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Reference:
Adriaenssens Elias, Geuens Thomas, Baets Jonathan, Echaniz-Laguna Andoni, Timmerman Vincent.- Novel insights in the disease biology of mutant small heat shock proteins in neuromuscular diseases
Brain - ISSN 0006-8950 - 140:10(2017), p. 2541-2549
Full text (Publisher's DOI): https://doi.org/10.1093/BRAIN/AWX187
To cite this reference: https://hdl.handle.net/10067/1468770151162165141

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Novel insights in the disease-biology of mutant small heat shock proteins in neuromuscular diseases.

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Running title: Small heat shock proteins and neuromuscular diseases

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Small heat shock proteins are molecular chaperones that exert diverse cellular functions. So far mutations in the coding regions of *HSPB1* (Hsp27) and *HSPB8* (Hsp22) were reported to cause distal Hereditary Motor Neuropathy and Charcot-Marie-Tooth disease. Recently, the clinical spectrum of *HSPB1* and *HSPB8* mutations was expanded now also including myopathies. Here we provide an update on the molecular genetics and biology of small heat shock protein mutations in neuromuscular diseases.

Mutations in the genes encoding for small heat shock protein *HSPB1* and *HSPB8* were reported to cause distal hereditary motor neuropathies (dHMN) and the axonal form of Charcot-Marie-Tooth neuropathy (CMT2) (Evgrafov *et al.*, 2004; Irobi *et al.*, 2004). Patients with *HSPB1* mutations usually present with progressive distal weakness of the legs often first manifesting under the form of bilateral foot drop. The age of onset is usually in the second decade of life, although much later disease onsets (up till the sixth/seventh decade of life) have also been described (Harding and Thomas, 1980; Capponi *et al.*, 2011; Echaniz-Laguna *et al.*, 2017; Rossor *et al.*, 2017). Disease progression is slow with a significant percentage of the patients also developing marked distal upper limb weakness and ultimately becoming wheel chair dependent. Transmission is dominant in the majority of kinships, but *de novo* mutations are frequent and recessive transmission has been observed in rare instances (**Table 1**). Mild sensory involvement consisting of mild feet paresthesia is observed in 30-50% of patients. However, the degree of sensory involvement may vary even between members of the same family (Rossor *et al.*, 2017). As a result, patients are usually diagnosed with distal hereditary motor neuropathy (dHMN) when symptoms are purely motor, and diagnosed with axonal Charcot-Marie-Tooth disease type 2 (CMT2) when symptoms are motor and sensory.

Other common features observed in patients with *HSPB1*-linked neuropathy include mildly elevated creatine kinase (CK) levels and foot deformities, including *pes cavus*. Frequently, thigh and hand weakness appear as the disease progresses and plantar flexion weakness of the ankle (rather than the dorsiflexion weakness typically observed in more common types of inherited peripheral neuropathies) was reported as a common feature for patients with *HSPB1* mutations (Rossor *et al.*, 2017). Interestingly, central nervous system (CNS) involvement is observed in 5-10% of cases, including pyramidal and cerebellar features (Echaniz-Laguna *et al.*, 2017). Fat infiltration in lower limb muscles is common and was observed even before clinical assessment identified signs of neuropathy (Chung *et al.*, 2008; Rossor *et al.*, 2017). Magnetic Resonance Imaging (MRI) may therefore provide a more sensitive method for early disease diagnosis. However, the degree of fatty infiltration only correlates with plantar flexion weakness, but not with the overall severity of the neuropathy (Rossor *et al.*, 2017).

Patients with *HSPB8*-linked neuropathy closely resemble patients with *HSPB1* mutations, with the exception that thigh weakness, CNS involvement and autosomal recessive transmission are rarely observed. Muscle pathology and electrophysiological examination of patients with *HSPB1*- and *HSPB8*-linked neuropathy usually shows a slowly progressive, mostly symmetrical and predominantly distal motor axonal neuropathy, with mild sensory involvement observed in a significant proportion of cases (Echaniz-Laguna *et al.*, 2017; Rossor *et al.*, 2017).

Two independent studies reported three different *HSPB1* mutations (p.Ser86Leu, p.Gln190His and p.Ala204fs*6) in two sporadic cases and one consanguineous family with Amyotrophic Lateral Sclerosis (ALS), suggesting that the disease spectrum of *HSPB1* may not be limited to CMT2/dHMN (Scarlato *et al.*, 2015; Capponi *et al.*, 2016). All patients had an Italian origin, but came from different regions of the country. The two male siblings (p.Ser86Leu) had a disease onset around their second and third decade of life respectively. They initially presented

with a typical dHMN/CMT2 phenotype characterized by a bilateral foot drop, distal weakness and wasting of the upper limbs without sensory symptoms. In his sixth decade of life, the older brother progressed rapidly with asymmetric worsening of lower limb strength. Within 18 months he developed hypophonia, dysphagia and respiratory distress requiring non-invasive ventilation. He also developed brisk reflexes, Babinski and Hoffmann signs. The patient died from myocardial infarction. The younger brother did not develop any signs reminiscent of motor neuron disease until now. For the sporadic cases, one of them (p.Gln190His) was diagnosed with ALS at the age of 58 and died from respiratory failure 24 months after onset of the symptoms. The other sporadic patient (p.Ala204fs*6) was diagnosed at the age of 73 with ALS plus extrapyramidal features and there was no longer worsening of the clinical phenotype at the age of 83. Both sporadic mutations target the third exon of *HSPB1* and affect the C-terminal region of the protein. This region was already shown to be important for stabilizing the interactions between monomers and thereby mediating oligomerization of HSPB1 proteins (Lindner *et al.*, 2000; Morris *et al.*, 2008; Vos *et al.*, 2008). Furthermore, dynamic subunit exchange from these large polydisperse oligomers is a key characteristic of small heat shock proteins (Bova *et al.*, 2000; Sobott *et al.*, 2002; Aquilina *et al.*, 2003, 2013; Shashidharamurthy *et al.*, 2005; Stengel *et al.*, 2010; Baldwin *et al.*, 2011). The C-terminal domain, containing the conserved IXI/V motif, is thought to play a key role in controlling the size distribution and kinetics of subunit exchange between these oligomeric complexes (Jehle *et al.*, 2010; Baldwin *et al.*, 2012; Delbecq *et al.*, 2012, 2015; Hilton *et al.*, 2013). Not surprisingly, mutations in the C-terminal domain were found to increase oligomerization and promote protein aggregation of HSPB1 (Ackerley *et al.*, 2006; Chalova *et al.*, 2014). Interestingly, C-terminal mutations seem to be clustered around the IXI/V motif (**Table 1**) and are typically linked to a more severe CMT2/dHMN phenotype (for review see (Benndorf *et al.*, 2014)). The newly discovered C-terminal mutant p.Ala204fs*6 was shown to capture the wild-type protein, in stable dimers, and

thereby decreases the overall HSPB1 chaperone activity (Capponi *et al.*, 2016). A similar alteration in the chaperone activity of HSPB1 was already reported before. An earlier study described a sporadic ALS patient with a rare genetic variant in the conserved Heat Shock Element (HSE) in the promoter of *HSPB1* (Dierick *et al.*, 2007). This variant led to a lower promoter activity and compromised stress response, possibly contributing to the ALS pathogenesis of this patient.

An alteration in the expression level of heat shock proteins has been associated with the pathogenesis of several neurodegenerative diseases. For example, alpha-B-crystallin (CRYAB) and HSPB1 were found upregulated in the spinal cord of mutant SOD1 mice (Vleminckx *et al.*, 2002; Wang *et al.*, 2005, 2008). In the muscle, HSPB8 expression levels were elevated at the post-symptomatic stage (Crippa *et al.*, 2013). In humans, HSPB1 and HSPB8 were both upregulated in the lumbar spinal cord of patients with ALS (Anagnostou *et al.*, 2010). Furthermore, levels of HSPB1 were increased in autopsy muscles of patients with pathogenic C9orf72 repeat expansions and pathogenic FUS mutations (Jesse *et al.*, 2016). In serum samples, from 58 patients diagnosed with ALS, protein levels of HSP70 and HSP90 were found elevated whereas HSPB1 levels were not altered (Miyazaki *et al.*, 2016). Decreased expression of CRYAB and HSPB1 on the other hand, was found to correlate with a faster disease progression in SOD1 mice (Maatkamp *et al.*, 2004; Marino *et al.*, 2015). Increased expression of small heat shock proteins thus seems to provide a protective mechanism against motor neuron disease. Indeed, overexpression of HSPB8 protects against neurotoxicity in models of mutant SOD1 and TDP-43 both *in vitro* and *in vivo* (Crippa *et al.*, 2010, 2016; Yerbury *et al.*, 2013). Overexpression of HSPB1 also delayed motor neuron degeneration in the early (but not late) stages of the disease in SOD1 mice (Sharp *et al.*, 2008). Finally, specific upregulation of a small heat shock protein reduced the neurotoxic effects of TDP-43 both in the photoreceptors and motor neurons of a TDP-43 *Drosophila* model (Gregory *et al.*,

2012). Together, these data underline the importance of small heat shock proteins in motor neuron proteostasis. Rare genetic variants in small heat shock proteins may therefore increase the overall susceptibility to develop motor neuron degeneration and may give rise to related neurodegenerative disorders.

Such expansion of the phenotypic spectrum towards other neurological disorders is a phenomenon observed for other disease entities as well. For example, it is well established for Frontotemporal Dementia (FTD) and ALS. Interestingly, literature suggests a similar overlap in the genes underlying ALS and other motor neuron disorders. For instance, the genes *VCP*, *FIG4*, *NEFH*, *SPG11*, *SETX, DNAJB2* and *CHCHD10* have all been reported to cause ALS and CMT2/dHMN (**Table 2**) as well as other neurological and even non-neurological disorders (e.g. *VCP* mutation causing Paget disease). Together, this provides evidence for an increasing overlap in the underlying genetic spectrum of these neurodegenerative diseases.

Although genetic variants in the *HSPB1* gene are a frequent cause of CMT2/dHMN with an estimated prevalence of 5%, *HSPB8* mutations are still rare and account for only 1% of patients (Dierick *et al.*, 2008; Capponi *et al.*, 2011; Echaniz-Laguna *et al.*, 2017). So far only autosomal dominant mutations were reported for *HSPB8* and, strikingly, all target the same amino acid residue (Lys141). This hot-spot residue therefore seems to play a key role in the structure and function of HSPB8. Similarly, the homologous Lys141 residue in HSPB1 also causes distal neuropathy (p.Lys141Gln) and is necessary to form a salt bridge with a negatively charged residue located on the interacting monomer (Nefedova *et al.*, 2013). In addition, HSPB8 also interacts with BAG3 (Carra *et al.*, 2008). This interaction is well characterized and found to be important for autophagy, a crucial degradative pathway for postmitotic cells like motor neurons. We found that mutations at the hot-spot Lys141 residue increase the binding affinity of HSPB8 towards BAG3 in a neuronal-like cell model (Echaniz-Laguna *et al.*, 2017). This is in contrast with two earlier studies where

mutations at the Lys141 resulted in decreased binding properties (Carra *et al.*, 2010; Shemetov and Gusev, 2011). It remains unclear why these studies gave different outcomes, but it is obvious that mutations at this lysine residue directly affect the interaction between HSPB8 and BAG3. In addition, we also report two missense mutations in *HSPB8* not targeting the Lys141 hot-spot (Echaniz-Laguna *et al.*, 2017). Both mutations (p.Pro90Leu and p.Asn138Thr) give rise to an identical motor-predominant phenotype. These phenotypes are consistent with those observed for *HSPB8* hot-spot mutations with broadly varying onset age from early (<10 years of age) to late (>60 years of age). Strikingly, the p.Pro90Leu and p.Asn138Thr mutations showed no altered affinity of HSPB8 towards BAG3, suggesting that their underlying molecular pathomechanisms may be different. This could indicate that the interaction with BAG3 is not relevant in disease context. However, our recent efforts to identify novel interacting proteins through immunoprecipitation-mass spectrometry (IP-MS) did not yield any additional binding partners, apart from BAG3, for wild type or mutant HSPB8 (unpublished data). The roles of these binding partners in autophagy, translational control and stress granule dynamics may therefore merit further exploration for mutant HSPB8 (Carra *et al.*, 2008, 2009; Ganassi *et al.*, 2016).

A frameshift mutation in *HSPB8* (p.Pro173fs*43) was recently reported to cause a motor neuropathy combined with distal myopathy (Ghaoui *et al.*, 2015). In addition, the same authors described a patient with a known *HSPB8* mutation (p.Lys141Glu) leading to a motor neuropathy but who developed a myopathy with rimmed vacuoles at a later stage of the disease. This suggests that dominant mutations in *HSPB8* are not restricted to CMT2/dHMN but may also cause a distal myopathy. This is interesting since dominant mutations in its molecular interactor BAG3 are also known to cause a myofibrillar myopathy often combined with an axonal neuropathy (Selcen *et al.*, 2009; Odgerel *et al.*, 2010). Therefore, these data suggest that the chaperone-assisted selective autophagy complex (CASA), of which HSPB8 and BAG3 are the main components, plays an

important role in maintaining the homeostasis of both the peripheral nerve and muscle (**Figure 1**). So far it is not established whether HSPB8 patients can also present with a distal myopathy only, however due to the tight molecular link with BAG3 this might still be possible. Thus it seems advisable to consider screening for mutations in *HSPB8* (or include *HSPB8* in gene panels) when patients present with a peripheral neuropathy and/or distal myopathy.

A similar case was recently reported for a novel missense mutation in *HSPB1* (p.Asp139Glu) in a three-generation Irish family that presented with both motor neuropathy and myopathy symptoms (Lewis-Smith *et al.*, 2016). The initial clinical presentation pointed towards a predominant myopathic process with early and severe fatty infiltration of all lower leg muscles. Neurological examination identified both reduced compound muscle action potentials and sensory nerve potentials. Although the role of HSPB1 in muscle tissues is not fully understood, a few interesting observations may provide hints. For example, genetic deletion of HSPB1 in the mouse results in phenotypically normal animals but with ultrastructural irregularities in the *Soleus* muscle (altered sarcomere structure with Z-line deformation but without nuclei internalization) (Kammoun *et al.*, 2016). Moreover, at the molecular level HSPB1 has been shown to interact with HSPB5 and BAG3 (Kato *et al.*, 1992; Rauch *et al.*, 2017), two proteins associated with myopathy (Vicart *et al.*, 1998; Selcen *et al.*, 2009). Although, the affinity of HSPB8 is much higher for BAG3 than that of HSPB1, as demonstrated by isothermal titration calorimetry (ITC), phosphomimetics of HSPB1 increased the affinity for BAG3 significantly (Rauch *et al.*, 2017). This may therefore suggest that the interaction between HSPB1 and BAG3 is context specific and depends on specific triggers (e.g. phosphorylation). The role of HSPB1 in muscle biology thus merits further investigation. Moreover, the identification of additional families with genetic variants in *HSPB1* linked to myopathic features will determine whether HSPB1-linked myopathies are an established phenomena.

In addition to *HSPB1* and *HSPB8*, *HSPB3* has also been described to cause dHMN. So far, only one mutation (p.Arg7Ser) has been identified in two affected siblings diagnosed with dHMN (Kolb *et al.*, 2010). Recently, one Czech family was identified with the same p.Arg7Ser mutation in a gene-panel analysis (Laššuthová *et al.*, 2016). Because it contains no introns, *HSPB3* is the smallest member of the small heat shock proteins. Recently, the first functional follow-up study of this mutation reported that no pathogenic effect of the p.Arg7Ser mutation could be appreciated when overexpressed in avian motor neurons (La Padula *et al.*, 2016). Since little is known about the biological function of HSPB3 at this moment, it is difficult to estimate the impact of the mutations. Like other small heat shock proteins, HSPB3 is able to form oligomeric structures, which are uniquely composed of tetramers. In contrast to HSPB1, this complex has a reduced chaperone-like activity and is composed of a well-defined hetero-oligomers between HSPB2 and HSPB3 in a 3:1 ratio (Sugiyama *et al.*, 2000; den Engelsman *et al.*, 2009). It is suggestible that the p.Arg7Ser mutation leads to a replacement of a positively charged arginine by a polar serine and therefore affects the ability of the protein to oligomerize and fulfil its biological function. However, as the normal function of HSPB3 remains unclear it is difficult to predict the possible consequences of the p.Arg7Ser mutation causing axonal motor neuropathy. The fact that the two largest cohort studies on small heat shock proteins could not detect additional mutations (Capponi *et al.*, 2011; Echaniz-Laguna *et al.*, 2017), questions the causality of *HSPB3* in CMT2/dHMN. It could therefore be of interest to re-evaluate the two *HSPB3* families for the presence of potential additional variants in other causal genes. Moreover, the presence of a multiple rare variants in neuropathy-associated genes was reported to appear more frequently than assumed in patients with inherited peripheral neuropathies (Gonzaga-Jauregui *et al.*, 2015).

In summary, the recent literature on the small heat shock proteins has shown that the phenotypic spectrum may not be restricted to CMT2/dHMN. Mutations in *HSPB1* were reported

in three cases of sporadic ALS. Although, it remains to be determined whether these mutations were truly pathogenic or simply increase the susceptibility to develop motor neuron disease. Further screening of ALS cohorts for mutations in *HSPB1* will aid in elucidating its precise role and contribution to the disease. For *HSPB8*, there were so far only reports with mutations at the hot-spot residue Lys141. Now the first publications report on mutations targeting other residues, for which a novel pathomechanism was suggested. In addition, the phenotypic spectrum was also extended for *HSPB8* after it was found in patients who developed a distal myopathy at a later stage of the motor neuropathy. This indicates that mutations in multifunctional small heat shock proteins can give rise to multiple neuromuscular disease phenotypes.

Acknowledgements:

Our research related to the small heat shock proteins was supported in part by the University of Antwerp, Fund for Scientific Research (FWO-Flanders), Medical Foundation Queen Elisabeth (GSKE), "Association Belge contre les Maladies Neuromusculaires" (ABMM), American Muscular Dystrophy Association (MDA) and the EC 7th Framework Programme under grant agreement number 2012-305121, 'Integrated European–omics research project for diagnosis and therapy in rare neuromuscular and neurodegenerative diseases (NEUROMICS)'. TG obtained a PhD fellowship from FWO-Flanders. JB is supported by a Senior Clinical Researcher mandate of the Research Fund - Flanders (FWO).

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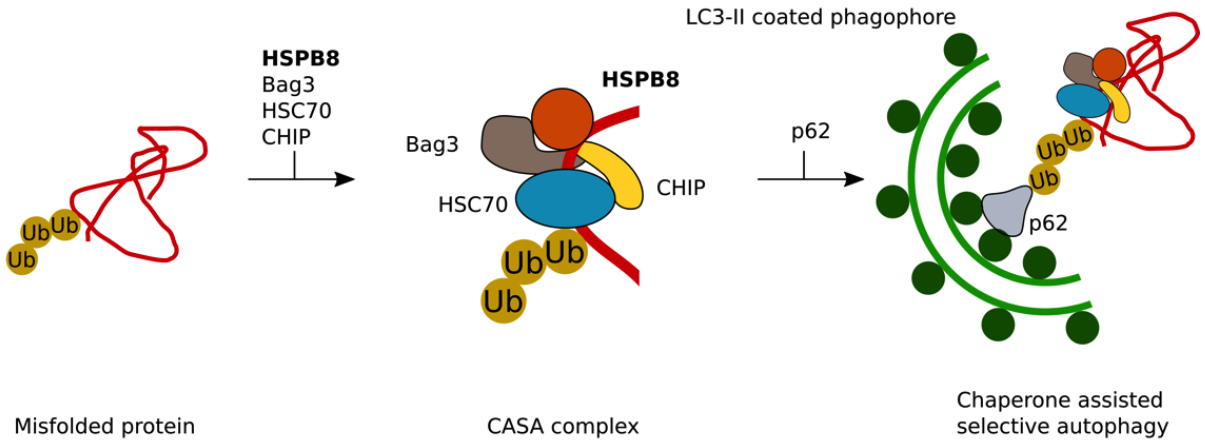
A



B

CASA complex	Link to disease by mutation	Reference
HSPB8	CMT2L and dHMN Distal myopathy	Irobi et al., 2004 Ghaoui et al., 2016
BAG3	Dilated cardiomyopathy Muscular dystrophy Giant axonal neuropathy	Arimura et al., 2011 Selcen et al., 2009 Jaffer et al., 2012
HSC70	Sporadic breast carcinoma Coronary heart disease	Bakkenist et al., 1999 He et al., 2010
CHIP/STUB1	Cerebellar ataxia	Depondt et al., 2014

Figure 1. Members involved in the chaperone assisted selective autophagy (CASA). Schematic overview of the protein complex involved in the recognition of misfolded proteins which are subsequently degraded by CASA (A). Key components of the CASA complex found to be mutated in multiple neurological and muscular diseases (B).

Gene	Disease	Mutation	Type of mutation	Domain	Number of patients *	Reference
HSPB1	CMT2/dHMN	P7S	Missense	N-terminal (WDPF motif)		Echaniz-Laguna et al., 2017
		G34R	Missense	N-terminal		Capponi et al., 2011
		P39L	Missense	N-terminal		Houlden et al., 2008; Capponi et al., 2011; Echaniz-Laguna et al., 2017
		E41K	Missense	N-terminal		Capponi et al., 2011
		G53D	Missense (Recessive)	N-terminal		Echaniz-Laguna et al., 2017
		L58fs*105	Frameshift	N-terminal		Echaniz-Laguna et al., 2017
		A61fs*100	Frameshift	N-terminal		Echaniz-Laguna et al., 2017
		G84R	Missense	N-terminal		Houlden et al., 2008; James et al., 2008
		L99M	Missense (Recessive)	alpha-crystallin		Houlden et al., 2008
		R127W	Missense	alpha-crystallin		Evgrafov et al., 2004; Tang et al., 2005; Dierick et al., 2008; Echaniz-Laguna et al., 2017
		R127L	Missense	alpha-crystallin		Ylikallio et al., 2015
		Q128R	Missense	alpha-crystallin		Echaniz-Laguna et al., 2017
		S135F	Missense	alpha-crystallin		Evgrafov et al., 2004; Chung et al., 2008; Houlden et al., 2008; Echaniz-Laguna et al., 2017
		S135C	Missense	alpha-crystallin		Benedetti et al., 2010; Oberstadt et al., 2016
		S135Y	Missense	alpha-crystallin		Ylikallio et al., 2014
		R136W	Missense	alpha-crystallin		Evgrafov et al., 2004
		R136L	Missense	alpha-crystallin		Capponi et al., 2011; Gaeta et al., 2012; Stancanelli et al., 2015
		R140G	Missense	alpha-crystallin		Houlden et al., 2008
		K141Q	Missense	alpha-crystallin		Ikeda et al., 2009; Maeda et al., 2014
		T151I	Missense	alpha-crystallin		Evgrafov et al., 2004; Dierick et al., 2008; Echaniz-Laguna et al., 2017
		S158fs*200	Frameshift	alpha-crystallin		Mandich et al., 2009; Capponi et al., 2011
		T164A	Missense	alpha-crystallin		Lin et al., 2011
		M169fs*2	Frameshift	C-terminal		Ylikallio et al., 2015
		Q175*	Nonsense	C-terminal		Rossor et al., 2012; Echaniz-Laguna et al., 2017
		T180I	Missense	C-terminal (IXI/V motif)		Luigetti et al., 2010; Capponi et al., 2011; Echaniz-Laguna et al., 2017
		P182L	Missense	C-terminal (IXI/V motif)		Evgrafov et al., 2004; Dierick et al., 2008
		P182S	Missense	C-terminal (IXI/V motif)		Kijima et al., 2005
		S187L	Missense	C-terminal (IXI/V motif)		Echaniz-Laguna et al., 2017
		R188W	Missense	C-terminal (IXI/V motif)		Capponi et al., 2011
	sporadic ALS	c.-217C	Promoter variant	Heat shock element		Dierick et al., 2007
		Q190H	Missense	C-terminal (IXI/V motif)		Capponi et al., 2016
		A204fs*6	Frameshift	C-terminal		Capponi et al., 2016
HSPB3	CMT2/dHMN	R7S	Missense	N-terminal		Kolb et al., 2010; Lassuthova et al., 2016
HSPB8	CMT2	P90L	Missense	N-terminal		Echaniz-Laguna et al., 2017
		N138T	Missense	alpha-crystallin		Echaniz-Laguna et al., 2017
		K141N	Missense	alpha-crystallin		Irobi et al., 2004; Tang et al., 2005; Dierick et al., 2008; Echaniz-Laguna et al., 2017
		K141M	Missense	alpha-crystallin		Echaniz-Laguna et al., 2017
		K141E	Missense	alpha-crystallin		Irobi et al., 2004; Dierick et al., 2008
		K141T	Missense	alpha-crystallin		Nakhro et al., 2013
	CMT2/distal myopathy	K141E	Missense	alpha-crystallin		Ghaoui et al., 2016
		P173fs*43	Frameshift	C-terminal		Ghaoui et al., 2016

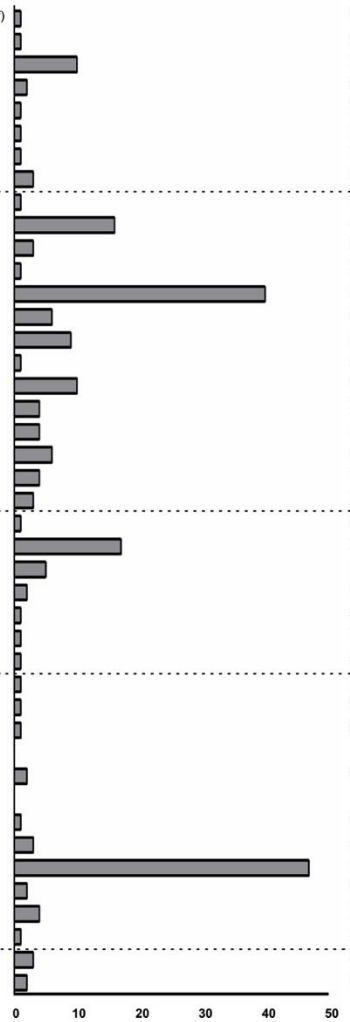


Table 1. Overview of all reported mutations in HSPB1, HSPB3 and HSPB8.

* The number of patients (reported in literature) was determined by incorporating all patients which have undergone neurological examination and for whom the mutation was confirmed by genetic testing.

Mutated gene	Neurological disorder		OMIM	Reference
	disease	subtype		
HSPB1	ALS+			Capponi et al., 2016
	CMT	CMT2F	606595	Evgrafov et al., 2004
	dHMN	dHMN2	608634	Evgrafov et al., 2004
VCP	ALS	ALS14	613954	Johnson et al., 2010
	CMT	CMT2Y	616687	Gonzalez et al., 2014
FIG4	ALS	ALS11	612577	Chow et al., 2009
	CMT	CMT4J	611228	Chow et al., 2007
NEFH	ALS	ALS	606002	Al-Chalabi et al., 1999
	CMT	CMT2CC	616924	Rebello et al., 2016
SPG11	ALS	ALS5	602099	Orlacchio et al., 2010
	CMT	CMT2X	616668	Montecchiani et al., 2016
	HSP	SPG11	604360	Stevanin et al., 2007
SETX	ALS	ALS4	602433	Chen et al., 2004
	dHMN			De Jonghe et al., 2002
	SCA	SCAR1	606002	Moreira et al., 2004
DNAJB2/HSJ1	ALS+			Frasquet et al., 2016
	CMT	CMT2		Gess et al., 2014
	dHMN		614881	Blumen et al., 2012
CHCHD10	ALS	ALS2	615911	Bannwarth et al., 2014
	CMT	CMT2		Auranen et al., 2015
	SMA	SMAJ	615048	Pentillä et al., 2015

Table 2. Genes reported to cause ALS and CMT2/dHMN as well as other neurological disorders.

ALS= Amyotrophic Lateral Sclerosis, ALS+= Amyotrophic Lateral Sclerosis-plus, CMT= Charcot-Marie-Tooth disease, HSP = Hereditary Spastic Paraplegia, SCA = Spinocerebellar Ataxia