Proteome-wide Changes in the *mdx-4cv* Spleen due to Pathophysiological Cross Talk with Dystrophin-Deficient Skeletal Muscle

**HIGHLIGHTS**

Biochemical and proteomic analyses have identified dystrophin isoform Dp71 in spleen

Normal Dp71 levels were found in the spleen of the *mdx-4cv* model of dystrophinopathy

The enzymes TGM2 and MMP-9 are drastically increased in *mdx-4cv* spleen

Apolipoproteins ApoE and ApoB are greatly reduced in *mdx-4cv* spleen

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Proteome-wide Changes in the mdx-4cv Spleen due to Pathophysiological Cross Talk with Dystrophin-Deficient Skeletal Muscle

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SUMMARY
Duchenne muscular dystrophy is primarily characterized by progressive muscle wasting due to deficiency in the membrane cytoskeletal protein dystrophin but is also associated with body-wide cellular disturbances in a variety of non-muscle tissues. In this study, we have focused on the comparative proteomic analysis of the spleen and established considerable changes in this crucial secondary lymphoid organ from the genetic mdx-4cv mouse model of dystrophinopathy. An apparent short isoform of dystrophin and associated glycoproteins were identified in spleen by mass spectrometry but appear not to be affected in muscular dystrophy. In contrast, the mdx-4cv spleen showed significant proteome-wide changes in other protein species that are involved in metabolism, signaling, and cellular architecture. Since the spleen plays a key role in the immune response, these proteomic alterations may reflect pathophysiological cross talk between the lymphoid system and dystrophic muscles, which are affected by both fiber degeneration and inflammation.

INTRODUCTION
The neuromuscular disorder Duchenne muscular dystrophy is due to primary abnormalities in the DMD gene (Guiraud et al., 2015), which encodes several different isoforms of the protein dystrophin (Muntoni et al., 2003). Large-scale and antibody-based surveys of the tissue-specific presence of individual proteins provide excellent information on the distribution of protein markers throughout the body (Thul and Lindskog, 2018). However, often the reliance on a single, albeit highly specific, antibody for antigen detection does not take into account the existence of multiple protein isoforms with greatly differing cellular expression patterns. This is especially apparent in the case of the extremely large 79 exon-spanning DMD gene, which contains seven promoters that drive the tissue-specific expression of various dystrophin isoforms of differing molecular mass (Muntoni et al., 2003). This includes three full-length isoforms of apparent 427 kDa, i.e., Dp427-M in contractile tissues, Dp427-B in the brain, and Dp427-P in Purkinje cells. Shorter dystrophin isoforms of approximately 116–260 kDa are represented by Dp116-S in Schwann cells, Dp140-B/K in brain and kidney, and Dp260-R in the retina. In addition, an ubiquitous proteoform named Dp71-G exists in the brain and various other tissues (Naidoo and Anthony, 2020).

Dystrophinopathies belong to a group of inherited degenerative diseases of muscles (Mercuri et al., 2019; Thompson et al., 2020) that are characterized by progressive changes in the skeletal musculature, including degeneration of contractile fibers, sterile inflammation, fatty tissue infiltration, and reactive myofibrosis (Allen et al., 2016; Dowling et al., 2020a; Shin et al., 2013; Smith and Barton, 2018; Tidball et al., 2018). The recent mass spectrometry-based proteomic profiling of the vastus lateralis muscle from patients with Duchenne muscular dystrophy has confirmed severe extracellular and cytoskeletal dysregulation in dystrophinopathy (Capitanio et al., 2020). Additional complications manifest themselves as metabolic abnormalities, hormonal disturbances, scoliosis, neuronal deficiencies, and late-onset cardio-respiratory impairments (Birnkrant et al., 2018; Meyers and Townsend, 2019; Saure et al., 2018). Thus, although X-linked muscular dystrophy is primarily a monogenetic disease of contractile tissues, the complexity of its secondary pathophysiology makes it a neuromuscular disorder with a high degree of vulnerability of other tissue and organ systems.
Animal models of Duchenne muscular dystrophy have been instrumental for studying the molecular and cellular details of progressive degeneration in dystrophin-deficient muscle tissues, as well as testing of novel experimental treatment strategies to counteract the dystrophic phenotype (McGrevey et al., 2015; Rodrigues et al., 2016; Wilson et al., 2017). One of the most frequently used models of dystrophinopathy is the mdx-23 mouse, which represents a naturally occurring mutant with a premature stop codon-inducing mutation in exon 23 of the DMD gene (Sicinski et al., 1989). Hence, in analogy to progressive forms of human dystrophinopathy, this animal model almost completely lacks the full-length dystrophin isoform Dp427-M (Partridge, 2013). An alternative mdx-type mouse model (mdx-4cv) was generated by chemical mutagenesis with N-ethyl-N-nitrosourea (Chapman et al., 1989) and resulted in a C to T transition at base 7,916 in exon 53, generating an ochre codon (Banks et al., 2010; Shin et al., 2011; Tichy and Mourkioti, 2017). Importantly, the mdx-4cv model is typified by an approximately 10-fold lower rate of dystrophin-positive revertant fibers as compared with the conventional mdx-23 mouse (Danko et al., 1992; Im et al., 1996; Judge et al., 2006). This feature of the genetic mouse model reflects more accurately the cellular characteristics of affected skeletal muscles from patients with Duchenne muscular dystrophy. Consequently the mdx-4cv musculature represents an attractive tissue source for the systematic evaluation of new therapeutic approaches, such as exon skipping following intramuscular injections of antisense oligomers (Mitrpant et al., 2009), viral vector injection into neonatal muscles for the stable restoration of dystrophin expression (Kimura et al., 2010), and CRISPR-Cas9-based dystrophin gene editing (Bengtsson et al., 2017).

Prior to the comparative mass spectrometric analyses of wild-type versus mdx-4cv mouse have established differential changes in the tissue proteome from various skeletal muscles, the heart, the brain, the liver, and the kidney (Murphy et al., 2015, 2016; 2018a; 2019a; 2019b; Dowling et al., 2020a) and have identified elevated levels of biofluid-associated markers in saliva, serum, and urine (Murphy et al., 2017, 2018a; Gargan et al., 2020). In this study, we have extended the proteomic characterization of body-wide alterations in the mdx-4cv mouse model of dystrophinopathy to the spleen, a secondary lymphoid organ that consists of two main types of tissue, the blood-filled red pulp and the lymphatic white pulp (Mebius and Kraal, 2005). The main functions of the spleen include the removal of abnormal erythrocytes, antigen detection, and antibody production (Lewis et al., 2019). In dystrophic organisms with an almost complete loss of the full-length Dp427-M isoform of dystrophin, abnormalities in the spleen were previously reported to include morphological adaptations in relation to lymph nodes in the white pulp region of the mdx mouse spleen (Santos et al., 2013), as well as altered levels of splenic inflammatory monocytes and increased migration of immune cells from the splenic reservoir to injured dystrophic fibers (Farini et al., 2016; Giordano et al., 2015; Mojumdar et al., 2014, 2016; Ouisse et al., 2019; Rizzo et al., 2020).

The proteomic survey presented here has initially identified the experimentally assessable protein constituents of the mouse spleen with the help of an Orbitrap Fusion Tribrid mass spectrometer. The established splenic protein catalog was screened for the presence of markers of spleen function and the potential presence of dystrophin. Previous mass spectrometric investigations focusing on the mdx-4cv mouse have established differential changes in the tissue proteome from various skeletal muscles, the heart, the brain, the liver, and the kidney (Murphy et al., 2015, 2016; 2018a; 2019a; 2019b; Dowling et al., 2020a) and have identified elevated levels of biofluid-associated markers in saliva, serum, and urine (Murphy et al., 2017, 2018a; Gargan et al., 2020). In this study, we have extended the proteomic characterization of body-wide alterations in the mdx-4cv mouse model of dystrophinopathy to the spleen, a secondary lymphoid organ that consists of two main types of tissue, the blood-filled red pulp and the lymphatic white pulp (Mebius and Kraal, 2005). The main functions of the spleen include the removal of abnormal erythrocytes, antigen detection, and antibody production (Lewis et al., 2019). In dystrophic organisms with an almost complete loss of the full-length Dp427-M isoform of dystrophin, abnormalities in the spleen were previously reported to include morphological adaptations in relation to lymph nodes in the white pulp region of the mdx mouse spleen (Santos et al., 2013), as well as altered levels of splenic inflammatory monocytes and increased migration of immune cells from the splenic reservoir to injured dystrophic fibers (Farini et al., 2016; Giordano et al., 2015; Mojumdar et al., 2014, 2016; Ouisse et al., 2019; Rizzo et al., 2020).

RESULTS

In order to evaluate the complex pathogenesis of X-linked muscular dystrophy, this study has focused on the proteomic characterization of potential secondary effects in a non-muscle organ, the spleen, in the established mdx-4cv mouse model of dystrophinopathy. Prior to the comparative mass spectrometric analysis of this secondary lymphoid organ, the spleen proteome was evaluated for the presence of dystrophin and spleen marker proteins.

Mass Spectrometric Identification of the Splenic Isoform of Dystrophin

To overcome the restricted specificity of protein isoform coverage by antibody screening using standard immunochemical methodology (Thul and Lindskog, 2018), we have employed here a more sensitive
proteomic screening approach with an Orbitrap Fusion Tribrid mass spectrometer for determining the potential presence of low levels of dystrophin in the spleen. As listed in Table 1, dystrophin and various dystrophin-associated proteins were clearly identified by this method, including an apparent short isoform of dystrophin, dystroglycan, beta-sarcoglycan, delta-sarcoglycan, epsilon-sarcoglycan, alpha-dystrobrevin, alpha-1-syntrophin, and beta-1-syntrophin. Dystrophin was recognized by six unique peptides, and these sections of the dystrophin sequence clearly aligned with the domain of the dystrophin protein that is close to the carboxy terminus, as shown in Figures 1A and 1B. Immunoblotting indicated that the expression of this short spleen-associated isoform of dystrophin is not affected in dystrophinopathy (Figures 1C and 1D), but the full-length Dp427-M isoform of dystrophin was clearly shown to be absent from mdx-4cv gastrocnemius muscle, as illustrated in Figures 2C–2E. The histological staining of mdx-4cv muscle depicts the typical hallmarks of X-linked muscular dystrophy, including variations in fiber diameter, a high degree of central nucleation, fibrosis, and inflammation (Figures 2A and 2B).

### Mass Spectrometric Profiling of the Mouse Spleen Proteome

Prior to carrying out a comprehensive proteome-wide comparison between wild-type and mdx-4cv spleen, it was essential to first establish how well the chosen proteomic approach covered the accessible splenic protein constituents using total tissue extracts. The biochemical cataloging of 12-month-old mouse spleen with the help of an Orbitrap Fusion Trbrid mass spectrometer resulted in the identification of 5,688 splenic protein species. This table was deposited as a supplementary multi-consensus file to the Open Science Framework repository under the following link: https://osf.io/f85ve/. In addition, individual files include data from the analysis of 14 separate mass spectrometric sample runs (representing 7 biological repeats of wild-type spleen preparations and 7 biological repeats of mdx-4cv spleen preparations, which were also deposited to the OSF entry ‘f85ve’) and consists of high-confidence peptides that were filtered based on Xcorr values. Importantly, a wide range of spleen-associated proteins were confirmed to be present in the examined tissue extracts.

Table 1. Proteomic Identification of Dystrophin and Dystrophin-Associated Proteins in Mouse Spleen

| Accession | Protein       | Gene | Coverage (%) | Peptides | Unique Peptides | Molecular Mass (kDa) |
|-----------|---------------|------|--------------|----------|----------------|---------------------|
| P11531    | Dystrophin Dp71 | Dmd  | 2            | 6        | 6              | 71                  |
| Q62165    | Dystroglycan   | Dag1 | 13           | 10       | 10             | 96.8                |
| P82349    | Beta-sarcoglycan| Sgcb | 5            | 1        | 1              | 34.9                |
| P82347    | Delta-sarcoglycan| Sgcd | 13           | 3        | 3              | 32.1                |
| O70258    | Epsilon-sarcoglycan| Sgce | 2            | 1        | 1              | 49.7                |
| Q9D2N4    | Alpha-dystrobrevin| Dtna | 6            | 2        | 2              | 84                  |
| Q61234    | Alpha-1-syntrophin| Snta1 | 6            | 2        | 1              | 53.6                |
| Q99L88    | Beta-1-syntrophin| Sntb1 | 6            | 3        | 1              | 58                  |

Figure 3 shows a pie chart that summarizes the results from the bioinformatic PANTHER analysis of protein families that were mass spectrometrically identified in mouse spleen preparations. Protein classes included a variety of enzyme families, such as hydrolases, oxidoreductases, and transferases, as well as enzyme modulators, nucleic acid-binding proteins, transcription factors, transporters, and cytoskeletal components. The identification of proteins by the highest percent of sequence coverage agreed with the involvement of the spleen with the removal of abnormal erythrocytes and the immune system. Spleen-associated proteins with a protein sequence coverage above 90% included hemoglobin subunit beta-1 (P02088), hemoglobin subunit beta-2 (P02089), and the immunoglobulin Ig kappa chain (P01654). Splenic proteins that were identified by more than 80 peptides included the crucial cytoskeletal proteins filamin, plectin, talin, myosin-9, myosin-11, dynein heavy chain, erythrocytic alpha/beta spectrin, and non-erythrocytic.
Proteomic identification of short dystrophin isoform in spleen

Figure 1. Mass Spectrometric Identification of a Short Isoform of Dystrophin in Mouse Spleen
(A) Diagrammatic presentation of the DMD gene and its 7 promoters.
(B) Overview of unique peptides determined by the mass spectrometric analysis of 12-month-old mouse spleen and their position within the carboxy terminal region of the dystrophin protein sequence.
(C and D) Comparative immunoblot analysis of the apparent spleen isoform Dp71 of dystrophin in wild-type versus mdx-4cv preparations. Lanes 1 and 2 contain wild-type and mdx-4cv specimens, respectively (C). In (D) the box plots of the immunoblot analysis are shown (Mann-Whitney U test; n = 5; none significant). The value of molecular mass standards (x10^-3 kDa) is marked on the left side of the gel. A representative protein gel of spleen extracts used to produce nitrocellulose replicas is shown in Figure 7A.

alpha/beta spectrin, as well as prolow-density lipoprotein receptor-related protein 1 and basement membrane-specific heparan sulfate proteoglycan core protein.

Of note, the proteomic survey of mouse spleen included the identification of major types of splenic receptors, enzymes, and lymphoid proteins, such as cell surface antigens. The spleen marker named CD5 antigen-like protein, which was previously shown to be one of the most highly expressed splenic proteins (Uhlen et al., 2015), was clearly identified as CD5L protein of 38.8 kDa (Q9QWK4; 47% coverage; 14 unique peptides). Other major spleen components that were covered by the mass spectrometric survey included the spleen-associated tyrosine kinase SYK of 71.3 kDa (P48025; 56% coverage; 25 unique peptides), the tyrosine-protein phosphatase non-receptor type substrate 1 SIRPA of 56.4 kDa (P97797; 20% coverage; 7 unique peptides), the abundant spleen marker protein FH1/FH2 domain-containing protein FHOD1 of 129.5 kDa (Q6P9Q4; 19% coverage; 17 unique peptides), the scavenger receptor stabilin STAB2 of 277.3 kDa (Q8R4U0; 20% coverage; 32 unique peptides), the tyrosine-protein phosphatase non-receptor type substrate 1 SIRPA of 56.4 kDa (P97797; 20% coverage; 7 unique peptides), and the alpha-tryptic inhibitor heavy chains 4, H1, H2, and H3; murinoglobulin; haptoglobin; serotransferrin; ferritin light and heavy chains; ceruloplasmin; plasminogen; the alpha, beta, and gamma chains of fibrinogen;
plasma kallikrein and von Willebrand factor, as well as the alpha-2-HS, alpha-1B, and beta-2 glycoproteins (Geyer et al., 2017; Moulder et al., 2018; Schwenk et al., 2017). A large number of the crucial class of serum lipid-binding proteins was also covered by the proteomic analysis of the spleen. This included the apolipoproteins of classes A to M, such as apolipoproteins A-I, A-II, A-IV, A-V, B-100, C-I, C-III, C-IV, D, E, F, and M (Table 3). Serum proteins with relevance to muscle diagnostics that are generally defined as "tissue leak markers" (Anderson and Anderson, 2002) were also identified, including myoglobin of 17.1 kDa (P04247; 60% coverage; 10 unique peptides), the muscle-specific Tnnc2 isoform of troponin TnC of 18.1 kDa (P20801; 15% coverage; 1 unique peptide), and M-type creatine kinase of 43 kDa (P07310; 49% coverage; 15 unique peptides).

Proteomic Profiling of the mdx-4cv Spleen

The comparative mass spectrometric survey of wild-type versus mdx-4cv spleen extracts identified 10.93% change in protein constituents. Of these components, 93 protein species were identified by a coverage of their amino acid sequence by at least 2 peptides and a minimum fold change of 1.5. A reduced expression was found to occur in 55 proteins, and 38 proteins were shown to be increased in their abundance, as listed in Table 4 and Table 5, respectively. The tables with the findings from the comparative proteomic analysis provide information on accession number, protein name, gene symbol, number of unique peptides, confidence score, adjusted p value, and fold change. In addition to changed proteins that were identified by at least 2 unique peptides, another 159 decreased proteins and 145 increased proteins were identified by only 1 unique peptide (not shown). Owing to their low sequence coverage, these proteomic hits were not included in the subsequent bioinformatic analysis of the mdx-4cv spleen. The most decreased and the most increased spleen-associated proteins were identified as apolipoprotein B-100 (E9Q414) and protein-glutamine gamma-glutamyl-transferase TGM2 (P21981), respectively, in mdx-4cv preparations. The heatmap of the comparative mass spectrometric survey is provided in Figure 4 and summarizes the differential expression pattern of changed proteins in wild-type versus the mdx-4cv spleen.

The bioinformatic PANTHER and STRING analyses depicted in Figure 5 summarize the proteome-wide changes in the mdx-4cv spleen, as well as potentially altered protein-protein interaction patterns of changed spleen-associated proteins. Both considerable decreases and increases were observed for...
metabolic interconversion enzymes, such as hydrolases, ligases, lyases, oxidoreductases, and transferases. Striking increases included protein modifiers such as proteases, protein modulators such as protease inhibitors, and transfer carriers. In relation to altered protein interactions, especially striking is the apolipoprotein hub with ApoE and ApoB of reduced protein species. Immunofluorescence microscopy clearly confirmed the drastic reduction of ApoE in the mdx-4cv spleen (Figures 6E, 6F, and 6J) and indicated that the expression of the short spleen-associated isoform of dystrophin is not affected in dystrophinopathy (Figures 6C, 6D, and 6I). No major histological changes (Figures 6A and 6B) and comparable expression levels of the molecular chaperone HspB2 (Figures 6G, 6H, and 6K) were observed in the mdx-4cv spleen.

Potentially excreted forms of proteins were identified as transglutaminase 2 (protein-glutamine gamma-glutamyl-transferase TGM2) and matrix metalloproteinase MMP9. The protein band pattern of gel electrophoretically separated spleen samples did not show major differences between wild-type and mdx-4cv preparations (Figure 7A). However, the increased abundance of TGM2 and MMP9 in the mdx-4cv spleen was confirmed by immunoblotting (Figures 7B, 7C, 7E, and 7F). In contrast, the spleen-associated alpha-subunit of the Na+/K+-ATPase showed no major changes in its density in the dystrophic phenotype (Figures 7Da and 7G).

**DISCUSSION**

The mdx-4cv Mouse Model of Dystrophinopathy

Spontaneous or bioengineered mdx-type mouse models of dystrophinopathy have been widely used in muscular dystrophy research (Partridge, 2013; Wilson et al., 2017), and there are ongoing efforts to improve specific aspects of dystrophic mice to better mirror the human pathology (Yucel et al., 2018). The mdx-4cv model has been successfully employed for the evaluation of experimental therapeutic approaches for the restoration of dystrophin (Mitropant et al., 2009; Kimura et al., 2010; Bengtsson et al., 2017) and the systematic identification of novel biomarker candidates to improve differential diagnosis, prognosis, and therapy monitoring (Hathout et al., 2014; Dowling et al., 2019). The cell biological characterization of the mdx-4cv mouse, which was generated by chemical mutagenesis (Chapman et al., 1989) and is characterized by a 10-fold lower rate of Dp427-positive revertant muscle fibers as compared with the naturally occurring mdx-23 mouse (Danko et al., 1992), has established distinct pathological alterations throughout the lifetime of this animal model (Latroche et al., 2015). This includes varying degrees of fiber degeneration, reactive myofibrosis, sterile inflammation, and metabolic disturbances in limb muscles (Murphy et al., 2019a), the diaphragm (Murphy et al., 2019b), and the heart (Murphy et al., 2016), as well as proteome-wide alterations in the brain (Murphy et al., 2015), the liver (Murphy et al., 2018a), and...
The kidney (Dowling et al., 2020b). The concentration of distinct protein biomarkers is also markedly affected in mdx-4cv serum (Murphy et al., 2017), saliva (Murphy et al., 2018b), and urine (Gargan et al., 2020). These complex and body-wide changes make the mdx-4cv mouse a suitable model system to study the molecular pathogenesis of dystrophinopathy.

### The Spleen Proteome and Muscular Dystrophy

The comparative mass spectrometric analysis presented in this report is based on an excellent coverage of the experimentally accessible spleen proteome in wild-type versus mdx-4cv extracts. Previous large-scale surveys using both systematic mass spectrometric and antibody-based methodology have established a wide range of spleen-associated proteins (Andersson et al., 2014; Dudekula and Le Bihan, 2016; Goltsev et al., 2018; Thul and Lindskog, 2018; Uhlén et al., 2015; Wilhelm et al., 2014). Many of these key marker proteins of the spleen were identified by a high degree of sequence coverage by unique peptides in this study. In agreement with established splenic changes in dystrophic animal models of Duchenne

| Accession | Protein | Gene | Coverage (%) | Unique Peptides | Molecular Mass (kDa) |
|-----------|---------|------|--------------|-----------------|---------------------|
| P07724    | Serum albumin | Alb  | 73           | 47              | 68.6                |
| P06909    | Complement factor H | Cfh  | 56           | 46              | 139                 |
| P01027    | Complement C3    | C3   | 50           | 67              | 186.4               |
| P01029    | Complement C4-B  | C4b  | 22           | 28              | 192.8               |
| P06684    | Complement C5    | C5   | 22           | 27              | 188.8               |
| Q8K182    | Complement C8 alpha | C8a  | 39           | 16              | 66                  |
| P06683    | Complement C9    | C9   | 37           | 19              | 62                  |
| A6X935    | Inter alpha-trypsin inhibitor, heavy chain 4 | Itih4 | 34           | 23              | 104.6               |
| Q61702    | Inter-alpha-trypsin inhibitor, heavy chain H1 | Itih1 | 26           | 16              | 101                 |
| Q61703    | Inter-alpha-trypsin inhibitor, heavy chain H2 | Itih2 | 24           | 16              | 105.9               |
| Q61704    | Inter-alpha-trypsin inhibitor, heavy chain H3 | Itih3 | 13           | 8               | 99.3                |
| P28665    | Murinoglobulin-1 | Mug1 | 38           | 43              | 165.2               |
| Q61646    | Haptoglobin | Hp   | 35           | 11              | 38.7                |
| Q92111    | Serotransferrin  | Tf   | 61           | 48              | 76.7                |
| P09528    | Ferritin heavy chain | Fth1 | 70           | 14              | 21.1                |
| P29391    | Ferritin light chain 1 | Fli1 | 66           | 12              | 20.8                |
| Q61147    | Ceruloplasmin | Cp   | 34           | 28              | 121.1               |
| P20918    | Plasminogen | Plg  | 62           | 33              | 90.7                |
| E9PV24    | Fibrinogen alpha chain | Fga  | 39           | 19              | 87.4                |
| Q8KOE8    | Fibrinogen beta chain | Fgb  | 69           | 33              | 54.7                |
| Q8VCM7    | Fibrinogen gamma chain | Fgg  | 64           | 23              | 49.4                |
| P26262    | Plasma kallikrein | Kllk1 | 12           | 6               | 71.3                |
| Q8CI28    | von Willebrand factor | Vwf  | 27           | 51              | 309.1               |
| P29699    | Alpha-2-HS-glycoprotein | Ahsg | 46           | 10              | 37.3                |
| Q19LI2    | Alpha-1B-glycoprotein | A1bg | 27           | 11              | 56.5                |
| Q01339    | Beta-2-glycoprotein 1 | Apg1 | 46           | 16              | 38.6                |

Table 2. Proteomic Identification of Serum Protein Markers in Mouse Spleen
muscular dystrophy, such as morphological adaptations in the white pulp domain of the spleen (Santos et al., 2013) and altered levels of immune cells in the spleen and enhanced migration of inflammatory cells from the splenic reservoir to dystrophic muscle tissues (Farini et al., 2016; Giordano et al., 2015; Mojumdar et al., 2014, 2016; Ouisse et al., 2019), this investigation has established considerable effects on the spleen proteome owing to deficiency in the full-length dystrophin isoform Dp427-M. Since the spleen acts as a dominant reservoir for inflammatory cells (Ingersoll et al., 2011) and splenic monocytes were recently shown to play an important role during chronic inflammation of dystrophic fibers (Rizzo et al., 2020), the novel proteomic findings are in agreement with the pathophysiological idea of a connection between the lymphoid system and dystrophic muscles with an inflammatory phenotype (Villalta et al., 2015; Tidball et al., 2018).

Preserved Expression of a Short Isoform of Dystrophin in the mdx-4cv Spleen

X-linked muscular dystrophy is a complex neuromuscular disorder that is characterized by necrosis, myofibrosis, and inflammation in the skeletal musculature, as well as late-onset cardiomyopathy, respiratory impairments, neurological deficiencies, scoliosis, and metabolic disturbances (Mercure et al., 2019; Thompson et al., 2020). The neuromuscular symptoms are due to primary abnormalities in the extremely large DMD gene, which encodes several isoforms of the protein dystrophin (Muntoni et al., 2003). The proteomic survey of the spleen presented here has identified unique peptides that belong to the dystrophin protein sequence. This not-well-characterized spleen-associated proteoform of the dystrophin protein is most likely a short version of the DMD gene product, such as the ubiquitously expressed Dp71-G isoform (Tokarz et al., 1998). The proteomic analysis found the presence of this dystrophin isoform in all analyzed spleen samples with high confidence; thus, the nonsense mutation in exon 53 of the DMD gene does not appear to affect the production of this short dystrophin isoform in the mdx-4cv mouse (Im et al., 1996). However, immunofluorescence microscopy clearly showed that the full-length Dp427-M isoform of dystrophin is absent from dystrophic mdx-4cv gastrocnemius muscle fibers (Murphy et al., 2019a), confirming the dystrophic phenotype.

Proteomic Identification of Dystrophin-Associated Proteins in the Spleen

The full-length isoform of dystrophin, Dp427-M, functions in contractile fibers as a membrane cytoskeletal component and forms a supramolecular assembly with a variety of sarcolemma-associated proteins. The dystrophin core complex, consisting of Dp427-M, dystroglycans, sarcoglycans, dystrobrevins, syntrophins, and sarcospan, links the extracellular matrix component laminin to the intracellular actin cytoskeleton (Ohlendieck, 1996). This trans-plasmalemmal structure plays a key role in lateral force transmission and the stabilization of the fiber surface during excitation-contraction-relaxation cycles (Murphy and Ohlendieck, 2015). In dystrophinopathy, the almost complete loss of Dp427-M causes a drastic reduction in the members of the dystrophin-associated glycoprotein complex, which in turn triggers sarcolemmal micro-

| Accession | Protein                  | Gene   | Coverage (%) | Unique Peptides | Molecular Mass (kDa) |
|-----------|--------------------------|--------|--------------|-----------------|---------------------|
| Q00623    | Apolipoprotein A-I       | Apoa1  | 53           | 19              | 30.6                |
| P09813    | Apolipoprotein A-II      | Apoa2  | 49           | 6               | 11.3                |
| P06728    | Apolipoprotein A-IV      | Apoa4  | 68           | 22              | 45                  |
| Q8C7G6    | Apolipoprotein A-V       | Apoa5  | 27           | 8               | 41.2                |
| E9Q414    | Apolipoprotein B-100     | Apob   | 19           | 69              | 509.1               |
| P34928    | Apolipoprotein C-I       | Apoc1  | 20           | 2               | 9.7                 |
| P33622    | Apolipoprotein C-III     | Apoc3  | 27           | 2               | 11                  |
| Q61268    | Apolipoprotein C-IV      | Apoc4  | 9            | 1               | 14.3                |
| P51910    | Apolipoprotein D         | Apod   | 6            | 1               | 21.5                |
| P08226    | Apolipoprotein E         | Apoe   | 63           | 20              | 35.8                |
| Q91V80    | Apolipoprotein F         | Apof   | 6            | 1               | 34.3                |
| Q921R3    | Apolipoprotein M         | Apom   | 12           | 2               | 21.3                |

Table 3. Proteomic Identification of Apolipoproteins in Mouse Spleen
| Accession | Protein                        | Gene                                | Unique Peptides | Confidence Score | Adjusted p value | Max Fold Change |
|-----------|--------------------------------|-------------------------------------|-----------------|------------------|------------------|-----------------|
| E9Q414    | Apolipoprotein B-100          | Apob                               | 2               | 5.4283           | 0.0176           | 296.2           |
| A6X935    | Inter alpha-trypsin inhibitor, heavy chain 4 | ItiH4                              | 2               | 4.6726           | 0.0172           | 163.3           |
| Q91ZX7    | Prolow-density lipoprotein receptor-related protein 1 | Lrp1                              | 2               | 4.9936           | 0.0453           | 48.3            |
| P08226    | Apolipoprotein E              | Apoe                               | 2               | 7.0306           | 0.0413           | 41.2            |
| Q91WT9    | Cystathionine beta-synthase    | Cbs                                | 4               | 9.0211           | 0.0168           | 21.1            |
| P03987    | Ig gamma-3 chain C region     | –                                  | 5               | 13.0469          | 0.0168           | 20.3            |
| Q6P8U6    | Pancreatic triacylglycerol lipase | Pnlip                            | 2               | 4.9116           | 0.0285           | 15.3            |
| P28665    | Murinoglobulin-1              | Mugi                               | 5               | 18.5175          | 0.0216           | 8.7             |
| P20918    | Plasminogen                   | Plg                                | 5               | 14.0486          | 0.0264           | 8.2             |
| P01027    | Complement C3                 | C3                                 | 2               | 6.9290           | 0.0265           | 5.8             |
| Q8K0C5    | Zymogen granule membrane protein 16 | Zg16                      | 2               | 4.7425           | 0.0205           | 5.3             |
| Q91X79    | Chymotrypsin-like elastase family member 1 | Cela1                        | 2               | 6.6288           | 0.0255           | 5               |
| Q64285    | Bile salt-activated lipase    | Cel                                | 2               | 4.0527           | 0.0213           | 4.4             |
| P32261    | Antithrombin-III              | Serpinc1                           | 4               | 13.3814          | 0.0174           | 4.2             |
| Q9D8U3    | Endoplasmic reticulum resident protein 27 | Erp27                        | 2               | 3.8992           | 0.0173           | 4.2             |
| Q504N0    | Carboxypeptidase A2           | Cpa2                               | 4               | 11.1796          | 0.0181           | 4               |
| Q9JK88    | Serpin I2                     | Serpini2                           | 6               | 15.7950          | 0.0174           | 3.7             |
| P07724    | Serum albumin                 | Alb                                | 3               | 9.5456           | 0.0140           | 3.7             |
| Q58KQ4    | Inactive pancreatic lipase-related protein 1 | Pnliprp1           | 5               | 12.4300          | 0.0237           | 3.6             |
| P0688     | Pancreatic alpha-amylase      | Amy2                               | 5               | 15.8377          | 0.0175           | 3.5             |
| A6H584    | Collagen alpha-5(VI) chain    | Col16a5                            | 4               | 12.5493          | 0.0174           | 3.3             |
| Q61024    | Asparagine synthetase (glutamine-hydrolyzing) | Asns                 | 5               | 12.0815          | 0.0172           | 3.2             |
| Q7TPZ8    | Carboxypeptidase A1           | Cpa1                               | 2               | 9.3357           | 0.0239           | 3               |
| Q9CR35    | Chymotrypsinogen B            | Ctrb1                              | 2               | 4.0493           | 0.0361           | 3               |
| D326P0    | Protein disulfide-isomerase A2 | Pdia2                             | 11              | 34.7523          | 0.0173           | 2.9             |
| P21614    | Vitamin D-binding protein     | Gc                                 | 4               | 10.5449          | 0.0298           | 2.9             |
| Q61838    | Pregnancy zone protein        | Pzp                                | 11              | 30.1861          | 0.0268           | 2.8             |
| P15947    | Kallikrein-1                  | Klk1                               | 3               | 7.0787           | 0.0276           | 2.8             |
| P49290    | Eosinophil peroxidase         | Epx                                | 5               | 13.9853          | 0.0169           | 2.7             |
| P07758    | Alpha-1-antitrypsin 1-1       | Serpina1                           | 4               | 13.4458          | 0.0233           | 2.5             |

Table 4. Decreased Proteins in the mdx-4cv Spleen as Determined by LC-MS/MS Analysis

(Continued on next page)
rupturing and calcium-induced proteolytic degradation (Guiraud et al., 2015). Here, we have extended the characterization of dystrophin-associated glycoproteins to the spleen and have identified by mass spectrometry the presence of dystroglycan, sarcoglycans, dystrobrevin, and syntrophins in this crucial secondary lymphoid organ.

Drastic Reduction of Apolipoproteins and Related Serum Proteins in the mdx-4cv Spleen
A variety of complex changes in the immunobiology of dystrophic skeletal muscles have been established that might be reflected by variations in the lymphoid system (Villalta et al., 2015; Lozanoska-Ochser et al.,

| Accession | Protein                                      | Gene          | Unique Peptides | Confidence Score | Adjusted p value | Max Fold Change |
|-----------|----------------------------------------------|---------------|-----------------|------------------|------------------|-----------------|
| P23953    | Carboxylesterase 1C                          | Ces1c         | 3               | 10.9205          | 0.0292           | 2.5             |
| Q8VDJ3    | Vigilin                                      | Hdlbp         | 2               | 5.4235           | 0.0301           | 2.5             |
| P50172    | Corticosteroid 11-beta-dehydrogenase isozyme 1 | Hsd11b1       | 2               | 5.8113           | 0.0207           | 2.3             |
| Q92111    | Serotransferrin                              | Tf            | 9               | 25.5997          | 0.0268           | 2.2             |
| Q00897    | Alpha-1-antitrypsin 1-4                      | Serpina1d     | 3               | 9.79536          | 0.0314           | 2               |
| Q64511    | DNA topoisomerase 2-beta                     | Top2b         | 2               | 4.0629           | 0.0477           | 1.9             |
| P15626    | Glutathione S-transferase Mu 2               | Gstm2         | 3               | 10.3769          | 0.0340           | 1.8             |
| Q9D855    | Cytochrome b-c1 complex subunit 7            | Uqcrb         | 3               | 8.1900           | 0.0126           | 1.8             |
| Q8R2E9    | ERO1-like protein beta                       | Ero1b         | 2               | 4.7424           | 0.0168           | 1.8             |
| Q62087    | Serum paraoxonase/lactonase 3                | Pon3          | 4               | 13.6453          | 0.0167           | 1.7             |
| Q99K85    | Phosphoserine aminotransferase               | Psat1         | 4               | 11.5566          | 0.0174           | 1.7             |
| Q9DBF1    | Alpha-aminoadipic semialdehyde dehydrogenase | Aldh7a1       | 4               | 10.4946          | 0.0308           | 1.7             |
| O08807    | Peroxiredoxin-4                              | Prdx4         | 3               | 8.6493           | 0.0172           | 1.7             |
| P61620    | Protein transport protein Sec61 subunit alpha isoform 1 | Sec61a1 | 3 | 11.7206 | 0.0167 | 1.7 |
| Q06138    | Calcium-binding protein 39                   | Cab39         | 2               | 5.5638           | 0.0338           | 1.7             |
| P10852    | 4F2 cell-surface antigen heavy chain         | Slc3a2        | 4               | 12.8087          | 0.0122           | 1.6             |
| P09103    | Protein disulfide-isomerase                  | P4hb          | 4               | 10.0713          | 0.0189           | 1.6             |
| P58252    | Elongation factor 2                          | Eef2          | 3               | 9.4214           | 0.0256           | 1.6             |
| Q62W07    | Signal peptidase complex subunit 3           | Spcs3         | 2               | 4.2641           | 0.0210           | 1.6             |
| P35564    | Calnexin                                     | Canx          | 2               | 5.9499           | 0.0312           | 1.6             |
| Q78PY7    | Staphylococcal nuclease domain-containing protein 1 | Snd1 | 5 | 13.1260 | 0.0295 | 1.5 |
| O35855    | Branched-chain-amino-acid aminotransferase, mitochondrial | Bcat2 | 2 | 6.0829 | 0.0174 | 1.5 |
| Q64674    | Spermidine synthase                          | Srm           | 2               | 5.6741           | 0.0172           | 1.5             |
| Q922Q8    | Leucine-rich repeat-containing protein 59     | Lrc59         | 2               | 4.9835           | 0.0170           | 1.5             |
| Q922R8    | Protein disulfide-isomerase A6               | Pdia6         | 2               | 6.9604           | 0.0234           | 1.5             |

Table 4. Continued

rupturing and calcium-induced proteolytic degradation (Guiraud et al., 2015). Here, we have extended the characterization of dystrophin-associated glycoproteins to the spleen and have identified by mass spectrometry the presence of dystroglycan, sarcoglycans, dystrobrevin, and syntrophins in this crucial secondary lymphoid organ.
| Accession | Protein                                             | Gene                        | Unique Peptides | Confidence Score | Adjusted p Value | Max Fold Change |
|-----------|-----------------------------------------------------|-----------------------------|-----------------|------------------|-----------------|----------------|
| P21981    | Protein-glutamine gamma-glutamyl-transferase 2      | Tgm2                        | 2               | 4.7403           | 0.0124          | 3.8            |
| P01878    | Ig alpha chain C region                             | –                           | 2               | 5.5789           | 0.0332          | 2.9            |
| Q8CJ91    | CD209 antigen-like protein B                        | Cd209b                      | 2               | 5.1658           | 0.0152          | 2.5            |
| P41245    | Matrix metalloproteinase-9                          | Mmp9                        | 2               | 5.9333           | 0.0175          | 2.3            |
| P08071    | Lactotransferrin                                    | Ltf                         | 15              | 49.8443          | 0.0136          | 2.1            |
| P54869    | Hydroxymethylglutaryl-CoA synthase, mitochondrial   | Hmgcs2                      | 3               | 7.7425           | 0.0130          | 2              |
| P21300    | Aldo-keto reductase family 1 member B7              | Akr1b7                      | 2               | 5.8779           | 0.0183          | 2              |
| P50608    | Fibromodulin                                        | Fmod                        | 2               | 4.9599           | 0.0190          | 1.9            |
| Q3UMY5    | Echinoderm microtubule-associated protein-like 4    | Eml4                        | 2               | 6.1649           | 0.0120          | 1.9            |
| P82343    | N-acetylglucosamine 2-epimerase                     | Renbp                       | 2               | 6.3056           | 0.0170          | 1.9            |
| O08692    | Neutrophil granule protein                          | Ngp                         | 2               | 5.4646           | 0.0185          | 1.9            |
| P26039    | Talin-1                                             | Tln1                        | 2               | 5.7934           | 0.0181          | 1.8            |
| Q9CWK8    | Sorting nexin-2                                     | Snx2                        | 2               | 6.9348           | 0.0183          | 1.8            |
| Q8VEE1    | LIM and cysteine-rich domains protein 1             | Lmcd1                       | 2               | 6.8011           | 0.0085          | 1.8            |
| P19070    | Complement receptor type 2                          | Cr2                         | 2               | 5.0366           | 0.0295          | 1.8            |
| P27870    | Proto-oncogene vav                                  | Vav1                        | 3               | 7.4085           | 0.0192          | 1.7            |
| Q9ESS2    | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 | Inpp5d                  | 3               | 8.2105           | 0.0152          | 1.7            |
| Q8R1G6    | PDZ and LIM domain protein 2                        | Pdlim2                      | 2               | 6.4459           | 0.0286          | 1.7            |
| Q6PBX1    | Sorting nexin-6                                     | Snx6                        | 2               | 6.9493           | 0.0176          | 1.7            |
| P49710    | Hematopoietic lineage cell-specific protein         | Hcls1                       | 2               | 6.3795           | 0.0295          | 1.7            |
| P63038    | 60 kDa heat shock protein, mitochondrial            | Hspd1                       | 2               | 4.9004           | 0.0317          | 1.7            |
| Q61823    | Programmed cell death protein 4                     | Pdcod4                      | 2               | 5.2918           | 0.0147          | 1.7            |
| Q6P4T2    | US small nuclear ribonucleoprotein 200 kDa helicase | Snnp200                     | 2               | 6.1287           | 0.0154          | 1.7            |
| Q62230    | Sialoadhesin                                        | Siglec1                     | 6               | 19.4232          | 0.0124          | 1.6            |
| Q8VCW8    | Medium-chain acyl-CoA ligase ACSF2, mitochondrial    | Acsl2                       | 5               | 16.0841          | 0.0118          | 1.6            |
| Q9QVP9    | Protein-tyrosine kinase 2-beta                      | Ptk2b                       | 3               | 11.6718          | 0.0265          | 1.6            |
| Q8K2T1    | NmrA-like family domain-containing protein 1        | Nmral1                      | 3               | 9.4830           | 0.0122          | 1.6            |
| Q921T2    | Torsin-1A-interacting protein 1                     | Tor1aip1                    | 2               | 4.2835           | 0.0135          | 1.6            |
| Q3UP87    | Neutrophil elastase                                 | Elane                       | 2               | 4.6109           | 0.0260          | 1.6            |

Table 5. Increased Proteins in the mdx-4cv Spleen as Determined by LC-MS/MS Analysis

(Continued on next page)
The most striking finding of this investigation is the drastic reduction in a variety of serum proteins in the mdx-4cv spleen. This includes proteins that are involved in lipid transport and metabolism (apolipoproteins ApoB-100 and ApoE, lipoprotein receptor Lrp1, and various lipases), the complement system (complement C3), metabolite transportation (albumin), digestion (amylase), and factors involved in the acute response and inflammation (kallikrein, inter alpha-trypsin inhibitor). Spleen-associated proteins with a drastic reduction in the mdx-4cv mouse model were previously shown to be also reduced in mdx-4cv serum (Murphy et al., 2017). This included the most significantly decreased protein species in the mdx-4cv spleen, apolipoprotein B-100, as well as antithrombin-III, complement C3, alpha-1-antitrypsin, murinoglobulin-1, plasminogen, inter-alpha-trypsin inhibitor heavy chain, chymotrypsin-like elastase, vitamin D-binding protein, and serotransferrin.

Apolipoproteins are crucial plasma proteins involved in the regulation of lipid homeostasis, and their concentration is a good indicator of the metabolic status of the organism (Geyer et al., 2017). Surprisingly, in the ApoE-null mdx mouse reduced atherosclerotic plaque formation was found, as well as decreased numbers of CD3+ cells in the spleen (Shami et al., 2015). In the case of the mdx-4cv model of dystrophinopathy, the reduced abundance of these plasma proteins in the spleen could reflect their lower concentration in the circulatory system. Impaired blood flow due to weakened cardiac output of the dystrophin-deficient heart might also be a crucial factor that causes a decrease in plasma components in the mdx-4cv spleen. In dystrophinopathy, late-onset cardiomyopathy causes cardiac weakness (Finsterer and Cripe, 2014) and this is reflected by drastic proteome-wide changes especially at the level of metabolic enzymes (Murphy et al., 2016). This cardiac impairment could cause a decreased rate of circulation of blood and concomitant chronic lack of proper supply of oxygen and nutrients to peripheral organs. The proteomic findings on the mdx-4cv spleen presented here agrees with this pathophysiological concept.

Increased Transglutaminase TGM2 and Matrix Metalloproteinase MMP9 in the mdx-4cv Spleen

The most increased protein in the mdx-4cv spleen was identified as protein-glutamine gamma-glutamyl-transferase TGM2, also more commonly referred to as transglutaminase 2, which is a multi-functional enzyme that mediates the cross-linking of proteins and catalyzes the conjugation of polyamines to proteins (Beninati et al., 2017). In addition, more recently transglutaminase 2 was shown to be intrinsically involved in the maintenance of proteostasis (D’Eletto et al., 2019). Since chronic cellular stress results in the increased occurrence of abnormal protein folding or detrimental protein aggregation, the regulation of chaperone function is upregulated under dystrophic conditions (Doran et al., 2006; Brinkmeier and Ohlendieck, 2014). Thus, the elevated levels of transglutaminase TGM2 might be involved in a key aspect of the regulation of protein homeostasis in the stressed mdx-4cv spleen. The increased abundance of the matrix metalloproteinase MMP9 agrees with the findings from the previous screening of serum from the mdx mouse and patients with Duchenne muscular dystrophy for minimally invasive biomarker candidates (Nadarajah et al.,...
The serum-associated proteoform of this member of the large family of matrix metalloproteinases was shown to be increased significantly in muscular dystrophy, which might explain its elevated levels in the *mdx-4cv* spleen. Although MMP9 probably plays a differential role during the progression of muscle degeneration (Shiba et al., 2015), its primary effect in muscular dystrophy appears to be the promotion of inflammation, tissue remodeling, and reactive myofibrosis (Kherif et al., 1999; Hindi et al., 2013; Shin et al., 2013). This confirms MMP9 as a good biomarker candidate for secondary changes in muscular dystrophy due to sterile inflammation and fibrotic changes.

**Figure 4. Heatmap of the Comparative Proteomic Analysis of Wild-Type versus mdx-4cv Spleen**

Shown are the findings from hierarchical clustering of the mean protein expression values of statistically significant differentially abundant spleen proteins.
Novel Biomarker Candidates of Muscular Dystrophy-Associated Changes in the Spleen

In conclusion, although the expression levels of a short spleen-associated dystrophin isoform and its associated proteins do not appear to be majorly affected in muscular dystrophy, the absence of the full-length dystrophin isoform in contractile tissues seems to trigger secondary effects in the lymphoid system. This includes a drastic reduction in the apolipoproteins ApoB-100 and ApoE and a concomitant increase in the enzyme protein-glutamyl gamma-glutamyl-transferase TGM2 and the matrix metalloproteinase MMP9, which might be useful candidates as novel biomarkers of dystrophinopathy-related changes in the spleen (Dowling et al., 2019; Al-Khalili Szigyarto, 2020). The proteomic findings suggest that pathophysiological cross talk and/or anatomical interconnectivity affects the spleen in muscular dystrophy, which substantiates the appropriateness of the mdx-4cv mouse model for studying secondary changes in non-muscle tissues in dystrophinopathy.

Limitations of the Study

In this report, we have characterized for the first time the secondary impact of dystrophin deficiency on the spleen and established considerable proteome-wide changes in this lymphoid organ. However, the study was carried out with a genetic animal model of dystrophinopathy, the mdx-4cv mouse, which shows a...
milder pattern of neuromuscular degeneration in most skeletal muscles as compared with patients with Duchenne muscular dystrophy. It will therefore be crucial to extend these studies in the future to the evaluation of secondary abnormalities in X-linked muscular dystrophy to patient specimens. Although comparative proteomics is an excellent bioanalytical approach to determine systematic changes in the dystrophic phenotype, mass spectrometric lists of altered protein expression levels do neither give detailed information on the pathobiochemical time course of cellular alterations nor provide a deep mechanistic understanding of disease progression. Thus, based on the comparative proteomic data supplied in this report, it will be crucial to further study the underlying disturbances that cause proteome-wide changes in the spleen in association with dystrophinopathy.

**Resource Availability**

**Lead Contact**

For further information, requests should be directed to the Lead Contact: Kay Ohlendieck (kay.ohlendieck@mu.ie).
Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The proteomic datasets from the mass spectrometric analysis of 14 separate spleen sample runs have been deposited to the Open Science Framework repository as OSF entry “f85ve” under the following link: https://osf.io/f85ve/. This link also features a multi-consensus file of the proteomic cataloging of mouse spleen with the help of an Orbitrap Fusion Tribrid mass spectrometer.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101500.

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AUTHOR CONTRIBUTIONS
P.D., D.S., and K.O. initiated the project and designed the research. S.G. and P.D. performed the biochemical experiments and analyzed the data. M.Z. carried out the preparation of tissue samples and cell biological analysis. M.H. and P.M. performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript.
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Supplemental Information

Proteome-wide Changes in the *mdx-4cv* Spleen due to Pathophysiological Cross Talk with Dystrophin-Deficient Skeletal Muscle

Paul Dowling, Stephen Gargan, Margit Zweyer, Michael Henry, Paula Meleady, Dieter Swandulla, and Kay Ohlendieck
Transparent Methods

Proteome-wide changes in the mdx-4cv spleen due to pathophysiological crosstalk with dystrophin-deficient skeletal muscle

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MATERIALS AND METHODS

Materials

For the mass spectrometry-based proteomic analysis of spleen preparations, general materials and analytical grade reagents were obtained from Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK), GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Sigma Chemical Company (Dorset, UK). Protease inhibitors were purchased from Roche Diagnostics (Mannheim, Germany). Protein digestion was carried out with sequencing grade-modified trypsin, Lys-C and Protease Max Surfactant Trypsin Enhancer from Promega (Madison, WI, USA). The Pierce 660-nm Protein Assay reagents and Invitrogen NuPAGE Novex Bis-Tris gels were purchased from ThermoFisher Scientific (Dublin, Ireland). Vivacon 500 filter units were purchased from Sartorius (Göttingen, Germany). Expedeon InstantBlue Coomassie Protein Stain was from Abcam (Cambridge, MA, USA). Primary antibodies were purchased from Antibody Genie, Dublin, Ireland (CAB16344 against apolipoprotein ApoE), Cell Signaling Technology, Leiden, The Netherlands (mAb23565 against the alpha-subunit of the Na⁺/K⁺-ATPase), Abbexa Ltd, Cambridge, UK (abx001907 against heat shock protein HspB2; abx000934 against transglutaminase TGM2), Leica Biosystems, Wetzlar, Germany (NCL-
DYS1 and NCL-DYS2 against dystrophin) and R&D Systems, Abingdon, UK (AF909 against matrix metalloproteinase MMP9; MAB4376 against transglutaminase TGM2). Anti-rabbit and anti-mouse peroxidase conjugated secondary antibodies were from Cell Signaling Technology (Leiden, The Netherlands). Normal goat serum, goat anti-mouse IgG RRX (Rhodamine Red-X), anti-rabbit Alexa Fluor 488 and Alexa 647 antibodies were purchased from Molecular Probes, Life Technologies (Darmstadt, Germany) and Jackson ImmunoResearch (West Grove, PA, USA), respectively. The embedding medium Fluoromount G was from Southern Biotech (Birmingham, AL, USA).

**Preparation of mouse tissue extracts for proteomic and biochemical analysis**

Mice were obtained from the Bioresource Unit of the University of Bonn (Murphy et al., 2019a), where they were kept under standard conditions and all procedures adhered to German legislation on the use of animals in experimental research (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany). The spleen and gastrocnemius muscle from 12-month old male wild type C57/BL6 mice and age-matched dystrophic male mdx-4cv mice were freshly dissected and quick-frozen in liquid nitrogen. Tissue specimens were transported to Maynooth University in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth) on dry ice and stored at -80°C prior to proteomic and biochemical analysis. For sample preparation, 25 mg of mouse tissues (wild type versus dystrophic phenotype) were lysed by homogenisation with 200µl of lysis solution (4% SDS, 100mM Tris-Cl pH 7.6, 0.1M dithiothreitol) and incubated at 95°C for 3 minutes and then sonicated for 30 seconds. The lysate was clarified by centrifugation at 16,000xg for 5 minutes. Tissue aliquots were used for gel electrophoretic separation and comparative immunoblotting. Spleen extracts were further processed for mass spectrometry. 10µl of lysate was mixed with
200µl of 8M urea, 0.1M Tris pH 8.9 in filter units (Sartorius, Vivacon 500, Product number: VN0H22) and centrifuged at 14,000xg for 15 minutes. Samples were subsequently processed according to the standardized FASP protocol for filter-aided sample preparation (Wiśniewski, 2009).

**Label-free liquid chromatography mass spectrometry**

For the proteomic profiling of the *mdx-4cv* spleen, reverse-phased capillary high-pressure liquid chromatography was performed with the help of the UltiMate 3000 nano system from Thermo Scientific coupled directly in-line with the Thermo Orbitrap Fusion Tribrid Mass Spectrometer (Murphy et al., 2019b). The digested protein samples (1µl ~ 800ng peptide) were loaded onto the trapping column (PepMap100, C18, 300 µm × 5 mm) at a flow rate of 25 µl/min with 2% (v/v) acetonitrile (ACN), 0.1% (v/v) trifluoroacetic acid (TFA) for 3 minutes before being resolved onto an analytical column (Acclaim PepMap 100, 75 µm × 50 cm, 3 µm bead diameter column). Peptides were eluted using the following binary gradient; solvent A (0.1% (v/v) formic acid in LC-MS grade water) and solvent B (80% (v/v) ACN, 0.08% (v/v) formic acid in LC-MS grade water) using 2-23% B for 75 minutes, 32-90% B for 5 minutes and holding at 90% B for 5 minutes at a flow rate of 300 nl/min. For peptide ionization, a voltage of 2.0 kV was applied and a capillary temperature of 320°C was used. Data-dependent acquisition with full scans in the 380-1500 m/z range was performed using an Orbitrap mass analyser with a resolution of 120,000 (at m/z 200), a targeted automatic gain control (AGC) value of 4E+05 and a maximum injection time of 50ms. The number of selected precursor ions for fragmentation was determined by the top-speed acquisition algorithm. Selected precursor ions were isolated in the Quadrupole with an isolation width of 1.6 Da. Peptides with a charge state of 2+ to 7+ were analysed and a dynamic exclusion was applied after 60s. Precursor ions were fragmented using higher energy collision-induced dissociation with a normalized
collision energy of 28%, and resulting MS/MS ions were measured in the linear ion trap. The typical MS/MS scan conditions were as follows: a targeted AGC value of 2E+04 and a maximum fill time of 35ms.

**Data analysis**

Qualitative data analysis, using mass spectrometric files (.raw), was performed by searching against the UniProtKB-SwissProt *Mus musculus* database with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific), and Percolator. The following search parameters were used for protein identification: (i) peptide mass tolerance set to 10 ppm, (ii) MS/MS mass tolerance set to 0.02 Da, (iii) an allowance of up to two missed cleavages, (iv) carbamidomethylation set as a fixed modification and (v) methionine oxidation set as a variable modification. Peptides were filtered using a minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge states, with peptide probability set to high confidence (Qu et al, 2017).

Quantitative label-free data analysis was performed using Progenesis QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle upon Tyne, UK), essentially as recommended by the manufacturer (www.nonlinear.com). Peptide and protein identification from this analysis were achieved with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific), and Percolator as described above and were imported into Progenesis QI software for further analysis.

Protein identifications were reviewed, and only those which passed the following criteria were considered differentially expressed between experimental groups with high confidence and statistical significance: (i) an ANOVA p-value of ≤0.01 between experimental groups; (ii) proteins with ≥2 unique peptides contributing to the identification. To calculate the maximum fold change for a protein, Progenesis QI calculates the mean abundance for that protein in each
experimental condition. These mean values are then placed in a condition-vs-condition matrix to find the maximum fold change between any two condition’s mean protein abundances.

The freely available software packages PANTHER (Mi et al., 2017; http://pantherdb.org/) and STRING (Szklarczyk et al., 2019; https://string-db.org/) were used to identify protein classes and characterise potential protein interactions, respectively.

**Gel electrophoresis and immunoblotting**

Protein fractions (12.5µg protein per lane for Coomassie Blue staining; and 25µg protein per lane for immunoblot analysis) were run on NuPAGE Novex Bis-Tris Gels under standard conditions (Murphy et al., 2019a). Protein concentration was determined using the Pierce 660-nm protein assay system (Antharavally et al., 2009). For comparative analyses, Coomassie staining of protein gels was carried out with InstantBlue Coomassie Protein Stain. Immunoblotting was then employed as an orthogonal method for the independent verification of changes in newly identified proteins in the mdx-4cv spleen. Proteins were transferred to nitrocellulose membranes, blocked and incubated with primary antibody overnight, followed by detection with peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence method (Murphy et al., 2019a). Densitometric scanning and statistical analysis of immunoblots was performed using a HP PSC-2355 scanner and ImageJ software (NIH, Bethesda, MD, USA) along with Microsoft Excel in which statistical significance was based on a p-value ≤ 0.05.

**Histological analysis and immunofluorescence microscopy**

Tissue cryo-sections from skeletal muscle and spleen were used for standardized histological and immunofluorescence microscopical analysis (Dowling et al., 2020). The mutant status of the mdx-4cv mouse was established by demonstrating the loss of the dystrophin isoform
Dp427-M in *gastrocnemius* muscle and correlated to potential changes in the spleen. Histological features of the analysed tissues were visualized by haematoxylin and eosin staining. For immunofluorescence microscopy, freshly dissected spleen and skeletal muscle specimens from 12-month old wild type and *mdx-4cv* mice were quick-frozen in liquid nitrogen-cooled isopentane and 10µm sections cut in a cryostat. For dystrophin immunostaining, unfixed cryosections were boiled in phosphate-buffered saline for 5 minutes as previously described in detail (Murphy et al., 2019b). For immuno-staining, sections were fixed in a 1:1 (v/v) mixture of methanol and acetone for 10 minutes at room temperature and then blocked with 1:20 diluted normal goat serum for 30 minutes at room temperature. Primary antibodies were diluted appropriately in phosphate-buffered saline for overnight incubation at 4°C. Tissue specimens were carefully washed and then incubated with fluorescently labelled secondary antibodies, using either 1:200 diluted anti-rabbit Alexa Fluor 488 or 647 antibodies or 1:200 diluted anti-mouse RRX antibody for 45 minutes at room temperature. Nuclei were counter-stained with 1µg/ml bis-benzimide Hoechst 33342. Antibody-labelled spleen and skeletal muscle sections were embedded in Fluoromount G medium and viewed under a Zeiss Axioskop 2 epifluorescence microscope equipped with a digital Zeiss AxioCam HRc camera (Carl Zeiss Jena GmbH, Jena, Germany). Immunofluorescence intensity was analysed using ImageJ software (NIH, Bethesda, MD, USA) along with Microsoft Excel in which statistical significance was based on a p-value ≤ 0.05.

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