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Proteomic profiling of the HSPB chaperonome: Mass spectrometric identification of small heat shock proteins in stressed skeletal muscles

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ABSTRACT

The continuing maintenance of protein homeostasis and the protection of proteomic integrity is essential for the survival of complex cellular systems under stressful conditions. Proteostasis is maintained by a complex system of protective pathways that involve several classes of molecular chaperones, now referred to as the chaperonome. The elaborate interplay of these components averts detrimental protein aggregation and supports proteins in resuming their functional fold. In skeletal muscle tissues, molecular chaperones protect contractile functions throughout fibre adaptations to changed physiological demands and prevent tissue damage during acute phases of protein misfolding or prolonged periods of harmful protein accumulation. This results in considerable changes in the expression profile of individual members of the large family of heat shock proteins. Systematic proteomic surveys of skeletal muscle tissues have revealed that the concentration of small heat shock proteins is especially affected following strenuous exercise, in various neuromuscular disorders and during the natural aging process. Of the 10 identified members of the small heat shock protein HSPB family, HSPB1 (Hsp25), HSPB2 (MKBP), HSPB3 (Hsp27), HSPB4 (αA-crystallin), HSPB5 (αB-crystallin), HSPB6 (Hsp20), HSPB7 (cardiovascular cvHsp) and HSPB8 (Hsp22) are clearly present in skeletal muscle tissues. This review outlines the proteomic identification of small heat shock proteins and their muscle-specific expression and induction patterns in health and disease. Since HSPB molecules are of relatively low molecular mass, belong to the markedly soluble type of proteins and represent critical pro-survival proteins that are intrinsically involved in the prevention of stress-induced fibre damage, they present ideal muscle-associated biomarker candidates for the establishment of superior diagnostic and therapy-monitoring approaches to assess stress-related skeletal muscle degeneration.

Keywords: Biomarker discovery; Heat shock protein; Molecular chaperone; Muscle disease; Neuromuscular disorder; Stress response.

Abbreviations

αBC: alpha-B-crystallin; **cvHSP**: cardiovascular heat shock protein; **CAL**: calreticulin; **CAX**: calnexin; **HSP**: heat shock protein; **MS**: mass spectrometry; **sHSP**: small heat shock protein; **PDI**: protein disulfide isomerase; **PPI**: peptidyl-prolyl cis-trans isomerase.

1. Introduction

The detailed analysis of puffing activity patterns in the salivary gland chromosomes of *Drosophila melanogaster* in relation to protein synthesis during heat shock [1-5] led to the foundation of the new scientific area of stress biology [6-8]. Following the initial identification of heat shock-inducible proteins, comprehensive investigations into the cellular response to stressful conditions has established the concept of molecular chaperoning and the classification of the large family of heat shock proteins (HSP) [9-11]. A large variety of HSP molecules have been categorized based on their molecular masses and protective functions following exposure of cells to oxidative stress, heat shock or toxic insults [12-14]. Molecular chaperones provide a variety of essential functions in relation to general cytoprotection and the prevention of deleterious side effects on protein function during stress. A rapid response to acute stressors or the up-regulation of sus-

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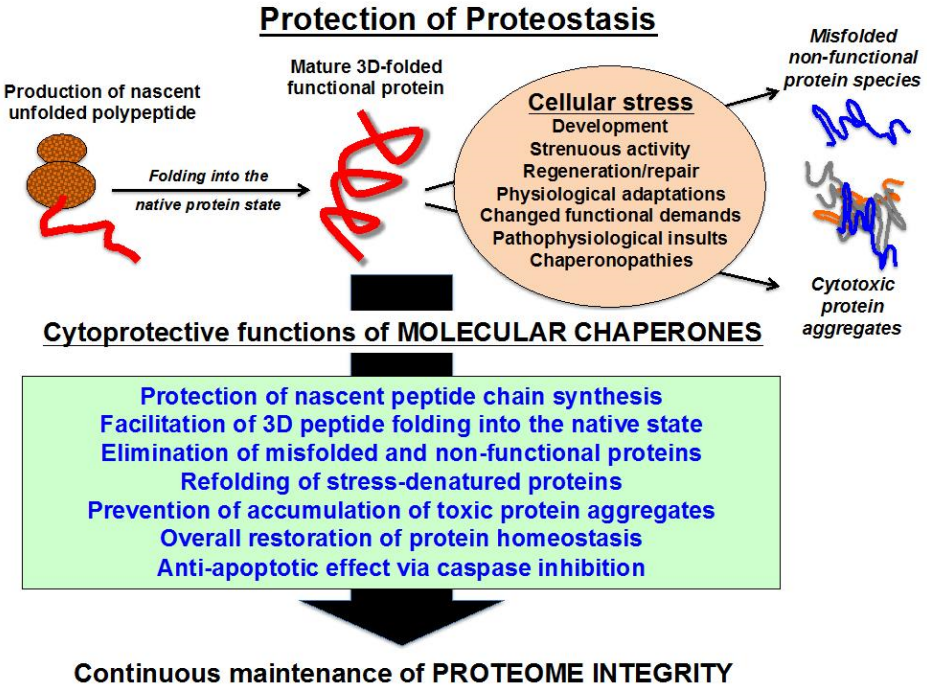

Figure 1. Overview of the cytoprotective roles of molecular chaperones.

tained protective pathways is provided by HSPs and related chaperoning biomolecules [10]. This includes the (i) protection of nascent peptide chain synthesis, (ii) the facilitation of proper peptide folding into the native protein state, (iii) the swift elimination of misfolded and non-functional protein species, (iv) the refolding of stress-denatured proteins, (v) the continuous prevention of the accumulation of misfolded proteins that might otherwise form toxic aggregates within cellular structures, (vi) anti-apoptotic effects via inhibition of the caspase system, and (vii) the restoration of proteostasis and thereby maintenance of proteome integrity [14]. Figure 1 outlines the concept of the cytoprotective role of molecular chaperones under stressful conditions in skeletal muscles, such as cellular development, strenuous activity, tissue regeneration, fibre repair, physiological adaptations to changed functional demands, secondary pathophysiological insults or primary abnormalities due to chaperonopathies [15].

2. The chaperonome and heat shock proteins

The human genome project has identified over 100 different molecular chaperones and based on OMICS-type investigations and systems biological approaches [16], the concept of the chaperonome has been developed [15]. The proteome-wide distribution of chaperones and their roles in maintaining and stabilizing the protein constituents of cells by facilitating the synthesis, transportation and macromolecular assembly of proteins, as well as peptide refolding following stress, essential protein degradation and proteotoxic aggregate dissociation, ensures cellular survival. The presence of molecular chaperones and their swift up-regulation during cellular stress balances protein synthesis and protein degradation, thereby providing proteostasis and proteome stability. In the past, HSPs have been classified based on their tissue-specific expression patterns, their molecular mass, their constitutive presence and/or their stress-related inducibility. In addition to the main HSPs, a variety of endoplasmic reticulum-associated proteins, co-chaperones and modifying enzymes are involved in cytoprotective pathways. The new nomenclature, as summarized by Kampinga et al. [13], categorizes molecular chaperones into several distinct protein families, including HSPA (HSP70) and co-chaperones, HSPB (αB-crystallin like small HSP), HSPC (HSP90), HSPD (HSP60), HSPE (HSP10), HSPH (HSP110), DNAJ proteins (HSP40), calreticulin (CAL), calnexin (CAX), peptidyl-prolyl cis-trans isomerases (PPI) and protein disulfide isomerases (PDI). As recently reviewed in detail in relation to skeletal muscle tissues, the different types of molecular chaperones provide a variety of protective functions [17].

HSPA molecules have a major chaperoning role by ensuring the correct folding of newly synthesized muscle proteins, by associating with misfolded and/or aggregated contractile proteins and by supporting the correct protein re-folding following cellular stress. The HSP70 family of proteins is widely distributed throughout muscle fibres, including inducible HSP70/72 and constitutive HSP73/Hsc70, as well as mitochondrial HSP75 and the HSP78 (GRP78) chaperone of the sarcoplasmic reticulum [18-20]. The large ATP-dependent molecular chaperones of the HSPC class function

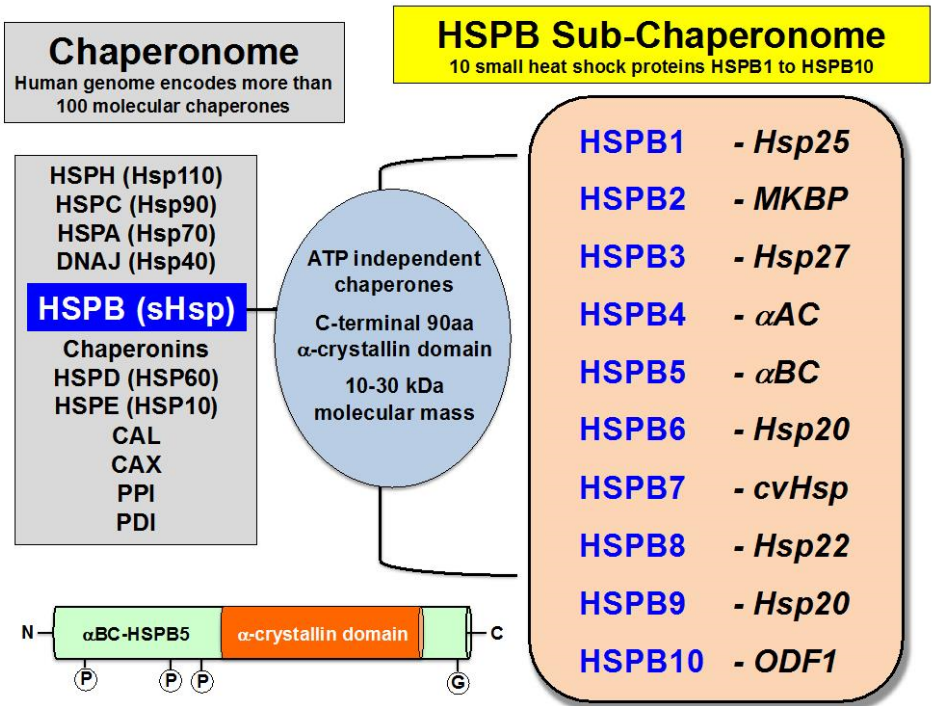

Figure 2. *Listing of the identified members of the chaperonome and the HSPB sub-chaperonome consisting of the family of small heat shock proteins.* The biochemical criteria for the classification of HSPB molecules are outlined and the domain structure of the characteristic α-crystallin domain within the prototype of a small heat shock protein, αB-crystallin (HSPB5), is shown.

down-stream of HSPA and bind to hormone receptors and kinases, whereby the interactions of HSP90α and HSP90β with co-regulators and co-chaperones is involved in the activation and stabilization of signalling proteins [21-23]. The HSP70-like unfolding proteins belonging to the category of disaggregating HSPH/HSP110 chaperones stabilize substrate proteins and actively dissociate stress-induced protein aggregates [24]. DNAJ/HSP40 proteins are primary co-chaperones that regulate the complex formation between HSPA and client proteins, and facilitate protein translation, protein folding, protein unfolding, protein translocation and protein degradation [25]. Another group of chaperonins that co-operate with HSPA molecules are HSPD/HSP60 molecules that support protein folding and re-folding patterns [26, 27]. The CAL/CAX chaperone system of the sarcoplasmic reticulum facilitates the correct folding of newly synthesized glycoproteins, especially those displaying N-linked glycan moieties, making it an essential part of the glycoprotein quality control system [28]. PPI enzymes preserve the correct conformation of distinct protein segments via catalysing the cis/trans isomerization of peptide-bonds besides proline residues [29] and PDI enzymes promote the correct disulfide-bridge formation and re-organization of disulphide -bridges in target muscle proteins [30].

3. Small heat shock proteins

The systems biological concept of the chaperonome is outlined in Figure 2 [15, 16], which specifically summarizes the biochemical criteria for the classification of the chaperone family of small heat shock proteins (sHSP). Several members of the sHSP/HSPB class of molecular chaperones respond swiftly to stressful stimuli during strenuous exercise or path-ophysiological insults [31-33]. The efficient disintegration of poly-disperse protein assemblies into smaller subunits is a key cytoprotective function of HSPB molecules and helps to counter-act the potentially harmful side effects from toxic protein aggregates [11]. The grouping of 10 distinct proteins, named HSPB1 to HSPB10 [13, 34], is based on (i) exhibiting distinct ATP-independent chaperoning activities [35-37], (ii) the presence of a conserved α-crystallin domain towards the carboxy-terminal region that spans approximately 90 residues [38-41], (iii) relatively low molecular masses ranging from approximately 10 to 30 kDa [34], and (iv) the capability of forming high-molecular-mass oligomers [42-44]. The 10 HSPB molecules and their tissue distribution are listed in Table 1. HSPB1, HSPB5, HSPB6 and HSPB8 are ubiquitously expressed throughout the body and HSPB9 and HSPB10 are restricted to testis [44]. HSPB1 to HSPB8, also referred to as HSP25, MKBP, Hsp27, αAC, αBC, HSP20, cvHSP, HSP22, respectively, are present at various concentrations in skeletal muscles and provide high-affinity binding platforms for partially misfolded or unfolded muscle proteins [45-48].

The expression of the cardiovascular cvHSP/HSPB7 chaperone is restricted to cardiac and skeletal muscles [47] and the highest concentration of αBC/HSPB5 among the non-lenticular cell types is in slow-twitching oxidative muscle fibres [45]. Major HSPB molecules are induced to prevent

Table 1. Summary of the human protein family of small heat shock proteins

Small heat shock protein	Protein Accession	Tissue distribution
HSPB1 (Hsp25, Hsp27)	P04792	Ubiquitous; high levels in muscle tissues
HSPB2 (MKBP)	Q16082	Skeletal, cardiac and smooth muscles
HSPB3 (Hsp27)	Q12988	Skeletal, cardiac and smooth muscles
HSPB4 (aA-Crystallin; aAC)	P02489	Highly abundant in the eye lens; low concentration in skeletal muscle tissues
HSPB5 (aB-crystallin; aBC)	P02511	Ubiquitous; high levels in skeletal and cardiac muscles
HSPB6 (Hsp20)	O14558	Ubiquitous; high levels in skeletal muscles
HSPB7 (cvHsp)	Q9UBY9	Skeletal and cardiac muscles
HSPB8 (Hsp22)	Q9UJY1	Ubiquitous; moderate levels in muscles
HSPB9 (Hsp20)	Q9BQS6	Testis
HSPB10 (ODF1)	Q14990	Testis

detrimental protein aggregation and are intrinsically linked to the association and modulation of the highly organized assembly of cytoskeletal protein networks, including actin, desmin, tubulin and vimentin [49-52]. HSPBs play a crucial role during myogenesis and the differentiation of mature contractile fibres in adult motor units [53-55]. Complex formation between HSPB2 and HSPB3 regulate myogenic differentiation steps [56] and modulate the muscle-specific transcription factor MyoD during fibre development [57]. In mature skeletal muscles, HSP molecules play a key role in preventing tissue damage during extensive repeats of excitation-contraction-relaxation cycles, which represents a major type of physiological stressor [58]. Strenuous exercise usually results in a robust and sustained up-regulation of molecular chaperones [59, 60], including especially HSPB molecules in skeletal muscle tissues [61, 62].

4. Proteomic profiling of small heat shock proteins from skeletal muscle

The highly adaptive neuromuscular system is heterogeneous in its molecular and cellular composition, extremely plastic in response to altered physiological demands, sensitive to mechanical unloading, vulnerable to traumatic injury and sensitive to altered metabolic states [63]. This dynamic nature of the musculature and the fact that muscle cells are highly abundant in the body makes contractile fibres and their supportive tissues exceedingly susceptible to various physiological and pathophysiological stressors [17]. Skeletal muscles therefore require a sophisticated and dynamic chaperoning system to prevent extensive cellular damage via protein unfolding and/or toxic protein aggregation [18-20]. The ATP-independent chaperones of the HSPB family of low-molecular-mass HSPs play a central role in these cytoprotective mechanisms [64] and provide considerable levels of stress tolerance by efficiently targeting misfolded muscle proteins for peptide refolding or degradation [17]. Comprehensive proteomic cataloguing studies that have focused on skeletal muscle preparations have identified a large number of molecular chaperones, including major representatives of the HSPB protein network [65-69]. This is illustrated here by the listings of major chaperoning molecules present in mouse skeletal muscle, as judged by the routine mass spectrometric evaluation of total muscle preparations versus the microsomal fraction.

An Ultimate 3,000 NanoLC system (Dionex Corporation, Sunnyvale, CA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Dublin, Ireland) was used for the label-free liquid chromatography mass spectrometric analysis of hind limb muscles from 6-month old C57BL6 mice, as recently described in detail [70]. Processing of the raw data generated from LC-MS/MS analysis was carried out using Progenesis QI for Proteomics software (version 3.1; Non-Linear Dynamics, a Waters company, Newcastle upon Tyne, UK). Data alignment was based on the LC retention time of each sample. The data was filtered using certain criteria prior to exporting the MS/MS data files to Proteome Discoverer 1.4 (Thermo Scientific): (i) peptide features with ANOVA < 0.05 between experimental groups, (ii) mass peaks with charge states from +1 to +5 and (iii) greater than one isotope per peptide [70]. A PepXML generic file was generated from all exported MS/MS spectra from Progenesis software. This file was used for peptide identification using Proteome Discoverer 1.4 against Mascot (version 2.3, Matrix Science, Boston, MA, USA) and Sequest HT (SEQUEST HT algorithm, licence Thermo Scientific, registered trademark University of Washington, USA) and searched against the UniProtKB-SwissProt database (taxonomy: *Mus musculus*). The following search parameters were used for protein identification: (i) peptide mass

Table 2. Routine identification of major chaperones in mouse hind limb muscle as revealed by the label-free mass spectrometric analysis of total tissue extracts..

Molecular chaperone	Protein Accession	Coverage (%)	Unique peptides	Molecular mass (kDa)
Heat shock protein HSPA1A (Hsp70, Hsp70A1A)	Q61696	9.98	1	70.0
Heat shock protein HSPA4 (Hsp70, HspA4)	Q61316	7.02	4	94.1
Heat shock protein HSPA5 (GRP78, mitochondrial HspA5)	P20029	30.08	15	72.4
Heat shock protein HSPA8 (Hsp70, Hsp71, HspA8)	P63017	49.69	25	70.8
Heat shock protein HSPA9 (GRP75, mitochondrial HspA9)	P38647	43.15	21	73.4
Heat shock protein HSPB1 (Hsp25, HspB1, Hsp beta-1)	P14602	51.20	8	23.0
Heat shock protein HSPB2 (MKBP, HspB2, Hsp beta-2)	Q99PR8	37.36	4	20.4
Heat shock protein HSPB6 (Hsp20, HspB6, Hsp beta-6)	Q5EBG6	59.26	6	17.5
Heat shock protein HSPB7 (cvHsp, HspB7, Hsp beta-7)	P35385	11.24	1	18.6
Heat shock protein HSPCA (Hsp90, Hsp90aa1, HspCA)	P07901	15.01	1	84.7
Heat shock protein HSPCB (Hsp90, Hsp90ab1, HspCB)	P11499	42.96	15	83.2
Heat shock protein HSPD1 (Hsp60, mitochondrial)	P63038	49.56	19	60.9
Heat shock protein HSPE1, (Hsp10, mitochondrial)	Q64433	52.94	6	11.0
DnaJ protein DNJA2 (Hsp40)	Q9QYJ0	6.80	2	45.7
Calreticulin (CAL, ERp60)	P14211	20.43	5	48.0
Protein disulfide isomerase A1 (PDI-A1, ERp59)	P09103	34.38	12	57.0
Protein disulfide-isomerase A3 (PDI-A3, ERp57)	P27773	10.69	4	56.6
Peptidyl-prolyl cis-trans isomerase A (PPI-A, PPIase A)	P17742	49.39	5	18.0
Peptidyl-prolyl cis-trans isomerase B (PPI-B, PPIase B)	P24369	10.19	2	23.7

tolerance set to 10 ppm, (ii) MS/MS mass tolerance set to 0.02 Da, (iii) up to two missed cleavages were allowed, (iv) carbamidomethylation set as a fixed modification and (v) methionine oxidation set as a variable modification [70]. For re-importation back into Progenesis LC-MS software for further analysis, only peptides with either ion scores of 40.00 or more (from Mascot) and peptides with XCorr scores >1.9 for singly charged ions, >2.2 for doubly charged ions and >3.75 for triply charged ions or more (from Sequest HT) were selected [70].

Crude muscle homogenates were shown to contain 19 major chaperones, including HSPA1A, HSPA4, HSPA5, HSPA8, HSPA9, HSPCA, HSPCB, HSPD1, HSPE1, DNJA2, CAL, PDI-A1, PDI-A3, PPI-A and PPI-B, as well as the sHSP molecules HSPB1, HSPB2, HSPB6 and HSPB7 (Table 2). The microsomal fraction, isolated by an optimized standard subcellular fractionation procedure for the depletion of the contractile apparatus and enrichment of the membrane fraction [70, 71], contained 28 chaperoning protein species. This included the sHSP molecules HSPB1, HSPB2, HSPB6 and HSPB7, as well as HSPA1A, HSPA1L, HSPA4, HSPA5, HSPA8, HSPA9, HSPCA, HSPCB, HSP90b1, HSPD1, HSPE1, Trap1, DNJA2, DNJA3, DJB11, DNJC3, DJC11, CAL, PPI-A, PPI-B, PDI-D, PDI-A1, PDI-A3 and PDI-A6 (Table 3). The fact that a considerable number of DNAJ and GRP proteins from the sarcoplasmic reticulum and mitochondria were identified to be present in the membrane fraction [25] underlines the successful application of subcellular fractionation [72]. The proteomic hits from the microsomal study were further characterized by standard bioinformatics using the STRING database of known and predicted protein interactions that include direct physical and indirect functional protein associations [73]. Figure 3 outlines the close interaction network of chaperoning proteins from skeletal muscles, including HSPB1, HSPB2, HSPB6 and HSPB7, emphasising the crucial importance of sHSP molecules for cytoprotection in tissues with a high degree of vulnerability to environmental, physiological or pathological stressors [74].

5. Comparative proteomic profiling of HSPB in skeletal muscles

Molecular chaperones are involved in a variety of neurodegenerative diseases, neuromuscular pathologies and the

Table 3. Identification of major chaperones in mouse hind limb muscle as revealed by the label-free mas spectrometric analysis of microsomes.

Molecular chaperone	Protein Accession	Coverage (%)	Unique peptides	Molecular mass (kDa)
Heat shock protein HSPA1A (Hsp70, Hsp70A1A)	Q61696	9.98	1	70.0
Heat shock protein HSPA1L (Hsp70, Hsp70A1L)	P16627	8.89	1	70.6
Heat shock protein HSPA4 (Hsp74, Hsp70RY, HspA4)	Q61316	7.25	4	94.1
Heat shock protein HSPA5 (GRP78, mitochondrial HspA5)	P20029	49.77	28	72.4
Heat shock protein HSPA8 (Hsp70, Hsp71, HspA8)	P63017	50.77	23	70.8
Heat shock protein HSPA9 (GRP75, mitochondrial HspA9)	P38647	40.35	20	73.4
Heat shock protein HSPB1 (Hsp25, HspB1, Hsp beta-1)	P14602	70.81	10	23.0
Heat shock protein HSPB2 (MKBP, HspB2, Hsp beta-2)	Q99PR8	43.96	5	20.4
Heat shock protein HSPB6 (Hsp20, HspB6, Hsp beta-6)	Q5EBG6	59.26	6	17.5
Heat shock protein HSPB7 (cvHsp, HspB7, Hsp beta-7)	P35385	38.46	3	18.6
Heat shock protein HSPCA (Hsp90, Hsp90aa1, HspCA)	P07901	9.28	1	84.7
Heat shock protein HSPCB (Hsp90, Hsp90ab1, HspCB)	P11499	30.39	12	83.2
Heat shock protein Hsp90b1 (Endoplasmin, GRP94)	P08113	30.92	15	92.4
Heat shock protein HSPD1 (Hsp60, mitochondrial)	P63038	58.12	23	60.9
Heat shock protein HSPE1, (Hsp10, mitochondrial)	Q64433	52.94	6	11.0
Trap1 (TNFR-associated protein 1, mitochondrial Hsp75)	Q9CQN1	4.11	1	80.2
DnaJ protein DNJA2 (Hsp40, DNJ3)	Q9QYJ0	7.77	1	45.7
DnaJ protein DNJA3 (mitochondrial Hsp40, mTid-1)	Q99M87	3.96	2	52.4
DnaJ protein DJB11 (ER Hsp40 co-chaperone of HSPA5)	Q99KV1	9.22	2	40.5
DnaJ protein DNJC3 (Hsp40, protein kinase inhibitor p58)	Q91YW3	5.95	1	57.4
DnaJ protein DJC11 (mitochondrial Hsp40, DNAJC11)	Q5U458	3.22	1	63.2
Calnexin (CAL, CALX, CNX)	P35564	11.34	4	67.2
Peptidyl-prolyl cis-trans isomerase A (PPI-A, PPIase A)	P17742	55.49	6	18.0
Peptidyl-prolyl cis-trans isomerase B (PPI-B, PPIase B)	P24369	10.19	2	23.7
Peptidyl-prolyl cis-trans isomerase D (PPI-D, PPIase D)	Q9CR16	4.32	1	40.7
Protein disulfide isomerase A1 (PDI-A1, ERp59)	P09103	44.79	15	57.0
Protein disulfide-isomerase A3 (PDI-A3, ERp57)	P27773	33.86	15	56.6
Protein disulfide-isomerase A6 (PDI-A6, TXNDC7)	Q922R8	12.05	3	48.1

natural aging process [17, 75-78]. Besides their essential neuroprotective functions, HSP molecule are associated with primary chaperonopathies and secondary alterations during pathophysiological insults. Neuromuscular disorders, metabolic diseases and neuropathies that are closely linked to altered expression levels in the HSPB family of molecular chaperones are myotonic dystrophy, myofibrillar myopathies, Duchenne muscular dystrophy, the dysferlinopathies

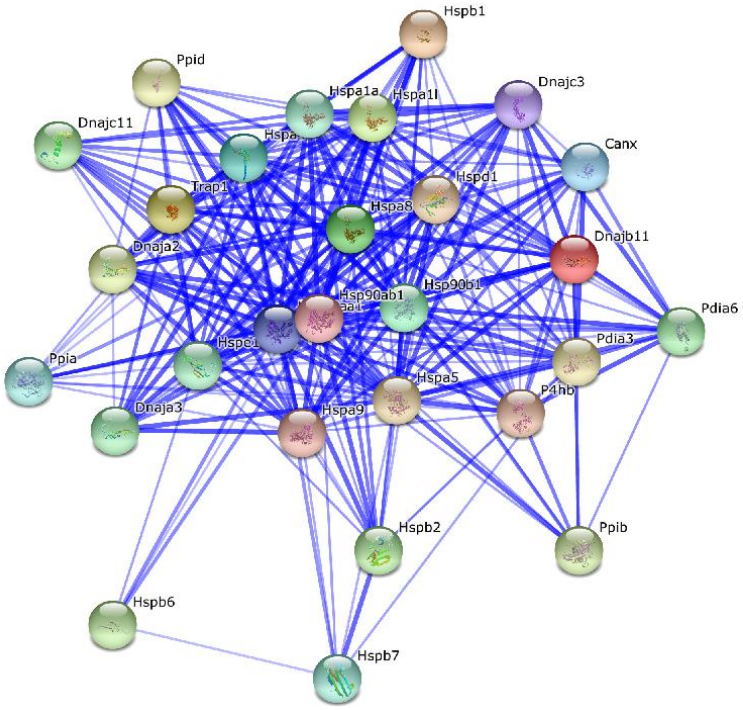

Figure 3. *Interaction map of the chaperoning protein system from mouse hind leg muscles.* The bioinformatics STRING database [73] was used to generate a protein interaction map with known and predicted protein associations that include direct physical and indirect functional protein linkages of mass spectrometrically identified molecular chaperones, including the sHSP molecules HSBP1, HSPB2, HSPB6 and HSPB7, as well as HSPA1A, HSPA1L, HSPA4, HSPA5, HSPA8, HSPA9, HSPCA, HSPCB, HSP90b1, HSPD1, HSPE1, Trap1, DNJA2, DNJA3, DJB11, DNJC3, DJC11, CAL, PPI-A, PPI-B, PDI-D, PDI-A1, PDI-A3 and PDI-A6 (Table 3).

termed Miyoshi myopathy and limb-girdle muscular dystrophy type LGMD2B, myotonia-related hyperexcitability, collagen VI myopathy, Charcot-Marie-Tooth disease type 2, distal hereditary motor neuropathy, motor neuron disease, type 2 diabetes-related muscle weakness and sarcopenia of old age, as listed in Table 4. Certain desmin-related myopathies (α-crystallinopathy) are characterized by abnormalities in HSPB5 [79-81]. HSPB1, HSPB3 and HSPB8 are primarily affected in distal motor neuropathy and the axonal form of Charcot-Marie-Tooth disease type 2 [82-86]. Figure 4 summarizes changes in HSPB molecules during physiological adaptations and in response to pathophysiological insults. A large number of comparative proteomic studies have focused on the molecular fate of HSP chaperones during stressful conditions. This has included the systematic analysis of myoblast differentiation and protein secretion during myogenesis [87-89], the fibre type specification in fast versus slow muscles [90-92], the physiological modifications following endurance exercise or resistance training [93-96], fast-to-slow muscle transformation following chronic low-frequency stimulation [97-99], skeletal muscle hypertrophy [100], muscular atrophy following immobilization or denervation [101-103], hypoxia-related stress [104] and sarcopenia of old age [105-110]. Exercise, fibre transitions, disuse atrophy and hypertrophy are clearly related to distinct changes in HSPB molecules.

Systematic surveys of changes in HSP chaperones in neuromuscular pathologies has included dysferlinopathy [111], Duchenne muscular dystrophy [17, 70, 71, 112-126], myofibrillar myopathies with abnormalities in HSPB5, desmin, filamin or myotilin [127-130], myasthenia gravis [131], myotonia-related hyperexcitability [132], motor neuron diseases including amyotrophic lateral sclerosis [133-137], hypokalemic myopathy [138], obesity-related muscular weakness and/or type 2 diabetes-associated insulin resistance [139-144], burn sepsis-related stress [145] and *post mortem* changes in skeletal muscle samples [146]. The differential expression of HSPB chaperones in pathological skeletal muscles is clearly related to the requirement of swiftly dispersing toxic protein aggregates and facilitating the degradation of misfolded muscle proteins under stressed conditions. The bioanalytical strategies used in the comparative proteomic profiling of HSP molecules in stressed skeletal muscles have relied on sophisticated labeling methods, efficient protein separation techniques and sensitive mass spectrometry. Often detergent- or urea-based extraction methods were used to prepare crude muscle extracts and the identification of changed proteins was conducted with various label-based or label-free approaches [17, 63].

In general, quantitative proteomic studies are routinely carried out with chemical or metabolic labeling and frequently include the isotopic tagging of peptides and proteins prior to mass spectrometric analysis. Cellular proteomics employs a variety of relative quantitation methods, including

Table 4. Major neuromuscular pathologies associated with primary or secondary abnormalities in small heat shock proteins.

Small heat shock protein	Pathological involvement
HSPB1 (Hsp25)	Charcot-Marie-Tooth disease type 2 Distal hereditary motor neuropathy Motor neuron disease Type 2 diabetes-associated muscle weakness Collagen VI myopathy Dysferlinopathy
HSPB2 (MKBP)	Myotonic dystrophy
HSPB3 (Hsp27)	Motor neuropathy Duchenne muscular dystrophy Myotonia-related hyperexcitability Sarcopenia of old age
HSPB5 (αBC)	Myofibrillar myopathies (desminopathy, crystallinopathy) Duchenne muscular dystrophy Sarcopenia of old age Motor neuron disease Muscular atrophy Type 2 diabetes-associated muscle weakness
HSPB6 (Hsp20)	Sarcopenia of old age
HSPB7 (cvHsp)	Duchenne muscular dystrophy Sarcopenia of old age
HSPB8 (Hsp22)	Charcot-Marie-Tooth disease type 2 Distal hereditary motor neuropathy

iTRAQ (isobaric Tags for Relative and Absolute Quantitation), ICAT (Isotope-Coded Affinity Tag) and SILAC (Stable Isotope Labeling with Amino acids in Cell culture) [147-149]. Subproteomic studies that focus on isolated organelles for the reduction of sample complexity involve advanced methods such as LCM (laser capture microscopy) [150] and LOPIT (Localization of Organelle Proteins by Isotope Tagging) [151]. Large-scale protein separation is usually achieved by gel electrophoretic methods and/or liquid chromatography. An efficient pre-electrophoretic labeling method for the comparative analysis of isolated proteomes is two-dimensional fluorescence difference in-gel electrophoresis (DIGE) [152-154]. Importantly, the extraordinary improvements of mass spectrometers in relation to mass accuracy, sensitivity, resolving power, dynamic range, throughput capacity and available fragmentation modes has greatly increased the coverage of the assessable proteome. Modern peptide mass analyzers rely on time-of-flight technology, linear ion traps, quadrupole, orbitrap or fourier transform ion cyclotron resonance methodologies [155-158]. Studies with a focus on HSP molecules have used many of these standard proteomic techniques. Since HSPB molecules are mostly low-molecular-mass proteins and relatively soluble as compared to many other protein species in skeletal muscles, they can be easily separated by gel electrophoretic procedures or liquid chromatography. For example, the application of the fluorescence DIGE technique has resulted in the identification of considerably increased levels of cvHSP/HSPB7 and αBC/HSPB5 in both dystrophic and senescent muscle fibres [107, 108]. Thus, although two-dimensional gel electrophoresis underestimates the presence of integral membrane proteins, high-molecular-mass proteins and low copy number proteins, it is highly suited for studying the large family of HSP molecules [17]. Label-free mass spectrometry has also been successfully employed to compare normal versus stressed muscle specimens and identified distinct changes in HSP molecules [70, 125, 126]. Since skeletal muscles contain some of the largest proteins in the human body, such as nebulin, obscurin and titin, alternative on-membrane digestion approaches have been developed to study these giant proteins [159-161]. In future studies, gradient gel electrophoretic separation in combination with on-

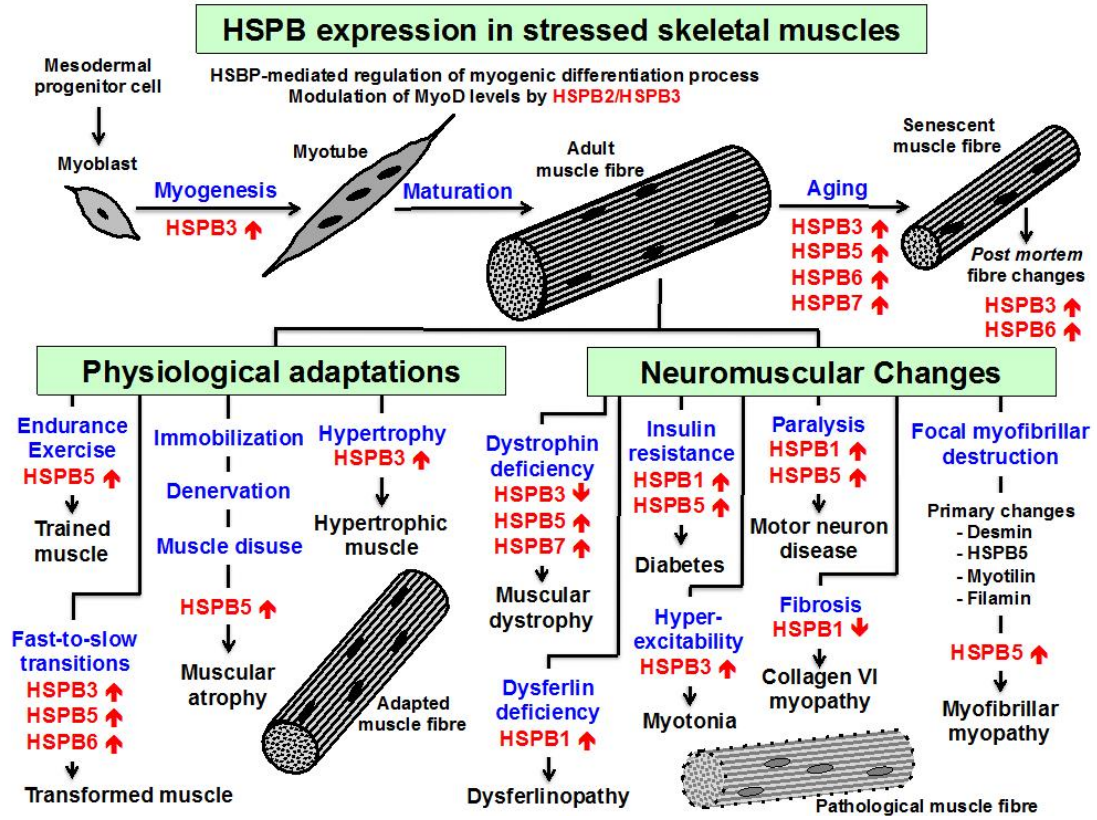

Figure 4. Overview of changes in HSPB molecules during physiological adaptations and in response to pathophysiological insults.

membrane digestion might also be useful to investigate the interaction patterns between HSPB species within supramolecular protein assemblies, such as the actomyosin apparatus or the Z-disc region of sarcomeres.

6. Concluding Remarks

The intrinsic role of HSPB molecules as chaperoning proteins makes them key components involved in the fine regulation of cellular proteostasis. In stressed skeletal muscles, HSPB chaperones are of central importance for the facilitation of various adaptive processes and cellular pro-survival mechanisms. This makes changes in their concentration, oligomerization, post-translational modifications and/or subcellular re-localization a characteristic feature of both stress tolerance and muscle damage pathways. These distinct alterations can be potentially exploited for the establishment of superior biomarker signatures of physiological adaptations and pathological changes [162-165]. Muscle-specific HSPB molecules are of low molecular mass and belong to the relatively soluble type of proteins. These biochemical and physicochemical properties make them good candidates for developing simple bioassays to predict, diagnose and evaluate stress-related skeletal muscle degeneration. Since HSPB chaperones are critical cytoprotective factors that prevent and reverse stress-related fibre damage, they are also potentially useful as therapy-monitoring biomarkers. In the future, the evaluation of disease model systems or routine testing in preclinical studies may profit from the usage of HSPB molecules as reliable and robust muscle-associated markers of cellular stress. This gives the detailed characterization of the HSPB chaperonome considerable importance for advancing several biomedical areas, including basic myology, applied physiology, sports medicine and neuromuscular pathology.

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