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Proteomic reference map for sarcopenia research: mass spectrometric identification of key muscle proteins located in the sarcomere, cytoskeleton and the extracellular matrix

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Abstract

Sarcopenia of old age is characterized by the progressive loss of skeletal muscle mass and concomitant decrease in contractile strength. Age-related skeletal muscle dysfunctions play a key pathophysiological role in the frailty syndrome and can result in a drastically diminished quality of life in the elderly. Here we have used mass spectrometric analysis of the mouse hindlimb musculature to establish the muscle protein constellation at advanced age of a widely used sarcopenic animal model. Proteomic results were further analyzed by systems bioinformatics of voluntary muscles. In this report, the proteomic survey of aged muscles has focused on the expression patterns of proteins involved in the contraction-relaxation cycle, membrane cytoskeletal maintenance and the formation of the extracellular matrix. This includes proteomic markers of the fast *versus* slow phenotypes of myosin-containing thick filaments and actin-containing thin filaments, as well as proteins that are associated with the non-sarcomeric cytoskeleton and various matrisomal layers. The bioanalytical usefulness of the newly established reference map was demonstrated by the comparative screening of normal *versus* dystrophic muscles of old age, and findings were verified by immunoblot analysis.

Key Words: aging, mass spectrometry, muscle proteomics, muscular dystrophy.

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In the matured human body, voluntary muscle fibers represent the most abundant cellular contributor to biomass,[1] and are intrinsically involved in generating contractile force for maintaining proper fitness levels and health status.[2-4] A high degree of skeletal muscle plasticity underlies the adaptative potential of the body to manage altered physiological demands and biochemical alterations, as well as fluctuations in metabolic and bioenergetic requirements,[5] emphasizing the importance of mobility medicine for supporting optimized body movements.[6] Skeletal muscle functions are diverse and include the promotion of coordinated locomotion via tightly controlled excitation-contraction-relaxation cycles, postural support to maintain muscle-skeletal balance and diaphragm-associated respiration.[3] Furthermore, skeletal muscle contractions are involved in the regulation of body thermogenesis, bioenergetic integration and metabolic adaptations, as well as facial and verbal communication.[4]

Key questions of basic and applied myology are concerned with the molecular and cellular mechanisms that underlie the response of the voluntary contractile system to stressful stimuli such as pathological insults, extreme physical strain, disuse-related atrophy and aging, and how muscle fiber dysfunction affects whole-body physiology.[7] Many of these physiological and pathophysiological issues are studied with the help of animal models. In general, animal models play a central role in the biomedical sciences,[8] including mice which are widely used in the neurosciences.[9] Various mouse tissues are routinely studied to improve our biological knowledge of development, differentiation, maturation, physiological adaptations, environmental effects, heat homeostasis, dietary requirements and disease mechanisms,[10-

[13] as well as the natural aging process.[14-16] This includes mouse models of skeletal muscle aging.[17-19]

Skeletal muscles are majorly affected during aging, now generally referred to as sarcopenia of old age,[20] and this process is characterized by the progressive loss of tissue mass and contractile strength.[21-23] Sarcopenia can result in fundamental consequences for the quality of life of aged individuals, since voluntary muscles are involved in a plethora of physiological functions in the body.[24] Unbiased and technology-driven approaches to carry out systematic surveys of aged tissues include omics-type techniques, such as genomics, transcriptomics, proteomics, metabolomics and cytomics.[25-27] Protein populations in senescent muscles can be conveniently determined by mass spectrometry-based proteomics.[28-31]

Skeletal muscle tissues from a variety of species have been extensively characterized by mass spectrometry using both bottom-up and top-down proteomics.[32-35] The combined biochemical cataloguing efforts of numerous mass spectrometric screening initiatives,[36-39] have been instrumental to establish an ever-growing catalog of proteoforms that constitute the dynamic skeletal muscle proteome.[40-42] Important studies in muscle proteomics have included the systematic analysis of fiber type specification,[43-46] fiber type shifting,[47-49] the effects of exercise,[50-53] the impact of primary muscle disease,[54-56] neuromuscular dysfunction due to co-morbidities,[57-59] and sarcopenia of old age.[60-65]

Building on these findings, we describe in this report the mass spectrometric profiling of total tissue extracts derived from the senescent mouse hindlimb musculature using bottom-up proteomics. The age of mouse models is usually categorized as 'mature adult' (3-6 months), 'middle-aged' (10-15 months) and 'old' (18-24 months).[66] Early effects of natural aging can already be observed in the middle-aged group, but severe alterations, such as a drastic decline in muscle mass and progressive dysfunction, are only observed in the senescent group.[15,67] Therefore, we have used 24 months old mouse skeletal muscle specimens for our proteomic mapping of the aged musculature.

Materials and Methods

Materials

The proteomic analysis of mouse hindlimb muscles was carried out with analytical grade chemicals from Sigma Chemical Company (Dorset, UK), GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK). Trypsin (MS-grade), protease inhibitors (cOmplete™, mini protease inhibitor cocktail) and spin filters (Vivacon 500, VN0H22; 30,000 MWCO) were obtained from ThermoFisher Scientific (Dublin, Ireland), Roche (Mannheim, Germany) and Sartorius (Göttingen, Germany), respectively. Protein assays were carried out with the Pierce 660 nm Protein Assay Reagent from ThermoFisher Scientific (Dublin, Ireland). For protein gel electrophoresis and immunoblot analysis, precast Invitrogen Bolt 4-12% Bis-Tris gels and Whatman nitrocellulose transfer membranes were purchased from Bio-Science Ltd. (Dun Laoghaire, Ireland). InstantBlue Coomassie Protein Stain was from Expedeon (Heidelberg, Germany). For immunoblotting, primary antibodies were obtained from ThermoFisher Scientific, Dublin, Ireland (mAb SD83-03 against collagen VI), Merck Life Science Ltd, Arklow, Ireland (mAb PARV-19 against parvalbumin), R&D Systems, Minneapolis, MN, USA (MAB5718 against glyceraldehyde-3-phosphate dehydrogenase), and Abcam, Cambridge, UK (mAb ERP5158 against tropomyosin TPM1, alpha). Secondary peroxidase-conjugated anti-IgG were from Sigma Chemical Company (Dorset, UK). The visualization of immuno-decorated bands was performed by the enhanced chemiluminescence (ECL) technique using an ECL kit from Roche (Mannheim, Germany).

Mouse hindlimb muscle

Hindlimb muscles were dissected from freshly prepared *post mortem* specimens from 24 months old wild type C57BL6 mice according to institutional regulations. Mouse populations were handled in strict adherence to local governmental and institutional animal care regulations and were approved by the Institutional Animal Care and Use Committee (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany).[39] Mice were kept under specific pathogen-free conditions in isolated, ventilated cages with free access to water and food. A maximum of 4 mice were kept per cage at a temperature of 21-22°C with a 12-hour light/12-hour dark cycle. Tissue samples were quick-frozen in liquid nitrogen and transported on dry ice to Maynooth University in accordance with the regulations of the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth).[68] The mass spectrometric analysis of total skeletal muscle preparations was performed with protein extracts from 6 wild type mice. For the comparison of aged wild type *versus* aged and dystrophic *mdx-4cv* muscles, which lack the membrane cytoskeletal protein dystrophin,[39] 24 months old muscles were used.

Protein extraction from skeletal muscle specimens

The bottom-up proteomic analysis of hindlimb muscles from wild type C57BL6 mice, as well as age-matched dystrophic *mdx-4cv mice*, was carried out with total protein extracts.[69] Tissue preparation, homogenization using pulverization of specimens via grinding in liquid nitrogen and protein extraction were performed by a standardized procedure.[70] Following homogenization in lysis buffer (0.1 M dithiothreitol, 4% (w/v) sodium dodecyl sulfate, 100 mM Tris-Cl, pH 7.6; supplemented with a protease inhibitor cocktail), suspensions were briefly treated in a sonicating water bath and then heated for 3 min at 95°C.[71] Samples were centrifuged at 16,000×*g* for 5 minutes and the protein-containing supernatant extracted for subsequent mass spectrometric analysis. The protein concentration was determined with the help of the Pierce 660 nm Protein Assay system.[72] For proteomics, samples were mixed with 8 M urea, 0.1 M Tris, pH 8.9 in Vivacon 500 spin filter units.[73] Following a centrifugation step at 14,000×*g* for 15 min, the further processing of samples, the switching of buffers and peptide generation by trypsin digestion was car-

ried out by the filter-aided sample preparation (FASP) technique, as described in detail by Wiśniewski.[74]

Mass spectrometry-based proteomic analysis

A Thermo Orbitrap Fusion Tribrid mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA) was used for the label-free liquid chromatography mass spectrometric analysis of aged hindlimb muscles from normal mice (n=6 biological repeats; n=2 technical repeats), as well as the comparison of wild type *versus* dystrophic *mdx-4cv* muscles (n=3 biological repeats; n=2 technical repeats).[56] The individual analytical steps (including FASP-based sample preparation, buffer composition, timing of preparative stages, data-dependent acquisition, and bioinformatic data handling) that were employed in the proteomic analysis, are described in detail in a recently published methods paper.[75]
A Thermo UltiMate 3000 nano system was used for reversed-phase capillary high-pressure liquid chromatography and directly coupled in-line with the Thermo Orbitrap Fusion Tribrid mass spectrometer. The qualitative data analysis of mass spectrometric files was carried out with the UniProtKB-SwissProt database (species: *Mus musculus*) with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator. Parameters for the mass spectrometric detection of senescent hindlimb muscle proteins were as follows: i) a value of 0.02 Da for MS/MS mass tolerance, ii) a value of 10 ppm for peptide mass tolerance, iii) variable modification settings for methionine oxidation, iv) fixed modification settings in relation to carbamidomethylation, and v) tolerance for the occurrence of up to two missed cleavages. Peptide probability was set to high confidence. A minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge state was employed for the filtering of peptides. The software analysis programme Progenesis QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle upon Tyne, UK) was used to carry out quantitative label-free data analysis. Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and a percolator were employed for the identification of peptides and proteins. Datasets were imported into Progenesis QI software for further analysis.
The multi-consensus MS file and listings of proteins in aged murine skeletal muscle that were generated by this proteomic study have been deposited under the title 'Proteomic analysis of senescent mouse hindlimb muscles' with the unique identifier '37txb' to the Open Science Foundation (OSF) (https://osf.io/37txb/).
The standard bioinformatic analysis tools PANTHER (http://www.pantherdb.org) and STRING (https://string-db.org) were used for the identification of protein classes,[76] and potential protein-protein interaction patterns,[77] respectively.

Immunoblot analysis

Comparative immunoblotting of preparations from aged wild type *versus* aged *mdx-4cv* mouse hindlimb muscle was carried out by an optimized procedure, as previously described in detail.[39] Protein separation was performed with precast Invitrogen Bolt 4-12% Bis-Tris gels. Separated protein bands were visualized with InstantBlue Coomassie protein stain. Following electrophoretic transfer onto Whatman nitrocellulose transfer membranes,[68] individual blots were labelled with 1:1000 diluted primary antibodies to glyceraldehyde 3-phosphate dehydrogenase, tropomyosin, parvalbumin or collagen isoform COL-VI. Membranes were washed and incubated with 1:1000 diluted secondary peroxidase-conjugated antibodies. Washed membranes were then treated with enhanced chemiluminescence (ECL) chemicals to visualize immuno-decorated protein bands.

Results

The findings from the mass spectrometric screening of total tissue extracts from the aged mouse hindlimb musculature are presented in this report. The major aim of this study was to establish a proteomic reference map of aged skeletal muscles for future sarcopenia research. The focus was on the expression patterns of key proteins located in the sarcomere, non-sarcomeric cytoskeletal networks and the extracellular matrix. To illustrate the bioanalytical value of the new proteomic data set, the mass spectrometric comparison of normal *versus* dystrophic skeletal muscles is presented, including comparative immunoblotting.

Mass spectrometric analysis of aged mouse hindlimb muscle extracts

Crude protein extracts from 100 mg muscle specimens were used as starting material to establish the optimum utilization of small tissue biopsy specimens. In order to minimize the number and complexity of preparative steps, the experimental protocol was conducted in a simple and streamlined way. The analysis pipeline used here represents a swift, economical and efficient proteomic procedure that avoids the introduction of excess bioanalytical artefacts and can be employed for the routine detection of key protein species in aged skeletal muscles, as shown by the results presented in below sections. All individual steps involved in the proteomic analysis pipeline, including a description of necessary materials, chemicals, buffers and the critical timing of preparative stages, as well as data-dependent acquisition and bioinformatic data handling, have been described in detail in a recently published methods paper.[78] The proper planning of experimental protocols, the optimized preparation of buffers and the continues maintenance of equipment are essential for the successful execution of a proteomic analysis. Streamlined sample processing can considerably reduce overall running costs and prevent the introduction of analytical artefacts.[32,75]
The proteomic profiling of the senescent mouse hindlimb musculature detected 3128 distinct protein species with 2 or more peptides. The multi-consensus MS file and listings of proteins in aged skeletal muscle that were generated by proteomics as outlined in this report have been deposited under the title 'Proteomic analysis of senescent mouse hindlimb muscles' with the unique identifier '37txb' to the OSF site (https://osf.io/37txb/). The results are based on multi-consensus data from 6 biological repeats and 2 technical repeats. The muscle-associated protein with the lowest molecular mass was identified as the actin-sequestering

protein thymosin beta-4 of 5.7 kDa and that with the highest molecular mass as the abundant sarcomeric protein titin of 3906.4 kDa. Besides the giant half-sarcomere spanning protein titin, other identified high-molecular-mass muscle proteins above 400 kDa included laminin, dystrophin, ankyrin-2, sacsin, dynein, plectin, the ryanodine receptor calcium release channel, epiplakin, nesprin, dystonin and obscurin.

As shown in Figure 1, the total number of peptides per detected protein ranged from 2 to 1807. The diagrams show the number of peptides of the top 1-20, top 21-100 and top 101-3128 detected protein species. An amino acid sequence coverage based on more than 120 individual peptides included titin, plectin, myosin-4, myosin-1, obscurin, ryanodine receptor isoform RYR1, myosin-8, myosin-7, dynein DYNC1H1 and myosin-9. Sequence coverage of identified proteins ranged from 0.4% (E3 ubiquitin-protein ligase RNF213 of 584.3 kDa) to 95.5% (cytoplasmic actin ACTG1 of 41.8 kDa). A sequence coverage above 90% was achieved in the case of cytoplasmic actin 1 and 2, skeletal muscle-specific myosin regulatory light chain MLC2/MYLPF, the 60S acidic ribosomal protein RPLP2, guanine nucleotide-binding protein subunit beta-2, dolichyl-diphospho-oligosaccharide-protein glycosyl-transferase subunit 4, peroxiredoxin-1, phosphatidyl-ethanolamine-binding protein PEBP1 and fructose-bisphosphate aldolase ALDOA.

Bioinformatic PANTHER analysis of protein families in aged skeletal muscle

The bioinformatic analysis tool PANTHER,[76] was used to generate a pie chart that displays the main types of identified protein families in aged skeletal muscles. As shown in Figure 2, the most abundantly identified muscle-associated proteins belong to the classes of metabolite interconversion enzymes, protein modifying enzymes, RNA metabolism proteins, cytoskeletal proteins, membrane traffic proteins, scaffold/adaptor proteins, transporters and translational proteins. In addition, skeletal muscle protein classes were identified as DNA metabolism proteins, calcium-binding proteins, cell adhesion molecules, cell junction proteins, molecular chaperones, chromatin, chromatin-binding proteins, defence proteins, immunity proteins, extracellular matrix proteins, gene-specific transcriptional regulators, intercellular signal molecules, protein-binding activity modulators, storage proteins, structural proteins, transfer proteins, carrier proteins and transmembrane signal receptors.

Proteomic profiling of sarcomeric proteins and contractile apparatus in aged skeletal muscle

Sarcomeres containing the contractile acto-myosin apparatus and auxiliary filaments, in conjunction with the extra-sarcomeric cytoskeletal network, constitute more than half

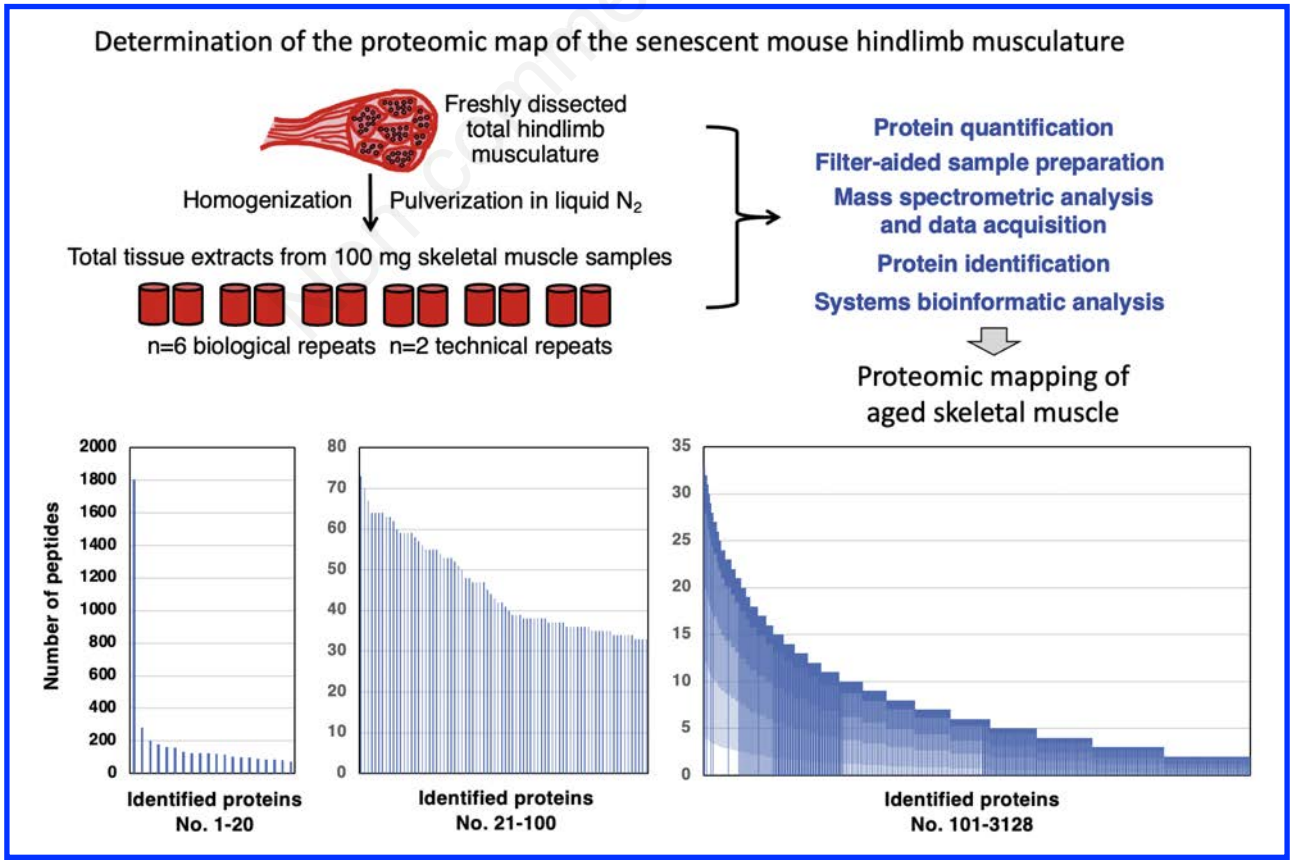

Figure 1. *Overview of the proteomic mapping of mouse skeletal muscle extracts.*

of the protein mass in voluntary muscles. The proteomic analysis of aged skeletal muscle clearly identified the main protein species that are associated with these subcellular structures that are involved in filament sliding and intracellular stabilization, including i) the myosin-containing thick filaments (myosin heavy chains, myosin light chains, myosin binding proteins), ii) the actin-containing thin filaments (muscle and heart actins) in combination with the troponin-tropomyosin regulatory complex (troponin subunits TnC, TnT and TnI, as well as tropomyosin TPM) and auxiliary proteins (F-actin-capping proteins, tropomodulins, Xin actin-binding protein and myopalladin), iii) the half-sarcomere spanning titin filaments (titin and the titin-associated muscle ankyrin repeat protein MARP), iv) the sarcomeric M-line complex (obscurin, myomesin), and v) the sarcomeric Z-line complex (alpha-actinin, filamin-C, telethonin and myozenin; Table 1).

Of note, the types of myosin light and heavy chains ranged from major slow and fast forms to developmental isoforms. This included myosin heavy chains (MyHC) of the fast type (MyHC-IIb, MyHC-IIx) and their slower counterparts (MyHC-I, MyHC-7b) and the mostly developmental types MyHC-embryonic and MyHC-perinatal. Myosin light chains (MLC) were identified as MLC1/MLC3 and muscle-specific MLC2, as well as the slow chains MLC2 and MLC3. In analogy, the regulatory troponin (Tn) subunits TnC, which is modified by calcium binding, TnT, which binds to tropomyosin, and TnI, which is involved in the inhibitory mechanism of acto-myosin interactions, were found to be present in both slow and fast isoforms. Tropomyosin was represented by its isoforms TPM1, TPM3 and TPM4 (Table 1). Previous studies of aged human muscles and animal models of sarcopenia have shown considerable changes in these identified sarcomeric proteins during the aging process,[60-65,79-86] indicating age-related fiber type shifting.[30] Graded muscle transformations involve a general trend of isoform transitions from faster types of MyHC and MLC, as well as TnC, TnI and TPM to their slower counterparts.[80,82,84] Thus, the proteomic catalog of aged muscle presented here can provide a suitable overview of protein isoform expression patterns that can be related to other aging studies and be highly useful as a reference databank for studying proteome-wide changes in senescent myofiber populations. As recently reviewed,[30] age-associated increases in distinct isoforms of sarcomeric proteins include MyHC-I, MLC-2s, MLC-1/3f, ACTA, TNT-3, MYOZ-1, MARP/ANKRD2, ACTC, and concomitant decreases were shown for MyHC-IIx, MLC-1/3, MLC2f, TnT-1, TnT-3, TnC-1, TPM-3, FLNC and TTN, as well as shifting of phosphorylated MLC-2f to MLC-2s isoforms.

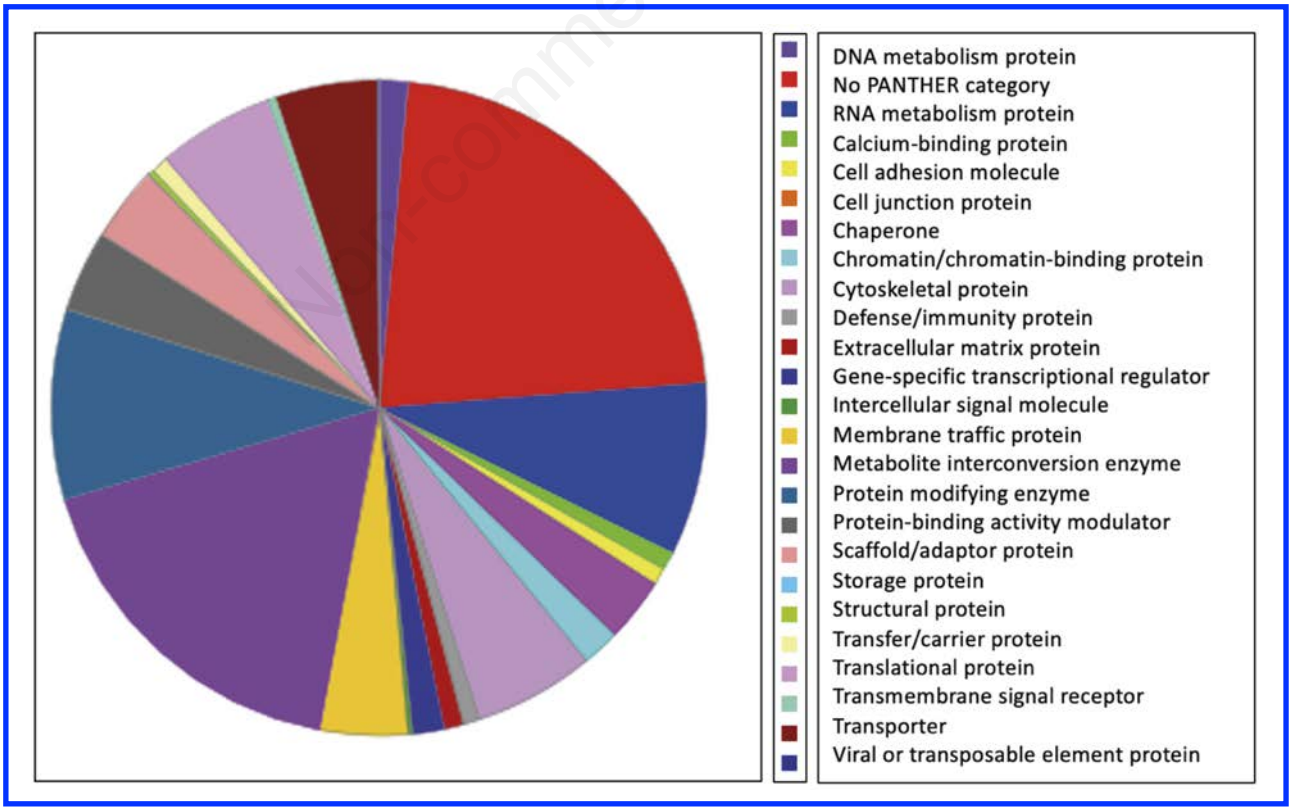

Figure 2. *Overview of protein families detected by the bottom-up proteomic analysis of total extracts from aged mouse skeletal muscle. The publicly available bioinformatic analysis tool PANTHER (http://www.pantherdb.org) was used for the identification of protein classes.*[76]

Table 1. *Mass spectrometry-based proteomic profiling of sarcomeric proteins in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Half-sarcomere spanning titin filament**					
A2ASS6	Titin	Ttn	1807	63.2	3906.4
Q9WV06	Titin-associated MARP	Ankrd2	10	42.1	36.7
(ii) **Sarcomeric M-line complex**					
A2AAJ9	Obscurin	Obscn	163	35.0	966.6
Q62234	Myomesin-1	Myom1	100	68.5	185.5
A2ABU4	Myomesin-3	Myom3	27	27.0	161.8
(iii) **Sarcomeric Z-line complex**					
Q7TPR4	Alpha-actinin-1	Actn1	55	70.0	103.1
Q9JI91	Alpha-actinin-2	Actn2	53	63.9	103.8
O88990	Alpha-actinin-3	Actn3	64	75.2	103.0
P57780	Alpha-actinin-4	Actn4	56	69.5	105.0
Q8VHX6	Filamin-C, muscle	Flnc	100	51.6	291.1
O70548	Telethonin	Tcap	8	55.1	19.1
Q9JK37	Myozenin-1	Myoz1	15	72.6	31.5
Q9JJW5	Myozenin-2	Myoz2	5	28.4	29.8
Q8R4E4	Myozenin-3	Myoz3	9	45.3	27.0
(iii) **Thick filament myosin complex**					
Q5SX39	Myosin MyHC-IIb, fast	Myh4	200	73.1	222.9
Q5SX40	Myosin MyHC-IIx, fast	Myh1	179	70.3	223.3
Q91Z83	Myosin MyHC-I(beta), slow	Myh7	126	56.9	222.9
A2AQP0	Myosin MyHC-7b, slow	Myh7b	20	6.7	221.5
P13541	Myosin MyHC-embryonic	Myh3	73	29.3	223.8
P13542	Myosin MyHC-perinatal	Myh8	133	50.0	222.7
P05977	Myosin MLC1/MLC3	Myl1	23	88.8	20.6
P97457	Myosin MLC2, muscle	Mylpf	18	92.9	19.0
P51667	Myosin MLC2, slow	Myl2	9	64.5	18.9
P09542	Myosin MLC3, slow	Myl3	15	77.5	22.4
Q8VCR8	Myosin light chain kinase 2	Mylk2	12	35.6	66.0
Q5XKE0	Myosin-binding protein MyBP-C	Mybpc2	73	71.8	127.4
P70402	Myosin-binding protein MyBP-H	Mybph	21	66.5	52.6

To be continued on next page

Table 1. *Continued from previous page.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(iv) **Thin filament actin complex and troponin/tropomyosin regulatory complex**					
P68033	Actin, alpha, cardiac muscle	Actc1	32	82.2	42.0
P68134	Actin, alpha, skeletal muscle	Acta1	32	81.2	42.1
P20801	Troponin TnC, muscle	Tnnc2	11	78.1	18.1
P19123	Troponin TnC, slow muscle	Tnnc1	6	41.0	18.4
O88346	Troponin TnT, slow muscle	Tnnt1	7	24.8	31.3
Q9QZ47	Troponin TnT, fast muscle	Tnnt3	17	36.8	32.2
Q9WUZ5	Troponin TnI, slow muscle	Tnni1	10	44.4	21.7
P13412	Troponin TnI, fast muscle	Tnni2	10	42.3	21.4
P58771	Tropomyosin, alpha-1	Tpm1	37	78.2	32.7
P21107	Tropomyosin, alpha-3	Tpm3	33	75.4	33.0
Q6IRU2	Tropomyosin, alpha-4	Tpm4	15	58.9	28.5
P47753	F-actin-capping protein, alpha-1	Capza1	11	59.4	32.9
P47754	F-actin-capping protein, alpha-2	Capza2	16	74.1	33.0
P47757	F-actin-capping protein, beta	Capzb	20	66.1	31.4
P49813	Tropomodulin-1	Tmod1	14	56.5	40.5
Q9JHJ0	Tropomodulin-3	Tmod3	7	22.2	39.5
Q9JLH8	Tropomodulin-4	Tmod4	10	40.3	39.3
O70373	Xin actin-binding protein 1	Xirp1	8	11.8	123.4
Q5DTJ9	Myopalladin	Mypn	8	10.2	144.1

Proteomic profiling of non-sarcomeric cytoskeletal proteins in aged skeletal muscle

Major extra-sarcomeric cytoskeletal proteins are listed in Table 2, including i) the major cytolinker component plectin, ii) adaptor proteins such as ankyrin, iii) membrane cytoskeletal elements such as spectrin and dystrophin, and iv) intracellular cytoskeletal proteins such as vimentin, vinculin, synemin, desmin and actin, as well as v) various tubulins that form microtubules. Differential effects of muscle aging have previously been observed to occur in extra-sarcomeric proteins.[60-65,79-86] Increases in the major intermediate filament protein desmin indicate a certain degree of remodeling of cytoskeletal networks in senescent myofibers. This could be a compensatory mechanism to stabilize the weakened intracellular structures of aged muscle fibers.

Proteomic profiling of extracellular matrix proteins in aged skeletal muscle

In skeletal muscles, the extracellular matrix is organized into three complex layers that form the endomysium, perimysium and epimysium, which are intrinsically involved in the provision of force transmission, as well as structural support, maintenance of the microenvironment of diverse myofiber populations and repair mechanisms. As listed in Table 3, the mass spectrometric analysis of aged mouse hindlimb muscles identified key components that are mostly found in the basal lamina (collagens COL-IV and COL-XV, laminin-211, perlecan, and the nidogen isoforms entactin and osteonidogen) or the endomysium (collagens COL-V and COL-VI). Proteins that are present throughout the endomysium, perimysium and epimysium were recog-

nized as dermatopontin, vitronectin, fibronectin, fibrinogens, prolargin and fibrillin (Table 3).
Members of the large family of small leucine-rich proteoglycans (SLRP) were clearly identified in aged muscles, including decorin, asporin, biglycan, fibromodulin and mimecan/osteoglycin. Additional protein species that are linked to the extracellular matrix are listed in Table 3, *i.e.* the sarcolemmal adhesion proteins/linkers alpha/beta-dystroglycan and alpha7/beta1-integrin, as well as the annexins ANXA2 and ANXA6. Markers of the myotendinous junction, tendon and cartilage are listed in the form of collagen COL-XII, collagen COL-I and cartilage-associated protein, respectively. Previous studies of aged muscle do not suggest a major increase in matrisomal proteins, such as collagens and proteoglycans,[60-65,79-86] so sarcopenia of old age is probably associated with a lower degree of myofibrosis as compared to severe neuromuscular disorders, such as X-linked muscular dystrophy.[56] This has been confirmed in this study, as can be seen in the below immunoblot analysis of the abundance of collagen isoform COL-VI in aged wild type *versus* aged and dystrophic *mdx-4cv* skeletal muscle preparations.

Table 2. *Mass spectrometry-based proteomic profiling of extra-sarcomeric proteins in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
Q9QXS1	Plectin (Plec-1d)	Plec	283	60.8	534.2
P16546	Spectrin a, non-erythrocytic 1	Sptan1	104	49.3	284.6
Q62261	Spectrin b, non-erythrocytic 1	Sptbn1	90	49.6	274.2
P20152	Vimentin	Vim	64	88.8	53.7
Q64727	Vinculin	Vcl	57	67.3	116.7
Q70IV5	Synemin	Synm	24	21.1	173.2
P31001	Desmin	Des	31	67.8	53.5
P60710	Actin, cytoplasmic 1	Actb	36	95.5	41.7
P63260	Actin, cytoplasmic 2	Actg1	36	95.5	41.8
P11531	Dystrophin (Dp427-M)	Dmd	5	1.6	425.8
P05213	Tubulin alpha-1B	Tuba1b	34	74.9	50.2
P68373	Tubulin alpha-1C	Tuba1c	34	75.1	49.9
P05214	Tubulin alpha-3	Tuba3a	23	51.3	50.0
P68368	Tubulin alpha-4A	Tuba4a	27	61.2	49.9
Q9JJZ2	Tubulin alpha-8	Tuba8	20	49.9	50.1
Q7TMM9	Tubulin beta-2A	Tubb2a	21	65.6	49.9
Q9CWF2	Tubulin beta-2B	Tubb2b	22	75.3	50.0
Q9ERD7	Tubulin beta-3	Tubb3	14	34.4	50.4
Q9D6F9	Tubulin beta-4A	Tubb4a	21	69.1	49.6
P99024	Tubulin beta-5	Tubb5	27	80.6	49.7
Q922F4	Tubulin beta-6	Tubb6	18	57.9	50.1
Q8C8R3	Ankyrin-2	Ank2	3	1.1	426.3
G5E8K5	Ankyrin-3	Ank3	5	4.1	214.1

Table 3. *Mass spectrometry-based proteomic profiling of matrisomal proteins in aged mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Basal lamina**					
P02463	Collagen IV, alpha-1	Col4a1	3	2.3	160.7
P08122	Collagen IV, alpha-2	Col4a2	7	6.9	167.3
O35206	Collagen XV, alpha-1	Col15a1	7	8.3	140.5
Q60675	Laminin-211, subunit alpha-2	Lama2	64	31.0	343.8
P02469	Laminin-211, subunit beta-1	Lamb1	36	24.9	197.1
P02468	Laminin-211, subunit gamma-1	Lamc1	52	42.8	177.3
Q05793	Perlecan (HSPG-2)	Hspg2	53	21.6	398.3
P10493	Nidogen-1 (entactin)	Nid1	23	24.3	136.54
O88322	Nidogen-2 (osteonidogen)	Nid2	8	7.8	153.91
(ii) **Endomysium**					
Q3U962	Collagen V, alpha-2	Col5a2	4	4.1	145.0
Q04857	Collagen VI, alpha-1	Col6a1	27	43.0	108.5
Q02788	Collagen VI, alpha-2	Col6a2	22	26.3	110.3
A6H584	Collagen VI, alpha-5	Col6a5	5	2.2	289.6
Q8C6K9	Collagen VI, alpha-6	Col6a6	8	5.2	246.3
(iii) **Endomysium, perimysium and epimysium**					
Q9QZZ6	Dermatopontin	Dpt	6	33.8	24.0
P29788	Vitronectin	Vtn	3	14.2	54.9
P11276	Fibronectin	Fn1	22	13.4	272.5
E9PV24	Fibrinogen, alpha	Fga	13	21.7	87.4
Q8K0E8	Fibrinogen, beta	Fgb	16	50.5	54.8
Q8VCM7	Fibrinogen, gamma	Fgg	14	33.7	49.4
Q9JK53	Prolargin	Prelp	14	40.7	43.3
Q61554	Fibrillin-1	Fbn1	59	28.9	312.3
(iv) **Small leucine-rich proteoglycans (SLRP type)**					
P28654	Decorin	Dcn	15	46.6	39.8
Q99MQ4	Asporin	Aspn	11	33.5	42.6
P28653	Biglycan	Bgn	11	38.2	41.6
P50608	Fibromodulin	Fmod	4	18.4	43.1
Q62000	Mimecan (Osteoglycin)	Ogn	11	42.6	34.0

To be continued on next page

Table 3. *Continued from previous page.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(v) **Sarcolemmal adhesion proteins/linkers**					
Q62165	Dystroglycan, alpha/beta	Dag1	12	15.5	96.9
Q61738	Integrin, alpha-7	Itga7	8	8.7	129.3
P09055	Integrin, beta-1	Itgb1	23	38.0	88.2
(vi) **Myotendinous junction, tendon and cartilage**					
Q60847 340.2	Collagen XII, a-1(myotendinous junction)		Col12a1	21	8.9
P11087	Collagen I, alpha-1 (tendon)	Col1a1	9	8.0	138.0
Q01149	Collagen I, alpha-2 (tendon)	Col1a2	10	10.1	129.6
Q9CYD3	Cartilage-associated protein	Crtap	8	18.5	46.2
(vii) **Annexins**					
P07356	Annexin A2	Anxa2	41	85.3	38.7
P14824	Annexin A6	Anxa6	47	65.4	75.9

Bioinformatic analysis of potential protein-protein interactions within protein clusters in aged skeletal muscle

The various muscle-associated protein complexes that were identified by mass spectrometry-based proteomics were further analyzed in the context of potential protein interaction patterns. As outlined in Figure 3, bioinformatic STRING analyses,[77] clearly demonstrated considerable interaction patterns within protein clusters that are involved in the contraction-relaxation cycle, the extrasarcomeric cytoskeleton and the complex arrangement of the matrisome in the extracellular matrix. Due to the complexity of functional and physical protein clustering and the large number of interacting protein species, the illustrations depicted in Figure 3 are not presented to give detailed information on individual protein interactions, but instead are shown to provide a general overview of complex formation of identified protein groupings in aged skeletal muscles.

Identification of proteomic markers of dystrophinopathy in aged mdx-4cv muscle

To demonstrate the usefulness of the newly established reference map of aged skeletal muscle, the comparison of wild type muscle *versus* dystrophic muscle was carried out using the established murine *mdx-4cv* model of Duchenne muscular dystrophy.[39] The marker proteins listed in Table 4 are molecular species that are absent *versus* present in the two different muscle specimens as judged by mass spectrometric surveys, analyzed using identical chromatography and protein identification parameters. These types of comparisons are ideal for single tissue sample classification purposes. In the case of the dystrophic samples, fibrotic markers such as the matricellular protein periostin and collagen isoform COL-XVIII, are present, in addition to moesin of the ezrin-radixin-moesin protein family that mediates linking of the plasmalemma to the actin cytoskeleton, and the cysteine protease cathepsin-B that is involved in intracellular proteolysis. In contrast, dystrophin isoform Dp427-M, which is primarily affected in X-linked muscular dystrophy,[68] is only present in wild type muscle, in conjunction with its associated glycoproteins dystroglycan and sarcoglycans. These clear proteomic differences between aged wild type *versus* aged *mdx-4cv* mouse hindlimb muscle demonstrate the suitability of simple data-based searches for tissue classification. The main advantage of this straightforward approach using established proteomic markers is the fact that it is considerably less time consuming as compared to software-driven analyses.

The proteomic characterization of the wild type *versus* dystrophic specimens confirms that the absence of dystrophin isoform Dp427-M and collapse of the dystrophin-associated glycoprotein complex are the primary trigger of sarcolemmal damage in this murine model of Duchenne muscular dystrophy. Increased levels of myonecrosis were previously shown to lead to fat substitution and chronic inflammation in dystrophic muscles.

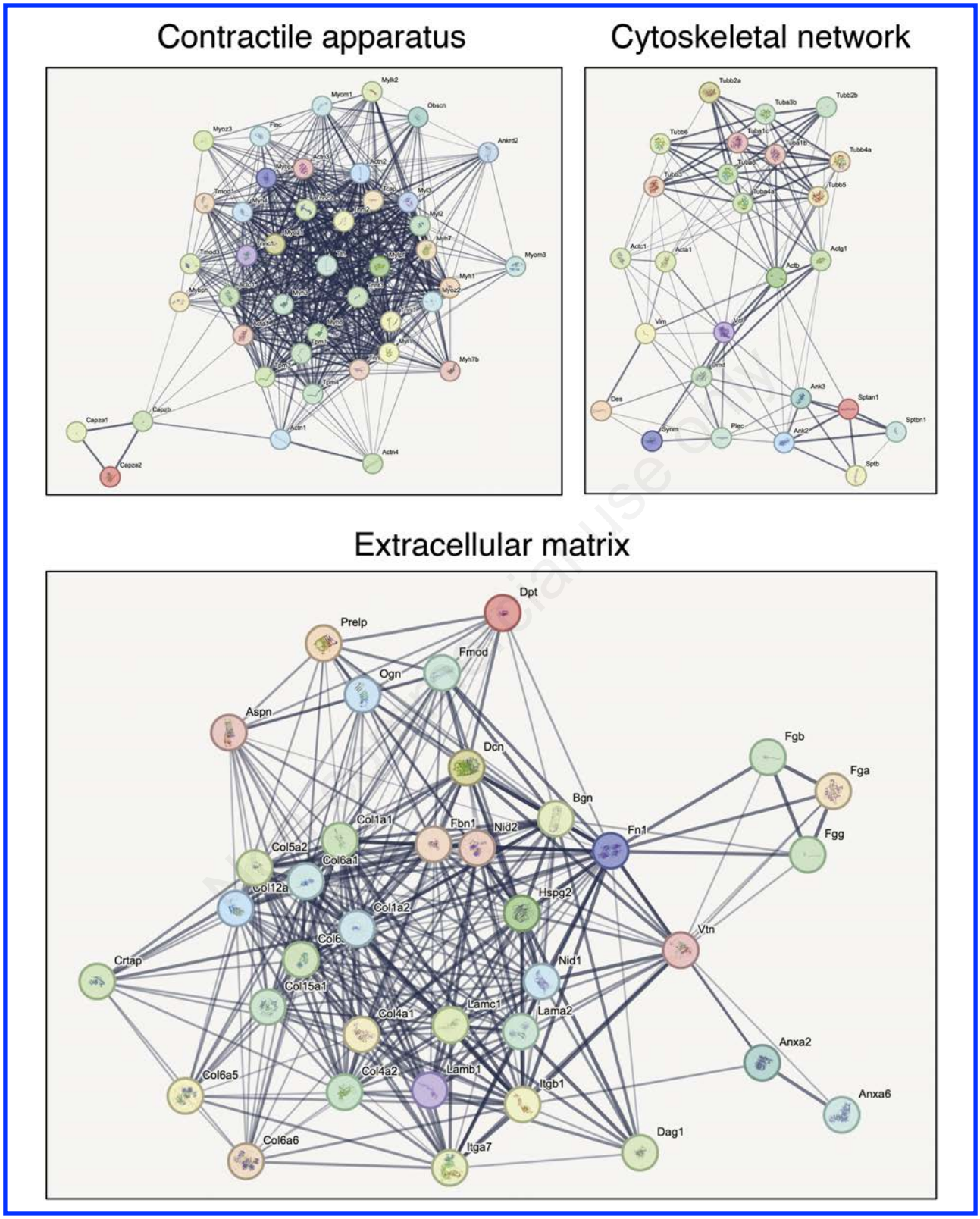

Figure 3. *Overview of potential protein-protein interactions within major protein complexes that were detected by bottom-up proteomics of total extracts from aged mouse skeletal muscle. The data used to generate the individual images are the listings of identified protein families that are linked to the contractile apparatus in sarcomeres (Table 1), the extra-sarcomeric cytoskeletal network (Table 2) and matrisomal proteins of the extracellular matrix (Table 3). The publicly available bioinformatic analysis tool STRING (https://string-db.org) was used for the analysis of protein interaction patterns.*[77]

Proteomics is an ideal bioanalytical tool to study the complexity of neuromuscular changes and multi-systems disturbances in dystrophinopathy, as recently discussed in a review on pathophysiological crosstalk in X-linked muscular dystrophy.[87] Reactive myofibrosis is a characteristic hallmark of Duchenne muscular dystrophy and this was confirmed here by the drastic increase in the matricellular component periostin and a specific isoform of collagen.[56] Since the presence of periostin, a protein that is almost absent from normal skeletal muscle, was clearly established in senescent *mdx-4cv* mouse hindlimb muscle, the dystrophic phenotype appears to be characterized by high levels of reactive myofibrosis. One of the most frequently employed fibrosis markers in the field of skeletal muscle pathobiology is the COL-VI isoform of collagen. As displayed in Figure 4, comparative immunoblotting showed an increase in the abundance of this key element of the extracellular matrix in the dystrophic and fibrotic *mdx-4cv* specimens. In contrast, CBB staining of gels did not show any major differences in protein expression patterns, and immunoblotting with antibodies to glyceraldehyde 3-phosphate dehydrogenase, tropomyosin and parvalbumin did not reveal changes in their abundance in aged wild type *versus* aged *mdx-4cv* mouse hindlimb muscle.

Discussion

This study has focused on the mass spectrometric profiling of total tissue extracts that were isolated from the aged mouse hindlimb musculature. The major aim of this investigation was to establish a proteomic reference map of senescent mouse skeletal muscles using a streamlined, economical and bottom-up proteomic workflow. The newly established proteomic catalogue displayed in this report includes key muscle proteins that are associated with the sarcomere, the extra-sarcomeric cytoskeleton and the extracellular matrix. These listings of unequivocally identified skeletal muscle proteins can now be employed as a biochemical reference for future investigations that will attempt to further elucidate the highly complex and multifactorial mechanisms that underlie sarcopenia of old age.[31] The processes that underlie sarcopenic changes are highly complex, including i) an increased rate of muscular atrophy in fast-twitching myofibers causing preferentially fast-to-slow muscle transitions, ii) neurodegeneration, which is characterized by the disintegration of motor neurons resulting in denervation and faulty patterns of reinnervation, as well as the progressive loss of the functionality of the neuromuscular junction system, iii) uncoupling between sarcolemmal excitation and contraction at the level of triad junctions, iv) abnormal calcium handling in the cytosol, sarcoplasmic reticulum and mitochondria, v) indirect bioenergetic shifting from glycolytic to more oxidative metabolism in slower contracting muscles, vi) proteotoxic effects triggering a sustained cellular stress response, vii) imbalanced proteostasis due to abnormal rates of protein turn-over and re-synthesis, viii) hormonal disturbances, including insulin resistance, and abnormal cellular signaling, affecting majorly the muscle-fat axis ix) a certain degree of myofibrosis that causes negative effects on myofiber elasticity and lateral force transmission, x) chronic inflammation, xi) epigenetic alterations, and xii) stem cell exhaustion that is associated with a reduction in regenerative capacity.[20-24,30]

Table 4. *Mass spectrometry-based proteomic profiling of protein markers in aged wild type* versus *aged* mdx-4cv *mouse hindlimb muscle.*

Accession	Protein name	Gene	Peptides	Coverage (%)	Molecular mass (kDa)
(i) **Proteins uniquely identified in aged and dystrophic *mdx-4cv* muscle**					
Q62009	Periostin	Postn	8	13	93.1
P39061	Collagen alpha-1(XVIII)	Col18a1	2	2	182.1
P26041	Moesin	Msn	9	12	67.7
P10605	Cathepsin B	Ctsb	5	24	37.3
(ii) **Proteins uniquely identified in aged wild type muscle**					
P11531	Dystrophin Dp427-M	Dmd	8	4	425.6
Q62165	Dystroglycan	Dag1	1	2	96.8
P82349	Sarcoglycan, beta	Sgcb	1	7	34.9
P82347	Sarcoglycan, delta	Sgcd	1	3	32.1

Skeletal muscle proteomics is an unbiased screening approach in discovery mode that can be used in bottom-up, middle-up/down or top-down fashion to identify and characterize distinct protein species and their post-translational modifications.[32] For the comprehensive protein biochemical analysis of complex muscle tissues, one of the most cost-effective and experimentally straightforward approaches is the label-free liquid chromatography-tandem mass spectrometric screening of total protein extracts from crude tissue homogenates.[31,88,89] Bottom-up proteomics is based on the identification of distinct protein species through the characterization of peptides that are produced from proteins of interest by controlled proteolysis.[90] This widely used approach ultimately detects and measures peptides, which represent ideal molecular species for the swift proteomic analysis due to the fact that they readily solubilize, separate and ionize.[91-93] Column chromatography is routinely employed for efficient protein separation, utilizing differences in charge (ion exchange – anion/cation), affinity (a specific binding affinity for peptide moieties or post-translational modifications) or size (gel filtration/size exclusion - resins are porous to molecules with a particular size range). A key step is the efficient digestion of proteins into peptides prior to MS-based analysis using either bottom-up proteomics or top-down proteomics. Trypsin is highly suitable for digesting proteins into small-size peptides,[94] which are more amenable to high-performance liquid chromatography separation and tandem mass spectrometric characterization. Alternative enzymes are available to be used alone or in combination with trypsin for the optimum generation of peptide populations prior to mass spectrometric analysis.[95-97] Once peptides have been generated after digestion, silica based octadecyl (C18) resins can be employed to purify and concentrate established peptides.

In bottom-up approaches, sophisticated liquid chromatography is instrumental for the optimum separation of peptides prior to mass spectrometry. Of note, the use of first dimension methods (*e.g.* size exclusion chromatography/SEC or ion exchange chromatography/IEX) coupled to compatible second dimension approaches (*e.g.* reversed-phase liquid chromatography/RPLC or hydrophilic interaction liquid chromatography/HILIC) and related techniques, greatly improves the resolving power and thereby decisively increases the number of peptides that can be analyzed per run.[98] The usage of untargeted label-free quantitation (LFQ) of protein species enables the determination of the relative abundance of peptides/proteins using both spectral counting and measuring MS1 signal intensities.[99-101] However, ion intensities are more accurate than spectral counts and have a greater dynamic range. A potential drawback of the LFQ approach is that run parameters, *e.g.* C18 column conditions, may change marginally between samples, having occasionally knock-on consequences with respect to sample alignment and analysis.

In contrast to a peptide-centric analysis of complex protein mixtures, as described here, an alternative strategy is represented by top-down proteomics, which has been widely

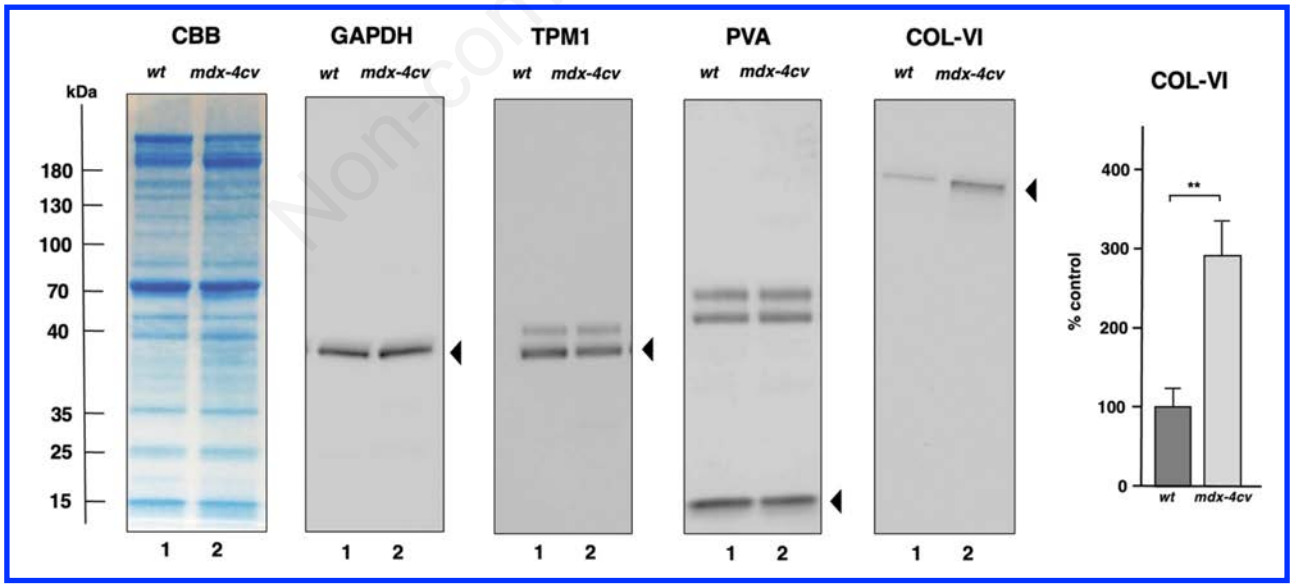

Figure 4. *Comparative immunoblot analysis of aged wild type* versus *aged* mdx-4cv *mouse hindlimb muscle. Shown is a CBB stained gel plus identical immunoblots labelled with antibodies to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sarcomeric protein tropomyosin TPM1-alpha (TPM1) of the actin-containing thin filaments, the cytosolic calcium-binding protein parvalbumin (PVA) and the extracellular matrix protein collagen VI (COL-VI). Lanes 1 and 2 contain protein extracts from 24 months old wild type* versus mdx-4cv *hindlimb muscles, respectively. A graphical representation of the immuno-decoration levels of collagen COL-VI are shown: Student's* t-*test, unpaired; n=4; **p<0.01. Molecular weight standards are marked on the left.*

used in skeletal muscle proteomics in the past,[102-104] including the large-scale analysis of aged myofibers.[79-86] Protein separation in top-down proteomics is often gel-based, *i.e.* two-dimensional gel electrophoresis is used prior to the digestion of intact proteoforms.[33] High-resolution gels can be used to facilitate the separation of hundreds to thousands of proteins on one 2D-gel. Of note, this technique has been optimized to separate the skeletal muscle proteome.[104,105] The advantage of gel-based top-down proteomics is the fact that this workflow can be effectively combined with other biochemical techniques, facilitating gel staining, followed by spot excision, de-staining, protein digestion, and the subsequent analysis of peptides by mass spectrometric analysis to unequivocally identify specific proteoforms.

However, a major analytical limitation of two-dimensional gel electrophoresis is the lack of sufficient electrophoretic mobility of very large proteins, which hinders the efficient movement of high-molecular-mass muscle protein species, such as dystrophin, plectin, obscurin, the ryanodine receptor calcium release channel, nebulin and titin, from the first dimension isoelectric focusing tube gels into the matrix of second dimension slab gels. In addition, accurate identification of proteins of very low abundance and/or species with extreme isoelectric points is not achievable using this technique. Gel-based techniques are considerably more time-consuming, costly and labor-intensive as compared to streamlined bottom-up proteomics. Importantly, since extensive subcellular fractionation steps can introduce considerable bioanalytical artefacts, especially in comparative studies,[106-109] it is advantageous to use crude extracts for initial studies. Thus, when starting material is scarce, bottom-up proteomics using total protein extracts is the method of choice for comprehensive biochemical surveys of skeletal muscle specimens.

The application of bottom-up proteomics in this study has identified the main protein components of the sarcomeres that are involved in the regulation of the contraction-relaxation cycle and force generation.[110-112] This includes a large array of fast and slow isoforms of myosin light and heavy chains in the thick filaments, which are characteristic for the presence of fast- *versus* slow-twitching myofibers.[43-45,113] In analogy, fast *versus* slow troponin isoforms were identified in the actin-containing thin filaments.[113,114] This shows that the presented analysis pipeline is suitable for studying myofiber type shifting during aging.[30] Crucial extra-sarcomeric cytoskeletal proteins were identified in skeletal muscles, including the central cytolinker plectin,[115] and members of costameres such as alpha7/beta1-integrin,[116] and alpha/beta-dystroglycan in conjunction with its membrane cytoskeletal anchor named dystrophin.[117,118] This would enable the in-depth analysis of alterations in the levels of lateral force transmission through costameric structures in aged skeletal muscles. Since the extracellular matrix is a crucial structural component of muscles,[119] and majorly involved in reactive myofibrosis in neuromuscular diseases,[120,121] it is encouraging that representative markers of the basal lamina, endomysium, perimysium, epimysium and tendon could be detected by proteomics in crude muscle tissue extracts.

In conclusion, the mass spectrometric screening of senescent mouse muscles has been successfully applied to establish a proteomic reference map for future sarcopenia research. As shown in this report, proteomic markers of dystrophic and fibrotic changes can easily be identified by simple data-based searches for tissue classification. For example, the lack of the membrane cytoskeletal protein dystrophin,[117] in conjunction with the increase in the matricellular protein periostin,[122] is a suitable way of confirming the dystrophic and fibrotic status of a particular muscle sample, such as aged *mdx-4cv* skeletal muscle tissues.[121]

List of abbreviations

ACT, Actin
CBB, Coomassie Brilliant Blue
COL, Collagen
Dp427-M, Dystrophin of 427 kDa
ECL, Enhanced chemiluminescence
FASP, Filter-aided sample preparation
FLNC, Filamin C
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
LFQ, Label-free quantitation
MARP, Muscle ankyrin repeat protein
MLC, Myosin light chain
MS, Mass spectrometry
MyBP, Myosin-binding protein
MyHC, Myosin heavy chain
OSF, Open Science Foundation
PVA, Parvalbumin
Tn, Troponin
TPM, Tropomyosin

Contributions

PD, DS and KO were involved in the conceptualization and initiation of this project, as well as the design of the research strategy. SG, MZ and PD were involved in the preparation of muscle tissues and performed the biochemical experiments and analyzed the data. MH and PM performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript.

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Conflict of interest

The authors declare no competing interests.

Ethics approval

Local governmental and institutional animal care regulations were followed and approved by the Institutional Animal Care and Use Committee (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany). Transportation and usage of tissue specimens was in accordance with the reg-

ulations of the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth). The study conforms with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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