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Review

Molecular, cellular, and clinical aspects of myofibrillar myopathy caused by HSPB8 frameshift mutations

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ABSTRACT

HSPB8 belongs to the small heat shock protein family, which comprises ten chaperones with molecular weights below 29 kDa. HSPB8 is broadly expressed across human tissues, with the highest levels in skeletal muscles, the cardiac muscle, and the nervous system. In muscles, HSPB8 plays a crucial role in chaperone-assisted selective autophagy (CASA), contributing to protein quality control and maintaining proteostasis. The most extensively studied mutations affecting the HSPB8 K141 codon are associated with autosomal dominant neuromuscular disorders such as Charcot-Marie-Tooth disease type 2L and distal hereditary motor neuropathy type 2 (dHMN2). Of note, recent findings have identified Myofibrillar Myopathy type 13 (MFM13) with Rimmed Vacuoles as a distinct disorder caused by frameshift (fs) mutations in the carboxy-terminus of HSPB8. This review focuses on the known HSPB8-fs mutations leading to MFM13, their associated clinical phenotypes and histological findings, and highlights the need to further understand the underlying etiologies and mechanisms.

Abbreviation list

aa	amino acid
ACD	α-crystallin domain
BAG3	BAG cochaperone 3
CASA	chaperone-assisted selective autophagy
CE	C-terminal extension
CK	creatine kinase
CMT2L	Charcot-Marie-Tooth disease type 2L
CTR	C-terminal region
dHMN2	distal hereditary motor neuropathy type 2
EIF2AK1/HRI	eukaryotic translation initiation factor 2 alpha kinase 1
fs	frameshift
HSP	heat shock protein
HSPB/sHSP	small heat shock protein
HSPB8	heat shock protein family B (small) member 8
ISR	integrated stress response
MAP1LC3B	microtubule associated protein 1 light chain 3 beta
mCTR	mutated CTR
MFM13	Myofibrillar Myopathy type 13
MTOC	microtubule-organizing center
MTOR	mechanistic target of rapamycin kinase
NMD	neuromuscular disease
NTR	N-terminal region
ORF	open reading frame
PQC	protein quality control
RNP	ribonucleoprotein
RVM	rimmed vacuolar myopathy
SG	stress granule
SQSTM1/p62	sequestosome 1

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TDP-43	TAR DNA binding protein 43
TIA1	TIA1 cytotoxic granule associated RNA binding protein
WT	wild-type

1. Introduction

The heat shock protein family B (small) member 8 (HSPB8) protein was first identified as an atypical serine-threonine protein kinase [1,2]. However, subsequent studies failed to support the kinase function of HSPB8, and demonstrated that HSPB8 is an HSPB1-binding protein in vitro; thus, HSPB8 was included in the human small heat shock protein (HSPB, or sHSP) family [3,4]. Located on chromosome 12 at position q24.23, the *HSPB8* gene consists of three exons and two introns, spanning over 53 kb, and encodes the HSPB8 protein composed of 196 amino acids [2,5]. Like other HSPBs, HSPB8 contains a conserved central α-crystallin domain (ACD), consisting of 92 amino acids (G80 to V172). The ACD is flanked by structurally disordered domains, a hydrophobic residue-rich N-terminal region (NTR) and a polar residue-enriched C-terminal region (CTR) [6].

Therefore, except for the ACD, HSPB8 is an intrinsically disordered protein able to flexibly interact with a variety of proteins [7,8]. In vitro, HSPB8 can be found as a monomer or homodimer [9]. Oligomers, or heterocomplexes generated by interactions of HSPB8 with other HSPBs, such as HSPB1, have been described, although these interactions might be dependent on the experimental conditions of the assay [10]. HSPB8 may also be associated with biological membranes and multiple other proteins [11–15]. Despite its involvement in various cellular functions - such as cell division, inflammation, immunity, and carcinogenesis [15–20] - HSPB8, like other HSPBs, is best known for its role in maintaining proteostasis, by preventing irreversible protein aggregation and promoting the removal of unfolded or detrimental pre-aggregated proteins through the cooperation with other heat shock proteins (HSPs) and the intracellular degradative pathways [21].

HSPB8 is a crucial component of the chaperone-assisted selective autophagy (CASA) complex, a selective degradation mechanism assisting the autophagy-lysosomal pathway in the disposal of damaged proteins. CASA intervenes in different conditions of proteotoxic stress, particularly when the accumulation of defective proteins overwhelms the degradative capacity of the proteasome or in case of inherent proteasomal dysfunction [22]. CASA-mediated degradation is achieved via the formation of a heteromeric complex of HSPB8 and its obligatory partner, BAG cochaperone 3 (BAG3), which allows the dynein-mediated transport of the targeted proteins to the microtubule-organizing center (MTOC), their compartmentalization into autophagosomes and subsequent lysosomal degradation [22–25]. For instance, HSPB8 prevents the detrimental accumulation of tau and β-amyloid proteins [26–30], aggregated α-synuclein [31], and proteins carrying the polyglutamine expansion, such as huntingtin and the androgen receptor, or carrying mutations enhancing misfolding and accumulation proneness [32–35]. By this function, HSPB8 plays a critical role in counteracting neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's diseases, spinal and bulbar muscular atrophy and amyotrophic lateral sclerosis [36].

Mutations in HSPB8 can compromise its function through the reduction in chaperone activity (loss of function), increased aggregation propensity, and sequestration of other proteins (gain of toxic function) [37–40]. HSPB8 deficiency in cell models of neurodegenerative diseases, such as amyotrophic lateral sclerosis, was shown to aggravate the accumulation of toxic aggregates, suggesting that HSPB8 loss of function may contribute to neurodegeneration [35,41]. On the other hand, gain of toxic function mutations often result in autosomal dominant disorders, including neuromuscular diseases (NMDs) or myopathies [42,43].

Among these mutations, the K141 hotspot mutation site in HSPB8 has been extensively studied. Missense mutations at this locus lead to codon changes resulting in a substitution of the positively-charged lysine residue with glutamic acid, asparagine, threonine, or methionine, which possess distinct biochemical properties [42,44–47]. These mutations are primarily associated with two autosomal dominant NMDs: Charcot-Marie-Tooth disease type 2L (CMT2L) and distal hereditary motor neuropathy (dHMN2) [42,44]. The clinical manifestations caused by these HSPB8 mutations are partially shared by those caused by HSPB1, HSPB3, and HSPB5 mutations, all of which tend to preferentially affect the neuronal and/or muscle tissues [47–49]. Both CMT2L and dHMN2 are genetically heterogeneous disorders with overlapping clinical features and, in some cases, overlapping classifications [50,51]. These two conditions are typically inherited in an autosomal dominant manner and often present with symptoms in late adolescence or adulthood. The clinical manifestations of both diseases include progressive distal muscle atrophy and weakness associated with motor neuron involvement, while sensory involvement is minimal or mild at most.

In contrast, HSPB8 frameshift (HSPB8-fs) mutations, first described by Ghaoui et al. in 2016 [46], are mainly associated with myofibrillar myopathy (MFM) with rimmed vacuoles. Since then, six additional studies involving eleven unrelated families have reported similar mutations [52–57] (Table 1). The increased number of subjects affected by this disease led to its recognition as Myofibrillar Myopathy type 13 with Rimmed Vacuoles in the OMIM database (MFM13, OMIM: #621078), as done previously with other 12 MFM types (1–13) based on the specific mutated gene (Table 2). Concerning MFM13, all identified HSPB8-fs mutations localize within the CTR and clinically manifest as myopathies, although some patients may also exhibit neurogenic involvement, respiratory insufficiency and cardiomyopathy. These clinical manifestations distinguish HSPB8-fs mutations from the missense K141 substitution, in which the myopathy phenotype usually manifests together with neuropathy, and the recently identified G62D mutation in the NTD, which has been reported in a single patient with neuromyopathy with no myofibrillar pathology [58,59]. Nonetheless, the overall overlapping clinical features suggest the existence of a spectrum of diseases linked to HSPB8 mutations (Fig. 1A). These disorders, either caused by HSPB8 -fs or missense mutations, exhibit a dominant inheritance pattern.

In this review, we summarize the clinical findings associated with MFM13 and HSPB8-fs mutations, discuss their potential pathological mechanisms, and differentiate them from the well-studied K141 mutations.

2. HSPB8-fs mutations

All the HSPB8-fs mutations identified so far occur at various locations within the CTR encoded by exon 3 and are caused by insertions or deletions of one or more nucleotides in this region (Fig. 1B). Depending on the number of nucleotides inserted or deleted, *HSPB8*-fs mutations can result in a shift on one of three possible reading frames (ORFs), each leading to different stop codon positions (indicated as * in Fig. 1). Frame 1 (+3/−3) terminates at the canonical stop codon, while frames 2 (+1/−2) and 3 (−1/+2) extend beyond it, terminating 53 bp or 148 bp downstream, respectively, and resulting in aberrant CTR extensions (CE, CE+19 or CE+49). Notably, the mutations are primarily clustered in two regions of the third exon: one comprised between c.508 and c.529 (corresponding to p.170 to p.176), and another one near the stop codon, spanning from c.562 to c.580 (from p.188 to p.194).

Mutations resulting in Frame 2 translation were first identified in a French-descendant Australian family [46] and later in a French-descendant American family [57]. Both cases involved a c.515C dupli-

Table 1
HSPB8-fs mutations: reported clinical cases.

Mutation	C-terminal Frame	Family	Clinical manifestation	Histology of the affected muscle	Onset age	Sex	Proband CK at the time of diagnosis (U/L)	Ref.
c.515dupC, p.Pro173Serfs*43	2	Family of French ancestry with 2 affected	Distal muscle weakness	myofibrillar aggregates and rimmed vacuolar pathology	45–50	2M	369	[46]
		Family of French ancestry with 3 affected	Distal muscle weakness and 2 with respiratory insufficiency	rimmed vacuoles, muscle fibre atrophy, and endomysial fibrosis	35–55	2M, 1F	430	[57]
c.508_509delCA, p.Gln170Glyfs*45	2	French family A with 4 affected	Onset: camptocormia, gradually with bilateral foot drop, then proximal lower and upper limb weakness	dystrophic features with atrophy, necrosis and regeneration, internal nuclei, fibrosis, fibre splitting and rimmed vacuoles	35–45	2M, 2F	449	[56]
		French family B with 1 affected			40	M	214	
		German family C: a sporadic proband			40	F	282	
c.577_580dupGTCA, p.Thr194Serfs*23	2	A Caucasian man	Onset: proximal lower limb weakness	rimmed vacuoles and myofibrillar pathology	19	M	1110	[54]
c.525_529delAACAT, p.Thr176Trpfs*38	2	Japanese family with 6 affected	Distal and proximal muscle weakness and degeneration, with severe respiratory failure	rimmed vacuolar myopathies	20s–50s	3M, 3F	NA	[55]
c.566_567delAC, p.Asp189Glufs*26	2	Indian patient. Not known if de novo	asymmetric limb–girdle syndrome. Upper and lower limb proximal weakness. Bulbar weakness and ptosis.	NA	23	M	NA	[60]
c.576_579delinsCAG, p.Glu192Aspfs*55	3	de novo mutation	Pediatric onset axial and limb-girdle muscle weakness, with respiratory dysfunction	classic myofibrillar myopathy (MFM) pathology characterized by myofibrillar disorganization and protein aggregation	6	F	726	[52]
c.515delC, p.Pro172Leufs*75	3	family of Ashkenazi Jewish descent with 3 affected	Distal muscle weakness, with 1 case of respiratory insufficiency. Proband's uncle carrying the mutation had cardiomyopathy	rimmed vacuoles myopathy (RVM)	teens	2M, 1F	237	[53]
c.562delC, p.Gln188Argfs*59	3	de novo mutation	Distal muscle weakness, respiratory complications, and cardiac involvement	rimmed vacuoles myopathy (RVM)	15–20	M	550	[53]
c.520_23delTACT, p.Tyr174Glnfs*72	3	de novo mutation	Progressive truncal and proximal lower limb weakness and cardiomyopathy	rimmed vacuoles myopathy (RVM)	<10	F	NA	[53]

M = male, F = female, NA = not assessed.

cation (p.Pro173Serfs*43), causing a shift to Frame 2 and resulting in a 24-amino acid (aa) alteration within the CTR (mutated CTR, mCTR), followed by the CE + 19 (Fig. 1C). Subsequently, Echaniz-Laguna et al. [56] reported a c.508_509CA deletion (p.Gln170Glyfs*45) in three unrelated European families, which also led to a Frame 2 shift. In the same region, a five-base (AACAT) deletion at c.525_529 (p.Thr176Trpfs*38) was identified, also resulting in a Frame 2 shift [55], while a deletion at c.520_533 (p.Tyr174Argfs*37) can be found in the ClinVar database but is not reported in the literature. Another Frame 2 mutation was found near the end of the HSPB8 ORF, involving a four-base (GTCA) insertion at c.577_580 (p.Thr194Serfs*23), resulting in a minimally altered (3 aa) mCTR and the same CE + 19 [54]. Finally, a Frame 2 mutation causing a two-base deletion at c.566_567AC (p.Asp189Glufs*26) was reported in a patient, resulting in 6-aa mCTR and the same CE + 19 [60].

Recently, two studies involving 4 families reported fs mutations that result in an elongated HSPB8 terminating at the Frame 3 stop codon (Fig. 1B) [52,53]. The first case involved a de novo mutation in a Chinese girl characterized by a four-base (AGTC) deletion and a three-base (CAG) insertion at c.576_579. This mutation, p.Glu192Aspfs*55, leads to the translation of a 4-aa mCTR and the CE + 49. The second study reported three cases, all sharing the same CE + 49, but showing variable modifications of the CTR. The first case involved a large Ashkenazi family with a single-base deletion (c.515delC) causing a Pro172Leu substitution, followed by 24-aa mCTR before the CE + 49. The second and third cases were de novo mutations identified in a male and a female, respectively. The male patient carried a single-base deletion (c.562delC), causing the Gln188Arg substitution, and 8-aa mCTR before the CE + 49. The female patient mutation consists in a c.520_523-TACT deletion, causing a Tyr174Gln substitution, followed by a 22-aa mCTR preceding the CE + 49 extension. Frame 3 mutations result in a CTR that is more than twice the length of the wild-type (WT) reference sequence (24 aa), with a shared 52-aa C-terminal sequence among all identified cases (Fig. 1C).

To date, no reports have identified deletion or duplication (−3/ + 3 or multiple) mutations that maintain the reading frame (Frame 1). Although in-frame mutations may merely shorten or extend the reference HSPB8 protein, it is not possible to predict their potential effect on HSPB8 activity and behavior, given the crucial role of the CTR in regulating HSPB8 dynamics.

In silico analyses reveal that the physical properties of the HSPB8-fs protein CTR and the entire mutant protein, including the isoelectric point, polarity, and solubility, are altered compared to WT HSPB8. As shown in Table 3, the reference WT CTR is highly acidic, whereas most mutant forms display a basic isoelectric point. Notably, two mutations clustering at the very C-terminus - c.577_580dupGTCA (p.Thr194Serfs*23, on Frame 2), and c.576_579delinsCAG (p.Glu192Aspfs*55, on Frame 3) - maintain an acidic isoelectric point, although it remains higher than that of the WT protein. All HSPB8-fs mutant proteins exhibit significantly reduced predicted water solubility, as calculated using the Protein-Sol tool [61] (Table 3). These findings are consistent with predictions made using CamSol and other solubility prediction software [52,55].

The increased length and aa composition of the alternative CTRs in HSPB8-fs mutants are likely to alter the tertiary structure of the protein, potentially leading to HSPB8 misfolding and enhanced aggregation propensity. Indeed, increased aggregation propensity was predicted for Frame 2 mutants [37]. In case of Frame 3 mutations, mCTR-induced

Table 2
Classification of MFM subtypes.

MFM subtype	Gene mutated	OMIM entry (disease)
MFM1	*DES*	https://omim.org/entry/601419
MFM2A	*CRYAB*	https://omim.org/entry/608810
MFM2B (infantile)	*CRYAB*	https://omim.org/entry/613869
MFM3	*MYOT*	https://omim.org/entry/609200
MFM4	*LDB3*	https://omim.org/entry/609452
MFM5	*FLNC*	https://omim.org/entry/609524
MFM6	*BAG3*	https://omim.org/entry/612954
MFM7	*KY*	https://omim.org/entry/617114
MFM8	*PYROXD1*	https://omim.org/entry/617258
MFM9 (with early respiratory failure)	*TTN*	https://omim.org/entry/603689
MFM10	*SVIL*	https://omim.org/entry/619040
MFM11	*UNC45B*	https://omim.org/entry/619178
MFM12	*MYL2*	https://omim.org/entry/619424
MFM13 (with rimmed vacuoles)	*HSPB8*	https://omim.org/entry/621078

structural changes were predicted in the c.576_579delinsCAG p.Glu192Aspfs*55 mutation, and they are linked to increased hydrogen bonding, polar interactions, and the generation of aberrant α-helical structures [52], which could ultimately lead to anomalous interactions with other cellular components and alterations in the biochemical functions of the HSPB8 protein. AlphaFold structural modeling was performed for the WT protein and all mutant variants. As summarized in Table 3, an additional α-helix was observed in the CTR of all mutant proteins except c.577_580dupGTCA (p.Thr194Serfs*23, Frame 2). In Frame 2, the α-helix is positioned closer to the ACD of the protein, whereas in Frame 3 it is located nearer the C-terminus. The p.Thr194Serfs*23 mutation occurs at the distal end of the WT CTR, which may account for the absence of an α-helical structure in this variant.

3. Clinical manifestation

As summarized in Table 1, the reported cases of HSPB8-fs mutations and their associated clinical manifestations involve at least 11 families and 24 individuals who exhibit varying degrees of symptoms. Individuals who have mutations, but do not present clinical symptoms at the time of corresponding studies, are not classified as "affected" and are not included in the table.

In contrast to HSPB8 K141 mutations, which are predominantly associated with neuropathy (i.e. CMT2L and dHMN2), HSPB8-fs mutations mainly lead to myopathic phenotypes, most notably rimmed vacuolar myopathy (RVM). These myopathies often affect distal and proximal muscles, such as limb-girdle and paraspinal muscles. Of note, some patients exhibit neurogenic involvement. Initial symptoms associated with HSPB8-fs mutations typically include muscle weakness in the distal or proximal lower limbs, progressively leading to steppage gait and inability to walk on heels, with no evidence of motor neuropathy in most cases. Approximately a third of the cases exhibit impaired respiratory function [53]. The age of onset varies, ranging from pediatric age to adulthood. For instance, all four newly reported Frame 3 mutation carriers exhibited early-onset disease during childhood or adolescence, often accompanied by cardiomyopathy or severe respiratory insufficiency.

Elevation of serum creatine kinase (CK) is considered a biological marker of myopathies. Among patients with available CK testing data (Table 1), seven out of nine showed elevated CK levels, consistent with chronic muscle damage observed in these cases. Mild CK elevation is more common in patients with chronic muscle damage, while those with early-stage or subclinical disease may exhibit normal CK levels. However, it is important to note that CK levels can fluctuate due to factors such as exercise [62], medications, infection, autoimmune diseases, and other conditions. It is also worth mentioning that CK was measured at the time of diagnosis rather than at disease onset, which may differ substantially, particularly if CK-lowering treatment had already been initiated before final diagnosis.

Although rimmed vacuoles and myofibrillar pathology, characterized by sarcomere disorganization and protein aggregation, are histological hallmarks of MFM13, the age of onset and the disease severity vary significantly among carriers. Some individuals carrying the mutation remain asymptomatic, suggesting that the disease exhibits incomplete expressivity or penetrance [52,55–57].

4. Etiology and molecular mechanisms

4.1. Aggregation propensity

As mentioned above, HSPB8 is a crucial component of the CASA complex and likely represents the limiting factor for its activity, given the fact that the other CASA members are normally expressed at much higher levels in cells. CASA is responsible for recognizing and degrading misfolded proteins, thereby contributing to cellular proteostasis. In muscles, HSPB8 plays a pivotal role in the structural maintenance of *Z*-disks, aiding in the degradation of damaged components such as filamin C. Convincing evidence for a direct interaction between HSPB8 and filamin C remains limited. Muranova and colleagues reported binding of HSPB7, but not HSPB8, to a human filamin C fragment containing immunoglobulin-like domains 22–24 [63], while Ulbrich and colleagues [64] observed only a very weak interaction between HSPB8 and immunoglobulin-like domains 19–21. However, experimental data support a role of HSPB8 partner BAG3 in inducing the release of damaged filamin C from the *Z*-disk [65], underscoring the central involvement of CASA in sarcomere proteostasis [66,67]. Notably, BAG3 participation in force-mediated protein quality control (PQC) pathways appears to require HSPB8 [68]. The CTR of HSPB8 might be essential for this function, as mutations affecting this region negatively affect CASA activity and may impair protein turnover at the Z-disk [65,69], while the NTR seems to be poorly involved in mediating HSPB8 pro-degradative activity [28]. WT HSPB8 CTR is short, hydrophilic, and poorly structured. The mCTR and the CE of the HSPB8-fs mutants display decreased polarity and decreased solubility (Table 3). It was first hypothesized that the presence of a new IXI/V-like motif in the CE + 19 could be responsible for the increased oligomerization and aggregation of HSPB8-fs mutants. Indeed, this motif plays a critical role in the association of homo- or hetero-oligomers of other HSPBs. For instance, HSPB1, HSPB5 and HSPB6 form large heterologous complexes that grow from dimers or oligomers primarily via the ACD of one monomer and the IXI/V motif in the CTR or NTR of another monomer [6,70–72]. The IXI/V motif binds to the hydrophobic groove formed by the β4 and β8 strands of a neighboring monomer [73]. WT HSPB8 lacks the IXI/V motif, resulting in a much-reduced tendency to form large homo- or hetero-oligomers compared to other HSPBs and a preferential binding to BAG3, through the IPV motifs present in the latter [9,69,74]. Frame 2 HSPB8-fs mutants exhibit an aberrant IXI/V motif, which may bind to the ACD of another HSPB monomer, and enhance HSPB8 oligomerization/aggregation propensity and the sequestration of other HSPBs, thereby counteracting their possible compensatory or specific mechanisms. These HSPB8-fs aggregates

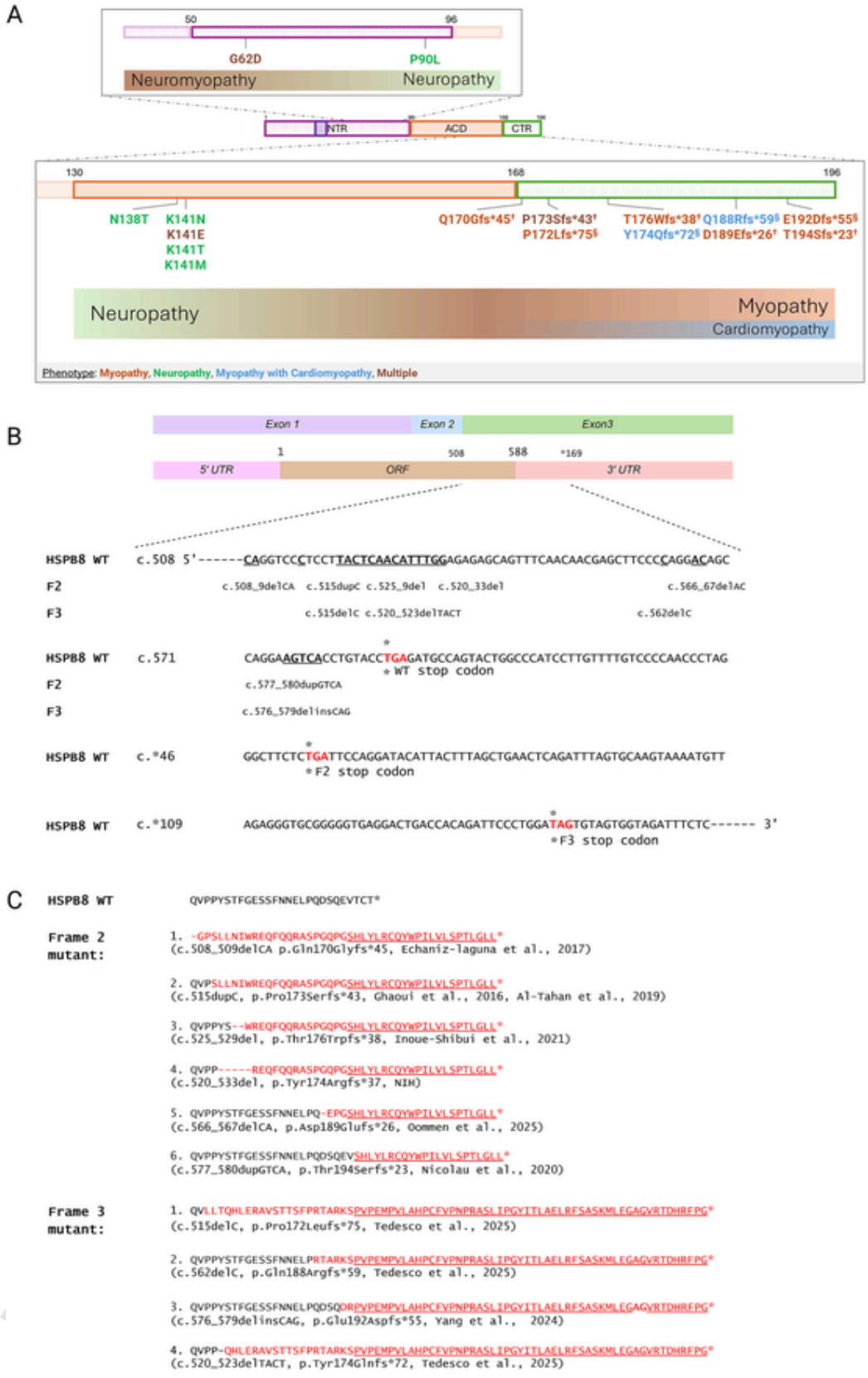

Fig. 1. HSPB8 mutations associate with a spectrum of diseases. A. Schematic representation of HSPB8 protein sequence and details on the HSPB8 fs mutations in the CTR in comparison to the missense mutations in the ACD and NTR. B. HSPB8 mRNA sequence from c.508 of exon 3 to the extended region. Frame 2 (F2) and Frame 3

Fig. 1.—*continued*
(F3) mutations with the novel stop codons indicated in red. C. CTR sequences of the HSPB8 wild type and fs mutants identified from p.Q170. NTR = N-terminal region, ACD = α-crystallin domain, CTR = C-terminal region. † = Frame 2 mutation, § = Frame 3 mutation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
In silico analyses of CTR and mutant protein solubility (PepCalc) https://pepcalc.com/ and mutant protein structures.

HSPB8 species	C-terminal frame	#residues/C-terminal/whole protein	Iso-electric Point (PI), C-terminal/whole protein	Net Charge at pH 7, C-terminal/whole protein	Predicted Protein-Solubility*/C-terminal/whole protein	Predicted Whole Protein Structure by AlphaFold[a]
WT	1	27/196	1.00/4.71	−4.1/−6.9	0.865/0.678	
c.508_509delCA, p.Gln170Glyfs*45	2	44/213	10.95/6.51	2/−0.8	0.481/0.329	
c.515dupC, p.Pro173Serfs*43	2	45/214	10.95/6.51	2/−0.8	0.478/0.345	
c.525_529delAACAT, p.Thr176Trpfs*38	2	43/212	10.36/6.51	2/−0.8	0.561/0.417	
c.520_33del, p.Tyr174Argfs*37	2	40/209	10.95/6.51	2/−0.8	0.535/0.429	
c.577_580dupGTCA, p.Thr194Serfs*23	2	46/215	4.29/5.0	−3/−5.8	0.557/0.530	
c.515delC, p.Pro172Leufs*75	3	76/245	11.32/7.84	4.2/1.4	0.595/0.418	
c.562delC, p.Gln188Argfs*59	3	76/245	10.20/6.63	2.1/−0.7	0.504/0.425	
c.520_23delTACT, p.Tyr174Glnfs*72	3	75/244	10.66/7.86	4.2/1.4	0.696/0.442	
c.576_579delinsCAG, p.Glu192Aspfs*55	3	76/245	5.63/5.45	−1.9/−4.7	0.515/0.494	

https://protein-sol.manchester.ac.uk/. The score is between 0 and 1, the higher it is, the more soluble the protein is in water (see [61] for details).
[a] https://alphafoldserver.com/.

can exert toxic effects either directly or indirectly, by their presence or by leaving unchaperoned misfolded proteins, further exacerbating cellular stress. Moreover, the HSPB8-fs IXI/V motif may promote competition with other HSPB8 binding partners, such as BAG3, thus disrupting the HSPB8-BAG3 interaction and impairing CASA function [75,76]. Regardless, experiments have shown that even HSPB8-fs mutants lacking the IXI/V motif form aggregates comparably to the original IXI/V-containing HSPB8-fs mutants. Furthermore, HSPB8-fs proteins carrying the CE + 49, which lack the IXI/V motif, still exhibit increased sequence disorder and reduced solubility, forming insoluble aggregates. These observations suggest that structural changes caused by the CE, rather than the presence of peculiar motifs, are the primary drivers of HSPB8-fs misfolding and aggregation [37].

4.2. Dominant negative effect and CASA impairment

Although HSPB8-fs proteins segregate into cytoplasmic aggregates, their ability to homodimerize with the WT HSPB8 and interact with BAG3 is retained. This results in the sequestration of CASA members, suggesting that a dominant negative effect on the HSPB8 WT protein and a broader impairment of CASA function stand at the basis of HSPB8 pathology [37,52]. In HSPB8-fs patients, histopathology analyses revealed abnormal aggregates, with an altered distribution of autophagy markers like sequestosome 1 (SQSTM1/p62), and proteins associated with the *Z*-disk, such as myotilin, desmin, and filamin C, which are known to be physiologically subjected to CASA-mediated clearance [52,54,77]. The presence of an altered distribution of structural components of the muscle cells suggests that the downstream effects of HSPB8-fs mutations might include compromised cytoskeleton maintenance. This is not surprising, since it is known that HSPBs are involved in the rearrangement and dynamics of the cytoskeletal components. Concerning HSPB8, biochemical and cellular studies demonstrated its ability to interact with actin and to regulate cell division [16,67,78].

Notably, the histological features of MFM with aggregates and rimmed vacuoles observed in all MFM13 patients closely resemble the pathology seen in myopathies caused by mutations in the CASA member BAG3 [23,79,80]. In addition, histological analyses also reveal signs of TAR DNA binding protein 43 (TDP-43) pathology, which seems to be a feature of myopathies caused by HSPB8 mutations [53,58]. TDP-43 is an RNA-binding protein involved in RNA maturation and splicing, whose dysregulation characterizes neurodegenerative and muscle diseases, such as amyotrophic lateral sclerosis and inclusion body myopathy [81,82]. Notably, TDP-43 and its disease-associated 35- and 25-kDa fragments are subjected to CASA or released with CASA components into extracellular vesicles upon blockage of degradative systems [83]. This indicates that CASA not only mediates the clearance of intracellular TDP-43 [34,84], but also controls its vesicular secretion in the extracellular environment when the intracellular PQC system is perturbed. Therefore, HSPB8-fs mutations could disrupt this process, further contributing to cellular dysfunction [58].

4.3. Haploinsufficiency: still an open question

Haploinsufficiency was the pathogenic mechanism initially proposed to be at the basis of HSPB8-fs pathology. Indeed, the absence of the elongated HSPB8 and the expected significant reduction in HSPB8 protein expression were also observed in muscle biopsies from a patient with the c.508_509delCA p.Gln170Glyfs*45 mutation (Frame 2, [56]).

Similarly, western blot analyses of patient fibroblasts carrying the c.515dupC p.Pro173Serfs*43 mutation – which is on the same Frame 2 - revealed up to a 50% reduction in HSPB8 protein expression levels compared to healthy controls and no HSPB8 mutant expression [57]. In addition, fibroblasts from affected patients exhibited increased expression of the autophagosome marker microtubule associated protein 1 light chain 3 beta (MAP1LC3B also known as LC3B) and the autophagy receptor SQSTM1/p62, compared to healthy controls, further suggesting impaired HSPB8 dynamics and deficiency in clearing stress-induced misfolded and toxic proteins, thereby compromising proteostasis maintenance.

These observations hint at some roles for HSPB8 haploinsufficiency in deficient CASA function and disease progression. However, the haploinsufficiency mechanism is not supported by in vivo observations on HSPB8 heterozygous or homozygous knockout mice, which do not exhibit a disease-associated phenotype [85]. This indicates that haploinsufficiency and impaired CASA function alone may not be sufficient to induce the pathology in muscles. Moreover, while previous analyses of biopsies from patients carrying HSPB8 Frame 2 mutations did not reveal the elongated HSPB8, a recent report of a patient with a Frame 3 mutation showed the presence of the elongated HSPB8 and its accumulation in atrophic muscle fibers, hence providing further evidence to subvert the previously suggested HSPB8 haploinsufficiency mechanism [52,53].

It is still unclear what the bases of this discrepancy are. One possible explanation might be related to the technical procedures used to process and analyze Frame 2 and 3 patient samples, such as protein extraction or detection (e.g., the antibody used). Alternatively, distinct cell types may differently regulate HSPB8 expression and turnover. For instance, the degradative power and the expression profile of HSPB8 differ between skin fibroblasts and muscle samples, possibly leading to a different turnover of HSPB8 in the two cell types [39]. In support of this second hypothesis, a previous study on BAG3 mutations, associated with neuromyopathy and prone to strong aggregation both in cell and animal models [86,87], required proteasome blockage to recapitulate BAG3 aggregation in patient-derived fibroblasts [79]. Similarly, patient-derived fibroblasts carrying a Frame 2 HSPB8 mutation displayed increased HSPB8 levels and altered distribution only after recovery from heat shock [57]. These observations strongly support the notion that fibroblasts might not rely on HSPB8 for their physiological maintenance, and that HSPB8 plays a major role when stress occurs. Instead, muscle tissues, being characterized by a constant exposure to mechanical tension, high metabolic activity and other types of stressors, require CASA complex activity and other components of the proteostasis network to continuously monitor and renovate the intracellular proteome. Also, cell turnover greatly differs between these two cell types: while muscle tissues exhibit low cell turnover and regenerative potential, fibroblasts have increased proliferative ability. One should also consider that biochemical evaluation of HSPB8 mutant protein levels and aggregation has been conducted on muscle biopsies or fibroblast cell cultures. Indeed, the difference in the detection of the HSPB8-fs mutants may be related to the analyses of a cell culture able to dilute the proteotoxic species with cell division. Moreover, cellular alterations leading to loss of protein inclusion buildup have been previously described for fibroblasts carrying an HSPB8 K141N mutation [39]. Notably, the exogenous expression of HSPB8 mutant representative of the two different frameshift mutations (p.Pro173Sfs*43, p.Gln188Argfs*59) in different cell models resulted in a similar accumulation of the two elongated proteins at the expected molecular weight [53]. Nevertheless, since the two CE differ highly in terms of length and aa composition, we cannot exclude that Frame 2 and Frame 3 HSPB8-fs mutants are subjected to a different turnover or engage different protein-protein interactions in the affected tissues.

4.4. Contribution of gain and loss of function to other HSPB8-related activities

So far, studies on the effects of HSPB8-fs have been focused on dysfunctions of the CASA complex. In contrast, additional cellular dysfunctions due to HSPB8-fs mutations probably exist but have not been investigated yet. Apart from the already mentioned role of HSPB8 in cytoskeletal maintenance, another relevant HSPB8 function is the regulation of mitochondrial homeostasis, metabolism and clearance (mitophagy). Indeed, nearly all HSPBs, including HSPB8, are imported into the intermembrane space, where they counteract protein aggregation [88]. Other studies linked HSPB8 to mitophagy. For instance, it has been shown that HSPB8 overexpression enhanced mitophagy, whereas its silencing inhibited this pathway in a neuronal cell model of oxygen-glucose deprivation/reperfusion [89]. In addition, although the HSPB8 KO mouse model is asymptomatic, evidence of accumulation of abnormal mitochondria with degenerating cristae in the distal muscle is reported, suggesting that HSPB8 deficiency might also be detrimental for muscle health. Instead, a transgenic mouse model expressing HSPB8 K141N displays mitochondria accumulation and signs of mitophagy in the sciatic nerve [85]. Given the role of HSPB8 in mitochondrial homeostasis in the skeletal muscle, it is likely that this protein also exerts a protective role in the cardiac muscle by acting on this organelle. For example, HSPB8 overexpression was shown to inhibit cytochrome *c* release from mitochondria, and consequently caspase-3 activation and the apoptotic pathway [90]. Other evidence supports that HSPB8 might also regulate mitochondrial metabolism: a mouse model overexpressing HSPB8 displayed senescence, reduced lifespan, and cardiac hypertrophy – the latter also observed in patients with HSPB8-fs mutation - due to exacerbated oxidative stress related to mitochondrial activity [91]. Other studies reported that HSPB8 overexpression or depletion in vivo resulted in an increase or reduction of cardiac mitochondrial respiration, respectively [92]. Similarly, a cardiac-specific HSPB8 K141N transgenic mouse exhibits mild cardiac hypertrophy and fibrosis, and molecular analyses demonstrated HSPB8 mutant interaction with mitochondrial proteins and reduction of mitochondrial oxidative phosphorylation [93]. All these pieces of evidence may explain prior observations and data on patients carrying HSPB8-fs mutations, who exhibit a type 1 (oxidative) to type 2 (glycolytic) switch in skeletal muscles [53] and possibly cardiac pathology.

The role of HSPB8 has also been described in other pathways relevant to cellular homeostasis. These include stress granule (SG) homeostasis and the Integrated Stress Response (ISR). SGs are cytoplasmic structures composed of ribonucleoproteins (RNPs) and RNAs that are generated to mitigate the stress load on cells until it subsides, by halting translation. RNPs identified in SGs in physiological or pathological conditions include TIA1 cytotoxic granule associated RNA binding protein (TIA1), TDP-43, and Fused in Sarcoma, whose dysfunction is linked to neuromuscular disorders. Muscle biopsies of carriers of HSPB8 mutations, either missense or fs, exhibit signs of RNP involvement. For instance, TIA1-positive aggregates have been observed in muscle biopsies of patients with HSPB8-linked myopathy [54,59]. TDP-43-aggregation and mis-splicing are other features of HSPB8-related disorders [53,58], and this RNP, as well as Fused in Sarcoma, are chaperoned by HSPB8. It is interesting to note that in the recovery phase after stress relief, most SGs disassemble in a process involving the HSPB8-BAG3-HSP70 chaperone complex, while only a small fraction of aberrant SGs is cleared through autophagy [94]. This granulostasis process seems to occur in two subsequent phases: the first begins with the HSPB8 recruitment into SGs to maintain misfolded proteins in competent state for further processing and also to prevent their irreversible aggregation inside SGs, whereas the second consists of the intervention of BAG3-HSP70 for the extraction of misfolded proteins and their CASA-mediated delivery to the MTOC for autophagy degradation [94]. SG formation is a cell response intimately connected to the ISR. The ISR is orchestrated by a

group of stress-activated kinases that promote a translational shutdown and the activation of a transcriptional program aimed at restoring cell homeostasis or inducing cell death. In this pathway, HSPB8 regulates the activity of one of the stress kinases, namely EIF2AK1/HRI (eukaryotic translation initiation factor 2 alpha kinase 1), by sequestering it in unstressed conditions and releasing it upon pathogen exposure to initiate the ISR [15,95].

All these diverse activities of HSPB8 in assuring cellular homeostasis strongly suggest that the pathogenic effects of mutant HSPB8, whether due to a loss or gain of toxic function, might not be simply related to protein aggregation and/or alterations in CASA-mediated protein disposal. Therefore, further studies are needed to elucidate and define novel therapeutic targets in MFM13 and other associated HSPB8-associated disorders.

4.5. Other factors

Besides the mechanisms directly related to the altered physical and biochemical properties of HSPB8-fs, other factors may contribute to disease development, influencing the variability in phenotypes, clinical expressivity, and incomplete penetrance. These factors may include physical activity, genetic background, other environmental or individual differences, and, possibly, biological sex.

4.5.1. Ethnic group

Most reported fs mutation cases are of Caucasian/European origin, with a few cases reported from Japan [55] and China [52], indicating a global distribution. However, due to the limited number of cases, it is challenging to assess the potential impact of ethnicity on the disease.

4.5.2. Genetic modifiers

The disease phenotype may not be solely driven by HSPB8 mutations; rather, the additive effects of less expressive mutations could underline the variability in the expressivity and penetrance of clinical manifestations. Conversely, some genes or gene variants may act to alleviate the effects of HSPB8 mutations [96]. Evidence of an effective contribution of other variants has not been clearly described in MFM13. Nevertheless, given the diverse roles of HSPB8 in cell homeostasis, it is expected that other variants in genes encoding factors of the proteostasis network, stress response, mitochondria turnover and metabolism might impact disease manifestation, phenotypes and progression. Concerning HSPB8, besides other variants in the gene itself that might influence its expression and turnover, it is worth mentioning that two alternatively spliced transcripts of the *HSPB8* gene have been identified in the liver, brain, and heart. These spliced variants have a smaller NTR, with one isoform predicted to localize predominantly to the nuclear region and the other to the cytoplasm [97]. The role of *HSPB8* mutations on its alternative spliced isoforms remains unknown, as does the functional significance of these variants.

4.5.3. Environmental factors

Like many other myopathies, NMDs or neurodegenerative diseases, adult onset of clinical symptoms suggests that lifestyle and environmental exposure may influence disease variability. Exercise-induced mechanical stress, particularly at the *Z*-disk, may lead to damage of proteins that rely on the CASA complex for clearance, thereby exacerbating the clinical phenotype in the case of *HSPB8* mutation and CASA dysfunction [64,65].

5. Therapeutic approaches: where do we stand?

To date, no targeted disease-modifying therapies are available for MFM13, underscoring the critical need for ongoing mechanistic and translational research. However, exploratory studies concerning other *HSPB* mutants and proteinopathies of distinct etiologies have identified potential strategies for therapeutic intervention which warrant further investigation and validation.

The most promising approaches concern those counteracting the toxic gain of function caused by the mutations and that are based on the stimulation of the PQC system to prevent mutated protein misfolding and aggregation or favor their disposal. For instance, a drug-repurposing study demonstrated that autophagy induction by piplartine is beneficial in counteracting axonal degeneration and neuronal stress caused by HSPB1 and HSPB8 missense mutants in CMT2 neuropathies [98]. The same article showed that pararosaniline pamoate was able to significantly rescue the altered neuronal phenotype. Conversely, cells carrying mutated HSPB1 or HSPB8 were almost unresponsive to autophagy enhancement through canonical mechanistic target of rapamycin kinase (MTOR) inhibition, and treatment with rapamycin or its analogs (generally shown to be protective in several proteinopathies [99]) led to impaired neuronal network development, mitochondrial stress and neuronal toxicity [98]. This is in line with other studies that already showed how rapamycin may induce several adverse effects [100], and reduce muscle contractile function in rats [101]. Thus, future research on MFM13 therapies should be focused on pro-autophagic compounds acting in MTOR-independent pathways. An interesting candidate is trehalose, which promotes autophagy without involving MTOR but instead activates the transcription factor EB, thereby promoting the lysosomal disposal of neurotoxic, aggregation-prone proteins. Of note, trehalose also induces HSPB8 expression [102]. Thus, its therapeutic applicability in MFM13 requires careful evaluation, as it could increase the expression of the endogenous mutated gene. A similar consideration applies to other compounds, e.g. geranylgeranylacetone, that enhances the PQC by inducing HSPs - inclunding *HSPB8* - and favoring their function as molecular chaperones [90,103].

Another interesting compound is the chemical chaperone 4-phenylbutyrate, which has already emerged as a promising therapeutic for epidermolysis bullosa simplex with muscular dystrophy, a subtype of MFM caused by plectin deficiency and characterized by desmin aggregation in skeletal muscle cells and disruption of the myofibrillar organization [104,105]. Indeed, both in vitro and in vivo studies demonstrated that 4-phenyl-butyrate reduces the accumulation of desmin-positive aggregates, improves sarcomere integrity and enhances muscle performance. Comparable proteostasis-restoring effects were reported in other proteinopathies, including Parkinson's disease [106] and cataract caused by mutant γD-crystallin [107]. Notably, 4-phenyl-butyrate is already clinically approved for other indications, thus it is an interesting pharmacological candidate of potential repurposing in MFM13 [108].

Similarly, doxycycline, a tetracycline-class antibiotic, emerged as a pharmacological chaperone capable of modulating proteostasis by inhibiting protein aggregation and facilitating the disruption of pre-existing aggregates. Its anti-aggregation activity was documented in amyloidosis, [109], oculopharyngeal muscular dystrophy [110] and desmin-related myofibrillar myopathy with cardiac involvement [111,112]. Notably, in zebrafish and murine in vivo models of desminopathy, doxycycline administration restored both skeletal muscle and cardiac function, providing a rationale for its putative future therapeutic utility in MFM13 [111,112].

As previously mentioned, HSPB8 is abundant and functionally active in mitochondria, and its deficiency or aberrant behavior is detrimental [88,92]. These findings support antioxidant-based therapeutic strategies. Interestingly, L-3-n-Butylphthalide, a compound found in Chinese celery seed extracts, has shown efficacy in the treatment of HSPB8 K141N-driven CMT2L by attenuating oxidative stress through modulation of reactive oxygen species -generating and scavenging pathways, thereby reducing mutant HSPB8-induced cytotoxicity [113]. Moreover, L-3-n-Butylphthalide decreases oxidative muscle damage in experimental autoimmune myositis models, further supporting its potential benefit for myopathy treatment [114].

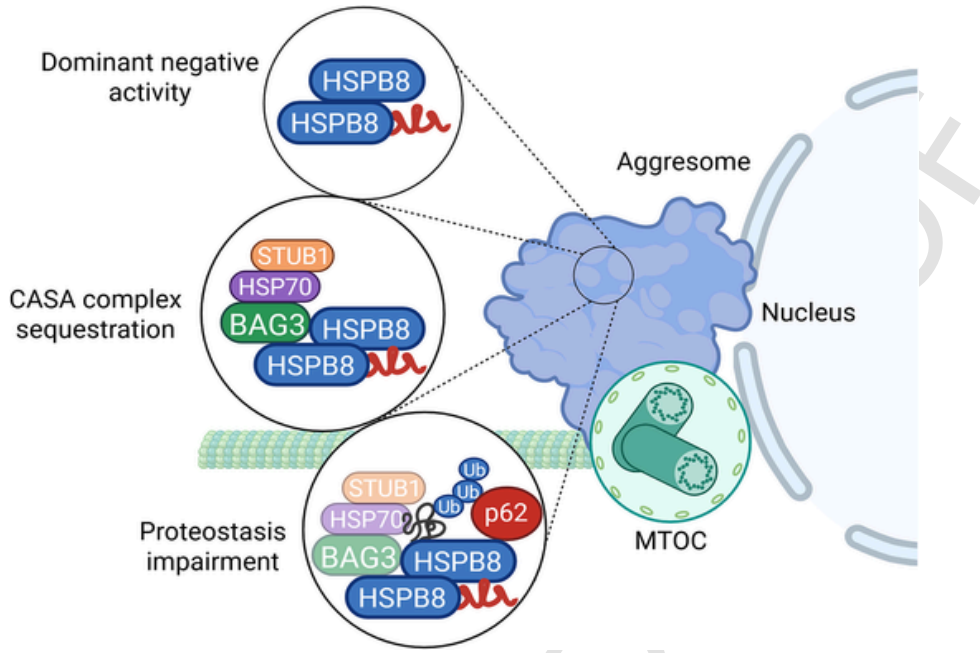

Fig. 2. Pathogenic mechanisms attributed to HSPB8-fs mutants. The HSPB8-fs pathogenic mechanisms consist of the dominant negative activity on the HSPB8 WT counterpart through its sequestration, together with the sequestration of other members of the CASA complex and factors involved in the PQC system (e.g., autophagy receptors such as SQSTM1/p62), leading to ubiquitinated substrate accumulation at the aggresomes and proteostasis impairment. Created in BioRender https://BioRender.com/l1d7v35.

Collectively, these data provide a putative multifaceted therapeutic framework - integrating proteostasis enhancement and antioxidant strategies, alone or in combination - to address the pathogenic mechanisms underlying MFM13, highlighting a compelling rationale and a promising avenue for further investigation.

6. Beyond MFM13

HSPB8-fs mutations concurrently lead to the impairment of CASA function and exert dominant negative and toxic gain of function effects. The combination of these phenomena likely underlies the development of rimmed vacuolar and myofibrillar myopathies observed in MFM13 patients. MFM13 exhibits incomplete penetrance and variable expressivity, highlighting the potential influence of additional factors such as genetic modifiers and environmental conditions. Pathogenic mutations associated with myofibrillar myopathies, distal myopathies, and other similar hereditary muscular disorders have been identified in more than 30 genes [80,115–118]. Most of these mutations are associated with an autosomal dominant inheritance pattern, and the encoded proteins are involved in diverse cellular activities such as RNA processing, protein chaperoning, lipid storage and metabolism, cell membrane interaction and trafficking, muscle fibre maintenance, and the autophagy-lysosome pathway [119]. Although these disorders share similar phenotypes, their molecular etiologies vary significantly. This heterogeneity complicates the initial proper diagnosis and the development of common treatments. However, broader approaches and collective research efforts could pave the way for effective therapeutic interventions to mitigate disease progression. Understanding the mechanisms underlying the newly classified MFM13 disorder is crucial for identifying optimal therapeutic approaches, including whether to restore CASA function or to inhibit the toxic gain of function caused by mutations. Additional efforts to ensure testing of the *HSPB8* gene in subjects showing rimmed vacuolar myopathies, and inclusion of the *HSPB8* gene into myopathy gene panels are necessary to correctly diagnose patients in a timely manner.

7. Summary

MFM13 is an ultrarare, monogenic, and slowly progressive muscle-wasting disorder caused by frameshift mutations in the exon 3 of the *HSPB8* gene. To date, MFM13 has been reported in 24 individuals from 11 families across eight case studies. These frameshift mutations result in a shift to either reading Frame 2 (+1/−2), which leads to a common 19-aa extension (CE+19), or Frame 3 (−1/+2), yielding a common 49-aa extension (CE+49), while each mutation has a different impact on the CTR sequence of the HSPB8. Clinically, MFM13 often presents from childhood to mid-adulthood with distal or proximal muscle weakness; cardiac involvement is also possible. The disorder shows incomplete penetrance and variable expressivity. Notably, the limited number of patients and the lack of a standardized scoring system to define disease severity prevent us from defining any genotype-phenotype correlation with a certain degree of confidence; even more so genetic modifiers or environmental factors may significantly influence disease manifestation. HSPB8 is a critical component of the CASA complex, and the mutations lead to protein aggregates, impaired proteostasis, ultimately contributing to myofiber degeneration. The pathogenic mechanism of MFM13 is under active investigation, with a dominant-negative effect and a toxic gain of function mechanism representing the leading hypotheses (Fig. 2). With the awareness that additional studies on in vitro and in vivo models, e.g. transgenic mice, are needed to better define the cellular consequences of HSPB8-fs mutations, the current view is that *HSPB8* should be included in diagnostic sequencing panels for myopathies. Currently, there are no disease-modifying therapies, therefore highlighting the importance of ongoing mechanistic and translational research.

CRediT authorship contribution statement

Wenli Zhou: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Veronica Marchesi:** Writing – review & editing, Writing – original

draft, Project administration, Methodology, Investigation, Conceptualization. **Matthew McLeod:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Anna Jolanta Kordala:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Sylwia Szwec:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Julia Anna Mielcarz:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Angelo Poletti:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Barbara Tedesco:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Angelo Poletti reports a relationship with NIH that includes: funding grants. Angelo Poletti reports a relationship with AFM-Téléthon that includes: funding grants. None. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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