frontiers in Neuroscience

REVIEW
published: 02 August 2019
doi: 10.3389/fnins.2019.00796

# The Regulation of the Small Heat Shock Protein B8 in Misfolding Protein Diseases Causing Motoneuronal and Muscle Cell Death

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**Specialty section:**
*This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience*

**Received:** *19 April 2019*
**Accepted:** *16 July 2019*
**Published:** *02 August 2019*

**Citation:**
*Cristofani R, Rusmini P, Galbiati M, Cicardi ME, Ferrari V, Tedesco B, Casarotto E, Chierichetti M, Messi E, Piccolella M, Carra S, Crippa V and Poletti A (2019) The Regulation of the Small Heat Shock Protein B8 in Misfolding Protein Diseases Causing Motoneuronal and Muscle Cell Death. Front. Neurosci. 13:796. doi: 10.3389/fnins.2019.00796*

Misfolding protein diseases are a wide class of disorders in which the aberrantly folded protein aggregates accumulate in affected cells. In the brain and in the skeletal muscle, misfolded protein accumulation induces a variety of cell dysfunctions that frequently lead to cell death. In motoneuron diseases (MNDs), misfolded proteins accumulate primarily in motoneurons, glial cells and/or skeletal muscle cells, altering motor function. The deleterious effects of misfolded proteins can be counteracted by the activity of the protein quality control (PQC) system, composed of chaperone proteins and degradative systems. Here, we focus on a PQC system component: heat shock protein family B (small) member 8 (HSPB8), a chaperone induced by harmful stressful events, including proteotoxicity. In motoneuron and muscle cells, misfolded proteins activate HSPB8 transcription and enhance HSPB8 levels, which contributes to prevent aggregate formation and their harmful effects. HSPB8 acts not only as a chaperone, but also facilitates the autophagy process, to enable the efficient clearance of the misfolded proteins. HSPB8 acts as a dimer bound to the HSP70 co-chaperone BAG3, a scaffold protein that is also capable of binding to HSP70 (associated with the E3-ligase CHIP) and dynein. When this complex is formed, it is transported by dynein to the microtubule organization center (MTOC), where aggresomes are formed. Here, misfolded proteins are engulfed into nascent autophagosomes to be degraded via the chaperone-assisted selective autophagy (CASA). When CASA is insufficient or impaired, HSP70 and CHIP associate with an alternative co-chaperone, BAG1, which routes misfolded proteins to the proteasome for degradation. The finely tuned equilibrium between proteasome and CASA activity is thought to be crucial for maintaining the functional cell homeostasis during proteotoxic stresses, which in turn is essential for cell survival. This fine equilibrium seems to be altered in MNDs, like Amyotrophic lateral sclerosis (ALS) and spinal and bulbar muscular atrophy (SBMA), contributing to the onset and the progression of disease. Here, we will review how misfolded proteins may affect the PQC system and how the proper activity of this system can be restored by

boosting or regulating HSPB8 activity, with the aim to ameliorate disease progression in these two fatal MNDs.

**Keywords: motoneuron diseases, amyotrophic lateral sclerosis, spinal and bulbar muscular atrophy, proteasome, autophagy, chaperones, misfolded proteins, HSPB8**

## INTRODUCTION

Proteotoxic stress associated with aberrantly folded (misfolded) protein production is one of the factors thought to be deeply involved in the pathogenesis of several neurodegenerative diseases (NDs), including motoneuron diseases (MNDs). Amyotrophic lateral sclerosis (ALS) and spinal and bulbar muscular atrophy (SBMA) are two different types of MNDs clearly linked to the aberrant folding behavior of proteins in their conformationally unstable wild type (wt) or mutated forms (Rusmini et al., 2017). These are resistant to folding, become misfolded, and are prone to aggregate, accumulating in motoneuronal cells as well as in their surrounding (glial) or target (skeletal muscle) cells. Postmitotic cells like neurons or skeletal muscle cells are highly prone to react to misfolded protein-induced stress and mount a potent intracellular response that includes chaperone overexpression and activation of the degradative pathways. These two systems work together and are referred to the protein quality control (PQC) system. The PQC system represents the first line of defense mechanism against misfolded protein toxicity; therefore, its modulation is considered as one of the best potential targets for a possible therapeutic approach aimed to counteract MND onset and/or progression, as well as neurodegeneration.

The PQC system comprises a large number of factors, which may act specifically in some subcellular compartments (i.e., chaperones located in the endoplasmic reticulum, mitochondria, lysosomes, and cytoplasm) or that are expressed in a cell and tissue specific manner. The chaperone pathways alone comprise more than 180 different chaperones and their co-regulators, while the two major degradative pathways involved in the PQC system comprise more than 600 components (in the case of the ubiquitin-proteasome system) and at least 30 different components (in the case of the autophagy system) (Hartl et al., 2011). The chaperone family comprises members that are grouped mainly on the basis of their size (small HSPs, HSP40s, HSP60s, HSP70s, HSP90s, and HSP100) and of their structure and/or function (Kampinga and Craig, 2010). Most chaperones act through the association with co-chaperones that are nucleotide exchange factors (NEFs) (Kampinga and Craig, 2010). A typical co-chaperone family is the BCL2-associated athanogene (BAG) family (Takayama and Reed, 2001). Different PQC system components, and particularly the chaperones, have been reported to be mutated and found to cause different neurodegenerative diseases. One example is represented by the small heat shock protein B8 (HSPB8), which has been found to be mutated in diseases involving motoneurons and/or muscle cells [like Charcot-Marie-Tooth type 2L disease, hereditary distal motor neuropathy type II (dHMN-II) or distal myopathy (Fontaine et al., 2006; Irobi et al., 2010; Ghaoui et al., 2016)]. This chaperone is widely expressed in almost all human tissues, and it has been proposed to be protective in ALS and SBMA (Carra et al., 2005, 2013; Crippa et al., 2010; Rusmini et al., 2013). HSPB8 is an essential member of a complex required for chaperone-assisted selective autophagy (CASA) (**Figure 1**). The CASA complex targets misfolded proteins to autophagy, and it is formed by two molecules of HSPB8, the HSP70 co-chaperone BAG3 (Carra et al., 2008b) and the HSP70 itself that can transiently associate to the E3-ubiquitin ligase CHIP/STUB1 (Arndt et al., 2010). Once the CASA complex is formed and associated with the misfolded target protein, the CHIP enzyme polyubiquitinates the misfolded substrate, which interacts with the autophagy receptor SQSTM1/p62. SQSTM1/p62 bridges the polyubiquitinated substrate proteins and the lipidated LC3 (LC3-II) protein, engulfing them into autophagosomes for degradation (Klionsky et al., 2016). The relevance of the CASA complex in the stress response to proteotoxicity and in neurodegenerative diseases is supported by a large body of evidence, including the finding that genetic mutations of HSPB8 (Irobi et al., 2004, 2010; Ghaoui et al., 2016) and of other three members of this complex have been linked to neurodegenerative or neuromuscular diseases. Mutant BAG3, for example, is implicated in dilated cardiomyopathy (Arimura et al., 2011), in muscular dystrophy (Selcen et al., 2009) and in giant axonal neuropathy (Jaffer et al., 2012), and STUB1/CHIP1 has been found mutated in Gordon Holmes syndrome (multisystemic neurodegeneration (Hayer et al., 2017) and in spinocerebellar ataxia 48 (SCA48) (Genis et al., 2018), while the protein has been reported to be destabilized in SCA16 in which six different variants have been reported (Pakdaman et al., 2017; Kanack et al., 2018). In addition, a missense mutation in the ubiquitin ligase domain of CHIP has been involved in the pathogenesis of spinocerebellar autosomal recessive 16 (SCAR16) (Shi et al., 2013, 2018). Finally, SQSTM1/p62 has been found to be mutated in some familial forms of ALS (fALS) (Fecto et al., 2011; Teyssou et al., 2013).

## THE ROLE OF HSPB8 IN THE SELECTION OF THE PROPER DEGRADATIVE SYSTEM FOR MISFOLDED PROTEINS IN MOTONEURON DISEASES

As mentioned in the introduction, ALS and SBMA are typically considered to be protein misfolding diseases, in which unstable proteins or their mutated forms tend to aggregate impairing motoneuronal or muscle functions.

From a clinical point of view, the two diseases present some difference, especially in the type of motoneurons affected. ALS is characterized by the loss of both upper and lower motoneurons and the regions affected are the brain motor cortex, the brainstem and anterior horns spinal cord motoneurons. In some fALS, alteration in the fronto-temporal regions are present and

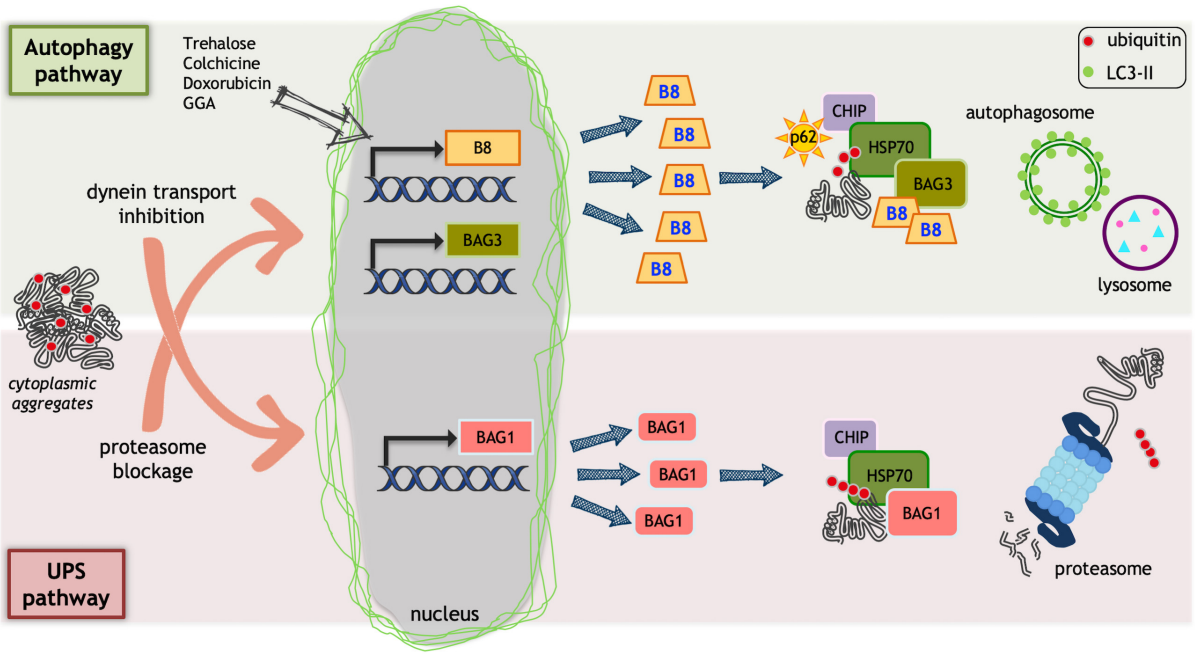


**FIGURE 1 |** Protein quality control (PQC) system. Ubiquitin proteasome system (UPS) and autophagy could be impaired by misfolded proteins that accumulate into aggregates. Aggregates can alter dynein mediated transport or block proteasome. This causes an inefficient protein homeostasis control. As a protective mechanism, HSPB8 (B8 in the figure) and BAG3 transcription is increased by proteasome blockage that, together with their partner HSP70, facilitates misfolded proteins degradation via autophagy. Trehalose, doxorubicin, and geranylgeranylacetone (GGA) selectively increase HSPB8 transcription facilitating misfolded proteins autophagic degradation. When autophagosome formation is reduced by dynein-mediated transport inhibition, the BAG1 transcription is activated. BAG1 interacts with HSP70/CHIP and allows the degradation of misfolded proteins via UPS.

correlates with a mixed phenotype involving motor dysfunction and frontolateral temporal dementia (FLTD) (Robberecht and Philips, 2013). In ALS, not only motoneurons are affected but also glial cells [astrocytes (Trotti et al., 1999; Boillée et al., 2006; Nagai et al., 2007), oligodendrocytes (Philips et al., 2013), Schwann cells (Lobsiger et al., 2009; Turner et al., 2010) and cells of the inflammatory response, like microglia (Philips and Robberecht, 2011)]. Recent data suggest that skeletal muscle cells can also be directly involved in disease onset and progression (Musarò, 2010; Onesto et al., 2011; Galbiati et al., 2014). Conversely, in SBMA, which is characterized by a much slower progression rate compared to ALS, the motoneurons affected are only the lower motoneurons in the bulbar region and in the anterior horns of the spinal cord. The motor and frontal cortex remain unaffected, and there are no clinical signs of dementia in SBMA patients. No signs of neuroinflammation involving microglial cells or of alteration in glial cells have been reported in SBMA, indicating that microglia is not involved (La Spada et al., 1991; Fischbeck, 1997; Soraru et al., 2008; Boyer et al., 2013; Malena et al., 2013; Cortes et al., 2014a; Lieberman et al., 2014). Interestingly, SBMA patients display sensory alteration due to the loss of dorsal root ganglia (DRG) sensory neurons. Finally, there are clear data indicating that, in SBMA, the skeletal muscle cells and several reproductive tissues containing androgen-target cells are directly affected (La Spada et al., 1991; Fischbeck, 1997; Adachi et al., 2005; Soraru et al., 2008; Boyer et al., 2013; Malena et al., 2013; Chua et al., 2014; Cortes et al., 2014a; Lieberman et al., 2014; Halievski et al., 2015; Sahashi et al., 2015; Xu et al., 2016, 2018).

ALS mainly appears as sporadic forms (sALS), but about 10% of the cases are inherited (fALS) and several mutated genes have already been described (**Table 1**; Cook and Petrucelli, 2019). Of note, mutations in these genes often result in the production of pathological misfolded/aggregating-prone proteins (Oskarsson et al., 2018). Moreover, these genes code for proteins that, even in their wt form, tend to be conformationally unstable (**Table 1**) forming misfolded species, which for unknown reasons aberrantly accumulate in sALS, causing cell death. This observation suggests the existence of common pathogenic mechanisms in fALS and sALS (Neumann et al., 2006; Daoud et al., 2009; Ju et al., 2009; Bosco and Landers, 2010; Tresse et al., 2010; Robberecht and Philips, 2013; Taylor et al., 2016). SBMA appears only in an inherited form, and it is associated to a CAG triplet repeat sequence expansion in exon 1 of the gene coding for the androgen receptor (AR). The coded AR protein contains a translated polyglutamine (polyQ) tract which confers toxicity to the ARpolyQ. Notably, this toxicity appears only when the ARpolyQ is bound to its endogenous ligands testosterone or dihydrotestosterone (DHT) (La Spada et al., 1991; Stenoien et al., 1999; Simeoni et al., 2000; Katsuno et al., 2002, 2003; Poletti, 2004).

At present, it remains unclear if protein misfolding is the primary toxic event in ALS, or if it reflects alteration of specific intracellular pathways (e.g., alterations of the PQC system).

**TABLE 1 |** List of genes related to ALS and SBMA.

| Gene | Name | Protein | Protein function | Aggregating/misfolded species |
|---|---|---|---|---|
| **ALS** | | | | |
| ALS2 | Alsin | ALS2 | Vesicle trafficking | |
| ANG | Angiogenin | ANG | Ribonuclease | |
| ANXA11 | Annexin A11 | ANXA11 | Vesicle trafficking, apoptosis, exocytosis, and cytokinesis | Mutated |
| ATXN2 | Ataxin 2 | ATXN2 | Endocytosis/RNA metabolism | Mutated; wt |
| C21orf2 | Chromosome 21 Open Reading Frame 2 | C21orf2 | Mitochondrial dysfunction, cytoskeletal dynamics | |
| C9orf72 | Chromosome 9 Open Reading Frame 72 | C9orf72 | Possible guanine nucleotide exchange factor-involved in autophagy | Dipeptide-repeat (DPR) proteins generated by ATG- independent transcription of the ALS/FTD-related abnormal GGGGCC expansion |
| CCNF | Cyclin F | CCNF | Catalyzes ubiquitin transfer to substrates for UPS degradation | |
| CHCHD10 | Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 | CHCHD10 | Mitochondrial protein | |
| CHMP2B | Charged multivesicular body protein 2B | CHMP2B | Protein degradation | |
| DLTNI | Dynactin subunit 1 | DCTN1 | Component of dynein motor complex | |
| EWSR1 | Ewing Sarcoma breakpoint region 1 | EWSR1 | RNA/DNA binding protein | Mutated; wt is intrinsically prone to aggregation |
| FIG4 | Phosphoinositide 5-phosphatase | FIG4 | Protein degradation | |
| FUS | Fused in Sarcoma | FUS | RNA binding protein | Mutated; wt FUS sequestered into pathological aggregates |
| HNRNPA1 | Heterogeneous nuclear ribonucleoprotein AI | HNRNPA1 | RNA-binding protein | Mutated |
| HNRNPA2/B1 | Heterogeneous nuclear ribonucleoprotein A2/B1 | HNRNPA2/B1 | RNA-binding protein | Mutated |
| KIF5A | Kinesin family member 5A | | Microtubule-based motor protein | |
| MATR3 | Matrin 3 | MATR3 | RNA-binding protein | Mutated and wt (rare inclusions) |
| NEFH | High molecular weight neurofilaments | NFH | Cytoskeletal component | Mutated |
| NEK1 | NIMA Related Kinase 1 | NEK1 | Cytoskeletal dynamics | |
| OPTN | Optineurin | OPTN | Autophagy adaptor | wt OPTN sequestered into pathological aggregates |
| PFN1 | Profilin 1 | PFN1 | Actin binding protein | Mutated |
| SETX | Senataxin | SETX | RNA/DNA helicase | |
| SOD1 | Cu-Zn superoxide dismutase 1 | SOD1 | Superoxide dismutase | Mutated; oxidized wild type (wt) SOD1 |
| SPG11 | Spatacsin | SPG11 | DNA damage repair | |
| SQSTM1 | Sequestosome 1 | SQSTM1 | Autophagy adaptor | Mutated; wt SQSTM1 sequestered into pathological aggregates |
| TAF15 | TATA box binding protein associated factor 15 | TAF15 | RNA-binding protein | Mutated; wt is intrinsically prone to aggregation |
| TARDBP | TAR DNA Binding Protein | TDP-43 | RNA-binding protein | Mutated TDP-43s; phopshorylated wt TDP-43; wt full-length TDP-43 and its C-terminal fragments (TDP-35andTDP-25) |
| TBK1 | Serine/threonine-protein kinase TBK1 | TBK1 | Innate immune response, autophagy, inflammation and cell proliferation | |
| TIA1 | T cell-restricted intracellular antigen 1 | TIA1 | RNA-binding protein | Mutated TIA1 showed altered stress granules dynamics |
| TUBA4A | Tubulin alpha 4a | TUBA4A | Microtubule subunit | Mutated |
| UBQLN2 | Ubiquilin 2 | UBQLN2 | Autophagy adaptor | Mutated; wt UBQLN2 sequestered into pathological aggregates |
| VAPB | VAMP/synaptobrevin-associated protein | VAPB | ER-membrane protein | Mutated |
| VCP | Valosin containing protein | VCP | Ubiquitin segregase | |

*(Continued)*

**TABLE 1** | Continued

| Gene | Name | Protein | Protein function | Aggregating/misfolded species |
|---|---|---|---|---|
| **SBMA** | | | | |
| AR | Androgen receptor | AR | Nuclear receptor that mediates male hormones effects | Mutant polyQ, ligand-dependent |

*The table illustrates the genes found mutated in ALS (upper part) and SBMA (lower part), listed in alphabetical order. For each gene, the name, the coded protein and its cellular function are indicated, if known. In the last column, the aggregation/misfolding propensity of the protein is depicted, specifying the affected species [wild-type (wt) and/or mutated]. In green the ALS genes that are most frequently mutated and collectively account for 60–70% of fALS and 10% of sALS cases.*

Several recent data underline that autophagy dysfunction is implicated in ALS (Valenzuela et al., 2018; Evans and Holzbaur, 2019; Nguyen et al., 2019) and SBMA (Cortes et al., 2014b), but its role in diseases is still debated (Chua et al., 2014; Cristofani et al., 2017, 2018). On one side, autophagy defects are clearly involved, since different genes found mutated in ALS code for proteins of the autophagic system, like SQSTM1/p62, OPTN, VCP, UBQLN2, TBK1 and C9orf72 (**Table 1**; Nguyen et al., 2019). Altered autophagic flux has furthermore been observed in ALS patients and confirmed in both cell and animal ALS models. On the other hand, an excessive autophagy seems to be related to the disease (Nguyen et al., 2019). Pharmacological manipulations of autophagy performed in different ALS models confirm this dual role of this degradative pathway in ALS (Valenzuela et al., 2018). For example, the treatment of SOD1-G93A mice with the mTOR-independent autophagy stimulator trehalose significantly prolonged life span and attenuated the disease signs, decreased SOD1 aggregates and enhanced motoneuron survival (Castillo et al., 2013; Zhang et al., 2014; Li et al., 2015). On the contrary, the mTOR-independent autophagy stimulator rilmenidine worsened motor neurons degeneration and symptom progression in SOD1-G93A mice (Perera et al., 2018). Similar results were observed also after treatment with the mTOR-dependent autophagy stimulator rapamycin, which exacerbated the pathological process of SOD1-G93A mice by accelerating the motor neurons degeneration, shortening the life span causing mitochondrial impairment and caspase-3 activation (Zhang et al., 2011).

With regards to SBMA, accumulation of autophagosome and reduced autophagic flux have been observed in cell and animal models of SBMA (Rusmini et al., 2010, 2013; Cortes et al., 2014a,b; Giorgetti et al., 2015; Cristofani et al., 2017). Interestingly, while the wild type AR positively regulates the activity of the transcription factor EB (TFEB, a master regulator of autophagy and lysosomal biogenesis), the mutant ARpolyQ interferes with TFEB activity, reducing its control on target genes and thus leading to autophagy dysregulation (Cortes et al., 2014b). Otherwise, in SBMA mice models, autophagy is upregulated in skeletal muscle during disease progression, indicating that tissue-specific aberrant activation of TFEB signaling might contribute to SBMA pathogenesis (Cortes et al., 2014a,b; Rusmini et al., 2015; Rocchi et al., 2016). In addition, a very recent observation suggests that alternative autophagic pathways can also be dysregulated. Indeed, the charged multivesicular body protein 7 (Chmp7) gene, which codes for an ESCRT-III related protein involved in autophagic flux and the endo-lysosomal sorting pathway is downregulated in induced pluripotent stem cells (iPSCs) derived from SBMA patients as well as in SBMA mice models even before disease onset. This suggests that CHMP7 may play a primary role protein in autophagic flux alteration observed in SBMA (Malik et al., 2019).

Several studies have proposed that HSPB8 is implicated both in ALS and SBMA, possibly by acting as a protective agent against disease onset and/or progression (Carra et al., 2005, 2008b, 2010; Crippa et al., 2010, 2013a,b, 2016a,b; Rusmini et al., 2013, 2019; Cristofani et al., 2017, 2018; Cicardi et al., 2018). It has been shown that, during disease manifestations and at the end stage of disease, HSPB8 is highly expressed in the spinal cord of the SOD1-G93A ALS mouse model and in spinal cord specimens of ALS patients (Anagnostou et al., 2010; Crippa et al., 2010). Moreover, HSPB8 was found upregulated in the lateral tract astrocytes of patients with short disease duration (Gorter et al., 2018). In the SOD1-G93A ALS mouse model, the high levels of HSPB8 are confined specifically in anterior horn spinal cord motoneurons that survive at the end stage of disease (Crippa et al., 2010). These data are of great interest, since in non-transgenic (NTg) normal mice, the expression of HSPB8 within the spinal cord typically decreases with age (Crippa et al., 2010). Thus, these motoneurons are potentially more vulnerable to the toxicity of mutant misfolded proteins, and HSPB8 overexpression in motoneurons of the SOD1-G93A affected mice could represent a cell response to damages induced by misfolded proteins. The increased HSPB8 levels may enhance proteotoxic stress tolerance of these surviving motoneurons.

In NTg mice, HSPB8 is also present at high levels in muscles; instead, in the SOD1-G93A ALS or SBMA AR113Q mouse models, HSPB8 expression is robustly increased paralleling disease progression (Crippa et al., 2013a,b; Rusmini et al., 2015). This is not unexpected, since skeletal muscle cells are a direct target of misfolded protein toxicity in both ALS and SBMA; therefore, the enhanced production of HSPB8 may serve to protect this tissue during disease progression. This interpretation is suggested by the finding that, in TDP-43 ALS *Drosophila melanogaster* models (TDP-43, TDP-35, and TDP-25), the overexpression of the HSPB8 functional ortholog (*HSP67Bc)* protects from misfolded protein toxicity, while its downregulation has the opposite effect (Crippa et al., 2016a). In line with these data is the observation that a viral homologue of HSPB8—the protein ICP10PK, carried by the herpes simplex virus type 2 (HSV-2)—when used to infect the SOD1-G93A ALS rat model is able to delay symptom onset and reduce the progression of the disease, thus enhancing the overall survival of the mice (Aurelian, 2012; Aurelian et al., 2012). Whether ICP10PK exerts similar functions in PQC compared to HSPB8

or whether its protective activity is due to other mechanisms that are not related to PQC and autophagy is still unknown.

Collectively, the data obtained using ALS and SBMA animal models corroborate the notion that HSPB8 is protective against misfolded protein toxicity in these diseases.

The observations performed at cellular and molecular levels parallel the animal data, showing that HSPB8 has a potent antiaggregant activity, and facilitates the removal of aggregating misfolded proteins from a variety of neuronal and muscle models of ALS and SBMA. For example, HSPB8 reduces the accumulation of several polyQ proteins, like polyQ of the mutant huntingtin and ataxin-3, as well as of the beta-amyloid protein, of the alpha-synuclein, and of a large number of ALS-associated mutant proteins (like the mutant SOD1-G93A or the mislocalized TDP-43 C-terminal fragments (TDP-35 and TDP-25) and the abnormally translated dipeptides (DPRs) produced from the expanded GGGGCC repeated sequence of the *C9orf72* gene causing ALS and/or FLTD) (Chavez Zobel et al., 2003; Wilhelmus et al., 2006; Carra et al., 2008a,b; Crippa et al., 2010, 2016b; Bruinsma et al., 2011; Seidel et al., 2012; Rusmini et al., 2013; Cristofani et al., 2017, 2018; Cicardi et al., 2018).

Very recently, it has been shown that HSPB8 is also able to maintain a correct dynamic behavior of stress granules (SGs), membraneless ribonucleoprotein (RNP) complexes that form via liquid-liquid phase separation (LLPS) (Banani et al., 2017).

In the past decade, researchers have sought to understand the principles that regulate the formation and dissolution of SGs, due to their potential implication in a number of neurological disorders [e.g., ALS, FLTD, Alzheimer's disease (AD), etc.] (Elden et al., 2010; Taylor et al., 2016). Similar to other types of membraneless organelles, such as PML bodies (Banani et al., 2017), SGs are highly dynamic and are induced by stress conditions, including oxidative stress, viral infection, and temperature upshift, but they tend to dissolve upon stress relief (Anderson and Kedersha, 2002). While SGs tend to dissolve rapidly after stress relief in healthy cells, SGs were reported to persist for longer time in cell models of ALS that express mutated, disease-causing forms of TDP-43, FUS, TIA-1, and hnRPNA1. TDP-43, FUS, TIA-1, and hnRPNA1 are all RNA-binding proteins recruited inside SGs. These RBPs can form liquid droplets *in vitro* that are unstable and can mature with time into amyloid-like aggregates (Molliex et al., 2015; Patel et al., 2015; Mackenzie et al., 2017). The ALS-associated mutated forms of TDP-43, FUS, TIA-1, and hnRPNA1 accelerate the conversion of the liquid droplets into a solid aggregated-like state *in vitro*, while they all confer rigidity to SGs in ALS cell models, thereby delaying their disassembly kinetics and favoring the co-aggregation of SGs with other misfolded aggregate-prone proteins (Ganassi et al., 2016; Taylor et al., 2016; Mateju et al., 2017). These observations have therefore, suggested the hypothesis that SGs play an important role in ALS. Recent data obtained using light-inducible SGs (or OptoGranules) showed that the repetitive induction of SGs leads to their conversion into neuronal aggregates that become enriched for phosphorylated TDP-43 forms, ubiquitin and SQSTM1/p62 (Zhang et al., 2019), typical hallmark of inclusions found in sALS and fALS (Neumann et al., 2009). Thus, SGs may contribute to the formation of the neuronal pathological inclusions (Zhang et al., 2019).

So far, two mechanisms that decrease SG dynamics have been identified: (1) the presence of unstable aggregate-prone RBPs (Molliex et al., 2015; Patel et al., 2015) and (2) the accumulation inside SGs of misfolded proteins [including defective ribosomal products (DRiPs) and ALS/FLTD-linked DPRs] (Ganassi et al., 2016; Lee et al., 2016; Mateju et al., 2017). Enhanced clearance of misfolded proteins, DRiPs or DPRs may, therefore, indirectly facilitate disassembly of SGs, restoring their physiological dynamics. Enhanced clearance of aberrant SGs may furthermore exert protective functions. Indeed, the accumulation of misfolded proteins inside SGs is prevented by the action of molecular chaperones such as VCP and the HSPB8-BAG3-HSP70 complex, which target misfolded proteins, DRiPs and DPRs to degradation (Verma et al., 2013; Seguin et al., 2014; Ganassi et al., 2016). In addition, ZFAND, VCP, and SQSTM1/p62 facilitate the degradation of persisting aberrant SGs via autophagy and the proteasome, respectively.

The formation of cytoplasmic aggregates containing phosphorylated TDP-43 may arise also with SG-independent mechanisms, and can be induced by fibrillar fragments of aggregated TDP-43 itself or FUS (Gasset-Rosa et al., 2019). These aggregates sequester nuclear transport factors, impairing the nucleocytoplasmic shuttling (Freibaum et al., 2015; Jovicic et al., 2015; Boeynaems et al., 2016; Woerner et al., 2016; Kim and Taylor, 2017; Chou et al., 2018). Considered together, these studies demonstrate that SG-dependent and SG-independent mechanisms contribute to ALS disease progression. They likewise suggest that SG-dependent and SG-independent mechanisms may converge so as to favor the formation of the pathological inclusions. Consequently, approaches that limit the cytoplasmic accumulation of aggregated TDP-43, such as overexpression of HSPB8 or induction of autophagy, may exert beneficial effects by acting both on SG-dependent and independent TDP-43 aggregates.

From the molecular point of view, the mechanism by which HSPB8 blocks the accumulation of misfolded proteins in cells affected in these MNDs is intriguing. As mentioned above, HSPB8 is a crucial component of the CASA complex. The intracellular levels of HSPB8 appear not to be sufficient to handle the large amounts of misfolded proteins that accumulate under certain stress conditions. In fact, the single overexpression of HSPB8 is able to restore a sufficient clearance of misfolded proteins, preventing their aggregation in cells. It is thus not surprising that the main activity of HSPB8 is to act as an autophagy facilitator. The first proof for this action comes from the observation that HSPB8 strongly interacts with the Ile-Pro-Val (IPV) domains of BAG3 forming a stable complex (Fuchs et al., 2010, 2015). Moreover, BAG3 might use the IPV domains to interact also with other HSPBs, like HSPB1, HSPB2 (Morelli et al., 2017), HSPB5 (αB-crystallin) (Hishiya et al., 2011), and HSPB6 (Fuchs et al., 2009; Rauch et al., 2017).

Like HSPB8, other members of the mammalian HSPB family have been linked to ALS progression. Indeed, two HSPB1 variants have been reported in a cohort of unrelated Italian ALS patients, while the loss of chaperone-like activity was demonstrated in

one of the mutant proteins (Capponi et al., 2016). Like HSPB8, also HSPB1, together with αB-crystallin, were found upregulated in the spinal cords of different symptomatic mutant SOD1 mice (G37R, G93A, G85R, H46R/H48Q), compared to control (Vleminckx et al., 2002; Wang et al., 2003). Notably, we observed in two similar SOD1-G93A mice strains that were characterized by a different progression rate (fast vs slow progression) that low basal expression of the αB-crystallin correlates with a fast progressing phenotype, whereas high αB-crystallin levels correlate with a more slowly progressing phenotype (Marino et al., 2015). This observation is indicative of a protective role of αB-crystallin in these animals. *In vitro* experiments also support this hypothesis, since both αB-crystallin and HSPB1 overexpression are able to suppress SOD1 aggregation (Yerbury et al., 2013). Focusing on HSPB1, its role in ALS is rather controversial; for example, HSPB1 overexpression is beneficial when tested in different SOD1-based ALS cell models (Patel et al., 2005; Krishnan et al., 2006; An et al., 2009; Yerbury et al., 2013; Heilman et al., 2017), but animal model experiments did not confirm this protective role. In its work, Krishnan et al. (2008) showed that the ubiquitous over-expression of human HSPB1 in SOD1-G93A mice [double transgenic SOD1(G93A)/hHSPB1 mice] did not affect disease duration, progression, motor neuron degeneration or SOD1 aggregation, although hHSPB1 overexpression alone (single transgenic hHSPB1 mice) protected against spinal cord ischemia (Krishnan et al., 2008). Slightly different results were obtained by Sharp et al. (2008), where the SOD1(G93A)/HSPB1 double transgenic mice showed an improvement in some pathological parameters compared to SOD1-G93A mice; this protective activity was present at the early stage of disease but was lost at later stages (Sharp et al., 2008). Interestingly, in these mice the expression of hHSPB1 protein in affected cells decreased during disease progression, although mRNA levels remained unchanged, and so far no explanation for this phenomenon have been provided, but this reduced translation/enhanced clearance of HSPB1 may help to explain the lack of protection at late stage of disease.

Part of the protective activity of HSPBs against neuronal loss may be due to their ability to interact with BAG3. In addition to the binding with different HSPBs, BAG3, with its BAG domain, can bind directly the HSP70 already involved in a heterodimer with CHIP (**Figure 1**). When HSPB8 binds, the CASA complex is formed, and this allows misfolded protein recognition. BAG3 is a scaffold protein, which also contains a PXXP motif for the binding to dynein, and this interaction is reinforced by the 14-3-3 protein, which binds in close proximity to the PXXP motif (McCollum et al., 2009; Merabova et al., 2015). Dynein has now the capability to move retrogradely the CASA complex with the misfolded protein (polyubiquitinated by CHIP) along microtubules to the MTOC, where aggresomes are formed and autophagosomes assembled.

Dynein mediated transport has been involved in ALS pathogenesis: (i) dynein has been detected in SOD1 aggregates and (ii) alteration of retrograde transport is present in transgenic SOD1-G93A mice even if the legs at odd angles (Loa) mutation in cytopasmic dynein could be protective in transgenic SOD1-G93A mice where it delays disease onset and extends the life span (Kieran et al., 2005; Zhang et al., 2007; Bilsland et al., 2010; El-Kadi et al., 2010). Moreover, DCTN1 and KIF5A motor protein and TUBA4, NEFH, and NEK1 cytoskeleton proteins are related to ALS (**Table 1**).

The polyubiquitinated misfolded proteins are then/finally recognized by the autophagy receptor (like SQSTM1/p62) and engulfed by the lipidated LC3-II into nascent autophagosome for clearance [see (Rusmini et al., 2017)]. This mechanism has been initially elucidated in physiological condition in muscle fiber subjected to extensive physical exercise, in which large amounts of damaged (carbonylated, nitrosylated, etc.) proteins are generated. Indeed, the CASA complex is essential for Z-disk maintenance in skeletal muscle (Arndt et al., 2010; Ulbricht et al., 2015). We proved that this mechanism takes place also in motoneurons, in pathological conditions due to the presence of ALS or SBMA-associated misfolded proteins (Crippa et al., 2010, 2016a,b; Rusmini et al., 2013; Cristofani et al., 2017, 2018; Cicardi et al., 2018). The CASA complex may also involve HSP40 (particularly DNAJB6) (Sarparanta et al., 2012), which acts as an HSP70 co-chaperone to block misfolded protein aggregation (Hageman et al., 2010). Like several members of the CASA complex, mutation in *DNAJB6* has been identified in human diseases linked to aberrant protein aggregation, like Limb-girdle muscular dystrophies (LGMDs) (Sandell et al., 2016). Of note, DNAJB6 aggregates in muscles of LGMD patients and sequesters BAG3, HSPB8, HSP70 and CHIP in inclusions of different sizes present in the cell cytoplasm. Curiously, in the same specimens DNAJB6 was also present in nuclear aggregates that were positive exclusively for HSPB8 (Sato et al., 2013), leading to the hypothesis that these two proteins may interact during CASA complex formation earlier, or at a different cell location, than with the other members of the CASA complex.

Even more intriguing is the existence of an alternative way to escape HSPB8/BAG3 recognition when the function of the CASA complex is blocked. This way reroutes substrates, including misfolded proteins, from autophagic to proteasomal degradation. For example, if the dynein mediated transport of the CASA complex is blocked, the heterodimer HSP70-CHIP does not interact with HSPB8-BAG3, but preferentially associates to an alternative interactor: BAG1 (Cristofani et al., 2017). Like BAG3, BAG1 is a NEF/BAG co-chaperone of HSP70, which after interacting with HSP70-CHIP routes cargoes, including misfolded proteins, to the proteasome (Arndt et al., 2010; Ulbricht et al., 2013; **Figure 1**). It is relevant to note that the blockage of the CASA complex transport to MTOC induces the transcriptional activation of the BAG1 gene. On the other hand, the blockage of proteasome induces the *de novo* synthesis of HSPB8 and BAG3 (Yew et al., 2005; Carra, 2009; Crippa et al., 2010; Carra et al., 2013; Cristofani et al., 2017). Therefore, this connected transcriptional regulation gives rise to a fine-tuned equilibrium between autophagy and proteasome and allows the selection of the proper degradative pathway during different types of proteotoxic stresses, which may differentially impact on one of the two systems (Arndt et al., 2010; Crippa et al., 2010; Behl, 2011, 2016; Gamerdinger et al., 2011a,b; Lilienbaum, 2013; Xu et al., 2013; Jia et al., 2014; Minoia et al., 2014; Merabova et al., 2015; Cristofani et al., 2017). An unbalanced

equilibrium between these two systems may thus account for the aberrant accumulation of misfolded proteins in MNDs and in NDs in general (Kakkar et al., 2014; Ciechanover and Kwon, 2015; Nikoletopoulou et al., 2015; Senft and Ronai, 2015; Xilouri and Stefanis, 2015).

The involvement of other HSPs, in particular HSP70 and HSP90, in ALS has been deeply reviewed in Kalmar and Greensmith (2017) and Lackie et al. (2017).

## IDENTIFICATION OF SMALL MOLECULES THAT REGULATE HSPB8 EXPRESSION IN MND-AFFECTED CELLS

In line with the data summarized above, it would be of interest to determine whether the expression of HSPB8, which is sufficient to restore autophagy, can be enhanced by acting at the level of its gene transcription, thus preventing its role of limiting factor for the CASA complex. Several small molecules have already been identified to act as HSPB8 inducers and could be an opportunity to be tested in clinical trials in MNDs. In a large, high-throughput screening, based on a luciferase reporter controlled by the human HSPB8 promoter, we were able to find several FDA-approved drugs capable of modulating HSPB8 gene expression. Among the list of hits, we selected colchicine and doxorubicin and showed that these compounds enhance the clearance of insoluble TDP-43 species (hallmark for ALS) in a HSPB8-dependent (even if not exclusive) manner (Crippa et al., 2016b). Since colchicine is a safe drug with a well-established pharmacokinetic, it is now under investigation in a phase II clinical trial on a large cohort of ALS patients (Mandrioli et al., 2019).

Other small molecules that are able to induce HSPB8 expression have been characterized. One is trehalose, a non-toxic natural compound well-known for its ability to induce autophagy (Rusmini et al., 2013), whose mechanism of action has been recently unraveled. TFEB is the main mediator of the effects of trehalose. Trehalose treatment correlates with the activation of the calcineurin/TFEB pathway by a rapid and transient lysosomal membrane permeabilization and, possibly, by lysosomal calcium release. This event triggers the induction of TFEB target genes leading to specific removal of damaged lysosomes by autophagy (called lysophagy) and the restoration of normal lysosomal homeostasis (Rusmini et al., 2019). Trehalose has been proven, both in cell and animal models, to be very efficient in the removal of misfolded proteins in many different NDs (Tanaka et al., 2004; Davies et al., 2006; Rodriguez-Navarro et al., 2010; Perucho et al., 2012; Schaeffer and Goedert, 2012; Castillo et al., 2013; Du et al., 2013; Sarkar et al., 2014; Zhang et al., 2014; He et al., 2016).

Estrogens and selective estrogen receptor modulators (SERMs) are also potent activators of HSPB8 expression (Sun et al., 2007; Piccolella et al., 2017), and this might help to explain the existence of gender differences in the risk to develop some age-related forms of NDs (Villa et al., 2016).

Geranylgeranylacetone (GGA), also known as teprenone, which is an inducer of several HSPs, is also a potent upregulator of HSPB8 expression, and has been proven to be able to decrease the formation of amyloid oligomer and aggregates in desmin-related cardiomyopathy (Sanbe et al., 2009).

Finally, one of the best known regulators of HSPB8 (and BAG3) is the NF-κB transcription factor, which is generally activated in the recovery period that follows a heat shock (Nivon et al., 2012). Even if the control of this pathway is still obscure, it is expected that modulators of the NF-kB pathway may also influence the expression of HSPB8 in cells. Whether or not this approach may have therapeutic perspectives remains to be elucidated.

## CONCLUSION

In conclusion, biochemical and immunohistochemical data obtained by using cell and animal (mouse and Drosophila) models of ALS and other neurodegenerative diseases support the interpretation that HSPB8 has a prominent role in counteracting the toxicity of misfolded proteins in MNDs and may be fundamental in the maintenance of the delicate equilibrium that regulates the routing of proteins to autophagy and to the proteasome. This is further suggested by the finding that HSPB8 and BAG3 are upregulated in the postmortem tissues from patients affected by several types of protein conformational diseases, specifically in the regions interested by neurodegeneration (Anagnostou et al., 2010; Seidel et al., 2012). HSPB8 may act as a limiting factor in this context, and its transcriptional induction or functional activation with small molecules may serve as a potential approach to counteract the onset and/or progression of these devastating NDs. Despite the fact that how HSPB8 works at the molecular level and how its expression is regulated have not yet fully been elucidated, the current literature highlights its relevance in several NDs, prompting us to investigate how to exploit the functions of this chaperone against neurodegeneration. The availability of safe drugs that are able to induce HSPB8 expression in MNDs may be the first step to clarify its potential protective role in these diseases.

## AUTHOR CONTRIBUTIONS

PR, RC, MG, VC, and AP designed and wrote the manuscript, and critically discussed all the sections of this minireview. RC prepared the figure. MEC, VF, BT, EC, MC, EM, MP, and SC critically revised the manuscript and figure. All authors have provided final approval of the version to be published.

## FUNDING

The following grants are gratefully acknowledged: Fondazione Telethon, Italy (Nos. GGP14039 and GGP19128 to AP), Fondazione Cariplo, Italy (Nos. 2014-0686 to AP and SC, and 2017_0747 to VC), Fondazione AriSLA, Italy (No. ALS_HSPB8, ALS_Granulopathy, and MLOpathy to AP and SC and Target_ALS to AP), Association Française contre les Myopathies, France (AFM Telethon No. 16406 to AP), Università

degli Studi di Milano e piano di sviluppo UNIMI – linea B (to VC and PR), Italian Ministry of Health (MinSal) (No. GR-2011-02347198 to VC and SC), Agenzia Italiana del Farmaco (AIFA) (Co_ALS to AP and SC), Fondazione Regionale per la Ricerca Biomedica (FRRB) (TRANS_ALS, Rif. No. 2015-0023 to AP), Regione Lombardia, Italy (to AP), Italian Ministry of University and Research (MIUR), PRIN - Progetti di ricerca di interesse nazionale (Nos. 2015LFPNMN and 2017F2A2C5 to AP and SC); MIUR progetto di eccellenza, Fondo per il Finanziamento delle Attività Base di Ricerca (FFABR-MIUR to PR, EM, and MG), European Molecular Biology Organization (EMBO), short term fellowship (No. 537 - 2015 to RC), Kennedy's Disease Association (KDA, United States to RC), International Brain Research Organization (InEurope short stay grant to MEC). This is an EU Joint Programme – Neurodegenerative Disease Research (JPND) project. This project is supported through the following funding organizations under the aegis of JPND – www.jpnd.eu. This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 643417 (Grant ID: 01ED1601A, CureALS to AP and SC).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.