Meeting Review

Small Heat Shock Proteins: multifaceted proteins with important implications for life.

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SUMMARY

Small Heat-Shock Proteins (sHSPs) evolved early in the history of life; they are present in archaea, bacteria and eukaryota. sHSPs belong to the superfamily of molecular chaperones: they are components of the cellular protein quality control machinery and are thought to act as the first line of defense against conditions that endanger the cellular proteome. In plants, sHSPs protect cells against abiotic stresses, providing innovative targets for sustainable agricultural production. In humans, sHSPs (also known as HSPBs) are associated with the development of several neurological diseases. Thus, manipulation of sHSP expression may represent an attractive therapeutic strategy for disease treatment. Experimental evidence demonstrates that enhancing the chaperone function of sHSPs protects against age-related protein conformation diseases, which are characterized by protein aggregation. Moreover, sHSPs can promote longevity and healthy aging in vivo. In addition, sHSPs have been implicated in the prognosis of several types of cancer. Here, sHSP upregulation, by enhancing cellular health, could promote cancer development; on the other hand, their downregulation, by sensitizing cells to external stressors and chemotherapeutics, may have beneficial outcomes. The complexity and diversity of sHSP function and properties, the need to identify their specific clients, as well as their implication in human disease have been discussed by many of the world's experts in the sHSP field during a dedicated workshop in Québec City, Canada, on the 26-29 of August 2018).

INTRODUCTION

Small Heat Shock Proteins (sHSPs) are ATP-independent molecular chaperones conserved across species, and expressed throughout the kingdoms of life from archaea to humans (Bult et al., 1996; Caspers et al., 1995; Eyles and Gierasch, 2010; Richter et al., 2010). sHSPs form dynamic oligomeric complexes that can exchange subunits and can dissociate into dimers and monomers; the latter can be influenced by post-translational modifications, including phosphorylation which tends to promote oligomer dissociation (Candido, 2002; Hilton et al., 2012; Kim et al., 1998; McDonald et al., 2012; van Montfort et al., 2001b). The flexibility of sHSP assembly is thought to be important in regulating their binding affinity for non-native and misfolded proteins, preventing irreversible protein aggregation. Once complexed with sHSPs, these non-native and aggregated proteins can be recovered and refolded with the assistance of ATP-dependent chaperones such as the HSP70 system. Thus, sHSPs can be considered as sponges that neutralize non-native and aggregate-prone intermediates, safeguarding the cellular proteome in response to stress. Importantly, sHSPs can be themselves part of proteinaceous inclusions; here, their role is to facilitate disaggregation (Mogk and Bukau, 2017; Mogk et al., 2018). Based on these findings, sHSPs act not only as “holdase” chaperones, as was thought for a long time, but they actively regulate the aggregation process, improving the ability of the cells to recover protein homeostasis following exposure to different stress conditions, such as temperature upshift or oxidative stress.

sHSPs are not only important in regulating the cell stress response and responding to changes in cellular conditions. Many sHSPs are expressed in normally growing cells, where they can regulate the interactions between native proteins, shaping protein-protein interactions; this, in turn,

influences disparate cellular functions, ranging from cell signalling to differentiation and apoptosis. Understanding how sHSP oligomerization and sHSP-substrate binding are regulated, how sHSP structure-function is influenced by post-translational modifications, and identifying selective and cell specific sHSP targets will shed light on their roles in thermotolerance, cell differentiation, development and cell death (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).

Due to their ability to interact promiscuously with a large variety of substrates and to their dynamic oligomerization, it is not surprising that deregulated expression of sHSPs and genetic mutations in several human sHSP/HSPB genes have been linked to different pathological conditions, including congenital cataracts, neuromuscular and age-related protein conformation diseases, as well as cancer (Evgrafov et al., 2004; Ghaoui et al., 2016; Irobi et al., 2004; Kolb et al., 2010; Morelli et al., 2017; Nam et al., 2018; Perng et al., 1999b; Vicart et al., 1998). Unravelling how disease-linked mutations in human HSPB genes affect their properties and functions will highlight potential drug targets for future therapeutic approaches.

sHSPs are also implicated in plant stress resistance and fungal and microbial infections (Haslbeck and Vierling, 2015; Mayer et al., 2012). Thus, dissecting the relationship between sHSP structure-function will open new avenues for biotechnological interventions aimed at improving plant stress

tolerance to abiotic insults, with the ultimate goal of ameliorating seed maturation and crop production, and developing pharmacological approaches for the treatment of human infectious diseases.

Here, we summarize recent advances in the field of sHSPs that were discussed by international experts during the Third Cell Stress Society International (CSSI) Workshop on sHSPs that was held in Québec, Canada (26-29 August 2018). This meeting followed on the Second International Workshop, organized in 2016 in Bertinoro, Italy by Serena Carra and Robert M. Tanguay (Carra et al., 2017), and further highlighted the need for building an interdisciplinary worldwide community for the advanced study of sHSPs.

sHSP structure: order among disorder

sHSPs belong to the superfamily of proteins with disordered domains, which represent up to 40% of the eukaryotic proteome and are present in all three kingdoms of life (Oates et al., 2013; Potenza et al., 2015; Ward et al., 2004). Disordered segments have been considered for a long time as protein fragments without any specific function, which are mainly required to link protein domains with well-defined three-dimensional structures. However, recent studies have demonstrated the biological importance of disordered sequences, adding the disorder-function paradigm to the existing structure-function paradigm (Dyson and Wright, 2005). Intrinsically disordered regions (IDRs) or low complexity domains are polypeptide segments enriched for polar or charged amino acids, with low/no hydrophobic amino acids and that lack a defined three-dimensional structure in their native state. IDRs can adopt extended or compact conformations and offer several advantages

to proteins. First, by exposing short linear motifs, disordered proteins have the ability to act as scaffolds and promiscuously interact with a large variety of substrates. This, in turn, enables the formation of dynamic macromolecular assemblies. Second, disordered proteins can acquire different conformations when interacting with different substrates, thereby functioning as signalling and regulatory proteins. Third, by being post-translationally modified, IDRs can regulate protein functionality. In agreement, many signalling molecules, kinases, splicing factors and transcription factors are disordered proteins (Babu, 2016; Jana et al., 2001).

From the structural point of view sHSPs are composed of three domains: the N-terminal domain (NTD), the middle alpha-crystallin domain (ACD) and the C-terminal domain (CTD) (van Montfort et al., 2001b). The ACD contains several β-strands that form two β-sheets arranged similarly to immunoglobulin, while the C- and N-terminal domains are intrinsically disordered (Sudnitsyna et al., 2011). In contrast to the ACD, which is conserved and represents the signature of the sHSP family, the C- and N-termini are only marginally conserved among the various sHSPs and across species. Together these three domains contribute to regulate the dynamic association of sHSP monomers, which have low molecular masses (13-43 kDa), into large oligomers, which can contain at the top range 24-40 subunits (Van Montfort et al., 2001a; van Montfort et al., 2001b).

The role of hetero-oligomerization on sHSP structure and function remains enigmatic. This aspect has been addressed by Dr. Buchner (Germany). He reported the structure of the eye lens specific sHsp alpha-A crystallin (HSPB4) solved by cryo-electron microscopy, together with Sevil Weinkauf's group (Germany). Besides identifying the principles of assembly the structure(s) also reveal the basis for the heterogeneity of the oligomeric ensemble.

10 different sHsps are encoded in the human genome. They differ in their oligomeric states and chaperone activities (Mymrikov et al., 2017). Some of them are expressed simultaneously in cells and are known to form mixed complexes (Haslbeck et al., 2018). This phenomenon can be considered as a means to regulate sHsps function. However, the impact of hetero-oligomers on the mechanism of sHsps remained enigmatic. Dr. Buchner and colleagues analyzed hetero-oligomer formation in vitro and in cells. They report that hetero-oligomerization affects the size distribution of the oligomers and also their chaperone activities.

sHSP oligomerization is a dynamic process that can be influenced by changes in pH (Fleckenstein et al., 2015) and post-translational modifications, such as phosphorylation (Aquilina et al., 2004; Arrigo and Gibert, 2012; Benn et al., 2002; Maitre et al., 2012). The phosphorylation sites are often located within the disordered N-terminal or C-terminal domain of many sHSPs. Phosphorylation induces conformational changes and influences sHSP hydrophobicity and binding affinity to other proteins. A direct link has been demonstrated between phosphorylation of specific sites located in disordered sHSP regions of sHSPs. Changes in oligomerization and chaperone activity have been shown using human HSPB1 and HSPB5 phosphomimicking mutants and non-phosphorylatable mutants, as well as deletion mutants or chimeric sHSP mutants. Together, these data point to the importance of disordered domains for the modulation of sHSP chaperone-like function (Delbecq and Klevit, 2013; Delbecq et al., 2015; Ecroyd et al., 2007; Giese et al., 2005; Giese and Vierling, 2002; Hilton et al., 2012; McDonald et al., 2012; McHaourab et al., 2002; Peschek et al., 2013; Stromer et al., 2004; Van Montfort et al., 2001a). This is further supported by studies using yeast Hsp42. The N-terminal domain of Hsp42 contains a prion-like domain (PrLD) and a canonical IDR that act in a coordinated manner to promote formation of

stress-induced macromolecular assemblies by recruiting misfolded proteins and regulating their aggregation. The ability of Hsp42 to sequester misfolded proteins into large proteinaceous aggregates is important to protect cells from proteotoxic insults and for cell fitness (Grousl et al., 2018).

However, the scenario for sHSPs is more complex, since also the disordered CTD and the folded ACD regulate sHSP oligomerization and, consequently, affinity for a given substrate, which can then vary based on the binding interfaces that are exposed by sHSP. In fact, recent studies from Dr. Reif (Germany) and Buchner, using state-of-the-art NMR spectroscopy, showed that while the ACD of HSPB5 preferentially binds to amyloid-like fibrils, such as Alzheimer disease linked Abeta1-40, the disordered N-terminal domain of HSPB5 preferentially captures amorphously aggregating substrates, such as e.g. lysozyme. The intrinsic structural plasticity of sHSP, which can expose different binding interfaces, confer on them the ability to bind to a wide range of structurally heterogeneous clients. This inherent structural plasticity represents a powerful way of regulating promiscuous interaction with a large variety of substrates, and timely regulating transient interaction with a given substrate. Indeed the oligomerization state of sHSPs is influenced by reversible and transient post-translational modifications, enabling the same sHSP to display differential affinity for a given substrate (Grousl et al., 2018; Mainz et al., 2015; McDonald et al., 2012; Sudnitsyna et al., 2011; van Montfort et al., 2001b). Thus, the common theme that is emerging from several studies performed using sHSP from various species and by mutating specific sites is that protein disorder contributes to regulate the dynamic assembly and disassembly of oligomers of variable size. This dynamic assembly, in turn, is a prerequisite for sHSP

functionality, since it confers transient and variable affinity for a heterogeneous class of proteins in response to external stimuli, stressors, and also developmental and pro-differentiation stimuli.

Another example in support of sHSP "disorder" as an important feature regulating their functionality is recent work discussed during this meeting by Dr. Carver (Australia). Together with his colleagues, Dr. Carver investigated the structure and molecular chaperone action of αB-crystallin (HSPB5) under in vitro macromolecular crowding conditions induced by the inert polysaccharide, Ficoll 400. HSPB5 and its partner small heat-shock protein αA-crystallin (HSPB4) are the predominant eye lens proteins that, with the unrelated β- and γ-crystallins, arrange themselves in an ordered supramolecular array that enables proper refraction and focussing of light onto the retina. The crystallins are present at very high concentrations in the eye lens (up to 400 mg/mL in the centre of aged lenses). Highly crowded conditions cause excluded volume effects that can alter protein structure, for example leading to unfolding and aggregation. In contrast to HSPB4, HSPB5 is present extensively outside the lens where it has a molecular chaperone role to stabilise proteins to prevent their unfolding, for example under conditions of cellular stress. Performing small-angle neutron scattering (SANS) of deuterated HSPB5 and protonated Ficoll 400 solutions, Dr. Carver and colleagues studied the conformation of HSPB5 under crowded conditions comparable to those in the lens (and in many other cells). Under these conditions at physiological temperatures over 30 hours, HSPB5's central, conserved β-sheet region (the ACD) unfolded, which was accompanied by co-association of the protein to form amorphous and subsequently fibrillar, possibly amyloid-like, aggregates. Despite these structural alterations, HSPB5 retained its chaperone ability to prevent the aggregation of destabilised proteins, implying that the unfolded state is the chaperone functional form of HSPB5 in intra- and extra-lenticular

environments. This conclusion is consistent with many other studies showing that HSPB5 functions effectively as a chaperone under destabilised conditions (for example low pH, high temperature and in the presence of denaturant) and when fragmented into its peptide components. Of note, changes in pH and high temperature regulate the dynamic assembly and liquid-demixing of many disordered proteins, with important biological implications (Alberti, 2017; Riback et al., 2017). This remarkable finding opens the question: To what extent, under these unfolded state conditions, do the intrinsically disordered terminal regions of HSPB5 contribute to regulating its chaperone activity? Thus, HSPB5 is a highly malleable protein that exhibits characteristics of an intrinsically disordered protein, including during its chaperone action.

Experimental evidence in support of the importance of disordered regions as regulators of chaperone function exists not only for sHSPs. We cite as examples Hsp33 and San1. Hsp33 possesses a redox-sensor domain that unfolds upon oxidative stress. Importantly, Hsp33 can bind to misfolded proteins only in this unfolded state, thereby inducing folding and stabilizing folding intermediates that can then be fully refolded by ATP-dependent foldases (Reichmann et al., 2012). San1 is a yeast nuclear E3 ubiquitin ligase that uses its disordered N- and C-terminal domains to recognize misfolded proteins and target them for proteasome-mediated destruction (Rosenbaum et al., 2011). Collectively these findings support the conclusion that IDRs are important functional regions for chaperones, including sHSPs. Identifying the molecular grammar that regulates sHSP oligomer dissociation and specific sites that may lock a given sHSP into a conformationally active or inactive state will advance our understanding of how sHSP recognize and bind specific substrates, regulating heterogeneous biological processes. We anticipate that such molecular grammar may differ from sHSP to sHSP, pointing to the need for careful study of each sHSP using

interdisciplinary in vitro and in vivo approaches. This, in turn, highlights the need to develop new strategies to study sHSP structure/function relationships.

Aware of this complexity, Dr. Klevit (USA) presented a talk entitled “sHSPs: the more we know, the more we don’t understand” in which she presented new structural and biochemical data on human HSPB1. Although ubiquitously expressed and implicated in several serious neuropathies, structural information on HSPB1 is lacking. Substitution of three serine residues in the disordered N-terminal domain (NTD) known to be phosphorylation sites under stress to aspartic acid and mutation of the IxI motif in the C-terminal region yield a fully dispersed dimeric form of HSPB1 that is an effective chaperone that delays aggregation of Tau (Baughman et al., 2018). This form, called HSPB1 dimer, is presumed to mimic a stress-activated HSPB1. Application of solution NMR and hydrogen-deuterium exchange/mass spectrometry (HDXMS) to HSPB1dimer and to wild-type HSPB1 oligomers offered a first structural glimpse at the disordered N-terminal domain (NTD). Although the NTD is dynamic and heterogeneous, the NMR results show that it is in contact with the structured alpha-crystallin domain that defines the dimer. In the phosphomimic HSPB1dimer, the phosphorylation sites are highly exposed, indicating that they are available for protein-protein interactions. Curiously, two missense mutations implicated in neuropathies, G34R and P39L, have distinct effects on both the local and global structure and dynamics of HSPB1 despite being only 5 residues apart in a disordered region of the NTD. These studies represent the first atomic-level information regarding the NTD of HSPB1, where a majority of disease-associated mutations are harboured and demonstrate the ability of hydrogen:deuterium exchange mass spectrometry (HDXMS) to provide novel structural and dynamic insights into sHSPs.

The complexity of structure/function relationships and identification of key molecular grammars is further increased when considering that different sHSPs can interact with each other forming hetero-oligomers. Dr. Boelens (The Netherlands) presented a recent study of HSPB2 and HSPB3 structure. These two vertebrate sHSPs interact in neuronal and muscular cells. They are components of skeletal and cardiac muscle and are upregulated during differentiation of myoblasts. Although their exact functions within the neuromuscular system are still enigmatic, variants and mutations of HSPB3 (R7S, R116P, A33fsX50 and Y118H) are associated with neuromuscular diseases, including axonal Charcot-Marie-Tooth disease (Kolb et al., 2010; Morelli et al., 2017; Nam et al., 2018). These findings strongly suggest that HSPB3, and its partner HSPB2 play important roles for the functionality of neuronal and muscular cells. HSPB2 and HSPB3 co-assemble into a tetramer with a 3:1 ratio, a unique composition within the sHsp family. Although this tetramer forms the building block for higher oligomer assemblies, consisting of 8-24 subunits, it is potentially a more tractable target for structural study than other human sHsps. By assessing the assembly and flexibility of HSPB2 and HSPB3 from both rat and human by means of size-exclusion chromatography, native mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, a human HSPB3 mutant was engineered that suppresses the formation of higher assemblies of HSPB2/B3. The resulting monodisperse hetero-tetramer was crystallized and solved at a resolution of 3.9 Å (Clark et al., 2018). In the HSPB2-HSPB3 tetramer, the four α-crystallin domains (ACDs) assemble to form a flattened tetrahedron that is pierced by two non-intersecting approximate dyads and portions of the unstructured N-terminus bind to the ACD grooves. Detailed description of the structure of HSPB2-HSPB3 assemblies can be found elsewhere (Clark et al., 2018). Future studies should aim at understanding how the heterogeneous interactions and plasticity of HSPB2-HSPB3 heteromers regulate their physiological functions.

Another typical feature of IDR-containing proteins is their ability to undergo liquid-liquid phase separation (LLPS) and form membraneless organelles or biomolecular condensates that have heterogeneous composition (Banani et al., 2017). Examples of membraneless organelles are cytoplasmic stress granules and P-bodies, and nuclear speckles, paraspeckles, PML bodies, Cajal bodies, as well as DNA damage foci (Banani et al., 2017). Although sHSPs possess IDRs, only limited evidence exists in support of their ability to undergo LLPS. For example, while the role of Hsp42-IDR in modulating its chaperone activity has been experimentally demonstrated, evidence for Hsp42 undergoing LLPS is still lacking (Grousl et al., 2018). Instead, Dr. Carra (Italy) presented recent evidence demonstrating that human HSPB2 undergoes concentration-dependent LLPS in mammalian cells. When the local concentration of HSPB2 reaches a critical threshold, HSPB2 forms nuclear membraneless compartments that sequester nuclear lamin-A, affecting its dynamics and function; this has functional consequences on chromatin organization and gene expression in mammalian cells, including HeLa and muscle cells (Morelli et al., 2017). HSPB2 phase separation is negatively regulated by its stoichiometric partner HSPB3, but not by two HSPB3 mutants linked to congenital myopathy. Based on these findings, it was suggested that deregulation of HSPB2 compartmentalization, due to decreased HSPB3 expression or HSPB3 mutations, could contribute to muscle aging and disease by affecting nuclear lamin distribution/function. Whether HSPB2 phase separation is required for its chaperone function is yet unclear. Whether lamin-A is a specific substrate of HSPB2 that is sequestered into the condensates observed in mammalian cells is not yet clear. Moreover, considering the regulatory role of HSPB3 on HSPB2 phase separation, it will be important to further understand how these proteins interact and what regulates their dynamic assembly/disassembly. Detailed understanding

of HSPB2/B3 co-assembly (Clark et al., 2018) could be exploited in the future to understand how these heterogeneous interactions influence HSPB2 LLPS, with potential functional implication on nuclear lamins.

Although direct demonstration of sHSP undergoing LLPS is currently limited, several independent experimental findings suggest a strong link between sHSP, membraneless compartments that originate via LLPS, stress response and disease. For example, human HSPB1 and HSPB8, as well as plant Hsp23 have been localized to stress granules (Arrigo et al., 1988; Ganassi et al., 2016; Jain et al., 2016; Kedersha et al., 1999; Mateju et al., 2017; Nover et al., 1989), while human HSPB1 and HSPB5 have been localized to nuclear splicing speckles (Adhikari et al., 2004; den Engelsman et al., 2005; van den et al., 2003; van Rijk et al., 2003). Recruitment of sHSPs inside these compartments seems to be regulated in response to stress conditions and has been proposed to enable the cell to adapt and respond to external stimuli. For example, HSPB1 and HSPB5 are recruited inside splicing speckles upon heat shock (Adhikari et al., 2004; den Engelsman et al., 2005). Of note, HSPB1 and HSPB5 recruitment is regulated by their phosphorylation and is impaired in disease-linked mutants, at least in the case of HSPB5 (Adhikari et al., 2004; den Engelsman et al., 2005). This finding opens the possibility that deregulated function of HSPB5 at the level of nuclear splicing speckles, due to the R120G mutation, might lead to HSPB5-related myopathies. One possible function of disordered regions could be that they enable sHSPs to partition into these membraneless organelles and regulate the solubility of their binding partners in these subcellular compartments. Future studies should address this aspect.

Concerning stress granules, it has been recently shown that they can accumulate misfolded proteins. When this occurs, stress granules acquire aberrant properties and tend to convert from

dynamic liquid-like compartments into solid-like aggregates (Ganassi et al., 2016; Mateju et al., 2017). The recruitment of sHSPs inside stress granules has been linked to the presence of misfolded proteins. Once recruited inside stress granules, sHSPs, along with other chaperones and co-chaperones such as HSP70, BAG3 and valosine containing protein (VCP), exert chaperone functions by inhibiting the irreversible aggregation of stress granules and promoting their disassembly (Alberti et al., 2017; Buchan et al., 2013; Seguin et al., 2014). This has important implications for human health, since accumulation of persisting stress granules seems to lie at the heart of several age-related neurodegenerative diseases (Kim et al., 2013; Taylor et al., 2016).

The ability of sHSPs to exert a chaperone function at the level of stress granules was further discussed during the meeting by Dr. Alberti (Germany), who presented unpublished data demonstrating the interaction of two sHSPs, HSPB8 and HSPB1, with the disordered protein Fused In Sarcoma (FUS), a component of stress granules. In vitro, FUS forms liquid droplets that undergo a transition into solid fibrils. This process of molecular aging is accelerated by mutations in FUS that have been linked to amyotrophic lateral sclerosis (ALS) (Patel et al., 2015). Drs. Alberti and Boczek used purified full-length proteins to reconstitute the quality control machinery of stress granules (SGs) in vitro. They find that HSPB8 plays a central role in maintaining the liquid properties of FUS droplets. In contrast, HSPB1 preferentially targets misfolded proteins that accumulate in the liquid droplet phase of SGs. These data are intriguing because they not only reinforce the link between sHSPs and membraneless compartments, but they seem to suggest that different sHSPs may exert distinct functions once recruited inside these compartments. Future studies are needed to understand the molecular events that lead to the enrichment of sHSPs inside these condensates and their exact function. As observed for splicing speckles, stress-induced post-

translational modifications of sHSPs, by regulating their oligomerization, may control sHSPs recruitment inside stress granules that acquire aberrant properties. The identification of the residues and motifs required for chaperone recruitment inside membraneless organelles such as speckles and aberrant stress granules may offer new therapeutic avenues in both age-related neurodegenerative diseases and HSPB-linked diseases.

sHSP and human disease

The implication of sHSP/HSPBs in human diseases represented a strong theme throughout the workshop, with several groups focusing on the understanding of how mutations in HSPB genes affect their structure and function, in vitro and in cells, and how to exploit HSPB chaperone activity for the treatment of age-related neurodegenerative diseases. Genetic analysis to identify either mutations or variants, as well as deregulated expression of HSPB are also being undertaken to expand the spectrum of diseases that are directly or indirectly associated with HSPB "malfunction".

The role of sHSPs in opposing toxic protein aggregation in cells was discussed by Dr. Lee (Canada), who presented unpublished data on cellular mechanisms that maintain the solubility of FUS, a stress granule protein that aggregates in ALS. Using Fluorescence Recovery After Photobleaching, Dr. Lee and colleagues demonstrated that depletion of HSPB1 and HSPB8, which have been implicated in stress granule dynamics, by RNAi alone or together does not affect the solubility of FUS in cells. However, their results indicate that HSPB8 does appear to function synergistically with ATP-dependent chaperones and aggregate clearance mechanisms to regulate

FUS solubility in cells. Interestingly, they report that the effect of HSPB8 is particularly pronounced in human neurons differentiated from induced pluripotent stem cells (iPSCs). Neurons exhibit significantly higher HSPB8 expression compared to iPSCs and neural progenitors. CRISPR/Cas9-mediated knock-out of HSPB8 leads to increased protein aggregation in neurons as measured by Filtration Retardation Assay and defects that promoted neurodegeneration. These results raise the intriguing possibility that mutations or defects in sHSP expression and decline in protein quality control machinery with age accelerate protein aggregation and drive degeneration of neurons in disease.

In line with these data, Dr. Poletti (Italy) discussed the possibility of exploiting pharmacological or genetic induction of HSPB8 expression to combat motor neuron diseases such as ALS and Kennedy's disease (or Spinal and Bulbar Muscular Atrophy/SBMA). HSPB8 associates with BAG3 HSP70 and CHIP (an ubiquitinating enzyme) to deliver misfolded protein to autophagosomes (Carra et al., 2008; Crippa et al., 2010; Gamerdinger et al., 2011). This specific form of autophagy is called Chaperone-assisted selective autophagy (CASA); therefore, the HSPB8-BAG3-HSP70 complex is also referred to as CASA complex (Arndt et al., 2010). HSPB8 is induced in response to several neuronal stresses such has proteotoxic and oxidative stresses (Crippa et al., 2010). Dr. Poletti reported that HSPB8 is highly induced in the two main targets of misfolded protein toxicity in transgenic mouse models of SBMA and ALS, the motorneurons and muscle. The pharmacological or genetic induction of HSPB8 expression is protective in motor neuron diseases, while its silencing has opposite effects. Therefore, pharmacological approaches that potentiate the HSPB8-BAG3 autophagic pathway could contribute to maintain proteostasis in motorneuron and muscle cells, with therapeutic implication in motor neuron diseases.

Both metabolic and neurodegenerative diseases are characterized by mitochondrial dysfunction (Mattson et al., 2008; Schon and Przedborski, 2011; Schrepfer and Scorrano, 2016). Several reports support the implication of sHSPs in mitochondria function. Examples include *Drosophila melanogaster* Hsp22, which is localized in mitochondria and whose overexpression in fruit flies extends life span by increasing resistance to oxidative stress (Morrow et al., 2000; Morrow et al., 2004) and HSPB2, whose knockout in mice reduces mitochondrial energetics following pressure overload, by as yet unclear mechanisms (Grose et al., 2015; Ishiwata et al., 2012). Dr. Timmerman and colleagues (Belgium) studied the role of human HSPBs in mitochondria. Given the mitochondrial transport defects in a mouse model of Charcot-Marie-Tooth (CMT) disease type 2F due to the HSPB1 mutations (S135F and P182L) (d'Ydewalle et al., 2011), Dr. Timmerman and colleagues investigated whether HSPB1 may participate in mitochondrial homeostasis and whether this role is altered by HSPB1-disease causing mutations. Mr. Adriaenssens from Dr. Timmerman's lab reported that a fraction of several HSPBs can be imported into mitochondria. Importantly, this process was disturbed by the C-terminal HSPB1-P182L mutation, likely due to the propensity of this mutant to form larger oligomeric complexes, compared to WT HSPB1. By contrast, mutations in the ACD of HSPB1 seemed to cause the opposite phenotype, as they were detected in higher amounts in mitochondrial fractions. Interestingly, none of these processes seemed to depend on the phosphorylation status of HSPB1. These preliminary studies highlight a potential link between yet unidentified mitochondrial functions of HSPB1 and CMT disease. Future studies are required to understand to what extent mitochondrial dysfunctions are directly or indirectly affected by HSPB1 mutations.

Next, Mrs. Vendredy from Dr. Timmerman's lab reported the generation and characterization of a mouse model to study HSPB8 implication in neuromuscular diseases. Dr. Timmerman previously identified mutations in the HSPB8 gene as one of the underlying genetic causes of autosomal dominant distal hereditary motor neuropathy (dHMN) which leads to progressive motor impairments. Interestingly, most of the identified mutations target the same amino acid residue (Lys141) in the HSPB8 protein. More recently, distal myopathy was also found to be associated with mutations in HSPB8. To delineate the molecular deficits and functional consequences of HSPB8 mutations, they generated a knock-in (KI) mouse model for the K141N missense mutation mimicking the human neuropathy genotype. They observed that homozygous mutant mice (Hspb8K141N/K141N) develop a progressive axonopathy, with decreased Compound Muscle Action Potential (CMAP) amplitudes, and loss of large and medium myelinated axons. This results in locomotor deficits with an impaired performance in the Rotarod test. At the ultrastructural level, mice accumulate mutant HSPB8 protein and display degenerative patterns similar to dHMN patients with the K141N mutation. Interestingly, these animals also develop a progressive myofibrillar myopathy (MFM) as observed in some patients with HSPB8 mutations (Bouhy et al., 2018). Additionally, Dr. Timmerman's group generated HSPB8 knock-out (KO) mice using the same targeting vector. Strikingly, the homozygous HSPB8-KO animals do not show any sign of axonopathy and display a much milder myopathy than the HSPB8-KI animals (Bouhy et al., 2018). Dr. Timmerman's team is currently investigating whether modifying the expression levels of HSPB8 can be exploited as a therapeutic strategy in motor neuron and muscle disease.

Dr. Tóth in collaboration with Dr. Miklós Sántha (Hungary), presented the hypothesis that increasing HSP expression and/or augmented stress response could be involved in the protective

mechanisms of physical activity. Therefore, they studied the functional, morphological and gene expression changes in transgenic mice in response to acute and regular exercise trainings, comparing normal and overweight animals. They observed differential changes in the expression of HSP genes in the two mice populations. Following acute exercise, Hspa1a expression showed a 4-fold increase in normal weight and a 9-fold increase in the overweight mice in skeletal muscles (m. quadriceps femoris). HSPB1 and HSPB5 were only slightly induced in the normal group; by contrast, their expression showed 12-13-fold increase in overweight mice after training. In addition, HSPB2 was induced only in the trained overweight group. These significant changes in gene expression were confirmed using Western blot analysis and immunohistochemistry. Together these results show that moderate exercise training induces the expression of HSPBs in the skeletal muscle and this effect strongly depends on body weight of the animals (unpublished data). Physical exercise has been suggested as a preventive or disease-modifying treatment of age-related diseases such as dementia and brain aging (Ahlskog et al., 2011). Whether part of the beneficial effects of physical exercise on cognition and neuronal cell survival also depends on the upregulation of HSPBs is still an open question.

Finally, Dr. Wu (Wuhan, China) discussed the implication of HSPB1 in cardiopulmonary diseases, which are the leading causes of morbidity and mortality worldwide and are caused by environments, genes and their interactions. Dr. Wu and colleagues reported that the functional HSPB1 promoter _1271G_C variant affected lung cancer susceptibility and survival by modulating endogenous HSPB1 synthesis (Guo et al., 2010). Dr. Wu further reported the association of the DNA methylation network with the risk of acute coronary syndrome (Li et al.,

2017). Finally, he suggested that the results obtained are promising, but need to be confirmed by prospective cohorts such as the Dongfeng-Tongji Cohort (Wang et al., 2013).

Ongoing studies, new hypotheses and future perspectives

Although the majority of the communications were focusing on human HSPBs, due to their expression among all kingdoms of life and their implications in the biology of many different organisms, the workshop also included selected reports on sHSP of bacterial, fruit fly and plant origins. Dr. Liberek (Poland) analyzed specific functional interplay between two *Escherichia coli* sHsps, IbpA and IbpB, in directing the protein aggregation process towards assembly formation. Dr. Liberek's results suggest that after an IbpA gene duplication event at the base of Enterobacteriales functional diversification post-duplication caused IbpA to specialize in efficient substrate binding upon aggregation while the second post-duplication sHsp (IbpB) became crucial for sHsp release from assemblies at the disaggregation step. In other bacteria possessing only one sHsp gene these functions are fulfilled by a single IbpA-like protein (*Vibrio harveyi, Erwinia amylovora*). Dr. Liberek inferred that the chaperone systems with two sHSPs, in contrast to a single sHsp, allows for substantially easier release of sHsps from assemblies without compromising assembly formation; this, in turn, would ensure lower demand for Hsp70 in disaggregation and refolding.

Next, Afrooz Dabbaghizadeh from Dr. Tanguay's laboratory (Canada) reported on the organization of *Drosophila melanogaster* Hsp22 (DmHsp22), focusing on the role of the ACD in oligomer assembly. In size exclusion chromatography DmHsp22 forms a single symmetric peak

with an apparent molecular weight of approximately 820 kDa. Dr. Tanguay and colleagues also examined the influence of arginine to glycine mutations in the conserved ACD region on the structure and function of DmHsp22. Mutation in R109G did not result in structural disruption of the oligomeric structure. By contrast, mutation of R110 induces the dissociation of DmHsp22 to smaller oligomers. While all mutants demonstrate the same efficiency as wild-type in a DTT-induced insulin aggregation assay, they all are more efficient chaperones in preventing aggregation of malate dehydrogenase (Dabbaghizadeh et al., 2017). Thus, dynamic oligomerization differentially affects the affinity and chaperone activity of this sHSP. Then, they identified the proteins that interact with DmHSP22, using immunoaffinity conjugation (IAC) with mass spectroscopy analysis. Since DmHsp22 is found in mitochondria (Morrow et al., 2000), the analysis was performed using mitochondria from HeLa cells transfected with DmHsp22. In two assays 139 and 72 proteins were found to be associated with various functional classifications. Most of the proteins interacting with DmHsp22 are transporters localized in the inner mitochondrial membrane. Among these, several ATP synthase subunits were found. Moreover, they reported that expression of DmHsp22 in transiently transfected HeLa cells increased mitochondrial oxygen consumption and ATP content, providing a mechanistic link between DmHsp22 and mitochondrial functions. Thus, DmHsp22 may be involved as a chaperone in assembly of complex V. Among the DmHsp22 interacting proteins, ATP synthase subunits alpha, beta and gamma were the most abundant peptides detected. DmHsp22 significantly increases oxidative capacity of the electron transport system. Mitochondrial O2 consumption rate was also somewhat increased in cells transfected with DmHsp22. Moreover, mitochondrial ATP levels increased upon expression of DmHsp22. Dr. Tanguay concluded that DmHsp22 could be involved in the modulation of ATP synthase (Dabbaghizadeh et al., 2018). Together with the current report

from Dr. Timmerman's group, who identified several human HSPBs in mitochondrial extracts, these data suggest potential unexplored functions of sHSP in mitochondrial homeostasis, which may be partly conserved throughout species, from fruit flies to humans.

Dr. Lockwood (USA) reported on the role of sHSPs in the physiological responses to sudden changes in temperature that allow organisms to cope with thermal variability in their natural environments (Lockwood et al., 2015). Dr. Lockwood showed examples in which the expression of sHSP genes causes large changes in whole-organism thermal tolerance, in both marine and terrestrial invertebrates. His work suggests that because sHSP genes are loci of potentially large phenotypic effect (Lockwood et al., 2017; Lockwood et al., 2010), these genes are likely to be targets of natural selection (Dilly et al., 2012; Healy et al., 2010; Lockwood et al., 2010) and may facilitate evolutionary responses to a warming world.

Plants express a unique set of sHSPs that have evolved independently from metazoan and bacterial sHSPs. They comprise nuclear genes encoding proteins targeted to every membrane bound cellular compartment, the cytoplasm, nucleus, endoplasmic reticulum, peroxisomes, mitochondria and chloroplasts. This diversity likely arose from new stresses encountered by plants on their movement to land. Studies of cytosolic plant sHSPs have been critical to developing the current model of sHSP chaperone activity, as well as important to defining conserved structural features of sHSPs (Basha et al., 2012). The crystal structure of the cytoplasmic, dodecameric Hsp16.9 from wheat (PDB:1GME) has also provided an excellent platform for modelling homologous plant sHSPs and for defining how sHSPs interact with denaturing substrates. Utilizing an extensive foundation of biochemical studies of plant cytosolic sHSPs, Dr. Vierling (USA) reported on in

vitro interaction of purified sHSPs from pea, Arabidopsis and wheat with heat-denaturing porcine malate dehydrogenase as substrate. Contacts between sHSPs and substrate were detected using an amine-amine crosslinker followed by mass spectrometry. Results with all three sHSPs support and extend previous work indicating that the substrate is only partially unfolded (Cheng et al., 2008) and that the N-terminal domain is involved in multiple substrate contacts (Jaya et al., 2009). To determine whether the interactions observed in this heterologous sHSP-substrate system reflect interactions in a homologous system, additional in vitro experiments were described with an Arabidopsis sHSP and the Arabidopsis enzyme fructose-bisphosphate aldolase (FBA). FBA was found associated with Arabidopsis sHSP during heat stress in vivo (McLoughlin et al., 2016) and may reflect an important sHSP substrate. Crosslinking and mass spectrometry data with these proteins support the conclusion that similar interactions occur with native substrates as observed with model substrates.

Several other aspects of sHSP biology and the development of new techniques to study sHSP structure and functions were discussed during this meeting. Dr. Benesch (UK) presented unpublished data obtained from advanced biophysical and structural biology methods that challenge the canonical view that sHSPs act as generalist interceptors of protein aggregation stemming from interaction with non-native states. Based on his recent findings, Dr. Benesch proposed the hypothesis that sHSPs also interact with mechanosensitive proteins to regulate physiological extension and contraction cycles. He also reported on another important type of native-state interaction made by sHSPs: their interaction with each other. In collaboration with Dr. Vierling, Dr. Benesch uncovered the balance co-assembly and selective self-assembly of these proteins that is a key step in evolving new sHSP function. He discussed how this work represents

a paradigm for understanding the biophysical basis for protein evolution (Hochberg et al., 2018). Building on this work, and capitalising on his group's recent advances in mass spectrometry and the development of mass imaging in solution (Young et al., 2018), Dr. Benesch and colleagues were able to quantify how sHSPs co-assemble to form a bewildering array of polydisperse hetero-oligomers, allowing speculation on their functional significance.

Next, the use of optical tweezers to study sHSP chaperone function was discussed by Dr. Cecconi (Italy), who investigated in vitro the effect of HSPB8 on the folding and aggregation processes of maltose binding protein (MBP), a previously published substrate (Mashaghi et al., 2013; Ungelenk et al., 2016). Optical tweezers stretch and relax polypeptides thereby enabling the study of rare and transient intermediate unfolding or refolding states of the substrate protein. Dr. Cecconi and colleagues mechanically denatured homotetramers of MBP and analyzed their folding and aggregation processes in the presence or absence of HSPB8. In line with the well-established role of sHSPs as chaperone holdases, their results reveal a strong holdase activity of HSPB8, which either prevents completely the aggregation of denatured MBP molecules or allows the substrate to form only small and mechanically weak aggregates. Importantly, a careful analysis of the data also discloses an unexpected foldase activity of HSPB8, which guides the folding process of denatured MBP domains into their native states. Their findings highlight new mechanisms of interaction between HSPB8 and its substrates and suggest a more complex physiological role for this chaperone than previously assumed, in line with data presented by Dr. Benesch using advanced biophysical and structural biology methods. Further information on the description of single-molecule approaches and their importance to study chaperone activity is summarized elsewhere (Avellaneda et al., 2017; Choudhary et al., 2019; Johnston et al., 2018).

Besides in vitro single molecule approaches, new methodologies to study sHSP chaperone and anti-aggregation activity in living cells were also discussed. Dr. Ecroyd (Australia) presented a new technique developed in collaboration with his colleagues called FloIT (Flow cytometric analysis of Inclusions and Trafficking). FloIT is a simple and rapid new flow cytometry-based method that enumerates, characterises and, if desired, can physically recover protein inclusions from cells (Whiten et al., 2016). Dr. Ecroyd used this technique to compare the ability of all ten human HSPBs to inhibit the intracellular aggregation of the model protein firefly luciferase (Fluc). Their unpublished work shows that HSPB4 and HSPB6 are the most potent suppressors of Fluc aggregation, whereas HSPB2 and HSPB3 enhance inclusion formation by Fluc. These tools are therefore providing the scientific community with new mechanistic and functional insights into the molecular chaperone action of sHSPs in cells.

Other aspects and technical issues were addressed during the workshop. For example, the importance of identifying and developing specific antibodies that efficiently and specifically detect post-translational modifications of sHSP was discussed. In particular, Dr. Gusev (Russia) put forward the need to develop reliable antibodies that detect methylglyoxal modification. Methylglyoxal (MGO) is a highly reactive dicarbonyl formed in the course of glucose metabolism. MGO-modification results in the formation of a number of different products such as hydroimidazolones, argpyrimidines, carboxymethyllysines and different cross-linked products. MGO levels are increased under certain pathological conditions, such as diabetes or carcinogenesis and MGO-modification of HSPB1 was previously reported and supposed to be important for HSPB1 function (Oya-Ito et al., 2011; Sakamoto et al., 2002). Interestingly, HSPB1 has been

implicated in the etiology of metabolic diseases and its expression levels are reduced in skeletal muscle of aged-insulin resistant animals (Gupte et al., 2008). Based on these findings, it will be important to develop specific tools to study HSPB1 (and other HSPB) MGO-modifications to then evaluate their impact (if any) on HSPB function and implication in metabolic diseases and cancer.

To accommodate the increased research efforts on sHSP, which have increasing implications in biology and human disease, as briefly summarized here, the fourth in this meeting series is planned for the year 2020.

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