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Isobaric Tagging-Based Quantification for Proteomic Analysis: A Comparative Study of Spared and Affected Muscles from mdx Mice at the Early Phase of Dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is the most common childhood myopathy, characterized by muscle loss and cardiorespiratory failure. While the genetic basis of DMD is well established, secondary mechanisms associated with dystrophic pathophysiology are not fully clarified yet. In order to obtain new insights into the molecular mechanisms of muscle dystrophy during earlier stages of the disease, we performed a comparative proteomic profile of the spared extraocular muscles (EOM) vs. affected diaphragm from the mdx mice, using a label based shotgun proteomic approach. Out of the 857 identified proteins, 42 to 62 proteins had differential abundance of peptide ions. The calcium-handling proteins sarcalumenin and casequestrin-1 were increased in control EOM compared with control DIA, reinforcing the view that constitutional properties of EOM are important for their protection against myonecrosis. The finding that galectin-1 (muscle regeneration), annexin A1 (anti-inflammatory) and HSP 47 (fibrosis) were increased in dystrophic diaphragm provides novel insights into the mechanisms through which mdx affected muscles are able to counteract dystrophy, during the early stage of the disease. Overall, the shotgun technique proved to be suitable to perform quantitative comparisons between distinct dystrophic muscles and allowed the suggestion of new potential biomarkers and drug targets for dystrophinopathies.

Introduction

Duchenne muscular dystrophy (DMD) is the most common and devastating of the human muscular dystrophies. It is characterized by progressive muscle weakness and death from cardiorespiratory compromise around the second or third decade of life [1]–[3]. In DMD and in the mdx mouse model of DMD [4], [5] the genetic abnormality is in the X chromosome, in which the nucleotide sequence responsible for the expression of the protein dystrophin is mutated. In the absence of dystrophin, instability of sarcolemma leads to progressive myonecrosis, followed by intense inflammation and fibrosis [1], [6].

Numerous proteomics analyses of dystrophic muscles in mdx and in DMD have been performed with the aim to unravel the molecular pathogenesis of muscular dystrophy [7]–[13]. Previous proteomic studies included the use of the differential gel electrophoresis (DIGE), which provided important data related to the nature of the dystrophic proteome and showed a great number of proteins that were differentially expressed in distinct dystrophic muscles and ages [7], [10]–[15].

The multidimensional protein identification technology (MudPIT) method is a gel free alternative [16]–[18] to conventional gel-based methods, which has revolutionized the proteomic field. Basically, complex proteins mixtures are digested to peptides, fractionated according to its different chemical properties and expressed, provided the original author and source are credited.

The authors have declared that no competing interests exist.

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tagging reagents, as tandem mass tags (TMT), allow multiple and independent measures of protein abundance in the same experiment, enabling statistical estimates of protein quantification and comparisons between different samples [24].

In order to obtain new insights into the molecular mechanisms of muscle dystrophy, we here used a label based shotgun proteomic approach, combining TMT labels and MudPIT method to analyze the diaphragm of 2-month-old mdx mice. We considered the age of 2 months as an early phase of dystrophy, similar to other proteomic studies [12], given the worsening of disease overtime. Signs of necrosis have been reported to occur at about 5 weeks of age in the mdx diaphragm [25]. At 2 months of age, diaphragm shows active muscle damage, regeneration and inflammation. However, significant histopathological signs of the disease, such as extensive fibrosis, are not seen at this age, suggesting an ability to compensate for muscle degeneration with cycles of muscle regeneration, as reported for limb muscles [26], [27]. Fibrosis will be present later in mdx diaphragm, increasing progressively from 6 months to 1 year of age and onwards, a timepoint when the muscle mostly resembles DMD myopathy [13], [26], [28]. In our proteomic analysis we have also searched for compensatory mechanisms involved in dystrophic muscle protection against myonecrosis, by comparing the proteomic profiles from dystrophic mdx diaphragm against the one from mdx extraocular muscles, which do not show muscle degeneration [29].

Materials and Methods

Animals

Males and females, 2-month-old mdx (C57BL/10ScSn-mdx/J) mice (n = 15) and age-match wild-type mice (C57BL/10SnJ, n = 15) were obtained from Jackson Laboratory and maintained in the animal facilities of Biomedical Center (Lund) according to the animal care guidelines. All mouse experimentation was approved by the Malmö/Lund (Sweden) ethical committee for animal research (permit numbers M62-09 and M122-10).

TMT labelling of mouse muscle samples

Protein extraction and preparing of the samples. Mice were sacrificed by cervical dislocation and the diaphragm (DIA; Figure 1) muscle and extraocular muscles (EOM; Figure 1) were dissected out, frozen in liquid nitrogen and reduced to powder using a mortar. Three different pools for each group (mdx mice and wild-type mice) were made, each composed of five animals. The muscles were lysed in assay lysis buffer (10 mM NaHCO₃, 5% SDS) containing freshly added protease and phosphatase inhibitors (Roche - Indianapolis, IN, USA). The samples were centrifuged for 10 min at 15,682 g, and the soluble fraction was removed. The protein concentration was determined using BCA Protein Assay Kit (Pierce).

The samples were processed according to the instructions of the TMT Isobaric Mass Tagging Kits and Reagents. In brief, 100 µg of protein per condition were mixed in six volumes of pre-chilled (−20°C) acetone and precipitated overnight. After centrifugation at 8,000 g for 10 minutes at 4°C, the pellet was dried. For protein digestion, 5 µl of 2% SDS, 45 µl of 200 mM TEAB were added to the sample and the final volume was adjusted to 100 µl with ultrapure water. Five microliter of 200 mM TCEP were added to the sample and incubated at 55°C for 1 hour. Then, 5 µl of the 375 mM iodoacetamide (with TEAB) were added and incubated for 30 min protected from light. To digest proteins, 2.5 µg of trypsin were added and kept overnight at 30–37°C. For protein labeling, 41 µl of the TMT Label Reagent were added to each sample and incubated for 1 h at room temperature. Eight microliter of 5% hydroxylamine were added and incubated for 15 min. Our labeling design allowed a label swap, in order to avoid possible bias due to technical errors (Table 1).

SCX fractionation of the pooled TMT labelled samples. The pooled TMT-labelled samples were fractionated by strong cation-exchange (SCX, Applied Biosystems) using 500 µl of buffer A with 30, 60, 90, 120, 240, 300, 420, 500 mM KCl, respectively and collect as fractions 1–8, respectively. The fractions were cleaned on Ultra Microspin C18 columns (The Nest Group, doi:10.1371/journal.pone.0065831.g001

| Table 1. Experimental setup for 2 muscles (EOM - extraocular and DIA - diaphragm) under 2 conditions (control and mdx) and with 3 biological replicates (Pool 1, 2 and 3). |
|---|---|---|
| Groups | TMT label |
| **Pool 1** (n = 5) | | |
| EOM control | 127 |
| DIA control | 128 |
| EOM mdx | 129 |
| DIA mdx | 130 |
| Internal standard | 126 |
| **Pool 2** (n = 5) | | |
| EOM control | 130 |
| DIA control | 129 |
| EOM mdx | 128 |
| DIA mdx | 127 |
| Internal standard | 126 |
| **Pool 3** (n = 5) | | |
| EOM control | 129 |
| DIA control | 130 |
| EOM mdx | 127 |
| DIA mdx | 128 |
| Internal standard | 126 |

The internal standard is a mixture of all samples. doi:10.1371/journal.pone.0065831.t001
LC-MS/MS Analysis on LTQ-OrbitrapXL. The fractions were analyzed on an LTQ-OrbitrapXL (Thermo Fisher Scientific) interfaced with an in-house constructed nano-LC column. Two-microliter sample injections were made with an HTC-PAL autosampler (CTC Analytics AG) connected to an Agilent 1200 binary pump (Agilent Technologies). The peptides were trapped on a pre-column (40 × 0.075 mm i.d.) and separated on a reversed phase column, 200 × 0.05 mm. Both columns were packed in-house with 3 μm Reprosil-Pur C18-AQ particles. The flow through the analytical column was reduced by a split to approximately 100 nl/min and the gradient was as follows: 0–6 min 0.1% formic acid, 6–76 min 7–35% acetonitrile, 0.1% formic acid, 76–79 min 40–90% acetonitrile 0.1% formic acid.

LTQ-OrbitrapXL settings were: spray voltage 1.4 kV, 1 microscan for MS1 scans at 60 000 resolutions (m/z 400), full MS mass range m/z 400–2000. The LTQ-Orbitrap XL was operated in a data-dependent mode with one MS1 FTMS scan of precursor ions followed by CID (collision induced dissociation) and HCD (high energy collision dissociation), MS2 scans of the three most abundant doubly, triply and quadruply protonated ions in each FTMS scan. The settings for the MS2 were as follows: 1 microscans for HCD-MS2 at 7500 resolution (at m/z 400), mass range m/z 100–2000 with a collision energy of 50%, 1 microscans for CID-MS2 with a collision energy of 30%. Dynamic exclusion of a precursor selected for MS2 was used for 120 s after one repeat, enabling most of the co-eluting precursors to be selected for MS2.

Database Search and TMT Quantification. MS raw data files from all 8 SCX fractions per one TMT set and 3 MS runs were merged for relative quantification and identification using Proteome Discoverer version 1.3 (Thermo Fisher Scientific, respectively). Database search was performed by Mascot search engine using the following criteria: *Mus musculus* in Swissprot protein database from April 2012; MS peptide tolerance as 10 ppm, MS/MS tolerance as 0.5 Da; trypsin digestion allowing 1 missed cleavages with variable modifications; methionine oxidation, Cys carbamidomethylation, and fixed modifications; N-terminal acetylation, cysteine methylthiol, and fixed modifications; Mascot identity threshold. Normalisation on protein median was used. The Mascot identity threshold in the software was set to a confidence using the FDR (false discovery rate) with estimated q-values was considered significantly different. For correction of false-positive values the FDR (false discovery rate) with estimated q-values was used.

Western blot analysis
Verification of the proteomic findings was carried out by comparative Western blot analysis. The levels of galectin-1 (GαI), annexin A5 (ANXA 5), β-dystroglycan (β-DG), calmodulin I (CaM) and calsequestrin-1 (CSQ) were quantified in DIA and EO muscles of control (n = 6) and mdx (n = 6) mice. The method was previously described [28], [31]. Muscles were lysed in assay lysis buffer containing freshly added protease and phosphatase inhibitors (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF, and 0.25 mM Na3VO4). The samples were centrifuged for 20 min at 12,581 g, and the soluble fraction was resuspended in 30 μl Laemmli loading buffer (2% SDS, 20% glycerol, 0.04 mg/ml bromophenol blue, 0.12 M Tris-HCl, pH 6.8, and 0.28 M β-mercaptoethanol). An amount of 60 μg of total protein homogenate was loaded onto 8%–15% SDS-polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane using a submersion electrotransfer apparatus (Bio-Rad Laboratories, Hercules, California, USA). Membranes were blocked for 2 h at room temperature with 5% skim milk/Tris-HCl buffered saline-Tween buffer (TBST; 10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20). The membranes were incubated with the primary antibodies overnight at 4°C, washed in TBST, incubated with the peroxidase-conjugated secondary antibodies for 2 h at room temperature, and developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, Illinois, USA).

To control for protein loading, Western blot transfer and nonspecific changes in protein levels, the blots were stripped and re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The signal from western blotting bands was captured (G:Box iChemi camera; Syngene, Cambridge, UK) and quantified using the software Gene Tools Version 4.01, Syngene, Cambridge, UK.

The following primary antibodies were used: annexin A5, galectin-1, calmodulin I and GAPDH (Santa Cruz Biotechnology, Santa Cruz, California, USA); β-dystroglycan (Novocastra Laboratories Ltd., Benton Lane, Newcastle Upon Tyne, UK); and calsequestrin-1 (Affinity Bioreagents, Golden, Colorado, USA). The corresponding secondary antibody was peroxidase-labeled affinity-purified mouse or rabbit IgG antibody (H+L) (KPL, Gaithersburg, Maryland, USA).

Results
Shotgun proteomic analysis of DIA and EOM
By using the shotgun with MudPIT and TMT methodology, a total of 857 proteins were identified [DIA plus EOM]. From this, about 48% (415 out of 857; Table S1) were common to the muscles studied, i.e., they were detected in both EOM and DIA, from both conditions (control and mdx) and in the three biological replicates. The criteria to identify proteins as being significantly changed between samples were proteins ratios with p-value ≤0.05 (Student’s t-test), q-value ≤0.025 and protein ratios smaller than −1.25 or greater than 1.25. About 10% (42 out of 415; Table 2) of the proteins showed an altered expression pattern in the dystrophic DIA compared with the control DIA. We did not observe any proteins that had differential abundance of peptide ions in the mdx EOM (compared to control EOM), according to our established criteria. Overall, the proteins identified could be grouped into several biological processes such as immune system processes, energy and metabolism, sarcomeric and cytoskeletal proteins.
Comparative proteomic profiling: mdx DIA versus control DIA

By comparing dystrophic DIA with control DIA, we found that most of the proteins detected were present in the sarcoplasm or in the cytoskeleton. Mitochondrion was the organelle that displayed the highest percentage of proteins with altered levels, followed by the nucleus and the sarcoplasmic reticulum (Table 3). The majority of the identified proteins belonged to the class of metabolic proteins or to the class of immune system processes. All 8 protein metabolism-related ribosomal proteins had increased expression in the mdx DIA in comparison to control DIA.

Table 2. The identified proteins that exhibit change in abundance in mdx diaphragm in comparison to control diaphragm.

| Accession | Description | MW [kDa] | Coverage | # Peptides | Fold Change |
|-----------|-------------|----------|----------|------------|-------------|
| Q5SX39    | Myosin-4    | 222.7    | 57.9     | 133        | −1.49       |
| P13707    | Glycerol-3-phosphate dehydrogenase [NAD+] cytoplasmic | 37.5 | 15.5 | 5 | −1.45 |
| Q8BW7S    | Amine oxidase [flavin-containing] B | 58.5 | 2.7 | 2 | −1.42 |
| Q80XN0    | D-beta-hydroxybutyrate dehydrogenase, mitochondrial | 38.3 | 8.8 | 3 | −1.35 |
| P19157    | Glutathione S-transferase P 1 | 23.6 | 12.9 | 2 | −1.33 |
| P10649    | Glutathione S-transferase Mu 1 | 26.0 | 28.9 | 6 | −1.32 |
| P28665    | Murinoglobulin-1 | 165.2 | 3.1 | 5 | −1.30 |
| P68134    | Actin, alpha skeletal muscle | 42.0 | 58.1 | 22 | −1.30 |
| P09542    | Myosin light chain 3 | 22.4 | 45.1 | 8 | −1.30 |
| Q924D0    | Reticulon-4-interacting protein 1, mitochondrial | 43.3 | 3.3 | 1 | −1.30 |
| P07310    | Creatine kinase M-type | 43.0 | 55.4 | 21 | −1.29 |
| Q9DF09    | Phosphoglucomutase-1 | 61.5 | 10.0 | 5 | −1.29 |
| Q09165    | Calsequestrin-1 | 45.6 | 23.8 | 10 | −1.29 |
| Q91V92    | ATP-citrate synthase | 119.7 | 3.2 | 3 | −1.29 |
| Q9C6Q2    | 2,4-dienoyl-CoA reductase, mitochondrial | 36.2 | 27.2 | 8 | −1.28 |
| P20801    | Troponin C, skeletal muscle | 18.1 | 37.5 | 5 | −1.27 |
| P05064    | Fructose-bisphosphate aldolase A | 39.3 | 44.5 | 18 | −1.26 |
| Q70250    | Phosphoglycerate mutase 2 | 28.8 | 24.5 | 8 | −1.26 |
| P14152    | Malate dehydrogenase, cytoplasmic | 36.5 | 28.1 | 7 | −1.25 |
| Q9CQ11    | Heat shock protein 75 kDa, mitochondrial | 80.2 | 4.4 | 2 | 1.26 |
| P19253    | 60S ribosomal protein L13a | 23.4 | 3.9 | 1 | 1.28 |
| P20029    | 78 kDa glucose-regulated protein | 72.4 | 10.2 | 5 | 1.28 |
| P35980    | 60S ribosomal protein L18 | 21.6 | 6.9 | 1 | 1.31 |
| P09103    | Protein disulfide-isomerase | 57.1 | 12.8 | 7 | 1.32 |
| Q8V0D5    | Myosin-9 | 226.2 | 6.3 | 11 | 1.33 |
| P47915    | 60S ribosomal protein L29 | 17.6 | 5.0 | 1 | 1.41 |
| Q9Z1N5    | Spliceosome RNA helicase Bat1 | 49.0 | 1.4 | 1 | 1.41 |
| Q9C3X8    | 40S ribosomal protein S19 | 16.1 | 18.6 | 3 | 1.42 |
| Q9D1R9    | 60S ribosomal protein L34 | 13.3 | 6.0 | 1 | 1.45 |
| Q91V65    | ATP-dependent RNA helicase DDX1 | 82.4 | 1.8 | 1 | 1.47 |
| P47955    | 60S acidic ribosomal protein P1 | 11.5 | 14.0 | 1 | 1.54 |
| P14148    | 60S ribosomal protein L7 | 31.4 | 12.2 | 3 | 1.59 |
| Q8C0P6    | Histone H2A type 1-H | 13.9 | 27.3 | 3 | 1.61 |
| P19324    | Serpin H1 | 46.6 | 10.8 | 3 | 1.62 |
| P48036    | Annexin A5 | 35.7 | 23.2 | 8 | 1.65 |
| P17742    | Peptidyl-prolyl cis-trans isomerase A | 18.0 | 35.4 | 6 | 1.71 |
| Q8C43     | Myosin light chain 6B | 22.7 | 34.3 | 7 | 1.75 |
| P20152    | Vimentin | 53.7 | 43.4 | 18 | 1.77 |
| P51881    | ADP/ATP translocase 2 | 32.9 | 38.9 | 12 | 1.95 |
| P16045    | Galectin-1 | 14.9 | 5.9 | 1 | 2.02 |
| Q61171    | Peroxiredoxin-2 | 21.8 | 14.7 | 2 | 2.03 |
| P10107    | Annexin A1 | 38.7 | 10.4 | 3 | 2.19 |

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S-transferase Mu1) were decreased in the mdx DIA compared to control DIA while 7 proteins were increased in the mdx DIA compared with control DIA, including proteins involved in responses to stress (heat shock protein 75 kDa, mitochondrial and 78 kDa glucose-regulated protein), induction of apoptosis (galectin-1), oxidative (ROS) processes (peroxiredoxin-2) and finally serine-type endoprotease inhibitors (serpin H1 and murinoglobulin-1). Proteins involved in cellular respiration were also decreased in the mdx DIA, with 3 engaged in glycolysis (phosphoglucomutase-1, fructose-bisphosphate aldolase A and phosphoglycerate mutase 2), 2 in tricarboxylic acid cycle (ATP-citrate synthase and malate dehydrogenase) and 1 from the respiratory electron transport chain (amine oxidase [flavin-containing] B).

**Comparative proteomic profiling: DIA versus EOM**

About 13% (54 out of 415; Table 4) of the proteins showed differential abundance of peptide ions when comparing control DIA with control EOM and 15% (62 out of 415; Table 5) increased or decreased in the mdx DIA compared with the mdx EOM. By performing a further double comparison (i.e., control DIA×control EOM with mdx DIA×mdx EOM), 21 proteins were found in common, and may represent constitutive proteins related to embryological, morphological or functional differences between DIA and EOM muscles, rather than related to the pathogenesis of dystrophy per se. Among these 21 proteins, only annexin A1 showed a distinct pattern of change (increased or decreased) depending on the comparison (39% decreased in control DIA vs. control EOM and 51% increased in mdx DIA vs. mdx EOM). The remaining 20 proteins, most [11] were decreased in DIA vs. EOM (collagen alpha-1 and alpha-2; myosin 3, 4 and 11; SERCA 1; calsequestrin 1; sarcalumenin; aspartate aminotransferase; tropomyosin alpha -3 and mitochondrial 2-oxoglutarate/malate carrier protein), with a few [9] proteins increased in DIA×EOM (voltage-dependent anion selective channel protein 1; myosin 1, regulatory light chain 2 and light chain 3; uncharacterized protein C1orf93 homolog; isocitrate dehydrogenase (NADP) mitochondrial; L-lactate dehydrogenase B chain; C-X-C chemokine receptor type 1, and SERCA 2).

Some proteins [41] were found exclusively in the comparison of the mdx DIA with the mdx EOM and therefore are more likely to be directly involved in the processes of muscle degeneration-regeneration or to the protection against myonecrosis. Regarding their biological processes classifications, most of them (37.2%) were related to carbohydrate, lipid or protein metabolism. Some were related to the immune system processes: in the dystrophic DIA, 3 proteins (HSP beta-6, glutathione peroxidase 3 and ceruloplasmin) were decreased and 5 proteins (HSP 90-beta, HSP beta-1, peptidyl-prolyl cis-trans isomerase A, elongation factor 1-gamma and galectin-1) were increased.

To select proteins that could be directly involved in dystrophic muscle degeneration we made a further double comparison (mdx DIA×vs. control DIA with mdx DIA×vs. mdx EOM) and found 19 proteins in common. Among these proteins, the majority (11 proteins; protein disulfide isomerase, 40S ribosomal protein S19; peptidyl-prolyl cis-trans isomerase; 60S ribosomal protein L7, L29 and L34; 60S acidic ribosomal protein P1; annexin A1; serpin H1; galectin-1 and ADP/ATP translocase 2) were increased in the mdx DIA (which presents muscle degeneration-regeneration in comparison to mdx EOM). Fewer (6 proteins; myosin-4, calsequestrin 1, ATP-citrate synthase, glyceral-3-phosphate dehydrogenase, fructose bisphosphate aldolase A, reticulon-1-interacting protein 1) were increased in control DIA and in the mdx EOM (which do not show muscle degeneration). Two myosins were increased or decreased depending on the comparison; myosin light chain 3 was 30% decreased in mdx DIA vs. control DIA and 124% increased in mdx DIA vs. mdx EOM and myosin light chain 6B was 76% increased in mdx DIA vs. control DIA and 37% decreased in mdx DIA vs. mdx EOM.

**Western blot analysis**

Western blot was performed in order to independently validate the identification and quantification of some proteins in DIA and EOM muscles of control and mdx mice (Figure 2). Western blot analysis indicated that galectin-1 levels were significantly higher in mdx DIA compared to control DIA (75% increase) and to mdx EOM (60% increase; p≤0.05, ANOVA; Figure 2). This is in agreement with the proteomic analysis showing a significant increase of galectin-1 in mdx DIA compared with control DIA (fold change of 2.02, Table 2) and with mdx EOM (fold change of 1.63, Table 5).

Annexin A5 also presented similar changes as those detected with MudPIT-TMT for most of the comparisons, mainly the increased (20%) levels of this protein in mdx DIA vs. control DIA (Table 2) and no differences in mdx EOM vs. control EOM (Figure 2). While Western blot analysis showed lower (20%) levels of annexin A5 in control DIA in relation to control EOM, proteomics showed comparable levels of annexin A5 between these muscles (Figure 2, Table 4).

The proteins related to calcium homeostasis, calmodulin I and calsequestrin-1, also displayed similar expression changes using both Western blot and proteomics analyses. Calmodulin levels

**Table 3.** Localization of shotgun identified proteins with altered expression in the comparisons: mdx diaphragm (DIA)×control (ct) DIA; ct DIA×ct extraocular (EO); mdx DIA×mdx EO.

|                | mdx DIA×ct DIA | ct DIA×ct EO | mdx DIA×mdx EO |
|----------------|---------------|--------------|---------------|
| Sarcoplasm     | 20.9%         | 19.4%        | 30.0%         |
| Cytoskeleton   | 30.2%         | 21.0%        | 14.3%         |
| Extracellular Matrix | 7.0%   | 3.2%         | 10.0%         |
| Mitochondrion  | 18.6%         | 29.0%        | 17.1%         |
| Nucleus        | 14.0%         | 9.7%         | 4.3%          |
| Sarcomatema    | 2.3%          | 8.1%         | 15.7%         |
| Sarcomplasm reticulum | 4.7% | 6.5%         | 8.6%          |
| Peroxisome     | 2.3%          | 3.2%         | 0.0%          |

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### Table 4. The identified proteins that exhibit change in abundance in control diaphragm in comparison to control extraocular muscle.

| Accession  | Description                                              | MW [kDa] | Coverage | # Peptides | Fold Change |
|------------|-----------------------------------------------------------|----------|----------|------------|-------------|
| P08121     | Collagen alpha-1(III) chain                              | 138.9    | 0.8      | 1          | -4.12       |
| P13541     | Myosin-3                                                  | 223.7    | 31.8     | 66         | -3.42       |
| Q08638     | Myosin-11                                                 | 226.9    | 4.6      | 8          | -2.44       |
| Q8C43      | Myosin light chain 6B                                     | 22.7     | 34.3     | 7          | -2.31       |
| Q01149     | Collagen alpha-2(II) chain                               | 129.5    | 4.7      | 6          | -2.28       |
| Q7T48      | Sarcalumenin                                              | 99.1     | 27.8     | 18         | -2.18       |
| Q9C62      | Mitochondrial 2-oxoglutarate/malate carrier protein       | 34.1     | 12.4     | 4          | -2.04       |
| Q5X39      | Myosin-4                                                  | 222.7    | 57.9     | 133        | -1.87       |
| Q8R429     | Sarcoplasmic/endoplasmic reticulum calcium ATPase 1       | 109.4    | 31.7     | 31         | -1.76       |
| Q6PE5      | Sodium/potassium-transporting ATPase subunit alpha-2      | 112.1    | 10.9     | 11         | -1.63       |
| P05202     | Aspartate aminotransferase, mitochondrial                 | 47.4     | 22.8     | 10         | -1.51       |
| P10922     | Histone H1.0                                             | 20.8     | 20.6     | 4          | -1.43       |
| P10107     | Annexin A1                                               | 38.7     | 10.4     | 3          | -1.39       |
| Q8CG6      | Histone H2A type 1-H                                      | 13.9     | 27.3     | 3          | -1.36       |
| P21107     | Tropomyosin alpha-3 chain                                 | 32.8     | 49.3     | 21         | -1.35       |
| O09165     | Calsequestrin-1                                           | 45.6     | 23.8     | 10         | -1.33       |
| Q00896     | Alpha-1-antitrypsin 1–3                                   | 45.8     | 22.3     | 11         | -1.28       |
| Q6P66      | Leucine-rich PPR motif-containing protein, mitochondrial  | 156.5    | 3.1      | 5          | -1.28       |
| Q60714     | Long-chain fatty acid transport protein 1                | 77.9     | 19.9     | 12         | -1.25       |
| Q8S346     | Troponin T, slow skeletal muscle                          | 31.3     | 7.6      | 2          | 1.25        |
| P97447     | Four and a half LIM domains protein 1                     | 31.9     | 16.1     | 5          | 1.26        |
| Q8BM51     | Trifunctional enzyme subunit alpha, mitochondrial        | 51.4     | 29.5     | 14         | 1.27        |
| P50544     | Very long-chain specific acyl-CoA dehydrogenase, mitochondrial | 70.8    | 13.0     | 7          | 1.27        |
| P51174     | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | 47.9     | 25.8     | 12         | 1.28        |
| Q07417     | Short-chain specific acyl-CoA dehydrogenase, mitochondrial | 44.9     | 10.2     | 3          | 1.28        |
| Q99JY0     | Trifunctional enzyme subunit beta, mitochondrial          | 82.6     | 25.7     | 19         | 1.32        |
| P10649     | Glutathione S-transferase Mu 1                            | 25.9     | 28.9     | 6          | 1.32        |
| P58771     | Tropomyosin alpha-1 chain                                 | 32.7     | 72.2     | 34         | 1.34        |
| Q9CQ62     | 2,4-dienoyl-CoA reductase, mitochondrial                 | 36.2     | 27.2     | 8          | 1.35        |
| Q99LK0     | Protein DJ-1                                             | 20.0     | 8.0      | 2          | 1.35        |
| P15626     | Glutathione S-transferase Mu 2                            | 25.7     | 16.1     | 4          | 1.35        |
| Q60932     | Voltage-dependent anion-selective channel protein 1      | 32.3     | 41.6     | 10         | 1.37        |
| Q9DB60     | Uncharacterized protein C1orf93 homolog                  | 21.7     | 4.0      | 1          | 1.39        |
| Q91WC3     | Long-chain-fatty-acid-CoA ligase 6                        | 78.0     | 2.7      | 2          | 1.39        |
| P07310     | Creatine kinase M-type                                    | 43.0     | 55.4     | 21         | 1.40        |
| Q294X2     | Carnitine O-palmitoyltransferase 1, muscle isofom        | 88.2     | 4.3      | 3          | 1.40        |
| Q99LC5     | Electron transfer flavoprotein subunit alpha, mitochondrial | 35.0    | 19.8     | 5          | 1.42        |
| Q9DCW4     | Electron transfer flavoprotein subunit beta               | 27.6     | 31.4     | 9          | 1.43        |
| P35550     | rRNA 2`-O-methyltransferase fibrillarin                   | 34.3     | 9.2      | 2          | 1.43        |
| Q291G7     | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | 68.0   | 2.4      | 2          | 1.44        |
| P41216     | Long-chain-fatty-acid-CoA ligase 1                        | 77.9     | 19.9     | 12         | 1.51        |
| P50247     | Adenosylhomocysteinate                                    | 47.7     | 9.5      | 4          | 1.59        |
| Q8BW75     | Amine oxidase [flavin-containing] B                       | 58.5     | 2.7      | 2          | 1.66        |
| Q8C51      | PDZ and LIM domain protein 5                             | 18.0     | 35.4     | 6          | 1.67        |
| Q91Z83     | Myosin-7                                                  | 222.7    | 36.5     | 81         | 1.73        |
| P19157     | Glutathione S-transferase P 1                             | 23.6     | 12.9     | 2          | 1.78        |
| P16125     | L-lactate dehydrogenase B chain                           | 36.5     | 31.1     | 12         | 2.07        |
| Q55X40     | Myosin-1                                                  | 223.2    | 59.9     | 148        | 2.15        |
similar results were described in a recent proteomic study [33].

Dystroglycan (mdx control compared with protein in the affected muscle (PLOS ONE | www.plosone.org 7 June 2013 | Volume 8 | Issue 6 | e65831 Table 2) and with reduced in agreement with our previous work [31]. Calsequestrin-1 was (significance only detected by Western blot analysis) (Figure 2), in
tendency to be increased in the mdx DIA compared with control DIA (fold change of −1.29, Table 2) and with mdx EO (fold change of −1.78, Table 5).

Western blot analysis of β-dystroglycan expression confirmed previous studies [32] demonstrating a persistent expression of this protein in the mdx EO concomitant with a significant decrease in the affected muscle (mdx DIA; Figure 2C). We also found that dystroglycan (α and β) expression was decreased in mdx DIA compared with mdx EO (−1.12) and control EO (−1.11) and similar results were described in a recent proteomic study [33].

Discussion

Shotgun proteomic analysis

By using the shotgun proteomic analysis, we have here identified a total of 857 proteins in the DIA and EOM from control and dystrophic mdx mice at 2 months of age. Out of the 857 proteins, 42 had differential abundance of peptide ions in the DIA of the mdx mice. Previous studies using the DIGE proteomic analysis detected 2398 2D spots of which 19 [7] or 33 [8] proteins showed a differential abundance of spots in mdx DIA compared to control DIA. By using the 2D gel-based proteomic technique, one protein made from one gene may exhibit an average of 10–15 different spots [34] due to post-translational modifications and protein degradation [35], [36]. Additionally, spots could appear in the gels due to disulfide bridges because the current DIGE protocol does not require alklylation during the isoelectric focusing step [34]. Therefore, the fact that multiple spots correspond to one protein may explain the difference between the number of spots detected by DIGE and the number of proteins detected in the present study. Nevertheless, the present study demonstrates that the shotgun methodology allowed the identification and quantification of most proteins present in a small amount of muscle (100 μg) and in a short period of time (one run in the mass spectrometer).

In the dystrophic DIA, we observed that some proteins already displayed abnormal levels at this early stage of the disease (2 months of age). Some of the changed proteins detected in the DIA had not been described before in other proteomic studies, such as galectin-1, annexin, serpin H1 and peristin. We also found proteins that had been described by other proteomic techniques. For instance, by using DIGE analysis, malate dehydrogenase, myosin light chain 3, myosin light chain 6B, myosin-4, myosin-9 and vimentin were found to be altered in the mdx diaphragm at 9

| Accession | Description | MW [kDa] | Coverage | # Peptides | Fold Change |
|-----------|-------------|----------|----------|------------|-------------|
| PS4071    | Isocitrate dehydrogenase (NADP), mitochondrial | 50.9 | 30.7 | 13 | 2.25 |
| QR0096    | 10-formyltetrahydrofolate dehydrogenase | 98.6 | 0.9 | 1 | 2.57 |
| PS1667    | Myosin regulatory light chain 2, ventricular/cardiac muscle isofrom | 18.9 | 22.9 | 3 | 2.67 |
| OS5143    | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 | 114.8 | 17.5 | 19 | 2.73 |
| PO1542    | Myosin light chain 3 | 22.4 | 45.1 | 8 | 3.28 |
| Q81046    | C-X-C chemokine receptor type 1 | 40.0 | 1.71 | 1 | 3.78 |

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were similar among the groups (blot and proteome), with a tendency to be increased in the mdx EO compared to control EO (significance only detected by Western blot analysis) (Figure 2), in agreement with our previous work [31]. Calsequestrin-1 was reduced in mdx DIA compared with control DIA (23% reduction) and with mdx EO (42% reduction; Figure 2) in line with the proteomic finding showing a significant decrease of calsequestrin-1 in mdx DIA compared with control DIA (fold change of −1,29, Table 2) and with mdx EO (fold change of −1,78, Table 5).

Proteins related to protection against myonecrosis

The comparison of EOM with DIA muscle revealed altered expression of cytoskeletal proteins (myosin and tropomin) and of extracellular matrix components, such as collagen. Furthermore, proteins related to calcium homeostasis and ion channels also exhibited different expression levels. The differential levels of calsequestrin1, SERCA1 and SERCA2 observed here, possibly related to fiber type, suggest a better calcium homeostasis, and consequent protection against myonecrosis, in the EOM compared with DIA. This finding is in agreement with previous observations in the mdx EOM using Western blotting analysis [31]. Other mdx spared muscles, such as the intrinsic laryngeals, also show higher levels of SERCA1 in comparison to normal ILM muscles [40]. Moreover, we observed that the calcium buffering proteins sarcalumenin and calsequestrin 1 [41], [42] were increased in EOM compared to DIA, even in the control group. Therefore, the present results support previous observations (with Western blotting, ELISA and immunocytochemistry techniques) that constitutional properties of the EOM compensate for the lack
Table 5. The identified proteins that exhibit change in abundance in *mdx* diaphragm in comparison to *mdx* extraocular muscle.

| Accession | Description | MW [kDa] | ΣCoverage | Σ# Peptides | Fold Change |
|-----------|-------------|----------|-----------|-------------|-------------|
| P08121    | Collagen alpha-1(III) chain | 138.9    | 0.8       | 1           | −3.64       |
| Q5SX39    | Myosin-4    | 222.7    | 57.9      | 133         | −3.46       |
| O08638    | Myosin-11   | 226.9    | 4.6       | 8           | −3.36       |
| P32848    | Parvalbumin alpha | 11.9    | 74.6      | 15          | −2.86       |
| P13541    | Myosin-3    | 223.7    | 31.7      | 66          | −2.73       |
| Q8R429    | Sarcoplasmic/endooplasmic reticulum calcium ATPase 1 | 109.4 | 31.7 | 31 | −2.03 |
| Q01149    | Collagen alpha-2(II) chain | 129.5 | 4.7 | 6 | −1.99 |
| P11087    | Collagen alpha-1(II) chain | 137.9 | 4.9 | 6 | −1.88 |
| O09165    | Calsequestrin-1 | 45.6 | 23.8 | 10 | −1.78 |
| Q91V92    | ATP-citrate synthase | 119.7 | 3.2 | 3 | −1.75 |
| P21550    | Beta-enzolase | 47.0 | 28.6 | 14 | −1.72 |
| Q9Z1E4    | Glycogen [starch] synthase, muscle | 83.9   | 7.6      | 5           | −1.63       |
| Q7TQ48    | Sarcalumenin | 99.1 | 27.8 | 18 | −1.63 |
| P13707    | Glycerol-3-phosphate dehydrogenase [NAD⁺], cytoplasmic | 37.5 | 15.5 | 5 | −1.62 |
| P05202    | Aspartate aminotransferase, mitochondrial | 47.4 | 22.8 | 10 | −1.62 |
| P21107    | Tropomyosin alpha-3 chain | 32.8 | 49.3 | 21 | −1.62 |
| P22599    | Alpha-1-antitrypsin 1–2 | 45.9 | 24.5 | 10 | −1.59 |
| P19096    | Fatty acid synthase | 272.3 | 3.2 | 7 | −1.53 |
| Q00898    | Alpha-1-antitrypsin 1–5 | 45.9 | 22.3 | 10 | −1.52 |
| Q5EBG6    | Heat shock protein beta-6 | 17.5 | 21.0 | 3 | −1.46 |
| P46412    | Glutathione peroxidase 3 | 25.4 | 15.9 | 3 | −1.44 |
| Q3MU70    | [Pyruvate dehydrogenase [acetyl-transferring]]-phosphatase 1, mitochondrial | 61.1 | 1.9 | 1 | −1.42 |
| Q6PB7     | Creatine kinase S-type, mitochondrial | 47.4 | 29.1 | 13 | −1.42 |
| P07759    | Serine protease inhibitor A3K | 46.8 | 17.5 | 6 | −1.42 |
| P05064    | Fructose-bisphosphate aldolase A | 39.3 | 44.5 | 18 | −1.41 |
| Q8C434    | Myosin light chain 6B | 22.7 | 34.3 | 7 | −1.37 |
| Q9WUB3    | Glycogen phosphorylase, muscle form | 97.2 | 34.6 | 30 | −1.32 |
| Q9CRB8    | Mitochondrial fission process protein 1 | 18.3 | 14.5 | 2 | −1.29 |
| Q61147    | Ceruloplasmin | 121.1 | 1.5 | 1 | −1.28 |
| Q9CR62    | Mitochondrial 2-oxoglutarate/malate carrier protein | 34.1 | 12.4 | 4 | −1.28 |
| Q8BH59    | Calcium-binding mitochondrial carrier protein Aralar1 | 74.5 | 31.2 | 15 | −1.25 |
| Q924D0    | Reticulon-4-interacting protein 1, mitochondrial | 43.3 | 3.3 | 1 | −1.25 |
| P11499    | Heat shock protein HSP 90-beta | 83.3 | 24.6 | 17 | 1.25 |
| P09103    | Protein disulfide-isomerase | 57.1 | 12.8 | 7 | 1.26 |
| Q60932    | Voltage-dependent anion-selective channel protein 1 | 32.3 | 41.6 | 10 | 1.29 |
| Q9CXX8    | 40S ribosomal protein S19 | 16.1 | 18.6 | 3 | 1.30 |
| Q62009    | Periostin | 93.1 | 1.7 | 1 | 1.33 |
| Q9CD5     | Mitochondrial-processing peptidase subunit beta | 54.6 | 3.7 | 2 | 1.34 |
| P14602    | Heat shock protein beta-1 | 23.0 | 27.3 | 5 | 1.35 |
| Q8VOD5    | Myosin-9 | 226.2 | 6.3 | 11 | 1.36 |
| P15864    | Histone H1.2 | 21.3 | 33.0 | 9 | 1.40 |
| P17742    | Peptidyl-prolyl cis-trans isomerase A | 18.0 | 35.4 | 6 | 1.43 |
| P14148    | 60S ribosomal protein L7 | 31.4 | 12.2 | 3 | 1.44 |
| Q90956    | Calsequestrin-2 | 48.2 | 12.1 | 5 | 1.45 |
| Q5SX40    | Myosin-1 | 223.2 | 59.9 | 148 | 1.46 |
| Q9D1R9    | 60S ribosomal protein L34 | 13.3 | 6.0 | 1 | 1.47 |
| P47955    | 60S acidic ribosomal protein P1 | 11.5 | 14.0 | 1 | 1.49 |
| Q9DK2     | Succinyl-CoA3-ketoacid-coenzyme A transferase 1, mitochondrial | 56.0 | 8.3 | 3 | 1.50 |
| P10107    | Annexin A1 | 38.7 | 10.4 | 3 | 1.51 |
| Accession | Description                                                                 | MW [kDa] | Coverage | # Peptides | Fold Change |
|-----------|------------------------------------------------------------------------------|----------|----------|------------|-------------|
| P19324    | Serpin H1                                                                     | 46.6     | 10.8     | 3          | 1.52        |
| P47915    | 60S ribosomal protein                                                         | 17.6     | 5.0      | 1          | 1.52        |
| Q9D8N0    | Elongation factor 1-gamma                                                     | 50.0     | 7.8      | 4          | 1.56        |
| P16045    | Galectin-1                                                                   | 14.9     | 5.9      | 1          | 1.63        |
| Q9D860    | Uncharacterized protein C1orf93 homolog                                       | 21.7     | 4.0      | 1          | 1.71        |
| P51881    | ADP/ATP translocase 2                                                         | 32.9     | 38.9     | 12         | 1.81        |
| P54071    | Isocitrate dehydrogenase [NADP], mitochondrial                               | 50.9     | 30.7     | 13         | 1.82        |
| P16125    | L-lactate dehydrogenase B chain                                              | 36.5     | 32.6     | 11         | 1.87        |
| P51667    | Myosin regulatory light chain 2, ventricular/cardiac muscle isoform           | 18.9     | 75.7     | 15         | 2.07        |
| P11404    | Fatty acid-binding protein, heart                                             | 14.8     | 48.9     | 7          | 2.19        |
| P09542    | Myosin light chain 3                                                          | 22.4     | 45.1     | 8          | 2.25        |
| Q810W6    | C-X-C chemokine receptor type 1                                               | 40.0     | 1.7      | 1          | 3.27        |
| O55143    | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2                           | 114.8    | 17.5     | 19         | 3.91        |

**Table 5.** Cont.

Figure 2. Western blot analysis of some proteins revealed by proteomics. Quantification of galectin-1 (Gal-1), annexin A5 (ANXA5), β-dystroglycan (β-DG), calmodulin I (CaM I) and calsequestrin-1 (CSQ-1) by Western blot analysis in crude extracts of diaphragm (DIA) and extraocular (EO) muscles from control (ct) and dystrophic (mdx) mice. In A, Western blot of proteins. In B, the same blot reprobed for GAPDH as a loading control. Graphs represent the level of proteins expressed in pixels. Bars represent standard deviation. a Significantly different from ct DIA (p < 0.05, ANOVA). b Significantly different from mdx EOM (p < 0.05, ANOVA).

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of dystrophin, allowing a better response against myonecrosis [11], [49].

Proteins related to degeneration and regeneration

Oxidative stress and fibrosis. Proteins involved in the oxidative stress response (HSP 75 kDa, 70 kDa glucose-related protein, serpin H1 or HSP 47 and peroxiredoxin-2) were increased in the mdx DIA compared to control DIA. While peroxiredoxins are antioxidant enzymes that control peroxide levels induced by cytokines [44], the HSPs have chaperone functions [45]. The higher levels of these proteins may reflect an attempt of the dystrophic DIA to control oxidative stress at this stage of the disease [46], [47]. However, HSP 47 is also related to increased collagen production and fibrosis [48], which will be morphologically prominent in the mdx DIA but only at later stages of the disease [29], as demonstrated by proteomics [13]. Interestingly, several proteins related to fibrosis that were demonstrated to be increased in the old mdx DIA muscle compared with younger mdx DIA, such as collagen x1 (VI) chain, minecan and actinin-92 [13] had no differential abundance of peptide ions at the age studied here (comparing mdx DIA×control DIA in the present study). This suggests that time-related changes of the proteomic profile occur and this may be of relevance for future studies of drug therapy for DMD.

Inflammation, apoptosis and regeneration. We detected an increased level of the annexins A1 and A5 in mdx DIA. The annexins bind to negative charged phospholipids in a calcium-dependent manner and participate in many physiological processes, such as cell shape changes, transport and organization of vesicles, exocytosis and endocytosis [49]. Annexin A1 prevents muscle degeneration due to sarcolemna resealing repair [50], [51]. Extracellular annexins act in fibrinolysis, coagulation and apoptosis [52], [53]. The annexins A1 and A2 are overexpressed in different forms of muscular dystrophies, possibly related to their anti-inflammatory activity [54]. Annexin A1 is a glucocorticoid-inducible protein [55], [56] able to mimic the anti-inflammatory effects of glucocorticoids in several experimental models of inflammation both in vivo and in vitro [57]. Besides participating in apoptotic processes, annexin A5 presents anti-inflammatory properties by inhibiting phospholipase A2 and phosphatidylserine-catalyzed inflammation [58], [59]. Therefore, the increased level of annexins early in the mdx DIA may suggest a potential ability of the dystrophic DIA to modulate inflammation.

Reticulon-1–interacting protein 1 is a mitochondrial protein that reduces the anti-apoptotic activity of Bcl-2 and Bcl-XL [60], [61]. annexin A5 and participates in muscle regeneration [71]–[76], and may be involved in the regenerative ability of the dystrophic DIA during this period, since at later stages fibrosis is a hallmark of diaphragm dystrophy. Galectin-1 also has a protective effect on skeletal muscle by reducing inflammation [77].

Conclusions

In the present study we demonstrated that the shotgun proteomics approach adds to the former proteomic techniques [7]–[10], [12], [13], [37] as a suitable alternative to track possible changes in the levels of proteins in dystrophic muscles, during the early phase of the disease. We would like to highlight some advantages of the technique that include the small amount of sample required, the relatively short time to accomplish the analysis and the possibility to perform qualitative and quantitative comparisons between distinct muscles and experimental groups.

The current proteomics study of the dystrophic DIA, in the phase prior to more advanced disease [13], [26], demonstrates an increase in proteins involved in muscle regeneration (galectin-1 [76], [77]), calcium handling (calsequestrin 1 [31]), inflammation (annexin A1 [50], [51], [54]) and fibrosis (HSP 47 [48]), making them valuable candidates for being potential drug targets and exploratory biomarkers.

Supporting Information

Table S1 Proteins identified in extraocular and diaphragm muscles of control and mdx mice in three biological replicates.

(XLS)

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Author Contributions

Conceived and designed the experiments: CYM MD BMdO MJM. Performed the experiments: CYM BMdO. Analyzed the data: CYM MD BMdO MJM. Contributed reagents/materials/analysis tools: MD. Wrote the paper: MJM CYM BMdO MD.

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