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SMALL HEAT SHOCK PROTEINS

The growing world of small heat shock proteins: from structure to functions

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Abstract Small heat shock proteins (sHSPs) are present in all kingdoms of life and play fundamental roles in cell biology. sHSPs are key components of the cellular protein quality control system, acting as the first line of defense against conditions that affect protein homeostasis and proteome stability, from bacteria to plants to humans. sHSPs have the ability to bind to a large subset of substrates and to maintain them in a state competent for refolding or clearance with the assistance of the HSP70 machinery. sHSPs participate in a number of biological processes, from the cell cycle, to cell

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differentiation, from adaptation to stressful conditions, to apoptosis, and, even, to the transformation of a cell into a malignant state. As a consequence, sHSP malfunction has been implicated in abnormal placental development and preterm deliveries, in the prognosis of several types of cancer, and in the development of neurological diseases. Moreover, mutations in the genes encoding several mammalian sHSPs result in neurological, muscular, or cardiac age-related diseases in humans. Loss of protein homeostasis due to protein aggregation is typical of many age-related neurodegenerative and neuromuscular diseases. In light of the role of sHSPs in the clearance of un/misfolded aggregation-prone substrates, pharmacological modulation of sHSP expression or function and rescue of defective sHSPs represent possible routes to alleviate or cure protein conformation diseases. Here, we report the latest news and views on sHSPs discussed by many of the world's experts in the sHSP field during a dedicated workshop organized in Italy (Bertinoro, CEUB, October 12–15, 2016).

Keywords Small heat shock proteins · Hsp27 · Protein conformation · Neurological diseases · Protein homeostasis · Protein aggregates

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Introduction

Small heat shock proteins (sHSPs) belong to the superfamily of HSPs and are expressed in all three kingdoms (Archaea, Bacteria, and Eukarya) and in viruses (Bult et al. 1996; Caspers et al. 1995; Eyles and Gierasch 2010; Richter et al. 2010). sHSPs are characterized by a small molecular weight, ranging from ca. 15 to 40 kDa and by a highly conserved domain, called the alpha-crystallin domain (ACD) that represents their identification label. From the structural point of view, sHSPs can exist in the form of monomers and dimers but can also assemble into large multimeric complexes that vary in size and contain up to 24-40 subunits (Candido 2002; 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 sHSP post-translational modification, including phosphorylation, which, in turn, regulates sHSP functions. sHSP functions are very diverse and include chaperone-like activity 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 participate indirectly in the regulation of complex processes such as the response and adaptation to cell stress, thermotolerance, cell differentiation, cell movement, cell apoptosis, and development (Arrigo 2000; Arrigo and Ducasse 2002; Arrigo and Gibert 2014; Balogi et al. 2008; Benjamin et al. 1997; Bruey et al. 2000; Doshi et al. 2009; Haslbeck et al. 2016; Hong and Vierling 2000; Kamradt et al. 2002, 2005; Lavoie et al. 1993; Lavoie et al. 1995; Litt et al. 1998; Nicholl and Quinlan 1994; Parcellier et al. 2006; Park et al. 2016; Perng et al. 1999a, b; 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 in a number of diseases and is the cause 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 their malfunction is linked mechanistically to disease will a have strong impact on cell biology and disease and will help identify potential drug targets.

In this review, we summarize the latest news and views concerning sHSP structural and functional properties that were presented and discussed by 29 international experts at the Second International Workshop of Cell Stress Society

International (CSSI) on sHSPs, entitled "The small HSP world," held in Centro Residenziale Universitario di Bertinoro, Italy (October 12–15, 2016). This meeting followed on the first Workshop held in Québec, Canada (Tanguay & Hightower 2015).

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

As mentioned earlier, sHSPs have a dynamic structure that spans from monomers/dimers to large oligomers (van Montfort et al. 2001). Oligomerization and dissociation 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 and identifying their preferred oligomerization state (in resting or stressful conditions), what regulates their transition from small oligomers into large oligomers, and how this influences their binding to clients are crucial in pinpointing sHSP-specific effects (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 an N-terminal domain (NTD), a C-terminal domain (CTD), and a highly conserved middle region, called the alpha-crystallin domain (ACD) (van Montfort et al. 2001). In contrast to the ACD, the CTD and the NTD of sHSPs are less conserved among the various members and across the various species. However, structural and functional studies have demonstrated that all three domains play an 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 that suggests a role for the N-terminal and C-terminal tails in the stabilization of oligomers and in the accessibility for interaction with clients of the oligomer itself (Ahrman et al. 2007a, b; Lambert et al. 2011). In particular, a structural model of Hsp21, obtained after homology modeling and fitting to cryo-EM, shows two hexameric discs rotated by 30° and separated by further a 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 dodecamer outside, as supported also by limited proteolysis, difference density maps, and NMR. Thus, even without subunit dissociation, they may be accessible for transient interaction with client proteins.

In contrast, using mass spectrometry and other biophysical methods, Justin Benesch (UK) showed how different sHSP classes found in the same cellular compartment manage, on a structural level, to avoid co-assembly (in collaboration with Elizabeth Vierling). 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 (Benesch et al. 2012; Painter et al. 2008; Stengel et al. 2010, 2012).

Regulation of sHSP co-assembly and self-oligomerization is indeed one way of regulating the binding affinity of sHSPs for a given substrate (Delbecq et al. 2015; Rajagopal et al. 2015). Rachel Klevit (USA) presented studies 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 her 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 capacity to delay the onset of amorphous aggregates (Rajagopal et al. 2015).

A comparative study performed by Robert M. Tanguay (Canada) highlighted the impact of the oligomerization state on sHsp functions and chaperone-like activity. Following on his 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 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/client). In contrast, the host cyanobacteria sHsp formed a small dimer and tetramer and showed no chaperone-like activity in the assays tested (MDH, CS, 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). 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 configurations with or without domain swapping. The

oxidation of human αA-crystallin, i.e., the formation of an intra-molecular disulfide bond as observed in vivo, seems to be coupled with conformational changes involving CTD swapping. Dr. Haslbeck concluded that oxidized protein shows enhanced subunit dynamics and an increased ability to suppress the aggregation of model substrates.

John Carver (Australia) presented recent work relating to the principal eye lens proteins, αA- and αB-crystallin (HSPB4 and HSPB5) (Cox et al. 2014, 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 crystallin adjustment with age to counter the extensive post-translational modifications that potentially affect lens transparency. 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 destabilization, unfolding, and aggregation of HSPB5. This study further highlights the dynamic nature of sHSPs and shows how external factors regulate their structure (and in turn, function), with consequences for 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 performed structural studies of human HSPB6, which is predominantly dimeric in isolation (Heirbaut et al. 2014, 2016; Weeks et al. 2014). Phosphorylation of HSPB6 within its intrinsically disordered N-terminal domain (NTD) results in a complex formation with the universal signaling hub 14-3-3, 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. 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 HSPB1/HSPB6 hetero-oligomer formation, which turns out to be driven by specific sequences within the NTD of HSPB6, has been discussed (Sluchanko et al. 2011, 2012, 2014).

Finally, André- Patrick Arrigo (France) further stressed the importance of changes in the phosphorylation and oligomerization state in the regulation of sHSP interaction with clients (Arrigo 2013). He summarized the modifications that occur in HSPB1 phosphorylation/oligomerization in cells undergoing changes in their physiology or exposed to environmental stressors. It was concluded that the changes in HSPB1 organization are highly condition-specific. 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 response to heat shock, damaged polypeptides are stored in HSPB1 structures that have a phosphorylation specific signature: P-ser15 and P-ser82. Importantly, these conformational changes are very transient and thereby 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, 2007; Arrigo and Gibert 2012).

These studies have far-reaching implications for the evolution of protein oligomers in general and illustrate the complexity of sHSP structure/function, with some sHSPs stabilizing oligomeric structures to favor 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 in the relationship 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 in the neighboring environment. As pointed out by André-Patrick Arrigo, cell-cell contact may also play a role in the regulation of sHSP structure, further increasing the level of complexity.

From the technical point of view, the main methods of studying sHSP structure include 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 specificity is determined for sHsps, with the use of gold nanoparticles. In addition, the use of different scattering techniques is also beneficial in characterizing the structural and functional properties of various sHSPs, as discussed by Stephanie Finet (France) who presented data concerning nuclear Hsp27 from *Drosophila melanogaster*, obtained in collaboration with R. M. 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 His-tag construct. The effects of mutations of the R residues in the ACD of the nuclear Hsp27 of Drosophila were also evaluated by Robert M. Tanguay (Canada) in collaboration with Stephanie Finet. While two 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 tag and its influence on sHSP structure and oligomerization was 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 useful new method of studying the dynamic oligomerization of sHSPs has been proposed by Wilbert Boelens (The Netherlands). In this method, 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 chaperoning activity. To date, most studies have focused on the dynamic behavior of the multimeric complexes rather than 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 understanding 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 and "hold" them, avoiding their irreversible aggregation. This in turn favors the processing of the sHSP-bound client by the downstream ATP-dependent chaperones such as, e.g., HSP70s and their fate (De Los Rios and Goloubinoff 2016; Vos et al. 2008). Thus, sHSPs, which bind to a large variety of substrates, act as the first line of defense for the maintenance of the 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 overexpressed large amounts of small HSPs IbpA/B, Trigger factor, and ATP-fuelled chaperones and proteases. The strong upregulation of sHSPs in this mutant further supports their requirement as a first line of defense under conditions that favor the accumulation of proteins in the non-native state. This can also occur 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-fueled unfoldases, assisted by small HSPs (De Los Rios and Goloubinoff 2016).

Intriguingly, Bernd Bukau (Germany) presented a comparative biochemical analysis of the two sHsps acting in the cytosol of *Saccharomyces 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 as a "disaggregase" (Ungelenk et al. 2016). In fact, it was discussed that Hsp26, which lacks aggregase function, is superior in facilitating Hsp70/Hsp100-dependent post-stress refolding. Instead, Hsp42 forms with misfolded protein 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 findings presented indicate that the sHSPs of a cell are functionally diversify in stress defense but share the working principle to promote sequestration of misfolding proteins for storage in native-like conformation. They also further highlight the need to cooperate with the Hsp70/Hsp100 machineries to ensure client refolding.

Krzysztof Liberek (Poland) presented recent advances in the understanding of how bacterial sHSP 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 solubilization 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 solubilization and refolding upon Hsp100 recruitment (Klosowska et al. 2016).

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

In vitro assays with pure recombinant sHSPs are fundamental for understanding the structural properties of sHSPs, their oligomerization 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 toward a specific subset of substrates, highlighting how structural differences in sHSPs result in different chaperone power. Johannes Buchner (Germany) reported the first comparative analysis of eight human sHSPs to determine their chaperone properties (Mymrikov et al.

2016). 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. Buchner then combined pure recombinant proteins to cell lysates to test and compare the activity of the pure sHSPs in the context of a whole cell lysate. A different picture emerged in cell lysate aggregation assays. Here, all sHSPs tested, except HSPB7, were active. These different properties depend in part on the structural organization and oligomerization of the various sHSPs and their ability to bind with different affinities to a large number of clients, properties that are to some extent linked. Substrate spectra of the sHSPs determined after immunoprecipitation by mass spectrometry identified a large number of interactors, which revealed general properties and functional classes among the eight 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 both similarities and 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 affecting the hydrodynamic properties of filaments. HSPB8 and especially HSPB6 weakly interacted with NFL but were less effective in the modulation of NFL polymerization. Interestingly, HSPBs did not interact with NFL tetramers; however, they influenced the 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. Justin Benesch (UK) showed how to use mass spectrometry successfully 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 has proven to be extremely useful approaches in providing detailed insights into sHSP structure and interaction with clients, with the identification of the functional significance of such interaction (HSP70-assisted refolding, holding, or assembly formation).

These results 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 whole prokaryotic or eukaryotic cells, where post-translational modification of sHSP or mutation plays 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, 12 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, 2014). They used a HSPB1-overexpressing transgenic mouse strain as a model system. 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 h 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 (Toth et al. 2010, 2014). This implies that HSPBs might play a specific function at the neuronal level, a question that has been addressed by Nikola Golenhofen and Britta Bartelt-Kirbach (Germany), who showed that HSPB5/αB-crystallin possesses powerful a 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 gain 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, Golehnofen'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 the 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 mediated by the facilitation of the autophagic system and by enhancing the routing of these various misfolded proteins to the autophagosomes (Data from Poletti lab).

Heath Ecroyd (Australia) extended this protective effect of HSPBs in yet another neurodegenerative disease model, Parkinson's disease, characterized by the accumulation of alpha-synuclein into amyloid fibrils. Previous work demonstrated that the protein deposits associated with diseases such as Parkinson's and other motor neuron diseases contain high levels of HSPB1 and HSPB5 (Ecroyd and Carver 2009). Ecroyd demonstrated 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, 2016; Hochberg et al. 2014; Treweek et al. 2015). Interestingly, Ecroyd proposed that these sHSPs should not be considered generic inhibitors of protein aggregation in cells, but rather that they would act in cells on specific intermediate states or substrates; in fact, 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 a model to study the properties and functions of sHSPs. In particular, he reported on the utility to use zebrafish as a model to study cataract and identify key players of the proteostasis network that are required to maintain lens transparency (Wu et al. 2016).

sHSP in cardiac and muscular diseases

The family of mammalian sHSPs (HSPBs) includes ten members (HSPB1–HSPB10). While some members are widely expressed such as HSPB1, HSPB5, and HSPB8, other members show a very restricted expression pattern; for example, HSPB4 is expressed only in the lens, 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 HSPBs: HSPB1, HSPB2, HSPB3, HSPB6, HSPB7, and HSPB8. Evidence supports their important role in the viability and function of myocardium, skeletal muscles, and neuromuscular systems. Moreover, mutations in HSPB1, HSPB3, HSPB,5 and HSPB8 have been directly associated with myofibrillar myopathy or motor neuron disease, further demonstrating the importance of these HSPBs in muscle and motor neuron maintenance (Boncoraglio et al. 2012). Recent studies confirm that these HSPB mutations can lead to disease both via a loss of function or by the addition of toxic function mechanisms, or both. 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 was reserved upon introduction of second mutations in the IxI motif or hydrophobic pocket in the alpha-crystalline domain. This suggests that the mutants act by dominant negative of hetero-oligomeric complex formation, which can be alleviated by weakening their interdimer interactions. Mutant aggregation could be alleviated by increasing the other HSPB members that hetero-oligomerize with HSPB5, including wild-type HSPB5 itself. Ivor J. Benjamin (USA) discussed the disease modeling 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 observed 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 classical loss-of-function mutation with our additional findings that the solubilization of 343delT by WT explained 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 a 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. Taken together, these studies highlight that imbalances in the expression levels and solubility of specific HSPBs due to aggregation propensity and deregulated association with other HSPBs or other clients could be the basis of complex diseases such as myopathies and neuromuscular diseases.

Conversely, upregulation of specific HSPBs may protect against cardiac and muscular cell dysfunction and atrophy. Bianca J. J. M. Brundel (The Netherlands) demonstrated the implication of specific HSPBs in atrial fibrillation (AF) (Ke et al. 2011). AF is the most common sustained clinical tachyarrhythmia and is a progressive condition; maintenance of normal rhythm and contractile function becomes more difficult the longer AF exists (den Hoed et al. 2013; Hoogstra-Berends et al. 2012). Recent investigations reveal that HSPB1, HSPB6, HSPB7, and HSPB8 attenuate the promotion of AF in both cellular and *D. melanogaster* experimental models (Hoogstra-Berends et al. 2012; Ke et al. 2011). Furthermore, studies in humans suggest a protective role for HSPBs against progression from paroxysmal AF to chronic, persistent AF. Therefore, manipulation of the HSP system may offer novel therapeutic approaches for the prevention of atrial remodeling.

In addition, the cross talk between specific HSPB and the intermediate filaments of the cytoskeleton and its implication in disease was discussed. Roy Quinlan (UK) demonstrated that mutations in HSPBs 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, b, 2004, 2016). Thus, Roy Quinlan proposed that HSPBs, 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. HSPBs would 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 implications in (cardiac and muscle) tissue maintenance/function.

Conclusions and future perspectives

The Bertinoro workshop brought together investigators representing core laboratories in the sHSP field to identify and discuss current and future trends in sHSP research. 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 presurgical states and post-surgical therapies in humans, and the expansion of the concept of sHSPs as polydisperse dynamic oligomers, to name several.

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 (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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