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Stress biology: Complexity and multifariousness in health and disease

Matthias P. Mayer[1,*] · Laura Blair[2] · Gregory L. Blatch[3,4] · Thiago J. Borges[5] · Ahmed Chadli[6] · Gabriela Chiosis[7,8] · Aurélie de Thonel[9,10,11] · Albena Dinkova-Kostova[12] · Heath Ecroyd[13] · Adrienne L. Edkins[14] · Takanori Eguchi[15] · Monika Fleshner[16] · Kevin P. Foley[17] · Sotirios Fragkostefanakis[18] · Jason Gestwicki[19] · Pierre Goloubinoff[20] · Jennifer A. Heritz[21,22,23] · Christine M. Heske[24] · Jonathan D. Hibshman[25] · Jenny Joutsen[26] · Wei Li[27] · Michael Lynes[28] · Marc L. Mendillo[29,30] · Nahid Mivechi[31] · Fortunate Mokoena[32] · Yuka Okusha[33] · Veena Prahlad[34] · Elizabeth Repasky[35] · Sara Sannino[36] · Federica Scalia[37,38] · Reut Shalgi[39] · Lea Sistonen[40,41] · Emily Sontag[42] · Patricija van Oosten-Hawle[43] · Anniina Vihervaara[44] · Anushka Wickramaratne[45] · Shawn Xiang Yang Wang[46] · Tawanda Zininga[47]

Abstract

Preserving and regulating cellular homeostasis in the light of changing environmental conditions or developmental processes is of pivotal importance for single cellular and multicellular organisms alike. To counteract an imbalance

Abbreviations: **CHAMPs**, Chaperone-mediated protein degraders; **DAMPs**, danger/damage-associated molecular patterns; **EM**, epithelial-to-mesenchymal transition; **EMT**, C-reactive protein; **EMVs**, extracellular macrovesicles; **ER**, endoplasmic reticulum; **EVs**, extracellular vesicles; **ESCRT**, endosomal sorting complexes required for transport; **Hsf**, heat shock transcription factor; **Hsp**, heat shock protein; **HSR**, heat shock response; **JDPs**, J-domain proteins (Hsp70 cochaperones); **MT**, metallothionein; **NCP**, neurochaperonopathy; **RSTS**, Rubinstein-Taybi syndrome; **TME**, tumor microenvironment

* Matthias P. Mayer
m.mayer@zmbh.uni-heidelberg.de

1 Center for Molecular Biology of Heidelberg University (ZMBH), DKFZ-ZMBH Alliance, Heidelberg, Germany

2 Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA

3 Biomedical Research and Drug Discovery Research Group, Faculty of Health Sciences, Higher Colleges of Technology, Sharjah, United Arab Emirates

4 Biomedical Biotechnology Research Unit, Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa

5 Department of Surgery, Center for Transplantation Sciences, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, USA

6 Georgia Cancer Center, Medical College of Georgia at Augusta University, Augusta, GA 30912, USA

7 Department of Medicine, Division of Solid Tumors, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

8 Chemical Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

9 CNRS, UMR 7216, 75250 Paris Cedex 13, Paris, France

10 Univeristy of Paris Diderot, Sorbonne Paris Cité, Paris, France

11 Département Hospitalo-Universitaire DHU PROTECT, Paris, France

12 Division of Cellular and Systems Medicine, Jacqui Wood Cancer Centre, School of Medicine, University of Dundee, Dundee, UK

13 Molecular Horizons and School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, New South Wales 2522, Australia

14 Biomedical Biotechnology Research Unit (BioBRU), Department of Biochemistry and Microbiology, Rhodes University, Makhanda, South Africa

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in cellular homeostasis transcriptional programs evolved, called the heat shock response, unfolded protein response, and integrated stress response, that act cell-autonomously in most cells but in multicellular organisms are subjected to cell-nonautonomous regulation. These transcriptional programs downregulate the expression of most genes but increase the expression of heat shock genes, including genes encoding molecular chaperones and proteases, proteins involved in the repair of stress-induced damage to macromolecules and cellular structures. Sixty-one years after the discovery of the heat shock response by Ferruccio Ritossa, many aspects of stress biology are still enigmatic. Recent progress in the understanding of stress responses and molecular chaperones was reported at the 12th International Symposium on Heat Shock Proteins in Biology, Medicine and the Environment in the Old Town Alexandria, VA, USA from 28th to 31st of October 2023.

Keywords Stress response · Heat shock response · Heat shock transcription factors · Heat shock proteins · Molecular chaperones · Protein folding diseases

Chaperone gene transcription and heat shock factors

In his seminal study on the effect of a temperature upshift on the polytene chromosomes in the salivary glands of *Drosophila busckii*, Ritossa noticed that some puffs disappeared and others appeared in response to the heat shock, indicating that transcription of some genes is upregulated whereas the transcription of other genes is repressed.[1] In fact, pro-seq data by Lis and Sistonen on mouse embryonic fibroblasts and human erythroleukemia K562 cells indicate that transcription of 700 to 1200 genes is upregulated on heat shock, but transcription of 6000 to 9000 genes is repressed.[2,3] An important fraction of the upregulated genes (29–48%) and some downregulated genes (2–8%) depend on the heat shock transcription factor Hsf1. In his opening key note lecture, *Matthias P. Mayer* (Heidelberg University,

[15] Department of Dental Pharmacology, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700–8525, Japan

[16] Department of Integrative Physiology, University of Colorado at Boulder, Boulder, CO 80309, USA

[17] Ranok Therapeutics, Waltham, MA 02451, USA

[18] Department of Biosciences, Molecular Cell Biology of Plants, Goethe University Frankfurt am Main, Frankfurt am Main 60438, Germany

[19] Institute for Neurodegenerative Diseases, University of California, San Francisco, CA 94158, USA

[20] Department of Plant Molecular Biology, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland

[21] Department of Urology, SUNY Upstate Medical University, Syracuse, NY 13210, USA

[22] Upstate Cancer Center, SUNY Upstate Medical University, Syracuse, NY 13210, USA

[23] Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY 13210, USA

[24] Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[25] Biology Department, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[26] Department of Pathology, Lapland Central Hospital, Lapland Wellbeing Services County, Rovaniemi, Finland

[27] Department of Dermatology and the Norris Comprehensive Cancer Center, University of Southern California Keck Medical Center, Los Angeles, CA 90033, USA

[28] Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

[29] Department of Biochemistry and Molecular Genetics, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA

[30] Simpson Querrey Center for Epigenetics, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA

[31] Molecular Chaperone Biology, Medical College of Georgia, Georgia Cancer Center, Augusta University, Augusta, GA 30912, USA

[32] Department of Biochemistry, North-West University, Mmabatho 2735, South Africa

[33] Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

[34] Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14203, USA

[35] Department of Hematology and Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, USA

[36] Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA

Germany) focused on the work of his lab on the molecular mechanism of the human heat shock transcription factor Hsf1. Studying the conformational dynamics of Hsf1 with hydrogen exchange mass spectrometry, they found that a previously identified heptad repeat region in Hsf1, which is important for keeping Hsf1 in a monomeric state,[4,5] unfolds in response to increasing temperatures, allowing heptad repeat region in Hsf1 trimerization, forming a triple leucin-zipper.[6,7] As this unfolding was proportional to the temperature and time of exposure, human Hsf1 qualifies as thermosensor, consistent with earlier findings by C. Wu and colleagues for *Drosophila* Hsf1.[8] Interestingly, HR-C unfolding and Hsf1 trimerization were also dependent on the concentration of Hsf1, suggesting that regulation of the concentration of Hsf1, for example, by increased expression of the HSF1 gene (in response to oxidative stress[9]) or by nuclear-cytoplasmic transport,[10] could adjust the set point of transition to its trimeric, active DNA-binding state. Hsf1 is dissociated from DNA by Hsc70-mediated monomerization of Hsf1 trimers,[11] consistent with an earlier publication.[12] Hsc70-mediated monomerization of Hsf1 depends on the J-domain cochaperone DNAJB1 and ATP and bears all hallmarks of a normal Hsp70 chaperone-client interaction. Hsf1 monomerization by Hsc70 is consistent with the entropic pulling concept[13] as moving the Hsc70 binding site away from the trimerization domain by just 10 to 20 residues abrogates monomerization and as binding of multiple Hsc70s to the binding sites in the Hsf1 trimer, creating local crowding, accelerates monomerization. In unpublished work, the Mayer lab shows that all cytosolic Hsp70s are able to monomerize Hsf1, albeit with different efficiency, and a small subset of cytosolic J-domain proteins assist in this process. How this network of Hsp70s and J-domain proteins integrates the information about the homeostasis of the cellular proteome will be the focus of future research.

Not only through a temporary heat shock, the homeostasis of the cellular proteome is constantly challenged by a great variety of extrinsic and intrinsic stressors, which triggers an extensive reprogramming of transcription.[14] The rewiring of gene and enhancer networks is driven by stress-regulated transcription factors, of which heat shock factors (HSFs) have been widely studied in response to proteotoxicity. However, the comprehensive repertoire of their targets is still poorly characterized. A recent study from the laboratory of *Lea Sistonen* (Åbo Akademi University, Finland) systematically investigated how two members of the mammalian HSF family, that is, Hsf1 and Hsf2 regulate transcription in cells exposed to either acute heat shock or oxidative stress. Although some targets were shared by these stress conditions, clearly distinct stress-specific transcription programs, involving also enhancers were identified that were dependent on either Hsf1 or Hsf2.[15] Intriguingly, the stress-specific binding of these HSFs was not due to differences in target DNA sequences, since both factors bind to canonical cis-acting elements. It remains to be demonstrated whether recruitment of stress-inducible co-factors and post-translational modifications allows the HSFs to access their specific targets upon different stress stimuli.

Transcription of a eukaryotic protein-encoding gene involves progression through a series of steps: (1) opening of the chromatin by chromatin remodeling complexes; (2) recruitment of the pre-initiation complex; (3) transcription initiation; (4) promoter-proximal pausing of RNA polymerase II; (5) release of RNA polymerase II from the promoter-proximal pause site; (6) productive elongation; (7) co-transcriptional pre-mRNA processing; (8) pre-mRNA cleavage and polyadenylation; (9) termination of transcription; and (10)

[37] Department of Biomedicine, Neuroscience and Advanced Diagnostics (BIND), University of Palermo, Palermo, Italy

[38] Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy

[39] Department of Biochemistry, Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa 31096, Israel

[40] Faculty of Science and Engineering, Cell Biology, Åbo Akademi University, Turku, Finland

[41] Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland

[42] Department of Biological Sciences, Marquette University, Milwaukee, WI 53233, USA

[43] Department of Biological Sciences, University of North Carolina at Charlotte, Charlotte, NC 28223, USA

[44] Department of Gene Technology, KTH Royal Institute of Technology, Science for Life Laboratory, Stockholm, Sweden

[45] Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[46] Developmental Therapeutics Program, VCU Comprehensive Massey Cancer Center, VCU Institute of Molecular Medicine, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298, USA

[47] Department of Biochemistry, Stellenbosch University, Stellenbosch 7602, South Africa.

recycling of RNA polymerase II. For successful progression through these steps, phosphorylation of serines in the C-terminal heptad repeat domain of the RBP1 subunit of RNA polymerase II is necessary. For heat shock genes, it is known that RNA polymerase II is stalled at the promoter-proximal pause site, ready to transit into the elongation phase of transcription and that Hsf1 triggers this release.[16–18] However, mechanistic details are still missing. For *C. elegans*, it was shown that histones H3 becomes methylated in the promoter region of heat shock genes within the first 12 h of adulthood, preventing subsequent initiation of the heat shock response (HSR),[19] emphasizing the importance of chromatin in the regulation of heat shock gene expression.

Anniina Vihervaara (KTH Royal Institute of Technology, Sweden) outlined strategies to track transcription and chromatin architecture and introduced a novel technique that reports modifications of engaged RNA polymerase II complexes at nucleotide resolution.[20] These techniques are currently uncovering how chromatin primes and times chaperone production upon acute heat shock (Rabenius and Vihervaara, in preparation).

Several studies have demonstrated that HSFs contribute to a variety of human pathologies, ranging from metabolic diseases to cancer and neurodegeneration.[21] *Jenny Joutsen* (Lapland Central Hospital, Finland) reported a comprehensive analysis of a selection of paraffin-embedded human tissue samples with immunohistochemistry, demonstrating a consistent and strong cytoplasmic localization of Hsf2 across all studied smooth muscle cells, including the vascular smooth muscle cells. Outstandingly, they found that Hsf2 localizes specifically at cell–cell adhesion sites in a broad selection of tissue types, including the cardiac muscle, liver, and epididymis. Previously, Joutsen and colleagues[22] had shown that, during proteotoxic stress, Hsf2 is essential for cell survival by maintaining cell–cell adhesion through triggering the expression of cadherin genes. The sequestration of Hsf2 to cell-cell adhesion sites could be a negative feedback loop adjusting the expression of cadherin genes to the demand and a mechanism for sensing stress-induced problems in these sites.

Neurodevelopmental disorders cause lifelong cognitive and behavioral challenges. Among the dysregulated biological pathways, *Aurélie de Thonel* (CNRS, Paris, France) focuses on stress response pathways as heat shock transcription factors (HSFs) respond to various stresses, manage proteostasis, and influence brain development. Using Rubinstein-Taybi syndrome (RSTS) as a model for neurodevelopmental disorder, the research of the de Thonel lab showcased the dysregulation of the CBP/EP300-Hsf2-N-cadherin cascade in neural models from RSTS patients-derived induced pluripotent stem cells. These models exhibit features linked to disrupted apical cohesion, indicating N-cadherin deficiency. Understanding the contribution of the Hsf2 pathway in RSTS could provide insights into the intricate molecular aspects of this complex pathology.

Hsf2 also plays an important role during tumorigenesis. Its precise function, however, remains elusive. *Nahid Mivechi* (Augusta University of Georgia, USA) reported that loss of Hsf2 leads to accelerated proliferation of T cell lymphoma in a mouse model. Her team found that Hsf2 loss is associated with a delay in DNA repair in hematopoietic stem cells.

As an important stem cell transcription factor, Oct4 levels must be tightly controlled. *Adrienne Edkins* (Rhodes University, South Africa) reported heat shock and recovery experiments that demonstrated an Hsf1-mediated increase in Oct4 mRNA post-stress. In addition, her team observed rapid shuttling of Oct4 protein between insoluble and soluble fractions and accumulation in nuclear puncta. These different mechanisms may function together to allow careful regulation of Oct4 levels during stress.

Toward inhibitors of heat shock proteins as therapeutics

One of the first molecular chaperones that came into the focus of drug discovery programs was the ATP-dependent dimeric Hsp90, as the first Hsp90 inhibitors were discovered by chance in a natural product screen for compounds that revert the Rous sarcoma virus-induced transformation to normal morphology, that is, inhibitors of the oncogenic kinase v-Src.[23–25] Although Hsp90 is essential in all eukaryotic cells, it seemed to be a promising candidate as many oncogenic drivers, like activated kinases and transcription factors, depend on continuous interaction with Hsp90, whereas non-oncogenic cellular counterparts seemed to be less Hsp90-dependent.[26,27] As Hsp90 interacts with some 400 client proteins, among them 60% of the human kinases, it is less likely that cancer cells might escape inhibition of Hsp90 by mutating the drug target, as often observed with kinase-specific inhibitors, or by switching to another driver kinase, as this would be very likely also an Hsp90 client. Furthermore, ATP-competitive Hsp90 inhibitors had favorable pharmacokinetics as they accumulated in malignant cells but not in non-malignant tissue and were cleared rapidly from the plasma.[28,29] Although many ATP-competitive Hsp90

inhibitors exhibited strong growth inhibitory effects on tumor cells in clinical trials, detrimental dose-limiting toxicity and metastasis formation prevented FDA approval.[30]

At this conference, *Wei Li* (University of Southern California, USA) presented data from cell lines and mouse organs, suggesting that it is hard to carry out "fair" toxicity studies of ATP-competitive Hsp90 inhibitors in human trials, due to heterogenous expression levels of Hsp90 and highly variable sensitivities of different organs and cell types to the inhibitors.[31]

Another problem of ATP-competitive Hsp90 inhibitors is that they induce the HSR, upregulating Hsp70 chaperones that support the survival of the cancer cells.[32] *Ahmed Chadli* (Augusta University, USA) reported that the cyclohexadepsipeptide enniatin A inactivates Hsp90 and destabilizes its client oncoproteins without inducing the HSR. Enniatin A triggers a $CD8^+$ T cell-mediated anticancer immunity in triple-negative breast cancer syngeneic mouse models by inducing immunogenic cell death and reducing the protein level of the immunosuppressive programmed cell death 1 ligand 1.[33]

Due to the difficulties of ATP-competitive Hsp90 inhibitors as monotherapy in cancer treatment, alternative usages of Hsp90 inhibitors are sought. *Christine Heske* (National Cancer Institute, USA) reported that Hsp90 inhibitor-drug conjugates, such as STA-8666/PEN-866, represent an antigen-independent mechanism for targeted drug delivery that takes advantage of the pharmacokinetic properties of Hsp90 inhibitors, resulting in extended release of the payload and tumor targeting with less effect on normal tissues. In multiple preclinical xenograft models, STA-8666/PEN-866, which delivers the topoisomerase I inhibitor payload SN-38, resulted in superior pharmacokinetic, pharmacodynamic, and efficacy measures as compared to the standard of care topoisomerase I inhibitor irinotecan, even in irinotecan-resistant models.[34,35] Data from an early phase clinical trial confirm the pharmacokinetic properties of PEN-866[36] and suggest that Hsp90 inhibitor-drug conjugates as a class may be a promising clinical application of Hsp90 biology.

A different approach was reported by *Kevin Foley* (Ranok Therapeutics, USA). Since the Hsp90 chaperone complex not only plays an important role in facilitating the folding of newly synthesized client proteins but can also mediate the degradation of misfolded proteins through interaction with ubiquitin E3 ligases, artificially directing proteins to Hsp90 might also lead to their degradation. Foley presented chaperone-mediated protein degraders (CHAMPs) that are hetero-bifunctional small molecules that bind to a target protein of interest and direct its interaction with the Hsp90 chaperone complex, thereby inducing degradation of the target protein via the ubiquitin proteasome system. CHAMPs have a number of advantages over other targeted protein degradation approaches, including an improved safety margin due to selective drug accumulation in tumors associated with Hsp90 activation. A CHAMP targeting the bromodomain transcription factor BRD4, RNK-05-047, is currently being tested in a phase 1 clinical trial in solid tumor and diffuse large B-cell lymphoma patients.[37]

Anushka Wickramaratne (National Institutes of Health, USA) presented her work on the formation of an intermediate Hsp90-J-domain protein complex in the pathway of protein remodeling. The interaction is conserved in both bacteria and yeast, and it is thought to be a mechanism of client transfer during protein remodeling.[38]

Hsp90 does not only appear to be a rewarding drug target for cancer therapy; inhibition of Hsp90 also seems to be a viable option for infectious diseases, in particular with eukaryotic pathogens. A first proof of concept was provided by the Lindquist lab that demonstrated that resistance of *Candida albicans* against anti-fungal drugs depended on Hsp90 and inhibition of Hsp90 resensitized previously resistant clinical isolates.[39] Another potential target parasite is *Plasmodium falciparum*, the cause of the most lethal and widespread form of malaria, resulting in up to 500,000 deaths annually, mostly in children under 5, and mostly in sub-Saharan Africa.[40] As with many other pathogens, rapidly spreading *P. falciparum* strains that are resistant to commonly used drugs make the quest for novel drug targets imperative. *Fortunate Mokoena* (North-West University, South Africa) presented a virtual screening protocol based on a pharmacophore model to identify chemically diverse inhibitors of *P. falciparum* Hsp90, a validated drug target in malaria, as it is essential for plasmodium proliferation in red blood cells. Although Hsp90 is highly conserved in evolution and the N-terminal ATP binding pocket belongs to the most conserved region in Hsp90, there are differences that could be exploited for drug selectivity. The Mokoena group identified several promising compounds that showed biological activity in anti-plasmodium assays and moderate to no toxicity toward mammalian cells. Biochemical and biophysical assays were used to show growth inhibition and binding to PfHsp90, respectively.[41]

A different approach was presented by *Tawanda Zininga* (Stellenbosch University, South Africa). He and his colleagues screened and characterized peptide-based inhibitors of the ER localized *Plasmodium falciparum* Hsp90 (PfGrp94) and Hsp70 (PfGrp78). Their study

focused on enriching decapeptides[42] with asparagine residues to mimic the malaria parasite's asparagine repeat-rich proteins that are processed through the ER for export into the host red blood cell.

Heat shock protein 60 (Hsp60, HSPD1) is a chaperonin that plays a key role in protein import and folding in the mitochondria. Multiple genetic studies have identified Hsp60 as a putative drug target in cancer and neurodegenerative disease, fueling interest in better understanding Hsp60's structure and function. At the CSSI meeting, *Jason Gestwicki* (UCSF, USA) discussed collaborative work with Dan Southworth (UCSF), which used cryo-EM to reveal the client-bound states of human Hsp60 for the first time.[43] These structures are being leveraged to design high-throughput chemical screens for the discovery of next-generation chemical inhibitors.

Environment and organismal stress responses

Pierre Goloubinoff (Lausanne University, Switzerland) discussed the mechanisms by which heat-induced Hsp70 chaperones, driven by J-domain cochaperones and assisted by thermo-protective osmolytes, can use energy from ATP to unfold stable aggregates of a multi-domain protein comprising a thermolabile luciferase core flanked by thermoresistant fluorescent proteins. Remarkably, ATP-fueled Hsp70-JDP action maintained active the luciferase core for hours under elevated non-equilibrium temperatures that otherwise readily denature luciferase.[44] Although such a function seems essential, especially for plants that cannot escape environmental stress conditions, plants can be selected to repress the HSR. Goloubinoff and colleagues demonstrated this by using a transgenic *Arabidopsis* plant expressing a toxic protein from a heat-inducible promoter[45] and isolated candidate mutant plants that failed to overproduce HSPs in response to heat stress. Yet, their heat-shock response defective phenotype mostly disappeared in mature plants. However, the repression of heat shock gene expression was nonetheless transmitted to up to 10% of their descendants. Goloubinoff concludes from these data that higher plants can activate a specific epigenetic program to block HSP production in response to heat, possibly to reduce the burden of unnecessary HSP overproduction, when on hot day, heat stress ends up not reaching a noxious level. These data are reminiscent to the shut-down of the HSR found in *C. elegans* within 12 h of adulthood.[19]

Sotirios (Akis) Fragkostefanakis (Goethe University Frankfurt, Germany) reported that, in tomato, the activity of the master regulator of the heat stress response, HSFA1a, is stimulated by interaction with other HSFs, such as HSFA2 and HSFA7, leading to the formation of complexes with strong transactivation activity.[46,47] Intron splicing in HSFA2 and HSFA7 pre-mRNAs produces protein isoforms that undergo rapid degradation, while intron retention yields mRNAs that are translated into stable protein isoforms, which are maintained for long periods in thermotolerant cells. In the case of HSFA2, selection of a haplotype that favors intron retention during tomato domestication is associated with increased acquired thermotolerance of modern tomato cultivars.[47]

Tardigrades are recognized for their ability to survive extreme stresses including desiccation, but the molecular protectants that mediate survival are not fully known. *Jon Hibshman* (The University of North Carolina at Chapel Hill, USA) presented a screening strategy to identify tardigrade proteins that could improve bacterial desiccation survival. This selection screen led to the discovery of mitochondrial single-stranded DNA-binding proteins as potent desiccation protectants, adding to the repertoire of protectants and approaches for promoting desiccation tolerance.[48]

In mammals, chronic and systemic stress seems to interface with the immune system.

Elizabeth Repasky (Roswell Park Comprehensive Cancer Center, USA) presented data showing that the mild, chronic stress caused by mandated cool environmental temperature in laboratory mouse colonies is sufficient to increase systemic norepinephrine, which is produced by sympathetic nerves driving additional thermogenesis.[49] This increase in norepinephrine signals through beta-adrenergic receptors on immune cells and was found to drive T cell exhaustion in the tumor microenvironment (TME). She also presented new preliminary data showing that the HSR in lung and heart tissue is mitigated in mice in which environmental stress has been reduced, suggesting a role for the level of baseline adrenergic stress in the regulation of the HSR.[50,51]

Monika Fleshner (University of Colorado Boulder, USA) presented data on systemic stress-evoked sterile inflammation. She has previously reported that exposure to acute intense psychological and physical stressors increases circulating danger/damage-associated molecular patterns (DAMPs), such as Hsp72.[52] These DAMPs facilitate innate immune responses such that organisms kill and clear bacterial challenges faster. She presented more recent work elucidating the role of extracellular macrovesicles (EMVs) in regulating stress-evoked inflammation. Specifically, she presented experimental evidence that after exposure

to a 90-min intermittent tail shock stressor, circulating EMVs have increased membrane-associated immunostimulatory endogenous DAMPs (e.g., Hsp72) and reduced intra-vesicle immunoinhibitory mi-RNA cargo compared to non-stressed controls.[53] These findings support the supposition that sterile inflammation is an elemental feature of the stress response and that circulating EMVs transporting immunomodulatory signals may play a fundamental role in immune homeostasis.

Chaperones in the extracellular and transcellular stress response

In recent years, it was demonstrated through research in *C. elegans* that the stress response is coordinated within an organism by transcellular signaling.[54] *Patricija van Oosten-Hawle* (University of North Carolina, USA) provided new mechanistic insights into the regulation of a zinc finger transcription factor called PQM-1 that is required for the maintenance of organismal proteostasis during aging and the regulation of stress responses.[55] The newly presented research demonstrates how caloric restriction and post-translational modifications of PQM-1 affect its activity and influence stress resilience in the nematode *C. elegans*.

In mammals, heat shock proteins including Hsp70s and Hsp90s are found in the extracellular space, non-conventionally secreted or released by damaged cells, are bound by specific receptors expressed on a wide variety of cells, including immune cells, and modulate the immune response context-dependent in pro-inflammatory and anti-inflammatory ways.[56–58] At this conference, *Thiago J. Borges* (Harvard Medical School, USA) reported that he and his colleagues identified an innate immune receptor through which the mycobacterial Hsp70 (MbDnaK) stimulates its regulatory profile on antigen-presenting cells. MbDnaK-induced immune-suppressive responses depend on the receptor Siglec-E. Studying the interplay between MbDnaK and Siglec-E, they found that Siglec-E is a novel inhibitory innate receptor that could be employed beneficially in organ transplantation.[59,60]

Michael Lynes (University of Connecticut, USA) reported on metallothionein, an "HSP wannabe" that shares several functional hallmarks with the Heat Shock Protein family, and the implications of an anti-metallothionein monoclonal antibody therapy for inflammatory diseases. The major isoforms of metallothionein (MT1 and MT2) are encoded by stress-response genes that are activated by a diverse range of agents and conditions, including both toxic and essential divalent heavy metal cations, endotoxin, glucocorticoids, acute phase cytokines, heat, and reactive oxygen species, agents that also can initiate HSP biosynthesis. MT biosynthesis can be increased during chronic inflammation, autoimmune disease, neoplasia, and infection. Intriguingly, also like some of the HSPs, MT can bind receptors in the LDLR family, and its effects on immune function are possibly due to those interactions.[61] Antagonizing extracellular MT interactions with a monoclonal anti-metallothionein antibody (clone UC1MT) diminished disease progression in several distinct animal models of autoimmune and chronic inflammatory diseases (inflammatory bowel disease, acetaminophen-induced liver injury, and type 1 diabetes). These observations suggest that this antibody may have a broad therapeutic potential.[62]

Shawn Xiang Yang Wang (VCU Health Center, USA) described a novel chaperone-based immunostimulatory agent for cancer immunotherapy by reprogramming the TME. Integration of this TME-targeting approach into the radiation therapy regimen can efficiently eradicate distant tumors when compared with radiation therapy alone, supporting the potential use of this agent to improve an abscopal response of cancer.

Takanori Eguchi (Okayama University, Japan) reported that his group found many heat shock proteins in extracellular vesicles (EVs) released from human carcinomas. He further showed that EVs released from cancer cells induce epithelial-to-mesenchymal transition (EMT) in head and neck carcinoma cells. Interestingly, triple siRNAs targeting Cdc37, Hsp90α, and Hsp90β reversed the EMT in these carcinoma cells and inhibited the EMT-initiating effect of carcinoma EVs.[63] He secondly presented work on the zinc-finger-less transcription factor SCAND1. By oligomerizing with SCAN zinc finger MZF1, SCAND1 transcriptionally suppresses chaperone genes (*CDC37* and *HSP90*), MAPK pathway genes (*MEKK, MAPK*...), and EMT driver genes (*ZEB1/2, TGFBR*, and *CTNNB1*) and thereby reverses EMT-based transformed neuroendocrine prostate cancer cells to a more normal, less invasive status and inhibits their proliferation, migration, and lymph node metastasis.[64]

Chaperone networks, co-chaperones in the stress response and as targets for therapy

As mentioned above, many ATP-competitive inhibitors of Hsp90 accumulate in a large variety of different cancer cell types but not in non-malignant normal tissue.[28,29,65] The reason for this observation was believed to be cancer-specific Hsp90 conformations and complexes with cochaperones and client proteins. Using ATP-competitive inhibitors developed in her lab, *Gabriela Chiosis* (Memorial Sloan Kettering, USA) was able

to obtain evidence for such Hsp90 complexes that she coined epichaperomes.[66] Chiosis proposed that, under pathological conditions, stressors trigger the formation of persistent assemblies comprised of tightly bound chaperones, co-chaperones, and other factors. Distinct from conventional chaperones that operate through dynamic one-on-one complexes, epichaperomes are supposed to serve as pathological scaffolding platforms, particularly observed in neurodegenerative disorders and cancers. In neurodegenerative disorders, epichaperome formation seems to impact proteins important for neuronal function, such as proteins involved in synaptic plasticity, cell-to-cell communication, protein translation, cell cycle re-entry, axon guidance, metabolic processes, and inflammation, leading to neuronal dysfunction and cognitive decline. In cancer, epichaperome formation is suggested to impact proteins crucial for maintaining context-dependent malignant phenotypes, such as proteins involved in signaling and metabolic or immune-regulatory pathways.[67]

In her presentation at the symposium, Chiosis provided evidence for the dependence of critical processes, such as the localization of nuclear mitotic apparatus protein 1 during mitosis and the formation and anchoring of mitotic spindles onto centrosomes and the cell cortex, on epichaperome activity. Notably, this mechanism is context-dependent, as epichaperomes are not present in normal cells and are also not universally found in all cancer cells, emphasizing their role as a cancer-specific maladaptation phenomenon rather than a general mechanism of mitosis regulation.[68]

Yuka Okusha (Okayama University, Japan) reported on the identification of a potent microRNA, miR-570 that could bind the 3′ untranslated regions of multiple heat shock proteins-encoding mRNAs and inhibit HSP synthesis. In addition, she determined a significant role for miR-570 in regulating markers of mammary tumor progression, including cell motility and invasion.[69] These data provide evidence for the principle that the tumor chaperome can be targeted by microRNAs, suggesting a potential therapeutic avenue toward cancer therapy.

Jennifer Heritz (State University of New York, USA) reported on an *in silico* approach her group used to screen and develop a selective inhibitor of serine/threonine protein phosphatase-5 (PP5), a co-chaperone of Hsp90 that promotes tumor progression and metastasis.[70,71] The data of the Heritz group suggest that PP5 promotes renal cancer survival by suppressing the extrinsic apoptotic pathway. Compound P053 competitively inhibits PP5 to activate this pathway, causing apoptosis in renal cancer.

Cochaperones that regulate the activity of chaperones are not only potential drug targets for cancer therapy. *Greg Blatch* (Higher Colleges of Technology, UAE) reported that *Plasmodium falciparum* J-domain proteins, essential cochaperones of the Hsp70 system, are the most prominent and diverse family of malarial proteins exported into parasite-infected human red blood cells. Certain exported *Plasmodium falciparum* J-domain proteins are essential for survival of *Plasmodium* (e.g., PFE0055c), and functionally regulate both the exported *P. falciparum* Hsp70-x (PfHsp70-x) and human Hsp70 (hHsp70), thereby enabling the trafficking, folding, and functioning of key malaria virulence factors.[72] Virtual screening conducted on drug repurposing libraries resulted in the identification of a number of drug-like small molecules that could specifically disrupt the interaction of PFE0055c with PfHsp70-x.[73]

Heath Ecroyd (University of Wollongong, Australia) presented work on the single-molecule fluorescence microscopy-based technique they developed to observe the interaction of molecular chaperone proteins with misfolded client proteins.[74] Using this technique, they have been able to observe the complete reaction cycle involved in Hsp70-mediated refolding of the model client protein, firefly luciferase, including multiple cycles of Hsp70 binding-and-release to an individual client protein. The work provides direct evidence for the unfoldase action of Hsp70 chaperones in facilitating client protein refolding. Confirming initial evidence from,[75] Ecroyd also presented preliminary direct single-molecule evidence that bacterial Hsp90 (HtpG) can facilitate the folding of protein intermediates to reach the native state that would otherwise remain trapped within the bacterial Hsp70-mediated unfolding cycle.

Protein quality control, autophagy, protein folding disorders, and aging

Albena Dinkova-Kostova (University of Dundee, UK) presented recent work from her laboratory showing that the stress-responsive transcription factor nuclear factor erythroid 2 p45-related factor 2 (Nrf2) facilitates mitochondrial function, bioenergetics, and adaptation to pro-inflammatory stimuli. They generated human lung cancer A549 cell lines in which Nrf2 is hyperactive or depleted by either siRNA or CRISPR/Cas9. The use of these cell lines revealed that, during autophagy, without affecting the formation of new autophagosomes, Nrf2 promotes the lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3) and mitolysosome degradation, thereby ensuring timely clearance of damaged mitochondria.[76,77]

Marc Mendillo (Northwestern University, USA) reported on recent work from his lab revealing a network of genes critical for cancer cell growth-associated stress revealing several important insights, including a cooperative relationship between Hsf2 and Hsf1 to drive a transcriptional program critical for cancer cell proliferation.[78] He also discussed a novel central coordination mechanism comprising the HAPSTR1/HUWE1 axis that functions as a rheostat in a network of pathways responsible for cellular adaptability.[79] More recently, Mendillo and his team extended these findings in unpublished work by combining a mammalian chemical-genetic profiling platform with their FIREWORKS computational pipeline[80] to identify components, function, and regulatory nodes within the mammalian stress response network. In doing so, they identified canonical functions of well-studied components within the cellular stress network along with functions of those whose activity was previously unknown. Most interestingly, they integrated these genetic data with short-term and long-term transcriptome studies to reveal fundamental principles for how cells cope with constitutive stresses, providing insights into the mechanisms by which stress response pathways are activated in cancers and mechanisms of resistance to anti-cancer therapies.

Veena Prahlad (Roswell Park Memorial Cancer Center, USA) discussed her lab's long-standing research on the role of the nervous system in the cephalic, anticipatory control over the conserved transcription factor, heat shock factor 1 (Hsf1).[81,82] Prahlad then focused on current, unpublished work from her lab, which shows that Hsf1 also acts as a repressor of transcription by recruiting chromatin modifiers that increase methylation of histone H3 at lysine 9 (H3K9me2) at regions across the genome[83] and by upregulating ncRNAs that act through post-transcriptional mechanisms to suppress global mRNA levels. Among the Hsf1-induced ncRNAs are Piwi-interacting RNAs (piRNAs)[84] that target transposase mRNA, which, intriguingly, are upregulated upon heat shock. Interestingly, Piwi and piRNA had been associated previously with the ability of Hsp90 to buffer genetic variations.[85] Prahlad's lab is investigating the role of these Hsf1-induced piRNAs in modulating transposition in the *C. elegans* germline.

Laura Blair (University of South Florida, USA) presented her group's findings identifying new molecular chaperone regulators of tau seeding, including multiple J-domain proteins (JDPs, also called Hsp40/DnaJ proteins), cochaperones of Hsp70, and cyclophilin C (PPIC).[86,87] Aberrant Tau accumulation is associated with neurodegeneration in Alzheimer's disease and other tauopathies. Of particular interest, DnaJB6b was found to reduce tau levels and interact with tau complexes.[86]

Emily Sontag (Marquette University, USA) reported on her work on the sequestration of misfolded proteins into compartments in the nucleus and cytoplasm. These compartments are transported to nucleus-vacuole junctions to facilitate vacuolar clearance via the ESCRT family of proteins. She showed high-resolution fluorescence microscopy images that suggest that clearance of nuclear aggregates occurs through a budding process into the juxtaposed vacuole.[88,89]

Genetic neurochaperonopathies (NCPs) are believed to be rare disorders, but this believe may result from a lack of knowledge and information and, consequently, an inability to recognize NCPs. Following a bibliographic and database search, *Federica Scalia* (University of Palermo, Italy) identified 65 genes encoding members of the chaperone system and their variants associated with NCPs.[90] Furthermore, Scalia presented a multidisciplinary method to study and recognize these rare disorders.

The Alfred Tissières Young Investigator Award of the Cell Stress Society International was awarded to *Sara Sannino* (University of Pittsburgh, USA) from J. Brodsky's laboratory. She presented her work on how tumor cells overcome proteotoxic stress by adjusting protein homeostasis—protein degradation, protein folding, and amino acid metabolism—using the integrated stress response. By using a specific allosteric Hsp70 inhibitor (MAL3-101), Sannino demonstrated that breast cancer cells exhibit different sensitivities to proteotoxic stress.[91] This Hsp70 inhibitor induced two sensors of the integrated stress response, GCN2 and PERK. GCN2 and autophagy induction prevented cancer cell death. In proteotoxic stress-resistant cells, the abundance of the amino acid arginine was reduced compared to stress-sensitive cells when exposed to Hsp70 inhibition. Arginine supplementation was sufficient to prevent autophagy and reduce GCN2 activation, favoring mTORC1-mediated cell death.[92] Therefore, amino acid abundance, GCN2, mTORC1, and autophagy represent integrated therapeutic targets whose modulation can regulate resistance to therapeutic stressors in cancer.

The Ferruccio Ritossa Early Career Award of the Cell Stress Society International was awarded to *Reut Shalgi* (Technion - Israel Institute of Technology, Israel). She presented her work on probing the chaperone network complexity in the context of protein aggregation diseases. Using a highly quantitative chaperone overexpression screen, Shalgi and her coworkers identified novel modulators of the aggregation of mutant FUS, which is related to amyotrophic lateral sclerosis (ALS),

revealing new roles for specific J-domain proteins (JDPs) in ALS-FUS. Specifically, a complex of the full-length isoforms of DNAJB14 and DNAJB12, together with Hsp70, potently inhibited mutant FUS aggregation in model cell lines and in primary neurons. Surprisingly, Shalgi's team found that different isoforms of DNAJB12 and DNAJB14 had opposing effects on aggregation inhibition for Huntington's chorea-related HTT-polyQ versus the ALS-related FUS: The naturally occurring isoform DNAJB12-short, lacking the J domain, significantly inhibited, whereas the full-length isoforms of DNAJB14 and DNAJB12 significantly enhanced HTT-polyQ aggregation. These findings[93] highlight three contributing features to the Hsp70-JDP network complexity: JDP isoforms, JDP heterodimers, and Hsp70-independent versus Hsp70-dependent functions of JDPs. Ongoing work in Shalgi's lab is currently seeking to systematically explore how these factors play a role in increasing the chaperone network's functional complexity.

Concluding remarks

This conference covered many areas of stress biology, from detailed insights into the physical-chemical basis of chaperone action and the molecular mechanism of chaperones at the single-molecule level to the complexity of chaperone networks, from regulation of stress responses at a cellular level to the cross-talk between cells within an organism, from adaptations in healthy cells to maladaptations in disease states. The increasing understanding of the molecular mechanisms of chaperones and stress response regulations reveals new targets for therapeutic interventions and paves the way to new drugs. Hsp90 drugs, which failed to deliver the promised benefits in monotherapeutic applications, are repurposed as conjugates for targeting toxins to cancer cells—a reincarnation of the "Zauberkugel" (magic bullet) concept originally proposed in the beginning of the 20th century by Paul Ehrlich[94]—or for targeting oncoproteins to Hsp90, where they are eventually ubiquitinated and degraded. Although molecular chaperones are in general highly conserved, parasite members of the chaperone family seem to be different enough to allow for the development of specific drugs which might be used for treating infectious diseases with much less side effects than experienced in the treatment of cancer. Thus, chaperone biology could help to alleviate health burdens of developing countries. Another highlight of the conference was how plants can tune their HSR and that, while selecting cultivars for improved food production, thermotolerance was increased by altering the splicing of some of the HSFs. Such discoveries will be paramount for the future adaptation of agriculturally valued plants to climate change.

The conference also uncovered future questions and challenges. We have only a rudimentary understanding of the complexity of the networks of feed-back and feed-forward loops regulating the stress response pathways. Transcription factors have many more recognition sequences within the genome than are actually bound by the transcription factor at any given moment and utilized to modulate transcription. Thus, elucidating the molecular mechanism of target site selection of transcription factors in general and Hsf1 and Hsf2 in particular, as examples for other stress responsive transcription factors, will be an important challenge and essential for understanding the wiring of stress response pathways in healthy cells and in disease states. Part of this mechanism might be the interfacing of the transcription factors with each other and with the chromatin. Novel methods for tracking changes in chromatin and posttranslational modifications in RNA-polymerase during the initiation of transcription with nucleotide resolution will help to address these questions. Another continuing challenge is the orchestration of cellular responses within an organism, including neuronal control over non-neuronal cellular responses, transcellular signaling, extracellular chaperones acting as chaperokines, and their interfacing with the innate immune system and inflammation.

Finally, a better understanding of adaptive and maladaptive stress responses might enable us to develop more targeted drugs for the treatment of many diseases including cancer, neurodegeneration, autoimmunity, and infections with bacterial and eukaryotic pathogens Figures 1 and 2).

Awards

In addition to the above-mentioned Alfred Tissières Young Investigator Award and the Ferruccio Ritossa Early Career Award, the Cell Stress Society International awarded a number of distinctions to members of the society and invited speakers for their contribution to the field of stress response and molecular chaperones and for their services to the society (Fig. 2). The CSSI Medallion for Career Achievement was bestowed on Stuart Calderwood for his many seminal contributions to the field, for his services to the society and organization of CSSI conferences including the organization of this symposium. Dimitra Bourboulia, Adrienne Edkins, Jill Johnson, and Mark Woodford were elected Fellow of the CSSI for reviewing

Fig. 1 Group photo of meeting attendees at the 12th International Symposium on Heat Shock Proteins in Biology, Medicine and the Environment inside the Hilton Old Town Alexandria, Alexandria, VA, USA.

Fig. 2 Matthias Mayer was honored as a CSSI Senior Fellow by CSSI founder Lawrence Hightower (left) and President Lea Sistonen (right).

many manuscripts for the CSSI journal *Cell Stress & Chaperones*, for contributing to the success of CSSI conferences and for organizing the CSSI conference on the Chaperone Code, which preceded the 12th International Symposium on Heat Shock Proteins in Biology, Medicine and the Environment in October 2023. Johannes Buchner, Heath Ecroyd, Matthias Mayer, Mehdi Mollapour, and Ariel Shabtay were elected Senior Fellows of the CSSI, for their seminal contributions in the field of chaperones and stress response, for reviewing numerous manuscripts for the CSSI journal, for contributing articles and meeting reports to the CSSI Journal, for organizing CSSI meetings on chaperones, and for serving as senior editors for the Society Journal (Ariel Shabtay).

Author contributions MPM, LB, GLB, TJB, AC, GC, AT, ADK, HE, ALE, TE, MF, KPF, SF, JG, PG, JAH, CMH, JDH, JJ, WL, ML, MLM, NM, FM, YO, VP, ER, SS, FS, RS, LS, ES, POH, AV, AW, SXYW, and TZ wrote the text. MPM, LB, and MLM organized and assembled the manuscript.

Declarations of interest

The authors declare no conflicts of interest.

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