exercise science does not hold as aging science.
- Encounter with CRYAB (protein which disappeared from anti gravitational muscle by lifting its own weight).
- "Knowing you", "Knowing Human", who can stand by holding its own weight and walk.
- Cells also carry their own weight and move.
- Three protein fiber systems to form shape Cytoskeleton: The three cytoskeletons are not equal.
- Localization of CRYAB and three cytoskeletons.
- *Muscle cells do not move, they stop and contract*.Finely-tuned striated muscles with subtle different function surviving our organism from CRYAB - tubulin relation.

**Physical Movement to Consider From 11 Clauses of Cellular Principle with Dynamic and Unstable Fiber Structure**

- Humans are multicellular animals.
- The body consists of 37 trillion cells and ECM secreted by the cells.

- Because humans are animals, the body is made to move.
- Cells are stimulated not only chemicals but also mechanical response to their own environment.
- Therefore, physical exercise is a mechanical stimulus (stress) to cells.
- A human being is a vertebrate living in a gravitational field.
- The cell has a protein dynamics response system.
- The movement that directly and dynamically connects the cell and the body becomes a living stimulus of the cell, however conversely the dynamically wrong movement exerts a hindrance on the joints of the knees and hips.
- Therefore, humans need to learn "using dynamically correct body" in the gravitational field (the spine composed of multi-joint variable structure is soft and unstable, and the trunk control which becomes the center of gravity is the point).
- Stress proteins that take care of flexible cells and proteins form the key to adaptation.
- Skeletal muscle that produces exercise of individual and *CRYAB*: Concept of total stress of exercise.

**Substrate Recognition by Small Hsp / Cryab for Mechanical Stress Response**

- Body adaptation determined by shape in three hierarchies.
- Domain structure and function of CRYAB ~ as a preface to CRYAB and tubulin / microtubule stories.
- Maintenance of "dynamic fiber structure" of tubulin / microtubule showing dynamic instability among complicated cells and support by CRYAB. CRYAB domain mutants and verify chaperone function against tubulin/microtubule.
- Use of pulsating myocardial cultured cells as a Non-stressed but constantly mechanical stressed model.

**Body-Mind Integrative Science from Small Hsp/Cryab**

- Even 10-min landing/day stimulates muscle and brain-behavior.
- Movement/exercise integrates a dual life unit consisting of activity-dependent life systems cell and body: Sophia and Phronesis.
- Knowing the role HSP / CRYAB that has played in the evolution and the its existence taught us the importance of correctly exercising and acting on us living in an aging society.

## 13.2 Introduction

As Japan is the first super-aging society, we are addressing a big challenge how to extend a healthy lifespan. One way is to induce HSP to maintain proteostasis. Among them, small HSP has been reported to contribute to this (Hsu et al. 2003; Morrow and Tanguay 2012; Walther et al. 2015). But research progress on the mechanism of small HSP is slow as compared with other HSPs. Although exercising is enforced, in fact, the population of people who does exercise does not increase and in Japan "inactivity is the third leading cause of death" is known (Ikeda et al. 2011). Why is exercise good for health? Knowing the reason why it is acceptable,

identifying the key factor, if there is any way that everyone can convince, it will be a savior of an aging society. We searched for such a crucial molecule and identified one of the small HSP, αB-crystallin (CRYAB). CRYAB disappears in the hindlimb suspension model released by its weight, and it does not decrease if stretching (Atomi et al. 1990). The reason is that the cytoskeleton is a substrate of CRYAB (Arai and Atomi 1997; Atomi 2015; Fujita et al. 2004; Jee et al. 2009; Ohto-Fujita et al. 2007; Sakurai et al. 2005; Shimizu et al. 2016).

People take on their weight in the standing position. Exercise tension with muscle contraction, create articulation, stand, walk and run. Cells also dynamically respond to a cytoskeleton that shapes and exerts tension. Both the cell and the body have weight. The cell maintains its weight by antagonistic/tensional contraction like tug of war with three cytoskeletal fibers. Myocardium (Arac et al. 2011; Atomi et al. 2000; Ito et al. 1997; Kato et al. 1994; Ray et al. 2001) and slow skeletal muscles (Atomi et al. 2000; Atomi et al. 1990; Atomi et al. 1991a, b; Brady et al. 2001; Kato et al. 1994; Koh and Escobedo 2004) and diaphragms (Atomi et al. 2000; Brady et al. 2001; Ito et al. 1997; Jee et al. 2009; Kato et al. 1994) with high CRYAB expression will keep as long as people live active. There is MTOC (mitotic organization center) in the cytoskeleton/microtubule, and the cells also control the balance (Prosser and Pelletier 2017). In this chapter we will introduce not only cellular balance control but also neuronal control of the body. The insula is the control center of the emotional system and the autonomic nervous system, which controls the balance of the unstable body of the standing person (Atomi et al. 2014). An appropriate balance control is necessary for an unstable upright body that is increased in the elderly, which requires humans will control. As mechanical stress continues to be applied, proteostasis continues to work. Learning to induce mild mechanical stress to the cell is also necessary for balance control. We have already reported that a mild thermal stress of 39 °C induces the inducer/coactivator PGC1α of the slow muscle which is the skeletal muscle responsible for health (Yamaguchi et al. 2010). Tubulin/ microtubules are exceptionally dynamic fibers called dynamic instability (Mitchison and Kirschner 1984), and CRYAB works as a chaperone. CRYAB supports that in an unstable, adjustable protein system. We have already made clear that the α-crystallin domain of CRYAB takes care of tubulin and the N-terminal takes care of MAPs (Fujita et al. 2004; Ohto-Fujita et al. 2007). CRYAB prevents pathological fibrosis such as Alzheimer's disease in the very early stages (Hochberg et al. 2014; Raman et al. 2005).

"Stand" and "move" are established by the will of the person himself. Humans practice as they are convinced. Scientific research to understand human characteristics is necessary. Cells were born in the Earth's gravitational field, and our humans gained languages by allowing us to live in standing position to produce the axis of the trunk, respiration and vibrant voice. We found that landing only for 10 min during the hind limb suspension for 24 h prevented the decrease of CRYAB and maintained activity. Humans need to acquire movement and exercise to support themselves standing balanced by their own weight. The importance of acquiring this skill is understood from the centroid control ability that cell microtubules have

acquired evolutionarily. Elderly policy is born by linking the ability to maintain tubulin / microtubule (MT) dynamics by CRYAB discovered by cells to proteostasis and further linking to human exercise and activities.

## 13.3 Geroscience from Proteostasis for Healthy Life

### *13.3.1 Inactivity Is the Third Cause of Death and 10 Years Bed-Ridden Life*

Based on World Health Statistics 2017 report, Japan's average life expectancy at birth still the highest in the world and health life expectancy is also the world's best. However, there is a difference of about 10 years between the average life expectancy and the healthy life expectancy. When comparing for males and females, the

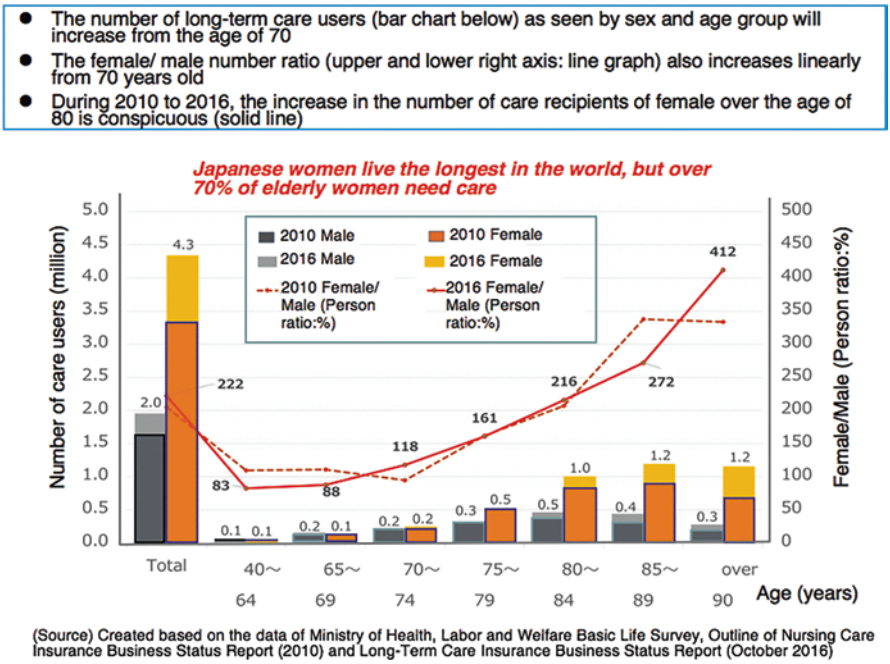


**Fig. 13.1 Changes in the Number of Long-Term Care Service Users by sex and age group, and female/male ratio.** A number of people who need long-term care (persons who need long-term care and need support) (the number of people requiring care (*left axis*) reflecting the number of men and women) and the ratio of women to men (*right axis: line graph*). The figures above the bar graph are real numbers of each man and woman (million people). The total number of long-term care needs is 6.3 million (October 2016)

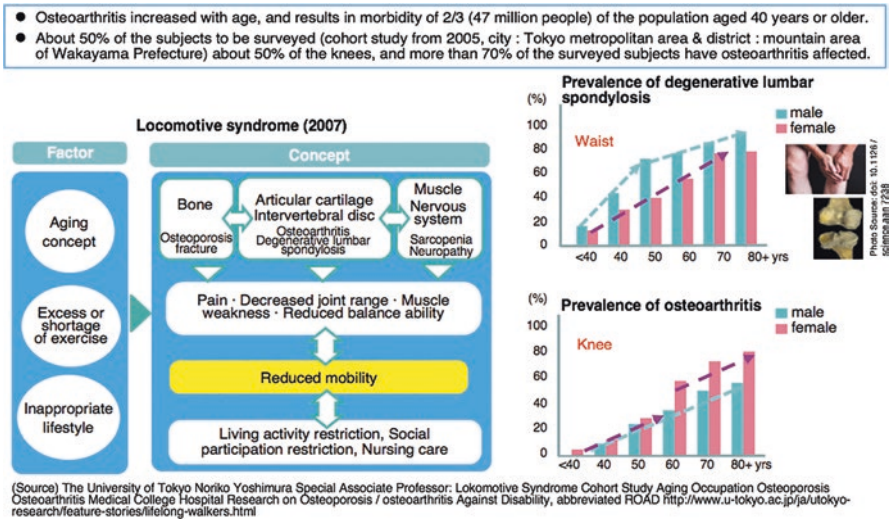


**Fig. 13.2 Can you walk on your feet and exercise?** Peoples are having "Locomotive syndrome," which has some troubles like knee and hip joint pain, etc., is increased linearly from over 40 years old

difference between the women is larger, and the number of years requiring nursing care is longer (Fig. 13.1). The years of the difference between these two lifespan means that there are people who cannot act freely (need nursing care). In fact, women elderly who were 50% at the age of 75 and over 70% at the age of 85 were required care (Atomi 2012). One of the reasons for this is the problem of osteoarthritis which you cannot do at all want to do exercise. Human beings who built original and unique culture with their standing position as a normal condition also had weak points. In a survey targeting Japanese, adults over 40 years of age have a linear increase in the population feeling pain in the hips and knees with age (Fig. 13.2).

### *13.3.2 Molecular Chaperone Supporting and Physical Exercise/Physical Activity*

Geroscience is a relatively new research area studying the relationship between aging and disease (Kennedy et al. 2014). In facing super-aging society worldwide, it is critical to extend healthy lifespan, and comprehensive geroscience should include human life science and physical education. Everyone knows that exercise is good for your health. Can we continue to carry on lifetime beyond 100 years in

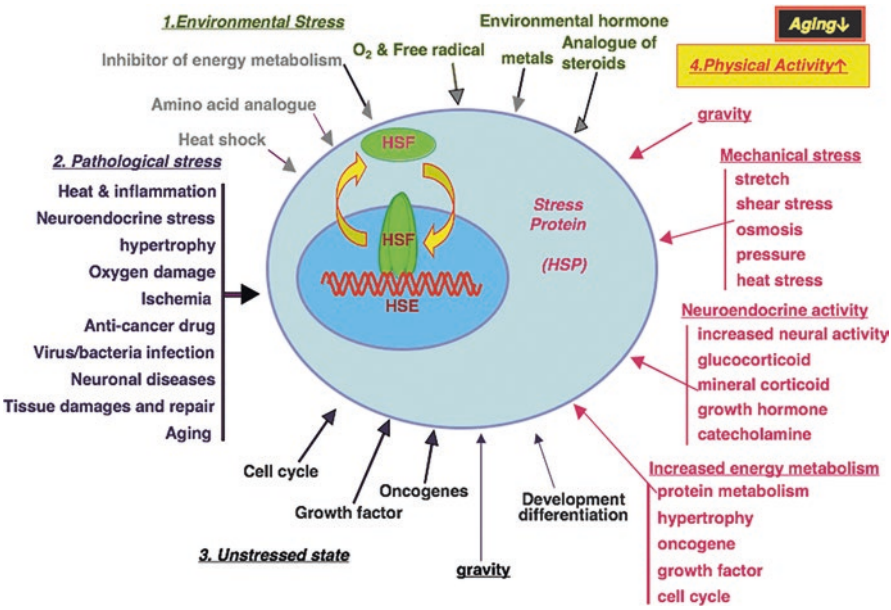


**Fig. 13.3** We can induce HSP with physical activity (Figure by Morimoto (Morimoto 2008) with modified Atomi (Atomi 2012))

a standing position? HSP express not only constitutively in adult but also induced during morphogenesis of fertilized egg because the process involved a lot of endogenous cytoskeletal tension and exogenous mechanical stress to shape the tissue and organ (Ulbricht et al. 2013; Ulbricht and Hohfeld 2013; Varlet et al. 2017) as shown in (Fig. 13.3). Since the organismal systems and cells emerged on the earth, they evolved to respond to all the global environmental factors. Especially in the gravity field of 1 G, the organism must be shaped using gravity. Stress generated in the body during exercise becomes "good" stress to almost all physiological functions of the body. If a strategy of elderly science to induce HSP and activate proteostasis actively, HSP related research should progress more. Among others, microtubule, which we have studied as a substrate of CRYAB, does not assemble under microgravity (Tabony and Job 1992). One of the small HSP, CRYAB was identified as a key molecule to explain the mechanism of exercise adaptation about 30 years ago (Atomi et al. 1990). It is only human beings that stand up against gravity and move all day in their standing position. With CRYAB and tubulin as a key word, we will introduce the principle of clarifying not only cells but also how to control the human body.

### 13.3.3 Moving Shapes of Cell and Human and One of Small HSP, CRYAB

Among adaptive responses of cells, research on mechanical stimulation is delayed. We would like to propose an aging strategy for sustainable standing posture, along with the history of encountering HSPs CRYAB who takes care of fibril-forming proteins, microtubule. Know-how at the scene of physiotherapists facing fine-tuned bodies, and martial arts developed in Asia since ancient times helped to overcome my knee pain and low back pain. Recently, mechanical axis control is proved to be necessary for correct movement in both cell and body, and CRYAB can give us theory for understanding. Both cells and body are in the gravitational field, and they cannot remove from the foundation, so body control is necessary to avoid falling. The animals desperately lived while moving in an environment of drastic change. Small HSPs, which are also highly expressed in bacteria and plants (de Jong et al. 1993; Kappe et al. 2002; Kim et al. 1998; Waters 1995), among them especially CRYAB, primary work to recognize neurofibrils in our human beings, not to make it worse further, and also contribute to immune responses (Steinman 2014). Both tubulin, a fibril-forming protein that produces microtubules, and Aβ have CRYAB domain (ACD) of CRYAB working (Mainz et al. 2015; Ohto-Fujita et al. 2007). When we tag the CRYAB in the cardiomyocytes that is pulsating fine with the tag of GFP, we recognize the substrate at a speed of 1 second or less (manuscript submitting). Humans become human by running endurance (Bramble and Lieberman 2004), if they run inappropriately, they will lose the freedom to move super-aged society with their own feet. That is a very wasteful thing. Living organism has emerged in the earth and successfully evolved by creating HSP. Especially, it is human beings who are most frequently and abundantly expressing endurance-compatible CRYAB among all species, so we should be able to establish an aging science strategy with CRYAB as the key. HSP science should contribute to extend healthy life.

### 13.3.4 Importance of Mild Stress: PGC1α/Coactivator Inducing Conversation to Slow Muscle at 39 °C Is Induced.

Figure (13.4) shows that the master gene myogenin and coactivator PGC1α are induced with mild stress at 39 °C during inducing culture from myotubes cells (Yamaguchi et al. 2010). Slow muscle fibers (cells) among skeletal muscle fiber type are responsible for aerobic exercise, which is a key to prevention of lifestyle-related diseases. On this condition, MyoD gene expression and also protein in nucleus, which is responsible to induce fast muscle fiber, decreased corresponding to the increase of the temperature of culture, observed in the photograph of mouse C2C12 cells and human muscle cells. In the usual experimental model of heat shock, the response is often verified at 42 ° C, 30 min. However, the orthopedic

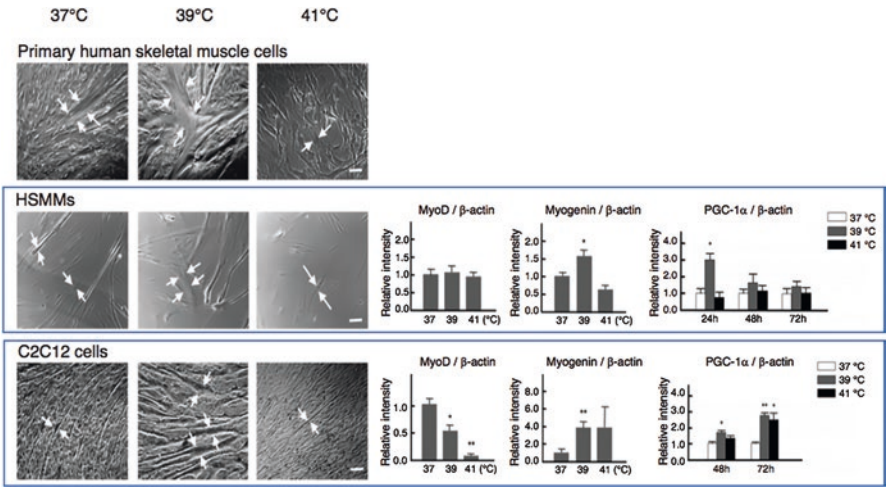


**Fig. 13.4** An example of mild stress to work as the threshold switching muscle fiber type: continuous mild heat stress at incubated at 39 °C induces slow muscle master gene (myogenin) and PGC1α but inhibits fast muscle MyoD (Yamaguchi et al. 2010)

surgeon Tetsuo Yamaguchi who carried out this study has experiences to cool and/or warm the affected part of the body as the first aid treatment at the hospital site, but he could not understood the principle to decide the temperature as heat therapy, and decided to do experiment and observed it by changing the temperature by 1 ° C at a time. In the field concrete science which can be applied actually is necessary. Diverse living things have evolved at each site due to the highly specific interaction of necessary proteins as well as CRYAB, as well as close to 20,000 proteins. Proteins that make myofibrils of skeletal muscles have many isotypes, and in order to demonstrate the performance according to the characteristics of the development to the growth process, and even the movement characteristics such as sports, depending on the quality and quantity of motion, various situation let the myogenic protein change including fiber-type changes according to the work that is needed. It became clear that the isotype of myosin that started from the study of skeletal muscle shows different isotypes in non-skeletal muscle cells, and it becomes clear that all the cells are created extremely dynamically and live doing finely-tuned motion. We are seeing prosperity of cell biology. Although skeletal muscle research has been done for a long time, the study of cytoskeleton in skeletal muscle research area has been delayed. People are deprived of their movements, but they are hardly noticed by the mechanisms that produce them. We do not know whether it is right even if I make own body movement, posture and walking style. It makes a fuss for the first time after hurting or falling over. It is extremely important to explore the movement science from HSP that elderly people can support their weight and move as long as they are alive. Supporting the bell-shaped population shown in (Fig. 13.5) is the development of science and technology for each person to induce HSP themselves, rather than medicine development.

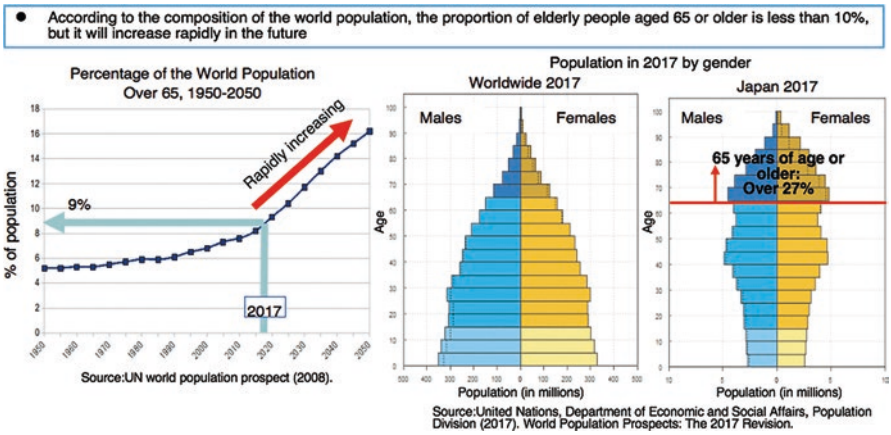


**Fig. 13.5** Trend of population and increase of elderly people

## 13.4 Learning from Experimental Model in Which One of Small HSP, CRYAB Disappears: Meaning to Take on Weight

### *13.4.1 Reason Why Current Exercise Science Does Not Hold as Aging Science*

We have studied exercise training by humans to elucidate the mechanism of "adaptation" by exercise, like the influence of aerobic capacity (Atomi and Miyashita 1974; Atomi and Miyashita 1980), glucose metabolism by running training in animals (Hatta et al. 1989), stretching effect on collagen synthesis using knee fibroblasts (Kim et al. 2002). Various changes occur with stimulation of exercise. In fact, in these experiments, various responses including changes in HSP are derived not only by the amount of stimulation but also by the strength and duration of exercise (Tabata et al. 1990). There are many experiments about this, and many reviews are done (Liu and Steinacker 2001; Locke and Noble 1995). However, from these experimental results, it is doubtful whether we can draw appropriate and necessary and sufficient exercise programs especially for the elderly. That is because the plan is very artificial. For animals, exercise is the survival strategy, and it uses mostly reflections. In fact, when people try to move with two legs, the muscles of the left and right feet alternately contract and produce rhythm. However, since it is a vertebrate, since the two feet are connected to the trunk through the hip joint, it is first necessary to make the trunk straight as a shaft against the force of gravity without paying special attention (Ohkawa et al. 2017). The number of skeletal muscles of a person is as many as 1000, including large and small. Four hundred of which actually connect 24 vertebrae to make 1 vertebra. It is used so that it can be flexed-extended and rotated left and right. These "voluntary muscles" support standing

position and realize movement. Skeletal muscle is innervated by spinal cord or central nervous system, it is systematized so as to locomotion. Developmental program progresses autonomously and automatically, but when human beings are born infants can't move by themselves. Although the development of the fetus progresses while floating in the amniotic fluid in a narrow environment, it is thrown out under the gravity of 1 G with the birth. The neonatal spine cannot resist gravity, so in the same horizontal position (ie recumbent position) as the other quadrupeds, the newborn borrows milk by borrowing hands from the surrounding people. The process until a newborn baby moves in a standing posture one year later is a process in which a body composed of a soft multi-segment structure ensures autonomous movement using reaction force obtained from contact with surrounding environment.

People do not remember these growth processes in a manner that they can talk about themselves. Although the circuit remains in the brain as the procedure memory all the process after birth, too. It is not possible to explain most of these processes with words. Aging science not only learns the substance process that deteriorates after aging but also the autonomous response system of the cell which always responds if worked up and the great possibility of proteostasis by HSP as its backup system. In aging science, it is not enough to learn the substance process that deteriorates with aging after growth. Everyone living in 100 years will need to learn the autonomous response system of the cell. If you work cells, they will surely respond through proteostasis maintaining system via HSP. Molecular biology of cells has revealed that the cell itself produces a highly autonomous moving system with autonomous association/disassociation of proteins. For example, Actin is part of the basic transcription machinery complex. Tubulin is also a constituent of cell membranes and mitochondrial membranes. Originally, both cytoskeletons were uniquely owned by other protozoans, but basically, they have three cytoskeletons in eukaryotes including humans. Through all processes of fertilization to development, fertilized eggs respond to the outer environment dynamically. We should keep in mind that morphogenesis progresses because there always a shape-dependent chemical reaction is proceeding. Animals and humans have the will to move themselves. The survival of cells is structured as "activity dependence". It is necessary as long as the individual is alive, to match the will of the individual with the cellular principle. Therefore, in addition to research using model animals, our researchers will need to study the shape, attitude and movement of the actual human body, as well as how to drive HSP responses and proteostasis.

### *13.4.2 Encounter with CRYAB (Protein Which Disappeared From Anti-Gravitational Muscle by Lifting Its Own Weight)*

Among various models studying exercise adaptation, the hind limb suspension model was provided an important viewpoint of health (Fig. 13.6). The model is in a state where the muscles of the feet that support the weight are not required to work.

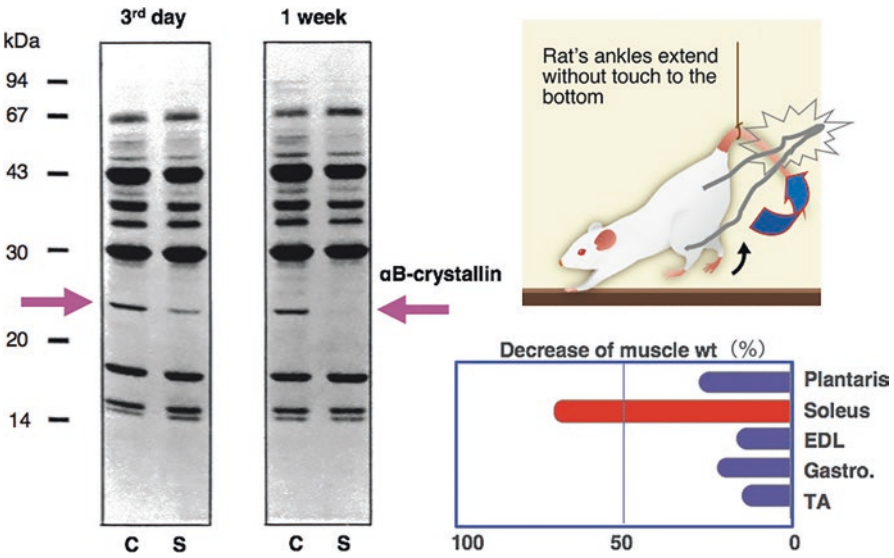


**Fig. 13.6** Discovery of small HSP, αB-crystallin/CRYAB as a key molecule to elucidate mechanism of muscle and brain atrophy. In rat's tail suspension model (S), the feet cannot touch the bottom, and maintain the body weight (Atomi et al. 1991a)

It was developed to study adaptive changes in the musculoskeletal system in quasi-zero gravity. Actually, even in aquatic animals, the vestibular system that balances with terrestrial animals is developed, but the necessity to support own body weight is extremely small compared to terrestrial animals. In our mammalian skeletal muscle, there are two kinds of muscles whose contraction speed and metabolism are different. Among them, slow muscles (red muscles) have large adaptation ability. It is humans that this slow anti-gravitational muscle is developed to the utmost. In many cases, slow muscle is described as red muscle, fast muscle is described as white muscle. This color difference is related to the degree of development of blood vessels and is related to the large amount of hemoglobin that red blood cells transport oxygen. That is, the red muscle is good at aerobic metabolism, and white muscle (fast muscle) is the one which metabolizes mainly on the glycolytic system which progresses anaerobically. The name "slow" and "fast" are the actual macroscale muscle contraction rate. Furthermore, this metabolic property and physiological output characteristic are linked with the rate of ATP hydrolysis of myosin, a motor protein. Differences in the amino acid sequence of the molecule have a strong correlation with the mechanical properties of skeletal muscle at the individual levels. Sports characteristics and the health problems are the real pleasure of skeletal muscle research, and aging science is necessary. Here, it is added that the slow muscle and the fast muscle are the names of the macroscopic muscle tissues. In fact, in the mammal, the slow muscles refer to a slow muscle cells (slow muscle fibers). The fast muscle refers to a muscle with many fast muscle fibers. That is, when the

slow muscle is biochemically analyzed, there are much quantity of types of myosin (Type I myosin), and fast muscle has a lot of fast myosin (Type II myosin). Furthermore, Type I myosin is identical to the major muscle fibers). The fast muscle refers to a muscle with many fast muscle fibers. Type II MHC is classified into IIa, IId and IIb subtypes, and the oxidative capacity of the fiber is lowered in this order. Basically, one cell (muscle fiber) expresses single myosin type. In mammals, these are arranged in a mosaic-like fashion. Detailed molecular biological research on these has just begun.

What we do not forget when studying movement and exercise is that we must maintain the balance of the body to support human beings and to support weight (Atomi et al. 2014). Bipedal standing on land was crucial for evolving human cultural activities freely using hands. But it required a systemic change of the cell to support the unstable posture with a lot of danger. We will introduce an interesting story to show difference between human and other mammalian probably relating to standing of human. As one of the substrates of molecular chaperone CRYAB, giant protein connectin/titin has been evolved lengthened the amino acid chain particularly increase in human gene. This protein was expected to be in skeletal muscle by a famous Japanese muscle researcher Reiji Natori. He gained insight that the muscle should have a structure that shows property of elasticity. Muscle researcher Kosaku Maruyama received this insight refined this large protein from the remaining residual fraction after extracted myosin and actin, and named it connectin (Maruyama 1976). About the same time US researcher Kuan Wang discovered the same protein a moment behind and named titin (Wang et al. 1979), but the name of titin which promoted genetic analysis is widely used. Not only soleus muscle in the triceps brachii muscle is important for human standing. The quantity increased to equal rate to gastrocnemius muscle, of which four leg animal rat in only shared one tenth. The vertebrate spine developed with a muscle spindle internal perceptual system to allow it to stand up against gravity while allowing fine movement. The body trunk with the spine has a foundation that allows extremely fine-tuned movement. Although it does not get deep here, it is essential for aging science, but it is very delayed. Researchers working on skeletal muscles also have a lot of people who do not know that the skeletal muscles have two types of muscles, slow muscle and fast muscle, and that contraction characteristics are significantly different in both striated muscles. In Olympic sports, short-distance and long-distance running, the physique and muscle development of the athlete differ greatly. Even if you are not interested in physiology, if you are a small HSP researcher, considering whether small HSP is more common in striated muscles, a new research perspective will be born. A massive array of vertically and horizontally oriented protein clusters produces a very large structural change called “contraction” in the population, so that a number of cells fuse together and do surprisingly that they function as one cell. The research model that I met CRYAB was in “skeletal muscle inactivity model” hanging the tail of the rat from the ceiling and attaching the hind limb to the floor. Protein with a molecular weight of 22 kDa was specifically depleted in one week (Atomi et al. 1991b). "What is the state of living?" “What is exercise?” "What changes and what support the effect by repeating" just estimates the function of the gene or molecule from the knockout experiment. It was an experimental model that made us

think about what happens unless it moves with gravity and moves. It is a model in which the stress protein disappears. Hind limb suspension itself must be stressful for individuals, but one of the HSP named as stress proteins also drastically decreases.

### *13.4.3 "Knowing you", "Knowing Human", Who Can Stand by Holding Its Own Weight and Walk*

This hind limb suspension model was originally developed as a model of bone atrophy in the microgravity and was then used by many researchers as a model of muscle atrophy. It is also used as a method to evaluate mental activity status of rats, mice and others later. In other words, an individual who quickly accepts a hanging resistance-impermissible state and whose movement decreases reflects the tendency for susceptibility to mental illness. I think it will be a glimpse of why the lack of exercise will be the third leading cause of death. It is equivalent to "the system does not work" of an animal that moves and carries its own weight. There will be no reason for the system of the central nervous system and immune system to function as an individual to function. The ecosystem of life has evolved with dependence on each other's relationship. The death of one human being is irreplaceable for humans. However, the positioning of animals as predators that have evolved in ecosystems is "activity-dependent." we think it is time to rethink the human wisdom beyond this gap and the science necessary to live as a human being. Education of science, knowing our existence "Knowing you" Education and science must be integrated. Human system was evolved during erect-standing and walking posture (Fig. 13.7).

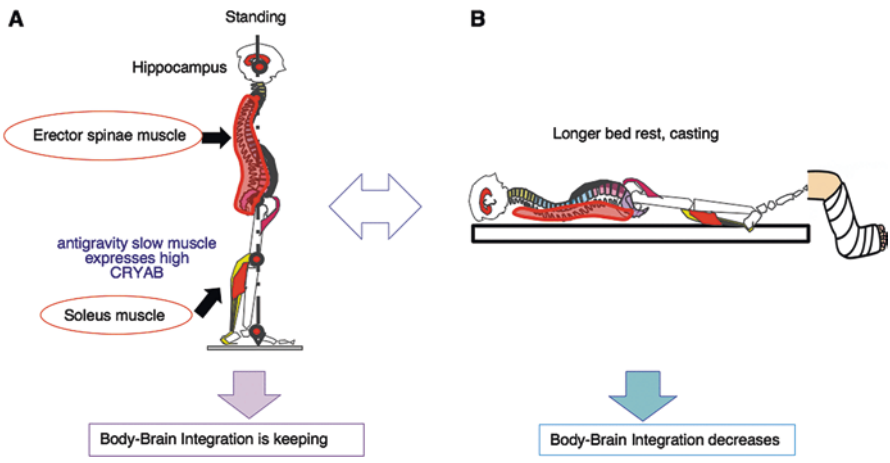


**Fig. 13.7 The human system was evolved during erect-standing & walking posture.** Antigravitational muscles develop well in standing human (**a**), but quantitative and qualitative deterioration occurs with hypokinesis (**b**)

As in this Figure, anti-gravitational muscles develop well in standing human but quantitative and qualitative deterioration occurs with hypokinesis. We need more science.

R. Edgerton of neurophysiologist UCLA, who is studying with interest in exercise, thinks there is a problem when studying separating both nerve and muscle activities. He is conducting research with a young group of researchers (neurologist, physiotherapist, IT engineer) who has made remarkable achievements in the spinal cord injury model using rats, with Courtine being the lead (Courtine et al. 2008; van den Brand et al. 2012). I wanted to exclude the influence of innervation in a rat hind suspension model long ago, I thought that gene expression of CRYAB, which was examined using a denervation model (Atomi et al. 1991a) which cuts the sciatic nerve. Gene expression pattern after nerve cut seemed to be ineffective condition as if released from suppression by neural innervation (Atomi et al. 1991a). As I talked about this at that time, it was a scholar who pointed out that cutting nerves cannot see the correct physiological response of muscle change in the experiment. This phenomenon is also consistent with the hypothesis that cells are functioning while communicating as one individual, while making mutual relations even when cells grow and divide in one individual. Although it is the above-mentioned spinal cord injury model, it seems that it is useless only by electric stimulation. It is a network of cells that has been made to move, and a network of cells. Treatment of spinal cord injury is to add regenerative stimulation by raising the chemical response between the central nervous system and the cells that make the muscle but this information is also great when you move the body mechanically, even passively. It is a result indicating that it has meaning.

Ohkawa's research in our laboratory, introduced above did not provide meaningful data in a special experimental model. In the end, we measured the free moment, which is a type of force occurring in the sole, using normal walking as an experimental task. As a result of correlation between free moment and movement of each body segment and joint movement, only the relative angle of pelvis and foot indicating the internal rotation angle of the hip joint was significantly correlated (Ohkawa et al. 2017). This suggests that flexibility and how to use of the hip joint are very important. This is also a question of how to use mechanical stress. Pain is cry of cells, so we need to respond appropriately. When we get older, my body gets harder and pain often goes on. This should not be taken for granted again, but this time it will not go deeper. The pain is the cry of the cells, so it is necessary to listen carefully and deal with it as soon as possible. Let's introduce the core research that connects the body and mind. Pain-related neural mechanisms are called "Pain-matrix", in which various brain regions are involved. Especially, insular cortex is an important area. The insular cortex connects the somatosensory nervous system, which is responsible for sensation related to physical exercise and equilibrium, to the autonomic nervous system, and is a place deeply related to human emotion. Actually, the insular cortex plays an important role also in controlling the balance of the body. Tomoaki Atomi, who specializes in physiotherapy and neuroscience, revealed that from a very unique research result using a mechanism of self - cognitive neural mechanism by functional magnetic resonance imaging (Atomi et al. 2014). Whatever

happens, we stand without falling and we can do whatever it is supposed to be because humans have connected the vestibular system and antigravity muscles with a reflection circuit and have evolved this regulation system to higher order.

### *13.4.4 Cells also Carry Their Own Weight and Move*

This hind limb suspension model drastically reduces soleus muscle, which is a slow anti-gravitational muscle. What are proteins doing in the cell that decreases by releasing self-weight? Research on the key molecule to solve the mechanism of "exercise adaptation" (one of small HSP, CRYAB) helps understand. What does it mean that we can do exercise? Controlling your weight well is actually quite difficult. The difficulty can be understood by those who experienced fractures, knee pain, back pain at least once. Those who are living a normal life think about how to do a good move, but I do not believe that I will control my weight. I decide to ask the cell what I do not understand like this question. A cell has proteins that create fibrous structures named "cytoskeleton". Actually, this is a fiber produced by protein assembly. Using that fiber, the cell creates a structure like a tent beam and maintains the tension by developing the tension. Cells that build up our bodies are adhesive, except for erythrocytes, they form a fulcrum in the foundation and organize the substances that are making themselves spatially to promote chemical reactions systematically. It is similar to the skeletal muscle exercising tension and creating articulation and movement as we move.

### *13.4.5 Three Protein Fiber Systems to Form Shape Cytoskeleton: The Three Cytoskeletons Are Not Equal*

Currently, there are three "cytoskeletons", named actin, tubulin/microtubule, and intermediate filament, fundamentally owned by multicellular animals. In these three fiber systems, proteins that are units as a unit are medium sized with a molecular weight of 40 to 50 kDa, and they assemble themselves to form fibers. Actin and tubulin require high-energy phosphate compounds ATP, GTP, respectively, for the formation of fibers. In both cases, however, when ATP and GTP are hydrolyzed to ADP and GDP during elongation of the fiber, the fiber becomes unstable and disassembles from the elongated end and starts shortening. This control is regulated by the concentration of free monomer or dimer. Most of the stimulus (stress) exerted by cells from the outside causes an increase in intracellular $Ca^{2+}$ concentration. Muscle contraction is also the same. As the concentration of $Ca^{2+}$ increases, the response to the assembly of actin is strengthened, but tubulin / microtubules conversely promote disassembly. Therefore, $Ca^{2+}$ oscillation is occurring in active striated muscle. In the myocardium and slow muscle with relatively slow contraction frequency, $Ca^{2+}$ concentration within the cell does not fall to the ground state, so the

translocation of transcriptional nuclear calcineurin by $Ca^{2+}$ continues to read the gene while it is transferred to the nucleus. It is conceivable that protein turnover will inevitably become high.

Of the three cytoskeletons, actin and tubulin may sometimes play a role by itself, but the main function is to interact with each specific motor protein to become a motor rail. The very unique point is that the rail itself is a rail that elongates and shortens. Cells are largely divided into cells that continue to divide depending on the timing of morphogenesis, growth, and where the cells have survived, and cells that continue to live by stopping division. Epithelial cells facing the outside world are bound to a special extracellular matrix called basement membrane. Since it is always exposed to stress from the outside world, basically one split remains as tissue stem cells and the other cell divides and continues to divide in a certain time. Sequential chemical reactions until cell division in the proliferative phase is doubled occurs in conjunction with the cytoskeleton. In other words, the cell needs to adhere to the fulcrum focal adhesion, and cannot divide unless the tension can be exerted. In addition, the differentiated cells also respond to the environment of new mechanical stress by recombining the cytoskeleton according to the role of each tissue. Not limited to contracting skeletal muscle cells, all cells are promoting chemical reactions in a structure-dependent manner while organizing the intracellular space by the dynamic structure generated by the cytoskeleton and motor proteins.

The partner of actin is a motor protein called myosin. The function based on the interaction between the two is not to convey the object, but when both are fixed, by exerting contraction force / traction force in the form of generating tension with respect to the fixed fulcrum is there. Characteristics of cytoskeleton, actin and tubulin-microtubule fiber change the shape of the cell as necessary by elongation and shortening produced due to assembly and disassembly. In addition, microtubules are thought to maintain mechanical homeostasis while sensing centrosomes and the mechanical properties of whole cells. For this, it is necessary to receive the tension from the actin filament which forms the fulcrum structure while lining and reinforcing the cell membrane at the fulcrum of the peripheral part of the cell. It is coordinating with protein such as plectin which binds both. In other words, the role of microtubules is to counter the passive force of cells and to extend in the necessary direction. This property is thought to act as antagonistic stress against the force generated by myosin - actin, but it does not produce contraction force like myosin and actin. Motor proteins, kinesin and dynein, which are companions of microtubules, transport goods from the center of the cell to the periphery, or vice versa.

Even among the differentiated skeletal muscle cells, there are several centers of microtubules (centrosome), and it appears that the role of placing the Golgi in the right place (Ralston et al. 1999) and the transport of goods/materials. By in 1999 Gundersen concluded that the function of microtubules was not necessary after myoblast fusion to myotube (Gundersen and Cook 1999). However, microtubules that are thought to play a central role in cellular regulation often develop in slow muscles (Ralston et al. 1999), supporting the active metabolism of slow muscles. The group of Spiegelman has made clear that the factor inducing slow muscle is a PGC1α coactivator up-regulates mitochondrial genes coded in the nuclear genome

and the function as well as the genes coding myofibrillar proteins of slow muscle fiber. So what is the factor regulating the expression of PGC1α? A comprehensive analysis of small molecules resulted in the involvement of drugs that promote microtubule dynamics (Arany et al. 2008). There are very few researchers who study microtubules in skeletal muscle. However, the author's view is that the strategy of the cell, even differentiated or modified, but the basis is not changed. Actually, the cell, the protein system called microtubule cooperates with actin, intermediate diameter filament, and changes the way of making cells according to their position in each tissue. In places where tissues and bodies face the outside, they become epithelial cells and undergo stress stimulation from various external worlds, almost all stress factors rise. It is important to induce stress proteins by mild exercise of endurance and to turn proteostasis.

### *13.4.6 Localization of CRYAB and Three Cytoskeletons*

In searching for the substrate of CRYAB, we found there are three cytoskeletal proteins actin, intermediate filament, and tubulin/ microtubule inside the cells. The cytoskeleton is a dynamic structure which produce tension and movement. Almost cells have three cytoskeletons, cooperates with actin, microtubule and intermediate diameter filament, and the dynamical system in which the cells themselves undergo developing tension and movement, advances chemical reaction interlocking these structures of the cytoskeleton. Skeletal muscle is a cell that produces a very special morphology in which one of the cytoskeleton, the actin protein and its partner myosin motor protein is aberrantly increased. However, CRYAB was perfectly localized in agreement with tubulin / microtubule of another name of three cytoskeletons (Fig. 13.8). And then it was the intermediate diameter filament vimentin that matched. Actin is the scaffold / focal contact of the cell whose cell is the fulcrum of force development. Although it may be interacting with actin in this case, a paper which takes care of FAK, one of the fulcrum producing proteins, was recently published (Pereira et al. 2014). There will be others. The protein complex interacts dynamically in fluctuations and is built and maintained. In the slow muscle, it was known that the Z structure of sarcomere corresponding to this fulcrum was long (wider) than the fast muscle from long ago. Why is it necessary to have a slow contraction compared to fast muscles and only a weak force to demonstrate why it is wide?

The myotubes that have differentiated to create a sarcomere structure will die away from the substrate due to the large contraction force exerted by them on the petri dish. Not only in the muscles but also in the gravitational field at the fulcrum when the force exercise regime disappears, the cells can not only maintain their shape, but they will die if peeled off. Ruoslahti, who discovered the integrin binding sequence to fibronectin "stretch is good for cells", briefly wrote the essence of cells in a short review in 1997 (Ruoslahti 1997). Both CRYAB-overexpressing and knockdown cell lines were prepared and analyzed as shown in (Fig. 13.9) (Shimizu et al. 2016). Compared with wild-type cells, CRYAB-overexpressing cells had a

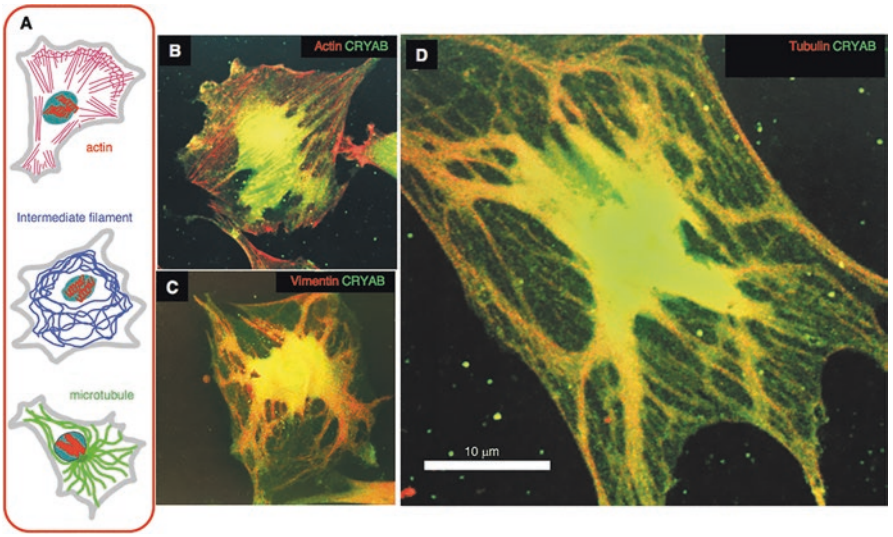


**Fig. 13.8** The three major types of cytoskeleton (**a**). Merged immunofluorescence micrographs of fixed myoblast cells visualized for CRYAB (*green*) with actin (*red*, **b**), vimentin (*red*, **c**), and tubulin (*red*, **d**)

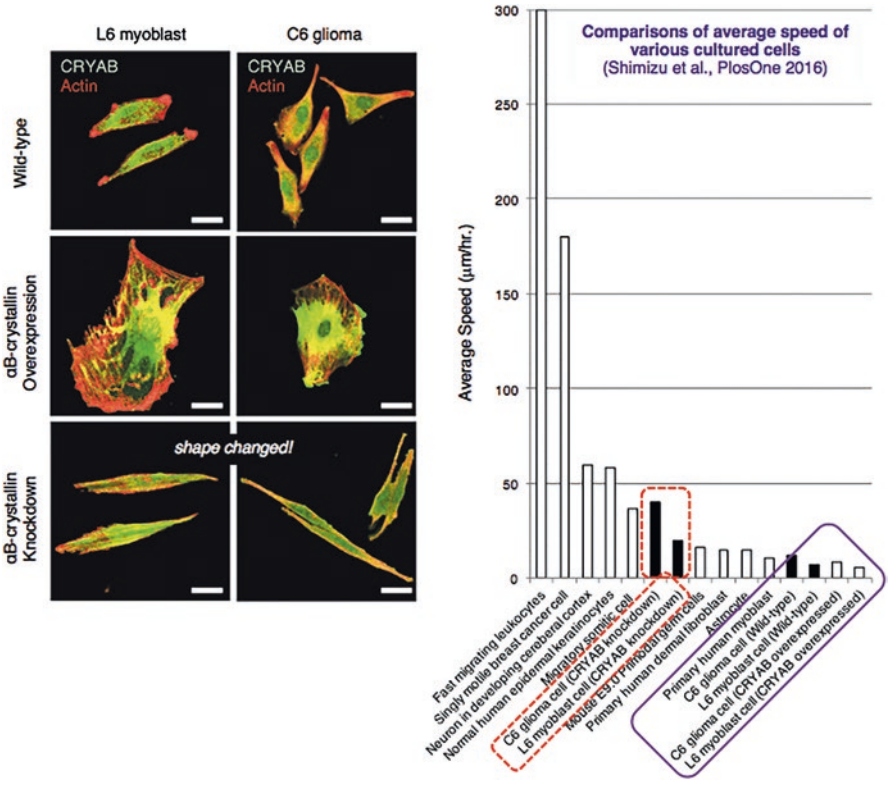


**Fig. 13.9** **Cell shape and movement. There is a structure which does not move.** (**a**) Both myoblast and glioma/glia cells don't migrate and show slow muscle cell-type tonic contraction," but very dynamic adhesion. (**b**) Migration speed of various cells (Shimizu et al. 2016)

more rounded and spread shape, and barely moved. On the other hand, CRYAB knockdown cells showed a characteristic narrow, fibroblast-like shape with extensively lengthened pseudopodia and migrated fast. In our laboratory, we tried to make a microscopic pillar (microarray) made of acrylamide, placing cells on it, to determine whether one cell of micro order really demonstrates its power. It is difficult to reproduce the same soft base as the living body with good reproducibility, but as shown in the cartoon in the later section (Fig. 13.16), myoblasts which reduced the expression of CRYAB cannot step on the foot and were buried and. Normal L6 myoblasts expressing CRYAB seemed to have the properties of muscle cells before differentiation, but they stepped on their legs and showed a traction of about 70 pico-Newtons. The traction of this single cell, as you go up the hierarchy to the differentiated muscle fiber, bundle of myofibrils, single muscle tissue, and the strength we exert, you can order ten or more orders of 9–10. Become. It is extremely difficult to connect this quantitatively, but if you can measure the quantity, type, and mechanical properties of extracellular matrix (collagen, proteoglycan, etc.) which are substances that connect cells and individuals, it can be determined according to the function and softness of the organization I believe that the range of appropriate force will be known.

Actually, what we move is that if joint angles change due to skeletal muscle contraction (not movement) and if there is a fulcrum on the ground, the skeletal muscles of the whole body collaboratively contract and extend, thereby utilizing gravity you can say that you can move. Skeletal muscle cells must not migrate but contract. Only the power of the interaction between myosin and actin has been studied as advanced science, and it is now supposed that coupling and divergence of both proteins are made utilizing different fluctuations (Funatsu et al. 1995). But with myosin and actin alone, in fact the power cannot be taken out. Unless it is connected with a fulcrum that does not move as much as possible due to the interaction between the molecules, force cannot be extracted and articulation does not occur. The reason why research on cell culture of skeletal muscle does not proceed is because no cell culture system has been established to strengthen adhesion which clears this gap.

### *13.4.7 Muscle Cells Do Not Move, they Stop and Contract (Fig. 13.10)*

It turned out that all the eukaryotic cells have three “cytoskeletons” since the research method of cultured cells was established. The history of skeletal muscle is older than the history of molecular biology of cells which see cells as units of life. The cytoskeleton in the skeletal muscle is a large protein such as nebulin or connecin (titin), which is a giant protein in the form of “supporting” suitable for the name of the cytoskeleton, and it supports the structure by one molecule itself. In the research field of skeletal muscle, microtubules are still almost none. However, in this gravity field of 1 G, life has evolved by constructing a system as a “form existence” in a

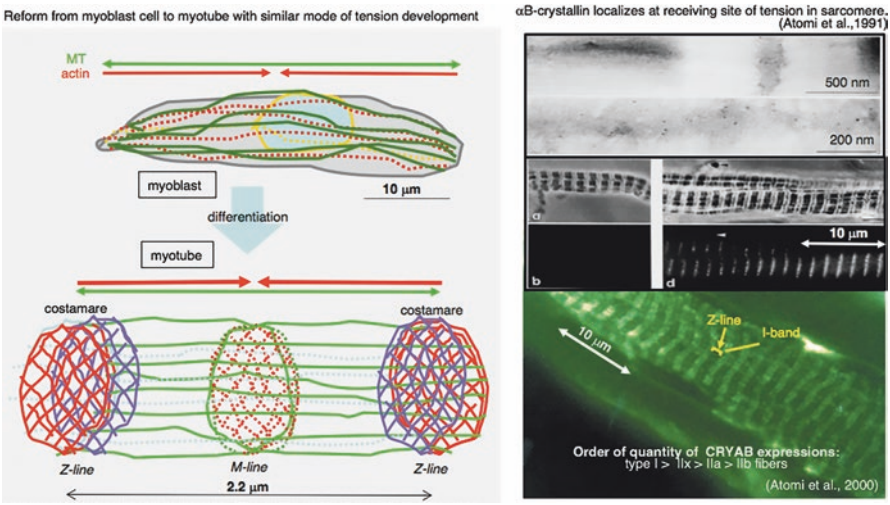


**Fig. 13.10** Cytoskeleton dynamics induction hypothesis that requires αB-crystallin/CRYAB in slow muscle cells, myoblast and glia cells. Dynamically antagonistic making of passive tension exertion of the cytoskeleton (microtubule and actin-myosin system) in striated muscle cells and myoblasts that exerts tension without exerting tension is a sustainable energy supply (mitochondrial Necessity) and maintenance of proteostasis. The third cytoskeleton/intermediate diameter filament maintains mechanical resistance by connecting to both via plectin

system that uses a dynamic system, filament / fiber structure extensively. Protein is also a form of life. In the fluctuating environmental chemical reactions in the form dynamics sustain the "living" state. From the Mitchison laboratory named "dynamic instability" in the dynamic behavior of microtubules within the cell (Mitchison and Kirschner 1985), a system excluding a part of the cells (system excluding lipids of the cell membrane). When establishing a system that activates the dynamic instability, since the intercellular distance becomes constant, they provide driving force to align intracellular proteins to the right place in the movement of assembly / disassembly of microtubules. An image saying that it seems to be made is presented, and the paper is being introduced (Ishihara et al. 2014).

### *13.4.8 Finely-Tuned Striated Muscles with Subtle Different Function Surviving Our Organism from CRYAB – Tubulin Relation (Fig. 13.11)*

In the skeletal muscle research field, we do not even know about the cytoskeleton of all the cells. There is no name of "cytoskeleton" also in the keyword of the research of academic society including the Japanese Physiological Society. It also has low

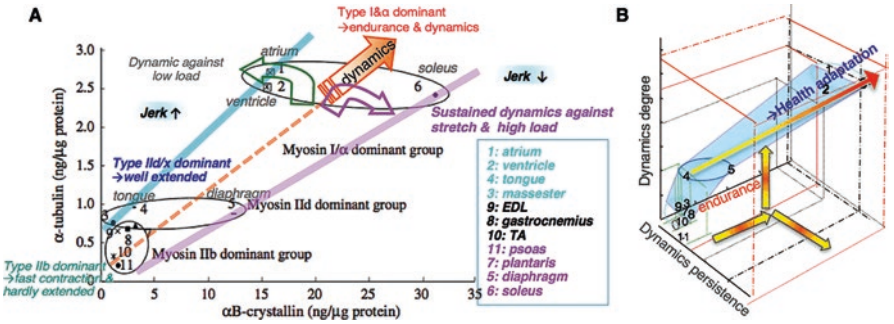


**Fig. 13.11** Consider the evaluation system of adaptability and exercise adaptation of mammalian striated muscle from the expression level of αB-crystallin/CRYAB and tubulin protein. (**a**) The relationship between the expressed CRYAB and α-tubulin contents in various muscles. Dominant MHC-isoform groups muscles. The attached numbers are for each striated muscle. There is a significant correlation ($P < 0.01$) between the quantity of CRYAB and that of α-tubulin ($n = 11$, Pearson correlation coefficient 0.837) ((Jee et al. 2009) modified by Atomi). Word of "Jerk" is the name that Mitchison gave first in place of "dynamic instability" for the phenomenon of microtubule assembly and disassembly. (**b**) Positioning is evaluating the contribution of various striated muscles in the body for fitness as seen from essential protein molecules (CRYAB and tubulin) that generate dynamic cell structure, endurance movement. Changes to the diagonally upper part of this figure become the direction of health adaptation. This trend tends to maintained in the region where the muscle is stretched even in the hind paw suspension state, but it is not limited in the state where the muscle contraction does not occur

recognition in the field of HSP research. Heat stress may have started. Since exercise also began with aerobics, oxygen is invisible and does not normally exist as a structure. That is, the form does not appear as a keyword. However, slow aerobic players developed muscles that can withstand the exertion of force as both troops continue to exert power like tug of war. Both armies tugging on muscle sarcomere are Actin - Myosin Army and Microtubule - Kinesin and/or Dynein Motor Force. The latter microtubule is like a pillar that continues to resist the applied force rather than exert its own force. This pillar existence does not exert its own power because it also works as a transportation route in the cell. Since it stretches and shortens while assembling and/or disassembling, its extension and shortening in the boundary structure of cells becomes giving and receiving of tension, creating a competing mechanical system. This antagonistic system works every time the heart is output, and in antigravity muscles, this antagonistic system operates alternately each time walking.

Since the heart keeps tug of war until it dies, we must take care. Antagonistic force demonstration will require mitochondria to constantly supply energy and will respond to "activity dependency" requirements of mitochondria creatures. It is a force under the edge to sustainably maintain the system. Therefore, the heart keeps on rhythm, it repeats eternal contraction as a pump, it is like a perpetual institution that recovers every beat. There are many mutations in CRYAB gene in its heart, and

the death period is actually accelerated in the case of diastolic heart disease and others. As long as we are alive, we hypothesized that CRYAB and its substrate tubulin / microtubule are supporting the muscles that carry oxygen and continue to consume, expressing both proteins in various ways we compare them with striated muscles that produce physiological functions (Fig. 13.11) (Jee et al. 2009). This figure shows that the eternal beat of the heart, the mysterious function of eternal breathing, and all the tongue streaks that we are moving so hard to speak the words are all striated muscles. Even with the same striated muscle, the magnitude of the output force, the required speed and fineness are different, not only related to the isotype of myosin but also adaptability of both proteins adaptively expanding our rich movements. It seems to be talking about the size of plasticity. You will be able to imagine the unique face of the cells that support the activities that will never stop as these days and you will feel that you will give them the right jobs and activities themselves. We refer to it as Body-Mind Integrative Science.

## 13.5 Physical Movement to Consider from 11 Clauses of Cellular Principle with Dynamic and Unstable Fiber Structure

It seems that it is almost never looking all over the world for the fields explaining human living while working actively every day with cell molecular biology and life science. Then we cannot save super aged society. Because the body moves, cells survive in activity dependence and turn on proteostasis even if cancer cells continue to grow, if you keep on running the immune system to eliminate it will not become a cancer of sickness. The protagonist who drives proteostasis is a cell. That cell exercises and organizes its structure and lives while working with extremely high order. Moreover, the protein that supports it is designed to be driven depending on the cytoskeletal system which contains the nature called shaking and dynamic instability. A scaffold is necessary for cells to exert their power. In this chapter, we do not intend to touch on extracellular matrix (ECM), which is a place to exercise strength, but it is indispensable as a concept. Moreover, the ECM itself is secreted and built by the cell itself. The cell senses its own shape and the place of force demonstration according to the environment to live and creates a place appropriately. Eleven clauses explaining the life principle that connects the body from cells that support body movement are explained with the help of illustration listed below. Finally, from the relationship between skeletal muscle that generates individual movement and CRYAB, we present a concept of total good stress of exercise..

Principle of Life as a Matter System has Following 11 Items

1) *Humans are multicellular animals* (Fig. 13.12). Almost cells in our body are adhesive, thus they contact each other or to ECM, which is secreted by themselves except red blood cells.

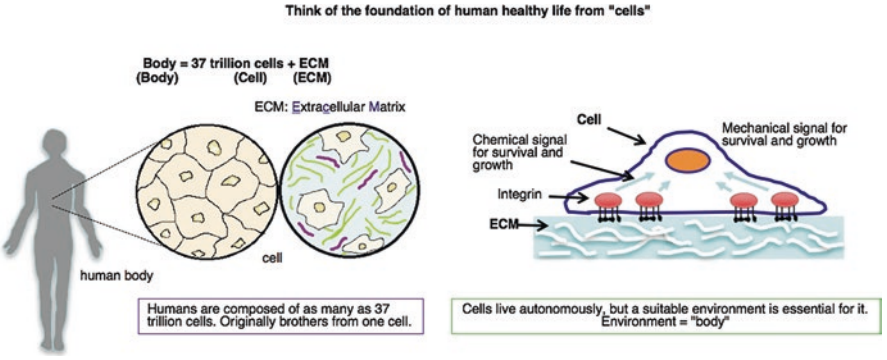


**Fig. 13.12 Body as a thing to consider from the molecular biology of a new "body" - cell relation via the mechanical environment.** Through research on the function of αB-crystallin, a HSP of the self-assembled protein system/cytoskeleton of cells, which is one of the molecular chaperones, the authors have studied from HSP system. We propose Body-mind integrative science" that connects the body - cells, producing the good adaptation

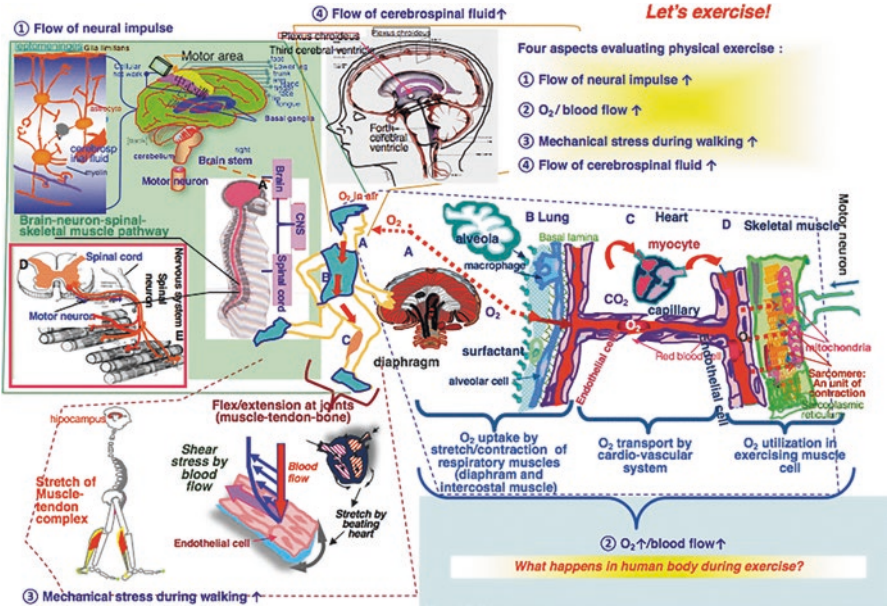


**Fig. 13.13 What happens in our exercising body?** The human body consists of sixty trillion cells and the ECM molecules that those cells secrete. When we walk and run, coordinated skeletal muscle contractions develop joint movements and support our posture and body weight. Aerobic exercise brings our body to increase four main aspects like 1) a flow of neuronal impulse, 2) a flow of oxygen/ bloodstream, 3) an activation of cells of our body by mechanical stimuli, and 4) a circulation of cerebrospinal fluid. All physical activities increase mechanical stress to relating cells in tissues and ECM (Atomi 2015)

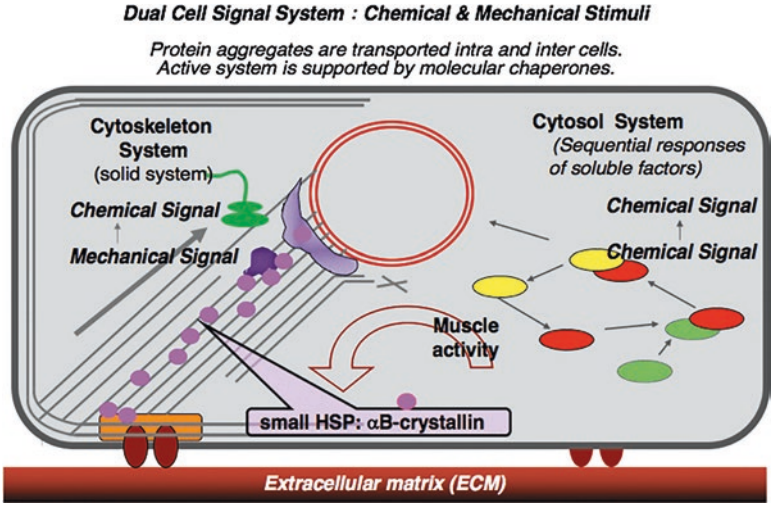


**Fig. 13.14 Dual Cell Signal System:Solid protein filament system of cytoskeleton and ECM organizes cellular metabolism responding both chemical and mechanical stimuli, even protein aggregates using aggresome.** Muscle activity facilitates proteostasis utilizing this system keeping filament system by itself to be dynamic (Figure originally created by Mikihito Tanaka)

2) The body consists of 37 trillion cells (Bianconi et al. 2013) and (ECM) secreted by the cells (26 trillion is red blood cells). Cell responds to its own environment not only chemically but also mechanically (Fig. 13.13): What is going on in human body during exercise? (Atomi 2012; Atomi 2015). Endurance exercise promotes collaboration among cells of different tissues via mechanically via ECM and connective tissue as well as neuronal and humoral stimuli.

3) Because humans are animals, the body is made to move on lands and takes oxygen in the air and circulates and transports it with red cells.

4) Cells are stimulated not only chemicals but also mechanical response to their own environment (Fig. 13.14). Mechanical stimulation is delivered to the cytoskeleton via a receptor that undergoes mechanical stress from the ECM. Altered proteins of conformational diseases such as neurofibrillary tangles which are problematic in the elderly are also transported through this cytoskeleton to the intracellular decomposition factory. Its decomposition plant named aggresome is located in the immediate vicinity of the nucleus. It is shown in the (Fig. 13.14).

5) Therefore, physical exercise is a mechanical stimulus (stress) to cells.

6) A human being is a vertebrate living in a gravitational field.

7) The cell has a protein dynamics response system (cytoskeleton - cell adhesion molecule - extracellular matrix) that dynamically controls the center of mass in cells (Fig. 13.15) (right) and develop tension against adhesion sites, which is a part of body, associating with mechanical stress produced by the exercise property. Exercising body is regulated by neuronal system working in the brain, which has both directions of cell to body and body to cell in exercise producing, supporting

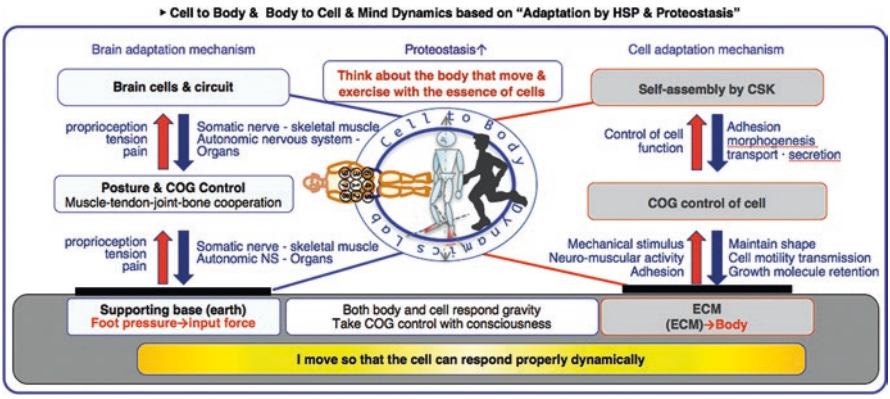


**Fig. 13.15 The body-mind integrating theory with proteostasis and proper physical activity.** We should know both the adaptation (learning) process of the brain and the cell, which dynamically can feel and respond to stimuli (stress). Exercise/action which could be produced by oneself integrate them. In the standing position, the body contacts the sole only, and in the supine position the supporting base surface increases. Cells are connecting with Fascia and skin inside of our body. Particular ECM structure including the basement membrane and other ECM consisting of connective tissue is also turned over by exercise by cells live in there. The act trying to produce right movement (neuro - muscle activity) is very important for connecting a muscle - tendon - joint – bone. We can activate the cells to cooperate and change the ECM composition

tissues under regulation somatic and autonomic nervous systems. All systems like the nervous system, the circulatory system, the endocrine system have been developed and evolved to be properly driven with physical exercise. Conventionally, in the viewpoint of studying physical exercise, a system that maintains and coordinates cooperative systems by exercising with this unique autonomous system is not yet incorporated yet. Repeated exercise produces adaptation via proteostasis for many cells of many tissues.

8) The movement that directly and dynamically connects the cell and the body becomes a living stimulus of the cell, however conversely the dynamically wrong movement exerts a hindrance on the joints of the knees and hips.

9) Therefore, humans need to learn "using dynamically correct body" in the gravitational field, The spine composed of multi-joint variable structure is soft and unstable, and the trunk control which becomes the center of gravity is the point.

10) HSP (molecular chaperones) that take care of flexible cells and proteins form the key to adaptation. Currently, our laboratory is studying the health effects of the eggshell membrane of chicken eggs. Unlike mammals, in order to connect to the land and to connect seeds with eggs, it is necessary to have the homeostasis function to maintain the living environment autonomously, although it is an open system similar to our body. We cannot expect supply of nutrition from the outside, we need ingenuity to keep moisture and not to dry, and it must be strong mechanically. There are devises to maintain necessary barriers and functions until the chick hatch, and the function of eggshell membrane mainly consisting of ECM seems to have an

immeasurable role. Among them, residential materials until the embryo develops seem to bring out materials that promote rejuvenation. Further study of ECM is likely to be a savior for aging science. Skeletal muscle that produces exercise of individual and CRYAB: Concept of total good stress of exercise.

11) Stimulation induced by exercise is firstly mechanical stress. As the duration of exercise increases, body temperature rises and other various homeostasis disorder occurs, so there are cellular activities in the whole body to normalize it, and small HSP must be induced first in it. Regarding CRYAB, it is thought that not only cytoplasmic HSP but also it also takes care of endoplasmic reticulum and Golgi proteins via probably highly hydrophobic N-terminal region. In addition, it has been reported that CRYAB interacts with nuclear speckle and RNA and it corresponds to emergency treatment of almost all biological substances. Since small HSP are evolutionarily old and have an α-crytallin domain with a structure similar to the IgG domain (Kim et al. 1998), CRYAB which is one of small HSP is also a multifunctional molecule so that IgG interacts with various molecules. However, as described below, CRYAB has no cysteine residue to be cross-linked.

Here, through the expression state and function of CRYAB relating to the cytoskeleton / tubulin and skeletal muscle that we have been pursuing so far, we would like to show possible cause of the diversity of human movement produced from our bodies and the hypothesis for health of human beings based on cell activation. Thus, we will suggest a method how to move in daily life. For examples that mutate in relation to lifestyle disease rather than genetic mutation, we must find a way to make sure that mutation does not occur. Small HSP are the most contributing factors in extending healthy lifespan (Kenyon et al. 1993). We think that there is only physical exercise. As mentioned at the outset, body movement/exercise is the activation of the animal's system. Not only does physical exercise improves circulation, it also increases the proteostasis of whole body cells. Currently, exercise is reported to be recommended for persons with visceral disorders, if the intensity is less than the lactate threshold (Atomi et al. 1986, 1987), which is a breaking point to keep homeostasis of acid-base balance and also hypothalamus-pituitary-adrenal (HPA) axis for stress response, below which stress hormone does not increase from resting level. Furthermore, as we will mention later, exercise works comprehensively enough to drive the animal's individual system even with 10-minutes-landing stimulation in the standing position (see also last section).

In search of key molecules for elucidation of the mechanism of exercise adaptation, we encountered CRYAB and met tubulin-microtubule as its substrate. Quinlan, who is studying the cytoskeleton / IF with a lens, reports that the lens is extremely dynamic (Quinlan 2002). Although it is rather difficult to show experimentally, researchers who are studying cytoskeletal proteins as small HSP, in particular as substrates for CRYAB, are eager to visualize the dynamics of small HSP structures. All exercises that are commonly going to be realized through realization of joint movement induced by muscle contraction. And everyone thought that the point of the effect on exercise cells is a mechanical stress like stretching. It was Vandenburgh who first tried to study the effect of stretching on the skeletal muscle

(Vandenburgh et al. 1990). He differentiated myocytes on the stretchy silicon membrane and gave a stretch stimulus. He differentiated myoblasts to myotube on the stretchy silicon membrane and gave a stretch stimulus. There is a memorable paper. If the stretch extension and contraction rate is fast or strong, the spindle type myoblasts will lie side by side to escape from the stress. However, if he stretches slowly and weakly like the time of occurrence in the muscle differentiation during embryo, do not escape from the direction of force and line up like in the living. Subsequently, many studies using myocardial cells and fibroblasts were announced. We also studied stretching using myoblast and myotube for several years, but when tandem sarcomere differentiates on a rigid culture dish and begin to contract, they dissociate from the culture dish by the magnitude of the force that they output. It is death. For this reason, research on the structure-dependent response mechanism to mechanical stimulation for skeletal muscle cells has not progressed much.

The importance of small HSP in geroscience is increasing. To clarify the dogma of small HSP (oligomerization is essential for the study of its chaperone activity), biochemical studies and approaches must be incorporated, including microscopic visualization techniques. We believe that research progress from life science should contribute to well-being. Molecular chaperone studies are considered superior when performed on model substrates and model animals. However, by itself, concrete measures for extending the healthy life of human beings are not provided. It is important to analyze the cell-animal-human results, interpret the relationship, and promote comprehensively studied HSP research. Unlike other animals, humans are rare and should have developed a long-lived cell line with cell adaptability, ie, a relationship with chaperones. Cells and humans need to think that the system will be read depending on the actual environment.

## 13.6 Substrate Recognition by Cryab for Mechanical Stress Response

### *13.6.1 Three Hierarchies of Adaptive Bodies that Shape Makes (Summarized in (Fig. 13.16)*

Alzheimer's disease and other neurofibrillary diseases called degenerative aggregation and accumulation in and out of the cell that cannot undergo proteolysis are called conformational diseases (Westerheide and Morimoto 2005). This is due to the denaturation of the protein form. How is the shape of the cells? There is an aging model of a cell that ages with the decline of division ability of human fibroblast called WI38 (Shay and Wright 2000). In this example, the β-GAL is used as an aging marker. Comparing this aged cell with a young cell, the younger one has a spindle shape. As increasing the number of divisions, the cell becomes thinner, and not spindle-shaped and spreads to an irregular shape. That is the shape changes.

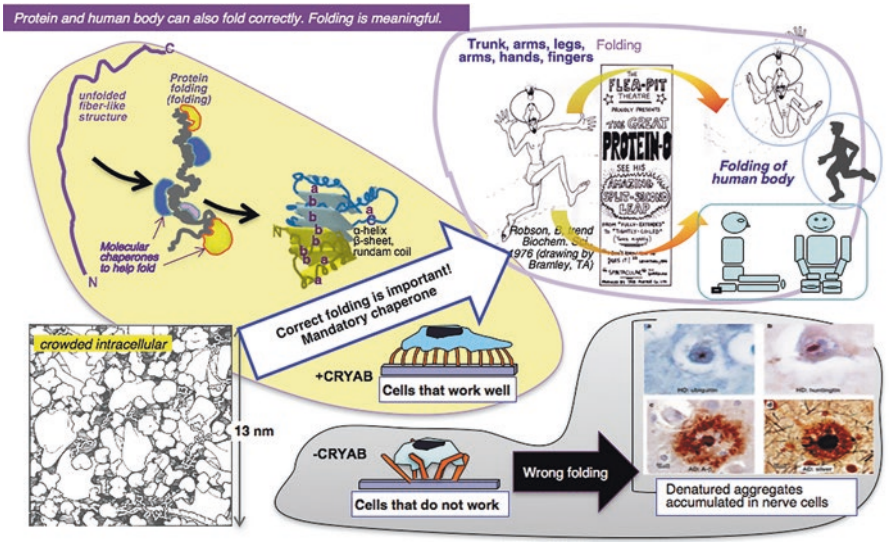


**Fig. 13.16 Thinking significance of folding from protein, cells, and the human body.** Inside of healthy cells is like crowded trains of proteins and other macromolecules (bottom left: (Goodsell 1991)), so caretaker / molecular chaperones are used for unstable proteins is necessary. We should know how to correct folding of own body scientifically

When cells of aged skin also lose elasticity, the cells which live there are even wanted to be flat. The aged cells may want to be flat as they cannot produce power. Myoblasts in which CRYAB is expressed correctly can pull the foundation but knock downed cell of CRYAB collapses (Fig. 13.16). Humans get stiffer as their body grows older, becomes elderly like shapes, and the range of motion of joints will also become smaller. However, there are significant individual differences in these changes, and at least exercise/movement is thought to have a preventive effect on these morphological changes.

Exercise and activities maintain and raise longevity genetic markers (Sharples et al. 2015) and improve energy metabolism. Not only that, but the exercise upgrades the proteostasis of the cytoskeleton and extracellular matrix proteins that make up the "shape and structure" of most cells and tissues in the body, such as tissues that produce mechanical stress, tissues that transmit tension, and tissues that receive will do. HSP including CRYAB supports proteostasis. Therefore, if you can make them work well, you will be able to delay at least the aging. By improving proteostasis of extracellular matrix which is often found in connective tissues due to aging suppresses fibrosis and also leads to the prevention of cancer, which is the most significant cause of death of elderly people. In research on lifespan using model animals, small HSP is essential among HSP, but the reason is yet to be understood. One of small HSP, CRYAB has a chaperone effect on the cytoskeleton tubulin/ MT which is also a transport pathway within the cell. Many subcellular organelles, including

mitochondria, are dynamically organized by cytoskeletons including microtubules. Even Alzheimer's disease can be prevented if it can induce CRYAB at an early stage.

Creatures, including humans, have their forms. Among them, the mechanical properties and physical properties of mammalian bodies are soft and elastic compared to other animals and plants. Among them, the human body is long and slender, but many joints can flex and extend well and can be folded softly (Fig. 13.16). Seiza, which is one of the sitting positions in Japan, sitting erect with one's legs folded beneath, has the effect of integrating mind and concentrating the mind. To use a person's skill of the left-right and front-to-rear balance well, and Seiza sitting is indispensable for the operation and document operation of the tea tool placed on the floor work. Proteins also have various folding style, each of which produces a different function, but it often depends on the localization environment (e.g., close to the membrane and/or cytosol, etc.).

Regardless of the Seiza sitting, the life sitting on the floor is considered to have strengthened the muscle strength, and stretched cells consisting of knee joints of daily life by the rising from the floor, and the rise in a narrow space was increasing the balance. As already shown above, the brain related to training balance is also the center of emotional system and pain. There may be not only the strengthening effect on the legs and trunk muscles but also the consolidation effect of the body and mind. Because the inside of the cell is crowded chaperones are necessary, and since it is too complicated, a device that does not diffuse molecules in cells is necessary. Small HSP is named Holdase (Eyles and Gierasch 2010). In other words, small HSP is designed to dynamically create groups, oligomers by interactions that are not separated from each other and require a variety of peers "in a sequence-dependent manner," and proteostasis at a place where dynamic must be functional. It must be making a significant contribution to creating the environment. From the standpoint of researching muscles, looking at this complicated situation, the wisdom of the striated muscles must be a fantastic ingenuity. Moreover, the length varies significantly in sarcomere units on the lines. We think that the striated muscle cannot be maintained without proteostasis, but small HSP are less expressed in fast muscle. If the way the contraction of the muscle is in a short time, it seems that the problem does not occur (although adaptation is difficult to occur). Given this, one part of the wisdom of life must be cached a glimpse.

### *13.6.2 Domain Structure and Function of CRYAB ~ as a Preface to CRYAB and Tubulin-Microtubule Stories*

CRYAB protein is a member of low molecular weight HSP, 10 kinds are known in humans, and the names of HSPB1 to HSPB10 are recently given (Kampinga et al. 2009). Mymrikov et al. (Mymrikov et al. 2017) added information on amino acid

chain length, PDB ID, and the number of cysteine residues considered to be involved in structural dynamics (Table 13.1). Although CRYAB is HSPB5, it is often used as CRYAB, as it is actively studied in fields other than chaperone researchers. Figure (13.17) shows the basic structure of CRYAB (Haslbeck and Vierling 2015). A variable length non-conserved N-terminal sequence (NTS), conserved α-crystallin domain (ACD), also called Hsp20 domain, PF00011 (conserved protein family http://pfam.xfam.org/family/PF00011), two And a non-conserved short C-terminal sequence (CTS) containing the conserved IxI motif necessary for the dimers to form oligomers. There are a maximum of 3 phosphorylation sites in NTS. The structure of ACD shared in small HSP is classified into two types (Fig. 13.17a). Since it cannot be strictly classified as prokaryotic or eukaryotic type, it will be appropriate to say β6-swap type (based on crystal structure analysis of Bacillus subtilismall HSP 16.5) and, β7 interface type (based on human CRYAB solid NMR analysis) (Haslbeck and Vierling 2015). N-terminal has not been able to analyze structure because it is considered to be dynamic (Patel et al. 2014; Uversky and Dunker 2010). Crystal structural analysis of proteins with dynamics controlled by lipid modification at N-terminus may be useful. CRYAB is the only small HSP that membrane-associates ubiquitously, and α-crystallin is palmitinated in lens at the interaction site with the membrane (Manenti et al. 1990). There is also a report that the chaperone of the lipid membrane is performed similarly to Synechocystis thylakoid small HSP 17 (Tsvetkova et al. 2002).

The basis of CRYAB substrate recognition may be a dimer. Oligomers are of the storage type and are assembled with substrates via dimers, resulting in high molecular weight aggregates. The N-terminus is essential for substrate recognition, but it is hidden in the oligomer state. Phosphorylation is necessary for oligomer removal (Haslbeck and Vierling 2015). Subunits are always exchanged, and dimers are always present to some extent to monitor substrate degeneration (Haslbeck and Vierling 2015). Proteins are not functional unless soluble. The fact that the associated form with the substrate is more soluble than the oligomer indicates the need for binding with HSP. There are not many studies showing interactions by actually purifying proteins as substrates for CRYAB (Bullard et al. 2004; Houck and Clark 2010; Song et al. 2008). Because CRYAB co-localizes with pathologic amyloid, we frequently see reports that CRYAB is the cause of the disease. But do not forget that cell systems can interpret that they are struggling to survive. Pathological mutations related to muscle diseases were map to CRYAB structure model (Mainz et al. 2015) and topology based on a structural analysis (Jehle et al. 2010) (Figs. 13.17b and 13.18). Although R120G with a mutation in ACD is famous, many mutations have been found in the NTS and CTS parts. Looking at the structure added by NTS and CTS pointed out by Mainz et al., It is unexpectedly large NTS and CTS made with this random coil, interface formed by ACD and flexible both termini may important for physiological function. CRYAB is expressed in all cells and recognizes early degeneration of various protein actions so that holdase retains its state, but thereafter releasing substrate to ATP dependence chaperone HSP70, 90, 40 as described in in vitro experiment (Haslbeck and Vierling 2015). However, when CRYAB recognizes small changes associated with free form association of cytoskeletal proteins,

**Table 13.1** Characteristics of human small HSP

| Name | Synonymous names | Length | Molecular weight of monomer, Da | Theoretical pI | Tissue specificity (cellular compartment) | Oligomeric state of an isolated protein | Domain | UniProt ID | Structure PDB ID | Number of Cystein residue |
|---|---|---|---|---|---|---|---|---|---|---|
| HSPB1 | Hsp27, Hsp25, Hsp28 | 205 | 22 783 | 5.98 | Ubiquitous (Cytoskeleton, Nucleus, Cytoplasm) | Large oligomers, a size depends on phosphorylation | ACD | P04792 | 2N3J, 3Q9Q, 3Q9P | 1 at ACD |
| HSPB2 | MKBP, myotonic dystrophy protein kinase binding protein | 182 | 20 233 | 5.07 | Cardiac and skeletal muscle (Nucleus, Cytoplasm) | Small oligomers, dependent on the concentration | ACD | Q16082, NP_000385.1 | | 1 at ACD |
| HSPB3 | | 150 | 16 966 | 5.66 | Cardiac and skeletal muscle (Nucleus, Cytoplasm) | Dimer or trimer (?) | ACD | Q12988, NP_006299.1 | | 1 at ACD |
| HSPB4 | αA-crystallin | 173 | 19 909 | 5.77 | Eye lens (Nucleus, Cytoplasm) | Large oligomers | N-terminal, ACD, putative dimer interface [polypeptide binding] | P02489 | 3L1F (Bovine) | 1 at ACD |
| HSPB5 | αB-crystallin | 175 | 20 159 | 6.76 | Ubiquitous, mainly in eye lens and muscles (Nucleus, secreted, Cytoplasm) | Large oligomers, a size depends on phosphorylation | N-terminal, ACD, C-terinal (IXI/V) | P02511 | 2klr, 3J07, 2N0K, 2Y22, 2Y1Y, 3L1G, 2WJ7 | No |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| HSPB6 | Hsp20, p20 | 160 | 17 136 | 5.95 | Ubiquitous, mainly in smooth and cardiac muscle (Nucleus, secreted, cytoplasm) | Dimer | N-terminal, ACD, putative dimer interface [polypeptide binding] | O14558 , NP_653218.1 | 5LUM, 5LU2 (complex with 14-3-3), 4JUS | No |
| HSPB7 | cvHsp, cardiovascular heat shock protein | 170 | 18 611 | 6.04 | Ubiquitous(?), mainly in skeletal and cardiac muscles (Nucleus, cajal body, cytoplasm) | Oligomer/dimer (?) | ACD, putative dimer interface [polypeptide binding], poly serine | Q9UBY9, NP_055239.1 | | No |
| HSPB8 | Hsp22, H11 protein kinase, product of E2IG1 gene | 196 | 21 604 | 5 | Ubiquitous, mainly in brain and muscles (Nucleus, Cytoplasm) | Dimer/monomer | ACD | Q9UJY1 | | 1 at N-terminal and 1 at ACD |
| HSPB9 | | 159 | 17 486 | 9.16 | Testis (Nucleus, Cytoplasm) | ? | ACD | Q9BQS6 | | 1 at N-terminal and 1 at ACD |
| HSPB10 | ODF1, ODFP1 | 250 | 28 366 | 8.46 | Testis (outer dense fiber, nucleus) | ? | ACD, putative dimer interface [polypeptide binding], 2 X 5 AA repeats of [RC]-C-L-C-D, C-X-P repeat | Q14990 | | 1 at N-terminal and 1 at ACD |

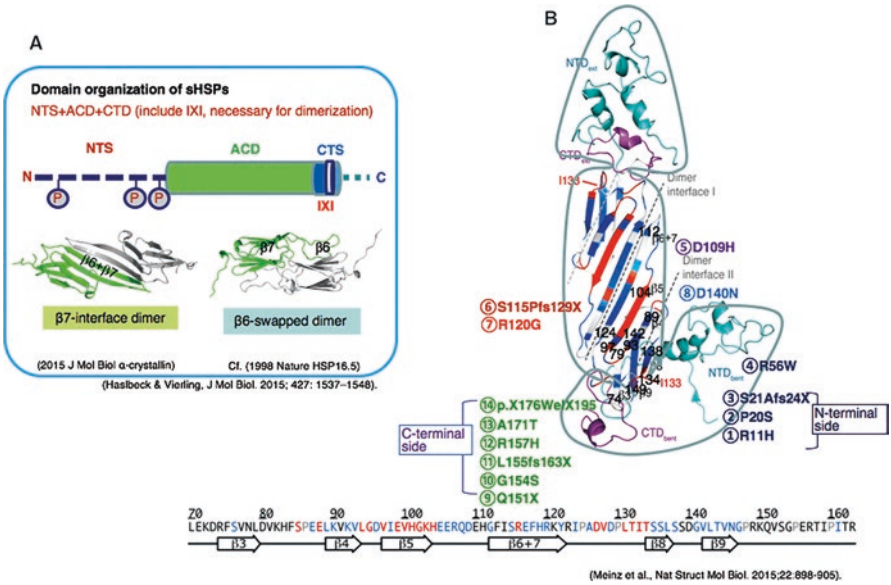


**Fig. 13.17 Domain organization of small HSP and αB-crystallin/CRYAB domain structure and its mutation**

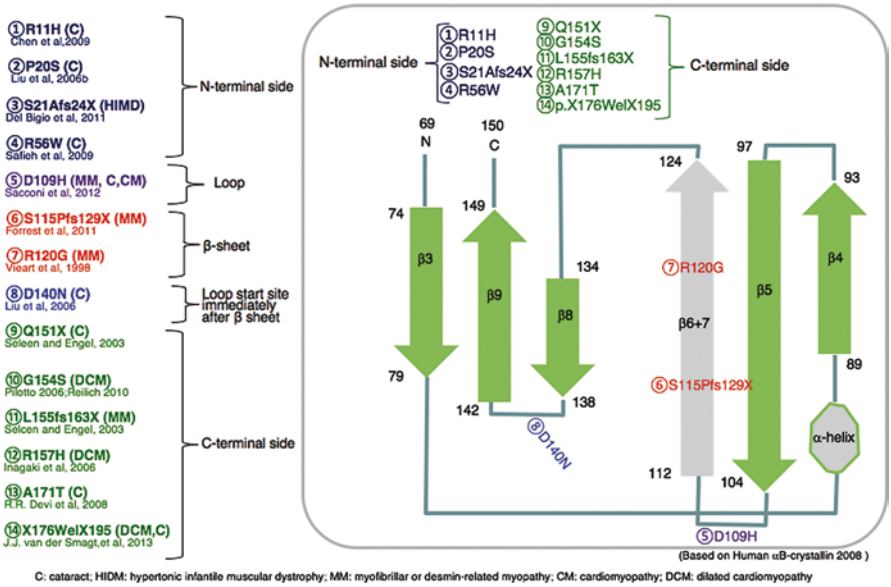


**Fig. 13.18** Topology of the secondary structure of a ACD domain (PDB 2klr, https://www.ebi.ac.uk/pdbe/) and location of mutation in muscle tissue

it supports more subtle conformational changes than refolding. At the same time, CRYAB also binds to the microtubule-associated protein, changes the cellular localization and direction, and can alter or maintain the dynamic state of microtubules, actin, IF according to the purpose of the cell. Cytoskeletal proteins differ from other fibril-forming proteins (especially actin and tubulin) and globular proteins self-associate to form a filament structure. In the presence of high-energy phosphate compound (ATP for actin and GTP for tubulin), dynamic state of the fiber is always maintained by association / disassociation while degrading them. In this way, the cytoskeletal protein produces a state in which cells can cope with environmental changes. It is not as simple as you imagine, but a system is needed that stores free-form proteins that can be constantly associated in the vicinity of microtubules. When you deposit money in a bank, you got interest earlier. If small HSP oligomers made by CRYAB exist like banks bound to microtubules, there is a high possibility that instead of attaching interest, it functions as a resting place to repair a small fray. CRYAB Banks are set up as materials for rails in cells or materials with lining structure of cell membranes, and constitute infrastructure. We are surprised about the multifunctionality of CRYAB. In small HSP, the domain structure of the protein may be functioning as a repairman / watchman, guardian of the cell system.

The interior of the cell is basically complicated, and among them, the striated muscle tissue is such that large molecules of proteins are aligned, so water molecules can not exist as well. In striated muscle tissue, migration occurs so that large proteins can be seen structurally with the naked eye. Small HSP do not interfere with their structure and take care by brief interactions with proteins at very early stages of minor changes and degeneration anytime anywhere. In our laboratory, FRAP analysis of CRYAB tagged with GFP was carried out in order to investigate the kinetics of CRYAB inside contracting myocardium. As a result, a CRYAB was detected in a short time within 1 second, bleaching recovered and FRET was observed between fluorescently labeled tubulin. Dynamics changed by nocodazole treatment which enhances disassembly of microtubules and taxol treatment which inhibits disassembly of microtubules. From these facts, it seems that, CRYAB is taking care of the cytoskeleton/ tubulin at least within the cell. Besides that, under stress conditions, it has been reported that taking care of the α-helix structure in order to avoid degradation of FAK by calpain (described later). The disease name of a person mutated in the middle part of R120G ACD is desmin myopathy. Looking at other CRYAB mutations in muscle (Figs. 13.17 and 13.18), myofibrils and myopathies caused by desmin are mentioned. Whether desmin is actually the causative gene or not, I do not know by desk discussion. It seems to be a function of CRYAB to keep necessary molecules ready for use at any time. What is actually happening in the pathology of muscle and cardiac muscle where mutation occurs in of CRYAB itself? Since CRYAB itself has a mutation, we cannot ask for a function as a chaperone. Furthermore, CRYAB itself undergoes co-aggregation denaturation depending on the mutation. Desmin Myopathy is the resulting naming, but perhaps there are a number of possible causes besides desmin. In the first place, most molecules are responsible for “transport” of “microtubule rails” such as kinesin and

dynein "carriers" proteins. The free form of desmin is also transported by microtubules. This transportation is not a hard rail, the rails themselves should also be kept dynamic. The pathological condition in skeletal muscle and myocardium caused by mutation of CRYAB may actually be involved in tubulin and microtubules. Sakurai et al. reported that fusion did not work on C2C12 myoblasts that reduced the expression of CRYAB (Sakurai et al. 2005). In recent studies on protein homeostasis of small HSP (Waters et al. 1996; Haslbeck and Vierling 2015), small HSP is conserved in eukaryotes, prokaryotes, plants and animals, but according to the form of the living organism, the N-terminal-ACD-C-terminal domain evolved independently and inevitably became such a structure (and sequence).

ACD is highly conserved and it has been shown that small HSP already exists in the last common ancestor of prokaryotes and eukaryotes (Kappe et al. 2002, Kriehuber et al. 2010, Waters 1995). We aligned the αB-crystallin homologs (including predicted) on the database and HSP 16.5 which analyzed the crystal structure of the ACD domain and the yeast homologue, Position of reported β-sheet (Fig. 13.19). CRYAB is preserved in animals after bilaterally symmetric animals, and recent research has reported roles in muscle development of Drosophila. CRYAB is considered to be an environmentally adaptive molecule after the birth of an individual. Since mechanical stress is also generated during embryonic cleavages and morphogenesis, possible involvement of CRYAB in developmental program has already been discussed above.

Small HSP is characteristically found in the cytoskeleton, nucleus, cytoplasm, exosome etc. (Sreekumar et al. 2010; Uhlen et al. 2015), respectively, for protection of the substrate. Since CRYAB is characterized by ubiquitous membrane localization, its function in lipid bilayer membranes (Huang et al. 2016) and mitochondrial membranes (McGreal et al. 2012) is of interest, so we take another opportunity. Substrate recognition diversity is at the N-terminal (and C-terminal), and the C-terminal I-x-I/V motif is required for oligomerization. The oligomer before substrate recognition, also called latent type, constantly exchanges subunits dynamically (McHaourab et al. 2009) and stepping on so that they can work anytime. In lower eukaryotes such as baker's yeast, two small HSP, Hsp26 and Hsp 42 act independently and do not form hetero-oligomers (Duennwald et al. 2012; Haslbeck et al. 2004; Specht et al. 2011). In humans (and other vertebrates), specific small HSP are hetero-oligomerized in vivo (for example, human αA-crystallin and CRYAB in crystalline lenses) because the function of homeostatic maintenance carried by small HSP is diverse in higher animals, which can act in parallel as a homooligomer. (Arrigo 2013; Datskevich et al. 2012). It is the philosophy of life to advocate that life evolves to survive and small HSP has been brought into the cell for it. Since small HSP cannot recognize fully denatured substrates, it needs to be assembled by capturing the initial structural changes close to denaturation as in the process of substrate denaturation. It does not require the energy of ATP, but phosphorylation is necessary for substrate recognition under stress conditions (Haslbeck and Vierling 2015). This is because the N-terminus is hidden during oligomer formation.

It is necessary for it to exist stoichiometrically over the substrate by more than 1: 1. Molecular dynamics simulations of NTS in dimeric form of wheat Hsp16.9

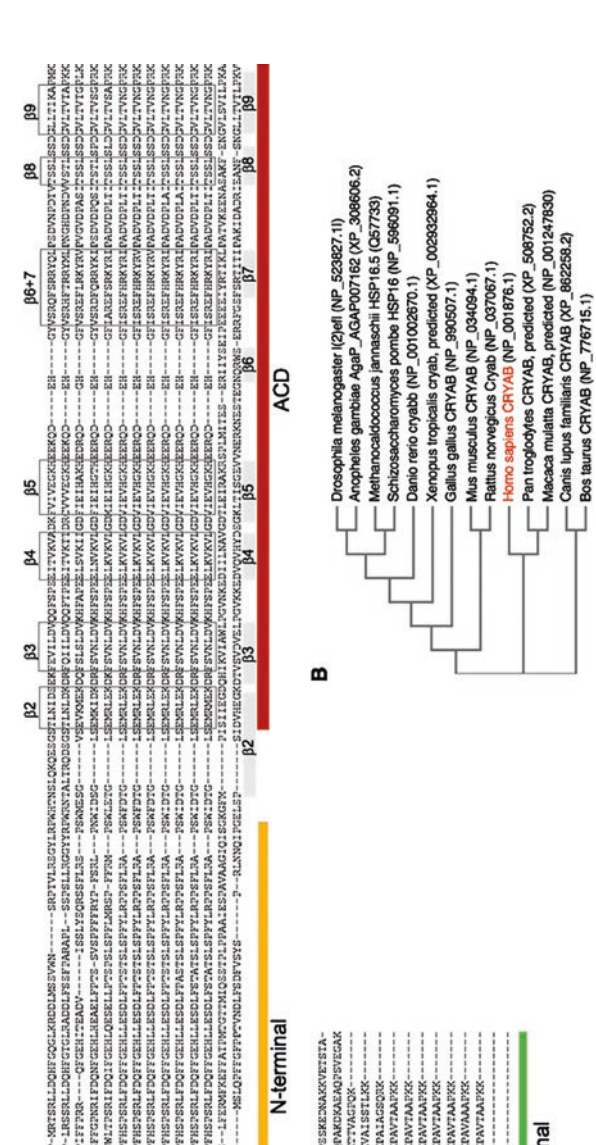


**Fig. 13.19 Homology comparison among species of αB-crystallin/CRYAB** (**a**) Multiple sequence alignment of twelve CRYAB homolog and two HSP16 and (**b**) Phylogenetic Tree (Neighbour-joining tree without distance corrections) created by Clustral Omega. β-sheets are highligted based on (Bagnéris et al. 2009). C-terminal IXI motif are soround by frame. Dlosophila and A homolog lacks IXI motif. CRYAB/HSPB5 is highly conserved in Bilateraia. Amino acid identities vs human by pairwise alignment: P.troglodytes (Pt) 99.4%, M.Mulatta (Mmul) 99.4%, C.lupus (Cl) 98.3%, B.taurus (Bt) 97.7%, M.musculus (Mm) 97.7%, R.norvegicus (Rn) 97.1%, G.gallus (Gg) 77%, D.rerio (Dr) 69.4%, D.Melanogaster (Dm) 63.2%, A.Gambiae (Ag) 47%, X.Tropicalis (Xt) 29.3%. Different dimer structures are reported for Human αB-crystallin (β7-interhace dimer) and M. jannaschii HSP16.5 (β6-swapped dimer) and it may reasoned by low amino acid identities (22%)

(1 GME) (and same pea Hsp18.1) showed hydrophobic surface patches in the range of 800 to 1700 Å 2 exposed on NTS, can access to the area of expanding substrate (Patel et al. 2014). Overall, there is extensive support for the main role of NTS in substrate binding. Independent evolution of NTS and CTS suggests that there is a change in the profile of the substrate recognized by evolutionally distant small HSP. The I-xI/V motif binds to the hydrophobic groove between the β4 and β8 chains within the ACD of the proximal monomer when the C-terminus is dissociated during oligomer degradation to expose the hydrophobic patch (Delbecq et al. 2012; Laganowsky et al. 2010; Treweek et al. 2010). The interaction between ACD and the substrate was hardly detected in crosslinking experiments and the β4 - β8 hydrophobic groove was less accessible to the hydrophobic surface for substrate binding than NTS (300 Å2) (Ahrman et al. 2007; Jaya et al. 2009; Patel et al. 2014). However, it has recently been shown that FAK binds to this area (Pereira et al. 2014). The hypothesis that withstands poor access is to use a structured β4 - β8 groove rather than the flexible N terminus for substrate protein recognition associated with cell death problems such as adhesion. Generally, N-terminal phosphorylation is necessary for activation, but we proved that the phenotype can be recovered without phosphorylation under non-stress conditions (Shimizu et al. 2016). CRYAB has been shown to interact with the FAK-focal adhesion targeting (FAT) domain in such an amount that phosphorylation is absent. The helical bundle structure of FAK-FAT domain is also contained in vinculin α-catenin (Hayashi et al. 2002) and we believe that these focal adhesion molecules are cared at the same time. Comprehensive analysis of recent astrocytes reports that cytoskeletal protein recognition by CRYAB does not require phosphorylation (Kuipers et al. 2017). CRYAB is always important to maintain homeostasis, adhesion integrity, even under stressful circumstances that can be easily imagined if it is mechanically stressed like a skeletal muscle. For caring for the cytoskeleton, activation by phosphorylation may not be required. Interestingly, there are no cysteine residues in HSPB5, 6, 7, it is impossible to form SS bonds which harden the structure. This suggests that the dynamics of these small HSP themselves may increase the efficiency of substrate recognition and also acquired greater chemical stabilities to oxidative stress. As we mentioned above, the small HSP dimers may be always monitoring the structural integrities of the structurally unstable substrate related to homeostasis.

### *13.6.3 Maintenance of "Dynamic Fiber Structure" of Tubulin / Microtubule Showing Dynamic Instability among Complicated Cells and Support by CRYAB: From Domain Mutant Analysis*

The subcellular localization of CRYAB and tubulin was completely consistent and co-precipitated by immunoprecipitation. Previously, we designed several truncation construct, expressed in bacteria, and examined the interaction between free tubulin

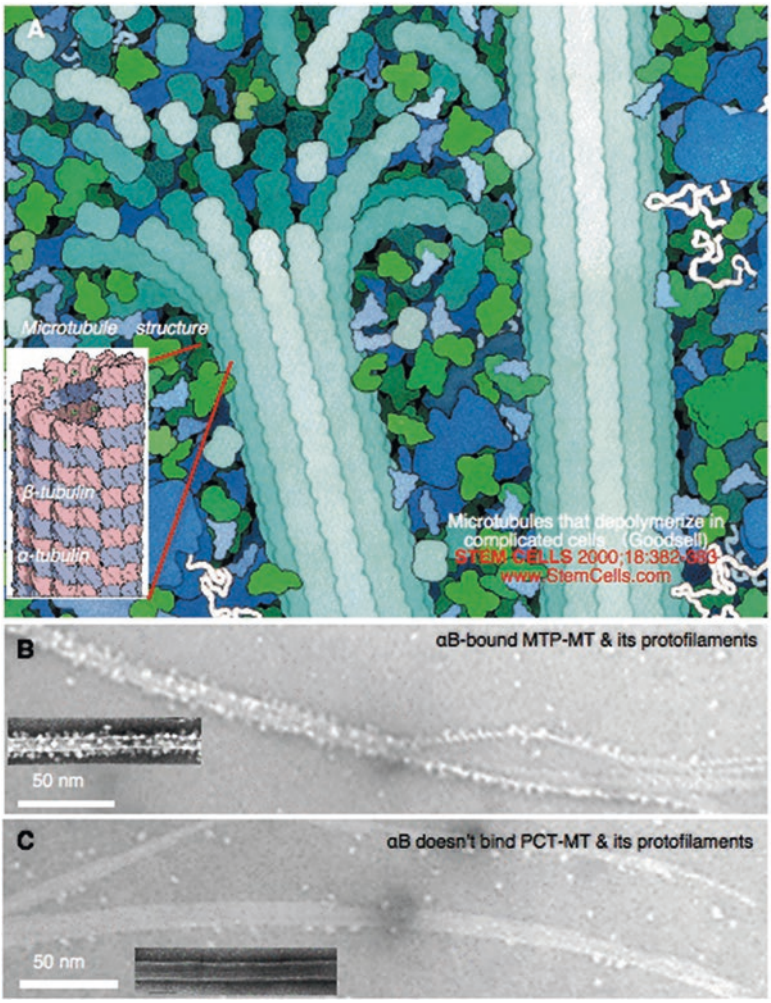


**Fig. 13.20 Model of interaction between αB-crystallin/CRYAB and tubulin/microtubule.** The reason that CRYAB binds to microtubules in cells in which dynamic instability is functioning: The interior of the cell is like a crowded train of macromolecules (**A**: Goodsell). For microtubules dynamic instability, it is necessary to place free tubulin in the vicinity without denaturation. CRYAB also binds to reassociated microtubules containing MAPs or to disassociated protofilaments (**B**). It hardly binds to microtubules and protofilaments associated with Taxol except for MAPs (**C**)

and one of three of crystallin domains (Fig. 13.20) (Ohto-Fujita et al. 2007). Other model substrates such as insulin showed no chaperone-like activity even if the N-terminus is missing. On the other hand, when tubulin is a substrate, even if the N-terminus of CRYAB is covered, even if there is no C-terminus, if ACD is present, the increase in turbidity due to heat denaturation can be suppressed. Although such truncated domain analysis is not usually done by small HSP researchers, our results

showed that substrate recognition for tubulin is obviously an ACD domain, but what kind of interaction it is still unknown. Fujita's data showing that CRYAB interacts with various model proteins at the N-terminus is consistent with lysozyme recognition by Mainz et al. (Mainz et al. 2015). It has been known that CRYAB has an effect of suppressing the aggregation of amyloid and α-synuclein so far, but this time it was found that ACD is an interface to Aβ by Mainz et al. (Mainz et al. 2015). It is interesting that ACD is used for tubulin which similarly fibrils. Clark et al. Synthesized various peptides of CRYAB, investigated the interaction with tubulin in vitro, found the region 47–49 in the β-tubulin sequences and the I-X-I/V motif of CRYAB β8, suggesting that tubulin monomers or dimers may be added to oligomer formation between small HSP (Houck and Clark 2010).

How is the actual assembly and disassembly of microtubules in complex cells (Fig. 13.20a). Myofibrillar proteins that produce sarcomere as striated muscles lined with narrowing. Furthermore, the muscles "contract". Even protein titin/ connectin that is responsible for elasticity of sarcomere changes elasticity, actually the length of sarcomere changes with increasing and decreasing $Ca^{2+}$ concentration each time it contracts. Elevation of $Ca^{2+}$ enhances F-actin formation, but microtubules tend to dissociate (Fujita et al. 2004). Being associated / disassociated with microtubules means that microtubules cannot be maintained unless unmodified tubulin, which is free of denaturation, is in the vicinity of microtubules. Figure (13.21b) is a photograph taken together with a photograph of an electron microscopy image CRYAB was attached to microtubules as previously reported (Fujita et al. 2004). When assembling microtubules in vitro from tubulin containing microtubule associating protein (MAP) it would probably be possible to produce protofilaments shown here while repeating association / dissociation. Many oligomers of CRYAB are bonded to protofilaments. If tubulin can be added to the CRYAB oligomer containing its small HSP, it is more convenient and functional (Houck and Clark 2010). It may be possible to establish a basis for maintaining and updating proteostasis that permanently withstands myocardial and contractile contractions only when this oligomer binds to microtubules and maintains its dynamic state. In fact, it has been reported that passive extension force increases as CRYAB binds to titin. Sarcomere is a dynamic structure and mutants of CRYAB that cannot form oligomers, binds to titin and does not leave immediately therefore it is reported to be an obstacle to contraction (Bullard et al. 2004).

To summarize the data of our laboratory (Fig. 13.17), not only CRYAB took care of the free tubulin at ACD but also was in charge of MAPs by N - terminal. In the small HSP research society, it is essential that small HSP form oligomers, so it is common sense not to perform deletion analysis. However, our findings are consistent with Mainz et al. (Mainz et al. 2015) and Pereira et al. (Pereira et al. 2014) showing FAK as a substrate of CRYAB molecular chaperon in vitro and in beating cardiomyocytes in vivo. Looking at those data, experiments detected the correct information. CRYAB that can interact with various biomaterials saving the aging society is believed to have supported and contributed to evolution from primates to humans. Evolution of the eye as an established organization has been on since the Cambrian era. The visibility and action strongly link. And CRYAB is a central molecular chaperone supporting visual and exercise for evolution into humans after

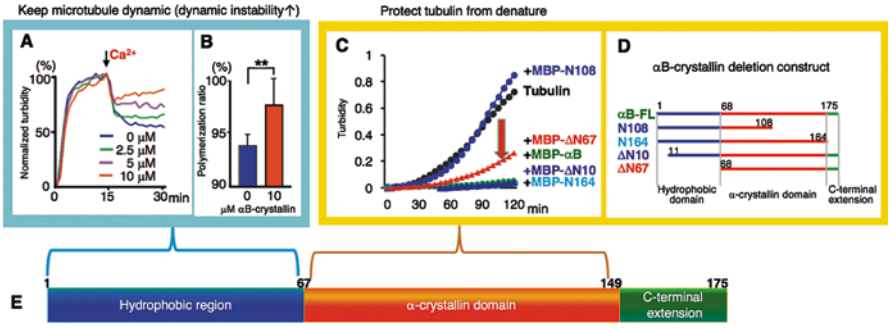


**Fig. 13.21 Chaperone-like activities of αB-crystalli/CRYAB for tubulin and microtubule relating to its domain structure.** (**A**, **B**) NTS (N-terminal side) of CRYAB acts to suppress diassembly for microtubules. (**A**) Inhibitory effect of microtubule diassembly during treatment with 1 mM calcium by CRYAB. After assembly of microtubules for 15 minutes in the presence of various concentrations of CRYAB, 2 mM calcium at final concentration (free calcium concentration is approximately 1 mM) was added (at the time of the arrow). The change in turbidity was shown (relative value with turbidity taken as 100% after 15 minutes from start of assembly). (**B**) Suppression of microtubule disassembly after treatment with 113 μM free calcium concentration in the presence or absence of CRYAB. Microtubules disassembled for 15 minutes in the presence of 0 or 10 μM CRYAB and then the change in turbidity was measured after addition of calcium (free calcium concentration is approximately 113 μM). Assembly amount was shown as the ratio of the average value of the turbidity immediately after addition of calcium. n = 6, ** $p < 0.01$. (**C**) ACD of CRYAB has chaperone activity and suppresses denaturation of free tubulin. Tubulin (1 mg/ml) and MBP fused deletion CRYAB (0.3 mg/ml) were incubated for 2 h in 80 mM PIPES (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP at 42 °C and the turbidity at 350 nm was measured. (**D**) Construction of deleted CRYAB. Structure of deleted CRYAB used in the experiment of (**C**). Each domain was color coded. The number of each construct indicates the amino acid number from the N terminus. MBP was designed to be attached to the N-terminal side of each deletion CRYAB. (**E**) Domain structure of CRYAB

living on land. Furthermore, muscles (slow muscle, cardiac muscle, diaphragm) with high CRYAB expression can be maintained healthy by appropriate exercise by human will. In order to prevent neurofibrils from becoming aggregates at an early stage, a specific method for inducing appropriate CRYAB is required. It can be said that it is an HSP which is the central axis of countermeasures for aging society.

### *13.6.4 Use of Pulsating Myocardial Cultured Cells as a Non-stressed but Constantly Mechanical Stressed Model*

CRYAB is thought to recognize a slight structural change of these proteins "initial structural change of fibrillated protein". We studied this by FLAP which shows the exchange of molecules of living cells Fig. (13.22) was studied by introducing GFP-CRYAB into pulsating cardiomyocytes, and ytescrystallin turnover was less than 1 second. As a preliminary experiment, CRYAB with tags for visualization and tubulin interact with each other by FRET analysis. Although this may reflect the

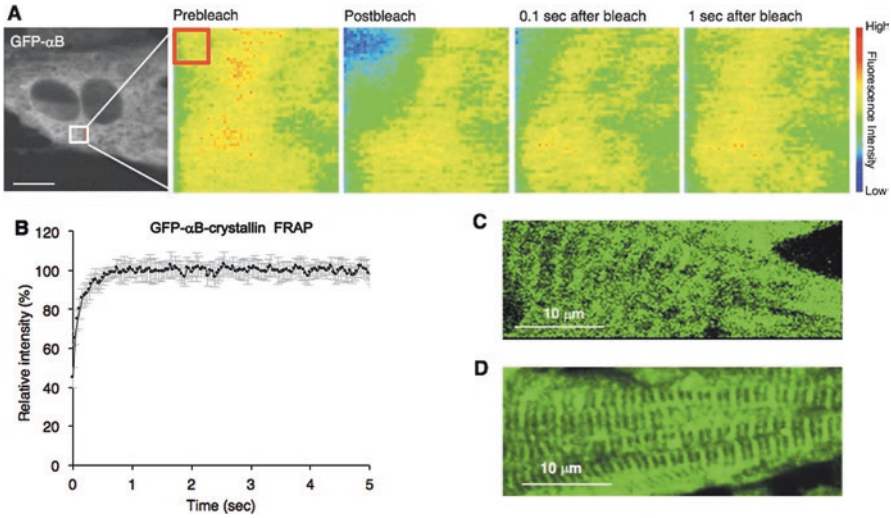


**Fig. 13.22 αB-crystallin/CRYAB recovered within 1 second at the substrate protein.** To investigate the kinetics of CRYAB inside contracting skeletal muscle, we performed a FRAP analysis of CRYAB tagged with GFP**.** (**a**) Cardiomyocyte transiently expressing GFP-αB-crystallin was imaged with a Zeiss LSM510 microscope. A typical single whole cell is shown in leftmost panel, and enlarged time-lapse views of the area indicated by the white square with pseudo-colors are shown to the right. GFP fluorescence was photobleached in the red-boxed area in the 'prebleach' image. Images were obtained before and immediately after photobleaching at 37°C. Scanning times were 124 ms and 39 ms for photobleaching and imaging, respectively. Bar = 10 μm. (**b**) The fluorescence intensity in each bleached area shown in top was plotted as a function of time. Mean ± SD. n = 10. (**c**) Striation of GFP-αB-crystallin in non-stressed beating cardiomyocyte. (**d**) Marked striated GFP-αB-crystallin after severe heat stress at 44.5 °C for 1 h

quick subunit exchange between the CRYAB oligomers, adding taxol or nocodazole, which is a microtubule dynamics modification reagent, changed the FRET profile by these reagents. Therefore, it can be considered that tubulin is contained in at least a substrate on which CRYAB n interacts (manuscript submitted).

## 13.7 Body-Mind Integrative Science From Small HSP/CRYAB

- Even 10 min landing/day stimulates muscle and brain-behavior.
- Movement/exercise integrates a dual life unit consisting of activity-dependent life systems cell and body: Sophia and Phronesis.
- Knowing the role SMALL HSP/CRYAB that has played in the evolution and the its existence taught us the importance of correctly exercising and acting on us living in an aging society.

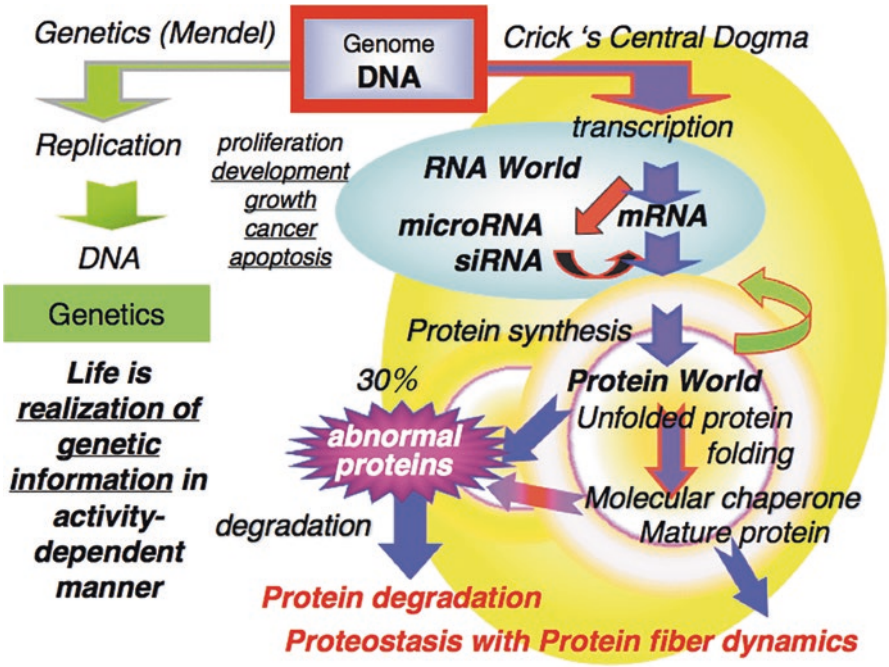


**Fig. 13.23** Exercise facilitates proteostasis through cytoskeleton protein dynamics

### *13.7.1 Even 10 minutes Landing/Day Stimulates Muscle and Brain-Behavior*

Rat hindlimb suspension is a slow muscle atrophy model. However, it can also be used as a research model for mental disorders. Therefore, the feet hanging on the hind legs were allowed to land for 10 minutes a day, and we observed the change in behavior by conducting an open field experiment in (Fig. 13.23). In rats with hindlimb suspension only, after one week, the free movement distance in the open field decreased. Not only did the expression level of CRYAB in soleus muscle of rats landed for just 10 minutes were significantly higher, but the activity amount in the open field was also not significantly different from the group with only hindlimb suspension, but it tended to be high was there. And, looking at the relation between the activity amount and the change amount in the open field of the rat before the experiment, there was no correlation in the group with only hindlimb suspension, but the relationship between both control and the group landing for 10 min was similar. That is rats that were active before the experiment tended to be active and continue to exist if hindlimb suspension conditions to land even for 10 minutes. Our test was rather small, and it is necessary to confirm the result in large scale. Also, if it gets bedridden, if you provide an appropriate program, you may be able to maintain its activity.

The aspects of mobility of individual activity were maintained by landing just for 10 minutes during rat hindlimb suspension as human bed rest model. However, it is possible to understand for us as follows; this result suggests that exercise is not only raising physical strength but also the possibility of growing active mind trying to live, especially in the case of humans possibly considering into "pursuing and cultivating "human will." Based on the rat's experiment, by educating the essence of activity for human beings, if the mind becomes active, that human seems to be able to create a circuit that changes the brain's circuitry and lives positively.

### *13.7.2 Movement/Exercise Integrates a Dual Life Unit Consisting of Activity-Dependent Life Systems Cell and Body: Sophia and Phronesis*

Molecular chaperone research should be categorized as the science of the bases of "adaptive life system." It is a fundamental academic field that can simultaneously present not only knowledge (Sophia) but also practical knowledge (Phronesis) which enhance one's homeostasis. Although there is growing attention to drug discoveries targeting HSP, HSP science should be heading towards a complete understanding of proteostasis mechanism which supports sustainable 100 years' life. We should move on to elucidate the adaptation mechanism of the system. The living is the continuous adaptation to the environment itself. That is, the biological system is possible to change because there are incredibly dynamic responding systems of the cells that make up the individual. The gene encoded by the sequence of the genomic DNA in the nucleus, cell transcribed a mRNA. Followed by translated a protein after receiving a signal corresponding to the stimulation/stress from inside and outside the cell and following many steps of reading the gene like transcription, translation with modification various RNA levels in an activity dependence. We will change our body. The first stimulus/stress does not come to genes cannot be read, just wait for decay. The ecological system circulates individuals decaying and changing to the ground (the earth). The life of one human being is the only one that cannot be replaced by anything. The recommendations of UNESCO in 2015 appeal to the essence of physical activity to create a world where each one can motivate themselves and actively. For that, it is necessary to have body-mind integrating science supported by HSP-based proteostasis system shown in (Fig. 13.24).

Here are words from distinguished scientists who amazed by a dynamic system of the cell. Gen Matsumoto, a formerly physicist, later brain scientist who made it possible to keep squid in the aquarium for the first time in the world, says animal cells are "output dependent" (Shigematsu et al. 1999). Matsumoto wanted to use big neuron of squid to start brain research. He understood our hypothesis to connect body and mind and the importance of exercise. Nobutaka Hirokawa, who is a cell biologist to study kinesin motor especially the brain and other cells, was surprised to find that kinesin protein was transit from the nucleus and work as a motor of the

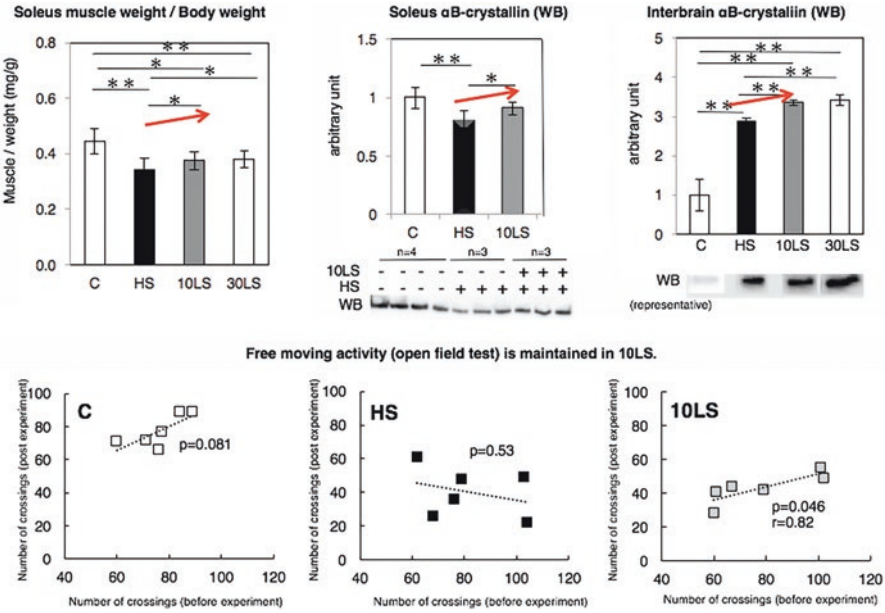


**Fig. 13.24 Good effects of landing stimulation to hindlimb rat on behavior during open field test.** In the hindlimb suspension (HS) group, 10-minutes-landing gave positive stimuli to rat and may improve soleus muscle atrophy, increased αB-crystallin/CRYAB expression in soleus muscle and also interbrain. It is postulated that possible significant relation between antigravitational muscle and interbrain to keep spontaneous activities, because positive relation between before and post-experiment was similar to control (C) group and 10 minutes loading (10LS) group. Landing stimulation is very important to keep active emotion

microtubule, immediately after neuron received calcium signal responding stimuli (Hirokawa 1998). Humans are multicellular animals, of course, adapt to an environment in the body as individuals. Only the brain and muscle alone can't stay alive. Even if you strengthen your neuromuscular workout, you will suffer joint pain, for example, and do exercise mood will be gone when it becomes difficult to move. Kids play actively using their bodies in anytime and anywhere as they like. Circuits connecting brain relations with the body will master in actual movement.

How to incorporate voluntary exercise as the foundation of an animal system in modern sedentary lifestyle is a big problem of the aged society. Aging science was born out of human problems. Each human has a will to live, will to exercise and act, and education is also possible which is a crucial difference because humans differ from other animals. The human brain is different from other animals because it makes a body capable of diverse activities. So it is convincing logic and background to maintain movement diversity research is necessary, and HSP should be a target. However, from the principle of life and the principle of adaptation of HSP, there is no research field which makes clear the essence of human movement at all. From the human life system itself, knowledge of life science, "exercise" is a stimulus

activate the life system, so it is clear from the stress response of the cell that body activity / physical activity is active. As the physical activity is declining as age, exercise will become the primary strategy for maintaining the healthy life.

In UNESCO's new international charter revised in 2015, not only physical education and sports but also "physical activity" was added to the wording. In fact, half a century ago WHO had a "human biology" perspective in the definition of health. But since the discovery of the DNA double helix structure in 1953 (Watson and Crick 1953), the viewpoint of seeing the whole human life science disappeared, reductionism and the science of science has become prosperous. "Exercise insufficiency" was born of a phenomenon in which low activity of daily life correlates with high mortality rate and prevalence, but it replaced words such as "lifestyle diseases" and "metabolic syndrome." "The second term of National Health Promotion Movement in the twenty-first century (Health Japan 21 (the second term))" (hereinafter National Movement) from 2013 fiscal year to 2022 financial year proposed Program to decide the amount and strength of exercise for improving energy metabolism and circulation. However, even if decides to exercise, he/she will not be able to exercise if the locomotive syndrome develops after the start of exercise. Exercise is an action that can be spontaneously performed only when the "moving system" of the body normally operates. There is no policy specialized for the elderly.

### *13.7.3 Knowing the Role of Small HSP, CRYAB that has Played in the Evolution and the its Existence Taught us the Importance of Correctly Exercising and Acting on us Living in an Aging Society*

The integrated medical project was running from early on in the United States because the state-of-the-art regenerative medicine took too much expense. But the plan was found to be terminated because less promising, and the health support program has begun instead. However, health support research also does not support health if it ends with phenomenology. Furthermore, although the merit of physical exercise is to activate the entire human body, physical exercise science follows reductionism same as modern life science, and it does not work for the integrated body. Improving only one muscle strength, it doesn't make people healthier. Also, both epidemiological and psychological research also don't contribute to individual well-being maybe because they don't give a practical program how and what to do. Exercise requires coordination between nervous and musculoskeletal system not to fall in the first place. There need to explore new exercise science to solve all the point above. Here we propose modern chaperone cell science which handle mechanical stress comes from daily exercise. During fertilized egg becomes an individual, the entire developmental process including early cleavages, embryogenesis, and morphogenesis are full of "mechanical stress" so there must have a biological function to handle it. We think it carried by HSP.

Many stars were born and disappeared in the universe, but it has become clear that there are not many stars where human beings-like creatures evolved. There was a physical and chemical environment namely stress to produce life on this planet. As "humans are born to run," it is natural to consider that mechanical stress also pushes the Homo sapience evolution forward. On the other hand, it's well known that HSP90 is a capacitor for evolution so why don't we include mechanical stress for HSP research. The living environment changes the way of Human movement. Our unstable bipedal standing posture can produce a tool. The shape of the body that counts the fingers is not only the tool but also the form of the body that creates the device. Human skills like touching and wandering may evolve from the shape of our body. Its amino acid sequence shapes protein. It is called Anphiensen's dogma. Each protein form determines the function of that protein. In cell HSP supports this creation. Small HSP CRYAB is still advancing evolution as a supporting entity supporting the standing body in the interaction with the environment.

## 13.8 Conclusions

Japan is the best longevity country in the world and health life expectancy is also the world's best. However, there is a difference of about 10 years between the average life expectancy and the healthy life expectancy. One of the reason may derived from lack of appropriate research area for solving world-wide health problems. In this review we described one of the small HSP, CRYAB, as an example of problem-solving research target. CRYAB supports control of the responsive dynamic structure of the cellular system. According to from the database (https://www.ncbi.nlm.nih.gov/gene/1410), CRYAB mainly express in lens / cardiac muscle / slow muscle / brain, however in this database data of slow skeletal muscle are not included. Especially for maintenance of QOL of a person, CRYAB is a core HSP. CRYAB was one of the subunits of a-crystallin discovered in lenses that support human vision in 1894 (Morner 1894). Muscles are filled longitudinally and laterally with myofibrils, and CRYAB is 5–6 mg/ml, but the lens is 800 mg/ml, 16 times more the muscle concentration (Seevaratnam et al. 2009). It seems essential that both lens (Barnes and Quinlan 2017) and muscle are dynamic. CRYAB, which supports a vision, also endorses the standing position of the person who was to provide a birds-eye viewpoint. Interestingly, the dynamics of "fiber" is the key for both lenses, muscles and conformation's diseases of the brain. We are waiting for the elucidation of the molecular mechanism of CRYAB that supports the dynamic fibrous structure. The practical knowledge Phronesis advocated by Aristotle can actually lead to the logic of the substance of life by connecting how to work correctly. We would like to point to the creation of a unified science that integrates the body and brain/mind, which makes own cells in the organization that is the core of human body efficiently activate/utilize resulting in creating humans from the insight of the global environment in which vertebrate animals that produced CRYAB were born.

**Acknowledgements** The works described here were partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Research grant from Japan Space Utilization Promotion Center, and Research grant from Japan Space Forum.

## References

Ahrman, E., Lambert, W., Aquilina, J. A., Robinson, C. V., & Emanuelsson, C. S. (2007). Chemical cross-linking of the chloroplast localized small heat-shock protein, Hsp21, and the model substrate citrate synthase. *Protein Science, 16*, 1464–1478. https://doi.org/10.1110/ps.072831607

Arac, A., Brownell, S. E., Rothbard, J. B., Chen, C., Ko, R. M., Pereira, M. P., Albers, G. W., Steinman, L., & Steinberg, G. K. (2011). Systemic augmentation of alphaB-crystallin provides therapeutic benefit twelve hours post-stroke onset via immune modulation. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 13287–13292. https://doi.org/10.1073/pnas.1107368108

Arai, H., & Atomi, Y. (1997). Chaperone activity of alpha B-crystallin suppresses tubulin aggregation through complex formation. *Cell Structure and Function, 22*, 539–544.

Arany, Z., Wagner, B. K., Ma, Y., Chinsomboon, J., Laznik, D., & Spiegelman, B. M. (2008). Gene expression-based screening identifies microtubule inhibitors as inducers of PGC-1alpha and oxidative phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 4721–4726. https://doi.org/10.1073/pnas.0800979105

Arrigo, A. P. (2013). Human small heat shock proteins: Protein interactomes of homo- and hetero-oligomeric complexes: An update. *FEBS Letters, 587*, 1959–1969. https://doi.org/10.1016/j.febslet.2013.05.011

Atomi, Y. (2012). Innovative science and education for understanding “Life” systems. *Trends In The Sciences, 17*(6), 86–93. https://doi.org/10.5363/tits.17.6_86

Atomi, Y. (2015). Gravitational effects on human physiology. *Sub-Cellular Biochemistry, 72*, 627–659. https://doi.org/10.1007/978-94-017-9918-8_29

Atomi, Y., & Miyashita, M. (1974). Maximal aerobic power of Japanese active and sedentary adult females of different ages (20 to 62 years). *Medicine and Science in Sports, 6*, 223–225.

Atomi, Y., & Miyashita, M. (1980). Effect of training intensity in adult females. *European Journal of Applied Physiology and Occupational Physiology, 44*, 109–116.

Atomi, Y., Iwaoka, K., Hatta, H., Miyashita, M., & Yamamoto, Y. (1986). Daily physical activity levels in preadolescent boys related to VO2max and lactate threshold. *European Journal of Applied Physiology and Occupational Physiology, 55*, 156–161.

Atomi, Y., Fukunaga, T., Hatta, H., & Yamamoto, Y. (1987). elationship between lactate threshold during running and relative gastrocnemius area. *Journal of Applied Physiology, 1985*(63), 2343–2347.

Atomi, Y., Yamada, S., & Hong, Y.-M. (1990). Dynamic expression of αB-Crystallin in skeletal muscle Effects of Unweighting, Passive Stretch and Denervation. *Proceedings of the Japan Academy, Series B, 66*, 203–208. https://doi.org/10.2183/pjab.66.203

Atomi, Y., Yamada, S., & Nishida, T. (1991a). Early changes of alpha B-crystallin mRNA in rat skeletal muscle to mechanical tension and denervation. *Biochemical and Biophysical Research Communications, 181*, 1323–1330.

Atomi, Y., Yamada, S., Strohman, R., & Nonomura, Y. (1991b). Alpha B-crystallin in skeletal muscle: Purification and localization. *Journal of Biochemistry, 110*, 812–822.

Atomi, Y., Toro, K., Masuda, T., & Hatta, H. (2000). Fiber-type-specific alphaB-crystallin distribution and its shifts with T(3) and PTU treatments in rat hindlimb muscles. *Journal of Applied Physiology, 1985*(88), 1355–1364.

Atomi, T., Noriuchi, M., Oba, K., Atomi, Y., & Kikuchi, Y. (2014). Self-recognition of one’s own fall recruits the genuine bodily crisis-related brain activity. *PLoS One, 9*, e115303. https://doi.org/10.1371/journal.pone.0115303

Bagneris, C., Bateman, O. A., Naylor, C. E., Cronin, N., Boelens, W. C., Keep, N. H., & Slingsby, C. (2009). Crystal structures of alpha-crystallin domain dimers of alphaB-crystallin and Hsp20. *Journal of Molecular Biology, 392*, 1242–1252. https://doi.org/10.1016/j.jmb.2009.07.069.

Barnes, S., & Quinlan, R. A. (2017). Small molecules, both dietary and endogenous, influence the onset of lens cataracts. *Experimental Eye Research, 156*, 87–94. https://doi.org/10.1016/j.exer.2016.03.024

Bianconi, E., Piovesan, A., Facchin, F., Beraudi, A., Casadei, R., Frabetti, F., Vitale, L., Pelleri, M. C., Tassani, S., Piva, F., Perez-Amodio, S., Strippoli, P., & Canaider, S. (2013). An estimation of the number of cells in the human body. *Annals of Human Biology, 40*, 463–471. https://doi.org/10.3109/03014460.2013.807878

Brady, J. P., Garland, D. L., Green, D. E., Tamm, E. R., Giblin, F. J., & Wawrousek, E. F. (2001). AlphaB-crystallin in lens development and muscle integrity: A gene knockout approach. *Investigative Ophthalmology & Visual Science, 42*, 2924–2934.

Bramble, D. M., & Lieberman, D. E. (2004). Endurance running and the evolution of homo. *Nature, 432*, 345–352. https://doi.org/10.1038/nature03052

Bullard, B., Ferguson, C., Minajeva, A., Leake, M. C., Gautel, M., Labeit, D., Ding, L., Labeit, S., Horwitz, J., Leonard, K. R., & Linke, W. A. (2004). Association of the chaperone alphaB-crystallin with titin in heart muscle. *The Journal of Biological Chemistry, 279*, 7917–7924. https://doi.org/10.1074/jbc.M307473200

Courtine, G., Song, B., Roy, R. R., Zhong, H., Herrmann, J. E., Ao, Y., Qi, J., Edgerton, V. R., & Sofroniew, M. V. (2008). Recovery of supraspinal control of stepping via indirect propriospinal relay connections after spinal cord injury. *Nature Medicine, 14*, 69–74. https://doi.org/10.1038/nm1682

Datskevich, P. N., Mymrikov, E. V., & Gusev, N. B. (2012). Utilization of fluorescent chimeras for investigation of heterooligomeric complexes formed by human small heat shock proteins. *Biochimie, 94*, 1794–1804. https://doi.org/10.1016/j.biochi.2012.04.012

de Jong, W. W., Leunissen, J. A., & Voorter, C. E. (1993). Evolution of the alpha-crystallin/small heat-shock protein family. *Molecular Biology and Evolution, 10*, 103–126.

Delbecq, S. P., Jehle, S., & Klevit, R. (2012). Binding determinants of the small heat shock protein, alphaB-crystallin: Recognition of the 'IxI' motif. *The EMBO Journal, 31*, 4587–4594. https://doi.org/10.1038/emboj.2012.318

Duennwald, M. L., Echeverria, A., & Shorter, J. (2012). Small heat shock proteins potentiate amyloid dissolution by protein disaggregases from yeast and humans. *PLoS Biology, 10*, e1001346. https://doi.org/10.1371/journal.pbio.1001346

Eyles, S. J., & Gierasch, L. M. (2010). Nature's molecular sponges: Small heat shock proteins grow into their chaperone roles. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 2727–2728. https://doi.org/10.1073/pnas.0915160107

Fujita, Y., Ohto, E., Katayama, E., & Atomi, Y. (2004). alphaB-Crystallin-coated MAP microtubule resists nocodazole and calcium-induced disassembly. *Journal of Cell Science, 117*, 1719–1726. https://doi.org/10.1242/jcs.01021

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., & Yanagida, T. (1995). Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature, 374*, 555–559. https://doi.org/10.1038/374555a0

Goodsell, D. S. (1991). Inside a living cell. *Trends in Biochemical Sciences, 16*, 203–206.

Gundersen, G. G., & Cook, T. A. (1999). Microtubules and signal transduction. *Current Opinion in Cell Biology, 11*, 81–94.

Haslbeck, M., & Vierling, E. (2015). A first line of stress defense: Small heat shock proteins and their function in protein homeostasis. *Journal of Molecular Biology, 427*, 1537–1548. https://doi.org/10.1016/j.jmb.2015.02.002

Haslbeck, M., Braun, N., Stromer, T., Richter, B., Model, N., Weinkauf, S., & Buchner, J. (2004). Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. *The EMBO Journal, 23*, 638–649. https://doi.org/10.1038/sj.emboj.7600080

Hatta, H., Atomi, Y., Yamamoto, Y., Shinohara, S., & Yamada, S. (1989). Incorporation of blood lactate and glucose into tissues in rats after short-term strenuous exercise. *International Journal of Sports Medicine, 10*, 275–278. https://doi.org/10.1055/s-2007-1024915

Hayashi, I., Vuori, K., & Liddington, R. C. (2002). The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nature Structural Biology, 9*, 101–106. https://doi.org/10.1038/nsb755

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science, 279*, 519–526.

Hochberg, G. K., Ecroyd, H., Liu, C., Cox, D., Cascio, D., Sawaya, M. R., Collier, M. P., Stroud, J., Carver, J. A., Baldwin, A. J., Robinson, C. V., Eisenberg, D. S., Benesch, J. L., & Laganowsky, A. (2014). The structured core domain of alphaB-crystallin can prevent amyloid fibrillation and associated toxicity. *Proceedings of the National Academy of Sciences of the United States of America, 111*, E1562–E1570. https://doi.org/10.1073/pnas.1322673111

Houck, S. A., & Clark, J. I. (2010). Dynamic subunit exchange and the regulation of microtubule assembly by the stress response protein human alphaB crystallin. *PLoS One, 5*, e11795. https://doi.org/10.1371/journal.pone.0011795

Hsu, A. L., Murphy, C. T., & Kenyon, C. (2003). Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science, 300*, 1142–1145. https://doi.org/10.1126/science.1083701

Huang, Y., Wang, Z., Liu, Y., Xiong, H., Zhao, Y., Wu, L., Yuan, C., Wang, L., Hou, Y., Yu, G., Huang, Z., Xu, C., Chen, Q., & Wang, Q. K. (2016). alphaB-Crystallin interacts with Nav1.5 and regulates ubiquitination and internalization of cell surface Nav1.5. *The Journal of Biological Chemistry, 291*, 11030–11041. https://doi.org/10.1074/jbc.M115.695080

Ikeda, N., Saito, E., Kondo, N., Inoue, M., Ikeda, S., Satoh, T., Wada, K., Stickley, A., Katanoda, K., Mizoue, T., Noda, M., Iso, H., Fujino, Y., Sobue, T., Tsugane, S., Naghavi, M., Ezzati, M., & Shibuya, K. (2011). What has made the population of Japan healthy? *Lancet, 378*, 1094–1105. https://doi.org/10.1016/S0140-6736(11)61055-6

Ishihara, K., Nguyen, P. A., Groen, A. C., Field, C. M., & Mitchison, T. J. (2014). Microtubule nucleation remote from centrosomes may explain how asters span large cells. *Proceedings of the National Academy of Sciences of the United States of America, 111*, 17715–17722. https://doi.org/10.1073/pnas.1418796111

Ito, H., Okamoto, K., Nakayama, H., Isobe, T., & Kato, K. (1997). Phosphorylation of alphaB-crystallin in response to various types of stress. *The Journal of Biological Chemistry, 272*, 29934–29941.

Jaya, N., Garcia, V., & Vierling, E. (2009). Substrate binding site flexibility of the small heat shock protein molecular chaperones. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 15604–15609. https://doi.org/10.1073/pnas.0902177106

Jee, H., Sakurai, T., Kawada, S., Ishii, N., & Atomi, Y. (2009). Significant roles of microtubules in mature striated muscle deduced from the correlation between tubulin and its molecular chaperone alphaB-crystallin in rat muscles. *The Journal of Physiological Sciences, 59*, 149–155. https://doi.org/10.1007/s12576-008-0014-6

Jehle, S., Rajagopal, P., Bardiaux, B., Markovic, S., Kuhne, R., Stout, J. R., Higman, V. A., Klevit, R. E., van Rossum, B. J., & Oschkinat, H. (2010). Solid-state NMR and SAXS studies provide a structural basis for the activation of alphaB-crystallin oligomers. *Nature Structural & Molecular Biology, 17*, 1037–1042. https://doi.org/10.1038/nsmb.1891

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B., & Hightower, L. E. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress & Chaperones, 14*, 105–111. https://doi.org/10.1007/s12192-008-0068-7

Kappe, G., Leunissen, J. A., & de Jong, W. W. (2002). Evolution and diversity of prokaryotic small heat shock proteins. *Progress in Molecular and Subcellular Biology, 28*, 1–17.

Kato, K., Goto, S., Inaguma, Y., Hasegawa, K., Morishita, R., & Asano, T. (1994). Purification and characterization of a 20-kDa protein that is highly homologous to alpha B crystallin. *The Journal of Biological Chemistry, 269*, 15302–15309.

Kennedy, B. K., Berger, S. L., Brunet, A., Campisi, J., Cuervo, A. M., Epel, E. S., Franceschi, C., Lithgow, G. J., Morimoto, R. I., Pessin, J. E., Rando, T. A., Richardson, A., Schadt, E. E., Wyss-Coray, T., & Sierra, F. (2014). Geroscience: Linking aging to chronic disease. *Cell, 159*, 709–713. https://doi.org/10.1016/j.cell.2014.10.039

Kenyon, C., Chang, J., Gensch, E., Rudner, A., & Tabtiang, R. (1993). A C. Elegans mutant that lives twice as long as wild type. *Nature, 366*, 461–464. https://doi.org/10.1038/366461a0

Kim, K. K., Kim, R., & Kim, S. H. (1998). Crystal structure of a small heat-shock protein. *Nature, 394*, 595–599. https://doi.org/10.1038/29106

Kim, S. G., Akaike, T., Sasagaw, T., Atomi, Y., & Kurosawa, H. (2002). Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. *Cell Structure and Function, 27*, 139–144.

Koh, T. J., & Escobedo, J. (2004). Cytoskeletal disruption and small heat shock protein translocation immediately after lengthening contractions. *American Journal of Physiology. Cell Physiology, 286*, C713–C722. https://doi.org/10.1152/ajpcell.00341.2003

Kriehuber, T., Rattei, T., Weinmaier, T., Bepperling, A., Haslbeck, M., & Buchner, J. (2010). Independent evolution of the core domain and its flanking sequences in small heat shock proteins. *The FASEB Journal, 24*, 3633–3642. https://doi.org/10.1096/fj.10-156992

Kuipers, H. F., Yoon, J., van Horssen, J., Han, M. H., Bollyky, P. L., Palmer, T. D., & Steinman, L. (2017). Phosphorylation of alphaB-crystallin supports reactive astrogliosis in demyelination. *Proceedings of the National Academy of Sciences of the United States of America, 114*, E1745–E1754. https://doi.org/10.1073/pnas.1621314114

Laganowsky, A., Benesch, J. L., Landau, M., Ding, L., Sawaya, M. R., Cascio, D., Huang, Q., Robinson, C. V., Horwitz, J., & Eisenberg, D. (2010). Crystal structures of truncated alphaA and alphaB crystallins reveal structural mechanisms of polydispersity important for eye lens function. *Protein Science, 19*, 1031–1043. https://doi.org/10.1002/pro.380

Liu, Y., & Steinacker, J. M. (2001). Changes in skeletal muscle heat shock proteins: Pathological significance. *Frontiers in Bioscience, 6*, D12–D25.

Locke, M., & Noble, E. G. (1995). Stress proteins: The exercise response. *Canadian Journal of Applied Physiology, 20*, 155–167.

Mainz, A., Peschek, J., Stavropoulou, M., Back, K. C., Bardiaux, B., Asami, S., Prade, E., Peters, C., Weinkauf, S., Buchner, J., & Reif, B. (2015). The chaperone alphaB-crystallin uses different interfaces to capture an amorphous and an amyloid client. *Nature Structural & Molecular Biology, 22*, 898–905. https://doi.org/10.1038/nsmb.3108

Manenti, S., Dunia, I., & Benedetti, E. L. (1990). Fatty acid acylation of lens fiber plasma membrane proteins. MP26 and alpha-crystallin are palmitoylated. *FEBS Letters, 262*, 356–358.

Maruyama, K. (1976). Connectin, an elastic protein from myofibrils. *Journal of Biochemistry, 80*, 405–407.

McGreal, R. S., Kantorow, W. L., Chauss, D. C., Wei, J., Brennan, L. A., & Kantorow, M. (2012). AlphaB-crystallin/sHSP protects cytochrome c and mitochondrial function against oxidative stress in lens and retinal cells. *Biochimica et Biophysica Acta, 1820*, 921–930. https://doi.org/10.1016/j.bbagen.2012.04.004

McHaourab, H. S., Godar, J. A., & Stewart, P. L. (2009). Structure and mechanism of protein stability sensors: Chaperone activity of small heat shock proteins. *Biochemistry, 48*, 3828–3837. https://doi.org/10.1021/bi900212j

Mitchison, T., & Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature, 312*, 237–242.

Mitchison, T. J., & Kirschner, M. W. (1985). Properties of the kinetochore in vitro. II. Microtubule capture and ATP-dependent translocation. *The Journal of Cell Biology, 101*, 766–777.

Morimoto, R. I. (2008). Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes & Development, 22*, 1427–1438. https://doi.org/10.1101/gad.1657108

Morner, C. T. (1894). Untersuchung der Proteinsubstanzen in den lichtbrechenden Medien des Auges (Examination of the protein-substances in the refractory media of the eye). *Hoppe-Seyler's Zeitschrift für Physiologische Chemie, 18*, 61–106.

Morrow, G., & Tanguay, R. M. (2012). Small heat shock protein expression and functions during development. *The International Journal of Biochemistry & Cell Biology, 44*, 1613–1621. https://doi.org/10.1016/j.biocel.2012.03.009

Mymrikov, E. V., Daake, M., Richter, B., Haslbeck, M., & Buchner, J. (2017). The chaperone activity and substrate Spectrum of human small heat shock proteins. *The Journal of Biological Chemistry, 292*, 672–684. https://doi.org/10.1074/jbc.M116.760413

Ohkawa, T., Atomi, T., Hasegawa, K., & Atomi, Y. (2017). The free moment is associated with torsion between the pelvis and the foot during gait. *Gait & Posture, 58*, 415–420. https://doi.org/10.1016/j.gaitpost.2017.09.002

Ohto-Fujita, E., Fujita, Y., & Atomi, Y. (2007). Analysis of the alphaB-crystallin domain responsible for inhibiting tubulin aggregation. *Cell Stress & Chaperones, 12*, 163–171.

Patel, S., Vierling, E., & Tama, F. (2014). Replica exchange molecular dynamics simulations provide insight into substrate recognition by small heat shock proteins. *Biophysical Journal, 106*, 2644–2655. https://doi.org/10.1016/j.bpj.2014.04.048

Pereira, M. B., Santos, A. M., Goncalves, D. C., Cardoso, A. C., Consonni, S. R., Gozzo, F. C., Oliveira, P. S., Pereira, A. H., Figueiredo, A. R., Tiroli-Cepeda, A. O., Ramos, C. H., de Thomaz, A. A., Cesar, C. L., & Franchini, K. G. (2014). AlphaB-crystallin interacts with and prevents stress-activated proteolysis of focal adhesion kinase by calpain in cardiomyocytes. *Nature Communications, 5*, 5159. https://doi.org/10.1038/ncomms6159

Prosser, S. L., & Pelletier, L. (2017). Mitotic spindle assembly in animal cells: A fine balancing act. *Nature Reviews. Molecular Cell Biology, 18*, 187–201. https://doi.org/10.1038/nrm.2016.162

Quinlan, R. (2002). Cytoskeletal competence requires protein chaperones. In M. W. E. G. Arrigo (Ed.), *Small Stress Proteins* (pp. 219–233). AP.

Ralston, E., Lu, Z., & Ploug, T. (1999). The organization of the Golgi complex and microtubules in skeletal muscle is fiber type-dependent. *The Journal of Neuroscience, 19*, 10694–10705.

Raman, B., Ban, T., Sakai, M., Pasta, S. Y., Ramakrishna, T., Naiki, H., Goto, Y., & Rao Ch, M. (2005). AlphaB-crystallin, a small heat-shock protein, prevents the amyloid fibril growth of an amyloid beta-peptide and beta2-microglobulin. *The Biochemical Journal, 392*, 573–581. https://doi.org/10.1042/BJ20050339

Ray, P. S., Martin, J. L., Swanson, E. A., Otani, H., Dillmann, W. H., & Das, D. K. (2001). Transgene overexpression of alphaB crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion. *The FASEB Journal, 15*, 393–402. https://doi.org/10.1096/fj.00-0199com

Ruoslahti, E. (1997). Stretching is good for a cell. *Science, 276*, 1345–1346.

Sakurai, T., Fujita, Y., Ohto, E., Oguro, A., & Atomi, Y. (2005). The decrease of the cytoskeleton tubulin follows the decrease of the associating molecular chaperone alphaB-crystallin in unloaded soleus muscle atrophy without stretch. *The FASEB Journal, 19*, 1199–1201. https://doi.org/10.1096/fj.04-3060fje

Seevaratnam, R., Patel, B. P., & Hamadeh, M. J. (2009). Comparison of total protein concentration in skeletal muscle as measured by the Bradford and Lowry assays. *Journal of Biochemistry, 145*, 791–797. https://doi.org/10.1093/jb/mvp037

Sharples, A. P., Hughes, D. C., Deane, C. S., Saini, A., Selman, C., & Stewart, C. E. (2015). Longevity and skeletal muscle mass: The role of IGF signalling, the sirtuins, dietary restriction and protein intake. *Aging Cell, 14*, 511–523. https://doi.org/10.1111/acel.12342

Shay, J. W., & Wright, W. E. (2000). Hayflick, his limit, and cellular ageing. *Nature Reviews. Molecular Cell Biology, 1*, 72–76. https://doi.org/10.1038/35036093

Shigematsu, Y., Okamoto, H., Ichikawa, K., & Matsumoto, G. (1999). Temporal event association and output-dependent learning: A proposed scheme of neural molecular connections. *Journal of Advanced Computational Intelligence, 3*, 234–244. https://doi.org/10.20965/jaciii.1999.p0234

Shimizu, M., Tanaka, M., & Atomi, Y. (2016). Small heat shock protein alphaB-Crystallin controls shape and adhesion of glioma and myoblast cells in the absence of stress. *PLoS One, 11*, e0168136. https://doi.org/10.1371/journal.pone.0168136

Song, S., Hanson, M. J., Liu, B. F., Chylack, L. T., & Liang, J. J. (2008). Protein-protein interactions between lens vimentin and alphaB-crystallin using FRET acceptor photobleaching. *Molecular Vision, 14*, 1282–1287.

Specht, S., Miller, S. B., Mogk, A., & Bukau, B. (2011). Hsp42 is required for sequestration of protein aggregates into deposition sites in Saccharomyces Cerevisiae. *The Journal of Cell Biology, 195*, 617–629. https://doi.org/10.1083/jcb.201106037

Sreekumar, P. G., Kannan, R., Kitamura, M., Spee, C., Barron, E., Ryan, S. J., & Hinton, D. R. (2010). alphaB crystallin is apically secreted within exosomes by polarized human retinal pigment epithelium and provides neuroprotection to adjacent cells. *PLoS One, 5*, e12578. https://doi.org/10.1371/journal.pone.0012578

Steinman, L. (2014). Immunology of relapse and remission in multiple sclerosis. *Annual Review of Immunology, 32*, 257–281. https://doi.org/10.1146/annurev-immunol-032713-120227

Tabata, I., Atomi, Y., Kanehisa, H., & Miyashita, M. (1990). Effect of high-intensity endurance training on isokinetic muscle power. *European Journal of Applied Physiology and Occupational Physiology, 60*, 254–258.

Tabony, J., & Job, D. (1992). Gravitational symmetry breaking in microtubular dissipative structures. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 6948–6952.

Treweek, T. M., Rekas, A., Walker, M. J., & Carver, J. A. (2010). A quantitative NMR spectroscopic examination of the flexibility of the C-terminal extensions of the molecular chaperones, alphaA- and alphaB-crystallin. *Experimental Eye Research, 91*, 691–699. https://doi.org/10.1016/j.exer.2010.08.015

Tsvetkova, N. M., Horvath, I., Torok, Z., Wolkers, W. F., Balogi, Z., Shigapova, N., Crowe, L. M., Tablin, F., Vierling, E., Crowe, J. H., & Vigh, L. (2002). Small heat-shock proteins regulate membrane lipid polymorphism. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 13504–13509. https://doi.org/10.1073/pnas.192468399

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A., Kampf, C., Sjostedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., Edqvist, P. H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J. M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., & Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*, 1260419. https://doi.org/10.1126/science.1260419

Ulbricht, A., & Hohfeld, J. (2013). Tension-induced autophagy: May the chaperone be with you. *Autophagy, 9*, 920–922. https://doi.org/10.4161/auto.24213

Ulbricht, A., Eppler, F. J., Tapia, V. E., van der Ven, P. F., Hampe, N., Hersch, N., Vakeel, P., Stadel, D., Haas, A., Saftig, P., Behrends, C., Furst, D. O., Volkmer, R., Hoffmann, B., Kolanus, W., & Hohfeld, J. (2013). Cellular mechanotransduction relies on tension-induced and chaperone-assisted autophagy. *Current Biology, 23*, 430–435. https://doi.org/10.1016/j.cub.2013.01.064

Uversky, V. N., & Dunker, A. K. (2010). Understanding protein non-folding. *Biochimica et Biophysica Acta, 1804*, 1231–1264. https://doi.org/10.1016/j.bbapap.2010.01.017

van den Brand, R., Heutschi, J., Barraud, Q., DiGiovanna, J., Bartholdi, K., Huerlimann, M., Friedli, L., Vollenweider, I., Moraud, E. M., Duis, S., Dominici, N., Micera, S., Musienko, P., & Courtine, G. (2012). Restoring voluntary control of locomotion after paralyzing spinal cord injury. *Science, 336*, 1182–1185. https://doi.org/10.1126/science.1217416

Vandenburgh, H. H., Hatfaludy, S., Sohar, I., & Shansky, J. (1990). Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. *The American Journal of Physiology, 259*, C232–C240.

Varlet, A. A., Fuchs, M., Luthold, C., Lambert, H., Landry, J., & Lavoie, J. N. (2017). Fine-tuning of actin dynamics by the HSPB8-BAG3 chaperone complex facilitates cytokinesis and contributes to its impact on cell division. *Cell Stress & Chaperones, 22*, 553–567. https://doi.org/10.1007/s12192-017-0780-2

Walther, D. M., Kasturi, P., Zheng, M., Pinkert, S., Vecchi, G., Ciryam, P., Morimoto, R. I., Dobson, C. M., Vendruscolo, M., Mann, M., & Hartl, F. U. (2015). Widespread proteome remodeling and aggregation in Aging C. elegans. *Cell, 161*, 919–932. https://doi.org/10.1016/j.cell.2015.03.032

Wang, K., McClure, J., & Tu, A. (1979). Titin: Major myofibrillar components of striated muscle. *Proceedings of the National Academy of Sciences of the United States of America, 76*, 3698–3702.

Waters, E. R. (1995). The molecular evolution of the small heat-shock proteins in plants. *Genetics, 141*, 785–795.

Waters, E. R., Lee, G. J., & Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany, 47*, 325–338.

Watson, J. D., & Crick, F. H. (1953). Molecular structure of nucleic acids; A structure for deoxyribose nucleic acid. *Nature, 171*, 737–738.

Westerheide, S. D., & Morimoto, R. I. (2005). Heat shock response modulators as therapeutic tools for diseases of protein conformation. *The Journal of Biological Chemistry, 280*, 33097–33100. https://doi.org/10.1074/jbc.R500010200

Yamaguchi, T., Suzuki, T., Arai, H., Tanabe, S., & Atomi, Y. (2010). Continuous mild heat stress induces differentiation of mammalian myoblasts, shifting fiber type from fast to slow. *American Journal of Physiology. Cell Physiology, 298*, C140–C148. https://doi.org/10.1152/ajpcell.00050.2009

# Chapter 14
# Heat Shock Protein 70 (Hsp70) in the Regulation of Platelet Function


**Rachel A. Rigg, Owen J. T. McCarty, and Joseph E. Aslan**

**Abstract** Heat shock protein 70 (Hsp70) and its family of molecular chaperones are critical mediators of protein folding, trafficking, and control. Platelets are known to express several members of the Hsp70 family at high levels, suggesting critical roles for Hsp70 in platelet function. Several studies have described Hsp70-associated activities in intracellular signaling events, including the regulation of the linker for activation of T cells (LAT) signalosome that initiates integrin conformational changes underlying platelet aggregation. Although other chaperones perform established extracellular functions on the platelet surface as well as in the circulation to mediate the activities of platelets and other hematopoietic cells in hemostasis, thrombosis, and inflammation, similar roles for Hsp70 in platelet regulation remain undefined. Given the extensive roles of Hsp70 in many platelet-related human disease states, from protective roles in cardiovascular disease and wound healing to pathological roles in cancer, inflammation, and metabolic diseases, the emerging importance of Hsp70 in platelet function offers numerous ramifications for human health and disease.

R. A. Rigg
Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR, USA

O. J. T. McCarty
Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR, USA

Department of Cell, Developmental & Cancer Biology, Oregon Health & Science University, Portland, OR, USA

Division of Hematology and Medical Oncology, Oregon Health & Science University, Portland, OR, USA

J. E. Aslan (✉)
Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, OR, USA

Knight Cardiovascular Institute, School of Medicine, Oregon Health & Science University, Portland, OR, USA
e-mail: aslanj@ohsu.edu



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_14

**Keywords** Chaperones · Hemostasis · Inflammation · Integrin · Platelets · Thrombosis

## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| APC | Activated protein C |
| ATP | Adenosine triphosphate |
| CLEC | C-type lectin |
| CRP | Collagen-related peptide |
| ER | Endoplasmic reticulum |
| GPCR | G-protein coupled receptor |
| GPVI | Glycoprotein VI |
| Grp | Glucose-regulated protein |
| GTP | Guanosine triphosphate |
| Hsp | Heat shock protein |
| ILK | Integrin-linked kinase |
| LAT | Linker for activation of T cells |
| NEF | Nucleotide exchange factor |
| PAR | Protease-activated receptor |
| PDI | Protein disulfide isomerase |
| $PGI_2$ | Prostacyclin |
| PLC | Phospholipase C |
| RAGE | Receptor for advanced glycation endproducts |
| TLR | Toll-like receptor |
| TRAP-6 | Thrombin receptor-activating peptide-6 |
| $TXA_2$ | Thromboxane $A_2$ |

## 14.1 Introduction

Platelets are the smallest cellular elements of circulating blood and serve critical roles in hemostasis as well as pathologies associated with inflammation and thrombosis (Gros et al. 2014; Smyth et al. 2009). Anuclear, discoid fragments of megakaryocytic cells, platelets patrol the vasculature and rapidly respond to injury by tethering to exposed extracellular matrix proteins such as collagen and laminin, changing shape, releasing chemical messengers, and recruiting additional platelets and blood cells to the site of injury. As such, platelets act at a nexus of coagulation and inflammation to defend the host from vascular injury. Platelet activation programs are initiated by a number of physiological agonists and stimuli, such as collagen from exposed extracellular matrix and the coagulation factor thrombin

generated following exposure of tissue factor on damaged endothelium. Thrombin cleaves and activates platelet G protein-coupled receptors such as PAR-1 and PAR-4, while collagen activates platelet receptor glycoprotein VI (GPVI) to activate distinct but overlapping intracellular signaling cascades (Watson et al. 2005). Activation of these pathways initiates platelet shape change, increasing the platelet surface area available to catalyze reactions and stabilizing platelet aggregates (Aslan 2017). Platelet activation also promotes secretion from platelet granules, which release a host of contents to attract and activate neighboring platelets, including ADP, thromboxane ($TXA_2$), and epinephrine, inflammatory chemokines such as CXCL4 and CXCL7, leukocyte-binding adhesion proteins such as P-selectin, and inhibitors such as $PGI_2$ (Golebiewska and Poole 2015). This sequence of events results in the formation of a hemostatic plug, which is transformed into an insoluble clot with the action of thrombin to convert fibrinogen into fibrin strands.

The diverse physiological functions of platelets are under the control of tightly regulated signaling networks downstream of specific receptor systems, ultimately resulting in cytoskeletal reorganization, secretion, and platelet-platelet aggregation (Aslan et al. 2012; Broos et al. 2011). While a number of more classical signaling proteins, including specific kinases, phospholipases, and small GTPases are widely studied in the context of platelet function, other biological mediators of platelet physiology remain less well characterized, particularly the numerous protein chaperones that are abundantly expressed in platelets. Some chaperone proteins, particularly the disulfide isomerases such as PDI and ERp57 are emerging as important regulators of platelet action (Furie and Flaumenhaft 2014); other classes such as heat shock protein (Hsp) family members are not yet well studied. In this chapter, we highlight some of the described roles of Hsp in platelet regulation while discussing some of the potential mechanisms by which Hsp may participate in platelet function.

## 14.2 Hsp Family Expression in Platelets

The heat shock protein 70 family, so named due to the approximate 70 kDa size of its members, is itself part of a larger family of heat shock proteins (Hsp) ranging in size from 10 to over 100 kDa. Several heat shock protein family members and associated proteins have been identified in platelets, including family members of Hsp27, Hsp60, Hsp70, and Hsp90 (Ma et al. 2011; Molins et al. 2010; Staron et al. 2011; Polanowska-Grabowska et al. 1997; Tokuda et al. 2015; Kageyama et al. 2013; Mateos-Caceres et al. 2010). Indeed, several mass spectrometry-based proteomics analyses of platelets have demonstrated that heat shock proteins are among the highest expressed proteins in platelets, as summarized in Table 14.1. For instance, quantitative proteomics analyses of human and mouse platelets have reported that the heat shock cognate 71 protein (HSP7C, HSPA8) is one of the most abundant proteins in platelets (abundance ranking #89 in human; #26 in mouse) (Burkhart et al. 2012; Zeiler et al. 2014). Heat shock proteins in platelets

**Table 14.1** Heat shock and other chaperone proteins identified in platelets

| Gene name (human/ mouse) | Other gene names (human) | Description | Rank (human) | Copy no. (human) | Rank (mouse) | Copy no. (mouse) |
|---|---|---|---|---|---|---|
| ACTB/Actb | None | Actin, cytoplasmic 1 | 1 | 795,000 | 1 | 882,635 |
| PF4/Pf4 | CXCL4, SCYB4 | Platelet factor 4 | 4 | 563,000 | 9 | 274,032 |
| ITGA2B/ Itga2b | GP2B, ITGAB | Integrin alpha-IIb | 51 | 83,300 | 49 | 106,624 |
| HSPA8/ Hspa8 | HSC70, HSP73, HSPA10 | Heat shock cognate 71 kDa protein | 89 | 39,400 | 26 | 160,062 |
| HSPA5/ Hspa5 | HSPA5 | 78 kDa glucose-regulated protein | 126 | 27,900 | 43 | 118,824 |
| HSPB1 | HSP27, HSP28 | Heat shock protein beta | 132 | 26,400 | *Not reported* | |
| PDIA3/Pdia3 | ERP57, ERP60, GRP58 | Protein disulfide-isomerase A3 | 144 | 24,300 | 68 | 78,982 |
| CALR/Calr | CRTC | Calreticulin | 167 | 20,300 | 62 | 81,052 |
| HSPA1A | HSP72, HSPA1, HSX70 | Heat shock 70 kDa protein 1A/1B | 176 | 19,100 | *Not reported* | |
| HSPA1B/ Hspa1b | HSP72 | Heat shock 70 kDa protein 1A/1B | 176 | 19,100 | 2315 | 908 |
| P4HB/P4hb | ERBA2L, PDI, PDIA1, PO4DB | Protein disulfide-isomerase | 185 | 18,000 | 75 | 72,809 |
| HSP90AA1/ Hsp90aa1 | HSP90A, HSPC1, HSPCA | Heat shock protein HSP 90-alpha | 189 | 17,100 | 175 | 32,241 |
| HSPD1/ Hspd1 | HSP60 | 60 kDa heat shock protein, mitochondrial | 201 | 15,900 | 215 | 26,987 |
| PDIA6/Pdia6 | ERP5, P5, TXNDC7 | Protein disulfide-isomerase A6 | 250 | 13,300 | 93 | 58,079 |
| HSP90AB1/ Hsp90ab1 | HSP90B, HSPC2, HSPCB | Heat shock protein HSP 90-beta | 301 | 11,000 | 97 | 57,186 |
| CANX/Canx | None | Calnexin | 321 | 10,400 | 159 | 35,051 |
| STIP1/Stip1 | None | Stress-induced-phosphoprotein 1 | 333 | 10,000 | 211 | 27,215 |
| HSPA9/ Hspa9 | GRP75, HSPA9B, mt-HSP70 | Stress-70 protein, mitochondrial | 357 | 9300 | 475 | 11,698 |
| TBCA/Tbca | None | Tubulin-specific chaperone A | 411 | 8400 | 375 | 14,963 |
| HSP90B1/ Hsp90b1 | GRP94, TRA1 | Endoplasmin | 227 | 14,400 | 91 | 59,304 |

(continued)

**Table 14.1** (continued)

| Gene name (human/ mouse) | Other gene names (human) | Description | Rank (human) | Copy no. (human) | Rank (mouse) | Copy no. (mouse) |
|---|---|---|---|---|---|---|
| HSP90AB2P | HSP90BB | Putative heat shock protein HSP 90-beta 2 | 457 | 7500 | *Not reported* | |
| HSPA4/ Hspa4 | APG2 | Heat shock 70 kDa protein 4 | 654 | 5200 | 301 | 19,452 |
| SYK/Syk | None | Tyrosine-protein kinase SYK | 684 | 4900 | 252 | 23,286 |
| CDC37/ Cdc37 | CDC37A | Hsp90 co-chaperone Cdc37 | 790 | 4300 | 442 | 12,501 |
| ST13/St13 | AAG2, FAM10A1, HIP, SNC6 | Hsc70-interacting protein | 1259 | 2500 | 285 | 20,313 |
| BAG5/Bag5 | KIAA0873 | BAG family molecular chaperone regulator 5 | 1551 | 1900 | 2060 | 1242 |
| AHSA1/ Ahsa1 | AHSA1 C14orf3, HSPC322 | Activator of 90 kDa heat shock protein ATPase homolog 1 | 1803 | 1600 | 642 | 8418 |
| F2RL3/F2rl3 | PAR4 | Proteinase-activated receptor 4 | 2405 | 1100 | 939 | 5195 |
| TRAP1/Trap1 | HSP75 | Heat shock protein 75 kDa, mitochondrial | 2428 | 1100 | 1909 | 1479 |
| BAG2/Bag2 | None | BAG family molecular chaperone regulator 2 | 2827 | 870 | 3015 | 399 |
| HSPBP1/ Hspbp1 | HSPBP, PP1845 | Hsp70-binding protein 1 | 3178 | 730 | 3675 | 161 |
| HSPA7 | HSP70B | Putative heat shock 70 kDa protein 7 | 3657 | <500 | *Not reported* | |

Selected heat shock protein family members, other protein chaperones and chaperone associated proteins identified in human and mouse platelets as well as their relative abundance rankings (Rank: #1 = most abundant; #4190 and #4376 = least abundant for human and mouse platelets, respectively) and estimated copy number per platelet as determined by proteomics analyses (Burkhart et al. 2012; Zeiler et al. 2014). Other selected proteins critical to platelet function (highlighted in gray) are also shown for purposes of comparison

have also been reported to be covalently modified by protein phosphorylation (Suttitanamongkol et al. 2002; Zhu et al. 1994; Kato et al. 2008; Kageyama et al. 2013) as well as lysine acetylation (Aslan et al. 2015). Given the number of Hsp proteins expressed at high levels in platelets, their covalent modification associated with responses to physiological stimuli, the diversity of functions associated with Hsp, and the number of proteins that associate with Hsp, it is would be remarkable if Hsp family members such as Hsp70 did not have critical or specialized roles in platelet biology.

## 14.3 Hsp90-Hsp70 System in Cellular and Platelet Regulation

The Hsp family members, most prominently Hsp70 and Hsp90, are highly conserved and abundantly expressed ATP-powered molecular machines that perform *de novo* protein folding along with other protein quality control tasks (Hartl et al. 2011). Hsp70 is considered a central actor in coordinating folding and control of nascent polypeptides, while Hsp90 acts downstream of Hsp70 to regulate conformation of larger proteins such as membrane receptors and kinases. Hsp70 has well-characterized roles in the coordination of folding emergent polypeptides, directing protein trafficking between compartments, inhibiting protein aggregation under temperature shock and other stressors, and assisting in the degradation of aberrant proteins (Mayer and Bukau 2005; Clerico et al. 2015; Saibil 2013; Boorstein et al. 1994; Pratt and Toft 2003). With the assistance of over 50 co-chaperones, Hsp70 acts in a tightly controlled and context-specific manner (Patury et al. 2009; Kampinga and Craig 2010). The localization of Hsp70 is also context-specific, and cellular activation can cause intracellular Hsp70 to translocate to the cell surface or be packaged for exosomal secretion (Lancaster and Febbraio 2005; Vega et al. 2008). Following translocation to the cell surface or secretion, Hsp70 can become an extracellular ligand and can activate toll-like receptors (TLRs) on neighboring blood cells, initiating their inflammatory signaling cascades and amplifying an immunological response (Anand 2010; Asea et al. 2002; Asea et al. 2000; Chen and Cao 2010; Theriault et al. 2005; Gutierrez and Simmen 2014).

In platelets, a number of Hsp70-associated proteins have been characterized for roles in protein sequestration and trafficking (Fig. 14.1). One Hsp70 family member, the classical endoplasmic reticulum (ER) chaperone BiP/Grp78, has been iden-

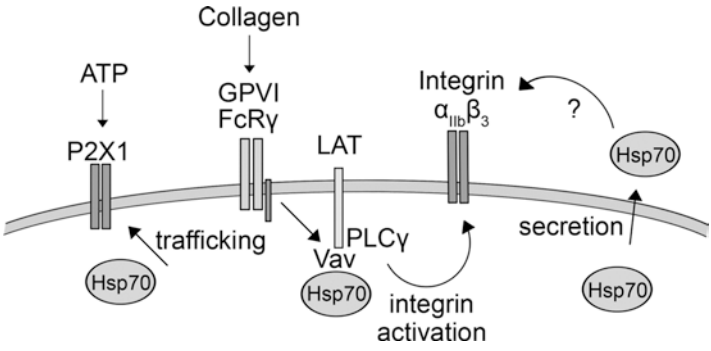


**Fig. 14.1** Summary of roles for Hsp70 family proteins in platelets. As described in the text, Hsp70 has roles in trafficking the platelet ATP receptor P2X1, as well as signaling events proximal to the LAT signalosome downstream involved in "inside-out" activation of integrin $\alpha_{IIb}\beta_3$. Platelets secrete Hsp70 family members and other chaperone proteins that may have autocrine roles in regulating extracellular events involved in platelet activation. Likewise, circulating Hsp70 family may have activating or inhibiting effects on platelets in physiological and disease specific contexts. See text for details

tified in the platelet cytosol, on the platelet surface, and in circulation, where it plays an antithrombotic role by sequestering pro-coagulant tissue factor (TF) on the platelet surface (Molins et al. 2010; Bertolotti et al. 2000). Hsp90 also facilitates surface expression and function of the platelet ATP receptor P2X1 (Lalo et al. 2012). Similarly, the Hsp90 family member Grp94 (endoplasmin) coordinates the assembly of the platelet glycoprotein Ib-IX-V receptor in the megakaryocyte endoplasmic reticulum (Staron et al. 2011). More recently, the platelet Hsp90/Hsp70 system has been hypothesized to regulate the intracellular trafficking and localization of the multidrug resistance protein MDR4/ABCC4 (Schaletzki et al. 2017). Hsp family members also play important roles in actomyosin filament assembly, a key step in platelet shape change and spreading. For example, Hsp27 has been specifically implicated as a downstream target of p38 MAPK in cytoskeletal assembly, and Hsp27 phosphorylation is required for platelet granule secretion (Gerthoffer and Gunst 2001; Tokuda et al. 2015; Kato et al. 2008; Pichon et al. 2004; Zhu et al. 1994). Together, these studies suggest intracellular as well as extracellular roles for Hsp70 and related chaperones in protein assembly, trafficking, and regulation in platelets.

In addition to regulating transit, as multifunctional chaperone proteins, Hsp can interact with and modulate cellular signaling pathways through a variety of mechanisms. Notably, heat shock proteins are critical to several signal transduction pathways, and several kinases are in complex with Hsp (Pratt and Toft 2003; Nollen and Morimoto 2002). For instance, the Hsp90/Cdc37 system is reported to interact with over 60% of the human kinome and is hypothesized to serve critical chaperone duties associated with a number of kinase functions (Verba and Agard 2017). While high-throughput analyses of platelets demonstrate that Cdc37 is expressed and modified by tyrosine as well as serine/threonine phosphorylation in platelets (Burkhart et al. 2012), its role in platelet regulation remains uncharacterized. Meanwhile, the Hsp70 family member Hsc70 has been identified in resting platelets in a complex with Hsp90 and protein phosphatase 1 (PP1), and this complex is dephosphorylated and dissociates following collagen-induced platelet activation, suggesting a role for Hsp70/90 in initiating platelet signaling (Polanowska-Grabowska et al. 1997). Similar to Hsp90/Cdc37, Hsp70 interacts with BAG proteins, nucleotide exchange factors (NEFs) that modulate Hsp70 activity to regulate a number of signaling mechanisms including Src signaling (Colvin et al. 2014), with implications for both physiology and disease (Sturner and Behl 2017; Kabbage and Dickman 2008). While proteomics studies have identified BAG-2 and BAG-5 in platelets, roles for Hsp70 interactors, including BAG proteins, are for the most part unknown in platelets.

## 14.4 Hsp70 in Platelet Signaling

Platelet tethering to extracellular matrix proteins initiates intracellular signaling and cytoskeletal remodeling events that generate filopodia and lamellipodia to enable firm platelet attachment (Watson 2009), involving a number of steps that require

protein control. Given the emerging involvement of Hsp in signaling regulation, our group has recently investigated the role of Hsp70 in the signaling systems of platelet activation (Rigg et al. 2016). We find that in human platelets spread on a surface of fibrinogen, both Hsp70 and Hsp90 are localized throughout the cytoplasm, and in partially but not fully spread platelets, Hsp70 colocalizes with actin, suggesting involvement of Hsp70 in cytoskeletal remodeling during the initial stages of spreading. Platelet spreading is diminished by the well-characterized Hsp70 inhibitors VER-155008 and MKT-077 (Williamson et al. 2009; Massey et al. 2010; Schlecht et al. 2013; Rousaki et al. 2011; Wadhwa et al. 2000) on surfaces of fibrinogen but not on fibrillar collagen, suggesting impairment of $\alpha_{IIb}\beta_3$ activation but not other pathways of activation. Hsp70 inhibition also decreases platelet aggregation by the GPVI agonist collagen-related peptide (CRP) but not thrombin receptor-activating peptide-6 (TRAP-6), revealing a role for Hsp70 downstream of ITAM-mediated signaling pathways. In flow cytometry assays, Hsp70 inhibition also decreases the activation marker P-selectin following stimulation by CRP but not thrombin, and Hsp70 inhibition diminishes activated integrin $\alpha_{IIb}\beta_3$ expression and binding to fluorescently-labeled fibrinogen while not affecting resting $\alpha_{IIb}\beta_3$ expression. Finally, Hsp70 inhibition drastically reduces platelet aggregate size in whole blood perfused at arterial shear rates over a surface of collagen, demonstrating the functional importance of Hsp70 in facilitating $\alpha_{IIb}\beta_3$-based aggregation.

Our data suggest a role for Hsp70 downstream of GPVI engagement associated with the "inside-out" activation of integrin $\alpha_{IIb}\beta_3$. Hsp70 inhibition decreases the phosphorylation of phospholipase Cγ2 (PLCγ2), a key component of the linker for activated T cells (LAT) signalosome involved in integrin activation downstream of GPVI (Hughes et al. 2008; Pasquet et al. 1999; Wonerow et al. 2002). Additionally, glutathione S-transferase (GST)-tagged recombinant Hsp70 captures LAT signalosome proteins from CRP-stimulated platelet lysates, including PLCγ2 and Vav1, and Hsp70 inhibition blocks immunoprecipitation of endogenous LAT with PLCγ2 and Vav1. Thus, Hsp70 appears to direct assembly and function of the LAT signalosome required for $\alpha_{IIb}\beta_3$ activation, and inhibition of Hsp70 impairs LAT functionality. This is supportive of work in other biological systems showing a role for Hsp70 in assembly of protein complexes near the intracellular membrane (Colvin et al. 2014; Pratt and Toft 2003). Overall, our study demonstrates a role of Hsp70 in healthy platelet function through assembly of the LAT signalosome downstream of GPVI. Intriguingly, a recent study by Durrant et al. finds that Hsp90-α (see Table 14.1) associates with the PI3K signalosome, which lies downstream of and proximal to the LAT signalosome in the regulation of platelet granule secretion and integrin activation (Durrant et al. 2017). Hsp70 has also recently been found to associate with the CLEC-14a receptor to promote angiogenesis, hinting at potential roles for Hsp70 in modulating CLEC-2 receptor activities in platelets also proximal to tyrosine kinase and PLC signaling (Jang et al. 2017).

## 14.5 Extracellular Hsp70 and Related Chaperones in Platelet Function

In contrast to the emerging roles for Hsp90/Hsp70 family members, oxidoreductase chaperones play more established roles in platelet function, regulating disulfide rearrangements to facilitate surface integrin conformational changes required for platelet-platelet interactions in thrombus formation. In particular, protein disulfide isomerase (PDI) and the homologous ERp5 and ERp57 are secreted from endothelial cells and platelets to facilitate thrombus formation and growth (Furie and Flaumenhaft 2014). Members of the PDI family, including PDI, ERp5, ERp57, and Ero1α bind integrin $\alpha_{IIb}\beta_3$ and are required for $\alpha_{IIb}\beta_3$ activation (Wang et al. 2013; Cho et al. 2012; Passam et al. 2015; Swiatkowska et al. 2010). These oxidoreductase chaperones physically interact with $\alpha_{IIb}\beta_3$ on the platelet surface to facilitate conformational changes required for activation (Passam et al. 2015; Wang et al. 2013; Cho et al. 2012; Furie and Flaumenhaft 2014; Crescente et al. 2016; Gibbins 2013; Schulman et al. 2016). Interestingly, Hsp70, like oxidoreductases, can be secreted from platelets, potentially playing roles in integrin conformational change and subsequent platelet activation. Indeed, recent in vitro studies demonstrate that extracellular Hsp72 can modulate platelet function, suggesting that Hsp may have roles outside of platelets in regulating platelet function and supporting platelet activation (Suzuki et al. 2017). Furthermore, studies in other cellular systems demonstrate that Hsp70 clusters with integrins (Chan et al. 2015) and that other Hsp70 family members can associate with PDI or Erp57 to fulfill chaperone activities and promote integrin activation (Delom et al. 2001; Gibbins 2013). Along these lines, platelets also express receptors for Grp94 (HSP90B1), and Grp94 binding to platelets increases in response to thrombin to regulate dendritic cell maturation (Hilf et al. 2002).

In addition to targeting platelet receptors such as integrins, some studies suggest roles for extracellular chaperones in mediating the organization and assembly of platelet receptor ligands such as fibrinogen/fibrin, collagen and other extracellular matrix components. Indeed, aspects of fibrinogen chain assembly require multiple chaperone activities (Roy et al. 1996). Emerging, more complex examples include interactions between Grp75 and the fibrinogen binding extracellular protein fibulin-1C, which is hypothesized to have roles in incorporating fibrin into blood clots (Hansen et al. 2015; Tran et al. 1995). In addition to the integrin/fibrinogen system, chaperones mediate platelet interactions with collagen. Activated platelets express surface Hsp47, a chaperone that associates with collagen, and this interaction is required for platelet activation by collagen (Kaiser et al. 2009). Given the rich literature linking the extracellular involvement of chaperones such as Hsp to inflammation, wound healing, fibrosis, diabetes, atherosclerosis, cancer and other pathologies, it is likely that roles for extracellular chaperone activities in physiological processes such as platelet activation are multifaceted, complex and only just beginning to be explored (Wyatt et al. 2013; Atalay et al. 2009; Bellaye et al. 2014).

## 14.6 Hsp70 in Thromboinflammatory Diseases

While cell biological and physiological models clearly leave room for chaperones such as Hsp70 in hypotheses concerning the regulation of platelet function, in vivo, roles for Hsp in hemostasis and thrombosis are likely more complex and context dependent. For instance, Allende et al. has reported that Hsp70 expression is highly downregulated in atrial fibrillation (AF) and stroke patients and that mice lacking Hsp70 develop thromboses more readily (Duerschmied and Bode 2016; Allende et al. 2016). Similarly, transgenic overexpression of Hsp72 in mice reduces infarct size in a left coronary artery occlusion model of thrombosis (Hutter et al. 1996). Interestingly, induction of Hsp70 expression also limits thrombosis in mice and is associated with higher circulating levels of activated protein C (APC) (Allende et al. 2016). Moreover, murine platelets do not reveal a change in aggregation with Hsp70 induction, suggesting Hsp70 can be upregulated without enhancing platelet reactivity. This anti-thrombotic effect of Hsp70 is further supported in murine thrombosis models showing that pharmacological Hsp70 induction delays thrombus formation without increasing bleeding (Allende et al. 2016). Similarly, drugs that upregulate Hsp70 overexpression have antithrombotic effects that do not interfere with hemostasis (Allende et al. 2017). Other studies suggest Hsp70 may also be protective in cerebrovascular atherosclerosis; levels of circulating Hsp70 are lower in atherosclerosis patients than controls (Galovic et al. 2016). Similarly, elevated levels of circulating Hsp70 mitigate atherosclerosis progression in individuals with hypertension, potentially through Hsp70-endothelial cell interactions (Pockley et al. 2009). Intracellular Hsp70 also plays a cardioprotective role in complex with integrin-linked kinase (ILK) in cardiomyocytes (Traister et al. 2013). In platelets, ILK promotes integrin $\alpha_{IIb}\beta_3$ activation (Tucker et al. 2008), yet a link between ILK and Hsp70 has not yet been investigated. Hsp70 family member Grp78 also plays an atheroprotective role by binding and inactivating tissue factor on the platelet surface (Molins et al. 2010). Hsp70 has also been found to facilitate wound healing by promoting angiogenesis in endothelial cells, although this pro-angiogenic property can also be hijacked to support cancer proliferation and metastasis (Kim et al. 2016). Similarly, ultrasound treatment upregulates protective Hsp70 expression in skin tissue, suggesting potential therapeutic applications for wound healing and drug delivery (Kruse et al. 2008). Overall, these results suggest that the antithrombotic and pro-angiogenic effects of Hsp70 induction occurs through multiple pathways, including platelet, leukocyte, and endothelial cell Hsp70 activity.

Hsp70 family members and platelets also converge in studies of innate immunity (Rondina et al. 2013). For instance, high levels of circulating Hsp70 may provide a biomarker for cellular injury, oxidative stress, or inflammation in many disease contexts (Molvarec et al. 2010; Inoue and Sawamura 2007; Mambula et al. 2007; Krepuska et al. 2011). Extracellular Hsp70 is also associated with the onset and progression of inflammation in diabetes, while intracellular Hsp70 activity is protective, suggesting multifaceted roles for Hsp70 in metabolic disorders (Rodrigues-Krause et al. 2012; Krause et al. 2015; Santos et al. 2015). Hsp70 binds misfolded

peptide segments during cellular stress, and these circulating Hsp70-peptide complexes can bind to CD40 receptor on immune cells, facilitating peptide uptake and immune priming (Inwald et al. 2003). Extracellular Hsp70 has also been shown to bind the receptor for advanced glycation endproducts (RAGE) in cancer cells, activating inflammatory signaling pathways and cytokine secretion (Somensi et al. 2017). Extracellular Hsp70/Hsp90 complexes as secreted from tumor cells have also been reported to be associated with detrimental conditions associated with cancer, such as cachexia and muscle wasting (Zhang et al. 2017). Likewise, extracellular, autocrine roles of the Hsp70/Hsp90 adaptor protein STIP1 in promoting tumor cell growth also suggest that circulating Hsp70 complexes have context-specific roles in disease progression (Chen et al. 2017). Indeed, it is intriguing to speculate how circulating Hsp70 may have roles in stimulating CD40, RAGE and other immune receptor systems in platelets to prime thromboinflammatory disease states. Along these lines, platelets are known to express receptors for heat shock protein Grp94 that neutralize Grp94 upon platelet activation and markedly diminish the immune response by dendritic cells, demonstrating an anti-inflammatory effect of Grp94 via platelets (Hilf et al. 2002). Platelets also store toll-like receptors (TLRs) in their alpha granules that are expressed on the platelet surface upon activation (Aslam et al. 2006; Shiraki et al. 2004). Whether platelets also express Hsp70 receptors that mediate pro- or anti-inflammatory roles remains an intriguing question. Moreover, the Hsp90/Hsp70 axis may also be a therapeutic target in sepsis. Hsp90 inhibition may have anti-inflammatory properties, as 17-DMAG improves outcomes of rats (sepsis/LPS) in an Hsp70-dependent manner (Wang et al. 2016). Interestingly, it has been proposed that administration of recombinant Hsp70 may help in treatment of sepsis by counteracting inflammatory responses (Vinokurov et al. 2012). Going forward, an approach targeting not only Hsp70, but specific co-chaperones that complex with Hsp70 may shift Hsp70 activity towards desired therapeutic outcomes (Assimon et al. 2013). Overall, a better understanding of the interplay of platelets, Hsp70, and its co-chaperones in thrombotic and inflammatory diseases is needed and will likely help to inform therapeutic approaches in the future.

## 14.7 Conclusions

The multifaceted roles of Hsp70 and its associated co-chaperones and other partnered proteins are of strong interest to studies of hemostasis, thrombosis, and inflammation, and the intersection of heat shock protein and platelet physiological studies offers a rich area of mechanistic and translational investigation. Future work will delineate contextual intracellular versus extracellular roles for Hsp70 in platelets and the resulting implications for health as well as more novel approaches towards understanding, detecting, and treating a range of associated disease states.

**Acknowledgements** The authors thank the Knight Cardiovascular Institute, the National Institutes of Health (R01HL101972 and R01GM116184 to O.J.T.M.), and the American Heart Association (17SDG33350075 to J.E.A. and 13EIA12630000 to O.J.T.M.) for support.

**Disclosures** The authors have no conflicts of interest to declare.

## References

Allende, M., Molina, E., Guruceaga, E., Tamayo, I., Gonzalez-Porras, J. R., Gonzalez-Lopez, T. J., Toledo, E., Rabal, O., Ugarte, A., Roldan, V., Rivera, J., Oyarzabal, J., Montes, R., & Hermida, J. (2016). Hsp70 protects from stroke in atrial fibrillation patients by preventing thrombosis without increased bleeding risk. *Cardiovascular Research, 110*, 309–318.

Allende, M., Molina, E., Lecumberri, R., Sanchez-Arias, J. A., Ugarte, A., Guruceaga, E., Oyarzabal, J., & Hermida, J. (2017). Inducing heat shock protein 70 expression provides a robust antithrombotic effect with minimal bleeding risk. *Thrombosis and Haemostasis, 117*, **1722**.

Anand, P. K. (2010). Exosomal membrane molecules are potent immune response modulators. *Communicative & Integrative Biology, 3*, 405–408.

Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., Koo, G. C., & Calderwood, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature Medicine, 6*, 435–442.

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A., & Calderwood, S. K. (2002). Novel signal transduction pathway utilized by extracellular HSP70: Role of toll-like receptor (TLR) 2 and TLR4. *The Journal of Biological Chemistry, 277*, 15028–15034.

Aslam, R., Speck, E. R., Kim, M., Crow, A. R., Bang, K. W., Nestel, F. P., Ni, H., Lazarus, A. H., Freedman, J., & Semple, J. W. (2006). Platelet toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production in vivo. *Blood, 107*, 637–641.

Aslan, J. E. (2017). Platelet shape change. In P. Gresele, N. S. Kleiman, J. A. Lopez, & C. P. Page (Eds.), *Platelets in thrombotic and non-thrombotic disorders: Pathophysiology, pharmacology and therapeutics: An update* (pp. 321–336). Cham: Springer.

Aslan, J. E., Itakura, A., Gertz, J. M., & McCarty, O. J. (2012). Platelet shape change and spreading. *Methods in Molecular Biology, 788*, 91–100.

Aslan, J. E., Rigg, R. A., Nowak, M. S., Loren, C. P., Baker-Groberg, S. M., Pang, J., David, L. L., & McCarty, O. J. (2015). Lysine acetyltransfer supports platelet function. *Journal of Thrombosis and Haemostasis, 13*, 1908–1917.

Assimon, V. A., Gillies, A. T., Rauch, J. N., & Gestwicki, J. E. (2013). Hsp70 protein complexes as drug targets. *Current Pharmaceutical Design, 19*, 404–417.

Atalay, M., Oksala, N., Lappalainen, J., Laaksonen, D. E., Sen, C. K., & Roy, S. (2009). Heat shock proteins in diabetes and wound healing. *Current Protein & Peptide Science, 10*, 85–95.

Bellaye, P. S., Burgy, O., Causse, S., Garrido, C., & Bonniaud, P. (2014). Heat shock proteins in fibrosis and wound healing: Good or evil? *Pharmacology & Therapeutics, 143*, 119–132.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., & Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biology, 2*, 326–332.

Boorstein, W. R., Ziegelhoffer, T., & Craig, E. A. (1994). Molecular evolution of the HSP70 multigene family. *Journal of Molecular Evolution, 38*, 1–17.

Broos, K., Feys, H. B., De Meyer, S. F., Vanhoorelbeke, K., & Deckmyn, H. (2011). Platelets at work in primary hemostasis. *Blood Reviews, 25*, 155–167.

Burkhart, J. M., Vaudel, M., Gambaryan, S., Radau, S., Walter, U., Martens, L., Geiger, J., Sickmann, A., & Zahedi, R. P. (2012). The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood, 120*, e73–e82.

Chan, Y. C., Greenwood, D. R., Yang, Y., Leung, E., & Krissansen, G. W. (2015). Leukocyte integrin α4β7 associates with heat shock protein 70. *Molecular and Cellular Biochemistry, 409*(1–2), 263–269.

Chen, T., & Cao, X. (2010). Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. *European Journal of Immunology, 40*, 1541–1544.

Chen, Z., Xu, L., Su, T., Ke, Z., Peng, Z., Zhang, N., Peng, S., Zhang, Q., Liu, G., Wei, G., Guo, Y., He, M., & Kuang, M. (2017). Autocrine STIP1 signaling promotes tumor growth and is associated with disease outcome in hepatocellular carcinoma. *Biochemical and Biophysical Research Communications, 493*(1), 365–372.

Cho, J., Kennedy, D. R., Lin, L., Huang, M., Merrill-Skoloff, G., Furie, B. C., & Furie, B. (2012). Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of beta3 integrins. *Blood, 120*, 647–655.

Clerico, E. M., Tilitsky, J. M., Meng, W., & Gierasch, L. M. (2015). How Hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology, 427*, 1575–1588.

Colvin, T. A., Gabai, V. L., Gong, J., Calderwood, S. K., Li, H., Gummuluru, S., Matchuk, O. N., Smirnova, S. G., Orlova, N. V., Zamulaeva, I. A., Garcia-Marcos, M., Li, X., Young, Z. T., Rauch, J. N., Gestwicki, J. E., Takayama, S., & Sherman, M. Y. (2014). Hsp70-Bag3 interactions regulate cancer-related signaling networks. *Cancer Research, 74*, 4731–4740.

Crescente, M., Pluthero, F. G., Li, L., Lo, R. W., Walsh, T. G., Schenk, M. P., Holbrook, L. M., Louriero, S., Ali, M. S., Vaiyapuri, S., Falet, H., Jones, I. M., Poole, A. W., Kahr, W. H., & Gibbins, J. M. (2016). Intracellular trafficking, localization, and mobilization of platelet-borne thiol isomerases. *Arteriosclerosis, Thrombosis, and Vascular Biology, 36*, 1164–1173.

Delom, F., Mallet, B., Carayon, P., & Lejeune, P. J. (2001). Role of extracellular molecular chaperones in the folding of oxidized proteins. Refolding of colloidal thyroglobulin by protein disulfide isomerase and immunoglobulin heavy chain-binding protein. *The Journal of Biological Chemistry, 276*, 21337–21342.

Duerschmied, D., & Bode, C. (2016). Hsp70 preventing thrombosis: Benefit without burden? *Cardiovascular Research, 110*, 291–292.

Durrant, T. N., Hutchinson, J. L., Heesom, K. J., Anderson, K. E., Stephens, L. R., Hawkins, P. T., Marshall, A. J., Moore, S. F., & Hers, I. (2017). In-depth PtdIns(3,4,5)P3 signalosome analysis identifies DAPP1 as a negative regulator of GPVI-driven platelet function. *Blood Advances, 1*, 918–932.

Furie, B., & Flaumenhaft, R. (2014). Thiol isomerases in thrombus formation. *Circulation Research, 114*, 1162–1173.

Galovic, R., Flegar-Mestric, Z., Vidjak, V., Matokanovic, M., & Barisic, K. (2016). Heat shock protein 70 and antibodies to heat shock protein 60 are associated with cerebrovascular atherosclerosis. *Clinical Biochemistry, 49*, 66–69.

Gerthoffer, W. T., & Gunst, S. J. (2001). Invited review: Focal adhesion and small heat shock proteins in the regulation of actin remodeling and contractility in smooth muscle. *Journal of Applied Physiology (1985), 91*, 963–972.

Gibbins, J. M. (2013). Platelets using proteins creatively. *Blood, 122*, 3553–3554.

Golebiewska, E. M., & Poole, A. W. (2015). Platelet secretion: From haemostasis to wound healing and beyond. *Blood Reviews, 29*, 153–162.

Gros, A., Ollivier, V., & Ho-Tin-Noe, B. (2014). Platelets in inflammation: Regulation of leukocyte activities and vascular repair. *Frontiers in Immunology, 5*, 678.

Gutierrez, T., & Simmen, T. (2014). Endoplasmic reticulum chaperones and oxidoreductases: Critical regulators of tumor cell survival and immunorecognition. *Frontiers in Oncology, 4*, 291.

Hansen, G. A., Ludvigsen, M., Jacobsen, C., Cangemi, C., Rasmussen, L. M., Vorum, H., & Honore, B. (2015). Fibulin-1C, C1 esterase inhibitor and glucose regulated protein 75 interact with the CREC proteins, calumenin and Reticulocalbin. *PLoS One, 10*, e0132283.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature, 475*, 324–332.

Hilf, N., Singh-Jasuja, H., Schwarzmaier, P., Gouttefangeas, C., Rammensee, H. G., & Schild, H. (2002). Human platelets express heat shock protein receptors and regulate dendritic cell maturation. *Blood, 99*, 3676–3682.

Hughes, C. E., Auger, J. M., McGlade, J., Eble, J. A., Pearce, A. C., & Watson, S. P. (2008). Differential roles for the adapters gads and LAT in platelet activation by GPVI and CLEC-2. *Journal of Thrombosis and Haemostasis, 6*, 2152–2159.

Hutter, J. J., Mestril, R., Tam, E. K., Sievers, R. E., Dillmann, W. H., & Wolfe, C. L. (1996). Overexpression of heat shock protein 72 in transgenic mice decreases infarct size in vivo. *Circulation, 94*, 1408–1411.

Inoue, N., & Sawamura, T. (2007). Lectin-like oxidized LDL receptor-1 as extracellular chaperone receptor: Its versatile functions and human diseases. *Methods, 43*, 218–222.

Inwald, D. P., McDowall, A., Peters, M. J., Callard, R. E., & Klein, N. J. (2003). CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circulation Research, 92*, 1041–1048.

Jang, J., Kim, M. R., Kim, T. K., Lee, W. R., Kim, J. H., Heo, K., & Lee, S. (2017). CLEC14a-HSP70-1A interaction regulates HSP70-1A-induced angiogenesis. *Scientific Reports, 7*(10666), 10666.

Kabbage, M., & Dickman, M. B. (2008). The BAG proteins: A ubiquitous family of chaperone regulators. *Cellular and Molecular Life Sciences, 65*, 1390–1402.

Kageyama, Y., Doi, T., Akamatsu, S., Kuroyanagi, G., Kondo, A., Mizutani, J., Otsuka, T., Tokuda, H., Kozawa, O., & Ogura, S. (2013). Rac regulates collagen-induced HSP27 phosphorylation via p44/p42 MAP kinase in human platelets. *International Journal of Molecular Medicine, 32*, 813–818.

Kaiser, W. J., Holbrook, L. M., Tucker, K. L., Stanley, R. G., & Gibbins, J. M. (2009). A functional proteomic method for the enrichment of peripheral membrane proteins reveals the collagen binding protein Hsp47 is exposed on the surface of activated human platelets. *Journal of Proteome Research, 8*, 2903–2914.

Kampinga, H. H., & Craig, E. A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews. Molecular Cell Biology, 11*, 579–592.

Kato, H., Takai, S., Matsushima-Nishiwaki, R., Adachi, S., Minamitani, C., Otsuka, T., Tokuda, H., Akamatsu, S., Doi, T., Ogura, S., & Kozawa, O. (2008). HSP27 phosphorylation is correlated with ADP-induced platelet granule secretion. *Archives of Biochemistry and Biophysics, 475*, 80–86.

Kim, T. K., Na, H. J., Lee, W. R., Jeoung, M. H., & Lee, S. (2016). Heat shock protein 70-1A is a novel angiogenic regulator. *Biochemical and Biophysical Research Communications, 469*, 222–228.

Krause, M., Heck, T. G., Bittencourt, A., Scomazzon, S. P., Newsholme, P., Curi, R., Homem de Bittencourt, P. I., & Jr. (2015). The chaperone balance hypothesis: The importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes, the effect of exercise, and the implications for clinical management. *Mediators of Inflammation, 2015*, 249205.

Krepuska, M., Szeberin, Z., Sotonyi, P., Sarkadi, H., Fehervari, M., Apor, A., Rimely, E., Prohaszka, Z., & Acsady, G. (2011). Serum level of soluble Hsp70 is associated with vascular calcification. *Cell Stress & Chaperones, 16*, 257–265.

Kruse, D. E., Mackanos, M. A., O'Connell-Rodwell, C. E., Contag, C. H., & Ferrara, K. W. (2008). Short-duration-focused ultrasound stimulation of Hsp70 expression in vivo. *Physics in Medicine and Biology, 53*, 3641–3660.

Lalo, U., Jones, S., Roberts, J. A., Mahaut-Smith, M. P., & Evans, R. J. (2012). Heat shock protein 90 inhibitors reduce trafficking of ATP-gated P2X1 receptors and human platelet responsiveness. *The Journal of Biological Chemistry, 287*, 32747–32754.

Lancaster, G. I., & Febbraio, M. A. (2005). Exosome-dependent trafficking of HSP70: A novel secretory pathway for cellular stress proteins. *The Journal of Biological Chemistry, 280*, 23349–23355.

Ma, C., Yao, Y., Yue, Q. X., Zhou, X. W., Yang, P. Y., Wu, W. Y., Guan, S. H., Jiang, B. H., Yang, M., Liu, X., & Guo, D. A. (2011). Differential proteomic analysis of platelets suggested possible signal cascades network in platelets treated with salvianolic acid B. *PLoS One, 6*, e14692.

Mambula, S. S., Stevenson, M. A., Ogawa, K., & Calderwood, S. K. (2007). Mechanisms for Hsp70 secretion: Crossing membranes without a leader. *Methods, 43*, 168–175.

Massey, A. J., Williamson, D. S., Browne, H., Murray, J. B., Dokurno, P., Shaw, T., Macias, A. T., Daniels, Z., Geoffroy, S., Dopson, M., Lavan, P., Matassova, N., Francis, G. L., Graham, C. J., Parsons, R., Wang, Y., Padfield, A., Comer, M., Drysdale, M. J., & Wood, M. (2010). A novel, small molecule inhibitor of Hsc70/Hsp70 potentiates Hsp90 inhibitor induced apoptosis in HCT116 colon carcinoma cells. *Cancer Chemotherapy and Pharmacology, 66*, 535–545.

Mateos-Caceres, P. J., Macaya, C., Azcona, L., Modrego, J., Mahillo, E., Bernardo, E., Fernandez-Ortiz, A., & Lopez-Farre, A. J. (2010). Different expression of proteins in platelets from aspirin-resistant and aspirin-sensitive patients. *Thrombosis and Haemostasis, 103*, 160–170.

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*, 670–684.

Molins, B., Pena, E., Padro, T., Casani, L., Mendieta, C., & Badimon, L. (2010). Glucose-regulated protein 78 and platelet deposition: Effect of rosuvastatin. *Arteriosclerosis, Thrombosis, and Vascular Biology, 30*, 1246–1252.

Molvarec, A., Tamasi, L., Losonczy, G., Madach, K., Prohaszka, Z., & Rigo, J.,. J. (2010). Circulating heat shock protein 70 (HSPA1A) in normal and pathological pregnancies. *Cell Stress & Chaperones, 15*, 237–247.

Nollen, E. A., & Morimoto, R. I. (2002). Chaperoning signaling pathways: Molecular chaperones as stress-sensing 'heat shock' proteins. *Journal of Cell Science, 115*, 2809–2816.

Pasquet, J. M., Gross, B., Quek, L., Asazuma, N., Zhang, W., Sommers, C. L., Schweighoffer, E., Tybulewicz, V., Judd, B., Lee, J. R., Koretzky, G., Love, P. E., Samelson, L. E., & Watson, S. P. (1999). LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. *Molecular and Cellular Biology, 19*, 8326–8334.

Passam, F. H., Lin, L., Gopal, S., Stopa, J. D., Bellido-Martin, L., Huang, M., Furie, B. C., & Furie, B. (2015). Both platelet- and endothelial cell-derived ERp5 support thrombus formation in a laser-induced mouse model of thrombosis. *Blood, 125*, 2276–2285.

Patury, S., Miyata, Y., & Gestwicki, J. E. (2009). Pharmacological targeting of the Hsp70 chaperone. *Current Topics in Medicinal Chemistry, 9*, 1337–1351.

Pichon, S., Bryckaert, M., & Berrou, E. (2004). Control of actin dynamics by p38 MAP kinase - Hsp27 distribution in the lamellipodium of smooth muscle cells. *Journal of Cell Science, 117*, 2569–2577.

Pockley, A. G., Calderwood, S. K., & Multhoff, G. (2009). The atheroprotective properties of Hsp70: A role for Hsp70-endothelial interactions? *Cell Stress & Chaperones, 14*, 545–553.

Polanowska-Grabowska, R., Simon, C. G., Jr., Falchetto, R., Shabanowitz, J., Hunt, D. F., & Gear, A. R. (1997). Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood, 90*, 1516–1526.

Pratt, W. B., & Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Experimental Biology and Medicine (Maywood), 228*, 111–133.

Rigg, R. A., Healy, L. D., Nowak, M. S., Mallet, J., Thierheimer, M. L., Pang, J., McCarty, O. J., & Aslan, J. E. (2016). Heat shock protein 70 regulates platelet integrin activation, granule secretion and aggregation. *American Journal of Physiology. Cell Physiology, 310*, C568–C575.

Rodrigues-Krause, J., Krause, M., O'Hagan, C., De Vito, G., Boreham, C., Murphy, C., Newsholme, P., & Colleran, G. (2012). Divergence of intracellular and extracellular HSP72 in type 2 diabetes: Does fat matter? *Cell Stress & Chaperones, 17*, 293–302.

Rondina, M. T., Weyrich, A. S., & Zimmerman, G. A. (2013). Platelets as cellular effectors of inflammation in vascular diseases. *Circulation Research, 112*, 1506–1519.

Rousaki, A., Miyata, Y., Jinwal, U. K., Dickey, C. A., Gestwicki, J. E., & Zuiderweg, E. R. (2011). Allosteric drugs: The interaction of antitumor compound MKT-077 with human Hsp70 chaperones. *Journal of Molecular Biology, 411*, 614–632.

Roy, S., Sun, A., & Redman, C. (1996). In vitro assembly of the component chains of fibrinogen requires endoplasmic reticulum factors. *The Journal of Biological Chemistry, 271*, 24544–24550.

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nature Reviews. Molecular Cell Biology, 14*, 630–642.

Santos, T. M., Sinzato, Y. K., Gallego, F. Q., Iessi, I. L., Volpato, G. T., Dallaqua, B., & Damasceno, D. C. (2015). Extracellular HSP70 levels in diabetic environment in rats. *Cell Stress & Chaperones, 20*, 595–603.

Schaletzki, Y., Kromrey, M. L., Broderdorf, S., Hammer, E., Grube, M., Hagen, P., Sucic, S., Freissmuth, M., Volker, U., Greinacher, A., Rauch, B. H., Kroemer, H. K., & Jedlitschky, G. (2017). Several adaptor proteins promote intracellular localisation of the transporter MRP4/ABCC4 in platelets and haematopoietic cells. *Thrombosis and Haemostasis, 117*, 105–115.

Schlecht, R., Scholz, S. R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J., Eggenweiler, H. M., Mayer, M. P., & Bukau, B. (2013). Functional analysis of Hsp70 inhibitors. *PLoS One, 8*, e78443.

Schulman, S., Bendapudi, P., Sharda, A., Chen, V., Bellido-Martin, L., Jasuja, R., Furie, B. C., Flaumenhaft, R., & Furie, B. (2016). Extracellular thiol isomerases and their role in thrombus formation. *Antioxidants & Redox Signaling, 24*, 1–15.

Shiraki, R., Inoue, N., Kawasaki, S., Takei, A., Kadotani, M., Ohnishi, Y., Ejiri, J., Kobayashi, S., Hirata, K., Kawashima, S., & Yokoyama, M. (2004). Expression of toll-like receptors on human platelets. *Thrombosis Research, 113*, 379–385.

Smyth, S. S., McEver, R. P., Weyrich, A. S., Morrell, C. N., Hoffman, M. R., Arepally, G. M., French, P. A., Dauerman, H. L., & Becker, R. C. (2009). Platelet functions beyond hemostasis. *Journal of Thrombosis and Haemostasis, 7*, 1759–1766.

Somensi, N., Brum, P. O., de Miranda Ramos, V., Gasparotto, J., Zanotto-Filho, A., Rostirolla, D. C., da Silva Morrone, M., Moreira, J. C. F., & Pens Gelain, D. (2017). Extracellular HSP70 activates ERK1/2, NF-kB and pro-inflammatory gene transcription through binding with RAGE in A549 human lung cancer cells. *Cellular Physiology and Biochemistry, 42*, 2507–2522.

Staron, M., Wu, S., Hong, F., Stojanovic, A., Du, X., Bona, R., Liu, B., & Li, Z. (2011). Heat-shock protein gp96/grp94 is an essential chaperone for the platelet glycoprotein Ib-IX-V complex. *Blood, 117*, 7136–7144.

Sturner, E., & Behl, C. (2017). The role of the multifunctional BAG3 protein in cellular protein quality control and in disease. *Frontiers in Molecular Neuroscience, 10*, 177.

Suttitanamongkol, S., Polanowska-Grabowska, R., & Gear, A. R. (2002). Heat-shock protein 90 complexes in resting and thrombin-activated platelets. *Biochemical and Biophysical Research Communications, 297*, 129–133.

Suzuki, H., Kosuge, Y., Kobayashi, K., Kurosaki, Y., Ishii, N., Aoyama, N., Ishihara, K., & Ichikawa, T. (2017). Heat-shock protein 72 promotes platelet aggregation induced by various platelet activators in rats. *Biomedical Research, 38*, 175–182.

Swiatkowska, M., Padula, G., Michalec, L., Stasiak, M., Skurzynski, S., & Cierniewski, C. S. (2010). Ero1alpha is expressed on blood platelets in association with protein-disulfide isomerase and contributes to redox-controlled remodeling of alphaIIbbeta3. *The Journal of Biological Chemistry, 285*, 29874–29883.

Theriault, J. R., Mambula, S. S., Sawamura, T., Stevenson, M. A., & Calderwood, S. K. (2005). Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. *FEBS Letters, 579*, 1951–1960.

Tokuda, H., Kuroyanagi, G., Tsujimoto, M., Enomoto, Y., Matsushima-Nishiwaki, R., Onuma, T., Kojima, A., Doi, T., Tanabe, K., Akamatsu, S., Iida, H., Ogura, S., Otsuka, T., Iwama, T., Tanikawa, T., Ishikawa, K., Kojima, K., & Kozawa, O. (2015). Release of phosphorylated HSP27 (HSPB1) from platelets is accompanied with the acceleration of aggregation in diabetic patients. *PLoS One, 10*, e0128977.

Traister, A., Walsh, M., Aafaqi, S., Lu, M., Dai, X., Henkleman, M. R., Momen, A., Zhou, Y. Q., Husain, M., Arab, S., Piran, S., Hannigan, G., & Coles, J. G. (2013). Mutation in integrin-linked kinase (ILK(R211A)) and heat-shock protein 70 comprise a broadly cardioprotective complex. *PLoS One, 8*, e77331.

Tran, H., Tanaka, A., Litvinovich, S. V., Medved, L. V., Haudenschild, C. C., & Argraves, W. S. (1995). The interaction of fibulin-1 with fibrinogen. A potential role in hemostasis and thrombosis. *The Journal of Biological Chemistry, 270*, 19458–19464.

Tucker, K. L., Sage, T., Stevens, J. M., Jordan, P. A., Jones, S., Barrett, N. E., St-Arnaud, R., Frampton, J., Dedhar, S., & Gibbins, J. M. (2008). A dual role for integrin-linked kinase in platelets: Regulating integrin function and alpha-granule secretion. *Blood, 112*, 4523–4531.

Vega, V. L., Rodriguez-Silva, M., Frey, T., Gehrmann, M., Diaz, J. C., Steinem, C., Multhoff, G., Arispe, N., & De Maio, A. (2008). Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *Journal of Immunology, 180*, 4299–4307.

Verba, K. A., & Agard, D. A. (2017). How Hsp90 and Cdc37 lubricate kinase molecular switches. *Trends in Biochemical Sciences, 42*(10), 799–811.

Vinokurov, M., Ostrov, V., Yurinskaya, M., Garbuz, D., Murashev, A., Antonova, O., & Evgen'ev, M. (2012). Recombinant human Hsp70 protects against lipoteichoic acid-induced inflammation manifestations at the cellular and organismal levels. *Cell Stress & Chaperones, 17*, 89–101.

Wadhwa, R., Sugihara, T., Yoshida, A., Nomura, H., Reddel, R. R., Simpson, R., Maruta, H., & Kaul, S. C. (2000). Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. *Cancer Research, 60*, 6818–6821.

Wang, L., Wu, Y., Zhou, J., Ahmad, S. S., Mutus, B., Garbi, N., Hammerling, G., Liu, J., & Essex, D. W. (2013). Platelet-derived ERp57 mediates platelet incorporation into a growing thrombus by regulation of the alphaIIbbeta3 integrin. *Blood, 122*, 3642–3650.

Wang, Y. L., Shen, H. H., Cheng, P. Y., Chu, Y. J., Hwang, H. R., Lam, K. K., & Lee, Y. M. (2016). 17-DMAG, an HSP90 inhibitor, ameliorates multiple organ dysfunction syndrome via induction of HSP70 in Endotoxemic rats. *PLoS One, 11*, e0155583.

Watson, S. P. (2009). Platelet activation by extracellular matrix proteins in haemostasis and thrombosis. *Current Pharmaceutical Design, 15*, 1358–1372.

Watson, S. P., Auger, J. M., McCarty, O. J., & Pearce, A. C. (2005). GPVI and integrin alphaIIb beta3 signaling in platelets. *Journal of Thrombosis and Haemostasis, 3*, 1752–1762.

Williamson, D. S., Borgognoni, J., Clay, A., Daniels, Z., Dokurno, P., Drysdale, M. J., Foloppe, N., Francis, G. L., Graham, C. J., Howes, R., Macias, A. T., Murray, J. B., Parsons, R., Shaw, T., Surgenor, A. E., Terry, L., Wang, Y., Wood, M., & Massey, A. J. (2009). Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. *Journal of Medicinal Chemistry, 52*, 1510–1513.

Wonerow, P., Obergfell, A., Wilde, J. I., Bobe, R., Asazuma, N., Brdicka, T., Leo, A., Schraven, B., Horejsi, V., Shattil, S. J., & Watson, S. P. (2002). Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cgamma2 regulation in platelets. *The Biochemical Journal, 364*, 755–765.

Wyatt, A. R., Yerbury, J. J., Ecroyd, H., & Wilson, M. R. (2013). Extracellular chaperones and proteostasis. *Annual Review of Biochemistry, 82*, 295–322.

Zeiler, M., Moser, M., & Mann, M. (2014). Copy number analysis of the murine platelet proteome spanning the complete abundance range. *Molecular & Cellular Proteomics, 13*, 3435–3445.

Zhang, G., Liu, Z., Ding, H., Zhou, Y., Doan, H. A., Sin, K. W. T., Zhu, Z. J., Flores, R., Wen, Y., Gong, X., Liu, Q., & Li, Y. P. (2017). Tumor induces muscle wasting in mice through releasing extracellular Hsp70 and Hsp90. *Nature Communications, 8*, 589.

Zhu, Y., O'Neill, S., Saklatvala, J., Tassi, L., & Mendelsohn, M. E. (1994). Phosphorylated HSP27 associates with the activation-dependent cytoskeleton in human platelets. *Blood, 84*, 3715–3723.

# Chapter 15
# Heat Shock Protein Responses in Septic Patients


**Marianna Miliaraki, Efrossini Briassouli, Stavroula Ilia, and George Briassoulis**

**Abstract** Sepsis is a maladaptive inflammatory process in response to infectious agents, related to severe complications and poor outcomes. Despite advances, sepsis care remains a crucial challenge for intensive care units. The heat shock response during a septic process is primarily characterized by a dramatic upregulation of heat shock proteins (HSP), which are molecular chaperokines (intracellular chaperones and extracellular cytokines) exhibiting sophisticated protection mechanisms for living organisms. The main HSP representatives in sepsis are the heat shock proteins 70 and 90, which are ubiquitous chaperones with anti-apoptotic and immunomodulatory functions. The HSP family seems to create networks associated with a state of oxidative stress, capillary leak syndrome, immunoparalysis and with the hormonal changes occurring in sepsis.

**Keywords** Heat shock · HSP · Intensive care · Oxidative stress · Sepsis

## Abbreviations

| | |
|---|---|
| ARDS | Acute respiratory distress syndrome |
| ATP | Adenosine-5-triphospate |
| eHSP | Extracellular HSP |
| HDL | High-density lipoprotein |
| hGRa | Human glucocorticoid receptor a |
| HLA | Human leukocyte antigen |
| HS | Heat shock |
| HSC70 | Heat shock cognate protein 70 |
| HSF1 | Heat shock factor 1 |

M. Miliaraki · S. Ilia · G. Briassoulis (✉)
Pediatric Intensive Care Unit, University Hospital, University of Crete, Heraklion, Greece
e-mail: ggbriass@otenet.gr

E. Briassouli
First Department of Pediatrics, Athens University, Athens, Greece



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_15

| | |
|---|---|
| HSP | Heat shock protein |
| ICU | Intensive care unit |
| IFN | Interferon-γ |
| iHSP | Intracellular HSP |
| IL | Interleukin |
| JDP | J-domain protein |
| LDL | Low-density lipoprotein |
| LPS | Bacterial lipopolysacharide |
| MHC | Major histocompatibility complex |
| mRNA | Messenger ribonucleic acid; NF-κB, nuclear factor kappa-B |
| SIRS | Systemic inflammatory response syndrome; SNPs, single nucleotide polymorphisms |
| TGF | Tissue growth factor |
| TLR | Toll like receptor |
| TNF-a | Tumor necrosis factor alpha |

## 15.1 Introduction

Sepsis is a life threatening multiple organ dysfunction process caused by a dysregulated, generalized inflammatory and procoagulant host response to infectious stimulants. As a common and life-threatening infectious syndrome, sepsis contributes significantly to morbidity and mortality in clinical settings. Severe sepsis and septic shock are the leading causes of death in adult and pediatric intensive care units (ICU) worldwide. Despite efforts in understanding sepsis pathophysiology and implementing effective treatment, its annual incidence has been projected to increase by 1.5% per year (Angus et al. 2001). Vascular endothelial injury and hyperpermeability play an important role in the development of sepsis-induced organ dysfunction. So far, most works have associated sepsis with redox imbalance and oxidative stress, but no correlation has been established between antioxidant-based therapies and reduction of sepsis mortality. This is also true for the role of alarmins called heat shock or stress proteins, which are increased in serum during sepsis, probably influenced by oxidative stress, and thus associated with severity of illness and a high risk for mortality (Gelain et al. 2011).

Heat shock proteins (HSP) are molecular chaperokines that prevent the formation of nonspecific protein aggregates and exhibit sophisticated protection mechanisms (innate immunity). Intracellularly, HSP act as cellular housekeepers (chaperones) during homeostasis. The stress-proteins response is primarily characterized by a dramatic upregulation of HSP, triggered by various kinds of stressful environmental insults, such as thermal stress, ischemia, heavy metals, nutrient deprivation, irradiation, infections, inflammation and exposure to organics or oxidants (Radons 2016). Thereby, the expression of HSP seems to promote cell survival upon exposure to different stressors. The most characteristic model for this response is in sepsis or

inflammation-induced conditions, resulting from complex interactions between host and infectious agents. This cytoprotective effect of HSP is mainly attributed to their intracellular ability to stabilize protein structures through their ATP dependent chaperone-like activity, although research has shown that their functions and roles are much more wider and complex. The HSP superfamily includes several different molecular weight class families. The main HSP representatives in sepsis are the heat shock proteins 70 and 90, which are ubiquitous chaperones with anti-apoptotic and immunomodulatory functions. They have also been identified as functional receptors for the bacterial lipopolysaccharide (LPS) on the surface of human macrophages. They can both serve as novel biomarkers for the characterization of the different stages of sepsis (Papadopoulos et al. 2017).

## 15.2 Heat Shock Protein Networks

Sepsis is associated with activation of proinflammatory mediators, including nuclear factor kappa-B (NF-κB), cytokines and chemokines, such as tumor necrosis factor (TNF)-α or interleukins (IL-6, IL-8 and IL-1β). Although these mediators are important for host defense against invading bacteria, their uncontrolled and excessive production ultimately contributes to multiple organ injury. Stress and heat shock seem to have an immune suppressive effect, possibly through heat shock factor 1 (HSF1) mediated competitive inhibition of NF-κB binding. It has been shown that the transcriptional activation of HSP genes is regulated by the stress-activated transcription factor HSF1, and that multiple chaperones, like HSP70, tend to form a complex with HSF1 monomers. It is also believed that the intracellular accumulation of misfolded proteins in response to stress serves as the universal signal, resulting in the upregulation of stress proteins (HSP). Certain molecules of host origin, designated alarmins, activate the immune response. Several candidate alarmins have been described, including uric acid, mitochondrial DNA and extracellular HSP (eHSP). Extracellular HSP70 exhibits immunomodulatory effects, being responsible for early immune hyperactivation and subsequent immunosuppression in the course of severe sepsis. Intracellular HSP70, on the other hand, exerts anti-apoptotic effects, protecting the structural integrity of cells and prolonging leukocyte survival (Gupta et al. 2013).

It has been suggested that stimulation of toll-like receptors (TLR) by LPS increases the HSP70 expression and its release from the intracellular to the extracellular environment. Many studies have reported the extracellular release of HSP in severe sepsis and the simultaneous reduction of intracellular HSP (iHSP). Acting as a TLR agonist, eHSP70 promotes inflammation, but its persistent action will eventually cause immune suppression by inducing tolerance to TLR agonists (Tulapurkar et al. 2015). In patients with non-infectious traumatic inflammation (systemic inflammatory response syndrome - SIRS), however, iHSP were found increased compared to healthy controls, indicating a protective function of iHSP during the acute phase of stress. On the other hand, the downregulation of iHSP in severe

sepsis seems to be a result of adaptation (or maladaptation) to the severity of illness (Vardas et al. 2014).

Extracellular HSP released from stressed cells serve to promptly activate immune competent cells through HSP/interleukin (IL) networks and the LPS-TLR4-CD14-dependent pathway, acting as "danger signals". Through complicated cascades these molecules can initiate both innate and adaptive immune responses and aid in immune surveillance via cytokine and chemokine production, although HSP upregulation has also been linked to worse outcomes and mortality (Fitrolaki et al. 2016). Their contrasting roles have been reported in various studies, emphasizing on the alarming role of HSP70, associated with severity of illness and poor outcome not only in septic patients, but also following cardio-pulmonary bypass or acute lung injury (Wheeler and Wong 2007). HSP have also been linked to the pathogenesis of several autoimmune diseases (Chen and Cao 2010). The release of extracellular HSP appears to be very complex and different mechanisms have been proposed for this phenomenon, such as translocation across the plasma membrane, release through lipid vesicles, passive release after cell death, or nonclassical exomal non-secretory pathways (Li et al. 2012).

### *15.2.1 Heat Shock Protein 70*

The HSP70 family consists one of the most conserved protein families in evolution and their members are present in all organisms and subcellular compartments. During periods of homeostasis, HSP70 levels are around 2% of the intracellular content, but during a physiological insult, cellular HSP70 levels can markedly increase to 20% (Marino et al. 2016). The human HSP70 consists of at least thirteen highly homologous members that differ from each other by the intracellular localization and expression pattern. In general, the HSP70 family includes the inducible HSP70 (also termed HSP72) and the constitutive heat shock cognate protein 70 (HSC70, also termed HSP73). HSP70 and HSC70 are highly homologous, but HSC70 is more abundantly expressed in most cell types (Hsu et al. 2014). Other examples include HSP70–5, which is located in the endoplasmic reticulum and mtHSP70 being expressed in the mitochondrial matrix, whereas the remaining HSP70 proteins reside mainly in the cytosol. HSP70 is expressed only at low or undetectable levels in most unstressed cells, but its expression is rapidly upregulated in a variety of physical and chemical stress states. Finally, HSPA6 (HSP70–6) shows no basal expression and is induced only by extreme stress factors (Rohde et al. 2005).

HSP70 are monomeric proteins that reside in any adenosine-5-triphosphate (ATP)-containing eukaryotic intracellular compartment and can also be found in cell membranes and the extracellular milieu as well as in bacteria. Stress-induced upregulation of HSP promotes cell survival in insults that have the potential to induce cell damage and death. There are studies that link the expression of HSP70 to several types of neoplasms, while HSP70 expression has being associated with resistance to therapy and poor clinical outcome (Radons 2016).

The major stress-inducible HSP70 comprise HSP70–1 (HSPA1A or HSP72) and HSP70–2 (HSPA1B), which are encoded by almost identical intronless genes. Multiple HSP70 isoforms are frequently co-expressed in the same cytosol, directed by different genes. HSP70 members have been suggested to serve as key determinants of mortality and play protective roles in an age-dependent response to sepsis (McConnell et al. 2011). HSP70, along with their co-chaperones of the J-domain protein (JDP) family, work in repairing misfolded proteins and their activity can be divided into three related tasks: prevention of aggregation, promotion of folding to the native state, and solubilization and refolding of aggregated proteins. Common client proteins are protein kinases and their biogenesis, stability and enzymatic activity are regulated by HSP (Mayer and Bukau 2005).

### *15.2.2 Heat Shock Protein 72 Isoform*

Heat shock protein 72 (HSP72) is the most stress-induced isoform in cells and tissues undergoing an inflammatory process of any kind, and is considered to be an important pro-inflammatory protein, since it protects cells from imminent danger. This is supported by recent studies showing higher HSP72 expression in mechanically ventilated critically ill patients with severe sepsis or with trauma, compared to healthy individuals (Briassouli et al. 2015). In vitro intracellular HSP72 responses to stress occur as early as 4 h after heat shock and persist for 24 h after the initial stress (Lee et al. 2017). Monocytes have been demonstrated to be the most sensitive blood cells for HSP72 expression analyses. The production of HSP72 has been shown to exert a protective effect against hypoxia and to facilitate crucial immunological responses in a severe stress state. In a "comparative study" model we showed that the balance between inducible HSP72 production and control of inflammatory cascades in sepsis differ unpredictably between human and animal studies (Briassoulis et al. 2014). Among septic human patients, the upregulation of HSP72 showed a low probability of protection or even a possible relation to mortality, as opposed to septic animals where studies have shown an apparent protective effect of HSP72 in sepsis. Furthermore, analysis of reviewed studies showed various forms of preconditioning in approaching the HSP72 implication in the septic process. Although still inconclusive, most results assume a protective in vitro HSP72 –effect or an in vivo HSP72 –mortality association (Fig. 15.1).

### *15.2.3 Heat Shock Protein 90*

The most widely studied chaperone, the intracellular HSP90 (iHSP90), is a homodimeric molecular chaperone constituting more than 3% of total cellular protein. It is thought to have a guarding and anti-inflammatory role, facilitating cell motility and tissue healing, through the LDL-receptor-related protein-1 (Jayaprakash et al. 2015). In septic myocardial models it was shown to stimulate apoptotic cascades

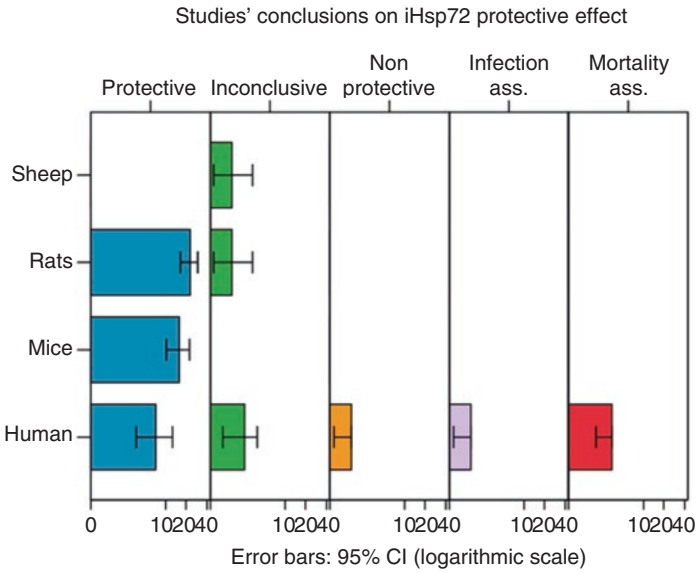


**Fig. 15.1** Comparative study model regarding the HSP72 protective effect in human and animal species (Briassoulis et al., Biomed Research Intern, 2014)

through caspase-3 activation (Li et al. 2013). HSP90 is a highly conserved protein and shows few differences between species. Human HSP90 has two isoforms in the cytoplasm: the constitutively expressed HSP90beta and the inducible HSP90alpha (equivalent to mouse HSP84 and HSP86 respectively). Although these isoforms share common functions, they have distinct characteristics, as HSP90b is primarily involved in signal transduction, growth and development, whereas HSP90a plays a role in the heat shock response (Zhao et al. 2013a).

Extracellular HSP90 (eHSP90) plays a crucial role during antigen presentation in dendritic cells, through the major histocompatibility complex (MHC) type 1, thus facilitating monocyte stimulation. HSP90 affects the cytoskeletal protein actin and enhances disease development in acute lung injury models. It has also been found that HSP90 regulates the expression of TNF-a, interferon-γ (IFN-γ) and IL-1, and that it also modulates the LPS induced inflammatory signaling pathway and the NF-κB activation. The inducible isoform HSP90a is primarily involved in the LPS-induced reactive oxygen species, cell apoptosis and the regulation of IL-1 gene expression (Briassouli et al. 2014). Another study has shown that the combination of LPS and HSP90 inhibition enhanced apoptosis, evidenced by increased caspase-3 activity, proportion of apoptotic nuclei and enhanced chromatin condensation of macrophages (Hsu et al. 2007).

In a recent study, Fitrolaki et al., showed that extracellular HSP90 and, to a lesser extent, HSP72 are markedly elevated in children with severe sepsis compared to non-infectious SIRS or controls, and independently related to severity of illness scores and risk of mortality. It was also shown that both HSP are inversely related to the low-LDL/low-HDL stress metabolic pattern, revealing their important role in

septic metabolic derangements. Another important observation of this study is a trend for an escalating response to stress (assuming DNA damage) of not only HSP90a, but also HSP72 in severe sepsis and SIRS compared to healthy children. Thus, the extracellular HSP90, acting as a "cytokine" or "alarmin", seems to potentiate an already active systemic inflammatory process in severe sepsis. This process probably concerns the patients that might enter a state of profound immunosuppression or persistent activation of innate immunity, with intractable outcomes and high risk of mortality (Fitrolaki et al. 2016).

## 15.3 Oxidative Stress and Heat Shock Protein Responses

In a recent work by Grunwald et al., analysis of serum oxidative parameters revealed that septic patients with pronounced oxidative damage in serum also had increased HSP70 levels, while septic patients with control-like serum oxidative parameters had lower HSP70 levels (Grunwald et al. 2014). There are also indications that oxidative stress is exacerbated in patients with poor outcome and that several anti-inflammatory proteins undergo oxidation by reactive species with impairment of their function. Researchers showed distinct alterations in HSP70 electrophoretic profile when submitted to an oxidative agent (hydrogen peroxide), suggesting that the oxidative environment either damaged the protein or oxidized specific residues. This process probably leads to changes of the protein's conformation and to the acquisition of secondary dysfunctional HSP structures. It was shown that the pre-oxidized HSP70 diminishes the proliferation and viability of the cells, while the phagocytic activity of the macrophages and TNF-α production are also reduced (Grunwald et al. 2014). These data suggest major cytotoxic effects with a lack of "danger signals" and inefficient activation of immune cells in the presence of HSP70 oxidized forms in septic patients. Based on current knowledge, studies imply that septic patients should be classified into two opposite phenotypes, one presenting an antioxidant profile and the other being pro-oxidant. Therefore, to avoid data misinterpretation, future research including the key-roles of HSP72 and 90 in sepsis, should not analyze septic patients as a homogenous group but based on oxidative parameters (Gelain et al. 2011).

### *15.3.1 Heat Shock Proteins and the Cardiovascular Responses in Sepsis*

A recent study focused on HSPA12B, the isoform of the HSP70 superfamily that is mainly expressed in vascular endothelial cells, probably controlling the capillary leak syndrome in the development of sepsis. Capillary leak syndrome, one of the causes of high mortality in early stages of sepsis, can exacerbate acute respiratory distress syndrome (ARDS) and septic shock. It is generally accepted that inflammatory

mediators, such as histamine, LPS and cytokines bind to their receptors on the endothelial surface, leading to the formation of paracellular gaps and increased transendothelial permeability for fluids and macromolecules. This is an important early step in the development of inflammatory pulmonary conditions, such as acute lung injury, ARDS and sepsis. HSPA12B is involved in the adhesion and migration of vascular endothelium cells and participates in angiogenesis and blood vessels stability. Data suggest that this isoform has a key role in protecting the endothelial barrier function after LPS stimulation and that downregulation of HSPA12B may increase pulmonary vascular permeability (Kang et al. 2016). Another study indicated that heat shock induced HSP70 promoted cardiovascular protection by suppressing NO production during endotoxemia. This study highlighted the functional significance of NF-κB activation in the manifestation of cardiovascular depression and its modulation by an upregulation of HSP70. The authors also insinuated an important protective role of HSP70 in central autonomic control of circulation, via potentiating the baroreceptor reflex response and the upregulation of glutamate receptors in the caudal medulla, together with the upregulation of HSP70 in the sympathetic nervous system (Chan et al. 2004).

### *15.3.2 Heat Shock Proteins and Immunoparalysis in Sepsis*

Sepsis is a struggle between pathogen-associated molecules and the host innate and acquired immunity. Proper immunologic balance between pro- and anti-inflammatory pathways is necessary for recovery. A state of acquired immunodeficiency, with persistence of marked compensatory anti-inflammatory immune responses in sepsis, is referred to the process of immunoparalysis and can be quantified through the measurement of monocyte cell-surface HLA-DR expression. HDA-DR has been shown to be negatively influenced by the presence of septic shock and to correlate with sepsis mortality. In fact, a threshold of <30% HLA-DR+ monocytes has become an accepted definition of immunoparalysis (Frazier and Hall 2008). Similar decreasing trends in the expression of CD14/HDL-DR were associated with repressed intracellular HSP70 and HSP90 levels in patients with sepsis compared to SIRS and healthy controls. These early-onset changes in HSP70 and HSP90 monocyte and neutrophil expressions are associated with increased pro-inflammatory cytokine levels, such as IL-6 and IL-10, and decreased IL-17 and IFN-γ levels in patients with sepsis (Papadopoulos et al. 2017).

## 15.4 Hormonal and Immune System Interactions

Sepsis is accompanied by major hormonal and immune system interactions. Heat shock proteins seem to reflect hormonal changes taking place during an inflammatory or septic process, presenting close correlations with the activation of the

hypothalamic-pituitary-adrenal axis and cortisol upregulation. The human glucocorticoid receptor-a (hGRa) mostly resides in the cytoplasm of cells as part of a hetero-oligomeric complex, which contains chaperone HSP 70 and 90. HSP90a and HSP72 work together as essential components for allowing hGRa to bind the incoming steroid hormone and for enhancing its affinity for the ligand. Upon ligand binding, hGRa dissociates from the HSP and translocates into the nucleus, in order to regulate the expression of target genes. Early expression of hGRa mRNA and hGR protein in monocytes have been found to be significantly higher in patients with sepsis compared to healthy controls or patients with trauma-associated SIRS (Vardas et al. 2017). This fact might imply that exogenous cortisol in the acute phase of sepsis probably decreases the hGR expression through a negative feedback mechanism, increasing the glucocorticoid resistance. Furthermore, responding pairs of hGR-HSP seem to be adequately expressed and simultaneously altered in the early phase of the septic process (Fig. 15.2). The increased expression of hGRa mRNA and hGR protein in sepsis, are associated with increased extracellular HSP72 and HSP90a, especially among non-survivors, while intracellular HSP are repressed. All these data probably imply a key role for the HSP 72 and 90a early in sepsis in converting the hGRa to the steroid- binding state, indicating that the high-risk patient prepares himself to respond to the "danger" by increasing the eHSP72 and eHSP90a and cortisol levels (Vardas et al. 2017).

## 15.5 Heat Shock Protein Genetic Polymorphisms

Polymorphisms of the HSP70 genes (HSPA1A, HSPA1B and HSPA1L) have been associated with the development of septic shock and a wide variety of infectious and inflammatory diseases. These three genes are located adjacent to each other within the MHC class 3 region on chromosome 6 and between genes encoding proteins known to be involved in the development of sepsis, such as the complement proteins or TNF. Polymorphisms have been described in the gene encoding for the stress-inducible isoform HSP70–2. A single nucleotide polymorphism (SNP) has been associated with variable expression of the stress-inducible HSP70–2 mRNA; most individuals have a G at position +1267, which is associated with lower mRNA levels after ex vivo stimulation. Although no association between this allele and susceptibility to severe sepsis has been demonstrated, patients with the AA genotype at the HSP70–2 + 1267 seem to be at greater risk for septic shock than those individuals who were either heterozygous or homozygous for the more common GG genotype (Pociot et al. 1993; Waterer et al. 2003). In another study, it was shown that homozygous individuals for the C allele of HSPA1B-179C > T were associated with lower HSPA1A and HSPA1B mRNA levels than HSPA1B-179CT after 8 h lipopolysaccharide stimulation (Temple et al., 2004). Recently, in subjects with confirmed puerperal group-A streptococcal infection, polymorphisms associated with the innate immune response to bacterial infection were analyzed. Allele as well as genotype frequencies were significantly different between subjects and controls for

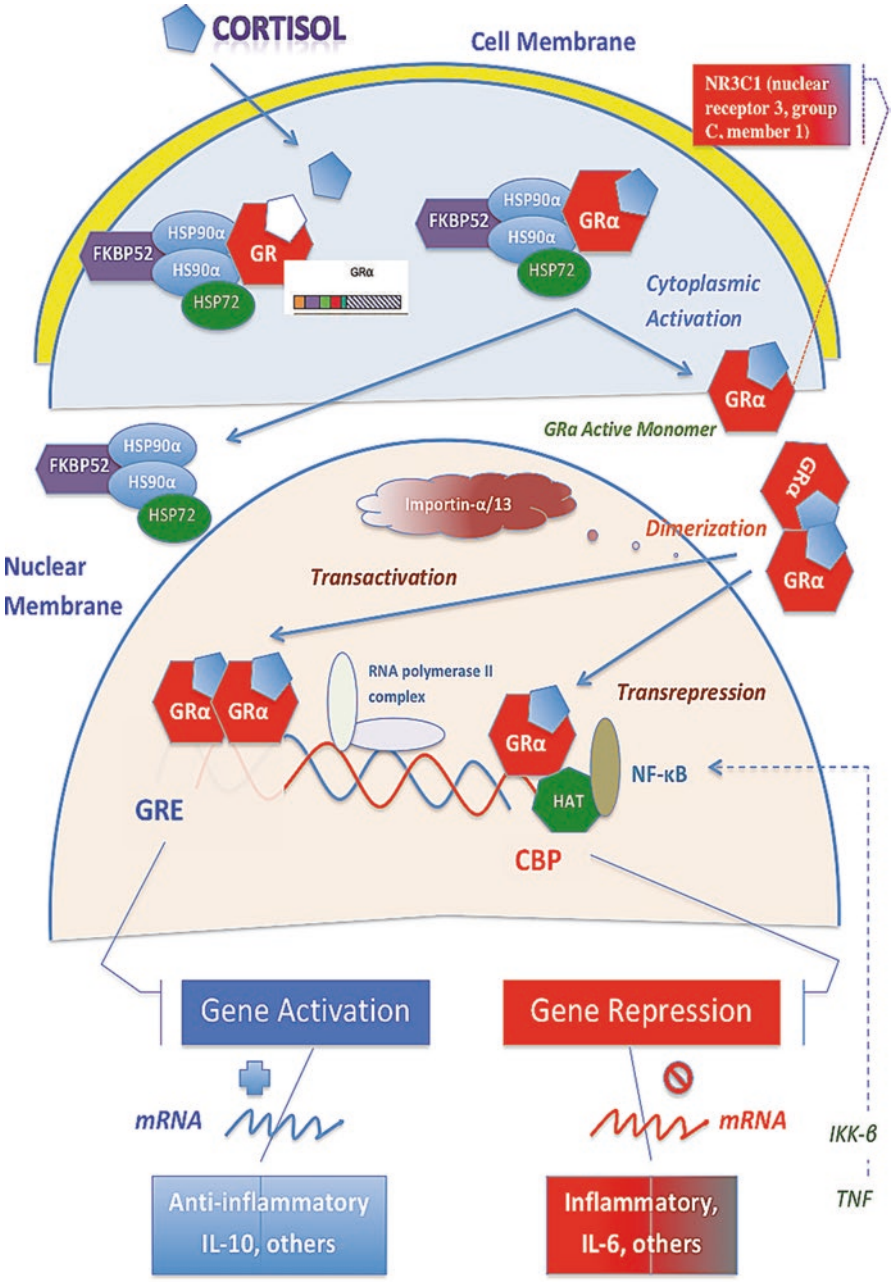


**Fig. 15.2** Glucorticoids diffuse across the cell membrane and bind to human glucocorticoid receptor in the cytoplasm. In a HSP heterocomplex, hGRa is activated (upon ligand binding), is release from HSP72 and HSP90a, and rapidly translocated into the nucleus, where the transcription of target genes is initiated. Through transactivation, binding of two hGRa molecules leads to the transcription of genes encoding anti-inflammatory mediators (i.e IL-10) and the inhibition of NF-κB. (Vardas et al. Intensive Care Medicine Experimental, 2017)

polymorphisms in HSP70–2 1267 (Davis et al. 2010). Despite the fact that HSP72 genotypes were suggested to influence the outcome in sepsis (Jabandziev et al. 2014), a recent study with genotype HSP72 analysis, in both adults and children, did not disclose any group differences regarding rs6457452 and rs1061581 in sepsis or outcome (Tavladaki et al. 2017). Similarly, another study showed that HSP72 mRNA and proteins were related to interleukins but were not influenced by HSP72 polymorphisms (Galic et al. 2010).

Other studies elaborate on the relationships between HSP genetic polymorphisms, inflammatory responses and organ dysfunction. Zhao et al., hypothetized that despite the fact that HSP90b is highly conserved, polymorphisms of this gene may still exist in humans. The researchers confirmed that these SNPs affected the transcription of HSP90b and that the expression of this gene impacts the inflammatory response. They also found that several SNPs were present in the promoter of the HSP90b gene, despite the fact that this chaperone is constitutively expressed in species. Therefore, SNPs located within regions important for transcriptional regulation (promoters), can lead to changes in protein levels and their functions. The authors conclude that specific SNPs alter HSP90b expression levels and are associated with the cellular inflammatory response and the severity of organ dysfunction following injury. HSP90b could also influence multiple inflammation-related genes simultaneously, thus showing more dramatic overall effects as a result of SNPs and could prove to be important in diagnosis and monitoring of high-risk patients (Zhao et al. 2013a).

## 15.6 Heat Shock Protein Agonists and Inhibitors in Sepsis

Research currently focuses on the probable effects of HSP inhibition, in an effort to track possible pharmaceutical targets and lead to favorable outcomes in sepsis. An example for this is a recent study by Zhao et al., which suggested that targeting HSP90 with its inhibitor (radicicol) seems to protect against intestinal inflammation and leakage, which is a key feature in sepsis, and this might be a useful strategy for preventing the development of the inflammatory cascade in sepsis (Zhao et al. 2013b). HSP90 inhibitors have been reported to possess anti-inflammatory effects, associated with activation of heat shock factor, leading to induction of HSP70 and inhibition of oxidative stress (Wang et al. 2016). Targeting HSP90 as an attractive therapeutic strategy for cancers is currently in clinical trials. Preclinical data suggest that HSP90 inhibition might be an effective treatment approach for alleviating chronic inflammatory diseases such as rheumatoid arthritis, since it has been found that HSP90 inhibition in vivo reduces proinflammatory cytokines (Ambade et al. 2012).

HSP90 inhibitors have long been used experimentally. Another example of HSP90 inhibition through geldanamycin has also been proven successful in attenuating tyrosine kinases activity through an indirect action, since tyrosine kinases are HSP90 client proteins. Studies suggest that not only inhibition of NF-κB activation

significantly improves survival in models of severe sepsis, but HSP90 inhibitors are also successful in inhibiting NF-κB activation in various cell lines in vitro (Chatterjee et al. 2007). Growing evidence indicates that HSP90 inhibitors prevent and restore endothelial cell permeability induced by several inflammatory mediators. Antonov et al., employed three HSP90 inhibitors: radicicol, the most effective HSP90 inhibitor in vitro and two geldanamycin analogs (17-AAG and 17-DMAG), which have undergone successful phase I and II clinical trials as adjunct therapies for various neoplasms. The authors elaborate on the protective and reparative effects of HSP90 inhibitors on endothelial cell hyperpermeability after stimulation with a crucial pro-inflammatory cytokine, TGF-β1. Therefore, it seems that HSP90 inhibitors have emerged as an attractive therapeutic modality, due their ability in blocking apoptotic pathways (Antonov et al. 2008).

On the other hand, it is remarkable that current studies also focus on HSP70 preconditioning in an effort to exploit the protective effects and mechanisms of HSP70 in sepsis. Notably, recent animal studies have shown that pretreatment with exogenous HSP70 can improve septic shock and reduce mortality -through the attenuation of endotoxin-induced cardiac and hepatic dysfunction- and could thus be considered as a novel strategy for the prophylaxis of sepsis. The limitation of this study was that only prophylactic but not therapeutic effects were determined because HSP70 was administered prior to but not after the initiation of sepsis (Hsu et al. 2014). Importantly, in vivo exogenous administration of r-AtHSP70 as a molecular modulator protected heart and liver against LPS-induced sepsis, by inhibiting IL-1β and TNFa, improving myocardial function and ameliorating liver dysfunction (Pasqua et al. 2015). Recently, long-term treatment with raloxifene, a selective estrogen receptor modulator, reduced the severity of sepsis in ovariectomized rats by exerting antioxidant and anti-inflammatory effects through the up-regulated HSP70 and HO-1 during bacterial infection (Shen et al. 2017). Further animal and clinical studies should substantiate these important clinical findings.

## 15.7 Glutamine Effects on Heat Shock Proteins in Sepsis

Parenteral administration of glutamine in ICU patients has been found to significantly enhance serum HSP72 expression and attenuate cytokine release after stimulation with bacterial endotoxin lipopolysaccharide, although inconsistent results in outcome have been reported (Briassouli et al. 2014). A recent study revealed a glutamine pretreatment depressive trend to HSP72 after exposure to LPS induced inflammatory signaling, but an enhancing trend after exposure to heat shock (HS) induction (Briassouli et al. 2015). This study also reported that HS but not LPS, induces HSP72 in leukocytes of critically ill patients. On the other hand, the LPS repression effect on HSP72 was also correlated with a synchronous strongly induced cytokine response, as opposed to the inhibitory effect of HS to cytokine release. These findings reveal different pathways activated in response to LPS or HS stress. However, genetic heterogeneity of the HSP and immune response across different patients makes the whole picture even more complicated. Enhanced HSP70

expression and improved survival was accompanied by a significant decrease in lung injury, attenuation of NF-κB activation, and proinflammatory cytokine expression in glutamine-treated HSP70(+/+) mice vs. HSP70(+/+) mice not receiving glutamine. In the HSP70(−/−) mice, glutamine's attenuation of lung injury, NF-κB activation, and proinflammatory cytokine expression was lost. These results indicate that HSP70 expression is required for glutamine's effects on survival, tissue injury, and the inflammatory response in sepsis (Singleton and Wischmeyer 2007).

## 15.8 Conclusions

Sepsis is associated with activation of proinflammatory mediators, through the transcriptional activation of HSP genes. Although these responses are important for host defense against invading bacteria, their uncontrolled and excessive production ultimately contributes to multiple organ injury. The main HSP representatives in sepsis are the heat shock proteins 70 and 90, which are ubiquitous chaperones with antiapoptotic and immunomodulatory functions. They can both serve as novel biomarkers for the characterization of the different stages of sepsis. In patients with sepsis decreasing trends in the expression of CD14/HDL-DR are associated with repressed intracellular HSP70 and HSP90 and increased pro-inflammatory cytokines. Early in sepsis, high-risk patients prepare themselves to respond to the "danger" by increasing the extracellular HSP 72, HSP 90a and cortisol levels by converting the hGRa to the steroid- binding state. There is accumulated evidence, however, that oxidative stress is exacerbated in patients with poor outcome and that several anti-inflammatory proteins including HSP undergo oxidation by reactive species with impairment of their function. Studies currently focus on the probable effects of HSP90 inhibition or HSP70 induction, in an effort to track possible pharmaceutical targets and lead to favorable outcomes in sepsis. Hopefully, more research in future might lead to a more individualized approach to the treatment of sepsis and septic shock, taking into account all the damaged metabolic and molecular pathways, as well as the balance of the immune-hormonal response.

**Acknowledgements** Authors' original research, on which this review is based upon, has been co-financed by the European Union (European Social Fund (ESF)) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF)-Research Funding Program: THALES.

## References

Ambade, A., Catalano, D., Lim, A., & Mandrekar, P. (2012). Inhibition of hsp90 attenuates proinflammatory cytokines and prevents LPS induced liver injury. *Hepatology, 55*, 1585–1595.

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J., & Pinsky, M. R. (2001). Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine, 29*, 1303–1310.

Antonov, A., Snead, C., Gorshkov, B., Antonova, G. N., Verin, A. D., & Catravas, J. D. (2008). Heat shock protein 90 inhibitors protect and restore pulmonary endothelial barrier function. *American Journal of Respiratory Cell and Molecular Biology, 39*, 551–559.

Briassouli, E., Goukos, D., Daikos, G., Apostolou, K., Routsi, C., Nanas, S., & Briassoulis, G. (2014). Glutamine suppresses Hsp72 not Hsp90α and is not inducing Th1, Th2, or Th17 cytokine responses in human septic PBMCs. *Nutrition, 30*, 1185–1194.

Briassouli, E., Tzanoudaki, M., Goukos, D., Routsi, C., Nanas, S., Vardas, K., Apostolou, K., Kanariou, M., Daikos, G., & Briassoulis, G. (2015). Glutamine may repress the weak LPS and enhance the strong heat shock induction of monocyte and lymphocyte HSP72 proteins but may not modulate the HSP72 mRNA in patients with sepsis or trauma. *BioMed Research International, 2015*, 806042.

Briassoulis, G., Briassouli, E., Fitrolaki, D.-M., Plati, I., Apostolou, K., Tavladaki, T., & Spanaki, A.-M. (2014). Heat shock protein 72 expressing stress in sepsis: Unbridgeable gap between animal and human studies--a hypothetical "comparative" study. *BioMed Research International, 2014*, 101023.

Chan, J. Y. H., Ou, C.-C., Wang, L.-L., & Chan, S. H. H. (2004). Heat shock protein 70 confers cardiovascular protection during endotoxemia via inhibition of nuclear factor-kappaB activation and inducible nitric oxide synthase expression in the rostral ventrolateral medulla. *Circulation, 110*, 3560–3566.

Chatterjee, A., Dimitropoulou, C., Drakopanayiotakis, F., Antonova, G., Snead, C., Cannon, J., Venema, R. C., & Catravas, J. D. (2007). Heat shock protein 90 inhibitors prolong survival, attenuate inflammation, and reduce lung injury in murine sepsis. *American Journal of Respiratory and Critical Care Medicine, 176*, 667–675.

Chen, T., & Cao, X. (2010). Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. *European Journal of Immunology, 40*, 1541–1544.

Davis, S. M., Clark, E. A. S., Nelson, L. T., & Silver, R. M. (2010). The association of innate immune response gene polymorphisms and puerperal group a streptococcal sepsis. *American Journal of Obstetrics and Gynecology, 202*, 308.e1–308.e8.

Fitrolaki, M.-D., Dimitriou, H., Venihaki, M., Katrinaki, M., Ilia, S., & Briassoulis, G. (2016). Increased extracellular heat shock protein 90α in severe sepsis and SIRS associated with multiple organ failure and related to acute inflammatory-metabolic stress response in children. *Medicine (Baltimore), 95*, e4651.

Frazier, W. J., & Hall, M. W. (2008). Immunoparalysis and adverse outcomes from critical illness. *Pediatric Clinics of North America, 55*, 647–668. xi.

Galic, S., Oakhill, J. S., & Steinberg, G. R. (2010). Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology, 316*, 129–139.

Gelain, D. P., de Bittencourt Pasquali, M. A., M Comim, C., Grunwald, M. S., Ritter, C., Tomasi, C. D., Alves, S. C., Quevedo, J., Dal-Pizzol, F., & Moreira, J. C. F. (2011). Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis. *Shock, 35*, 466–470.

Grunwald, M. S., Pires, A. S., Zanotto-Filho, A., Gasparotto, J., Gelain, D. P., Demartini, D. R., Schöler, C. M., de Bittencourt, P. I. H., & Moreira, J. C. F. (2014). The oxidation of HSP70 is associated with functional impairment and lack of stimulatory capacity. *Cell Stress & Chaperones, 19*, 913–925.

Gupta, A., Cooper, Z. A., Tulapurkar, M. E., Potla, R., Maity, T., Hasday, J. D., & Singh, I. S. (2013). Toll-like receptor agonists and febrile range hyperthermia synergize to induce heat shock protein 70 expression and extracellular release. *The Journal of Biological Chemistry, 288*, 2756–2766.

Hsu, H.-Y., Wu, H.-L., Tan, S.-K., Li, V. P.-H., Wang, W.-T., Hsu, J., & Cheng, C.-H. (2007). Geldanamycin interferes with the 90-kDa heat shock protein, affecting lipopolysaccharide-mediated interleukin-1 expression and apoptosis within macrophages. *Molecular Pharmacology, 71*, 344–356.

Hsu, J.-H., Yang, R.-C., Lin, S.-J., Liou, S.-F., Dai, Z.-K., Yeh, J.-L., & Wu, J.-R. (2014). Exogenous heat shock cognate protein 70 pretreatment attenuates cardiac and hepatic dysfunction with associated anti-inflammatory responses in experimental septic shock. *Shock, 42*, 540–547.

Jabandziev, P., Smerek, M., Michalek, J., Fedora, M., Kosinova, L., Hubacek, J. A., & Michalek, J. (2014). Multiple gene-to-gene interactions in children with sepsis: A combination of five gene variants predicts outcome of life-threatening sepsis. *Critical Care, 18*, R1.

Jayaprakash, P., Dong, H., Zou, M., Bhatia, A., O'Brien, K., Chen, M., Woodley, D. T., & Li, W. (2015). Hsp90α and Hsp90β together operate a hypoxia and nutrient paucity stress-response mechanism during wound healing. *Journal of Cell Science, 128*, 1475–1480.

Kang, Q., Chen, Y., Zhang, X., Yu, G., Wan, X., Wang, J., Bo, L., & Zhu, K. (2016). Heat shock protein A12B protects against sepsis-induced impairment in vascular endothelial permeability. *The Journal of Surgical Research, 202*, 87–94.

Lee, E. C.-H., Muñoz, C. X., McDermott, B. P., Beasley, K. N., Yamamoto, L. M., Hom, L. L., Casa, D. J., Armstrong, L. E., Kraemer, W. J., Anderson, J. M., et al. (2017). Extracellular and cellular Hsp72 differ as biomarkers in acute exercise/environmental stress and recovery. *Scandinavian Journal of Medicine & Science in Sports, 27*, 66–74.

Li, W., Sahu, D., & Tsen, F. (2012). Secreted heat shock protein-90 (Hsp90) in wound healing and cancer. *Biochimica et Biophysica Acta, 1823*, 730–741.

Li, X., Luo, R., Jiang, R., Meng, X., Wu, X., Zhang, S., & Hua, W. (2013). The role of the Hsp90/Akt pathway in myocardial calpain-induced caspase-3 activation and apoptosis during sepsis. *BMC Cardiovascular Disorders, 13*, 8.

Marino, L. V., Pathan, N., Meyer, R. W., Wright, V. J., & Habibi, P. (2016). An in vitro model to consider the effect of 2 mM glutamine and KNK437 on endotoxin-stimulated release of heat shock protein 70 and inflammatory mediators. *Nutrition, 32*, 375–383.

Mayer, M. P., & Bukau, B. (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences, 62*, 670–684.

McConnell, K. W., Fox, A. C., Clark, A. T., Chang, N.-Y. N., Dominguez, J. A., Farris, A. B., Buchman, T. G., Hunt, C. R., & Coopersmith, C. M. (2011). The role of heat shock protein 70 in mediating age-dependent mortality in sepsis. *Journal of Immunology, 186*, 3718–3725.

Papadopoulos, P., Pistiki, A., Theodorakopoulou, M., Christodoulopoulou, T., Damoraki, G., Goukos, D., Briassouli, E., Dimopoulou, I., Armaganidis, A., Nanas, S., et al. (2017). Immunoparalysis: Clinical and immunological associations in SIRS and severe sepsis patients. *Cytokine, 92*, 83–92.

Pasqua, T., Filice, E., Mazza, R., Quintieri, A. M., Carmela Cerra, M., Iannacone, R., Melfi, D., Indiveri, C., Gattuso, A., & Angelone, T. (2015). Cardiac and hepatic role of r-AtHSP70: Basal effects and protection against ischemic and sepsis conditions. *Journal of Cellular and Molecular Medicine, 19*, 1492–1503.

Pociot, F., Rønningen, K. S., & Nerup, J. (1993). Polymorphic analysis of the human MHC-linked heat shock protein 70 (HSP70-2) and HSP70-Hom genes in insulin-dependent diabetes mellitus (IDDM). *Scandinavian Journal of Immunology, 38*, 491–495.

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress & Chaperones, 21*, 379–404.

Rohde, M., Daugaard, M., Jensen, M. H., Helin, K., Nylandsted, J., & Jäättelä, M. (2005). Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. *Genes & Development, 19*, 570–582.

Shen, H.-H., Huang, S.-Y., Cheng, P.-Y., Chu, Y.-J., Chen, S.-Y., Lam, K.-K., & Lee, Y.-M. (2017). Involvement of HSP70 and HO-1 in the protective effects of raloxifene on multiple organ dysfunction syndrome by endotoxemia in ovariectomized rats. *Menopause, 24*, 959–969.

Singleton, K. D., & Wischmeyer, P. E. (2007). Glutamine's protection against sepsis and lung injury is dependent on heat shock protein 70 expression. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 292*, R1839–R1845.

Tavladaki, T., Spanaki, A. M., Dimitriou, H., Kondili, E., Choulaki, C., Georgopoulos, D., & Briassoulis, G. (2017). Similar metabolic, innate immunity, and Adipokine profiles in adult and pediatric sepsis versus systemic inflammatory response syndrome-a pilot study. *Pediatric Critical Care Medicine, 18*(11), e494–e505.

Tulapurkar, M. E., Ramarathnam, A., Hasday, J. D., & Singh, I. S. (2015). Bacterial lipopolysaccharide augments febrile-range hyperthermia-induced heat shock protein 70 expression and extracellular release in human THP1 cells. *PLoS One, 10*, e0118010.

Temple, S. E. L., Cheong, K. Y., Ardlie, K. G., Sayer, D., & Waterer, G. W. (2004). The septic shock associated HSPA1B1267 polymorphism influences production of HSPA1A and HSPA1B. *Intensive Care Medicine, 30*, 1761–1767.

Vardas, K., Apostolou, K., Briassouli, E., Goukos, D., Psarra, K., Botoula, E., Tsagarakis, S., Magira, E., Routsi, C., Nanas, S., et al. (2014). Early response roles for prolactin cortisol and circulating and cellular levels of heat shock proteins 72 and 90α in severe sepsis and SIRS. *BioMed Research International, 2014*, 803561.

Vardas, K., Ilia, S., Sertedaki, A., Charmandari, E., Briassouli, E., Goukos, D., Apostolou, K., Psarra, K., Botoula, E., Tsagarakis, S., et al. (2017). Increased glucocorticoid receptor expression in sepsis is related to heat shock proteins, cytokines, and cortisol and is associated with increased mortality. *Intensive Care Medicine Experimental, 5*(10), 10.

Wang, Y.-L., Shen, H.-H., Cheng, P.-Y., Chu, Y.-J., Hwang, H.-R., Lam, K.-K., & Lee, Y.-M. (2016). 17-DMAG, an HSP90 inhibitor, ameliorates multiple organ dysfunction syndrome via induction of HSP70 in Endotoxemic rats. *PLoS One, 11*, e0155583.

Waterer, G. W., ElBahlawan, L., Quasney, M. W., Zhang, Q., Kessler, L. A., & Wunderink, R. G. (2003). Heat shock protein 70-2+1267 AA homozygotes have an increased risk of septic shock in adults with community-acquired pneumonia. *Critical Care Medicine, 31*, 1367–1372.

Wheeler, D. S., & Wong, H. R. (2007). Heat shock response and acute lung injury. *Free Radical Biology & Medicine, 42*, 1–14.

Zhao, Y., Tao, L., Jiang, D., Chen, X., Li, P., Ning, Y., Xiong, R., Liu, P., Peng, Y., & Zhou, Y.-G. (2013a). The -144C/a polymorphism in the promoter of HSP90beta is associated with multiple organ dysfunction scores. *PLoS One, 8*, e58646.

Zhao, Y., Huang, Z.-J., Rahman, M., Luo, Q., & Thorlacius, H. (2013b). Radicicol, an Hsp90 inhibitor, inhibits intestinal inflammation and leakage in abdominal sepsis. *The Journal of Surgical Research, 182*, 312–318.

# Chapter 16
# Heat Shock Protein 70 (HSP70) Family in Dengue Virus Infection

**Rattiyaporn Kanlaya and Visith Thongboonkerd**

**Abstract** Dengue virus (DENV) infection is a mosquito-borne disease and remains one of the major public health problems worldwide, particularly in tropical and subtropical regions. In most cases, the infection causes only mild and self-limiting illness, namely dengue fever (DF). The infection, however, can develop to severe and life-threatening disease known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Recent studies have suggested that host factors, while protect against the invaded virus, can be modulated and utilized by DENV for its survival and propagation within host cells, leading to infection. Heat shock protein 70 (HSP70) family is one of the key components of host machinery for protein homeostasis (proteostasis). Molecular chaperone function of HSP70 family is achieved by cooperative networking of HSP70 family members (HSP70s) and their co-chaperones to regulate ATP-ADP cycling. According to their fundamental importance, HSP70s have been discovered as the host factors hijacked by many viruses, including DENV, for their efficient infection. This chapter provides the current knowledge on how DENV manipulates host HSP70s in multiple stages of the viral life cycle to accomplish the infection. The potential of HSP70s-based antiviral therapeutics is also discussed.

**Keywords** Chaperone · Dengue · HSP70 · Stress response · Viral entry · Viral replication

## Abbreviations

| | |
|---|---|
| ADE | Antibody-dependent enhancement |
| APCs | Antigen presenting cells |
| ATF6 | Activating transcription factor 6 |

R. Kanlaya · V. Thongboonkerd (✉)
Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_16

| | |
|---|---|
| BiP | Binding immunoglobulin protein |
| C | Capsid |
| DC | Dendritic cells |
| DC-SIGN | Dendritic cell-specific ICAM-grabbing non-integrin |
| DENV | Dengue virus |
| DF | Dengue fever |
| DHF | Dengue hemorrhagic fever |
| DSS | Dengue shock syndrome |
| DVHFs | Dengue viral host factors |
| E | Envelope |
| eIF2α | Eukaryotic translation initiation factor 2α |
| ER | Endoplasmic reticulum |
| ERp44 | Endoplasmic reticulum protein 44 |
| GRP78 | 78 kDa glucose-regulated protein |
| Hsc70 | Heat shock cognate 70 |
| HSP70 | Heat shock protein 70 |
| Hsp70i | Inducible HSP70 |
| HSP70s | HSP70 family members |
| HSPA5 | Heat shock 70 kDa protein 5 |
| ICAM | Intercellular adhesion molecule |
| IRE1 | Inositol-requiring protein 1 |
| JEV | Japanese encephalitis virus |
| M | Membrane protein |
| miRNA | microRNA |
| NS | Non-structural protein |
| PERK | Protein kinase R-like ER kinase |
| prM | Premembrane protein |
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| siRNA | Small interfering RNA |
| UPR | Unfolded protein response |
| VOPBA | Viral overlay protein binding assay |
| VSR | Viral suppressor of RNA silencing |
| WNV | West Nile virus |
| XBP1 | X-box binding protein 1 |

## 16.1 Introduction

Dengue virus (DENV) infection is a common disease worldwide with approximately 400 million infections per annum (Bhatt et al. 2013). This mosquito-borne pathogen is divided into four distinct serotypes (DENV1, DENV2, DENV3 and DENV4) that can cause a wide range of clinical manifestations, including

asymptomatic infection, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Among these, the classical form of DF with self-limiting illness is the most common manifestation, whereas much fewer cases develop severe disease with complications manifested by extravasation, spontaneous bleeding, vascular leakage, low platelet count, circulatory failure and shock (i.e. DHF/DSS) (Bhatt et al. 2013). Ignorance, misdiagnosed infection and inappropriate treatment can lead to mortality. Although the disease has been well-recognized for a long time, the pathogenic mechanisms of DHF/DSS remain poorly understood and should be further elucidated.

## 16.2 Overview of DENV and Its Infection

DENV is a positive single-stranded RNA virus in the family *Flaviviridae* and genus *Flavivirus* with a genome size of approximately 11 kilobases. The viral genome contains one open reading frame encoding a polyprotein precursor that is co-translated and post-translationally modified by virus and host proteases into ten proteins (Harris et al. 2006). These include three structural proteins (core/capsid (C), premembrane (prM)/membrane (M), and envelope (E) proteins) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Harris et al. 2006). Each viral protein plays different roles in each step of DENV life cycle, starting from virion attachment and entry into host cells, genome uncoating, viral replication, viral assembly, and finally release. To establish the infection, DENV attaches to host cells through various specific cellular receptors, e.g., heparin sulfate, DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin, where ICAM is intercellular adhesion molecule), CD14, HSP70, etc., or non-specific Fc receptors (especially in case of dengue-immune complex) leading to the viral entry via receptor-mediated endocytosis (Cruz-Oliveira et al. 2015). The acidic pH of endosome subsequently triggers conformational change of E protein, allowing uncoating of nucleocapsid into the cytoplasm (Modis et al. 2004). Thereafter, the viral RNA is processed for viral protein synthesis by host translational machinery in rough endoplasmic reticulum (ER) to form a viral replication complex. Once the replication complex is formed, the viral RNA translation is terminated and viral replication is switched on. Following the packaging of newly synthesized viral RNA into nucleocapsid, viral assembly continues to take place on the ER surface and is budded into the ER lumen (Kuhn et al. 2002). The immature virion is sorted through the trans-Golgi network where it meets furin protease for proteolytic cleavage of prM to become mature (or partly mature) and ready to release (Kuhn et al. 2002; Yu et al. 2008).

Several hypotheses have been proposed to explain the pathogenesis of DHF/DSS, e.g., antibody-dependent enhancement (ADE) (Halstead 2014), aberrant T cell response during secondary heterotypic infection (Mongkolsapaya et al. 2006), defective T cell response by original antigenic sin (Mongkolsapaya et al. 2003), and direct infection of myeloid and endothelial lineages (Avirutnan et al. 1998; Chen

et al. 2007). Of these, ADE hypothesis is mostly and widely accepted. ADE is a mechanism by which protective antibody against DENV generated during primary infection turns to cause severe form of the disease (DHF/DSS) since it has low avidity or titer falls below neutralizing threshold. Instead of neutralizing the virus, the diluted antibody turns to facilitate viral entry into Fc receptor-bearing cells during the secondary heterotypic DENV infection (Halstead 2014). This phenomenon raises a great concern in vaccine design.

In addition, T cell response in DENV infection has emerging role in the dengue pathogenesis (Malavige and Ogg 2013; Screaton et al. 2015). Difference in T cell response between mild and severe forms of the disease has been reported. Functional study of T cells has revealed that DENV-specific $CD8^+$ T cells are highly degranulated but produce low levels of proinflammatory cytokines in mild DF (Duangchinda et al. 2010). In contrast, those isolated from DHF/DSS show defective degranulation but produce high levels of TNF and IFN-γ (Duangchinda et al. 2010). These findings imply that DENV-specific $CD8^+$ T cells found in the former group are potentially involved in viral clearance, while those found in the latter have no effect on such clearance, but are rather associated with immunopathology of severe infection (Duangchinda et al. 2010). Moreover, the extent of response to both $CD4^+$ and $CD8^+$ T cells is positively correlated with the disease severity and $CD8^+$ T cells are cross-reactive during the secondary infection (Duangchinda et al. 2010; Mongkolsapaya et al. 2003). High expansion of T cell population found during the secondary infection has been shown to be cross-reactive to the pre-infecting virus serotype during the primary infection in addition to the current virus serotype (Mathew et al. 2014; Mongkolsapaya et al. 2003; Mongkolsapaya et al. 2006). However, the theory of original antigenic sin in T cell response cannot explain severe disease found in infants.

## 16.3 Involvement of Human Hsp70 Family in Viral Infection

The HSP70 family is one among the most evolutionary conserved protein families. In humans, 13 gene products have been reported, including those encoded by *HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA5, HSPA6, HSPA7, HSPA8, HSPA9, HSPA12A, HSPA12B, HSPA13,* and *HSPA14* (Brocchieri et al. 2008; Radons 2016). Subcellular localizations and tissue distributions of these HSP70 family members (HSP70s) are quite varied. HSP70s comprise N-terminal domain (also known as nucleotide-binding domain) possessing ATPase activity to hydrolyze ATP and C-terminal part holding a substrate-binding domain (Flaherty et al. 1990). The chaperone function of HSP70s is driven by the transition between ATP-bound and ADP-bound states, which have low and high affinities to the substrate, respectively. At basal state, ATP hydrolysis activity of HSP70s is very low; however, it is activated by binding with substrate and co-chaperone proteins, thereby switching from ATP-bound to ADP-bound state and increasing affinity to the substrate. The ATPase cycle of HSP70s is thus controlled by interacting with their co-chaperones, which

are typically DNAJ proteins and nucleotide exchange factors (Dekker et al. 2015; Kampinga and Craig 2010).

The network of HSP70s and co-chaperones plays a central role in protein homeostasis, including nascent protein folding, protein translocation across membranes, and protein-complex assembly under optimal condition (Hartl and Hayer-Hartl 2009; Kampinga and Craig 2010). However, under sustained environmental stresses induced by various stimuli (e.g., thermal stress, nutrient starvation, chemical toxicity, oxidative stress, hypoxia, inflammation, infection, etc.), chaperones and their partners may facilitate protein degradation when they cannot cope with massive misfolded proteins (Alberti et al. 2002). In addition to intracellular HSP70s, cell surface and extracellular HSP70s have been shown to have immuno-modulatory effects on both innate and adaptive immune responses (De Maio 2014; Fong et al. 2015; Henderson and Pockley 2010; Pockley et al. 2014). In this context, HSP70s released from tumor cells bind to surface receptors on antigen presenting cells (APCs) and elicit tumor-specific killers by means of antigen cross-presentation (Salimu et al. 2015; Zhu et al. 2016). Additionally, extracellular HSP70s can also regulate cytokine production by dendritic cells (DCs), underscoring a connection between innate and adaptive immune responses modulated by HSP70s (Chen and Cao 2010).

According to their fundamental importance in many aspects, HSP70s have been discovered as the host factors for protein surveillance to protect host cells against virus(es). On the other hand, HSP70s are occasionally hijacked by many viruses to achieve their infection. HSP70 can bind to viral capsid protein derived from West Nile virus (WNV) (Oh and Song 2006). Overexpression of HSP70 can inhibit translocation of viral capsid into nucleoli. Although this interaction has not yet been investigated in natural infection, the *in vitro* findings have suggested that HSP70 may act as a negative regulator of viral capsid protein to protect host cells against WNV infection by abolishing cytotoxic effects induced by the viral capsid (Oh and Song 2006). In addition, recent studies have revealed that Japanese encephalitis virus (JEV) requires HSP70s in particular stage of its life cycle for survival and establishment of infection, including viral entry (Thongtan et al. 2012; Zhu et al. 2012), replication (Nain et al. 2017; Ye et al. 2013), and maturation (Wu et al. 2011). These findings underscore the significant roles of HSP70s in many virus infections. Whereas additional details of the involvement of molecular chaperones, particularly HSP70s, in viral infections have been reviewed elsewhere (Xiao et al. 2010), this chapter will focus on the roles of HSP70s in DENV infection.

## 16.4 Role of Hsp70 in DENV Infection

### *16.4.1 HSP70 as the Receptor for DENV Viral Entry*

Several studies have reported that HSP70s serve as putative DENV receptors in both mammalian and mosquito cells. In mammalian, 78 kDa glucose-regulated protein (GRP78) (also known as heat shock 70 kDa protein 5 (HSPA5) or binding immunoglobulin protein (BiP)) has been previously identified as a DENV2 receptor expressed on hepatocytic cell line (HepG2) by using viral overlay protein binding assay (VOPBA) coupled to mass spectrometry (Jindadamrongwech et al. 2004). Blocking GRP78 on the cell surface by specific antibody against N-terminus of GRP78 causes moderate decrease in DENV production. However, blocking at its C-terminus, on the other hand, enhances virus production in a dose-dependent manner (Jindadamrongwech et al. 2004). These findings suggest that binding of the antibody at the C-terminus of GRP78 may induce conformational change of the receptor and thus can facilitate DENV binding and finally enhance DENV replication inside the cells (Jindadamrongwech et al. 2004). Although precise mechanism has not yet been investigated, this study provides evidence that the non-Fc protein receptor GRP78 can serve as DENV receptor in hepatocytes (Jindadamrongwech et al. 2004). Moreover, to overcome the limitation of VOPBA in which proteins can be denatured during gel electrophoresis, another study has developed DENV2-affinity chromatography to identify DENV2-binding proteins from HepG2 cell membrane fraction (Upanan et al. 2008). Western blotting has shown that GRP78 is one of the DENV2-bound proteins eluted from the affinity column (Upanan et al. 2008), consistent with the data obtained from the VOPBA approach in the aforementioned study (Jindadamrongwech et al. 2004).

In addition, DENV receptor complex has been isolated from neuroblastoma and monocytic cell lysates by using DENV E protein affinity chromatography (Reyes-Del Valle et al. 2005). Mass spectrometric analysis has identified 84-kDa protein eluted from the column as HSP90. Furthermore, a 72-kDa protein found in the eluate has been proven to be HSP70 by immunoblotting (Reyes-Del Valle et al. 2005). His-tag pull down assay using DENV E protein along with cell surface biotinylation and indirect immunofluorescence assay has confirmed that both HSP90 and HSP70 are expressed on the cell surface and thus reasonably interact with DENV E protein. Interestingly, competitive assay by incubating DENV particles with recombinant HSP90 or HSP70 prior to infection causes significant reduction of DENV infection in a dose-dependent manner. Similarly, blocking HSP90 and HSP70 on the host cell surface before DENV infection shows the same result (Reyes-Del Valle et al. 2005). In addition, perturbation of lipid rafts using cholesterol-depletion agent prior to infection markedly inhibits DENV infectivity (Reyes-Del Valle et al. 2005). Collectively, these data suggest that HSP90 and HSP70 are members of DENV receptor complex and reside on lipid rafts of human monocytes, which in turn may induce intracellular signaling pathways involving in the dengue pathogenesis (Reyes-Del Valle et al. 2005). Interestingly, the same group of investigators have

also reported that heat shock stress can enhance DENV replication in differentiated U937 cells by a mechanism related to the increased HSP90 and HSP70 expression on the cell surface especially in the lipid raft microdomains (Chavez-Salinas et al. 2008). Surprisingly, the increased expression of surface HSP90 and HSP70 during heat stress does not enhance DENV binding, but facilitates virus entry as shown by the increased cells with internalized DENV immediately after viral challenge. These findings suggest that both HSP90 and HSP70 are required for DENV entry and heat stress provides the optimal stress for viral replication in U937 cells (Chavez-Salinas et al. 2008).

Subsequent study has revealed that HSP70 is required for DENV life cycle, including viral entry, viral replication, and virion production (Taguwa et al. 2015). To address whether HSP70 is required for DENV entry, a specific HSP70 inhibitor (JG40) has been employed. Pretreating or co-treating the cells with such inhibitor significantly reduces viral RNA and production comparable to heparin, which is the well known inhibitor of DENV entry. Additionally, electroporation of the cells to introduce *in vitro* transcribed viral genome to bypass viral entry step in the presence of JG40 causes a reduction of viral RNA production, while heparin has no effect. These findings strongly confirm the significant role of HSP70 in both viral entry and post-entry steps in DENV infection (Taguwa et al. 2015).

Recently, inducible HSP70 (Hsp70i), which is the closely related homolog to heat shock cognate 70 (Hsc70) protein, has been demonstrated to be increased during DENV4 infection and be employed by the virus during its entry (Howe et al. 2016). Using an inhibitor (HS-10) specific to HSP90 also results in the increased Hsp70i level on surface of the infected cells and subsequently the increased infectivity in U937 + DC-SIGN cells. Interestingly, pretreatment with an inhibitor (HS-72) specific to Hsp70i prior to infection markedly reduces DENV infectivity. Proximity ligation assay indicates the interaction between Hsp70i and E protein or DC-SIGN, which can be disrupted by HS-72 (Howe et al. 2016).

Attempts have been also made to identify DENV receptors on mosquito cells using similar approach (Cao-Lormeau 2009; Munoz et al. 1998; Salas-Benito et al. 2007; Salas-Benito and Del Angel 1997). Hsc70 is one among the identified DENV2-interacting proteins in mosquito cell membranes (Paingankar et al. 2010). Nonetheless, the mRNA expression of HSc70 is unchanged in DENV2-infected mosquito cells. Additionally, HSP70/Hsc70 and BiP (also known as GRP78) have been identified as the proteins involved in DENV4 binding and entry in C6/36 cells (Vega-Almeida et al. 2013). During DENV4 infection, these proteins are translocated from cytoplasm to the cell surface (to serve as the DENV receptors) without significant changes in their total levels. Blocking their surface expression by specific antibodies prior to the virus challenge causes significant decrease in DENV binding and infectivity. In addition to these two proteins, HSP90, protein disulfide isomerase (PDI) and endoplasmic reticulum protein 44 (ERp44) have been also identified as DENV E-binding proteins and also play significant roles in DENV binding and entry (Vega-Almeida et al. 2013).

### *16.4.2 HSP70 as the Enhancer for DENV Viral Replication*

In addition to the role as DENV receptors, HSP70s can also facilitate DENV viral replication. BiP (GRP78) interacts directly to Domain III of DENV E protein in Vero cells (Limjindaporn et al. 2009). Knockdown of BiP using small interfering RNA (siRNA) causes a significant reduction of the virus titer (Limjindaporn et al. 2009). Additionally, HSP70 is increased in the DENV-infected THP-1 monocytic cells (Padwad et al. 2010). Moreover, the infectivity of DENV is abolished when HSP70 is knocked down by siRNA (Padwad et al. 2010). Moreover, HSP70 requires several DNAJ proteins as co-chaperones (i.e., DnaJA2, DnaJB6, DnaJB7, DnaJB11 and DnaJC102) for post-entry steps as shown by the decreased viral RNA production in DNAJ-knockdown experiments (Taguwa et al. 2015).

Recent study has demonstrated that DENV nonstructural protein NS3 acts as a viral suppressor of RNA silencing (VSR) by interacting with Hsc70, a part of RNA interference (RNAi) machinery, thereby interrupting host antiviral system by RNAi pathway and subsequently enhancing DENV replication (Kakumani et al. 2015a). In addition, the complex between NS3 and Hsc70 can destabilize RNA-induced silencing complex (RISC)-loading complex, resulting in reduction of microRNA (miRNA) biogenesis and alteration in mRNA profiling of DENV-infected cells. Interestingly, mRNA profiling of NS3-overexpressed cells shows up-regulation of known dengue viral host factors (DVHFs), which in turn promote DENV replication (Kakumani et al. 2015a). Taken together, these data indicate that NS3 interferes antiviral RNAi pathway by interacting with Hsc70 to modulate host miRNA and mRNA levels that subsequently enhance DENV replication.

More recently, there is an additional evidence demonstrating that DVHFs involving in miRNA processing, in particular dicer complex, may play role in controlling DENV replication by RNAi pathway (Kakumani et al. 2016). GRP75 (also known as HSPA9 or mortalin) is one of the dicer complex proteins as shown by its ability to interact with known dicer proteins, i.e. HADHA (trifunctional enzyme subunit alpha protein) (Kakumani et al. 2015b). While premature forms of miRNAs are decreased, their mature forms (in particular hsa-miR-126-5p, which has been reported to be altered by dengue NS3 (Kakumani et al. 2015a)) are increased in the GRP75-overexpressed cells. Knockdown of GRP75 enhances viral replication, while the increased hsa-miR126-5p markedly suppresses viral replication as indicated by the decreased viral genome as well as viral titer (Kakumani et al. 2016). These data indicate that GRP75, a component of dicer complex, acts as the protein surveillance to restrict DENV replication through the action of hsa-miR126-5p, strengthening the role of antiviral RNAi pathway against DENV infection (Kakumani et al. 2016).

### *16.4.3 HSP70 as the Regulatory Chaperone to Cope with DENV-Induced Stresses*

DENV can trigger numerous signaling pathways in host cells (Fischl and Bartenschlager 2011), as well as the unfolded protein response (UPR) (Diwaker et al. 2015; Paradkar et al. 2011; Umareddy et al. 2007). The master regulator of UPR is BiP (GRP78), which normally keeps all three branches of UPR initiation molecules or transmembrane protein sensors (i.e., protein kinase R-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring protein 1 (IRE1)) inactive during the basal state (or another word, when there is no ER stress induced). Once infected, the increased DENV viral proteins initiate host ER stress, leading to UPR activation for cell survival through the dissociation of BiP and UPR initiation molecules (Perera et al. 2017). DENV modulates host UPR pathway for many reasons that eventually contribute to its pathogenesis (e.g., to facilitate viral replication and to inhibit apoptosis by modulating autophagy and induction of inflammation) (Perera et al. 2017).

The correlation between degree of UPR and disease severity of DENV infection has been demonstrated (Ooi et al. 2011). In addition, levels of mRNA involving UPR in THP-1 infected with clinical isolates are correlated well with degree of DENV viral replication and disease severity (Ooi et al. 2011). Interestingly, DENV can modulate UPR activation in a sequential manner to prolong the viral life cycle by allowing cellular adaptation to cope with the infection-induced ER stress (Pena and Harris 2011). In the early phase of infection, UPR is transiently induced by PERK pathway resulting in phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) and subsequently translational attenuation. However, it is then suppressed by the virus shortly after. This transient event allows viral protein synthesis and accumulation that finally trigger UPR by IRE1-XBP1 (X-box binding protein 1) axis in the mid-phase of infection (Pena and Harris 2011). This results in the increased expression of GRP78 to facilitate protein folding and also the increased expression of GADD34 (growth arrest and DNA damage 34), which dephosphorylates eIF2α, and thus allowing protein translation to be continued. The increased GRP78 also prevents cellular apoptosis-mediated by CHOP (pro-apoptotic protein during stress persistence). Finally, in late phase, the increased viral proteins transiently trigger ATF6 arm of UPR to provide the active spliced XBP1 for sustaining UPR activation (Pena and Harris 2011).

Recently, proteomics has emerging role in studying virus-host interactions. In addition to BiP, other members of HSP70 family have been reported to have altered levels in response to DENV infection in both mosquito cells and various human cell lines (Chiu et al. 2014; Martinez-Betancur et al. 2014; Pando-Robles et al. 2014; Patramool et al. 2011; Rungruengphol et al. 2015; Zhang et al. 2013). These findings, although inconclusive due to a lack of functional validation, appear to confirm the importance roles of HSP70s and UPR response to some extent upon DENV infection. Besides cellular proteome of DENV-infected cells, secretome analysis of DENV-infected HepG2 liver cells also shows some chaperones, including HSP70

and HSP90, as the secreted products of the cells (Higa et al. 2008). Moreover, both HSP70 and HSP90 have been recognized to be exported from the cells via exosomal pathway (Olver and Vidal 2007). Nonetheless, the functional relevance of such chaperones in the secretome of DENV-infected cells remains largely unknown and should be further elucidated.

### *16.4.4 HSP70 as the New Therapeutic Target for Management of DENV Infection*

Several studies have demonstrated the potential role of HSP70s as new therapeutic targets for DENV infection. For example, direct targeting of HSP70 and GRP78 by gene manipulation or by using a small inhibitor (HS-72) significantly reduces DENV viral replication, indicating the important role of these chaperones in DENV infection (possibly through viral antigen production and assembly) (Howe et al. 2016; Limjindaporn et al. 2009; Padwad et al. 2010; Wati et al. 2009). In addition, it has been shown that the antiviral RNAi pathway can be manipulated by DENV infection. DENV can hijack Hsc70, a component of host RNAi machinery, by forming the interaction between Hsc70 and viral NS3, thereby interfering host miRNA biogenesis and facilitating DENV replication (Kakumani et al. 2015a). Moreover, GRP75 has been illustrated to act as a component of dicer complex to control DENV replication through the host antiviral RNAi pathway (Kakumani et al. 2016). Therefore, additional study to provide insights into the interplays between host RNAi machinery and viral proteins would be another strategy to cope with DENV infection.

Interestingly, recent study has shown that subnetworks between HSP70 and selective DNAJ proteins play important role in multiple steps of DENV life cycle, including viral entry, replication, protein production, and particle biogenesis (Taguwa et al. 2015). Importantly, inhibition of HSP70 activity by allosteric drug JG40 significantly declines DENV activity in all steps of the virus life cycle without any cytotoxic effect. JG40 also reduces proinflammatory cytokines and chemokines that contribute to the development of DHF/DSS. In addition, the antiviral activity of JG40 is also applicable to different DENV serotypes and other flaviviruses (i.e., WNV, Yellow fever virus, Tick-borne encephalitis virus) (Taguwa et al. 2015). These findings suggest a potential role of HSP70s as the new therapeutic targets for management of DENV infection.

DENV can manipulate host UPR in a time-dependent manner for its efficient infectivity (Pena and Harris 2011). Pharmacological disruption of UPR by inhibition of protein translation using salubrinal markedly reduces DENV replication (Umareddy et al. 2007). Additionally, inhibition of GRP78 by a specific inhibitor (VER-155008) can activate all three branches of UPR and subsequently suppresses DENV replication (Diwaker et al. 2015). These findings suggest that extensive investigations of host UPR during DENV infection and manipulations of chaperones

or components of UPR pathway by using specific inhibitors at the right time are promising strategy to fight against DENV infection.

## 16.5 Conclusions

HSP70s play many important roles in various viral infections. In DENV infection, HSP70s serve as DENV receptors for viral entry, enhancers for viral replication, and regulatory chaperones to cope with stresses induced by DENV infection. Because HSP70s are involved in UPR and interplays between host RNAi machinery and viral proteins, HSP70s thus may serve as the promising new therapeutic targets for management of DENV infection in the future.

**Acknowledgements** This work was supported by Mahidol University research grant and the Thailand Research Fund (IRN60W0004 and IRG5980006).

## References

Alberti, S., Demand, J., Esser, C., Emmerich, N., Schild, H., & Hohfeld, J. (2002). Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. *The Journal of Biological Chemistry, 277*, 45920–45927.

Avirutnan, P., Malasit, P., Seliger, B., Bhakdi, S., & Husmann, M. (1998). Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. *Journal of Immunology, 161*, 6338–6346.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., et al. (2013). The global distribution and burden of dengue. *Nature, 496*, 504–507.

Brocchieri, L., Conway, D. M., & Macario, A. J. (2008). *hsp70* genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evolutionary Biology, 8*, 19.

Cao-Lormeau, V. M. (2009). Dengue viruses binding proteins from Aedes Aegypti and Aedes Polynesiensis salivary glands. *Virology Journal, 6*, 35.

Chavez-Salinas, S., Ceballos-Olvera, I., Reyes-Del Valle, J., Medina, F., & Del Angel, R. M. (2008). Heat shock effect upon dengue virus replication into U937 cells. *Virus Research, 138*, 111–118.

Chen, T., & Cao, X. (2010). Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. *European Journal of Immunology, 40*, 1541–1544.

Chen, H. C., Hofman, F. M., Kung, J. T., Lin, Y. D., & Wu-Hsieh, B. A. (2007). Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. *Journal of Virology, 81*, 5518–5526.

Chiu, H. C., Hannemann, H., Heesom, K. J., Matthews, D. A., & Davidson, A. D. (2014). High-throughput quantitative proteomic analysis of dengue virus type 2 infected A549 cells. *PLoS One, 9*, e93305.

Cruz-Oliveira, C., Freire, J. M., Conceicao, T. M., Higa, L. M., Castanho, M. A., & Da Poian, A. T. (2015). Receptors and routes of dengue virus entry into the host cells. *FEMS Microbiology Reviews, 39*, 155–170.

De Maio, A. (2014). Extracellular Hsp70: Export and function. *Current Protein & Peptide Science, 15*, 225–231.

Dekker, S. L., Kampinga, H. H., & Bergink, S. (2015). DNAJs: More than substrate delivery to HSPA. *Frontiers in Molecular Biosciences, 2*, 35.

Diwaker, D., Mishra, K. P., & Ganju, L. (2015). Effect of modulation of unfolded protein response pathway on dengue virus infection. *Acta Biochimica et Biophysica Sinica (Shanghai), 47*, 960–968.

Duangchinda, T., Dejnirattisai, W., Vasanawathana, S., Limpitikul, W., Tangthawornchaikul, N., Malasit, P., Mongkolsapaya, J., & Screaton, G. (2010). Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 16922–16927.

Fischl, W., & Bartenschlager, R. (2011). Exploitation of cellular pathways by Dengue virus. *Current Opinion in Microbiology, 14*, 470–475.

Flaherty, K. M., DeLuca-Flaherty, C., & McKay, D. B. (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature, 346*, 623–628.

Fong, J. J., Sreedhara, K., Deng, L., Varki, N. M., Angata, T., Liu, Q., Nizet, V., & Varki, A. (2015). Immunomodulatory activity of extracellular Hsp70 mediated via paired receptors Siglec-5 and Siglec-14. *The EMBO Journal, 34*, 2775–2788.

Halstead, S. B. (2014). Dengue antibody-dependent enhancement: Knowns and unknowns. *Microbiology Spectrum, 2*. https://doi.org/10.1128/microbiolspec.AID-0022-2014

Harris, E., Holden, K. L., Edgil, D., Polacek, C., & Clyde, K. (2006). Molecular biology of flaviviruses. *Novartis Foundation Symposium, 277*, 23–39.

Hartl, F. U., & Hayer-Hartl, M. (2009). Converging concepts of protein folding in vitro and in vivo. *Nature Structural & Molecular Biology, 16*, 574–581.

Henderson, B., & Pockley, A. G. (2010). Molecular chaperones and protein-folding catalysts as intercellular signaling regulators in immunity and inflammation. *Journal of Leukocyte Biology, 88*, 445–462.

Higa, L. M., Caruso, M. B., Canellas, F., Soares, M. R., Oliveira-Carvalho, A. L., Chapeaurouge, D. A., Almeida, P. M., Perales, J., Zingali, R. B., & Da Poian, A. T. (2008). Secretome of HepG2 cells infected with dengue virus: Implications for pathogenesis. *Biochimica et Biophysica Acta, 1784*, 1607–1616.

Howe, M. K., Speer, B. L., Hughes, P. F., Loiselle, D. R., Vasudevan, S., & Haystead, T. A. (2016). An inducible heat shock protein 70 small molecule inhibitor demonstrates anti-dengue virus activity, validating Hsp70 as a host antiviral target. *Antiviral Research, 130*, 81–92.

Jindadamrongwech, S., Thepparit, C., & Smith, D. R. (2004). Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. *Archives of Virology, 149*, 915–927.

Kakumani, P. K., Rajgokul, K. S., Ponia, S. S., Kaur, I., Mahanty, S., Medigeshi, G. R., Banerjea, A. C., Chopra, A. P., Malhotra, P., Mukherjee, S. K., et al. (2015a). Dengue NS3, an RNAi suppressor, modulates the human miRNA pathways through its interacting partner. *The Biochemical Journal, 471*, 89–99.

Kakumani, P. K., Shanmugam, R. K., Kaur, I., Malhotra, P., Mukherjee, S. K., & Bhatnagar, R. K. (2015b). Association of HADHA with human RNA silencing machinery. *Biochemical and Biophysical Research Communications, 466*, 481–485.

Kakumani, P. K., Medigeshi, G. R., Kaur, I., Malhotra, P., Mukherjee, S. K., & Bhatnagar, R. K. (2016). Role of human GRP75 in miRNA mediated regulation of dengue virus replication. *Gene, 586*, 7–11.

Kampinga, H. H., & Craig, E. A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews. Molecular Cell Biology, 11*, 579–592.

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., et al. (2002). Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. *Cell, 108*, 717–725.

Limjindaporn, T., Wongwiwat, W., Noisakran, S., Srisawat, C., Netsawang, J., Puttikhunt, C., Kasinrerk, W., Avirutnan, P., Thiemmeca, S., Sriburi, R., et al. (2009). Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. *Biochemical and Biophysical Research Communications, 379*, 196–200.

Malavige, G. N., & Ogg, G. S. (2013). T cell responses in dengue viral infections. *Journal of Clinical Virology, 58*, 605–611.

Martinez-Betancur, V., Marin-Villa, M., & Martinez-Gutierrez, M. (2014). Infection of epithelial cells with dengue virus promotes the expression of proteins favoring the replication of certain viral strains. *Journal of Medical Virology, 86*, 1448–1458.

Mathew, A., Townsley, E., & Ennis, F. A. (2014). Elucidating the role of T cells in protection against and pathogenesis of dengue virus infections. *Future Microbiology, 9*, 411–425.

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature, 427*, 313–319.

Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., Sawasdivorn, S., Duangchinda, T., Dong, T., Rowland-Jones, S., et al. (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nature Medicine, 9*, 921–927.

Mongkolsapaya, J., Duangchinda, T., Dejnirattisai, W., Vasanawathana, S., Avirutnan, P., Jairungsri, A., Khemnu, N., Tangthawornchaikul, N., Chotiyarnwong, P., Sae-Jang, K., et al. (2006). T cell responses in dengue hemorrhagic fever: Are cross-reactive T cells suboptimal? *Journal of Immunology, 176*, 3821–3829.

Munoz, M. L., Cisneros, A., Cruz, J., Das, P., Tovar, R., & Ortega, A. (1998). Putative dengue virus receptors from mosquito cells. *FEMS Microbiology Letters, 168*, 251–258.

Nain, M., Mukherjee, S., Karmakar, S. P., Paton, A. W., Paton, J. C., Abdin, M. Z., Basu, A., Kalia, M., & Vrati, S. (2017). GRP78 is an important host factor for Japanese encephalitis virus entry and replication in mammalian cells. *Journal of Virology, 91*, e02274–e02216.

Oh, W. K., & Song, J. (2006). Hsp70 functions as a negative regulator of West Nile virus capsid protein through direct interaction. *Biochemical and Biophysical Research Communications, 347*, 994–1000.

Olver, C., & Vidal, M. (2007). Proteomic analysis of secreted exosomes. *Sub-Cellular Biochemistry, 43*, 99–131.

Ooi, A., Wong, J. C., Petillo, D., Roossien, D., Perrier-Trudova, V., Whitten, D., Min, B. W., Tan, M. H., Zhang, Z., Yang, X. J., et al. (2011). An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. *Cancer Cell, 20*, 511–523.

Padwad, Y. S., Mishra, K. P., Jain, M., Chanda, S., & Ganju, L. (2010). Dengue virus infection activates cellular chaperone Hsp70 in THP-1 cells: Downregulation of Hsp70 by siRNA revealed decreased viral replication. *Viral Immunology, 23*, 557–565.

Paingankar, M. S., Gokhale, M. D., & Deobagkar, D. N. (2010). Dengue-2-virus-interacting polypeptides involved in mosquito cell infection. *Archives of Virology, 155*, 1453–1461.

Pando-Robles, V., Oses-Prieto, J. A., Rodriguez-Gandarilla, M., Meneses-Romero, E., Burlingame, A. L., & Batista, C. V. (2014). Quantitative proteomic analysis of Huh-7 cells infected with Dengue virus by label-free LC-MS. *Journal of Proteomics, 111*, 16–29.

Paradkar, P. N., Ooi, E. E., Hanson, B. J., Gubler, D. J., & Vasudevan, S. G. (2011). Unfolded Protein Response (UPR) gene expression during antibody-dependent enhanced infection of cultured monocytes correlates with dengue disease severity. *Bioscience Reports, 31*, 221–230.

Patramool, S., Surasombatpattana, P., Luplertlop, N., Seveno, M., Choumet, V., Thomas, F., & Misse, D. (2011). Proteomic analysis of an Aedes albopictus cell line infected with Dengue serotypes 1 and 3 viruses. *Parasites & Vectors, 4*, 138.

Pena, J., & Harris, E. (2011). Dengue virus modulates the unfolded protein response in a time-dependent manner. *The Journal of Biological Chemistry, 286*, 14226–14236.

Perera, N., Miller, J. L., & Zitzmann, N. (2017). The role of the unfolded protein response in dengue virus pathogenesis. *Cellular Microbiology, 19*. https://doi.org/10.1111/cmi.12734

Pockley, A. G., Henderson, B., & Multhoff, G. (2014). Extracellular cell stress proteins as biomarkers of human disease. *Biochemical Society Transactions, 42*, 1744–1751.

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress & Chaperones, 21*, 379–404.

Reyes-Del Valle, J., Chavez-Salinas, S., Medina, F., & Del Angel, R. M. (2005). Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *Journal of Virology, 79*, 4557–4567.

Rungruengphol, C., Jaresitthikunchai, J., Wikan, N., Phaonakrop, N., Keadsanti, S., Yoksan, S., Roytrakul, S., & Smith, D. R. (2015). Evidence of plasticity in the dengue virus: Host cell interaction. *Microbial Pathogenesis, 86*, 18–25.

Salas-Benito, J. S., & Del Angel, R. M. (1997). Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. *Journal of Virology, 71*, 7246–7252.

Salas-Benito, J., Reyes-Del Valle, J., Salas-Benito, M., Ceballos-Olvera, I., Mosso, C., & Del Angel, R. M. (2007). Evidence that the 45-kD glycoprotein, part of a putative dengue virus receptor complex in the mosquito cell line C6/36, is a heat-shock related protein. *The American Journal of Tropical Medicine and Hygiene, 77*, 283–290.

Salimu, J., Spary, L. K., Al Taei, S., Clayton, A., Mason, M. D., Staffurth, J., & Tabi, Z. (2015). Cross-presentation of the Oncofetal tumor antigen 5T4 from irradiated prostate cancer cells–a key role for heat-shock protein 70 and receptor CD91. *Cancer Immunology Research, 3*, 678–688.

Screaton, G., Mongkolsapaya, J., Yacoub, S., & Roberts, C. (2015). New insights into the immunopathology and control of dengue virus infection. *Nature Reviews. Immunology, 15*, 745–759.

Taguwa, S., Maringer, K., Li, X., Bernal-Rubio, D., Rauch, J. N., Gestwicki, J. E., Andino, R., Fernandez-Sesma, A., & Frydman, J. (2015). Defining Hsp70 subnetworks in Dengue virus replication reveals key vulnerability in Flavivirus infection. *Cell, 163*, 1108–1123.

Thongtan, T., Wikan, N., Wintachai, P., Rattanarungsan, C., Srisomsap, C., Cheepsunthorn, P., & Smith, D. R. (2012). Characterization of putative Japanese encephalitis virus receptor molecules on microglial cells. *Journal of Medical Virology, 84*, 615–623.

Umareddy, I., Pluquet, O., Wang, Q. Y., Vasudevan, S. G., Chevet, E., & Gu, F. (2007). Dengue virus serotype infection specifies the activation of the unfolded protein response. *Virology Journal, 4*, 91.

Upanan, S., Kuadkitkan, A., & Smith, D. R. (2008). Identification of dengue virus binding proteins using affinity chromatography. *Journal of Virological Methods, 151*, 325–328.

Vega-Almeida, T. O., Salas-Benito, M., Nova-Ocampo, M. A., Del Angel, R. M., & Salas-Benito, J. S. (2013). Surface proteins of C6/36 cells involved in dengue virus 4 binding and entry. *Archives of Virology, 158*, 1189–1207.

Wati, S., Soo, M. L., Zilm, P., Li, P., Paton, A. W., Burrell, C. J., Beard, M., & Carr, J. M. (2009). Dengue virus infection induces upregulation of GRP78, which acts to chaperone viral antigen production. *Journal of Virology, 83*, 12871–12880.

Wu, Y. P., Chang, C. M., Hung, C. Y., Tsai, M. C., Schuyler, S. C., & Wang, R. Y. (2011). Japanese encephalitis virus co-opts the ER-stress response protein GRP78 for viral infectivity. *Virology Journal, 8*, 128.

Xiao, A., Wong, J., & Luo, H. (2010). Viral interaction with molecular chaperones: Role in regulating viral infection. *Archives of Virology, 155*, 1021–1031.

Ye, J., Chen, Z., Zhang, B., Miao, H., Zohaib, A., Xu, Q., Chen, H., & Cao, S. (2013). Heat shock protein 70 is associated with replicase complex of Japanese encephalitis virus and positively regulates viral genome replication. *PLoS One, 8*, e75188.

Yu, I. M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G., & Chen, J. (2008). Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science, 319*, 1834–1837.

Zhang, M., Zheng, X., Wu, Y., Gan, M., He, A., Li, Z., Zhang, D., Wu, X., & Zhan, X. (2013). Differential proteomics of Aedes albopictus salivary gland, midgut and C6/36 cell induced by dengue virus infection. *Virology, 444*, 109–118.

Zhu, Y. Z., Cao, M. M., Wang, W. B., Wang, W., Ren, H., Zhao, P., & Qi, Z. T. (2012). Association of heat-shock protein 70 with lipid rafts is required for Japanese encephalitis virus infection in Huh7 cells. *The Journal of General Virology, 93*, 61–71.

Zhu, H., Fang, X., Zhang, D., Wu, W., Shao, M., Wang, L., & Gu, J. (2016). Membrane-bound heat shock proteins facilitate the uptake of dying cells and cross-presentation of cellular antigen. *Apoptosis, 21*, 96–109.

# Chapter 17
# Roles and Regulation of the Heat Shock Proteins of the Major Human Pathogen *Helicobacter pylori*


**Davide Roncarati and Vincenzo Scarlato**

**Abstract** The ability of pathogens to gauge the surroundings and modulate gene expression accordingly is a crucial feature for bacterial survival. In this respect, the heat-shock response, a universally conserved mechanism of protection, allows bacterial cells to adapt rapidly to hostile environmental conditions and to survive during stress. The major human pathogen *Helicobacter pylori* employs a collection of highly conserved heat-shock proteins (HSP) to preserve cellular proteins and to maintain their homeostasis, allowing the pathogen to adapt and survive in the hostile niche of the human stomach. Moreover, various evidences suggest that some chaperones of *H. pylori* may play also non-canonical roles, as for example in the interaction with the extracellular environment. In *H. pylori*, the regulation of HSP expression is orchestrated by two dedicated transcriptional repressors, named HspR and HrcA, as well as via a chaperones-dependent posttranscriptional feedback control acting on the activity of HspR and HrcA regulators themselves. This chapter briefly reviews our understanding of the roles and regulation of the most important HSP of *H. pylori*, which represent a crucial virulence factor for bacterial infection and persistence in the human host.

**Keywords** Heat-shock response · *Helicobacter pylori* · HrcA repressor · HspR repressor · Transcriptional regulation

D. Roncarati (✉) · V. Scarlato
Department of Pharmacy and Biotechnology (FaBiT), University of Bologna, Bologna, Italy
e-mail: davide.roncarati@unibo.it



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_17

## Abbreviations

| | |
|---|---|
| bp | Base-pairs |
| CIRCE | Controlling inverted repeat of chaperone expression |
| ERK | Extracellular signal-regulated kinase |
| HAIR | HspR associated inverted repeat |
| HSP | Heat-shock proteins |
| i2-FFL | Incoherent type-2 feed forward loop |
| MAPK | Mitogen-activated protein kinase |
| TLR | Toll-like receptor |
| WHO | World Health Organization |

## 17.1 Introduction

*Helicobacter pylori*, a Gram-negative, spiral-shaped, flagellated bacterium, was isolated for the first time in 1983 from a gastric biopsy of a patient suffering from duodenal cancer (Warren and Marshall 1983). Following many years of intense research, today we know that *H. pylori* is one of the most widespread and successful human pathogens, infecting the gastric mucosa of about 50% of the population in the world. Infected people carry this bacterium for decades or even for life, if untreated. *H. pylori* can cause chronic gastritis that can remain clinically silent due to the dynamic equilibrium between the bacterium and the host, or evolve into more severe diseases such as atrophic gastritis, peptic ulcer, lymphoma of the mucosa-associated lymphoid tissue (or MALT-lymphoma) or gastric adenocarcinoma. It is the only bacterium that has been classified as a class 1 carcinogen by the World Health Organization (WHO) (Vogiatzi et al. 2007). Despite its declining incidence rate, gastric cancer remains the fifth most common malignancy in the world and the third leading cause of cancer-related death (Sugano et al. 2015). *H. pylori* infections can be treated with antibiotics: unfortunately, the available therapies are beginning to loose efficacy because of insurgence of antibiotic resistance. For example, due to a constant increase in *H. pylori* resistance to clarithromycin, the triple clarithromycin-based treatment has become progressively less efficacious. For this reason, the WHO has recently included *H. pylori* in a global priority list of 12 antibiotic-resistant bacterial pathogens to help in prioritizing the research, discovery, and development of new antibiotics (World Health Organization 2017).

In order to establish a persistent infection *H. pylori* relies on many different virulence factors, defined as the effectors that allow the bacterium to contact, enter and persist into the host and respond to the particularly adverse conditions typical of the human stomach (Peterson 1996). One of the most important factors involved in the initial phase of infection is the flagellar apparatus that allows bacteria to move in the stomach lumen and through the viscous mucus layer overlying the gastric epithelium (Suerbaum 1995). Once reached this niche, *H. pylori* exploits an arsenal of

adhesion molecules to adhere tightly to epithelial cells. Another bacterial factor essential for host colonization is represented by the urease enzyme, which hydrolyses urea into ammonia and carbon dioxide and leads to a pH increase (Labigne et al. 1991; Cussac et al. 1992). Several other bacterial factors contribute to the process of infection and colonization of the gastric epithelium, including the vacuolating toxin VacA, the cytotoxin-associated protein CagA and various mechanisms of molecular mimicry that allow the bacterium to elude the host immune response (Covacci et al. 1997; de Bernard et al. 1995; Moran et al. 1997; Andersen-Nissen et al. 2005). Among *H. pylori* virulence factors, the highly conserved class of stress-induced proteins, known as HSP, has to be included (Kao et al. 2016). These proteins, through their canonical roles in protecting cellular proteins and in maintaining cellular homeostasis, allow the pathogen to adapt and survive in the hostile niche represented by the human stomach. Moreover, several evidences show that the HSP of *H. pylori* play also non-canonical roles, as for example in the interaction with the host (Kao et al. 2016). This chapter focuses on what we know about the major HSP of *H. pylori*, with a particular emphasis on their typical and atypical functions and on the regulatory strategies adopted by the pathogen to precisely tune their expression in response to environmental cues.

## 17.2 Heat-Shock Proteins of *H. pylori*

Typically, HSP accumulate following different kind of stress insults and are involved in several cellular processes, including assisting the folding of newly synthesized polypeptides, preventing aggregation of proteins under stress conditions, and recovering proteins that have been partially or completely unfolded by stresses (Roncarati and Scarlato 2017). During stress, in fact, spontaneous folding of newly synthetized proteins is inefficient and error-prone, and a predominant fraction of nascent polypeptides undergoes a chaperone-mediated folding process (Mogk et al. 1999). Also, a large fraction of already folded proteins gets partially or completely denatured and becomes incline to form deleterious aggregates (Mogk et al. 1999).

The list including the major HSP encoded by the *H. pylori* genome is reported in Table 17.1. In general, they can be divided in two main functional groups: chaperone proteins that assist protein folding and assembly, and stress proteases whose function in the cell includes the removal of misfolded/aggregated polypeptides. GroEL and DnaK, the bacterial representatives of the two major chaperone families Hsp60 and Hsp70, respectively, play a key role in protein folding even during non-stressed growth conditions, although their action becomes more important during stress. In *H. pylori*, the genes encoding almost all the members of these two chaperones families map in three multicistronic operons represented in Fig. 17.1. Specifically, while the GroES-GroEL chaperonin-coding genes belong to the same bi-cistronic operon, the coding sequences of the DnaK system members are transcribed by different transcription units. DnaK and the co-chaperone GrpE encoding genes are in the same operon together with the *hrcA* gene (coding for the heat-shock

**Table 17.1** HSP of *Helicobacter pylori*

| Protein | Genomic locus | Function |
|---|---|---|
| GroEL (Hsp60, Cpn60) | *HP0010* | 60 kDa molecular chaperonin, protein folding |
| GroES (Hsp10, Cpn10) | *HP0011* | 10 kDa co-chaperone of GroEL chaperonin |
| DnaK (Hsp70) | *HP0109* | 70 kDa molecular chaperone, protein folding |
| DnaJ (Hsp40) | *HP1332* | Co-chaperone of DnaK |
| GrpE | *HP0110* | Co-chaperone and nucleotide exchange factor of DnaK |
| CbpA | *HP1024* | Co-chaperone of DnaK and putative nucleoid associated protein |
| HtpG (Hsp90) | *HP0210* | Chaperone protein |
| FtsH | *HP1069* | ATP-dependent zinc metalloprotease |
| HtpX | *HP0927* | Membrane-localized metallo-protease |
| Lon | *HP1379* | ATP-dependent protease |
| ClpB (Hsp100) | *HP0264* | ATP-dependent protease |
| HslV (ClpQ) | *HP0515* | ATP-dependent protease, proteolytic subunit |
| HslU (ClpY) | *HP0516* | ATPase subunit, binds and translocates substrate to HslV |
| ClpP | *HP0794* | ATP-dependent protease, proteolytic subunit |
| ClpA | *HP0033* | ATP-dependent specificity component of the ClpAP protease |
| ClpS | *HP0032* | ATP-dependent Clp protease adaptor for the ClpAP protease |
| ClpX | *HP1374* | ATP-dependent specificity component of the ClpXP protease |

List of HSP of *H. pylori*. Names reported in the leftmost column refer to the currently used nomenclature. Alternative names for some HSP are reported in brackets. Names of genomic loci (central column) have been assigned according to the annotated genome sequence of *H. pylori* strain 26695 (Tomb et al. 1997). HSP sharing common functions or belonging to the same machinery/system are grouped together

repressor HrcA), while the gene coding for the principal DnaK co-chaperone DnaJ is located in a different genomic locus, not represented in Fig. 17.1. The operon encoding the other DnaK co-chaperone CbpA, harbours also the sequences encoding the heat-shock master repressor HspR and a protein with a still poorly defined function. The canonical roles of these chaperone systems are well conserved throughout all kingdoms of life (Roncarati and Scarlato 2017). GroEL and DnaK proteins bind hydrophobic patches of unfolded proteins and, upon interaction with their co-chaperones (GroES and DnaJ-GrpE, respectively) and ATP hydrolysis, they promote the acquisition of proper folding by the substrate polypeptides through different mechanisms that reflect their different architectures. While fourteen GroEL monomers assemble into a cylindrical complex to form two heptameric rings that generate the cavity in which the entire substrate protein is enclosed and folded with no interaction with other proteins, the DnaK chaperone exerts its action as a monomer, binding to brief surface-exposed hydrophobic amino-acid regions (Mogk et al. 1999).

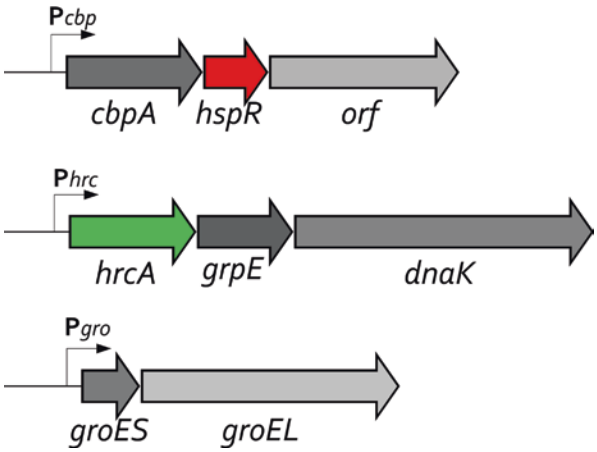


**Fig. 17.1** Schematic representation of the three multicistronic operons containing the major chaperone genes of *H. pylori*. Grey arrows indicate chaperone genes and the putative helicase encoding gene (here named *orf*), while coloured arrows indicate regulatory genes. The P*cbp* promoter controls the transcription of the *cbpA-hspR-orf* operon, coding for the DnaJ homolog and DNA binding protein CbpA, for the heat-shock repressor HspR and for a protein putative helicase activity (*orf*). The P*hrc* promoter transcribes an mRNA encoding the heat-shock repressor HrcA, the DnaK chaperone and its co-chaperone GrpE. The P*gro* promoter drives the transcription of a bi-cistronic operon encoding the components of the GroE chaperonin system. Bent arrows depict the transcription start sites

The other functional group of HSP owned by *H. pylori* consists of a set of proteins with protease activity. The principal function of these proteins is essentially to remove mis-folded polypeptides from stressed cells. Some proteases are multicomponent systems, in which a catalytic subunit (e.g. ClpP and HslV) associates to partner subunits with a role in substrate recognition (ClpA or ClpX for ClpP and HslU for HslV). These are ATP-dependent co-chaperones that are able to remodel polypeptides and deliver them to proteolytic degradation (Missiakas et al. 1996; Wawrzynow et al. 1996). While these heat-shock proteases assemble into complex structures, other members of this group combine on a single polypeptide both chaperone and proteolytic activities (e.g. Lon, FtsH and the membrane bound metalloprotease HtpX). A different role, fundamental for the survival of cells during severe stress, is played by the oligomeric chaperone ClpB. This chaperone does not associate with a peptidase and does not act by itself in proteolysis. Its activity, in cooperation with DnaK, DnaJ and GrpE, consists in the resolubilization of protein aggregates. Specifically, protein binding stimulates ATP hydrolysis, which leads to the unfolding/disaggregation of the denatured protein aggregates (Haslberger et al. 2010).

## 17.3 Regulation of Heat-Shock Proteins in *H. pylori*

The HSP of *H. pylori* have been studied in detail in the last two decades because of their importance in protecting the pathogen from adverse environmental cues, but also for their proposed involvement in several steps of the infectious process (see below). These studies provided a large amount of biochemical and immunological data concerning the specific role of some heat-shock proteins of *H. pylori*. In addition, many efforts have been made also to characterize the transcriptional regulation of the major chaperone genes of this gastric pathogen.

### *17.3.1 Transcriptional Regulation by Heat-Shock HspR and HrcA Repressors*

In general, transcription of heat-shock genes is tightly regulated, with a basal level of transcription during physiological growth condition that ensures the production of chaperones required for normal cellular functions. Following the perception of a stress signal, the transcription of heat-shock genes is up-regulated leading to a transient accumulation of heat-shock and chaperone proteins in the cell. In general, transcriptional regulation of heat-shock genes could be either positive or negative, according to the kind of regulator involved (Roncarati and Scarlato 2017). Specifically, positive regulation relies on alternative heat-shock σ factor of the RNA polymerase, while negative regulation is orchestrated by transcriptional repressors. The *H. pylori* genome lacks a homologue of the *Escherichia coli* heat-shock sigma factor $\sigma^{32}$, but encodes two heat-shock repressors, which are homologues to *Bacillus subtilis* HrcA and to *Streptomyces coelicolor* HspR (Tomb et al. 1997). The involvement of these two repressors in controlling the transcription of the three chaperone-encoding multicistronic operons, represented in Fig. 17.1, was demonstrated by comparing their transcription in the *H. pylori* wild-type strain and in isogenic *hrcA*- or *hspR*-mutant background strains. Specifically, it turned out that the transcription of all the heat-shock operons is derepressed in the *hspR*-mutant background with respect to the wild type strain, while the *hrcA* deletion specifically leads to strong increase in the transcripts associated with *groES-groEL* and *hrcA-grpE-dnaK* operons only (Spohn and Scarlato 1999; Spohn et al. 2004). These observations led to the conclusion that HspR alone is able to repress its own transcription, while both heat-shock regulators HspR and HrcA are required to repress transcription of the *groES-groEL* and *hrcA-grpE-dnaK* operons. The direct binding of HrcA and HspR to the promoter regions controlling heat-shock operons' transcription was analyzed *in vitro* through high-resolution DNase I footprinting assays. HspR was found to bind all the three operons promoters, protecting a large DNA region of ca. 75 bp (Spohn and Scarlato 1999). Intriguingly, whereas on the promoter of the *cbpA-hspR-helicase* operon (controlled solely by HspR itself) HspR binding occurs in a region overlapping the −35 and −10 core promoter elements (essential for the RNA

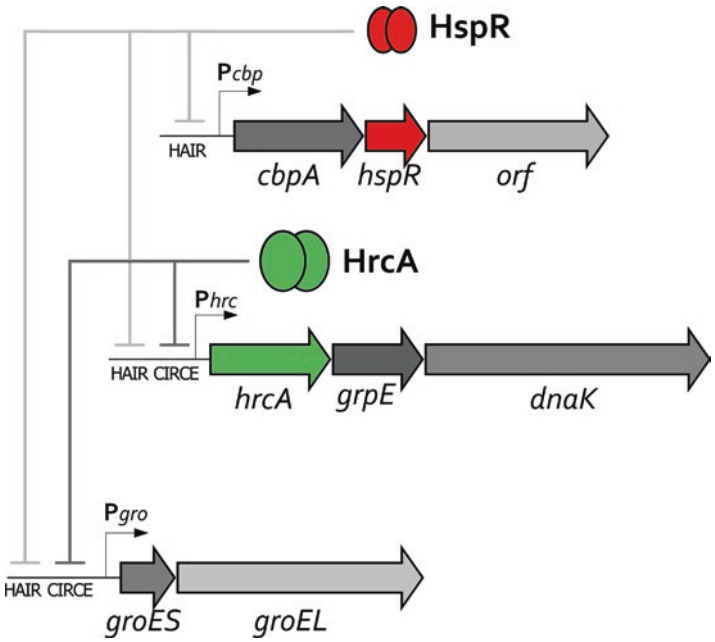


**Fig. 17.2** Transcriptional regulation of the major heat-shock genes of *H. pylori* by the concerted action of HspR and HrcA regulatory proteins. HspR repressor (red oval) binds and represses, alone, the transcription of the P*cbp* promoter (DNA-binding and repressive functions are represented by the grey lines ending with a hammerhead). Both HspR and HrcA (green oval) bind adjacent DNA regions on P*hrc* and P*gro* promoters, repressing transcription under physiological growth condition (HrcA binding is depicted by black lines). HAIR and CIRCE indicate the conserved binding sequences for HspR and HrcA, respectively (see text)

polymerase to bind and initiate a new round of transcription), the HspR binding sites on the promoters of *groES-groEL* and *hrcA-grpE-dnaK* operons map upstream the core promoter region, being centred 72 and 117 bp upstream their specific transcription start site, respectively. On HspR-HrcA coregulated promoters, the core promoter regions, led unoccupied by HspR, are bound by its partner HrcA: this repressor covers a compact DNA region of about 30 bp, overlapping the core promoter elements and the transcription start site (Roncarati et al. 2007a; Roncarati et al. 2007b). For both transcriptional repressors, the protected DNA regions on the heat-shock operons promoters encompass sequences that resemble the well characterized HspR and HrcA recognition sequences, known as HAIR (for HspR Associated Inverted Repeat) and CIRCE (for Controlling Inverted Repeat of Chaperone Expression), respectively. The model of HspR and HrcA mediated transcriptional repression of the major chaperones genes of *H. pylori* is schematically represented in Fig. 17.2. During physiological growth conditions, the transcription of the three heat-shock operons is maintained repressed by the concerted action of HspR and HrcA. Following stress signals, the repression is released, leading to

accumulation of HSP in the cell cytoplasm. In addition to responding to a temperature upshift, the transcription of *H. pylori* chaperones-encoding operons is induced also by other stress insults, such as high osmolarity and puromycin treatment (Homuth et al. 2000; Spohn et al. 2002). The exposure of bacterial cells to puromycin leads to accumulation of truncated polypeptides in the cytoplasm. The finding that transcription of chaperones genes is induced by this treatment suggests that misfolded proteins represent the intracellular signal perceived by the transcriptional machinery.

*H. pylori* chaperones regulatory circuit (Fig. 17.2) represents a rare example in which two dedicated transcriptional repressors combine in a peculiar and complex way to control gene expression. In fact, as described above, HspR combines with HrcA to control *groES-groEL* and *dnaK* operons. Moreover, considering that *hrcA* is the leading gene of the *dnaK* operon, this results in the HrcA regulon being entirely embedded within the HspR regulon. An interesting explanation for the peculiar interaction of heat-shock repressors found in *H. pylori* can be inferred by considering a logical scheme involving both regulators. In this circuit, the master regulator HspR directly controls expression of the HrcA regulator and the *groES-groEL* operon, which, in turn, is under the transcriptional control of both HrcA and HspR. Moreover, considering that all three regulatory interactions are repressive, it has been proposed that these circuits are rare examples of incoherent type-2 feed forward loops (i2-FFL) (Alon 2007; Danielli et al. 2010). Possibly, this peculiar network motif is employed to speed up the transcriptional response of target genes upon stress signals and it could be evolved by bacteria for a better adaptation to their specific ecological niches.

### *17.3.2 Feedback Regulation Mediated by Chaperones*

A common theme in heat-shock genes regulation is the involvement of chaperone proteins themselves in the feedback modulation of the activity of transcriptional regulators. The homeostatic control of *E. coli* heat-shock sigma factor $\sigma^{32}$ activity and stability by both GroE chaperonin and DnaK system is the best-characterized example (Roncarati and Scarlato 2017). However, similar feedback regulatory functions of chaperones modulating HrcA and HspR heat-shock repressors' DNA binding activity have been described in different bacterial species, like *B. subtilis*, *Chlamydia trachomatis*, *S. coelicolor* and *Mycobacterium tuberculosis* (Mogk et al. 1998; Wilson et al. 2005; Bucca et al. 2000; Parijat and Batra 2015). In this context, *H. pylori* represents a peculiar example in which two distinct chaperones modulate the activity of both HrcA and HspR transcriptional regulators. Firstly, the DNA binding affinity of HrcA for its CIRCE-like operators appears to be significantly increased in the presence of GroESL chaperonin (Roncarati et al. 2007b; Roncarati et al. 2014), an observation that parallels well with the GroE-HrcA interplay characterized in *B. subtilis* (Mogk et al. 1998). According to the "titration model" proposed for *B. subtilis*, the chaperonin GroE might interact with *H. pylori* HrcA to

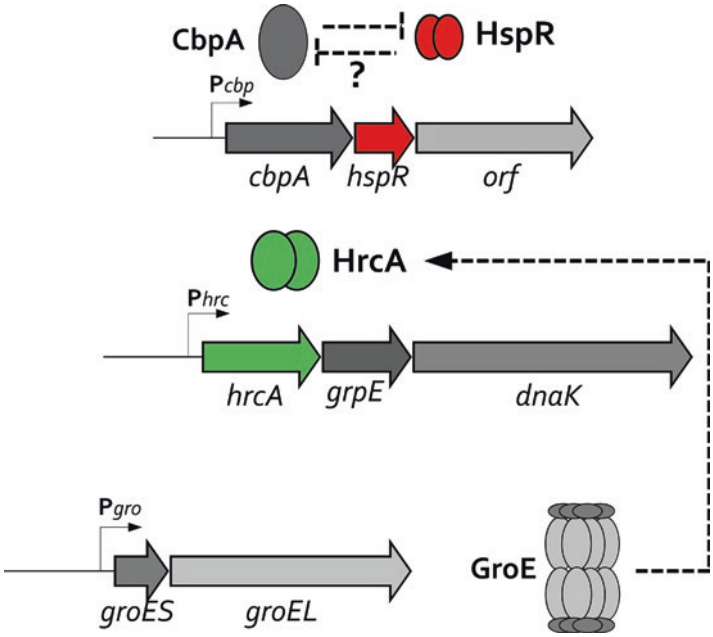


**Fig. 17.3** Schematic representation of the posttranscriptional protein-protein feedback regulation of the heat-shock repressors exerted by chaperone proteins. Positive modulation of HrcA DNA-binding activity by the GroE chaperonin is highlighted by a black dashed arrow, while the negative regulation of HspR by the co-chaperone and DNA-binding protein CbpA is represented by a black dashed hammerhead. The hypothetical negative regulation of CbpA activities upon interaction with HspR is depicted by a black dashed hammerhead marked with a question mark

enhance its DNA binding activity, thereby playing a feedback role in the transcriptional repression of chaperone genes. Following stress stimuli, GroE would be titrated away by increasing amounts of misfolded polypeptides, relieving HrcA transcriptional repression of heat-shock target promoters (Fig. 17.3). In addition, in *H. pylori* the DNA-binding activity of the second chaperones repressor HspR is negatively modulated by the heat-shock protein CbpA, rather than by DnaK as seen for example in *S. coelicolor* (Fig. 17.3). In analogy with the homologous protein of *E. coli* (Chae et al. 2004; Bird et al. 2006), the HspR-repressed *cbpA* gene is thought to encode a dual-function protein, acting both as a DnaJ-like co-chaperone of DnaK and as a nucleoid-associated protein involved in nucleoid structuring functions. Intriguingly, it appears that upon direct protein-protein interaction, CbpA negatively modulates HspR binding to target promoters *in vitro*. This negative regulation is exerted by CbpA when the HspR repressor is in solution and with no contacts with the DNA. Accordingly, cells overexpressing CbpA show deregulation of heat-shock response (Roncarati et al. 2011). These findings would suggest new perspectives in heat-shock gene regulation as the negative effect of CbpA on HspR DNA-binding is opposite to the modulation exerted by DnaK or GroE on HspR and HrcA

(positive effect). Therefore, the accepted model for chaperone feedback regulation of transcriptional repressors is insufficient to explain CbpA-HspR functional interactions in *H. pylori*. Possibly, CbpA regulation of HspR binding activity is required to fine-tune the shutoff response of the heat-shock genes in *H. pylori* (Roncarati et al. 2011). Furthermore, preliminary results suggest that CbpA of *H. pylori* possesses both co-chaperone and nucleoid-associated functions (D. Roncarati, unpublished results). Consequently, it would be interesting to characterize the functional interplay between HspR and CbpA. That is, the heat-shock regulator HspR might influence the co-chaperone and/or nucleoid-associated activity of CbpA by direct protein-protein interaction (Fig. 17.3). This would represent a novel example of intersection between heat-shock gene regulation and other cellular functions, as for example the maintenance and regulation of the bacterial nucleoid.

### *17.3.3 Signal Sensing*

In order to respond rapidly to sudden perturbations of their living niche, bacteria employ an arsenal of specific biomolecules that are able to detect environmental cues and transduce them into coordinated gene expression patterns. In general, bacteria can measure and detect environmental fluctuations by exploiting all different classes of biomolecules including nucleic acids, proteins and lipids (Kortmann and Narberhaus 2012; Schumann 2016; Roncarati and Scarlato 2017). In the context of heat-shock genes' regulation, a central question is how a sudden temperature upshift is decoded and how the appropriate response pathways are activated. Temperature sensing mechanisms can be either direct or indirect. Direct temperature sensing affects the activity of the sensing biomolecule, while indirect sensing is a consequence of a sudden temperature increase (such as the accumulation of misfolded proteins in the cytoplasm). It has been demonstrated that in *H. pylori* the transcriptional repressor HrcA is the direct heat-sensor of the chaperone regulatory circuit (Roncarati et al. 2014), a rare function for a regulator of transcription initiation and observed just in few other cases (Servant et al. 2000; Hurme et al. 1997; Herbst et al. 2009; Elsholz et al. 2010). Specifically, HrcA has the ability to intrinsically sense temperature upshift: temperature, in fact, specifically provokes a major and irreversible structural change of HrcA, thereby inducing a complete loss of its DNA-binding capabilities. Accordingly, upon heat-shock and concomitant loss of HrcA DNA-binding affinity for its operators, the transcription of chaperones genes is rapidly induced. Interestingly, while *in vitro* assays suggest that heat-treated HrcA is irreversibly inactivated, experimental evidences suggest that *in vivo* HrcA is able to recover its repressive capabilities after the temperature challenge (Roncarati et al. 2014). The recovery of HrcA proper folding and binding activity is mediated by GroE, whose chaperone function is exploited in this case to restore HrcA role in heat-shock promoters' repression. The model depicted in Fig. 17.4 recapitulates all these findings. Upon heat-shock, HrcA undergoes a major conformational change and loses DNA binding activity, leading to promoter derepression and increased

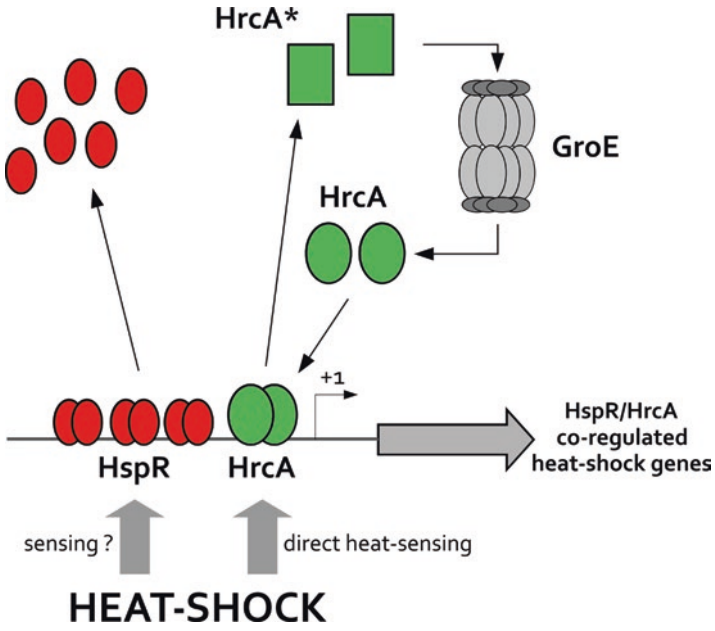


**Fig. 17.4** Model for heat sensing in *H. pylori*. On co-regulated promoters (P*gro* and P*hrc*), HspR and HrcA bind adjacent operators and repress transcription under normal conditions of growth. Upon temperature upshift, HrcA goes through a major structural change and acquires an inactive conformation (represented by a green rectangle and here named HrcA*), detaching from its operator and relieving transcriptional repression. Following a direct interaction with the GroE chaperonin, HrcA is refolded to its native conformation (green oval), retrieving its DNA-binding capabilities. The mechanism of temperature sensing adopted by the heat-shock repressor HspR (red oval) is still unknown

expression of chaperone genes. Once the environmental temperature returns to a physiological level, at least a fraction of heat-inactivated HrcA interacts with and is actively refolded by the GroE chaperonin. This fraction of refolded protein, together with newly synthetized HrcA, restores the transcriptional repression of target promoters. Considering the central role of GroE chaperonin in modulating HrcA binding activity upon heat-challenge, this interaction could be crucial in *H. pylori* to adjust the transcriptional response under various stress conditions. In other words, depending on the severity of a particular stress stimulus, the amount of GroE chaperonin available for the functional interaction with HrcA will change, influencing the restoration of target gene regulation. In addition, this combined sensing system that involves direct sensing (HrcA intrinsic heat sensing) and indirect sensing (GroE-mediated sensing of unfolded proteins amounts) could allow *H. pylori* to respond differentially, depending on the intensity and on the kind of stress perceived.

While HrcA acts as an intrinsic protein thermometer, its partner heat-shock repressor HspR appears to be rather a stable protein, whose conformation and DNA binding activity are unaffected by temperature fluctuations (Roncarati et al. 2014).

Considering that the *cbpA-hspR-helicase* operon, repressed exclusively by HspR (Fig. 17.2), is rapidly and strongly derepressed by heat-shock and other stresses (Spohn et al. 2002), the current working hypothesis is that HspR sensing mechanism maybe indirect, probably mediated by an interacting partner, whose identity is, so far, still unknown.

## 17.4 Non-Canonical Roles of Heat-Shock Proteins in *H. pylori*

An interesting aspect concerning HSP function is that, besides their canonical roles in protecting cellular proteins from environmental cues (as described above), some of them seem to have undertaken additional and diverse functions. Several examples in this sense have been described in various bacterial species (Henderson and Martin 2011), and *H. pylori* makes no exception. The GroE chaperonin is one of the most conserved heat-shock player in all kingdoms of life, with a key role in assisting the proper folding of a myriad of cellular substrate polypeptides (see above and Roncarati and Scarlato 2017). However, in *H. pylori* both GroES and GroEL proteins seem to have evolved other functions, involving them in separate cellular processes and in the interaction with the extracellular space. For example *H. pylori* GroES has a highly conserved region (N-terminal, named domain A) and a unique C-terminal extension (domain B), which is absent from all other GroES-like proteins, except the closely related *Helicobacter acinonychis* (Cun et al. 2008; Schauer et al. 2010). Interestingly, domain B contains a histidine-rich motif with eight histidine residues, which bind two nickel ions per GroES monomer. This metal binding ability is associated with storage and trafficking of nickel ion, a crucial virulence determinant for the human pathogen (Kansau et al. 1996; Schauer et al. 2010; de Reuse et al. 2013). Additionally, the *H. pylori* co-chaperonin GroES can be classified also as a gastric cancer-associated virulence factor, due to its ability to bind Toll-like receptor 4 (TLR-4) exposed on the host cells membrane (Su et al. 2015). The HpGroES-TLR4 interaction, dependent on the proper folding of GroES domain B that, in turn, is stabilized by binding of nickel ions and triggers the production of the pro-inflammatory interleukin-8 in gastric epithelial cells (Lin et al. 2006; Lee et al. 2016). Besides GroES, a number of other virulence factors of *H. pylori*, able to induce the production of interleukin-8 by the host have been identified (such as CagA and BabA among the others), including the chaperone protein and GroES-partner GroEL (Brandt et al. 2005; Rad et al. 2002; Zhao et al. 2007). Specifically, it has been shown that *H. pylori* GroEL induces the production of interleukin-8 by monocytes through ERK and p38 MAPK signaling, events that are linked to the recognition of TLR-2 (Zhao et al. 2007). Finally, although relatively controversial, it has been proposed that GroEL, together with the other major chaperone protein DnaK, could be associated to the bacterial outer membrane, and this extracellular

surface localization could be involved in the modification of the glycolipid binding specificity of *H. pylori* at low pH (Dunn et al. 1997; Huesca et al. 1996; Phadnis et al. 1996).

## 17.5 Conclusions

Bacteria respond to adverse environmental conditions by inducing the synthesis of a class of highly conserved HSP, which allow cells to counteract imminent cellular damages. The major gastric pathogen *H. pylori* exploits an arsenal of chaperones and HSP to defend itself from the adverse conditions that the microorganism undergoes during the infectious process and the long persistence in the human stomach. As detailed above, *H. pylori* harbours an almost complete set of chaperones and proteases (Table 17.1), whose expression is enhanced upon several and different stress cues. During the last two decades, many efforts have been made to dissect the mechanisms adopted by the gastric pathogen to regulate the expression of the major HSP in response to environmental stress. *H. pylori* employs a highly complex strategy to control the expression of HSP, orchestrated at the transcriptional level by the combination of two dedicated repressors (Fig. 17.2). Moreover, protein-protein feedback regulations add additional post-transcriptional mechanisms to achieve a fine modulation of the expression of chaperones (Fig. 17.3). Although many aspects of these regulatory mechanisms have been dissected, several questions remain to be answered. Firstly, the mechanism by which *H. pylori* controls the expression of all stress proteases is still unknown. The amount of proteases is expected to increase in response to different stress insults encountered by the pathogen, but their promoters do not apparently belong to HrcA and/or HspR regulons (Roncarati et al. 2007b). An interesting hypothesis considers the existence of post-transcriptional or post-translational strategies, which would provide enhanced levels of these crucial players during adverse conditions of growth. Another aspect that deserves further clarifications concerns the existing link between heat-shock genes' regulation and motility of *H. pylori* cells. It has been shown through genome-wide whole transcriptome analysis that *hspR* gene disruption led to significantly lower expression of many genes coding for proteins involved in the regulation and assembly of the flagellar apparatus. Accordingly, the *hspR*-mutant strain showed reduced motility on soft agar plates (Roncarati et al. 2007b). A similar scenario has been described in the closely related bacterium *Campylobacter jejuni* (Andersen et al. 2005). An intriguing hypothesis to explain the intersection of heat-shock genes' regulation and motility functions could be that aberrant accumulation of one or more components of the HspR regulon (for example GroELS or DnaK chaperones) could interfere with the highly regulated assembly of some flagellar structures, which in turn establish a proper transcriptional response.

**Acknowledgements** This work was supported by Grants from the University of Bologna.

## References

Alon, U. (2007). Network motifs: Theory and experimental approaches. *Nature Reviews. Genetics, 8*(6), 450–461.

Andersen, M. T., Brøndsted, L., Pearson, B. M., Mulholland, F., Parker, M., Pin, C., Wells, J. M., & Ingmer, H. (2005). Diverse roles for HspR in Campylobacter jejuni revealed by the proteome, transcriptome and phenotypic characterization of an hspR mutant. *Microbiology, 151*(Pt 3), 905–915.

Andersen-Nissen, E., Smith, K. D., Strobe, K. L., Barrett, S. L., Cookson, B. T., Logan, S. M., & Aderem, A. (2005). Evasion of toll-like receptor 5 by flagellated bacteria. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 9247–9252.

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., Fontana, A., Rappuoli, R., & Montecucco, C. (1995). Low pH activates the vacuolating toxin of Helicobacter pylori, which becomes acid and pepsin resistant. *The Journal of Biological Chemistry, 270*, 23937–23940.

Bird, J. G., Sharma, S., Roshwalb, S. C., Hoskins, J. R., & Wickner, S. (2006). Functional analysis of CbpA, a DnaJ homolog and nucleoid-associated DNA-binding protein. *The Journal of Biological Chemistry, 281*(45), 34349–34356.

Brandt, S., Kwok, T., Hartig, R., König, W., & Backert, S. (2005). NF-kappaB activation and potentiation of proinflammatory responses by the helicobacter pylori CagA protein. *Proceedings of the National Academy of Sciences of the United States of America, 102*(26), 9300–9305.

Bucca, G., Brassington, A. M., Schönfeld, H. J., & Smith, C. P. (2000). The HspR regulon of Streptomyces coelicolor: A role for the DnaK chaperone as a transcriptional co-repressor. *Molecular Microbiology, 38*(5), 1093–1103.

Chae, C., Sharma, S., Hoskins, J. R., & Wickner, S. (2004). CbpA, a DnaJ homolog, is a DnaK co-chaperone, and its activity is modulated by CbpM. *The Journal of Biological Chemistry, 279*(32), 33147–33153.

Covacci, A., Falkow, S., Berg, D. E., & Rappuoli, R. (1997). Did the inheritance of a pathogenicity island modify the virulence of Helicobacter pylori? *Trends in Microbiology, 5*(5), 205–208.

Cun, S., Li, H., Ge, R., Lin, M. C., & Sun, H. (2008). A histidine-rich and cysteine-rich metal-binding domain at the C terminus of heat shock protein a from Helicobacter pylori: Implication for nickel homeostasis and bismuth susceptibility. *The Journal of Biological Chemistry, 283*(22), 15142–15151.

Cussac, V., Ferrero, R. L., & Labigne, A. (1992). Expression of Helicobacter pylori urease genes in Escherichia coli grown under nitrogen-limiting conditions. *Journal of Bacteriology, 174*(8), 2466–2473.

Danielli, A., Amore, G., & Scarlato, V. (2010). Built shallow to maintain homeostasis and persistent infection: Insight into the transcriptional regulatory network of the gastric human pathogen Helicobacter pylori. *PLoS Pathogens, 6*(6), e1000938.

Dunn, B. E., Vakil, N. B., Schneider, B. G., Miller, M. M., Zitzer, J. B., Peutz, T., & Phadnis, S. H. (1997). Localization of Helicobacter pylori urease and heat shock protein in human gastric biopsies. *Infection and Immunity, 65*(4), 1181–1188.

Elsholz, A. K., Michalik, S., Zühlke, D., Hecker, M., & Gerth, U. (2010). CtsR, the Gram-positive master regulator of protein quality control, feels the heat. *The EMBO Journal, 29*(21), 3621–3629.

Haslberger, T., Bukau, B., & Mogk, A. (2010). Towards a unifying mechanism for ClpB/Hsp104-mediated protein disaggregation and prion propagation. *Biochemistry and Cell Biology, 88*(1), 63–75.

Henderson, B., & Martin, A. (2011). Bacterial virulence in the moonlight: Multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity, 79*(9), 3476–3491.

Herbst, K., Bujara, M., Heroven, A. K., Opitz, W., Weichert, M., Zimmermann, A., & Dersch, P. (2009). Intrinsic thermal sensing controls proteolysis of Yersinia virulence regulator RovA. *PLoS Pathogens, 5*(5), e1000435.

Homuth, G., Domm, S., Kleiner, D., & Schumann, W. (2000). Transcriptional analysis of major heat shock genes of Helicobacter pylori. *Journal of Bacteriology, 182*(15), 4257–4263.

Huesca, M., Borgia, S., Hoffman, P., & Lingwood, C. A. (1996). Acidic pH changes receptor binding specificity of Helicobacter pylori: A binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. *Infection and Immunity, 64*(7), 2643–2648.

Hurme, R., Berndt, K. D., Normark, S. J., & Rhen, M. (1997). A proteinaceous gene regulatory thermometer in Salmonella. *Cell, 90*(1), 55–64.

Kansau, I., Guillain, F., Thiberge, J. M., & Labigne, A. (1996). Nickel binding and immunological properties of the C-terminal domain of the Helicobacter pylori GroES homologue (HspA). *Molecular Microbiology, 22*(5), 1013–1023.

Kao, C. Y., Sheu, B. S., & Wu, J. J. (2016). Helicobacter pylori infection: An overview of bacterial virulence factors and pathogenesis. *Biomedical Journal, 39*(1), 14–23.

Kortmann, J., & Narberhaus, F. (2012). Bacterial RNA thermometers: Molecular zippers and switches. *Nature Reviews. Microbiology, 10*(4), 255–265.

Labigne, A., Cussac, V., & Courcoux, P. (1991). Shuttle cloning and nucleotide sequences of Helicobacter pylori genes responsible for urease activity. *Journal of Bacteriology, 173*(6), 1920–1931.

Lee, H., Su, Y. L., Huang, B. S., Hsieh, F. T., Chang, Y. H., Tzeng, S. R., Hsu, C. H., Huang, P. T., Lou, K. L., Wang, Y. T., & Chow, L. P. (2016). Importance of the C-terminal histidine residues of Helicobacter pylori GroES for toll-like receptor 4 binding and interleukin-8 cytokine production. *Scientific Reports, 6*, 37367.

Lin, Y. F., Wu, M. S., Chang, C. C., Lin, S. W., Lin, J. T., Sun, Y. J., Chen, D. S., & Chow, L. P. (2006). Comparative immunoproteomics of identification and characterization of virulence factors from Helicobacter pylori related to gastric cancer. *Molecular & Cellular Proteomics, 5*(8), 1484–1496.

Missiakas, D., Schwager, F., Betton, J. M., Georgopoulos, C., & Raina, S. (1996). Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in Escherichia coli. *The EMBO Journal, 15*(24), 6899–6909.

Mogk, A., Völker, A., Engelmann, S., Hecker, M., Schumann, W., & Völker, U. (1998). Nonnative proteins induce expression of the Bacillus subtilis CIRCE regulon. *Journal of Bacteriology, 180*(11), 2895–2900.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., & Bukau, B. (1999). Identification of thermolabile Escherichia coli proteins: Prevention and reversion of aggregation by DnaK and ClpB. *The EMBO Journal, 18*(24), 6934–6949.

Moran, A. P., Lindner, B., & Walsh, E. J. (1997). Structural characterization of the lipid A component of Helicobacter pylori rough- and smooth-form lipopolysaccharides. *Journal of Bacteriology, 179*, 6453–6463.

Parijat, P., & Batra, J. K. (2015). Role of DnaK in HspR-HAIR interaction of Mycobacterium tuberculosis. *IUBMB Life, 67*(11), 816–827.

Peterson, J. W. (1996). Bacterial pathogenesis. In S. Baron (Ed.), *Medical microbiology* (4th ed.). Galveston: University of Texas Medical Branch at Galveston. Chapter 7.

Phadnis, S. H., Parlow, M. H., Levy, M., Ilver, D., Caulkins, C. M., Connors, J. B., & Dunn, B. E. (1996). Surface localization of Helicobacter pylori urease and a heat shock protein homolog requires bacterial autolysis. *Infection and Immunity, 64*(3), 905–912.

Rad, R., Gerhard, M., Lang, R., Schöniger, M., Rösch, T., Schepp, W., Becker, I., Wagner, H., & Prinz, C. (2002). The Helicobacter pylori blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *Journal of Immunology, 168*(6), 3033–3041.

de Reuse, H., Vinella, D., & Cavazza, C. (2013). Common themes and unique proteins for the uptake and trafficking of nickel, a metal essential for the virulence of Helicobacter pylori. *Frontiers in Cellular and Infection Microbiology, 3*, 94.

Roncarati, D., & Scarlato, V. (2017). Regulation of heat-shock genes in bacteria: From signal sensing to gene expression output. *FEMS Microbiology Reviews, 41*, 549. https://doi.org/10.1093/femsre/fux015

Roncarati, D., Spohn, G., Tango, N., Danielli, A., Delany, I., & Scarlato, V. (2007a). Expression, purification and characterization of the membrane-associated HrcA repressor protein of Helicobacter pylori. *Protein Expression and Purification, 51*(2), 267–275.

Roncarati, D., Danielli, A., Spohn, G., Delany, I., & Scarlato, V. (2007b). Transcriptional regulation of stress response and motility functions in Helicobacter pylori is mediated by HspR and HrcA. *Journal of Bacteriology, 189*(20), 7234–7243.

Roncarati, D., Danielli, A., & Scarlato, V. (2011). CbpA acts as a modulator of HspR repressor DNA binding activity in Helicobacter pylori. *Journal of Bacteriology, 193*(20), 5629–5636.

Roncarati, D., Danielli, A., & Scarlato, V. (2014). The HrcA repressor is the thermosensor of the heat-shock regulatory circuit in the human pathogen Helicobacter pylori. *Molecular Microbiology, 92*(5), 910–920.

Schauer, K., Muller, C., Carrière, M., Labigne, A., Cavazza, C., & De Reuse, H. (2010). The Helicobacter pylori GroES cochaperonin HspA functions as a specialized nickel chaperone and sequestration protein through its unique C-terminal extension. *Journal of Bacteriology, 192*(5), 1231–1237.

Schumann, W. (2016). Regulation of bacterial heat shock stimulons. *Cell Stress & Chaperones, 21*(6), 959–968.

Servant, P., Grandvalet, C., & Mazodier, P. (2000). The RheA repressor is the thermosensor of the HSP18 heat shock response in Streptomyces albus. *Proceedings of the National Academy of Sciences of the United States of America, 97*(7), 3538–3543.

Spohn, G., & Scarlato, V. (1999). The autoregulatory HspR repressor protein governs chaperone gene transcription in Helicobacter pylori. *Molecular Microbiology, 34*(4), 663–674.

Spohn, G., Delany, I., Rappuoli, R., & Scarlato, V. (2002). Characterization of the HspR-mediated stress response in Helicobacter pylori. *Journal of Bacteriology, 184*(11), 2925–2930.

Spohn, G., Danielli, A., Roncarati, D., Delany, I., Rappuoli, R., & Scarlato, V. (2004). Dual control of Helicobacter pylori heat shock gene transcription by HspR and HrcA. *Journal of Bacteriology, 186*(10), 2956–2965.

Su, Y. L., Yang, J. C., Lee, H., Sheu, F., Hsu, C. H., Lin, S. L., & Chow, L. P. (2015). The C-terminal disulfide bonds of Helicobacter pylori GroES are critical for IL-8 secretion via the TLR4-dependent pathway in gastric epithelial cells. *Journal of Immunology, 194*(8), 3997–4007.

Suerbaum, S. (1995). The complex flagella of gastric Helicobacter species. *Trends in Microbiology, 3*(5), 168–170.

Sugano, K., Tack, J., Kuipers, E. J., Graham, D. Y., El-Omar, E. M., Miura, S., Haruma, K., Asaka, M., Uemura, N., Malfertheiner, P., & faculty members of Kyoto Global Consensus Conference. (2015). Kyoto global consensus report on Helicobacter pylori gastritis. *Gut, 64*(9), 1353–1367.

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., & Venter, J. C. (1997). The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature, 388*(6642), 539–547. Erratum in: *Nature* 389(6649)**,** 412.

Vogiatzi, P., Cassone, M., Luzzi, I., Lucchetti, C., Otvos, L. Jr., & Giordano, A. (2007). Helicobacter pylori as a class I carcinogen: Physiopathology and management strategies. *Journal of Cellular Biochemistry, 102*(2)**,** 264–273.

Warren, J. R., & Marshall, B. (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet, 1*(8336), 1273–1275.

Wawrzynow, A., Banecki, B., & Zylicz, M. (1996). The Clp ATPases define a novel class of molecular chaperones. *Molecular Microbiology, 21*(5), 895–899.

Wilson, A. C., Wu, C. C., Yates, J. R. 3rd, & Tan, M. (2005). Chlamydial GroEL autoregulates its own expression through direct interactions with the HrcA repressor protein. *Journal of Bacteriology, 187*(21), 7535–7542.

World Health Organization (2017) Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. p. 1–7. (www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/).

Zhao, Y., Yokota, K., Ayada, K., Yamamoto, Y., Okada, T., Shen, L., & Oguma, K. (2007). Helicobacter pylori heat-shock protein 60 induces interleukin-8 via a toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes. *Journal of Medical Microbiology, 56*(Pt 2), 154–164.

# Chapter 18
# Heat Shock Protein Expression in Various Tissues in Thermal Stress


**Elke Doberentz and Burkhard Madea**

**Abstract** Heat shock proteins (Hsp) have a cytoprotective function and support the cell to survive in (sub) lethal conditions. Expression of Hsp is stimulated by several stress conditions, including hypo- and hyperthermia. Immunohistochemical expression of Hsp is studied in forensic medicine, mainly in victims of hypothermia, fire, sudden infant death syndrome (SIDS), and amphetamine-related deaths. Hsp expression has been observed in cardiac tissue in fire victims, in hypothermia, and in amphetamine-related deaths, but at a lower degree compared with in the kidney or pulmonary tissue. Hsp27 expression is present in cases of fatal hypothermia in the pituitary gland. In SIDS victims, there is no Hsp expression in cardiac tissue, but low expression is found in pulmonary and renal tissues. In fire-related fatalities, an extensive and different expression pattern of Hsp27 and Hsp70 according to the survival time can be observed. Hsp27 is rapidly expressed in short-term survivors and Hsp70 is higher in long-term survivors. In the future, Hsp expression in cardiac tissue in myocardial infarction and coronary insufficiency should be studied.

**Keywords** Amphetamine-associated death · Heat shock protein · Hyperthermia · Hypothermia · SIDS · Thermal stress

## Abbreviations

| | |
|---|---|
| A | Amphetamine |
| ATP | Adenosine triphosphate |
| eHSP | Extracellular heat shock protein |
| HSP | Heat shock protein |
| MA | Metamphetamine |
| SIDS | Sudden infant death syndrome |

E. Doberentz · B. Madea (✉)
Institute of Legal Medicine, Rheinische Friedrich-Wilhlems University Bonn, Bonn, Germany
e-mail: b.madea@uni-bonn.de



A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6_18

## 18.1 Introduction

Heat shock proteins (Hsp) are a group of proteins that belong to molecular chaperones (Feder and Hofmann 1999). Hsp were first described in 1962 (Ritossa 1962). They have a cytoprotective function and support the cell to survive in (sub)lethal conditions. Hsp control protein biosynthesis by supporting accurate protein synthesis, preventing aggregation of newly folded proteins, and stabilizing various cell structures (Brinkmeier and Ohlendieck 2014; Deshaies et al. 1988; Jeng et al. 2015), Macario and Conway de Macario 2005, Walter and Buchner 2002, Wilson et al. 2015) Hsp are expressed in cells when there is exposure to variable stressful stimuli. Hsp expression is stimulated by hypo- or hyperthermia, as well as by every other external and internal physiological stress. These stresses include a lack of energy (ATP), oxidative stress, heavy metals, ischemia, UV light, chemicals, injuries, mechanical stress, chemotherapeutic substances, and cytokines, which increase the number of these proteins in the cellular cytoplasm (Benndorf and Bielka 1997; Brinkmeier and Ohlendieck 2014; Feder and Hofmann 1999; Sunderland and Emery 1981). Hsp are also expressed in erythrocytes, macrophages, and leukocytes (Benndorf and Bielka 1997; Brinkmeier and Ohlendieck 2014; Feder and Hofmann 1999; Wang et al. 2014). As a direct result of hypo- and hyperthermia, metabolism is increased with a consecutive higher requirement of oxygen. This results in metabolic acidosis with destruction of cellular structures (Kramme 2006; Lantry et al. 2012, Larsen 2009, Madea et al. 2004, Pomara et al. 2010, Turk 2010, Xu et al. 2005).

Important Hsp are Hsp27 and Hsp70 (Clerico et al. 2015; Wang et al. 2014), named according to their molecular weight (Table 18.1, Fig. 18.1). Hsp70 is an established marker for cellular stress in heat and fire deaths in the tissue of the upper respiratory tract (Marschall 2005; Nollen et al. 1999). Whether other organ systems (heart and kidney) express Hsp in cases of antemortem hyperthermic stress is a research area of interest. Identifying a valid vitality marker, which could complete

**Table 18.1** The main Hsp, their localization and main function

| Hsp family | Localization | Main function |
|---|---|---|
| Hsp27 | Cytosol and nucleus | Stabilization of microfilaments |
| Hsp60 | Mitochondria | Protection of proteins and repair of proteins |
| Hsp70 | Cytosol and nucleus | Protein folding and cytoprotection |
| Hsp72 | Cytosol and nucleus | Protein folding and cytoprotection |
| Hsp73 | Cytosol and nucleus | Protein translocation |
| Hsp75 | Mitochondria | Protein translocation |
| Hsp78 | Endoplasmic reticulum | Cytoprotection and protein translocation |
| Hsp90 | Cytosol, nucleus, endoplasmic reticulum | Protein translocation and regulation of receptors |
| Hsp100–104 | Cytosol | Protein folding |

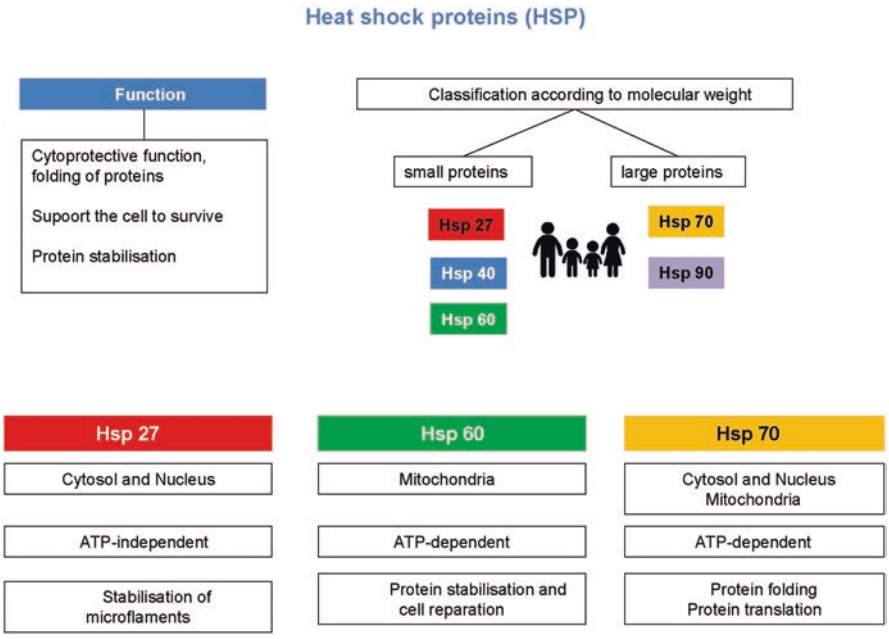


**Fig. 18.1** Heat shock proteins. Classification, function and localization

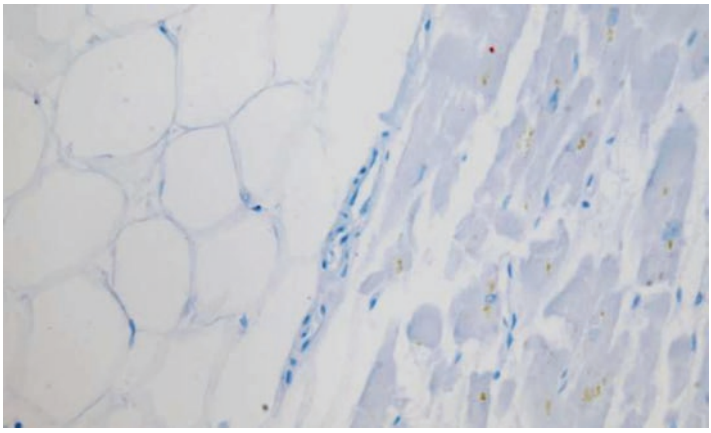

**Fig. 18.2** Heart, control group, Hsp70, myocytes and vessels grade 0, x 400

the well-known markers for soot aspiration and ingestion, as well as elevated carbon monoxide blood levels, could be important. Expression of Hsp has been extensively studied in renal tissue (Fig. 18.2), especially in cases of hypo- and hyperthermia (Doberentz et al. 2017b; c; Doberentz et al. 2016; Doberentz et al. 2014; Preuß et al. 2008). Hsp are expressed in renal tissue in cases of lethal hypothermia. In a study group of 100 cases of death due to hypothermia, renal glomerular Hsp 70 expression

was observed in 80% of cases and renal tubular expression was found in 89% of cases compared with a control group (n = 50) (Preuss et al. 2008). Additionally, in the control group, there were short and long agonal periods where no renal glomerular expression was found in 84% of cases and no renal tubular expression was observed in 66% of all cases. Hsp27 and Hsp70 expression has also been studied in the pituitary gland in cases of fatal hypothermia (Doberentz et al. 2017a). The pituitary gland is an endocrine gland and plays an important role in thermoregulation. When the core body temperature drops, the pituitary gland is activated by stimulation of hypothalamic hormones. Several morphological alterations of the anterior lobe of the pituitary gland, such as hemorrhage, vacuolization, and hyperemia, have been previously described in fatal hypothermia (Baillif 1938, Büchner 1943, Doberentz and Madea 2017, Doberentz et al. 2011, Hirano and Shiino 1994, Ishikawa et al. 2008a, Ishikawa et al. 2004, Ishikawa et al. 2008b, Verdiccio et al. 2006).

Furthermore, Hsp expression in SIDS has been investigated. In industrialized countries, sudden infant death is a rare cause of death in young children, but it still occurs and the etiology is still unresolved. Pathognomonic findings of premortem hyperthermia do not exist. Although the prone sleeping position is a well-known risk factor of SIDS (Blair et al. 2008, Fleming et al. 1992, Mitchell and Krous 2015, Rhode et al. 2013, Sawczenko and Fleming 1996), hyperthermia might also be important. Hyperthermia and overheating are risk factors of SIDS because high ambient temperatures have been found at the place of death and inadequate warm covering or clothing of the infants has been found (Fleming et al. 1990, Gilbert et al. 1992, Kleemann et al. 1996, Pfeifer 1980, Ponsonby et al. 1992, Stanton, 183, Sunderland and Emery 1981). Furthermore, core body temperatures have been measured (Kleemann et al. 1996; Stanton 1984; Sunderland and Emery 1981). During stress, including thermal effects, Hsp expression increases. Therefore, whether hyperthermia is a contributing or pathogenic factor for SIDS needs to be investigated.

Hyperthermia has also been observed in amphetamine/methamphetamine abuse (Akerfelt et al. 2010; Armenian et al. 2013; Greene et al. 2003; Henry et al. 1992). Well-recognized side effects of amphetamine (Doberentz et al. 2017a, b, c) and methamphetamine (MA) abusers at autopsy are fatty liver, moderate coronary artery disease, cirrhosis, pneumonia, myocardial fibrosis, severe coronary artery disease, emphysema, and hepatitis (Bora et al. 2016; Campbell and Rosner 2008; Chandra et al. 2016; García-Repetto et al. 2003; Jochum et al. 2017; Kalant and Le 1983; Vennemann et al. 2009). The hearts of amphetamine/MA and cocaine users essentially show the same microscopic features, such as hypertrophy, interstitial fibrosis, and microvascular disease (Karch and Chih-Hsieng 1999, Karch and Drummer 2016, Milroy 2011, Mizia-Stec et al. 2008, Pilgrim et al. 2008, Wöllner et al. 2015a, Wöllner et al. 2015b). However, acute myocardial infarction is much more common in cocaine abusers than in MA abusers, even though the extent of disease present appears to be approximately parallel. MA-related infarcts are much less common than in cocaine users (Karch and Drummer 2016). MA induces production of Hsp, but cocaine does not. According to Karch and Drummer 2016, for decades, Hsp

production has been shown to be an adaptive myocardial response that occurs within 24 h after short episodes of cardiac ischemia and that production of Hsp (which of the proteins predominate is unclear) increases myocardial resistance to infarction. Production of Hsp is a logical explanation of the known ability of MA, and most other amphetamines, to cause hyperthermia.

There have been only a few studies on immunohistochemical expression of Hsp in cardiac tissue (Doberentz et al. 2017b, c; Doberentz et al. 2016; Doberentz et al. 2014; Madea et al. 2017). Western blotting has shown that Hsp content of cardiac tissue is increased by either ischemic or thermal stress. Hsp60 was preferentially elevated by ischemic pretreatment (Marber et al. 1993). In the heat stressed group, rectal temperatures in anaesthetized rabbits was raised to at least 42 °C for 15 min by wrapping the rabbits in an electric warming blanket. Substantial myocardial Hsp72 induction was possible after sublethal thermal pretreatment.

The aim of the present studies was to investigate Hsp expression in various tissues in thermal stress (1) in cases of death due to fire (lung, kidney, heart), (2) in cases of fatal hypothermia (pituitary gland), (3) in cases of SIDS (heart, kidney, lung), and (4) in amphetamine-associated fatalities (heart).

## 18.2 Materials and Methods

### *18.2.1 Cases of Death Due to Fire*

The study group consisted of 48 fire fatalities, with 37 (77.7%) males and 11 (22.9%) females, and a mean age of 59.3 years (3–83 years). In most cases, the cause of death was either burning or flue gas intoxication, or a combination of possible causes of death in fire-related fatalities. The control group consisted of 100 cases of a natural and non-natural manner of death without any evidence of antemortem thermal exposure. This group included 86 males (86.0%) and 14 females (14.0%), with a mean age of 49.09 years (5–99 years). Causes of death are listed in Table 18.2. All data were collected from autopsy protocols or protocols of toxicological analysis of the Institutes of Legal Medicine of the Universities of Bonn and Greifswald from a time period of 9 years. The study group and the control group cases were divided into two subgroups of different survival times. The group of

**Table 18.2** Control group with causes of death and survival times

| Cause of death | Short-term survival (cases) | Long-term survival (cases) | Total number (cases) |
|---|---|---|---|
| Shotgun wounds | 38 | 1 | 39 |
| Suffocation | 28 | 0 | 28 |
| Myocardial infarction | 0 | 24 | 24 |
| Multiple trauma | 8 | 1 | 8 |

short-term survival included sudden death within seconds up to a few minutes (n = 40). Those who were categorized in the group of long-time survival died on the way to hospital or at the hospital within hours or days (n = 8) (Table 18.2). In the control group, there were 26 long-term survivors and 74 short-term survivors. Myocardial, pulmonary, and renal tissues were examined.

### *18.2.2 Hypothermia*

The study group consisted of 11 cases of fatal hypothermia. The diagnosis of fatal hypothermia was based on circumstances of the case, the type of discovery of the body, autopsy findings (mainly Wischnewski's spots and frost erythema), and the lack of concurrent causes of death. In the study group, all cases showed Wischnewski's spots as autopsy findings and four cases had frost erythema. The study group contained cases with at least one of these findings. The study group contained five women and six men aged between 44 and 88 years. In all cases, a complete histological investigation and toxicological screening, including determination of blood alcohol concentration were performed. In the control group, pituitary glands from 10 autopsy cases were taken. The control group contained five women and five men aged between 7 and 83 years. Pituitary glands were examined.

### *18.2.3 Cases of SIDS*

The study group consisted of 120 SIDS cases (46 girls and 74 boys). The diagnosis of SIDS as a cause of death was based upon the criteria of the San Diego Consensus Definition from 2004 (Krous et al., 2004). Most cases belonged to category Ib. The minimum age was 7 days and the maximum was 344 days (mean age, 125 days). The control group consisted of 29 cases of death in children due to unnatural causes (13 girls and 16 boys), with a minimum age of 4 days and a maximum age of 354 days. Myocardial, pulmonary, and renal tissues were examined.

### *18.2.4 Amphetamine-associated Fatalities*

A total of 19 amphetamine-associated fatalities were investigated where either amphetamine intoxication alone, mixed intoxication, and intoxication together with amphetamine-related preexisting diseases were the causes of death. The study group contained three women and 16 men aged between 22 and 58 years (mean age, 34.5 years) (Table 18.3). Myocardial, pulmonary, and renal tissues were examined.

**Table 18.3** Causes of death in amphetamine/methamphetamine-related deaths

| Case number | Autopsy number | Cause of death | A/MA Contribution to death | A Heart blood (ng/ml) | A Femoral vein blood (ng/ml) | MA Femoral vein blood (ng/ml) | Hsp/grade of Hsp |
|---|---|---|---|---|---|---|---|
| 1 | 168/09 | Amphetamine and preexisting cardiac disease | Yes | | 246.5 | | Negative |
| 2 | 284/09 | Mixed intoxication | No | | 35.01 | | Negative |
| 3 | 137/10 | Amphetamine and preexisting cardiac disease | Yes | | 47.73 | | Negative |
| 4 | 139/11 | Mixed intoxication/heroin | No | | 52.3 | | Negative |
| 0.5 | 190/11 | Mixed intoxication | ? | | 165.71 | | Negative |
| 6 | 291/11 | Amphetamine and preexisting cardiac disease | Yes | | 360.95 | | Negative |
| 7 | 321/11 | Mixed intoxication MDMA | Yes | | 519 | 1353 | Negative |
| 8 | 147/12 | Mixed intoxication, amphetamine | Yes | 147.69 | | | Negative |
| 9 | 240/12 | MDMA intoxication | Yes | | 249.5 | 1300 | – |
| 10 | 275/13 | Amphetamine intoxication | Yes | | 1084 | | Negative |
| 11 | 373/13 | Mixed intoxication/heroine | No | | 89.34 | | 27 (heart) [2], 70 (heart) [1] |
| 12 | 453/13 | Mixed intoxication amphetamine and preexisting cardiac disease | ? | | 67.9 | | Negative |
| 13 | 174/14 | Mixed intoxication | Yes | | 222.51 | | Negative |
| 14 | 412/14 | Amphetamine intoxication | Yes | Positive | 304 | | 27 (heart) [3], 27 (kidney) [1] |
| 15 | 004/15 | Mixed intoxication amphetamine/heroin | Yes | | 93.6 | | 27 (heart) [1] |
| 16 | 022/15 | Amphetamine and preexisting cardiac disease | Yes | | 255 | | Negative |
| 17 | 288/15 | Mixed intoxication MDMA | Yes | | 238 | 8142 | 27 (heart) [2] |
| 18 | 066/16 | Amphetamine and preexisting cardiac disease | Yes | Positive | 170 | | 27 (heart) [1] |
| 19 | 076/16 | Amphetamine intoxication | Yes | | 107 | | Negative |

The cause of death was mostly intoxication or intoxication together with preexisting heart disease. Hsp-positive cases are highlighted in red (grade is shown in brackets). MDMA - 3,4-Methylendioxy-N-methylamphetamin, A – amphetamine, MA – methamphetamine

### *18.2.5 Immunohistochemistry*

Tissue samples of the heart, lungs, kidney, and pituitary glands were removed from the bodies during forensic autopsy and fixed in +8–10% formalin. After fixation, samples were embedded in paraffin wax, sliced (3–4 μm), and stained with Hsp27 and Hsp70 antibodies (monoclonal mouse antibodies NCL-HSP27 and NCL-HSP70; Menarini/Novocastra), as well as with hematoxylin–eosin (H&E). For every staining procedure, positive controls of tonsillar tissue were used. In amphetamine and MA-associated deaths Hsp60 staining (Mouse anti-HSP60 clone 1; Sigma-Aldrich) was additionally performed. To avoid evaluation of false-positive cases, every staining run had negative controls. Using this method, in every staining procedure, one sample of tonsillar tissue was used without primary antibody and one without secondary antibody as negative controls. Every slide was examined by a light microscope at ×400 magnification in 30 visual fields for reddish stained Hsp in tissues. Evaluation of the following organ structures was performed: heart with epicardium, myocytes, intercalated discs, perinuclear space, vessels, and fibrocytes (Table 18.4). The immunohistochemical reaction of the tissue was graded semi-quantitatively in a four-degree scale according to Preuss et al. 2008. The number of positively reddish stained structures/cells in relation to all investigated structures/cells, which were visible in each visual field, was estimated on a percentage basis. For every analyzed slice and structure, a mean value averaged over all 30 visual fields was calculated and graded according to the pattern shown in Table 18.5. Examples for the grading of Hsp expression in cardiac tissue are shown in Figs. 18.3, 18.4, and 18.5.

**Table 18.4** Investigated organ structures

| Organs | Structures |
|---|---|
| Heart | Myocytes, fibrocytes, vessels |
| Lung | Peripheral and central bronchial tubes, vessels (endothelium, lumen), Inter-alveolar septa, pleura, peribronchial glands, peribronchial connective tissue, ciliated epithelium |
| Kidney | Glomeruli, tubuli, vessels, connective tissue |

**Table 18.5** Grades of Hsp27 and Hsp70 expression

| Percentage of reddish stained structures in total | Grades | Explanation |
|---|---|---|
| 0 | Grade 0 | No reaction (Fig. 18.3) |
| >0 to 29.99 | Grade 1 | Weak staining (Fig. 18.4) |
| 30 to 59.99 | Grade 2 | Moderate staining (Fig. 18.5) |
| 60 to 100 | Grade 3 | Intensive staining (Fig. 18.6) |
| | Grade 4 | Analyzed structures were not present in the section of the tissue sample (i.e., central bronchus was not found in the sample) |

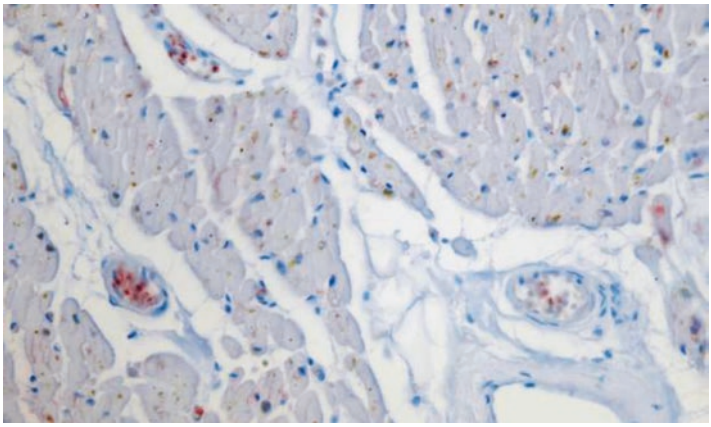

**Fig. 18.3** Heart, study group, death due to fire, cardiac tissue, Hsp70, myocytes and vessels grade 1, x 400

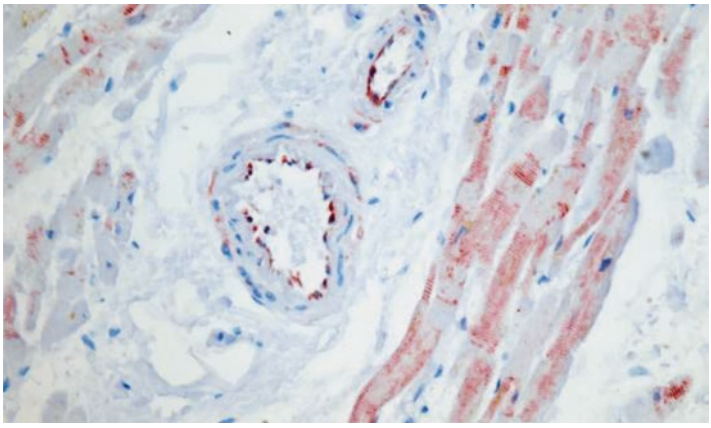

**Fig. 18.4** Heart, study group, death due to fire, cardiac tissue, Hsp27, vessels grade 2, myocytes grade 3, x 400

## 18.3 Results

Positive Hsp27 staining in amphetamine-associated deaths is shown in Figs. 18.6 and 18.7. Hsp expression in hypothermia is shown in Figs. 18.8 and 18.9. In Fig. 18.10, a typical case of fatal hypothermia is shown. Positive findings in pulmonary tissue, the kidney, and the pituitary gland are shown in Figs. 18.11–18.14.

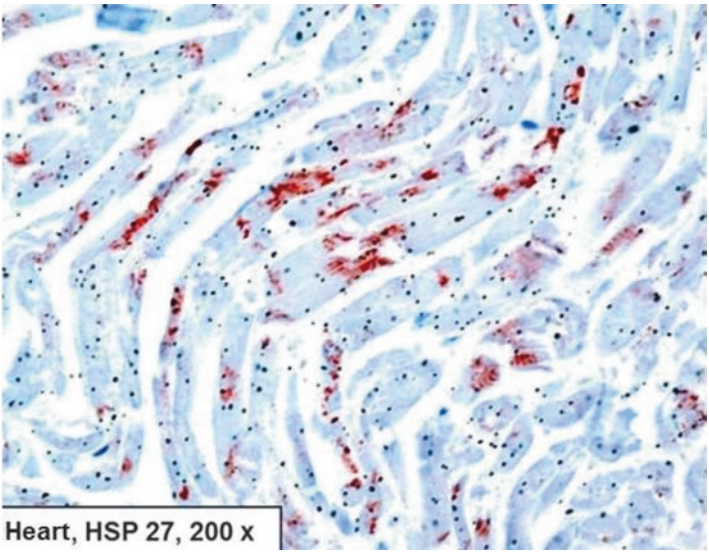


**Fig. 18.5** Heart, amphetamine associated death, cardia tissue, Hsp27, grade 2, x 200

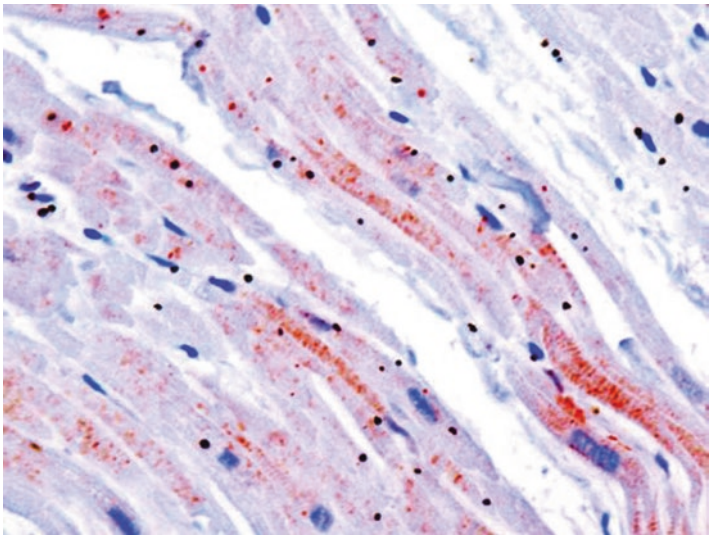

**Fig. 18.6** Heart, amphetamine associated death, cardiac tissue, Hsp27, grade 3, x 400

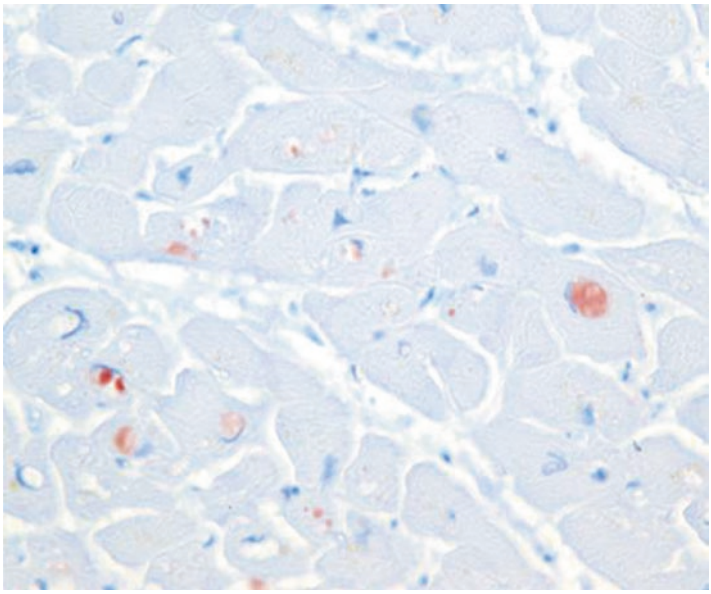

**Fig. 18.7** Fatal hypothermia, slightly positive Hsp27-expression

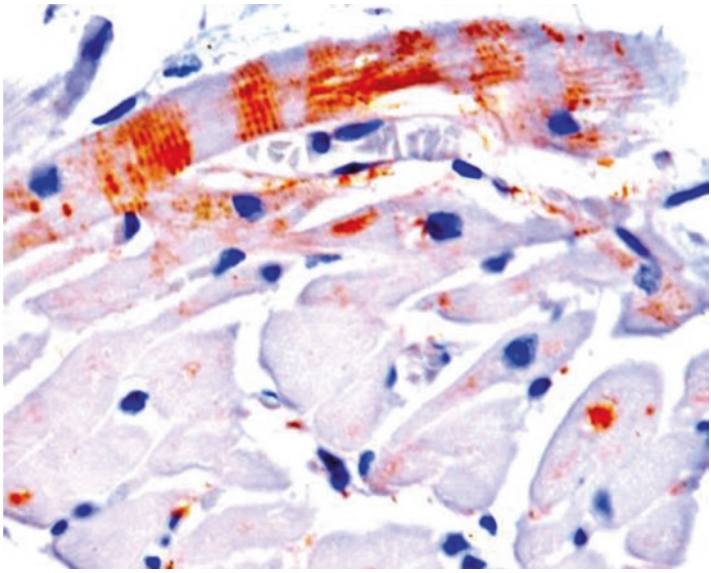

**Fig. 18.8** Fatal hypothermia in amphetamine intoxication, strongly positive Hsp27-expression

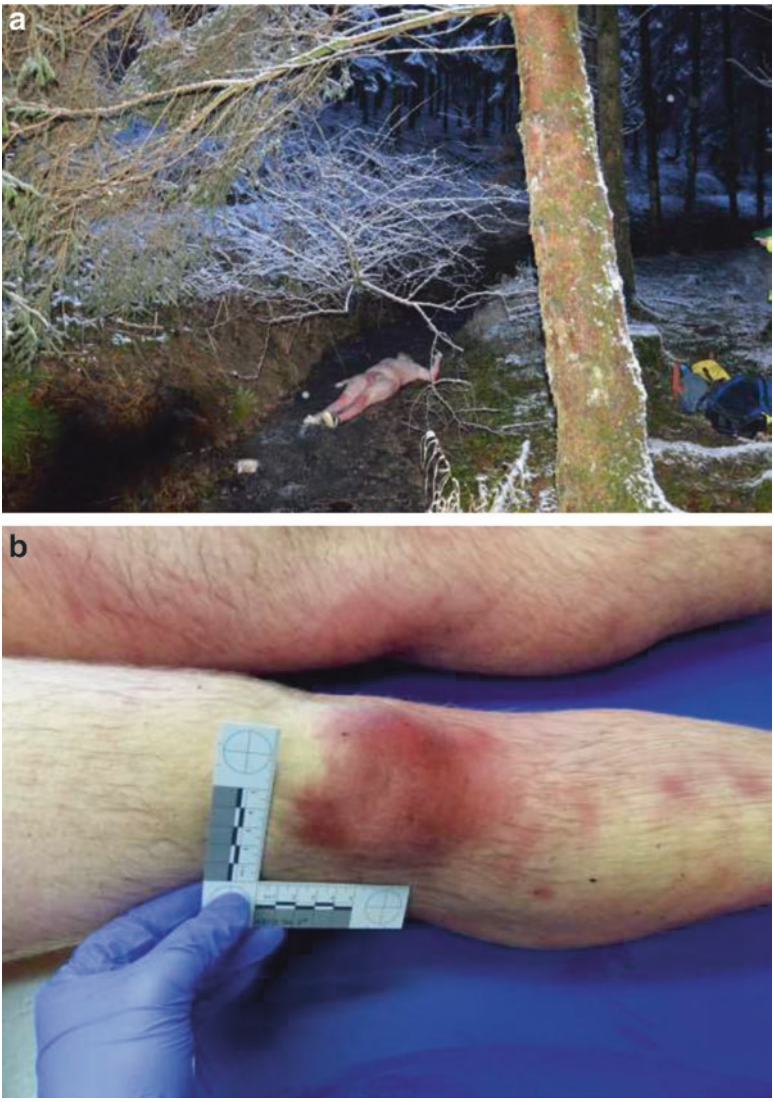


**Fig. 18.9** (A) A 24 years-old man was found undressed in December in a river. Mouth and nose above the water level. Body weight 102.7 kg, body length 185 cm. Some meters away the clothing of the deceased was found. (B) Main autopsy findings: Frost erythema above the knee joints, the elbow joints on both sides. Hemorrhagic gastric erosions. No further preexisting diseases. Cause of death: Death due to hypothermia. Blood alcohol concentration: negative. Urine: positive immunological test for amphetamine, metamphetamine and ecstasy. Amphetamine about 84,965 ng/ml, metamphetamine 22.0 ng/ml. Femoral blood: amphetamine 304 ng/ml, olanzapine 56.0 ng/ml, BAC negative

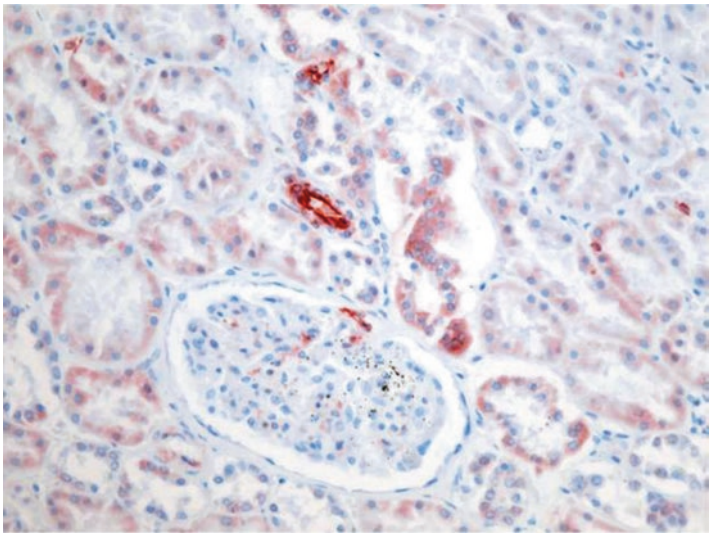

**Fig. 18.10** Kidney, control group. Hsp27, positive tubular cells (grade 2), glomeruli (grade 1), and renal vessels (grade 3), x 400

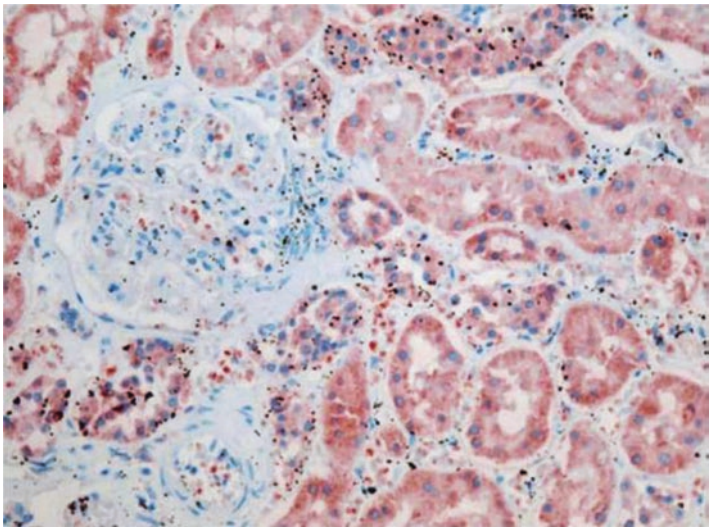

**Fig. 18.11** Kidney, renal tubuli, Hsp70, grade 3, x 400

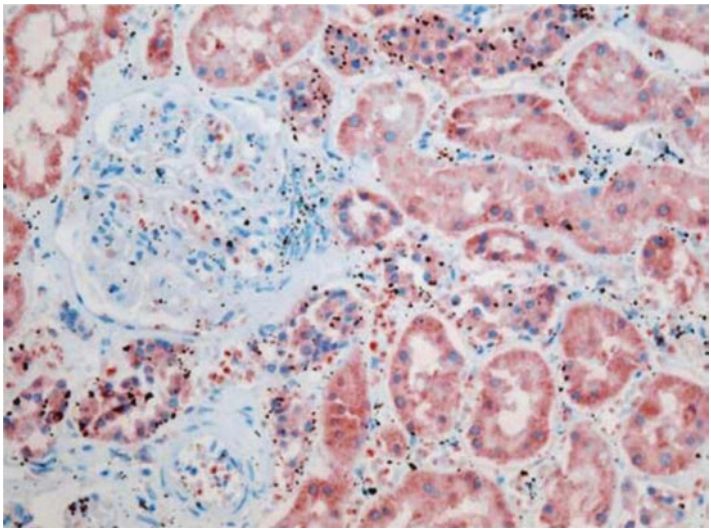

**Fig. 18.12** Pulmonary tissue, study group. Positive blood vessels (grade 3) and positive bronchial tubes (grade 3), Hsp27, x 400

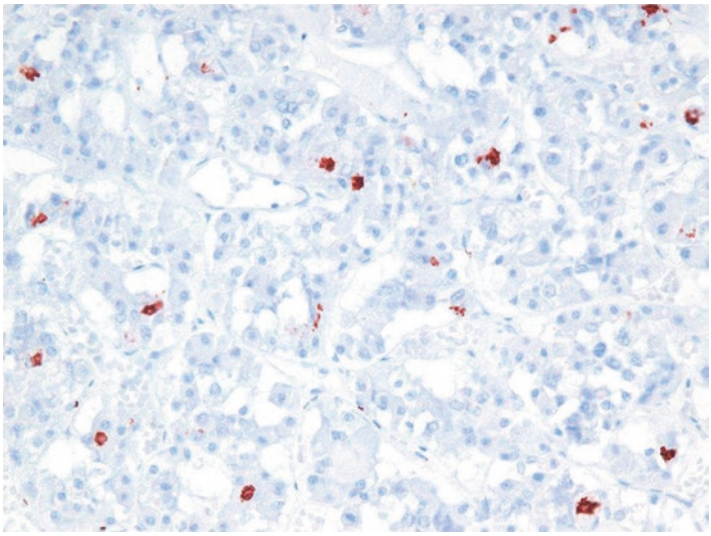

**Fig. 18.13** Anterior lobe of the pituitary gland, study group. Hsp27, grade 1, x 400

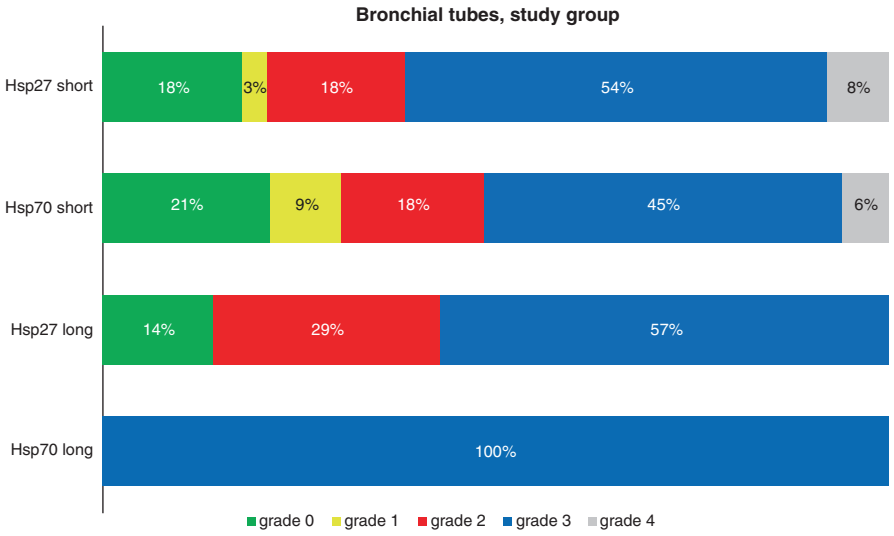


**Fig. 18.14** Percental expression rate of Hsp27 and 70 in bronchial tubes. Study group

### *18.3.1 Death Due to Fire*

Hsp27 and Hsp70 were expressed within seconds and minutes after exposure, and the survival time affected Hsp expression in the study group cases. In pulmonary tissue of fire-related cases, Hsp27 and Hsp70 expression in the bronchial tubes showed a similar expression pattern in all short-term survivors (Fig. 18.15). With increasing survival time, Hsp70 expression greatly increased, and the long-term survivors had the highest expression levels in all cases. Similar results were found in the pulmonary vessels (Fig. 18.16). The expression patterns of Hsp27 and Hsp70 were similar in short- and long-term survival, but the expression of Hsp70 was increased relative to the survival time. In contrast, the majority of cases in the control group showed negative results. In the control group, Hsp expression in the bronchial tubes decreased with survival time, whereas the relation of cases with lower graduation of hsp expression increased (Fig. 18.17). Interestingly, the pulmonary vessels showed similar results in the study group, with a considerable increase in Hsp70 expression relative to survival time (Fig. 18.18). In the short-term survivors, Hsp27 was expressed more rapidly and at higher levels compared with the long-term survivors.

The expression patterns of Hsp27 and Hsp70 in the renal tubules and renal vessels in the study group differed from that in pulmonary tissue (Figs. 18.19 and 18.20). In short-term survival, Hsp27 expression was dominant, whereas Hsp70 expression increased with a longer survival time. In the control group, the majority of cases showed negative results (grade 0 expression of Hsp) in the renal tubules, and Hsp27 and Hsp70 expression decreased with the survival time (Fig. 18.21).

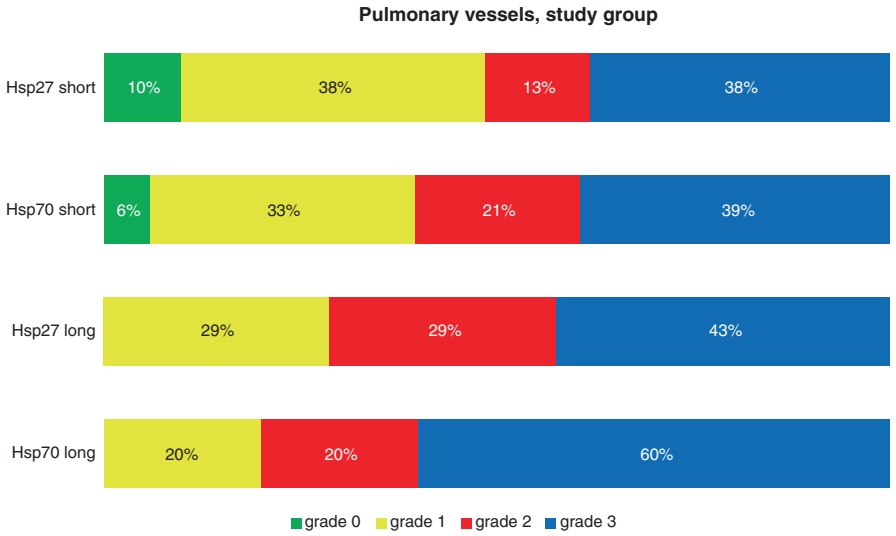


**Fig. 18.15** Percentual expression rate of Hsp27 and 70 in pulmonary vessels. Study group

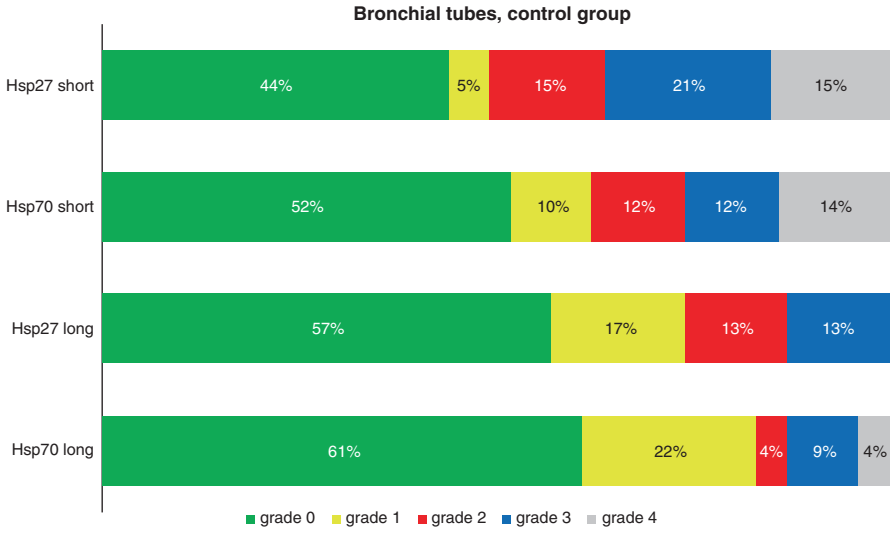


**Fig. 18.16** Percentual expression rate of Hsp27 and 70 in bronchial tubes. Control group

In renal vessels, Hsp27 was dominant in short- and long-term survival, and most cases were grades 2 and 3 (Fig. 18.22). Myocardial tissue in the study group showed completely different results (Figs. 18.23 and 18.24) between lung and kidney tissue. In short- and long-term survival, Hsp27 expression was low. In short-term survival,

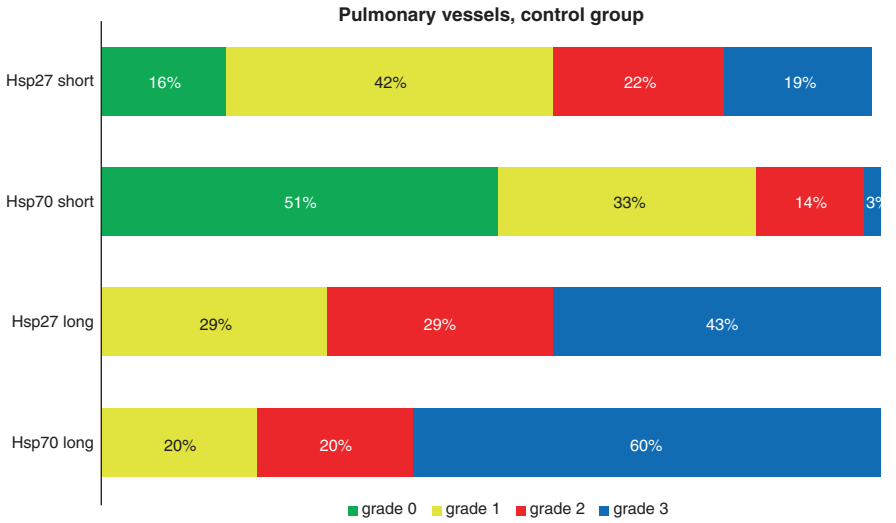


**Fig. 18.17** Percentual expression rate of Hsp27 and 70 in pulmonary vessels. Control group

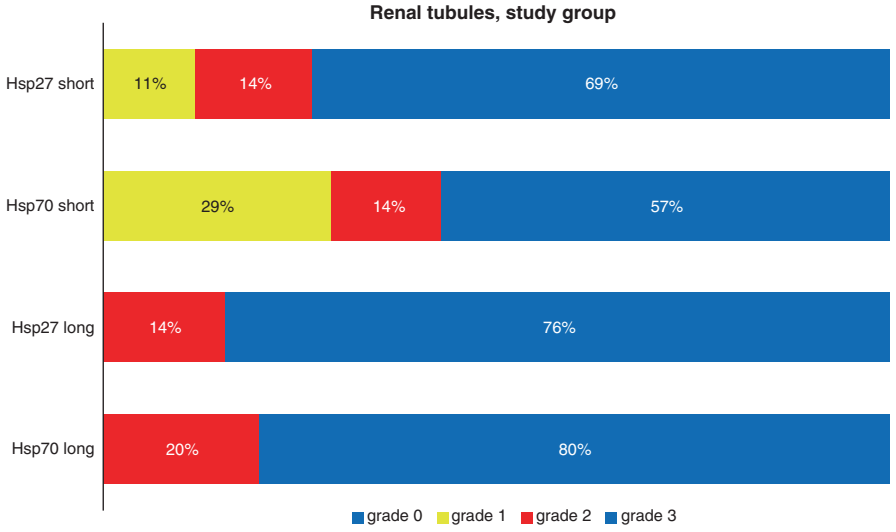


**Fig. 18.18** Percentual expression rate of Hsp27 and 70 in renal tubules. Study group

70% of cases were not stained with Hsp70 antibodies and even 40% of the long-term survivor cases were negative. In the control group, the majority of cases did not show Hsp expression in the myocardial vessels or even in myocytes (Figs. 18.25 and 18.26). Hsp27 expression showed a higher grading of immunohistochemical reaction.

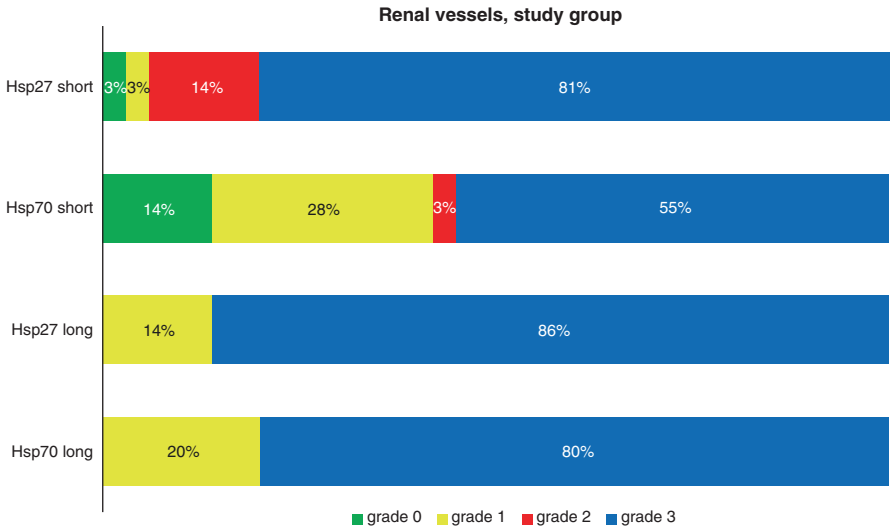


**Fig. 18.19** Percentual expression rate of Hsp27 and 70 in renal vessels. Study group

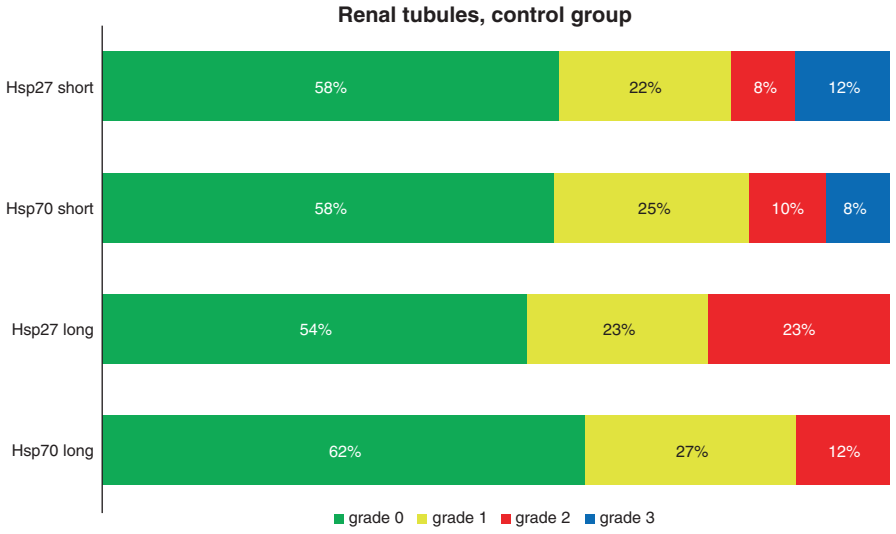


**Fig. 18.20** Percentual expression rate of Hsp27 and 70 in renal tubules. Control group

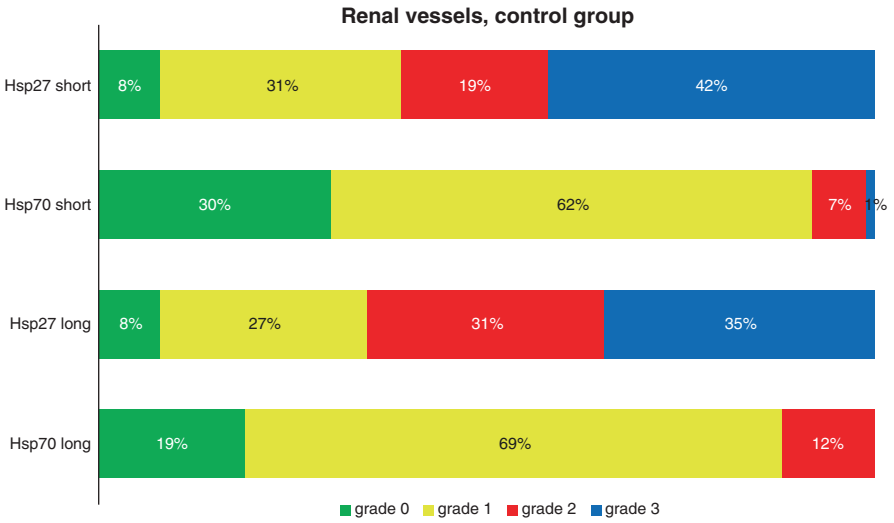


**Fig. 18.21** Percentual expression rate of Hsp27 and 70 in renal vessels. Control group

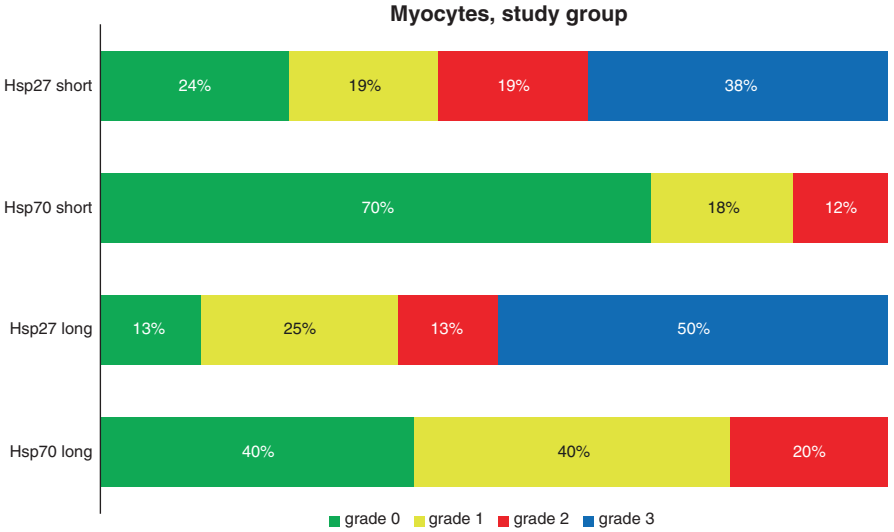


**Fig. 18.22** Percentual expression rate of Hsp27 and 70 in myocytes. Study group

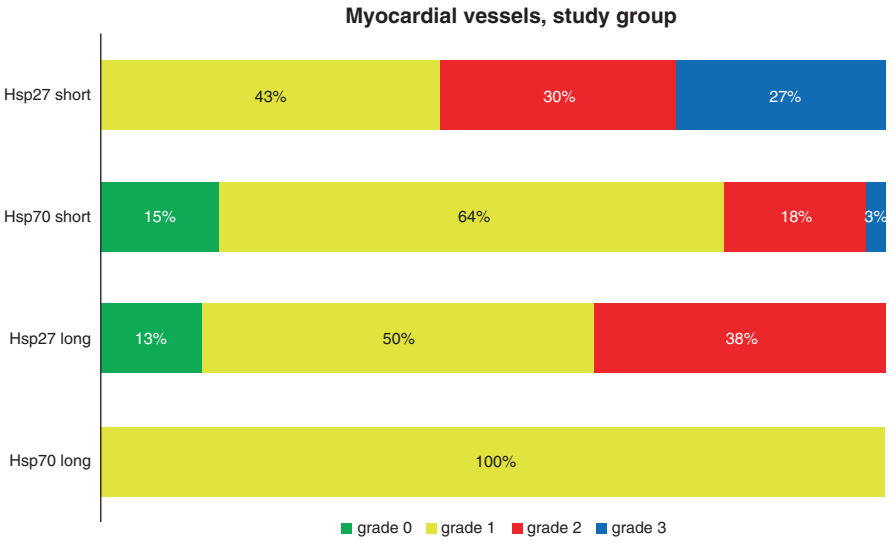


**Fig. 18.23** Percentual expression rate of Hsp27 and 70 in myocardial vessels. Study group

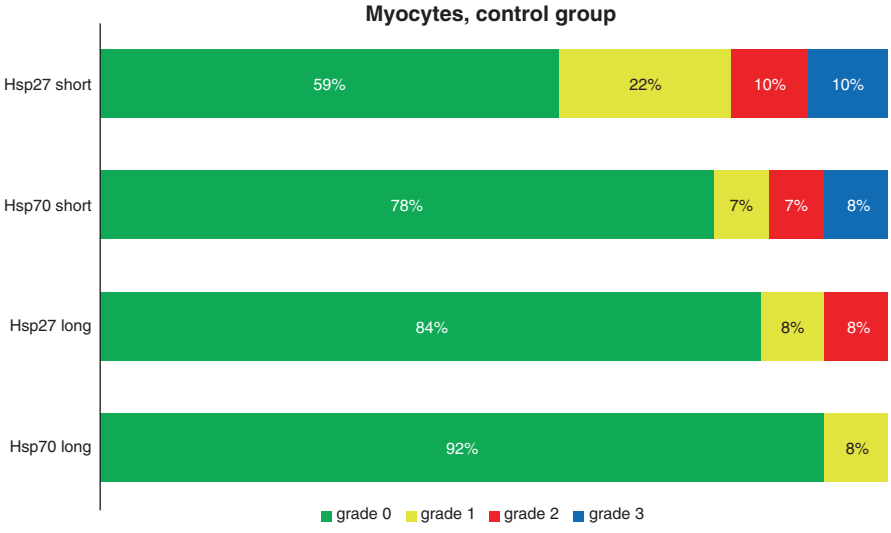


**Fig. 18.24** Percentual expression rate of Hsp27 and 70 in myocytes. Control group

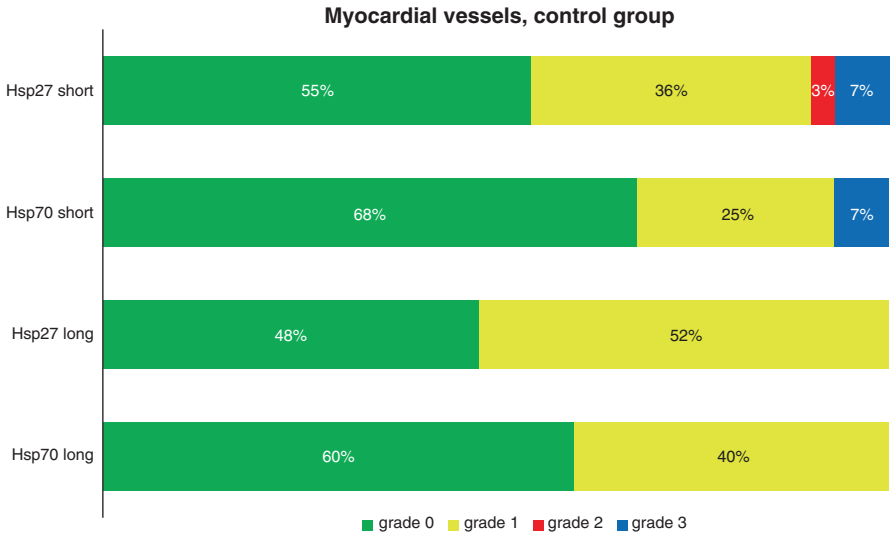


**Fig. 18.25** Percentual expression rate of Hsp27 and 70 in myocardial vessels. Control group

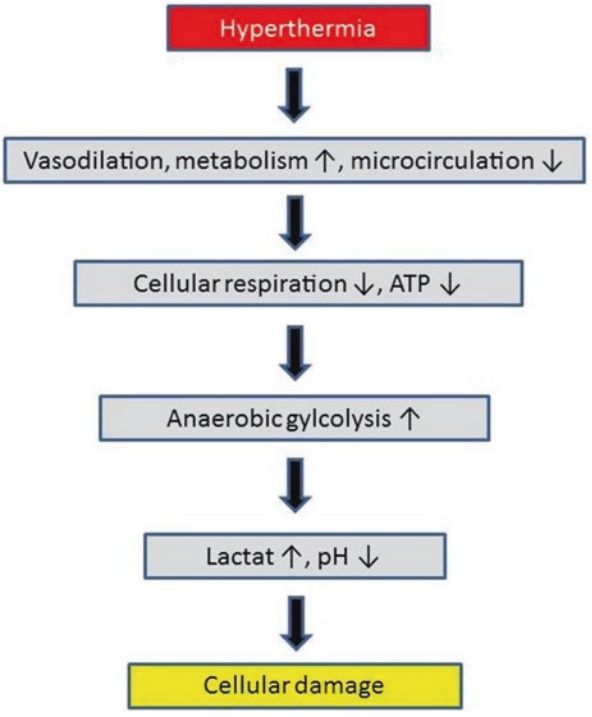


**Fig. 18.26** Reaction in tissue due to hypothermia

### 18.3.2 Pituitary Glands in Cases of Fatal Hypothermia

Hsp27 expression was detected in 27.3% of cases of fatal hypothermia and in 10% of control cases, whereas Hsp70 expression was not detected in any case. Additionally, Sudan staining was performed to quantify fatty degeneration. Positive Sudan stainging was found in 45.5% of the study group and in 10% of the control group. This finding indicates that fatty degeneration might be a useful marker when other macroscopic signs of hypothermia are absent.

### 18.3.3 SIDS Cases

Tables 18.6 and 18.7 show immunohistochemical analysis results for Hsp27 and Hsp70 in the cellular structures of each organ. In all 120 cases of SIDS, H&E-stained sections showed no remarkable abnormal structures in tissues. H&E-stained sections also showed no abnormal structures in tissues in all 29 control cases. Hsp70 expression was not found in any case (Table 18.7). Hsp27 expression was detected in both groups. In myocardial tissue, Hsp27 expression was not found in any SIDS cases. In one case in the control group, myocardial vessels showed Hsp27 expression of grade 2. In pulmonary and renal tissues, some cases with positive expression were found in both groups. In 27% and 19% of SIDS cases, lung respiratory

**Table 18.6** Results for Hsp27 staining

| | Study group ($n$ = 120) | Control group ($n$ = 29) |
|---|---|---|
| Heart | Hsp-negative | 4% of endothelial cells were positively stained |
| Kidneys | 1% of tubular cells were positively stained | 8% of tubular cells were positively stained<br>4% of vascular endothelial cells were positively stained |
| Lungs | 27% of respiratory epithelial cells were positively stained<br>19% of vascular endothelial cells were positively stained | 8% of respiratory epithelial cells were positively stained<br>29% of vascular endothelial cells were positively stained |

**Table 18.7** Results for Hsp70 staining

| | Study group ($n$ = 120) | Control group ($n$ = 29) |
|---|---|---|
| Heart | Negative | Negative |
| Kidneys | Negative | Negative |
| Lungs | Negative | Negative |

epithelial cells and vascular endothelial cells were positively stained for Hsp27, respectively (Table 18.6). In the SIDS group in 1% of the cases, kidney tubular cells were positively stained for Hsp27. In 8% and 29% of control cases, lung respiratory epithelial cells and vascular endothelial cells were positively stained for Hsp27, respectively (Table 18.6). In 8% and 4% of control cases, kidney tubular cells and vascular endothelial cells were positively stained for Hsp27, respectively.

### *18.3.4 Amphetamine-associated Deaths*

Among 19 cases, five had positive Hsp27 staining in cardiac tissue. Hsp60 staining was not found in any cases. In pulmonary tissue, positive Hsp27 and 70 expression was not detected. In one case, positive Hsp27 expression of grade 1 in renal tissue was found.

## 18.4 Discussion

Hsp are always present, even in the absence of stress, in cells of the body in low or very low concentrations, especially in the cytoplasm (Akerfelt et al. 2010, Brinkmeier and Ohlendieck 2014, Feder and Hofmann 1999, Hartl 1996, Jäättelä 1999, Kampinga et al. 2009, Multhoff et al. 1995, Wu et al. 1993). Under normal physiological conditions, Hsp bind to heat shock factor (hsf), which is a transcriptional regulator of Hsp, leading to elevated synthesis of Hsp (Inouye et al. 2003). The most important hsf is hsf-1, which regulates the response to different types of stress. In case of stressful conditions, the number of damaged or misfolded proteins increases (Doberentz et al. 2017a, b, c; Doberentz et al. 2014; Inouye et al. 2003; Walter and Buchner 2002). Hsp binds to these misfolded proteins and dissociates from hsf. The rising level of free hsf induces expression of new Hsp (Akerfelt et al. 2010; Brinkmeier and Ohlendieck 2014; Inouye et al. 2003; Wang et al. 2014; Welch and Feramisco 1984). In this manner, when heat shock occurs, transcription of heat shock genes increases (Akerfelt et al. 2010, Brinkmeier and Ohlendieck 2014, Inouye et al. 2003, Wang et al. 2014, Welch and Feramisco 1984). Synthesis of Hsp immediately increases within several minutes after stress influence (Beissinger and Buchner 1998; Doberentz et al. 2017a; b; c; Doberentz et al. 2014; Khan et al. 1996; Wu et al. 1993). Hsp are expressed in a large number in damaged cellular structures to guarantee protection of the cell. The rate of Hsp expression correlates with the severity of heat stress. The increasing amount of these special proteins becomes visible with immunohistochemical staining of Hsp.

In hyperthermic stress (Fig. 27), when tissue is heated, reflexive dilatation of blood vessels is induced. Hyperemia is followed by an increase in metabolism. In case of persistent hyperemic effects or a further increase in temperature, failure of the microcirculation will occur (e.g., with formation of emboli). Consequently,

hypoxemia occurs with an increase in oxidative cellular breathing. In this manner, production of ATP is reduced. Anaerobic glycolysis occurs and lactate concentrations in tissue increase. The pH level decreases with damage of cellular structures (Cavaliere et al. 1967; Dewey et al. 1971). Temperature levels above 42 °C or long-lasting hyperthermia have direct cytotoxic effects and lead to protein denaturation (Cavaliere et al. 1967; Kennedy et al. 2014).

In case of hypothermic stress, vasoconstriction and an increase in metabolism due to shivering, with an increase in oxygen demand and later hypotonia and bradycardia, result in hypoxia in tissues. Additionally, pH is decreased with damage of cellular structures (Kirsch and Gunga 2004; Madea et al. 2004; Turk 2010; Xu et al. 2005). In this process, both opposing thermal influences lead to increased oxygen demand with disturbance of acid balance, a decrease in pH, and protein degradation.

Human tissue shows variations in Hsp because of different localization in human tissue, as observed in the present study. In our study, different expression patterns of Hsp27 and Hsp70 were found with a predominance of rapid Hsp27 expression in cases of short-term survival. We also observed elevation of Hsp, especially Hsp70 expression in cases of long-term survival in pulmonary and renal tissues. These expression patterns are based on different functions of the Hsp families and Hsp themselves (Table 18.1), and probably on dependence of energy. The anti-apoptotic Hsp27 and Hsp70 belong to the most studied Hsp (Clerico et al. 2015; Wang et al. 2014). The small Hsp27 (molecular weight is between 25 and 30 kDa) is expressed in all human tissues, but mainly in skeletal, smooth, and cardiac muscle cells, and functions independently of metabolic energy sources (Akerfelt et al. 2010; Beissinger and Buchner 1998; Brinkmeier and Ohlendieck 2014; Hartl 1996; Jäättelä 1999; Kampinga and Garrido 2012; Kampinga et al. 2009; Sugiyama et al. 2000). Hsp27 is always present at a certain expression level. Hsp27 has the function of binding unfolded or misfolded proteins to prevent aggregation and pass to Hsp70, which is mainly responsible for refolding. Hsp27 is required because Hsp are essentially involved in stabilizing the cytoskeleton.

The Hsp70 family comprises 13 members and their function depends on their cellular localization. Hsp70 (Hsp72 or HspA1) belongs to the four major proteins of the family. The Hsp70 family plays a major role in the responses to cellular stress and thermal stress (Jäättelä 1999; Matz et al. 1996). Hsp70 activity (molecular weight of 70 kDa) is dependent on co-chaperones and different factors (J-domain co-chaperones Hsp40, nucleotide exchange factor, and TPR domain co-chaperones), which regulate ATPase activity, substrate binding, and hydrolysis (Akerfelt et al. 2010, Beissinger and Buchner 1998, Clerico et al. 2015, Hasday and Singh 2000, Inouye et al. 2003, Khan et al. 1996, Pakanen et al. 2011, Radons 2016, Wang 2013). ATP is necessary for Hsp70 to bind to degraded proteins and to stabilize them. Hsp70 is also required to release proteins. Two main states of Hsp70 activity are present. There is low affinity for peptides when ADP is bound and high affinity for peptides when ATP is bound (Khan et al. 1996). In this, manner, ATP is required in cellular stress. When a heat shock response begins, Hsp70 levels increase, but cellular ATP levels decrease because of higher consumption what restricts the

function of Hsp70. Furthermore, Hsp70 is usually not expressed in the absence of stressful conditions (Maloyan et al. 2005; Maloyan et al. 1999; Nollen et al. 1999) or has only low cellular levels and acts as a molecular chaperone. However, in case of cellular stress, Hsp70 is rapidly expressed within 1 h. This expressed level can persist for several days.

Hsp72 is particularly responsive to heat stress and exercise. Several authors have observed upregulation of extracellular Hsp72 (eHsp72) expression after prolonged exercise. A strong relationship between eHsp72 and final core temperature has been shown. Measurements taken 24 h postexercise underscore the transient nature of this increase with a return in eHsp72 to basal levels (Périard et al. 2012). Hsp27, a small member of the Hsp family, also rapidly increases in healthy human subjects following maximal cycling exercises. Expression of eHsp72 and eHsp27 is upregulated under physiological stress. Extracellular eHsp72 expression during exercise–heat stress might increase with the level of hyperthermia attained. Périard et al. 2012 investigated the effect of exercise of various intensities to determine this relationship and investigated the association between eHsp72 and eHsp27. They observed a significant increase and correlation between eHsp72 and eHsp27 concentrations at exhaustion. Expression of eHsp72 was highly correlated with the core temperature attained and the rate of increase in core temperature. The similarity in expression of eHsp72 and eHsp27 using moderate- and high-intensity exercise may relate to the duration (i.e., core temperature attained) and intensity (i.e., rate of increase in core temperature) of exercise.

When the core body temperature drops, the hypothalamus, as the center of thermoregulation, activates the adenohypophysis (Romanowsky 2007). Adrenocorticotropic hormone and thyroid-stimulating hormone are initially released and act as stimuli for the metabolic production of heat. While the body is cooling down later, hormone production decreases (Ishikawa et al. 2008a; b). This is part of a self-regulatory mechanism in homoeothermic organisms to maintain a constant body temperature. The adenohypophysis is immediately activated in cases of hypothermia-induced systemic stress.

Increased Hsp expression in renal tissue following hypothermia was investigated by Preuss et al. 2008. They found positive Hsp70 expression in 89% of the renal tubular epithelium in the study group compared with 34% in the control group. Furthermore, they found positive Hsp expression in 80% of glomeruli in the study group compared with 16% in the control group. Kita 2000, evaluated Hsp70 expression in the cerebral cortex in cases of fatal cold exposure. Other studies have also reported cerebral Hsp expression (Kita 2000). In the present study, no Hsp70 expression was detected in tissue of the anterior pituitary. Hsp27 expression alone was found in 27.3% of cases in the study group and in 10.0% of cases in the control group in the anterior pituitary.

The most investigated risk factor of SIDS is probably the prone sleeping position. This sleeping position increases the risk of hypercapnia, hypoxia, and hyperthermia (Blair et al. 2008, Fleming et al. 1992, Mitchell and Krous 2015, Nelson et al. 1989, Rhode et al. 2013, Sawczenko and Fleming 1996). However, in addition to the inevitable and continual discussion about the consequent obstruction of

respiration and cerebral blood flow, a prone sleeping position per se can lead to a disturbance in regulation of physiological temperature. Because of limited mobility in the prone sleeping position and a relatively large contact area of the head and body with the contact surface, heat emission can be impeded and heat accumulation can result. This requires a physical mechanism to maintain the core body temperature at a constant level (Blair et al. 2008; Franco et al. 2000; Nelson et al. 1989; Ponsonby et al. 1992; Rhode et al. 2013). In case of an additional adverse factor (e.g. warm covering of an infant), decompensation of thermoregulation with an increase in core body temperature may result. This hyperthermic stress can lead to death in apnea-induced hypoxia. The predominantly negative results of the present study, without evidence of increased Hsp expression in SIDS cases, lead to the question of whether hyperthermia is a risk factor or whether hyperthermia possibly even affects death. However, an increase in core body temperature is not associated with objectifiable Hsp expression.

Previous studies have shown that the core body temperature in infants who were diagnosed with SIDS after autopsy should have been 38–40 °C (Kleemann et. al., 1993, Stanton 1984, Sunderland and Emery 1981). Indeed, Hsp expression is dependent on the level of the core temperature or the temperatures to which cells are directly exposed. The physiological core body temperature in humans is 37 °C and can vary by up to 1 °C. At 37 °C, there is physiologically low Hsp expression in all cells, and this can vary in level between the different Hsp families.

In case of fever accompanying infections, the balance between heat generation and elimination is disturbed. The heat shock response is induced with expression of Hsp with an increase in core temperature of higher than 4 °C above the physiological temperature level (Feder and Hofmann 1999; Hasday and Singh 2000). Nevertheless, some tissues are more sensitive than others and may even react to lower core temperatures (brain and kidney). In conclusion, fever can be determined by postmortem evidence of Hsp expression. However, above 40 °C, induction of Hsp expression occurs, whereby 41.8 °C to 42.5 °C should be the optimal stimulus for Hsp expression (Maloyan et al. 2005; Multhoff et al. 1995; Nollen et al. 1999). Becker 2004, also showed that at temperatures below 41.8 °C, Hsp are not greatly expressed in healthy cells. After heat greater than 41.8 °C, Hsp expression increases 20-fold. The present results and the circumstances of death in infants suggest that no Hsp expression is expected at body core temperatures between 38 °C and 40 °C because no cellular stress occurs.

Hsp27 was expressed in our study, which was expected because Hsp27 is always present at a certain level in all human tissues. Hsp70 is usually not expressed under stressful conditions. This could explain the negative results in the study and control groups for Hsp70 in our study. In conclusion, core temperatures below 40 °C or 41 °C do not induce a heat shock response. Finally, while healthy cells are able to be protected by Hsp and their repair mechanisms, heat above 42 °C leads to direct cytotoxic effects.

The causes of death varied considerably among amphetamine-related fatalities in our study (Tables 18.3 and 18.8). Mono-intoxication was rare, and the predominant causes of death were mixed intoxications and long-term use of amphetamines

**Table 18.8** Categorization of amphetamine cases

| Group | Cause of death | Drugs involved |
|---|---|---|
| A1 | Hemorrhage and presence of amphetamines | Amphetamines and no other relevant drugs |
| A2 | Amphetamine toxicity and heart disease | Amphetamines and no other relevant drugs |
| A3 | Amphetamine toxicity leading to serotonin syndrome | Amphetamines and other relevant serotonin-active drugs |
| A4 | Amphetamine toxicity | Amphetamines only |
| B | Drug overdose | Death caused predominantly by drugs other than amphetamines |
| C | Mechanical injury | Amphetamines and other drugs |

associated with heart disease. In line with the different causes of death, the agonal periods may also have differed considerably. A dramatic increase in sympathetic stimulation and development of malignant hyperthermia may have occurred in some, but not all, cases. Positive Hsp expression in some cases in the current study may support the hypothesis of Karch (Karch and Chih-Hsieng 1999; Karch and Drummer 2016) who postulated hyperthermia-induced Hsp expression increases myocardial resistance to infarction. The high levels of Hsp expression that was observed in cardiac tissue in some of the current cases were not observed in cases of death due to fire or hypothermia.

All of the current cases were negative for Hsp60, while five were positive for Hsp27 and one for Hsp70. The reasons for this expression pattern remain unclear. However, Hsp are located in various cellular compartments and have different functions. An analysis of Hsp in pulmonary tissue from fire-related fatalities indicated that Hsp27 was the predominant Hsp in short-term survivors compared with Hsp70 in long-term survivors. Further studies are required to determine the patterns of Hsp expression in amphetamine-related fatalities, especially in cases where amphetamines are the leading cause of death, and in cases for which details of the terminal period and body core temperature are available.

In deaths due to fire with ambient temperatures >700 °C and a rapid elevation in core body temperature, Hsp are rapidly expressed after the beginning of thermal stress (Doberentz et al. 2017a; b; c; Doberentz et al. 2014). In our study, in short-term survival, Hsp27 was expressed more rapidly and higher than Hsp70. Additionally, in death due to hypothermia and amphetamine-associated deaths, Hsp expression in cardiac tissue was observed, while in SIDS cases, almost no Hsp expression was observed.

Interestingly, in different thermal stress conditions, different organs are involved concerning Hsp expression. In death due to fire, pulmonary and renal tissues are the main organs with a positive reaction to stress, especially in bronchial tubes and pulmonary vessels. In hypothermia, renal tissue is the most prominently involved organ. High Hsp expression was also observed in renal tissue in death due to fire in our study (nearly 70% positive expression in the highest grade for short- and long-

term survivors). Interestingly, in short-term survivors, Hsp27 expression was higher, and in long-term survivors, Hsp70 expression was higher. Hsp expression and the grade of expression in cardiac tissue (myocytes and vessels) were lower compared with pulmonary and renal tissues.

There is a considerable difference in Hsp expression between myocardial, pulmonary, and renal tissues. In contrast to pulmonary and renal tissue, no higher expression of Hsp70 in long-term survivors could be detected. In amphetamine-associated deaths, only five of 19 cases showed Hsp expression in cardiac tissue. This does not support the hypothesis that amphetamine-induced hyperthermia increases with production of Hsp myocardial resistance to infarction. A further explanation for the low Hsp expression in cardiac tissue in amphetamine-related deaths may be that amphetamine-related elevation of the core body temperature was not as pronounced as expected, and did not reach a threshold value of 41.8 °C (Becker 2004; Horowitz 1998; Multhoff et al. 1995). Cardiac ischemia results in high Hsp production. However, to the best of our knowledge, systematic studies on Hsp expression in cardiac tissue in cases of myocardial infarction, coronary insufficiency, and coronary thrombosis are lacking. Future studies are required to investigate these conditions.

## 18.5 Conclusions

Heat-related fatalities with different expression patterns of Hsp27 and Hsp70 are dependent on the survival time. The highest Hsp expression is found in pulmonary tissue, and especially Hsp27 is rapidly expressed in a large amount. Hsp are rapidly expressed within seconds or minutes after stressful effects on cells. However, their expression pattern also depends on organ structure. In fire-related fatalities, expression of Hsp is low in cardiac tissue. Cardiac Hsp expression was found in five of the amphetamine-related fatalities in our study, and high expression was found in a case of fatal hypothermia in amphetamine intoxication. Relevant premortem hyperthermic effects with immunohistochemical evidence of Hsp27 and Hsp70 expression could not be achieved in SIDS cases. However, a lack of expression of Hsp27 and Hsp70 does not exclude slight premortem hyperthermia and its effect on death. A physiological increase in core body temperature, in accordance with the results of other studies, causes no relevant cellular stress associated with an increase in Hsp. However, hyperthermia is not likely to have a considerable effect on the death rate of infants in SIDS when Hsp expression is absent. In case of decompensation of the tolerance period, an increase in Hsp expression, which would be detectable, would be expected in the reverse conclusion.

## References

Akerfelt, M., Morimoto, R. I., & Siston, L. (2010). Heat shock factors: Integrators of cell stress, development and lifespan. *Nature Reviews. Molecular Cell Biology, 11*, 545–555.

Armenian, P., Mamantov, T. M., Tsutaoka, B. T., et al. (2013). Multiple MDMA (ecstasy) overdoses at a rave event: A case series. *Journal of Intensive Care Medicine, 28*, 252–258.

Baillif, R. N. (1938). Die Erfrierung-Untersuchungen über ihre pathologische Anatomie und Pathogenese. *The American Journal of Physiology 1938; cited in Staemmler M (*1944*).* Georg-Thieme Verlag Leipzig 62, 475.

Becker, V. T. (2004). Einfluss der Temperatur auf die Lungenfunktionsparameter im Rahmen der isolierten hyperthermen Lungenfunktion. Medizinische Dissertation, Universität Jena, 10.

Beissinger, M., & Buchner, J. (1998). How chaperones fold proteins. *Biological Chemistry, 379*, 245–259.

Benndorf, R., & Bielka, H. (1997). Cellular stress response: Stress proteins-physiology and implications for cancer. *Recent Results in Cancer Research, 143*, 129–144.

Blair, P. S., Mitchell, E. A., Heckstall-Smith, E. M., & Fleming, P. J. (2008). Head covering - a major modifiable risk factor for sudden infant death syndrome: A systematic review. *Archives of Disease in Childhood, 93*, 778–783.

Bora, F., Yılmaz, F., & Bora, T. (2016). Ecstasy (MDMA) and its effects on kidneys and their treatment: A review. *Iran Journal of Basic Medical Sciences, 19*, 1151–1158.

Brinkmeier, H., & Ohlendieck, K. (2014). Chaperoning heat shock proteins: Proteomic analysis and relevance for normal and dystrophin-deficient muscle. *Proteomics Klin Appl, 8*, 875–895.

Büchner, F. (1943). Die Pathologie der Unterkühlung. *Klinische Wochenschrift, 22*, 89–92.

Campbell, G. A., & Rosner, M. H. (2008). The agony of ecstas: MDMA (3,4 methylendioxymethamphetamine) and the kidney. *Clinical Journal of the American Society of Nephrology, 3*, 1852–1860.

Cavaliere, R., Ciocatto, E. C., Giovannella, B. C., et al. (1967). Selective heat sensitivity of cancer cells. Biochemical and clinical studies. *Cancer, 20*, 1351–1381.

Chandra, Y. G., Shetty, A. R., Jayanth, S. H., Hugar, B. S., Praveen, S., & Harish, S. (2016). A death due to ecstasy - a case report. *The Medico-Legal Journal, 84*, 46–48.

Clerico, E. M., Tilitsky, J. M., Meng, W., & Gierasch, L. M. (2015). How Hsp70 molecular machines interact with their substrates to mediate diverse physiological functions. *Journal of Molecular Biology, 427*, 1575–1588.

Deshaies, R. J., Koch, B. D., Werner-Washbourne, M., Craig, E. A., & Schekman, E. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature, 332*, 800–805.

Dewey, W. C., Westra, A., Miller, H. H., & Nagasawa, H. (1971). Heat induced lethality and chromosomal damage in synchronized chinese hamster cells treated with 5-bromodeoxyuridine. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine, 20*, 505–520.

Doberentz, E., & Madea, B. (2017). Microscopic examination of pituitary glands in cases of fatal accidental hypothermia. *Forensic Sciences Research*. published online 7 June 2017, https://doi.org/10.1080/20961790.2017.1330804

Doberentz, E., Preuss-Wössner, J., Kuchelmeister, K., & Madea, B. (2011). Histological exmaniation of pituitary glands in cases of fatal hypothermia. *Forensic Science International, 207*(1–3), 46–49.

Doberentz, E., Genneper, L., Böker, D., Lignitz, E., & Madea, B. (2014). Expression of heat shock proteins (hsp) 27 and 70 in various organ systems in cases of the death due to fire. *International Journal of Legal Medicine, 128*(6), 967–978.

Doberentz, E., Führing, S., & Madea, B. (2016). Sudden infant death syndrome: No significant expression of heat-shock proteins (HSP27, HSP70). *Forensic Science, Medicine, and Pathology, 12*, 3–9.

Doberentz, E., Markwerth, P., Wagner, R., & Madea, B. (2017a). Expression of hsp27 and 70 and vacuolization in pituitary glands in cases of fatal hypothermia. *Forensic Science, Medicine, and Pathology*., published online 19 June 2017. https://doi.org/10.1007/s12024-017-9884-3

Doberentz, E., Genneper, L., Wagner, R., & Madea, B. (2017b). Expression times for hsp27 and hsp70 as an indicator of thermal stress during death due to fire. *International Journal of Legal Medicine*. Published online Feb 2017. https://doi.org/10.1007/s00414-017-1566-x

Doberentz, E., Führing, S., & Madea, B. (2017c). Hsp27 and 70 expression in heart, lung and kidney in SIDS. *Rom. Journal of Legal Medicine, 24*, 247–252.

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology, 61*, 243–282.

Fleming, P. J., Gilbert, R. E., Azaz, Y., et al. (1990). The interaction between bedding and sleeping position in sudden infant death syndrome: A population-based case-control study. *British Medical Journal, 301*, 85–89.

Fleming, P. J., Azaz, Y., & Wigfield, R. (1992). Development of thermoregulation in infancy: Possible implications for SIDS. *Journal of Clinical Pathology, 45*, 17–19.

Franco, P., Szliwowski, H., Dramaix, M., & Kahn, A. (2000). Influence of ambient temperature on sleep characteristics and autonomic nervous control in healthy infants. *Sleep, 23*, 401–407.

García-Repetto, R., Moreno, E., Soriano, T., Jurado, C., Giménez, M. P., & Menéndez, M. (2003). Tissue concentrations of MDMA and its metabolite MDA in three fatal cases of overdose. *Forensic Science International, 135*, 110–114.

Gilbert, R., Rudd, P., Berry, P. J., et al. (1992). Combined effect of infection and heavy wrapping on the risk of sudden unexpected infant death. *Archives of Disease in Childhood, 67*, 171–177.

Greene, S. L., Dargan, P. I., O'Connor, N., Jones, A. L., & Kerins, M. (2003). Multiple toxicity from 3,4-methylenedioxymethamphetamine ("ecstasy"). *The American Journal of Emergency Medicine, 21*, 121–124.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature, 381*, 571–580.

Hasday, J. D., & Singh, I. S. (2000). Fever and the heat shock response: Distinct, partially overlapping processes. *Cell Stress & Chaperones, 5*, 471–480.

Henry, J. A., Jeffreys, K. J., & Dawling, S. (1992). Toxicity and deaths from 3,4-methylenedioxy methamphetamine ("ecstasy"). *Lancet, 340*, 384–387.

Hirano, N., & Shiino, M. (1994). The large vacuoles have appeared in the anterior lobe of the hamster hypophysis in a low temperature environment. *Acta Anat Nippon, 69*, 350.

Horowitz, M. (1998). Do cellular heat acclamatory responses modulate central thermoregulatory acticity? *News in Physiological Sciences, 13*, 218–225.

Inouye, S., Katsuki, K., Izu, H., et al. (2003). Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. *Molecular and Cellular Biology, 23*, 5882–5895.

Ishikawa, T., Miyaishi, S., Tachibana, T., Ishizu, H., Zhu, B. L., & Maeda, H. (2004). Fatal hypothermia related vacuolation of hormone-producing cells in the anterior pituitary. *Legal Medicine, 6*, 157–163.

Ishikawa, T., Michiue, T., Zaho, D., Komatsu, A., & Azuma, Y. (2008a). Evaluation of postmortem serum and cerebrospinal fluid levels of thyroid stimulating hormone with special regard to fatal hypothermia. *Legal Medicine (Tokyo), 11*, S228–S230.

Ishikawa, T., Quan, L., Li, D. R., et al. (2008b). Postmortem biochemestry and immunohistochemestry of adrenocorticotropic hormone with special regard to fatal hypothermia. *Forensic Science International, 179*, 147–151.

Jäättelä, M. (1999). Heat shock proteins as cellular lifeguards. *Annals of Medicine, 31*, 261–271.

Jeng, W., Lee, S., Sung, N., Lee, J., & Tsai, F. (2015). Molecular chaperones: Guardians of the proteome in normal and disease states. *F1000Res*. https://doi.org/10.12688/f1000research.7214.1

Jochum, M., Oeding, J., Lackner, D., & Lienhart, H. (2017). Ecstasy-Intoxikation mit disseminierter intravasaler Gerinnung und Multiorganversagen. *Anästhesiologie, Intensivmedizin, Notfallmedizin, Schmerztherapie, 52*, 145–151.

Kalant, H., & Le, A. D. (1983). Effects of ethanol on thermoregulation. *Pharmacology & Therapeutics, 23*, 313–364.

Kampinga, H. H., & Garrido, C. (2012). HSPBs: Small proteins with big implications in human disease. *The International Journal of Biochemistry & Cell Biology, 44*, 1706–1710.

Kampinga, H. H., Hageman, J., Vos, M. J., et al. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress & Chaperones, 14*, 105–111.

Karch, S. B., & Chih-Hsieng, H. (1999). Medamphetamine-related deaths in San Francisco: Demographic, pathologic, and toxicologic profiles. *Journal of Forensic Sciences, 44*, 359–368.

Karch, S. B., & Drummer, O. H. (2016). Synthetic stimulants. In S. B. Karch & O. H. Drummer (Eds.), *Karch's pathology of drug abuse* (5th ed., pp. 273–364). Boca Raton/London/New York: CRC Press.

Kennedy, D., Jager, R., Mosser, D. D., & Samali, A. (2014). Regulation of apoptosis by heat shock proteins. *IUBMB Life, 66*, 327–338.

Khan, W., Mc Guirt, J. P., Sens, M. A., Sens, D. A., & Todd, J. H. (1996). Expression of the heat shock protein 27 in developing and adult human kidney. *Toxicology Letters, 84*, 69–79.

Kirsch, K. A., & Gunga, H. C. (2004). Physiological aspects of accidental hypothermia. In M. Oehmichen (Ed.), *Hypothermia. Clinical, pathomorphological and forensic features* (pp. 43–54). Lübeck: Schmidt-Römhildt.

Kita, T. (2000). The role of heat shock proteins on the disordered tissues: Implication for the pathogenesis and diagnostics in the forensic practice. *Nihon Hoigaku Zasshi, 54*, 367–371.

Kleemann, W. J., Schlaud, M., Poets, C. F., Rothämel, T., & Tröger, H. D. (1996). Hyperthermia in sudden infant death. *International Journal of Legal Medicine, 109*, 139–142.

Kramme, R. (2006). *Medizintechnik* (3rd ed.p. 475). Berlin: Springer.

Krous, H. F., Beckwith, J. B., Byard, R. W., Rognum, T. O., Bajanowski, T., Corey, T., Cutz, E., Hanzlick, R., Keens, T. G., Mitchell, E. A. (2004). Sudden infant death syndrome and unclassified sudden infant deaths: A definitional and diagnostic approach. *Pediatrics, 114*, 234–238.

Lantry, J., Dezman, Z., & Hirshon, J. M. (2012). Pathophysiology, management and complications of hypothermia. *British Journal of Hospital Medicine (London, England), 73*, 31–37.

Larsen, R. (2009). *Anästhesie und Intensivmedizin in der Herz-, Thorax und Gefäßchirurgie* (7th ed.pp. 79–95). Heidelberg: Springer.

Macario, A. J. L., & Conway de Macario, E. (2005). Sick chaperones, cellular stress, and disease. *The New England Journal of Medicine, 353*, 1489–1501.

Madea, B., Preuß, J., & Lignitz, E. (2004). Unterkühlung: Umstände morphologische Befunde und ihre Pathogenese. *Rechtsmedizin, 14*, 41–57.

Madea, B., Wagner, R., Markwerth, P., & Doberentz, E. (2017). Heat shock protein expression in cardiac tissue in amphetamine-related deaths. *Romanian Journal of Legal Medicine, 25*, 8–13.

Maloyan, A., Palmon, A., & Horowitz, M. (1999). Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. *The American Journal of Physiology, 276*, R1506–R1515.

Maloyan, A., Eli-Berchoer, L., Semenza, G. L., Gerstenblith, G., Stern, M. D., & Horowitz, M. (2005). HIF-1alpha-targeted pathways are activated by heat acclimation and contribute to acclimation-ischemic cross-tolerance in the heart. *Physiological Genomics, 23*, 79–88.

Marber, M. S., Latchman, D. S., Walker, J. M., & Yellon, D. M. (1993). Cardiac stress protein elevation 24 hours after brief ischemia or heat stress in associated with resistance to myocardial infarction. *Circulation, 88*(3), 1264–1272.

Marschall, S. (2005). Expression von Hitzeschockprotein 70 (Hsp 70) in den Atemwegen und Lungen bei Brandtodesfällen. Dissertation Freiburg 11, 51–62.

Matz, J. M., Lavoi, K. P., & Blake, M. J. (1996). Adrenergic regulation of the heat shock response in brown adipose tissue. *The Journal of Pharmacology and Experimental Therapeutics, 277*, 1751–1758.

Milroy, C. M. (2011). "Ecstasy" associated deaths: What is a fatal concentration? Analysis of a case series. *Forensic Science, Medicine, and Pathology, 7*(3), 248–252.

Mitchell, E. A., & Krous, H. F. (2015). Sudden unexpected death in infancy: A historical perspective. *Journal of Paediatrics and Child Health, 51*, 108–112.

Mizia-Stec, K., Gąsior, Z., Wojnicz, R., et al. (2008). Severe dilated cardiomyopathy as a consequence of ecstasy intake. *Cardiovascular Pathology, 17*, 250–253.

Multhoff, G., Boltzer, C., Wiesnet, M., et al. (1995). A stess-inducible 72-kDa heat shock proetein (hsp72) is expressed on the surface of human tumor cells, bur not on normal cells. *International Journal of Cancer, 61*, 272–279.

Nelson, E. A. S., Taylor, B. J., & Weatherall, I. L. (1989). Sleeping position and infant bedding may predispose to hyperthermia and the sudden infant detah syndrome. *Lancet, 1*, 199–202.

Nollen, E. A., Brunsting, J. F., Roelofsen, H., Weber, L. A., & Kampinga, H. H. (1999). In vivo chaperone activity of heat shock protein 70 and thermotolerance. *Molecular and Cellular Biology, 19*, 2069–2079.

Pakanen, L., Kortelainen, M. L., Särkioja, T., & Porvari, K. (2011). Increasedadrenaline to noradrenaline ratio is a superior indicator of antemortemhypothermia compared with separate catecholamine concentrations. *Journal of Forensic Sciences, 56*, 1213–1218.

Périard, J. D., Ruell, P., Caillaud, C., & Thompson, M. W. (2012). Plasma Hsp72 (HSPA1A) and Hsp27 (HSPB1) expression under heat stress: Influence of exercise intensity. *Cell Stress and Chaperones, 17*, 375–383.

Pfeifer, K. (1980). Bedeutung der Rektaltemperaturmessung und der Umgebungsuntersuchungen beim plötzlichen Kindstod. *Deutsche Medizinische Wochenschrift, 105*, 1065.

Pilgrim, J. L., Gerostamoulos, D., Drummer, O. H., & Bollmann, M. (2008). Insolvent of amphetamines in sudden and unexpected death. *Journal of Forensic Sciences, 54*, 478–485.

Pomara, C., D'Errico, S., Zummo, L., Cappello, F., & Li Volti, G. (2010). MDMA administration and heat shock proteins response: Foreseeing a molecular link. *Current Pharmaceutical Biotechnology, 11*, 496–499.

Ponsonby, A. L., Dwyer, T., Gibbons, L. E., Cochrane, J. A., Jones, M. E., & Mc Call, M. J. (1992). Therm environment and sudden infant death syndrome: Case-control study. *BMJ, 304*, 277–282.

Preuss, J., Dettmeyer, R., Poster, S., Lignitz, E., & Madea, B. (2008). The expression of heat shock protein 70 in kidneys in cases of death due to hypothermia. *Forensic Science International, 176*, 236–247.

Radons, J. (2016). The human HSP70 family of chaperones: Where do we stand? *Cell Stress & Chaperones, 21*, 379–404.

Rhode, M. A., Corydon, T. J., Hansen, J., Pedersen, C. B., Schmidt, S. T., Gregersen, N., & Banner, J. (2013). Heat stress and sudden infant death syndrome-stress gene expression after exposure to moderate heat stress. *Forensic Science International, 232*, 16–24.

Ritossa, F. (1962). A new puffering pattern induced by heat shock and DNP in drosophila. *Experimentia, 8*, 571–573.

Romanowsky, A. A. (2007). Thermoregulation: Some concepts have changed. Functional architecture of the thermoregulatory system. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 292*, R37–R46.

Sawczenko, A., & Fleming, P. J. (1996). Therm stress, sleeping position, and the sudden infant death syndrome. *Sleep, 19*, S267–S270.

Stanton, A. N. (1984). Sudden infant death. Overheating and cot death. *Lancet, 2*, 1199–1201.

Sugiyama, Y., Suzuki, A., Kishikawa, M., et al. (2000). Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. *The Journal of Biological Chemistry, 275*, 1095–1104.

Sunderland, R., & Emery, J. (1981). Febrile convulsions and cot death. *Lancet, 318*, 176–178.

Turk, E. E. (2010). Hypothermia. *Forensic Science, Medicine, and Pathology, 6*, 106–115.

Vennemann, M. M., Bajanowski, T., Brinkmann, B., Jorch, G., Sauerland, C., & Mitchell, E. A. (2009). GeSID study group. Sleep environment risk factors for sudden infant death syndrome: The German sudden infant death syndrome study. *Pediatrics, 123*, 1162–1170.

Verdiccio, M., Rosen, T., Orndal, C. (2006). Hypothalamo-pituitary haemorrhages in hypothermia, poster presentation, 16th nordic conference on forensic medicine, 15–17 June 2006, Turku, Finland.

Walter, S., & Buchner, J. (2002). Molecular chaperones – Cellular machines for protein folding. *Angewandte Chemie (International Ed. in English), 41*, 1098–1113.

Wang, X. (2013). Hsp70 chaperone dynamics and molecular mechanism. *Trends in Biochemical Sciences, 38*, 507–514.

Wang, X., Chen, M., Zhou, J., & Zhang, X. (2014). HSP27, 70 and 90, anti-apoptotic proteins in clinical cancer therapy (review). *International Journal of Oncology, 45*, 18–30.

Welch, W. J., & Feramisco, J. R. (1984). Nuclear and nucleolar localizytion of the 72,000-dalton heat shock protein in heat-shocked mammalian cells. *The Journal of Biological Chemistry, 259*, 4501–4513.

Wilson, J., Sukyeong, L., Nuri, S., Jungsoon, L., & Francis, T. F. T. (2015). Molecular chaperones: Guardians of the proteome in normal and disease states. *F1000Res*. https://doi.org/10.12688/f1000research.7214.1

Wöllner, K., Mußhoff, F., Heß, C., & Madea, B. (2015a). Retrospektive Analyse von Todesfällen durch Amphetamin- und Ecstasy-Konsum. *Archiv für Kriminologie, 236*, 199–207.

Wöllner, K., Stockhausen, S., Musshoff, F., & Madea, B. (2015b). Todesfälle nach der Einnahme von Amphetamin/Ecstasy: zwei Fallvorstellungen. *Archiv für Kriminologie, 235*, 53–61.

Wu, B., Gu, M. J., Heydari, A. R., & Richardson, A. (1993). The effect of age on the synthesis of two heat shock proteins in the hsp70 family. *Journal of Gerontology, 48*, B50–B56.

Xu, X., Tikuisis, P., Gonzales, R., & Giesbrecht, G. (2005). Thermoregulatory model for prediction of long-term cold exposure. *Computers in Biology and Medicine, 35*, 287–298.

# Index





A. A. A. Asea, P. Kaur (eds.), *Regulation of Heat Shock Protein Responses*, Heat Shock Proteins 13, https://doi.org/10.1007/978-3-319-74715-6