The growing world of small Heat Shock Proteins: from structure to functions

Serena Carra[1 *], Simon Alberti[2], Patrick A Arrigo[3], Justin LP Benesch[4], Ivor J Benjamin[5], Wilbert Boelens[6], Britta Bartelt-Kirbach[7], Bianca JJM Brundel[8], Johannes Buchner[9], Bernd Bukau[10], John A Carver[11], Heath Ecroyd[12], Cecilia Emanuelsson[13], Stephanie Finet[14], Nikola Golenhofen[7], Pierre Goloubinoff[15], Nikolai Gusev[16], Martin Haslbeck[9], Lawrence E Hightower[17], Harm H Kampinga[18], Rachel E Klevit[19], Krzysztof Liberek[20], Hassane S Mchaourab[21], Kathryn A McMenimen[22], Angelo Poletti[23], Roy Quinlan[24], Sergei V Strelkov[25], Melinda E Toth[26], Elizabeth Vierling[27], and Robert M Tanguay[28 #]

AFFILIATIONS

[1] Department of Biomedical, Metabolic and Neural Sciences, and Centre for Neuroscience and Nanotechnology, University of Modena and Reggio Emilia, via G. Campi 287, Modena 41125, Italy.

[2] Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany.

[3] Apoptosis, Cancer and Development Laboratory, Lyon Cancer Research Center, INSERM U1052-CNRS UMR5286, Claude Bernard University Lyon 1, Lyon, France.

[4] Department of Chemistry, Physical & Theoretical Chemistry Laboratory, University of Oxford, Oxford OX1 QZ, United Kingdom.

[5] Department of Biochemistry, University of Utah, Salt Lake City, Utah 84112-5650, USA.

[6] Biomolecular Chemistry, 284, Radboud University, PO Box 9101, 6500 HB, Nijmegen, The Netherlands.

[7] Institute of Anatomy and Cell Biology, University of Ulm, 89081 Ulm, Germany.

[8] Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands.

[9] Technische Universitat Munchen, Germany.

[10] Center for Molecular Biology of the University of Heidelberg (ZMBH), DKFZ-ZMBH Alliance, Im Neuenheimer Feld 282, Heidelberg D-69120, Germany and German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg D-69120, Germany.

[11] Research School of Chemistry, The Australian National University, Acton, Australian Capital Territory 2601, Australia.

[12] Illawarra Health and Medical Research Institute and School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522, Australia.

[13] Department of Biochemistry and Structural Biology, Center for Molecular Protein Science, Lund University, 221 00 Lund, Sweden.

[14] IMPMC UMR7590, CNRS, UPMC Paris 6, 4 place Jussieu, Paris, France.

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

[16] Department of Biochemistry, School of Biology, Moscow State University, Moscow 119991, Russia.

[17] Dept. of Molecular & Cell Biology, University of Connecticut, 91 North Eagleville Road, Storrs, CT 06269-3125, USA.

[18] Department of Cell Biology, University Medical Center Groningen, University of Groningen, 9713 GZ Groningen, the Netherlands.

[19] Department of Biochemistry, University of Washington, Seattle, Washington, 98195, USA.

[20] Department of Molecular and Cellular Biology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and the Medical University of Gdańsk, Gdańsk, Poland.

[21] Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232

[22] Department of Chemistry, Mount Holyoke College, South Hadley, MA, USA.

[23] Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB), Centro di Eccellenza sulle Malattie Neurodegenerative, Università degli Studi di Milano, Milano, Italy.

[24] Department of Biosciences and the Biophysical Sciences Institute, University of Durham, United Kingdom.

[25] Laboratory for Biocrystallography, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium.

[26] Laboratory of Animal Genetics and Molecular Neurobiology, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary.

[27] Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003 and Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, USA.

[28] Laboratory of Cell & Developmental Genetics, IBIS, and Department of Molecular Biology, Medical Biochemistry and Pathology, Medical School, Université Laval, Québec (Qc), Canada G1V 0A6.

[*] [#] To whom correspondence can be addressed at:

Dr. Serena Carra Department of Biomedical, Metabolic and Neural Sciences, and Centre for Neuroscience and Nanotechnology, University of Modena and Reggio Emilia, via G Campi 287, Modena 41125, Italy

Email: serena.carra@unimore.it

Tel: +39-0592055265

Or

Dr. Robert M. Tanguay Laboratory of Cell & Developmental Genetics, IBIS, and Department of Molecular Biology, Medical Biochemistry and Pathology, Medical School, Université Laval, Québec (Qc), Canada G1V 0A6

Email: Robert.tanguay@fmed.ulaval.ca

Tel: 418 656-3339

SUMMARY

Small Heat-Shock Proteins (sHSPs) are present in all kingdoms of life and play fundamental roles in cell biology. sHPSs are key components of the cellular protein quality control system, acting as a first line of defence against conditions that affect protein homeostasis and proteome stability, from bacteria to plants and humans. Due to their ability to bind to a large subset of client proteins, maintaining them in a state competent for refolding or clearance, with the assistance of the HSP70 machinery, sHSPs participate to a number of biological processes, from cell cycle, to cell differentiation, from adaptation to a stress, to apoptosis, and even in the transformation of a cell into a malignant state. As a consequence, their malfunction has been implicated in various disorders e.g. abnormal placental development and pre-term deliveries, the prognosis of several types of cancer, and the development of neurological diseases. Moreover, mutations in the genes encoding for several mammalian sHSPs result, in humans, in neurological, muscular and/or cardiac age-related diseases. In contrast, considering that loss of protein homeostasis due to protein aggregation is typical of many age-related neurodegenerative and neuromuscular diseases, and in light of the role of sHSPs in the clearance of un/misfolded aggregation-prone clients, pharmacological modulation of sHSP expression or function, and rescue of defective sHSPs represent possible routes to alleviate or cure protein conformational diseases. Here, we report the latest news and views on sHSPs that were discussed by many of the world's experts in the sHSP field during a dedicated workshop organized in Italy (Bertinoro, Centro Residenziale Universitario Bertinoro/CEUB, 12-15 October 2016).

INTRODUCTION

Small Heat Shock Proteins (sHSPs) belong to the superfamily of HSPs and are expressed in all three domains (viruses, archaea, bacteria and eukaryota) (Bult et al. 1996; Caspers et al. 1995; Eyles and Gierasch 2010; Richter et al. 2010). sHSPs are characterized by a small molecular subunit mass, ranging from ca. 15-40 kDa and by a highly conserved central domain, called the alpha-crystallin domain (ACD) that represents their identification label. sHSPs exist as monomers and dimers, which can also assemble into large multimeric complexes that vary in size and can contain up to 24-40 subunits (Candido 2002; Hilton et al. 2012; Kim et al. 1998; McDonald et al. 2012; van Montfort et al. 2001). Association of monomers into large complexes, as well as dissociation of large oligomers into dimers and monomers, is modulated by post-translational modification, including phosphorylation, which in turn, regulates sHSP functions. sHSP functions are very diverse and include molecular chaperone-like activities and modulation of cytoskeleton stability (Aquilina et al. 2004; Arrigo 2013; Bryantsev et al. 2002; den Engelsman et al. 2005; Ecroyd et al. 2007; Gaestel 2002; Lambert et al. 1999; Lavoie et al. 1995; Mehlen et al. 1997; Morrison et al. 2003; Morrow et al. 2015; Rogalla et al. 1999; Rouse et al. 1994; Theriault et al. 2004; Webster 2003).

As a consequence of their role as chaperones towards diverse clients, which influences client fate (refolding or degradation) and due to their role as stabilizing agents of the cytoskeleton, sHSPs indirectly participate in the regulation of complex processes such as the response and adaptation to cell stress, including thermotolerance, cell differentiation, cell death and development (Arrigo 2000; Arrigo and Ducasse 2002; Arrigo and Gibert 2014; Balogi et al. 2008; Benjamin et al. 1997; Bruey et al. 2000; Haslbeck et al. 2016; Hong and Vierling 2000; Kamradt et al. 2002; Kamradt et al. 2005; Lavoie et al. 1993; Lavoie et al. 1995; Litt et al. 1998; Nicholl and Quinlan 1994; Parcellier et al. 2006; Park et al.; Perng et al. 1999a; Perng et al. 1999b; Qian et al. 2009; Quinlan and Van Den Ijssel 1999; Takayama et al. 2003; Tanguay and Hightower 2015; Webster 2003). Thus, malfunction of sHSPs can have adverse effects and these have been found to be causative of a wide range of pathologies including cardiomyopathy, myofibrillar myopathy, motor neuron diseases and cataracts (Evgrafov et al. 2004; Ghaoui et al. 2016; Irobi et al. 2004; Kolb et al. 2010; Perng et al. 1999b;

Vicart et al. 1998). Understanding how sHSP function is regulated and elucidating how, mechanistically, their malfunction is linked to disease will a have strong impact on cell biology and disease and will help in the identification of potential drug targets.

In this review, we summarize the latest news and views concerning the structural and functional properties of sHSP that were presented and discussed by 29 international experts during the Second International Workshop of Cell Stress Society International (CSSI) on sHSPs, entitled "The small HSP World" that was held in Centro Residenziale Universitario di Bertinoro, Italy (12-15 October 2016). This meeting followed up on the first Workshop held in Québec, Canada (Tanguay & Hightower, 2014).

New insights into the structure of sHSPs and their interaction with clients

As mentioned above, sHSPs have a dynamic structure that spans from monomers/dimers to large oligomers (Hilton et al. 2012; van Montfort et al. 2001). Oligomerization and dynamics affect their binding affinity to specific subsets of clients and, therefore, sHSP functions (Delbecq and Klevit 2013; Delbecq et al. 2015; Ecroyd et al. 2007; Giese et al. 2005; Giese and Vierling 2002; McDonald et al. 2012; McHaourab et al. 2002; Stromer et al. 2004). Thus, understanding how sHSPs oligomerize, identifying their preferred oligomerization state (in resting conditions or upon stress), what regulates their transition from small oligomers into large oligomers and how this influences their binding to clients is crucial to pinpoint sHSP function (Mainz et al. 2015). This information will also help in designing strategies and drugs that may stabilize one conformation, thereby modulating specific sHSP functions, with potential application in cell stress response, apoptosis and disease.

sHSPs are composed of a N-terminal domain (NTD), a C-terminal domain (CTD) and a middle highly conserved region, called the alpha-crystallin domain (ACD) (van Montfort et al. 2001). In contrast to the alpha-crystallin domain, the C-terminal and the N-terminal domains of sHSPs are poorly conserved amongst the various members and across the various species. However, structural and functional studies have demonstrated that all these three domains play important role in sHSP oligomerization and function (Mainz et al. 2015; McDonald et al. 2012; van Montfort et al. 2001).

Cecilia Emanuelsson (Sweden) presented data on Hsp21, the chloroplast-localized sHSP, and suggested a role for the N-terminal and C-terminal tails in the stabilization of oligomers and in the accessibility for interaction of the oligomer itself with clients (Ahrman et al. 2007a; Ahrman et al. 2007b; Lambert et al. 2011). In particular, a structural model of Hsp21, obtained after homology modelling and fitting to cryo-EM, shows two hexameric discs rotated by 30 degrees and separated further by 35 Å compared to the crystal structure of the cytosolic homologue Hsp16.9. This model suggests that the Hsp21 dodecamer is stabilized by the C-terminal tails, which are shorter than in Hsp16.9 and human HSPB5, and with the IXI-motif extended to IXVXI. The flexible N-terminal arms, unusually long and with functionally important and conserved methionines, appear on the outside of the dodecamer, as supported by limited proteolysis, difference density maps and NMR. Thus, even without subunit dissociation, they may be accessible for transient interaction with client proteins.

The theme of combining structural and biophysical methods to elucidate structural details on sHSPs was continued by Justin Benesch (UK). With native mass spectrometry experiments acting as a thread, he presented new data from a collaboration with Elizabeth Vierling that explained how different evolutionary classes of sHSPs from plants that are found in the same cellular compartment manage to avoid co-assembly (Basha et al. 2010; Painter et al. 2008; Sobott et al. 2002). This presentation highlighted how rich and complex the self- and co-assembly processes of sHSPs are, and the bewildering heterogeneity of the complexes resulting from chaperone activity (Stengel et al. 2012; Stengel et al. 2010).

Regulation of sHSP homo- and hetero-oligomerization is indeed one way of regulating the binding affinity of sHSPs for a given client (Delbecq et al. 2015; Rajagopal et al. 2015). In line with this concept, Rachel Klevit (USA) presented studies aimed at defining how HSPB5 interacts with a destabilized client protein. Klevit's group compared three mutant forms of HSPB5 that each mimic a different mode of activation to "unactivated" (wild-type) HSPB5. Interactions between destabilized α-lactalbumin and HSPB5 were analyzed in four different biochemical/biophysical assays that allow detection of species at different stages along the aggregation pathway. Based on the presented results, Dr. Klevit suggested a model in which unactivated HSPB5 interacts transiently with very early

species, while activated forms of HSPB5 can also interact with client species that are further along the aggregation pathway, providing them with additional capability to delay the onset of amorphous aggregates (Rajagopal et al. 2015).

A comparative study performed by Robert M Tanguay (Canada) further highlighted the impact of the oligomerization state on sHsp function and chaperone-like activity. Following up on their previous *in silico* report of small Hsps in viruses of the Synechococcus cyanobacteria, Robert Tanguay described the properties of the viral and bacterial sHsps protein using size-exclusion chromatography (SEC), native gels, dynamic light scattering (DLS) and chaperone assays (Bourrelle-Langlois et al. 2016; Maaroufi and Tanguay 2013). The cyanophage sHsp forms large oligomers and shows a polydisperse profile (in collaboration with Dr. Stephanie Finet); it exerts chaperone-like activity through the formation of stable and soluble hetero-oligomeric complexes (sHSP:substrate/client). In contrast, the host cyanobacteria sHsp formed a small dimer and tetramer and showed no chaperone-like activity in the assays tested (malate dehydrogenase/MDH, citrate synthase/CS, luciferase/Luc) (Bourrelle-Langlois et al. 2016).

The regulation of the oligomerization state of sHSPs can be influenced by several factors, including post-translational modifications, but also oxidation and crowding agents (Haslbeck et al. 2016). Indeed, on behalf of Sevil Weinkauf´s lab, Martin Haslbeck (Germany) presented the oligomer structures of human αA-crystallin (HSPB4) obtained by cryo-electron microscopy (Peschek et al. 2009). The oligomers form barrel-like structures consisting of tetrameric units. As seen in the pseudo-atomic model, the tetramers assemble mainly via N-terminal interactions while the C-terminal tails exist in 3D non-domain swapped and domain swapped configurations. The oxidation of HSPB4, i.e. the formation of an intra-molecular disulfide bond as observed in vivo, seems to be coupled with conformational changes involving C-terminal domain swapping. They concluded that the oxidized protein shows enhanced subunit dynamics and increased ability in suppressing the aggregation of model substrates.

Next, John Carver (Australia) presented recent work relating to the principal eye lens proteins, HSPB4 and αB-crystallin (HSPB5) (Cox et al. 2014; Cox et al. 2016; Hochberg et al. 2014). Firstly,

he described structural and functional studies of Q147E HSPB4, a major site of deamidation in age-related cataract which leads to a slight reduction in chaperone ability, enhanced temperature stability and a small increase in oligomeric mass. Similar observations for other deamidated crystallins may reflect a general evolutionary adjustment to crystallins with age to counter the extensive post-translational modifications that potentially affect lens transparency (Ray et al., submitted for publication). Secondly, he presented small-angle neutron scattering analysis of deuterated HSPB5 in the presence of crowding agents at high concentration comparable to that of crystallins in the centre of the lens. It was concluded that these conditions lead to destabilisation, unfolding and aggregation of HSPB5. This study further highlights the dynamic nature of sHSPs and how external factors are important to regulate their structure (and in turn, function), with consequences on cell viability and fitness (Treweek et al. 2015).

The importance of structural studies for the understanding of sHSP binding to clients and, as a consequence, function, has been further highlighted by Sergei Strelkov (Belgium), who reported on structural studies of human HSPB6, which is predominantly dimeric in isolation (Heirbaut et al. 2014; Weeks et al. 2014). Phosphorylation of HSPB6 within its intrinsically disordered N-terminal domain (NTD) results in a complex formation with the universal signalling hub 14-3-3 protein (Sluchanko et al. 2011), a process that can trigger smooth muscle relaxation. In collaboration with Nikolai Gusev and Nikolai Sluchanko, the Strelkov lab has succeeded in determining the crystal structure of the entire 14-3-3/pHSPB6 heterotetrameric complex (Sluchanko et al. 2017). As a result, the first-ever atomic resolution snapshot of a mammalian small HSP in a functional state has been obtained. Interestingly, formation of the complex results in partial ordering of the NTD. In addition, the formation of the HSPB1/HSPB6 hetero-oligomer formation, which turns out to be driven by specific sequences within the NTD of HSPB6, was discussed (Heirbaut et al. 2014; Sluchanko et al. 2011).

Finally, André-Patrick Arrigo (France) further stressed the importance of changes in the phosphorylation, oligomerization state and native molecular size in the regulation of sHSP interaction with clients (Arrigo 2013). He summarized the modifications that occur in HSPB1 phosphorylation/oligomerization and native state in cells undergoing changes in their physiology or being exposed to environmental stressors. It was concluded that the changes in HSPB1 organization

are highly condition-specific and phosphorylation and oligomerization, which dynamically react to different cell conditions, were suggested as key factors involved in the generation of HSPB1 platforms that can recognize specific clients, such as F-actin or damaged proteins. In particular, in response to heat shock, damaged polypeptides are stored in HSPB1 large structures that have a phosphorylation specific signature: P-Ser15, P-Ser82. Another important point that was discussed is that these conformational changes are very transient, and, thereby, they likely trigger signals, through transient and specific interaction with selected clients; such changes in the HSPB1-client partnerships would allow cells to react and adapt (Arrigo 2000; Arrigo 2007; Arrigo and Gibert 2012).

Combined, these works further illustrate the complexity of sHSP structure/function, with some sHSPs stabilizing oligomeric structures to favour binding to specific clients and other sHSPs avoiding co-assembly to bind to other subsets of clients (or other conformations/aggregation states of the same client); the level of complexity of the relation between sHSP configuration and its binding to a given substrate is further increased when considering that these structural and functional aspects can all be influenced by post-translational modifications or oxidation of the sHSP itself and by crowding conditions of the neighbouring environment. As pointed out by André-Patrick Arrigo, cell-cell contact also may play a role in the regulation of sHSP structure, further increasing the level of complexity.

From the technical point of view, the main methods employed to study sHSP structure include e.g. mass spectrometry, electron microscopy, NMR and cryo-EM (Baldwin et al. 2012; Benesch et al. 2006; Kondrat et al. 2015; Lambert et al. 2011; Mainz et al. 2015; Shi et al. 2012). Kathryn McMenimen (USA) presented data using HspB1 N-terminal region peptides conjugated to gold nanoparticles and showed that these peptides exhibited concentration-dependent chaperone activity toward citrate synthase (CS) and malate dehydrogenase (MDH). These studies seek to understand the role of oligomerization in chaperone activity and how substrate/client specificity is determined for sHsps, with the use of gold nanoparticles. In addition, the use of different scattering techniques is also beneficial to characterize the structural and functional properties of various sHSPs, as discussed by Stephanie Finet (France). She presented data concerning nuclear Hsp27 from Drosophila

melanogaster, obtained in collaboration with Robert Tanguay's group (Michaud et al. 2008; Moutaoufik et al. 2016). The use of these techniques demonstrated that the major population of DmHsp27 obtained after a two-step chromatography purification was monodisperse and compatible with globular oligomers constituted by 18-20 SU, similar to the smallest population previously produced with a His-tag construct. The effects of mutations of the arginine residues in the ACD of the nuclear Hsp27 of Drosophila were also evaluated by Robert M Tanguay (Canada) in collaboration with Stephanie Finet. While 2 forms of the WT were seen in native gels and in SEC, mutants of R122G, R131G and R135G showed only one peak. The mutants also had the same chaperone-like activity. Finally, the use of the tags and their influence of sHSP structure and oligomerization were also discussed and the general consensus is that comparison between untagged and tagged sHSP forms must be performed, especially in test-tube assays, to avoid possible misinterpretations due to the tag.

From the methodological point of view, a new method that may become useful to study the dynamic oligomerization of sHSPs has been proposed by Wilbert Boelens (The Netherlands). In particular, he presented a method by which the interaction of monomers and dimers with HSPB5 complexes can be analyzed using surface plasmon resonance (Bruinsma et al. 2011). HSPB5 complexes contain between 20 and 40 subunits, which are in equilibrium with monomers and dimers. This dynamic behavior is highly regulated and is crucial for the chaperoning activity. Up to now most studies have focused on the dynamic behavior of the multimeric complexes and not so much on the subunits. By analyzing how monomers and dimers interact with HSPB5 complexes more insight will be obtained into the dynamic behavior of HSPB5. The use of optical tweezers to study biophysical properties of sHSPs and their ability to partition may also represent a promising new approach to understand sHSP structure and function, as suggested by Simon Alberti (Germany) (Sudnitsyna et al. 2011).

Lessons from in vitro studies: chaperone-like activity, cooperation with the HSP70 machine and fate of the sHSP-bound client

In contrast to HSP70, HSP90 and HSP100, sHSPs do not possess ATPase activity. Thus, while chaperones with ATPase activity are classified as chaperone “foldases”, sHSPs are classified as chaperone “holdases”, since they can recognize and bind to unfolded and/or misfolded substrates/clients, and “hold” them avoiding their irreversible aggregation, although this is not their only mechanism of chaperone action (Ecroyd 2015). This holdase-action in turn favours the processing of the sHSP-bound client by the downstream ATP-dependent chaperones such as e.g. HSP70s (De Los Rios and Goloubinoff 2016; Vos et al. 2008). Thus, sHSPs, which bind to a large variety of client proteins, and in doing so act as the first line of defence for the maintenance of cellular proteostasis (Haslbeck and Vierling 2015). In this context, Pierre Goloubinoff (Switzerland) presented results from quantitative proteomic studies from an *E. coli* knockout mutant of DnaK/J (de Marco et al. 2005). He showed that this mutant constitutively over-expressed massive amounts of small HSPs IbpA/B, Trigger factor and ATP-dependent chaperones and proteases. The strong upregulation of sHSPs in this mutant further supports their requirement as a first line of defence under conditions that favour the accumulation of proteins in the non-native state. This can occur also in resting cells, where labile proteins that spontaneously tend to misfold would be constantly restored and maintained in a metastable native state by an active network of ATP-fuelled unfoldases, assisted by small HSPs (De Los Rios and Goloubinoff 2016; Veinger et al. 1998).

Intriguingly, Bernd Bukau (Germany) presented a comparative biochemical analysis of the two sHsps acting in the cytosol of *S. cerevisiae*, Hsp26 and Hsp42. The results reveal that heat denatured model substrates are retained in near native state and kept physically separated when complexed with either sHsp, while being unfolded when aggregated without sHsps. However, the fate of the sHSP bound substrate largely depends on its cooperation with the Hsp70/Hsp100 machineries, and on sHSP ability to act in conjunction with this machinery in disaggregation (Ungelenk et al. 2016). In fact, it was discussed that Hsp26, which lacks disaggregase function, is superior in facilitating Hsp70/Hsp100-dependent post-stress refolding. Instead, Hsp42 forms with misfolded proteins light scattering aggregates *in vitro* and microscopically visible large assemblies in the yeast cytosol; formation of these aggregates specifically ensures cellular fitness during repeated heat stress (Ungelenk et al. 2016). The presented findings indicate that the sHSPs of a cell functionally diversify in stress defence,

but share the working principle to promote sequestration of misfolding proteins for storage in native-like conformation. They also further highlight the requirement to cooperate with the Hsp70/Hsp100 machineries to ensure client refolding.

Krzysztof Liberek (Poland) presented recent advance in the understanding of how bacterial sHSPs cooperate with the HSP70 machinery (Strozecka et al. 2012). Under heat stress conditions sHSPs form assemblies with misfolded proteins, preventing them from further aggregation and keeping them in a refoldable state that facilitates subsequent solubilisation and refolding by ATP-dependent Hsp70 and Hsp100 chaperones. The refolding of substrates from sHsp-substrate assemblies requires the disruption of sHSP association with trapped misfolded proteins. This process depends on Hsp70. Hsp70 acts in a passive manner by outcompeting sHSP molecules that dynamically interact with the surface of sHSP-substrate assemblies. Hsp70 binding to assemblies preserves their architecture following dissociation of sHSPs and allows for superior substrate solubilisation and refolding upon Hsp100 recruitment (Żwirowski S. et al. 2017).

Functional studies of sHSPs: from in vitro assays to combined in vitro/cell assays to understand sHSP cellular activities

In vitro assays with pure recombinant sHSPs are fundamental to understand the structural properties of sHSPs, their oligomerizaton state and how post-translational modifications or disease-linked mutations affect them. Test-tube studies are also required to test and compare the chaperone-like activity of the various sHSPs towards a specific subset of client proteins, allowing how structural differences in sHSPs result in different chaperone efficacy to be highlighted. In particular, Johannes Buchner (Germany) reported the first comparative analysis of 8 human sHSPs with a view to determine their relative chaperone properties (Mymrikov et al. 2016). Amorphous aggregation assays using several model substrate proteins under standard conditions revealed differences between assays and sHSPs. Generally, the large oligomeric sHSPs (HSPB1, HSPB4 and HSPB5) and also HSPB3 proved to be promiscuous chaperones suppressing the aggregation of various substrate proteins. Then, he combined pure recombinant proteins to cell lysates to test and compare the activity of the pure sHSPs to prevent heat-induced protein aggregation in the context of a whole cell lysate. A different

picture emerged in this cell lysate aggregation assays. Here, all sHSPs tested, except HSPB7, were active. These different properties between sHSPs were dependent on, of course, the structural organization and oligomerization of the various sHSPs, as well as their ability to bind with different affinities to a large number of clients, properties that are, to some extent, linked. In line, substrate spectra of the sHSPs determined after immunoprecipitation by mass spectrometry identified a large number of interactors, which revealed general properties and functional classes amongst the 8 human sHSPs compared (Mymrikov et al. 2016).

Similarly, Nikolai Gusev (Russia) compared the interaction of mammalian HSPBs with the light component of neurofilaments (NFL), further highlighting similarities but also differences. HSPB1, HSPB5, HSPB6 and HSPB8 were equally effective in preventing NFL bundling and decreased the quantity of filaments pelleted after low speed centrifugation (Nefedova et al. 2016). HSPB1 and HSPB5 affected kinetics of NFL polymerization and decreased NFL pelleting after high speed centrifugation, probably by affecting hydrodynamic properties of filaments. HSPB8 and especially HSPB6 weakly interacted with NFL but were less effective in modulation of NFL polymerization. Interestingly, sHSPs did not interact with NFL tetramers; however they influenced transition from tetramers to mature filaments, hydrodynamic properties of filaments and their bundling (Nefedova et al. 2016).

Once a client is identified by co-immunoprecipitation and interaction studies, more thorough analysis can be done to understand in detail the biophysical nature of such interaction. In fact, Justin Benesch (UK) showed how to successfully use mass spectrometry and other biophysical methods to elucidate the structure, and quantify the underpinning thermodynamics and kinetics, of sHSP interactions with target proteins. He first revealed a molecular mechanism by which HSPB5 interacts with titin, the giant muscle protein, and modulates the stiffness of heart tissue (Zhu et al. 2009).

Thus, a combination of *in vitro* and combined *in vitro*/cell assays have proven to be extremely useful approaches to provide detailed insights in sHSP structure and interaction with clients, with the

identification of the functional significance of such interaction (HSP70-assisted refolding, holding or assembly formation).

The information obtained from these approaches will help in the interpretation and understanding of functional studies in more complex systems such as endogenous or exogenous expression of a given sHSP in prokaryotic or eukaryotic cells, where post-translational modification of sHSPs or mutation play a role in their regulation or even in whole organisms.

Functional studies of sHSPs in cells and organisms: dissecting their role in keeping the balance and beyond

Although some sHSPs are constitutively expressed, their levels can be increased upon diverse stress conditions, supporting their implication in the cell and organismal stress response. Elizabeth Vierling (USA) presented data concerning higher plants, which express 11 or more distinct gene families of sHSPs, including multiple cytosolic proteins and proteins targeted to every cellular organelle. Vierling used RNAi in transgenic Arabidopsis plants to suppress expression of either cytosolic Class I or II sHSPs, which are the most abundant sHSPs produced during heat stress (McLoughlin et al. 2016). The transgenic plants are sensitive to severe heat stress, consistent with a non-redundant function of these sHSPs. CI and CII proteins also showed distinct biochemical behavior *in vitro* and *in vivo*. Thirty six proteins that were specifically associated with affinity-tagged CI sHSPs during heat stress *in vivo* were identified by mass spectrometry. Of these, twelve are involved in translation and have previously been identified as components of heat stress granules in yeast. These data support the hypothesis that CI sHSPs are involved in recovery of translation after heat stress (McLoughlin et al. 2016).

A similar question (the role of sHSPs in stress response) was addressed from a different perspective and using a different model by Melinda Toth (Hungary), who, in collaboration with Miklós Sántha's and László Vígh's groups studied the role of HSPB1 in the regulation of neuroinflammation (Toth et al. 2010; Toth et al. 2014). As a model system they used a HSPB1 overexpressing transgenic mouse strain. In their latest studies, neuronal damage was induced by a single day of ethanol treatment in 7 day-old mice. Expression levels of inflammatory cytokines and markers of astrocyte and microglia

activation were doubled in the wild-type animals 24 hours after the ethanol treatment, while some of them showed a much higher increase in the ethanol treated transgenic mice, suggesting that HSPB1 can promote cytokine response in the brain *in vivo*, under acute brain injury. This implies that sHSPs might play a specific function at the neuronal level, an area that has been addressed by Nikola Golenhofen and Britta Bartelt-Kirbach (Germany), who showed that HSPB5 possesses powerful neuroprotective capacity in the brain (Bartelt-Kirbach et al. 2016; Golenhofen and Bartelt-Kirbach 2016; Schmidt et al. 2016). They overexpressed HSPB5 in hippocampal neurons to get insights into its function particularly in neurons. Whereas axon length and synapse density were not affected, HSPB5 increased significantly the complexity of the dendritic tree. This stimulating effect of HSPB5 on dendritic branching might become especially relevant during neurodegenerative diseases in dysfunctional neurons with reduced dendritic complexity. Based on their results, Golenhofen's group hypothesized that endogenous upregulation of HSPB5 may serve to maintain dendritic structure and neuronal connectivity (Bartelt-Kirbach et al. 2016).

Further evidence for a protective role of HSPBs in neuronal cells comes from the work presented by Angelo Poletti (Italy), who found a specific upregulation of HSPB8 in motor neurons that survive at end stage of disease in the spinal cord of mice carrying a mutation in the SOD1 gene linked with amyotrophic lateral sclerosis (ALS) (Crippa et al. 2010). Mechanistic studies in cell models expressing a variety of disease associated mutant proteins (mutant SOD1, TDP-43, ARpolyQ) (Crippa et al. 2010; Rusmini et al. 2013) or dipeptides generated by abnormal translation of the C9ORF72 gene product showed that HSPB8 overexpression counteracts their accumulation. This HSPB8 activity is likely mediated by the facilitation of the autophagic system and by enhancing the routing of these various misfolded proteins to the autophagosomes (Cristofani et al, Autophagy 2017 in press).

Heath Ecroyd (Australia) extended this protective effect of HSPBs in yet another neurodegenerative disease model, Parkinson's disease, which is characterized by the accumulation of alpha-synuclein into amyloid fibrils. Previous work already demonstrated that the protein deposits associated with diseases such as Parkinson's disease and other motor neuron diseases contain high levels of e.g. HSPB1 and HSPB5 (Cox et al. 2014; Ecroyd and Carver 2009). Heath Ecroyd presented data demonstrating that HSPB1 and HSPB5 bind stably to amyloid fibrils formed by alpha-synuclein and,

in doing so, prevent the cytotoxicity associated with the fibril. Moreover, HSPB1 and HSPB5 can prevent the aggregation of alpha-synuclein in cells (Cox et al. 2014; Cox et al. 2016). Interestingly, Ecroyd proposed that these sHSPs should not be considered generic inhibitors of protein aggregation in cells, but rather act in cells on specific intermediate states or substrates; in support of this he reported that HSPB1 and HSPB5 had no effect on the intracellular aggregation of the substrate firefly luciferase.

Finally, Hassane Mchaourab (USA) presented data concerning the use of zebrafish as model to study the properties and functions of sHSPs. In particular, he reported on the utility to use zebrafish as model to study cataract and identify key players of the proteostasis network that are required to maintaing lens transparency (Wu et al. 2016).

sHSP in cardiac and muscular diseases

The family of mammalian sHSPs (HSPBs) includes 10 members (HSPB1-HSPB10). While some members are widely expressed, such as e.g. HSPB1, HSPB5 and HSPB8 other members show a very restricted expression pattern; for example, HSPB4 is mainly expressed in the lens, although it was recently also found in pancreatic cells, where it negatively regulates tumorigenesis (Kappe et al. 2003; Liu et al. 2016). HSPB2 and HSPB3 are specifically expressed in differentiating and mature cardiac and muscle cells, while HSPB9 and HSPB10 are testis specific (Fontaine et al. 2003; Kappe et al. 2003; Sugiyama et al. 2000; Suzuki et al. 1998; Verschuure et al. 2003). Cardiac and skeletal muscle cells express the largest variety of sHSPs: HSPB1, HSPB2, HSPB3, HSPB6, HSPB7 and HSPB8. Evidence exists supporting their important role for the viability and function of myocardium, skeletal muscles and neuromuscular systems. Moreover, mutations in HSPB1, HSPB3, HSPB5 and HSPB8 have been directly associated with myofibrillar myopathy or motor neuron disease, providing further evidence for the importance of these sHSPs in muscle and motor neuron maintenance (Boncoraglio et al. 2012). sHSPs mutations could lead to disease both via a loss of function or gain of toxic function mechanisms, or both. During this meeting, Harm H Kampinga (The Netherlands) showed data about mutations in HSPB5 related to dominantly inherited (cardio)myopathies (Bova et al. 1999; Hishiya et al. 2010; Perng et al. 1999b; Rajasekaran et al. 2007; Simon et al. 2007; Treweek et al. 2005; Vicart et

al. 1998). Expression of these mutants in cardiomyocytes leads to the accumulation of HSPB5 aggregates that are positive for ubiquitin and are associated with contractile dysfunction. Aggregation reverted upon introduction of second mutations in the IXI motif or hydrophobic pocket in the alpha-crystallin domain. This suggests that the mutants act via a dominant negative effect on hetero-oligomeric complexe dynamics, which can be alleviated by weakening their inter-dimer interactions (Vonk et al., in preparation). This is in line with published findings that aggregation of mutant HSPB5 forms can be alleviated by increasing the levels of those other HSPB members that hetero-oligomerize with HSPB5, including wildtype HSPB5 itself (Hussein et al. 2015). Ivor J. Benjamin (United States) discussed the disease modelling of early childhood myofibrillar myopathy caused by a homozygous recessive mutation in HSPB5, 343delT (Mitzelfelt et al. 2016). Both standard cell culture systems and induced pluripotent stem cells (iPSCs), derived from the 343delT patient (343delT/343delT), were used to characterize HSPB5 343delT protein dynamics in skeletal myotubes (iSKMs) and cardiomyocytes (iCMs). The truncated protein found in the patient was extremely insoluble under basal conditions and was only observed after HSPB5 343delT overexpression with induction of a cellular stress response. HSPB5 343delT is a classic loss of function mutation; the additional findings that the solubilization of 343delT by WT could explain the absence of symptoms in carrier individuals (Mitzelfelt et al. 2016). Serena Carra (Italy) reported the identification of two novel mutations in the HSPB3 gene in patients affected by myopathy (Carra unpublished). The two mutations of HSPB3 identified by Carra's group lead to protein aggregation or truncation and destabilization, respectively. Interestingly, both mutations, directly or indirectly, abrogate HSPB2-HSPB3 complex formation, leading to an exceeding free pool of HSPB2 that tends to mislocalize inside the cells (Carra unpublished). Dr. Carra discussed the possibility that deregulated HSPB2-HSPB3 interaction would lead to deregulation of HSPB2, with potential consequences on myoblast function and viability.

Combined these studies highlight that imbalances in the expression levels and solubility of specific sHSPs, due to aggregation propensity and deregulated association with other sHSPs or other clients, could be the basis of complex diseases such as myopathies and neuromuscular diseases.

Conversely, upregulation of specific sHSPs may protect against cardiac and muscular cell dysfunction and atrophy. Bianca Brundel (The Netherlands) presented work demonstrating the implication of

specific HSPBs in atrial fibrillation (AF) (Ke et al. 2011). AF is the most common sustained clinical tachyarrhythmia. AF is a progressive condition as demonstrated by the finding that maintenance of normal rhythm and contractile function becomes more difficult the longer AF exists (de Groot et al. 2010; Hoogstra-Berends et al. 2012). Recent investigations reveal that HSPB1, HSPB6, HSPB7 and HSPB8 attenuate the promotion of AF in both cellular and Drosophila melanogaster experimental models (Hoogstra-Berends et al. 2012; Ke et al. 2011). Furthermore, studies in humans suggest a protective role for sHSPs against progression from paroxysmal AF to chronic, persistent AF (Brundel et al. 2006). Therefore, manipulation of the HSP system may offer novel therapeutic approaches for the prevention of atrial remodelling.

In addition, the cross-talk between specific sHSPs and the intermediate filaments of the cytoskeleton and its implication in disease was discussed. Roy Quinlan (UK) highlighted the fact that mutations in sHSPs cause cataract, myopathies and neuropathies and these are phenocopied by mutations in intermediate filament proteins (Toivola et al. 2010). The histopathological aggregates that typify such diseases always contain both intermediate filaments and sHSPs. Nevertheless the bigger picture is that HSPs, like intermediate filaments, are stress responsive and together the chaperones and cytoskeleton integrate every aspect of cell biology. Mutations subvert this role and diseases ensue (Perng et al. 1999a; Perng et al. 2016; Perng et al. 1999b; Perng et al. 2004). Thus, Roy Quinlan proposed that sHSPs, like other chaperones, are evolutionary capacitors given their role in cell proliferation, cell death, shape and function, redox potential and ATP levels. However, they should not only be looked at as factors that prevent and control protein misfolding and/or their subsequent aggregation; sHSPs cooperate with the intermediate filament cytoskeleton to act as a transcellular network that not only partitions efficiently the intracellular space of individual cells, but also integrates the individual cell into the context of the tissue, with far more complex implication in (cardiac and muscle) tissue maintenance/function.

Conclusions and future perspectives

The purpose of the Bertinoro workshop was to bring together investigators representing core laboratories in the sHSP field in order to identify and discuss current and future trends in sHSP research. The workshop was successful in that new trends were featured including characterizations of a rapidly expanding repertoire of binding partners, association with a variety of diseases, elaboration of the roles of sHSPs as stability sensors, development of drugs to induce sHSPs, creating protected pre-surgical states and post-surgical therapies in humans, expanding the concept of sHSPs as polydisperse dynamic oligomers, to name several but not all.

Another example of the growing interest in this field is the recent establishment of a database (sHSPdb) for analyses of sHSPs by Jaspard and Hunault (Jaspard and Hunault 2016). To accommodate the increased research efforts on sHSPs, the third in this meeting series will be held in Québec, Canada, in 2018. It will be expanded from a workshop into a CSSI symposium of about 100 participants, principally organized by Robert M. Tanguay.

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