Chapter 21
Role of HSPB8 in the Proteostasis Network: From Protein Synthesis to Protein Degradation and Beyond

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Abstract Proper protein folding is crucial for protein stability and function; when folding fails, due to stress or genetic mutations, proteins may become toxic. Cells have evolved a complex protein quality control (PQC) system to protect against the toxicity exerted by aberrantly folded proteins, that may aggregate accumulating in various cellular compartments perturbing essential cellular activities, ultimately leading to cell and neuron death. The PQC comprises molecular chaperones, degradative systems (proteasome and autophagy) and components of the unfolded protein response. Prevention of protein aggregation, clearance of misfolded substrates and attenuation of translation, which decreases the amount of misfolding clients to levels manageable by the molecular chaperones, are all key steps for the maintenance of proteostasis and cell survival. In parallel, alterations of proteostasis may also (indirectly) influence RNA homeostasis; in fact, RNA-containing aggregates, known as stress granules, accumulate in cells with impaired PQC and autophagy colocalizing with proteinaceous aggregates in several neurodegenerative diseases. Among the different molecular chaperones, here we will focus on the small heat shock protein HSPB8, which is expressed in neurons in basal conditions and upregulated in response to misfolded protein accumulation. HSPB8 exerts protective functions in several models of protein conformation neurodegenerative diseases. The putative sites of action of HSPB8 that confer HSPB8 pro-survival and anti-aggregation functions are discussed, as well as its potential role at the crossroad between proteostasis and ribostasis.

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ATX2	Ataxin 2
eIF2α	Eukaryotic initiation factor 2 on Ser51 of the α subunit
FTLD-U	Frontotemporal lobar degeneration with ubiquitin-positive inclusions
FUS	Fused in sarcoma
HD	Huntington's disease
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HSP	Heat shock protein
IBMPFD	Inclusion body myopathy with early-onset Paget disease and fronto-temporal dementia
JNK	c-Jun N-terminal kinase
KD	Kennedy's disease
LAMP2A	Lysosome-associated membrane protein 2A
LC3	Microtubule-associated protein 1A/1B-light chain 3
MKK7	Mitogen-activated Protein Kinase Kinase 7
MSP	Multisystem proteinopathy
NBR1	Neighbor of BRCA1 gene 1
PD	Parkinson's disease
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
polyQ	Polyglutamine
PQC	Protein quality control
RACK1	Receptor for Activated C Kinase 1
rhoA	Ras homolog gene family member A
RNPs	Ribonucleic proteins
ROCK1	Rho-associated coiled-coil containing protein kinase 1
SCA3	Spinocerebellar ataxia 3
SG	Stress granule
SOD1	Superoxide dismutase 1
SQSTM1	Sequestosome 1
TDP-43	TAR DNA-binding protein 43
TIA-1	T-cell intracytoplasmic antigen
TRAF2	TNF receptor-associated factor 2
UPS	Ubiquitin proteasome system
VCP	Valosin containing protein

21.1　Protein Quality Control: Guardian of Cellular Health

21.1.1　The Physiological Activation of the Protein Quality Control System

Protein homeostasis refers to the ability of cells to maintain an appropriate balance among protein synthesis, folding, assembly, translocation and clearance (Morimoto and Cuervo 2014). Protein homeostasis is ensured and modulated by the protein quality control (PQC) system and is essential for the long-term viability and health of the cells. Malfunction and deregulation of the PQC system are severe risk factors for the development of protein conformation diseases characterized by the accumulation of aggregated proteins (Bence et al. 2001; Douglas and Dillin 2010).

The PQC system includes molecular chaperones, degradative systems and stress-inducible pathways. Molecular chaperones (typically heat shock proteins, HSPs) are expressed in multiple cell compartments and survey protein quality by either assisting protein folding (both co- and post-translational folding) or directing aberrant proteins to degradation and thereby protecting against misfolding (Hartl 1996). Molecular chaperones also assist assembly and disassembly of macromolecular complexes, as well as protein translocation (Hartl and Hayer-Hartl 2002; Deuerling and Bukau 2004; Ron and Walter 2007). Unfolded substrates are repeatedly bound and held by molecular chaperones to avoid their irreversible aggregation (Hartl et al. 2011). There are several classes of molecular chaperones, which may also work with the assistance of co-chaperones and/or interactors of different nature; some chaperones are ATP-independent (e.g. small heat shock proteins, sHSPs/HSPBs) (Gobbo et al. 2011), while other are ATP-dependent (Hsc70/HSPA8 and Hsp70/HSPA1A) (Hartl et al. 2011). Both ATP-independent and ATP-dependent chaperones can bind to unfolded substrates. Instead, folding of the bound client to its native state is regulated by Hsp70 and ATP hydrolysis (Hartl et al. 2011). Nucleotide binding to Hsp70 and hydrolysis coupled to the release of the folded substrate is further regulated by co-chaperones including proteins of the Bag family (BAG1-BAG6), DNAJ/Hsp40 and Hip (Hartl et al. 2011), which also provide the specificity for interaction of chaperones with client proteins. When misfolding cannot be prevented (e.g. due to protein damage, denaturation, oxidation or a genetic mutation), proteins cannot mature into their fully active state, lose their function and become aggregation-prone, eventually acquiring toxic functions. Under these conditions, as well as when refolding fails, molecular chaperones assist the degradation of the bound client acting in concert with two major degradative systems: the ubiquitin-proteasome system (UPS) and lysosome-based degradation. The latter includes macroautophagy (here referred to as autophagy) and chaperone-mediated autophagy (CMA) (Cuervo and Wong 2014; Parzych and Klionsky 2014).

The UPS is a low capacity, but highly specific and selective proteolytic system that degrades short-lived proteins labeled with ubiquitin moieties (Ciechanover 2005; Ciechanover and Brundin 2003). The proteasome has a barrel shape, so that to be degraded by this system proteins must first be ubiquitinated and unfolded, in

order to acquire the capability to enter its narrow central cavity; globular or irreversibly aggregated proteins cannot be processed by the proteasome. Among the molecular chaperones and co-chaperones that assist the proteasomal mediated degradation of ubiquitinated substrates are HSPAs/DNAJs and HSPA/BAG1 complexes (Alberti et al. 2002; Demand et al. 2001; Kampinga and Craig 2010).

Autophagy is a high capacity and non-specific multi-step process in which cytosolic material is sequestered in a double-membrane vesicle, the phagophore/autophagosome; the latter then fuses with the lysosome delivering its inner content for degradation by enzymatic hydrolysis, greatly facilitated by the acidic environment of the resulting intracellular compartment (Parzych and Klionsky 2014). Although autophagy has been initially described as a bulk nonspecific degradation process that mainly clears long-lived proteins and organelles, recent evidence has highlighted that autophagy plays a crucial role in the selective elimination of unwanted components such as dysfunctional organelles, pathogens and also aberrant protein aggregates (Klionsky and Emr 2000; Hara et al. 2006; Komatsu et al. 2006). In particular, it has emerged that autophagy can also selectively degrade ubiquitinated proteins (Bjorkoy et al. 2005). The recognition of ubiquitinated substrates is done by autophagy adaptors including SQSTM1 (also known as p62), NBR1 and valosin containing protein (VCP), which bind both to ubiquitin and the autophagosome-specific proteins members of the LC3/GABARAP/Gate16 family (Johansen and Lamark 2011). Specific chaperones and co-chaperones can participate in the delivery of bound clients to autophagosomes, including BAG3 (Carra et al. 2008a). Another example of the tight cooperation between chaperones and lysosome-based degradation is CMA, which removes a specific subset of proteins containing the pentapeptide lysosome-targeting motif (KFERQ). These substrates are directly translocated into the lysosome after docking to the LAMP2A and unfolding by a chaperone complex containing HSPA8 and the co-chaperones BAG1, HSPA8-interacting protein (Hip), Hsp-organising protein (Hop) and HSP40/DNAJB1 (Cuervo and Wong 2014).

21.1.2 Protein Damage and the PQC System

When cells are exposed to different types of acute or chronic stress, protein homeostasis is challenged and the protein-folding equilibrium is altered. Cells respond by increasing the expression of genes that protect against proteotoxic stress, including HSPs and do so by instantaneously stimulating the heat-shock response (HSR) (Anckar and Sistonen 2011; Morimoto 2008). The HSR is often mediated by specific sensors that activate transcription factors, such as the heat shock factor 1 (HSF-1), which controls several genes of the HSR. Existing and newly synthesized molecular chaperones capture the folding intermediates to prevent misfolding and aggregation and to facilitate refolding or degradation, and the entire PCQ system machinery is potentiated under these conditions. In parallel, cells temporarily attenuate translation allowing a decrease in the total intracellular levels of misfolding

proteins to amounts manageable by the molecular chaperones (Tsaytler et al. 2011). Translation attenuation is triggered by the accumulation of unfolded proteins within the endoplasmic reticulum (ER) (Ron and Walter 2007). This, in fact, leads to PERK-mediated phosphorylation of eukaryotic initiation factor 2 on Ser51 of the α subunit (eIF2α), thereby resulting in the temporary sequestration of mRNAs encoding for "housekeeping" functions in cytoplasmic foci called stress granules (SGs) and inhibiting their translation (Kedersha and Anderson 2002). Sequestration of mRNAs encoding for "housekeeping" functions within SGs also prioritizes the synthesis of chaperones and enzymes needed for the stress response, while protecting and storing mRNAs during stress (Kedersha and Anderson 2002). Potential cell damage associated with the response to increased levels of misfolded proteins can be attenuated by the sequestration into SGs of scaffold proteins (e.g. JNK, MKK7, rhoA) and pro-apoptotic proteins (e.g. RACK1, ROCK1, TRAF2), thus. These proteins respectively modulate signaling cascades and inhibiting apoptosis during stress, generating a well-integrated stress response system (Buchan and Parker 2009; Kedersha et al. 2000; Takahashi et al. 2012). Once the stress is relieved, SGs, which are highly dynamic, disassemble, restoring proper translation within cells. Persistent SGs will be removed by autophagy, with the assistance of the ubiquitin chaperone VCP (Buchan et al. 2013), thereby also contributing to the recovery of protein homeostasis.

21.2   Protein Aggregation and Proteostasis Imbalance in Neurodegenerative Diseases

Misfolding and aggregation are common molecular events that affect several organs and tissues and are responsible for a large number of human diseases, including neurodegenerative and neuromuscular diseases. All the diseases that are characterized by the presence of proteinaceous aggregates have been called conformational diseases (Chiti and Dobson 2006). Conformational diseases affecting the brain include repeat expansion diseases, such as CAG-repeat/polyglutamine (polyQ) related diseases (e.g. Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), and spinocerebellar ataxias (SCAs)), C9ORF72/GGGGCC related diseases (e.g. the most frequent familial forms of amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), etc.) as well as non-repeat expansion diseases including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), prion disease, and Alzheimer's disease (AD), which may appear both in sporadic or familial forms. In the latter cases, the inheritance is associated to mutations of a specific gene whose product cannot fold properly. Little is known on the sporadic forms, but often alteration of components of the PCQ system is present, supporting a direct link between imbalance in proteostasis and disease onset and/or progression-severity. Depending on the type of disease (and type of disease-related protein) aggregates/fibrils accumulate in different neuronal populations/brain areas, which correlate with different symptoms and are always associated with a progressive (fatal) clinical course

(Cummings and Zoghbi 2000). However, it is important to underline that not all aggregated species are toxic, as neuronal death does not always correlate with the presence of aggregates. Protein aggregation is a multi-step process in which pre-fibrillar detergent soluble species assemble into large fibrillar non-detergent soluble species (Chiti and Dobson 2006). The neurotoxicity of the different forms of these aggregates/inclusions is still largely debated and in general large macromolecular aggregates are nowadays believed to be protective, while intermediate species would exert high toxicity. Macroscopic aggregates (inclusion bodies) would exert a protective role by trapping the early neurotoxic species into a specific subcellular compartment, waiting for their clearance from the cells. Microaggregates/intermediate species can exert toxicity acting at different steps, such as sequestration of cellular components, particularly transcription factors which are essential for neuronal survival (McCampbell et al. 2000; Kikis et al. 2010), alteration of the intracellular trafficking of molecules and organelles, possibly affecting axonal transport (Sau et al. 2011; Lee et al. 2004). Independent of the type of aggregate and/or of the precise step at which misfolded proteins exert their toxicity, the overall reduction or the prevention of protein misfolding and/or aggregation in neurons, as well as the enhanced clearance of the aggregating/aggregated species are considered to be neuroprotective. Decreased aggregation of misfolded proteins and increased clearance can be achieved a) by potentiating the molecular chaperones, which act in concert with the degradation systems UPS and/or autophagy and b) by directly stimulating autophagy with pharmacologic agents. Indeed, the protective role of specific molecular chaperones and co-chaperones (e.g. members of the HSPA, DNAJ and HSPB families) has been well established using both cellular and animal models of protein conformational neurodegenerative disorders or by potentiating autophagy-mediated clearance (Carra et al. 2008a; Crippa et al. 2010; Hageman et al. 2010; Ravikumar et al. 2004; Rubinsztein 2006; Vos et al. 2010).

21.3 Ribostasis, Stress Granules and Protein Conformation Diseases

Recent observations demonstrated that several neurodegenerative diseases are characterized by the accumulation of nuclear or cytoplasmic RNA-protein (RNP) aggregates. These RNP aggregates often contain stress granule markers and can colocalize with proteinaceous fibrillar aggregates. Most notably, in sporadic and familial forms of ALS, frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U), multisystem proteinopathy (MSP), HD, AD (Schwab et al. 2008; Wilson et al. 2011) the nuclear RNA-binding protein, TDP-43 redistributes from the nucleus to cytoplasm, where it colocalizes with proteinaceous inclusions. These observations strongly suggest that a tight connection/cross-talk between protein and RNA homeostasis exists and that altered RNP/SG assembly, as well as impaired SG clearance may contribute to protein conformation diseases. This is further suggested by the fact that mutations in TDP-43 and in a number of other

mRNA-binding proteins, including (e.g.: FUS, hnRNPA1, ATX2) are associated with neurodegenerative diseases, namely ALS, MSP, spinocerebellar ataxia type 2 (SCA2) or constitute a risk factor in other diseases (e.g. ATX2 is a risk factor for ALS (Laffita-Mesa et al. 2013)). Intriguingly, when mutated these mRNA-binding proteins aberrantly assemble into SGs upon stress, pointing to altered SG dynamics (assembly and disassembly) as important pathomechanism (Acosta et al. 2014). In fact, SGs play a crucial role in protecting and storing mRNAs during stress, as well as indirectly modulating signaling pathways (e.g. by sequestration of specific factors); altered SG dynamic may in turn affect RNA homeostasis. Thus, with this mechanism SG aggregates could alter the population of mRNAs available for translation, which may also contribute to disease.

What molecular events lead to persistence of SGs and colocalization of SG components with proteinaceous inclusions in protein conformation diseases is still largely unknown. Curiously, many of the mRNA-binding proteins involved in SG assembly (or recruited to SGs) contain prion-like domains (e.g. TIA-1, TDP-43, hnRNPA1), which allow them to self-aggregate/polymerize, and trigger SG assembly (Gilks et al. 2004; Li et al. 2013). However, unlike prionogenic fibrillar aggregates, which are irreversible, during normal SGs metabolism, the prion-like domains present in the mRNA-binding proteins reversibly assemble and disassemble ensuring their dynamic nature. The presence of deregulated expression or of mutations in RNA-binding proteins may confer the tendency to form stable amyloid structures mediated by the prion-like domains, which would cause a defective SG disassembly. Accumulation of persistent altered SGs may not only alter RNA metabolism and homeostasis, but also favor protein aggregation; in fact, improperly disassembled SGs or partly disassembled SGs may act as seed for aggregation, due to the presence of proteins with highly aggregation-prone prion-like domains. Alternatively, but not mutually exclusively, defects in clearance mechanisms, deregulated autophagy and accumulation of other aggregate-prone proteins, as it occurs in protein conformation diseases, could contribute to an increase in the frequency of amyloid initiation at SGs. This may change SG assembly dynamics and composition, favoring SG accumulation and/or coalescence with aggregates/inclusion bodies (which would participate in disease progression). In both scenarios, cells rely on an efficient PQC system and autophagy flux to properly clear unassembled SGs, thereby maintaining both RNA and protein homeostasis. In line with this hypothesis, mutations of VCP are associated with ALS and MSP (Inclusion body myopathy with early-onset Paget disease and frontotemporal dementia/IBMPFD), characterized by the accumulation of ubiquitinated inclusions that colocalize with the SG marker TDP-43 (Ju et al. 2008; Ju and Weihl 2010). Intriguingly, VCP is a ubiquitin chaperone involved in the autophagy-mediated clearance of SGs (Buchan et al. 2013), as well as in the modulation of autophagosome formation and autophagy-mediated ubiquitinated client degradation (Ju et al. 2009). This further points to a tight connection between altered proteostasis and RNA processing in disease and suggests that boosting molecular chaperones and degradative systems may be beneficial in preventing RNA aggregation and dysfunction (besides decreasing protein aggregation and aggregate-mediated toxicity).

21.4 eIF2α: Master Regulator of Protein Synthesis, SGs and Autophagy

As previously mentioned, upon stress cells activate a sophisticated integrated response aimed to conserve energy and divert cellular resources toward survival. This includes the temporary shut-down of translation (to decrease the load of unfolding chains for the existing molecular chaperones), upregulation of molecular chaperones (which are essential to avoid irreversible protein aggregation, as well as protein-RNA aberrant aggregation) and activation of autophagy (to clear the misfolded accumulating proteins) (Ron and Walter 2007). Key to these events is the phosphorylation of eIF2α. In fact, upon stress phosphorylation of eIF2α on one hand induces the conversion of LC3-I into LC3-II, which corresponds to the lipidated autophagosome-anchored form, and on the other hand induces the expression of key autophagy genes (e.g. ATG5) (Kouroku et al. 2007). Impairment in the activation of the eIF2α stress response renders the cells more vulnerable to the toxicity mediated by aggregate-prone proteins such as polyQ proteins, leading to accumulation of proteinaceous aggregates and activation of apoptosis (Kouroku et al. 2007). In parallel, phosphorylation of eIF2α promotes SG assembly (although SG formation can occur also independently of eIF2α phosphorylation) (Anderson and Kedersha 2002; Mazroui et al. 2007). SG assembly is triggered after translation shut-down and polyribosome disassembly and is not required for translation attenuation (Anderson and Kedersha 2008). While the beneficial role of autophagy stimulation and translation attenuation upon stress has been well documented, it has not yet been elucidated whether the SG response induced by phospho-eIF2α contributes directly, as an early event, in protein homeostasis and to what extent cross-talk between SG response and PQC exists. Interestingly, the ubiquitin chaperone VCP participates in SG clearance and several players of the PQC system are components of SGs or even modulate their assembly (e.g. ubiquitin and HDAC6, respectively) (Buchan et al. 2013; Kwon et al. 2007). This strongly points to a close interplay between the sensors of proteotoxicity (unfolded protein response, UPR), specific molecular chaperones, eIF2α phosphorylation, SG response and autophagy-mediated clearance. Thus, alteration in the activation of the eIF2α pathway or dysfunction of key players upstream or downstream of this pathway could also alter SG response (and indirectly RNA metabolism and/or stability upon stress), favouring protein overload and protein misfolding/accumulation. In parallel, due to the inefficient activation of autophagy, persistent SGs would accumulate (Buchan et al. 2013). In combination, these observations suggest that modulation of target proteins that can induce phospho-eIF2α, thereby promoting or facilitating temporary translation shut-down, autophagy and, eventually also SG response, may be beneficial in restoring and/or maintaining cell health. Interestingly, specific molecular chaperones such as HSPB8 can modulate (indirectly) via phospho-eIF2α both events, thereby exerting a protective function under proteotoxic stress conditions (see later) (Carra et al. 2009). To what extent molecular chaperones participate in the modulation of SG response and dynamic and whether/how this correlates with

their protective/pro-survival role is still largely unknown and is certainly a field to be investigated in the future.

21.5   HSPBs: Implication in Neurodegenerative and Neuromuscular Diseases

Small HSPs (or HSPB) belong to the superfamily of ATP-independent chaperones. The mammalian HSPB protein family comprises ten members (HSPB1-10) (Fontaine et al. 2003; Kappe et al. 2003). From a functional point of view, two major functions have been attributed to HSPBs. First, some HSPB proteins can stabilize the cytoskeleton (actin based microfilaments and intermediate filaments), especially under stress conditions (see chapter by J. Lavoie and J. Landry) (Lavoie et al. 1995). Second, HSPB proteins participate in the maintenance of protein homeostasis by assisting the refolding (when possible) of misfolded aggregate-prone proteins, preventing their irreversible aggregation, and/or participating in their clearance (Carra et al. 2005; Vos et al. 2010). Due to their role in PQC, upregulation of some HSPBs has been implicated, indirectly or directly, in several neurodegenerative and neuromuscular disorders. Indeed, immunohistochemical studies done on post-mortem human tissues from patients suffering from e.g. PD, AD, HD show upregulation of HSPB1, HSPB5, HSPB8 in the areas characterized by neuronal damage/death and by reactive gliosis (Carra and Landry 2006; Seidel et al. 2012). Reactive gliosis is a reaction of the astrocytes to brain injury, resulting in morphological and functional changes of astrocytes and aimed at protecting the surrounding neuronal population from toxic insults and maintaining neuronal homeostasis. Interestingly, the highest expression levels of HSPB proteins are often observed in reactive astrocytes, which provide essential activities that preserve neuronal function. The pathological significance of HSPBs up-regulation in areas characterized by reactive gliosis and neurodegeneration is still largely not understood (Carra and Landry 2006); it is however assumed that it could be part of the stress response to neuronal damage to prevent/decrease the toxicity mediated by the aggregated mutated proteins (e.g. mutated polyQ proteins, mutated SOD1) (Carra et al. 2005; Crippa et al. 2010). This is further suggested by the findings that the transient overexpression of several HSPBs, in vitro, in mammalian cells and in Drosophila melanogaster models of protein conformation diseases, attenuates the aggregation of mutated proteins and protects against their mediated cytotoxicity (see also later: *HSPB8, autophagy and protein degradation*) (Carra et al. 2010; Vos et al. 2010; Gregory et al. 2012). Furthermore, transgenic flies overexpressing members of the Drosophila melanogaster (Dm) HSPBs are protected against mutated polyglutamine-induced neurodegeneration, as well as aging-related decline in locomotor behavior (extension of life span and protection against oxidative stress have been found in Dm-Hsp22 transgenic flies) (Morrow et al. 2004a, b). In combination these data suggest that several members of the HSPB family display protective functions and are important player for

maintaining neuronal and muscular cell viability. This is further supported by the fact that mutations of several HSPB proteins (HSPB1, HSPB3, HSPB4, HSPB5, HSPB8) are associated with muscular and neurological disorders, including hereditary sensory and/or motor neuropathy (e.g. HSPB1, HSPB3, HSPB8), myofibrillar myopathy (HSPB5) and congenital cataract (HSPB4) (Boncoraglio et al. 2012; Irobi et al. 2004; Evgrafov et al. 2004; Kolb et al. 2010; Litt et al. 1998; Vicart et al. 1998).

In this chapter we will focus on the potentially protective function exerted by HSPB8 in neurodegenerative and neuromuscular diseases and we will highlight how HSPB8 may act at the crossroad of both protein synthesis and protein degradation, thereby participating in the maintenance of proteostasis.

21.6 HSPB8: At the Crossroad Between Protein Synthesis and Protein Aggregation

Differently from other members of the HSPB family that mainly exist as homo and/or hetero-oligomers containing other HSPB partners (e.g. HSPB1, HSPB5), in cells, HSPB8 forms a stable and stoichiometric complex with the HSPA8 co-chaperone Bcl-2 associated athanogene BAG3 that contains a well-defined 2:1 HSPB8:BAG3 ratio (Carra et al. 2008a). The different behavior of HSPB8, as compared to other "classical" HSPBs, was recently confirmed in vitro, using fluorescently labeled HSPBs and monitoring the type of hetero-oligomeric complexes formed. In particular, while HSPB1, HSPB5 and HSPB6 formed heterogeneous high molecular weight complexes and exchanged subunits, HSPB8 did not form stable complexes with either HSPB1 or HSPB5 (Datskevich et al. 2012). In mammalian cells, stability of HSPB8 is enhanced by its association with BAG3 (Carra et al. 2008a); this was confirmed in vitro studies, that also demonstrated that this interaction of HSPB8 and BAG3 leads both to an increase of thermal stability and an increased resistance to limited chymotrypsinolysis of HSPB8 (Shemetov and Gusev 2011). This makes HSPB8 an "atypical" member of the HSPB family, at least from the structural point of view. Although interaction of other members of the HSPB family with BAG3 has been reported (e.g. HSPB5 and HSPB6, but not HSPB1, can interact with BAG3) (Fuchs et al. 2010; Hishiya et al. 2010), the strength of these interactions was weaker as compared to HSPB8 affinity for BAG3 (Shemetov and Gusev 2011).

21.6.1 HSPB8, Autophagy and Protein Degradation

Concerning the functions of HSPB8, results from our laboratories, using cellular and Drosophila disease models of polyglutamine diseases, show that HSPB8, together with BAG3, reduced the aggregation of mutated polyglutamine proteins such as huntingtin, androgen receptor (AR) and ataxin 3, which are associated with HD, KD and SCA3 (Carra et al. 2005, 2008a, 2010). Similarly, the HSPB8-BAG3

complex inhibited the formation of insoluble species generated by misfolded mutated SOD1 and by a truncated form of TDP-43, associated/involved in familial or sporadic ALS (Crippa et al. 2010). Both the levels of high molecular weight oligomeric species and the insoluble aggregated species generated by mutated SOD1 and truncated TDP-43 were decreased by HSPB8 overexpression, suggesting that HSPB8 exerts its function on these misfolded proteins independently on their oligomeric state (Crippa et al. 2010). The reduced aggregation of misfolded proteins in cells overexpressing HSPB8 is due to the facilitation of the autophagic process (Carra et al. 2008a). In fact, in autophagy deficient cells (ATG5−/−) or upon inhibition of autophagy with 3-methyladenine and wortmannin, HSPB8 and BAG3 were no longer able to inhibit the aggregation/accumulation of mutated polyQ proteins, SOD1 and TDP-43 (Carra et al. 2005; Crippa et al. 2010). From the mechanistic point of view, such anti-aggregation and pro-degradative function of HSPB8 depends on its association with the partner BAG3, as knockdown of the latter diminishes HSPB8 ability to inhibit mutated protein aggregation (Carra et al. 2008b). Moreover, the work of several independent laboratories demonstrated that the HSPB8-BAG3-HSPA8 complex not only facilitates autophagy flux, but is involved also in client binding and targeting to autophagosomes for degradation (Arndt et al. 2010; Carra et al. 2008a; Gamerdinger et al. 2011). In particular, BAG3 possesses a dynein binding domain, which allows binding of the HSPB8-BAG3 complex to dynein and transport of the bound cargo to the microtubule organization center (MTOC) (Gamerdinger et al. 2011). Here, at the MTOC, autophagosomes are mainly assembled and highly concentrated, favouring cargo engulfing and degradation within autophagosomes. If not efficiently degraded, the bound cargo is targeted to the aggresome. Indeed, the aggresome is a dynamic structure that forms, at the MTOC, in response to an overload of improperly folded proteins (Kopito 2000). Thus, when autophagy flux is insufficient or dysregulated, as it occurs in neurodegenerative diseases, the dynein-mediated retrograde transport may not be fully paralleled by the rate of autophagosome formation; this would lead to the typical accumulation of aggresomes found in protein conformation diseases.

Concerning the stimulation of autophagy, overexpression of HSPB8 and/or BAG3 in mammalian cells (HeLa, HEK293T, but not in motoneuronal NSC34 cells) induces the LC3-II (autophagosome-anchored)/LC3-I ratio, which corresponds to an increased formation of autophagosomes (Carra et al. 2008b, 2009). The complex also increases the fusion of autophagosomes with lysosomes, as measured using specific inhibitors of the fusion step, thereby favoring autophagy-mediated degradation of different substrates (Carra et al. 2008b). In contrast, knocking down the HSPB8-BAG3 complex leads to a decreased activation of autophagy in basal conditions particularly under proteotoxic stress, thus rendering the cells more vulnerable to proteotoxicity (Carra et al. 2008b; Rapino et al. 2013); this is accompanied by a large increase in the accumulation of insoluble proteins within the cells. The activation of autophagy by HSPB8-BAG3 observed in the cell lines tested (e.g. HEK293T, HeLa) is a consequence of the induction of the phosphorylation of eIF2α. In fact, on one hand co-transfection of HSPB8 (and BAG3) with GADD34, which promotes the dephosphorylation of eIF2α, abrogates HSPB8 (and BAG3) mediated induction

of autophagy (Carra et al. 2009). On the other hand, overexpression of HSPB8 (and BAG3) induces the phosphorylation of eIF2α, both in cells and in vitro. Such induction of phospho-eIF2α upon HSPB8-BAG3 overexpression is generally observed prior to obvious effects on the LC3-II/LC3-I ratio. In particular, while we observed induced phospho-eIF2α typically between 16 and 24 h after transfection of the chaperone complex, the maximal effects on autophagy were observed between 24 and 48 h post-transfection in cells overexpressing HSPB8-BAG3 ((Carra et al. 2009) and Carra, unpublished). As mentioned above phospho-eIF2α induces the expression of key autophagy genes, including ATG5, thus explaining the delay between these two HSPB8-BAG3-mediated effects.

Concerning client binding and targeting, the HSPB8-BAG3-HSPA8 complex interacts with the E3 ligase CHIP (Arndt et al. 2010; Crippa et al. 2010) and with the autophagy receptor protein p62/SQSTM1 (sequestosome1) (Gamerdinger et al. 2009). CHIP would ubiquitinate the HSPA8-bound substrates, while SQSTM1 is a multi-adaptor protein that simultaneously binds to ubiquitin and the autophagosome-associated protein LC3 (Bjorkoy et al. 2005; Pankiv et al. 2007), thereby linking polyubiquitinated proteins to the autophagic machinery. Actually, recent findings from our group show that BAG3 interacts, via HSPA8, with (poly)ubiquitinated proteins; whether all these clients are ubiquitinated via CHIP is however still unknown (Minoia et al. 2014). Once bound by the HSPB8-BAG3-HSPA8 complex, these clients are sequestered into cytoplasmic puncta that are labeled with the autophagic adapter/linker proteins SQSTM1, but also WIPI-1 and LC3. The sequestration of the ubiquitinated clients into SQSTM1-positive cytoplasmic puncta would avoid their proteasome-mediated degradation, thereby favoring their re-routing towards autophagy (Minoia et al. 2014). It is of note that while HSPB8 can colocalize with BAG3 and HSPA8 in these ubiquitin-SQSTM1-containing cytoplasmic puncta, it is not strictly required for BAG3 binding to ubiquitinated proteins as well as for BAG3-induced sequestration into cytoplasmic puncta, which depends entirely on BAG3 interaction with HSPA8. However, we observed that HSPB8 can also pull-down ubiquitinated proteins (Carra, unpublished), similarly to HSPA8; this opens the possibility that HSPB8 may exert an additive role to the one of HSPA8 and participate in the targeting of specific clients to degradation. Alternatively, since we found that HSPB8 colocalizes with BAG3 in ubiquitin-positive cytoplasmic puncta especially after prolonged inhibition of the proteasome, our data suggest that HSPB8 may cooperate with HSPA8 and BAG3 in client re-routing especially under severe stress conditions (Carra, unpublished).

21.6.2   HSPB8 and Protein Synthesis

As previously mentioned, we found that overexpression of HSPB8 in mammalian cells leads to phosphorylation of eIF2α (Carra et al. 2009), which acts as a dominant inhibitor of the guanine nucleotide exchange factor eIF2B and prevents the recycling of eIF2 between successive rounds of protein synthesis. As a consequence, translation

is attenuated. A number of conditions result in eIF2α phosphorylation including heat shock, viral infection, nutrient deprivation, iron deficiency, and accumulation of unfolded or denatured proteins (Kimball 1999). In the case of cells overexpressing HSPB8, increased levels of phospho-eIF2α is accompanied by the induction of key transcription factors (e.g. ATF4) and of autophagy (Carra et al. 2009). Both attenuation of translation and induction of autophagy participate in decreasing the accumulation and, subsequently, the aggregation of mutated misfolded proteins mediated by HSPB8 (Carra et al. 2009). The ability of HSPB8 to inhibit protein synthesis has been further demonstrated in vitro, using recombinant HSPB8 and cDNAs encoding for different substrates, thereby allowing a generalization of its effect (Carra et al. 2009). From the mechanistic point of view, it is still largely unknown how HSPB8 can trigger the phosphorylation of eIF2α. HSPB8 shows sequence similarity to the protein kinase coding domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) and displays a Mn^{2+}-dependent protein kinase activity (Smith et al. 2000). This autokinase activity of HSPB8 depends on the lysine 113 and permits it to phosphorylate specific substrates such as e.g. myelin basic protein (Chowdary et al. 2004; Depre et al. 2002). Its putative autokinase, both wildtype HSPB8 and the kinase-dead K113G mutant forms of HSPB8 could induce the phosphorylation of eIF2α, thereby excluding any direct role of HSPB8 as kinase at the level of eIF2α (Carra et al. 2009).

In parallel to the eIF2α signaling pathway, the phosphoinositide-3 kinase (PI3K) transduction pathway is also activated in response to a wide range of stresses (Bang et al. 2000). Recently it has been reported that a cross-talk between these pathways exists and that the eIF2α kinase PKR, which leads to phosphorylation of eIF2α, also acts upstream of PI3K and turns on the Akt/PKB-FRAP/mTOR pathway. From the mechanistic point of view, the activation of the PI3K pathway is indirect and requires the inhibition of protein synthesis by phospho-eIF2α. As a result, the apoptotic and protein synthesis inhibitory effects exerted by phospho-eIF2α are counterbalanced/antagonized by the Akt/PKB-FRAP/mTOR pathway, which promotes cell proliferation and survival. Curiously, HSPB8 increases the phosphorylation of Akt and acts as survival and differentiation factor in hippocampal neurons (Ramirez-Rodriguez et al. 2013). Whether the induction of eIF2α phosphorylation is upstream of and required for the activation of Akt mediated by HSPB8 is currently unknown. Understanding how upregulation of HSPB8 leads to phosphorylation of eIF2α and whether this is linked to its role in cell survival (Akt activation) will unravel new targets whose modulation helps maintain proteostasis and boosts cell survival in stress and disease.

An inhibitory role in protein synthesis has been suggested also for another member of the HSPB family, HSPB1 (Cuesta et al. 2000). However, this seems mechanistically unrelated to phosphorylation of eIF2α. During heat shock, HSPB1 binds to the eukaryotic initiation factor eIF4G, promotes eIF4G insolubilization and prevents translation (Cuesta et al. 2000). Following heat shock, HSPB1 accumulates together with eIF4G in heat shock granules; similarly to HSPB1, we observed accumulation of HSPB8 in heat-shock granules (Carra, unpublished). What is the

functional role of HSPB8 at the level of heat-shock granules and whether, similarly to HSPB1, HSPB8 can interact with eIF4G is not known.

21.6.3   HSPB8, eIF2α Phosphorylation and Stress Granules

As previously mentioned, upon stress, phosphorylation of eIF2α promotes SG assembly; however, it is of note that SGs can also be triggered in an eIF2α-independent manner, e.g. following inhibition of the proteasome or robust heat shock (Grousl et al. 2009; Mazroui et al. 2007). Formation of SGs is part of the integrated stress response aimed at cell survival and alteration in the dynamics of SGs has been associated with a number of pathological conditions. In particular, in protein conformation diseases, such as CAG/polyQ diseases or in ALS, colocalization of SG components with proteinaceous inclusions has been shown (Wolozin 2014; Ramaswami et al. 2013). This may result from the presence of mutated proteins that by itself can trigger SGs with altered dynamics (e.g. persistent SGs can be triggered by ALS-associated mutated FUS/TLS) as well as by the aberrant clearance of SGs, which, in turn, can derive from impairment of the PQC system (inhibition of autophagy and VCP) (Ramaswami et al. 2013; Buchan et al. 2013); the two processes are not mutually exclusive, and may coexist. Whether HSPB8 also influences the SG response, due to its action on phospho-eIF2α and whether, with its pro-autophagic activity, it participates in the clearance of persistent SGs, is currently unknown. However, considering that SG assembly is triggered by the self-aggregation of proteins that contain prion-like domains, it is plausible that specific chaperones are recruited into SGs to avoid irreversible aggregation of their components, thereby maintaining their dynamic nature. We found that HSPB8 is recruited into SGs following several stress conditions, including heat shock, treatment with arsenite and inhibitors of the proteasome (Fig. 21.1a and Carra, unpublished). HSPB8 also colocalizes with mutated ALS-associated FUS R518K in SGs (Fig. 21.1b). Combined, these observations open the possibility that HSPB8 may participate in the PQC at the level of SGs. HSPB8 may contribute to avoid irreversible protein aggregation within SGs. When irreversible aggregation occurs and/or in the presence of mutated or damaged components of SGs, HSPB8 may cooperate with BAG3 to target such components (or damaged and persistent SGs) to autophagy for degradation (Fig. 21.2). Indeed, SGs can be degraded by autophagy with the assistance of the ubiquitin chaperone VCP (Buchan et al. 2013).

   Alternatively, but not mutually exclusive, HSPB8 may be recruited into SGs to serve specific functions, such as to regulate the activity of specific mRNA-binding proteins; for example HSPB8 interacts with two RNA-binding proteins: DEAD box protein Ddx20 (gemin3, DP103) and Src associated in mitosis of 68 kDa (Sam68) (Sun et al. 2010; Badri et al. 2006). The DEAD box proteins form a subgroup of the
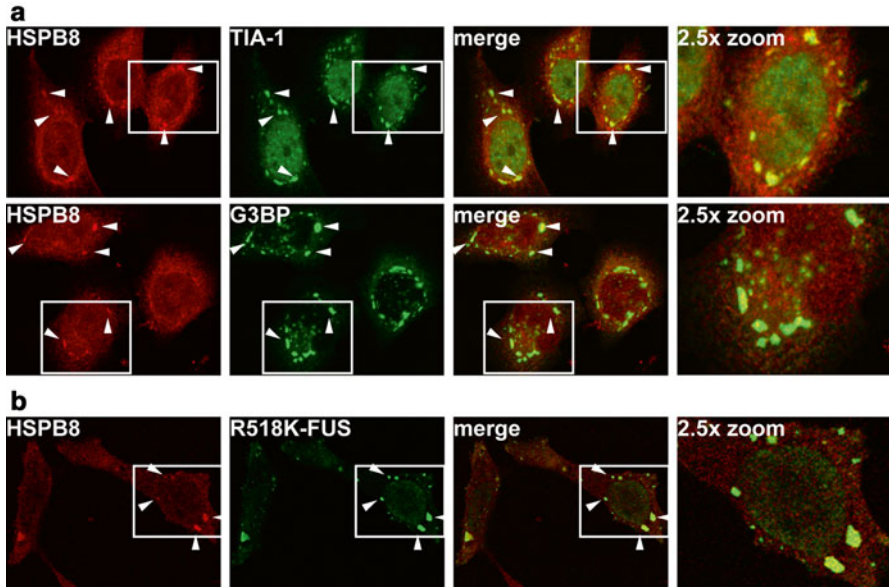
Fig. 21.1 HSPB8 is recruited into stress granules. (**a**) HeLa cells were heated at 43.5 °C for 45 min, fixed in 4 % formaldehyde for 10 min at room temperature, followed by permeabilization with cold acetone for 5 min. Cells were processed for immunofluorescence with anti-HSPB8, anti-TIA-1, anti-G3BP and DAPI. TIA-1 and G3BP were used as markers of stress granules. (**b**) HeLa cells were transfected by calcium phosphate with a cDNA encoding for HA-tagged R518K-FUS (kindly provided by Dr. U. Pandey). Twenty four hours post-transfection cells were fixed as described in A and processed for immunofluorescence with anti-HSPB8, anti-HA and DAPI. (**a**, **b**) A 2.5× magnification of the selected area is shown

DExD/H box family of helicases and have an ATP-dependent RNA unwinding (helicase) activity (Rocak and Linder 2004), and are involved in pre-mRNA processing, RNA turnover, RNA transcription, RNA export. In particular, Ddx20 associates with a protein, the survival of motor neuron (SMN) protein, that when mutated is responsible for spinal muscular atrophy (SMA). Also SMN localizes to SGs and its overexpression induces SGs (Charroux et al. 1999; Hua and Zhou 2004). SMN complexes are also involved in assembly and processing of diverse ribonucleoparticles (RNPs), including snRNPs (spliceosomes), snoRNPs, hnRNPs, transcriptosomes, and miRNPs (Pellizzoni et al. 2002). Sam68, associates with T-cell intracellular antigen-1 (TIA-1), a core component of SGs and localizes to SGs following oxidative stress (Henao-Mejia and He 2009; Henao-Mejia et al. 2009). At present, the functional significance of HSPB8 interaction with both Ddx20 and Sam68 is still largely unknown and may reflect a yet unraveled role of HSPB8 in modulating the function of these specific RNA-binding proteins, rather than their recruitment to SGs.
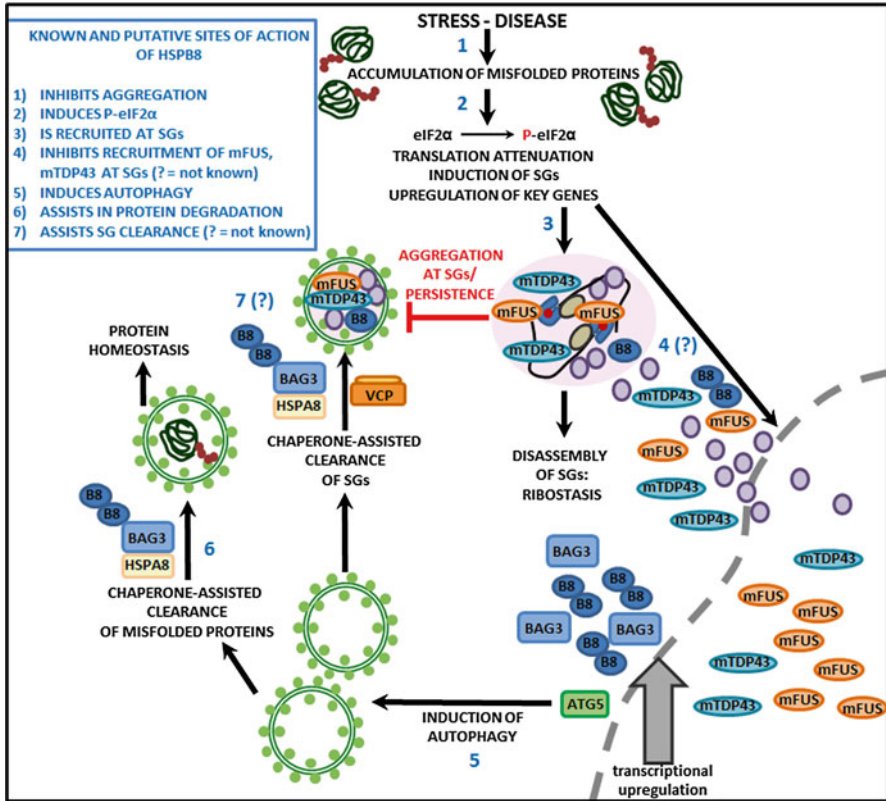
Fig. 21.2 Schematic model of the known and putative sites of action of HSPB8. (*1*) Upon proteotoxic stress conditions, due to either external insult, ageing or genetic mutations, the amount of aberrantly folded substrates exceeds the capacity of the cells to properly assist their folding or clearance; as a result aggregation-prone proteins accumulate, leading to the activation of the protein quality control system. (*2*) Phosphorylation of eIF2α represents an early event and has several consequences: it allows to temporarily attenuate translation; it induces the assembly of SGs (*3*) and it induces the expression of specific genes, including essential autophagy genes (ATG5). SG assembly is triggered by the self-aggregation of RNA-binding proteins that contains a prion-like domain, such as TIA-1, which translocates from the nucleus to the cytoplasm upon stress. Translocation into the cytoplasm and redistribution into SGs can also occur as a consequence of disease-associated mutations; in fact, TDP-43 and mutated FUS "aberrantly" redistribute into SGs. The molecular chaperone HSPB8 is also recruited at SGs but its function at this level is still largely unknown (*4*). (*5*) HSPB8, together with BAG3, is amongst the genes upregulated following proteotoxic stress (e.g. inhibition of the proteasome). HSPB8 and BAG3 participate in the stimulation of the autophagic flux and assist the targeting of damaged clients to autophagy (*6*), thereby allowing to restore proteostasis. HSPB8, which is recruited into SGs and where it colocalizes with mutated FUS, may also participate in the maintenance of SG dynamics. HSPB8 may prevent aberrant protein aggregation at SGs or may target altered and persistent SGs or components thereof to autophagy for clearance (*7*)

21.6.4   Mutated HSPB8 Is Associated with Motor Neuron Diseases

At present, three mutations (K141E, K141N and K141T) of HSPB8 have been associated with hereditary motor neuropathy (HMN) or with Charcot-Marie-Tooth type 2L (CMT-2L) disease and specifically target motor neurons (Irobi et al. 2004; Nakhro et al. 2013). The specific vulnerability of motor neurons to mutated HSPB8 has been confirmed by overexpression studies in primary neuronal motor neuron cultures, where K141E and K141N HSPB8 caused neurite degeneration (the K141T mutation has been discovered recently and little experimental information is available concerning its properties) (Irobi et al. 2010). Instead, no significant toxicity was observed in primary sensory neurons, cortical neurons or glial cells overexpressing mutated HSPB8 (Irobi et al. 2010). Although it is still unclear why motor neurons are particularly sensitive to mutated HSPB8, recent data obtained by our group suggest that K141E and K141N are mainly characterized by loss of function (LOF) rather than by a toxic gain of function (GOF). In fact, the anti-aggregation and pro-degradative activities exerted by wildtype HSPB8 on misfolded mutated proteins such as mutated AR, huntingtin, ataxin-3, SOD1, TDP-43 and the mutated form P182L of HSPB1, which is also associated with HMN, were significantly decreased by its mutations (Carra et al. 2005, 2010). The observation that mutated HSPB8 is characterized by a loss of function in PQC was further confirmed in vivo, using a Drosophila model of the CAG/polyQ disease SCA3 (Carra et al. 2010). The reduced ability of mutated HSPB8 to block the accumulation of aggregate-prone substrates may be due to a (partial) loss of HSPB8 function at the level of protein synthesis regulation (phosphorylation of eIF2α), autophagy induction and/or targeting of clients to autolysosomes for degradation. The latter is supported by experimental finding showing that overexpression of mutated HSPB8 caused the accumulation of autophagosomes that colocalise with protein aggregates, but fail to fuse with the lysosomes (Kwok et al. 2011). Instead, overexpression of wildtype HSPB8 increased the autophagic flux and colocalisation of autophagosomes with lysosomes (Kwok et al. 2011). Binding affinity of mutated HSPB8 to BAG3 was found to be decreased both in cells and in vitro, using recombinant proteins (Carra et al. 2010; Shemetov and Gusev 2011). In parallel, our recent data demonstrate that within the HSPB8-BAG3-HSPA8 complex, BAG3 seems to play a crucial role in client binding and re-routing towards autophagy as well as in stimulation of autophagic flux, while HSPB8 seems to act as helper (Minoia et al. 2014). In light of these observations, it is possible that the decreased ability of mutated HSPB8 to clear misfolded proteins and induce autophagic flux is a consequence of its reduced interaction/cooperation with BAG3 and of decreased HSPB8 stability (mutated HSPB8 by itself tends to aggregate) (Irobi et al. 2004). This would contribute to motor neuropathy. In fact, defective autophagosome maturation and autophagic vacuole accumulation have been shown in a number of protein conformational diseases as well as in HMNs/CMT disease. Moreover, genes directly involved in the endolysosomal and lysosomal pathway are mutated in HMNs (e.g. mutations in Rab7, in

lipopolysaccharide-induced tumor necrosis factor-alpha factor/small integral membrane protein of lysosome/late endosome, LITAF/SIMPLE) (Verhoeven et al. 2003; Street et al. 2003; Saifi et al. 2005), further supporting that deregulated autophagy contributes to motor neuron diseases and knockout of key autophagy genes causes neurodegeneration with accumulation of ubiquitin-positive inclusions (Komatsu et al. 2006). Whether a decreased autophagy induction also occurs in cells expressing mutated HSPB8 as a consequence of impaired modulation of phospho-eIF2α and whether such mutant forms of HSPB8 may also indirectly affect SG response/dynamics under stress is still unknown. However, it is of note that interplay between autophagy and SG clearance exists (Buchan et al. 2013) and that both deregulated autophagy and accumulation of SG components are hallmarks of motor neuron diseases, including ALS, IBMPFD (Wolozin 2014; Ramaswami et al. 2013). Thus, altered clearance of SGs as a consequence of decreased autophagic flux may also occur and contribute to HSPB8-associated motor neuropathy. Future studies are needed to understand the exact mechanisms responsible for motor neuronal death in HSPB8-associated motor neuropathy and to explain what makes specifically motor neurons vulnerable to mutated HSPB8.

21.7   Conclusions and Perspectives

Accumulation of aggregated proteins is a hallmark of many neurodegenerative and muscular diseases, confirming that imbalance of protein homeostasis is deleterious for cell survival. To survive proteotoxic stress, due to external insults or genetic mutations, cells have evolved a well-orchestrated system, the protein quality (PQC) system. The PQC system avoids or limits irreversible protein aggregation, thereby maintaining normal protein homeostasis under many different intracellular or extracellular insults that affect protein stability and function. Key players of the PQC system are molecular chaperones, including both the heat shock proteins (HSPs) and the degradative pathways, mainly the proteasome and autophagy systems. Upregulation of chaperones that can boost protein degradation, is normally beneficial in protein conformation models, but the protective functions exerted by specific molecular chaperones may be linked to their action not only at the level of proteins, but also in ribonucleoprotein complexes. In fact, in several neurodegenerative diseases, RNA-containing SGs colocalize with proteinaceous aggregates; thus, imbalance of ribonucleoprotein homoestasis may, concomitantly with proteostasis imbalance, contribute to disease and cell death. Intriguingly, the formation of these SGs is triggered, upon proteotoxic stress, by the self-reversible aggregation of RNA-binding proteins that contain prion-like domains; this suggests that chaperones may also assist SG dynamics, avoiding irreversible RNA/protein aggregation at SGs (and SG persistence, which can be observed in neurodegenerative diseases). Alternatively, but not mutually exclusive, chaperones can recognize aberrant/damaged SGs and target them (or components thereof, such as mutated FUS, TDP-43) to autophagy for degradation. How and to what extent molecular chaperones act at

the level of misfolded proteins, ribonucleoproteins and SGs, as well as on translation attenuation (which precedes SG formation) has attracted the attention of researchers. HSPB8 is an example of a chaperone, with multifaceted roles, which may act at different sites to help maintaining both protein and RNA homeostasis. In fact, HSPB8 promotes the autophagy-mediated clearance of aggregated proteins, induces the phosphorylation of eIF2α, which in turns triggers SGs and is itself recruited within SGs. Future research should investigate in more detail the link between protein and RNA homeostasis as well as the specific sites of action of chaperones, including HPB8. This will help unravel to what extent HSPB8 protective functions in protein conformation diseases are linked to its role in translation control, whether it assists SG dynamics or targets SG components to autophagy, which would also contribute to cell protection, or rather whether HSPB8 modulation of eIF2α phosphorylation and recruitment at SGs represent different, unrelated, activities. Therefore, a detailed characterization of the different partners and/or interactors recruited by HSPB8 to trigger one or all pathways acting synergistically to maintain protein and RNA balance, as well as the identification of small molecules capable of inducing HSPB8 expression in a cell-specific manner will be fundamental to finding innovative approaches to counteract human diseases associated with proteotoxicity.

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