Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder caused by a mutation in the dystrophin gene. DMD is characterized by progressive weakness of skeletal, cardiac, and respiratory muscles. The molecular mechanisms underlying dystrophy-associated muscle weakness and damage are not well understood. Quantitative proteomics techniques could help to identify disease-specific pathways. Recent advances in the in vivo labeling strategies such as stable isotope labeling in mammals (SILAM) with $^{15}$N have enabled accurate quantitative analysis of the proteomes of whole organs and tissues as a function of disease. Here we describe the use of the SILAC mouse strategy to define the underlying pathological mechanisms in dystrophin-deficient skeletal muscle. Differential SILAC proteome profiling was performed on the gastrocnemius muscles of 3-week-old (early stage) dystrophin-deficient mdx mice and wild-type (normal) mice. The generated data were further confirmed in an independent set of mdx and normal mice using a SILAC spike-in strategy. A total of 789 proteins were quantified; of these, 73 were found to be significantly altered between mdx and normal mice ($p < 0.05$). Bioinformatics analyses using Ingenuity Pathway software established that the integrin-linked kinase pathway, actin cytoskeleton signaling, mitochondrial energy metabolism, and calcium homeostasis are the pathways initially affected in dystrophin-deficient muscle at early stages of pathogenesis. The key proteins involved in these pathways were validated by means of immunoblotting and immunohistochemistry in independent sets of mdx mice and in human DMD muscle biopsies. The specific involvement of these molecular networks early in dystrophic pathology makes them potential therapeutic targets. In sum, our findings indicate that SILAC mouse strategy has uncovered previously unidentified pathological pathways in mouse models of human skeletal muscle disease. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.023127, 1061–1073, 2013.

Dystrophin is an essential skeletal muscle protein that interacts with other glycoproteins such as the dystroglycans and sarcoglycans to form the dystrophin glycoprotein complex. This complex links the extracellular matrix and the cytoskeleton of the myofiber via F-actin, thereby protecting the skeletal muscle membrane against contraction-induced damage (1). The absence of this complex due to the lack of expression of dystrophin makes the myofiber membrane susceptible to damage, which in turn activates various pathogenic processes and aberrant signaling cascades (2–4). Immune-mediated mechanisms are considered one of the key contributors to muscle degeneration in dystrophin-deficient subjects. However, the explicit role of specific pathogenic processes in this disease has not been thoroughly investigated.

Dystrophin-deficient mdx mice are one of the most widely used animal models for studying disease pathophysiology and testing various therapeutic regimens (5). The mdx-23 mouse model on a C57BL/10 background is a spontaneous mutant with a point mutation in exon 23 of the dystrophin gene that eliminates the expression of dystrophin (6). However, this mutation does not disrupt the expression of shorter isoforms that are also expressed from the dystrophin gene through differential promoter usage. Another mutant mdx mouse model, mdx-52 on a C57BL/6 background, has been generated by disrupting the dystrophin gene through gene targeting and the deletion of exon 52. The mdx-52 mice lack both dystrophin and the shorter dystrophin isoforms (Dp140 and Dp260). The skeletal muscles of mdx-52 mice exhibit a pathological profile similar to that of mdx-23 mice (7). These mdx strains show a mild phenotype relative to the human disease, but nevertheless display substantial myofiber degeneration, muscle weakness, elevated serum creatine kinase,
and extensive inflammatory infiltrates in the muscle tissue (5, 8). Initial disease onset in mdx mice occurs around 3 weeks of age, with recurring bouts of myofiber degeneration and regeneration. These bouts are limited by 12 to 16 weeks of age, but the tissue infiltration and muscle weakness continue for the remainder of the animal’s life. Thus, this model continues to be important for studying the consequences of dystrophin deficiency and alteration in the molecular events that lead to muscle pathology. For our studies, we have used mdx-52 mice that are on the C57BL/6 background.

The investigation of protein dynamics and their involvement in signaling pathways in the course of dystrophinopathy can provide valuable insight into its pathogenesis. Protein modulations can be monitored using mass-spectrometry-based quantitative strategies. In the past, two-dimensional gel electrophoresis and fluorescence difference in gel electrophoresis methods have been used to study protein changes in the muscle of dystrophic mdx mice. Traditional proteomic techniques (2-DE) suffer from a disadvantage in that they detect alterations predominantly in abundantly expressed proteins. Furthermore, prior studies using the mdx mouse model focused on established disease instead of early disease, in which limited pathways drive the pathology. Proteomic profiling of established disease has identified perturbations in Ca²⁺ handling and bioenergetic pathways but not specific mechanisms that are upstream in the pathogenesis (9–12). Therefore, we performed proteomic studies of early disease stages in the mdx mouse model.

The stable isotope labeling by amino acids in cell culture (SILAC) strategy introduced by Ong et al. has been successfully implemented in various cell culture systems and has been proven to be the most accurate method for proteome profiling (13–17). More recently, this powerful technique has been extended to in vivo studies to develop heavy-stable-isotope-labeled mammals. This method has been validated in both mice (SILAC mouse) and rats (SILAM) (18–20). In the SILAC mouse strategy, the mice feed consists of a balanced synthetic feed labeled with 13C6-lysine, whereas in SILAM, the feed consists of 15N-labeled algae. These techniques enabled accurate measurement of differentially expressed proteins in different organs and tissues of mice and rats under different conditions (18, 19, 21–23). Here we have extended the use of the SILAC mouse strategy to study the underlying molecular alterations in gastrocnemius muscle in the early phase of dystrophic muscle disease. To our knowledge, this is the first proteomics study of dystrophic skeletal muscle using an in vivo labeling strategy. With this method, we have not confirmed the previously identified pathways (mitochondria and energy metabolism) that are differentially modulated in Duchenne muscular dystrophy (DMD), but also uncovered novel pathways such as actin cytoskeletal and integrin-linked kinase (ILK) signaling pathways that are implicated in dystrophic pathology.

**MATERIALS AND METHODS**

*Animals and Feed—*C57BL/6 control mice and dystrophin-deficient mdx-52 mice weighing 20 to 25 g were used for breeding to generate SILAC-labeled and unlabeled mice using custom-made mouse feed as described below. The mdx-52 mice were on a C57BL/6 background (7). All animals were handled according to Institutional Animal Care and Use Committee guidelines at the Children’s National Medical Center (Approved Protocol No. 199-07-01).

*Generating 13C-lysine-C57BL/6-SILAC Mice—*We followed the method described by Kruger et al. to generate SILAC mice (18). Mouse-Express feed containing “heavy” L-lysine (13C6, 99%) or “light” L-lysine (12C6, 99%) at the 1% level that adhered to standard laboratory mouse nutritional standards was purchased from Cambridge Isotope Laboratories (Andover, MA). In this study, we arbitrarily chose to label wild-type C57BL/6 mice. Breeding pairs were set up, and after the confirmation of pregnancy, dams were fed the custom 13C6-lysine diet and breeding was continued to obtain F2-generation litters. In parallel, dystrophin-deficient mdx-52 breeding pairs were maintained on unlabeled custom feed (12C6-lysine) and bred to obtain F2-generation mdx litters.

For all validation experiments, an independent set of C57BL/6 and mdx-52 mice (n = 3/group) that had been maintained on non-custom (normal) feed was used. All animals were housed in an individually vented cage system under a controlled 12-hour light/dark cycle with free access to feed and water.

*Sample Collection—*SILAC mice and age-matched mdx mice were perfused with phosphate-buffered saline to remove excess blood from organs and tissues and were then euthanized using CO₂. All organs including muscle tissues were harvested and flash-frozen in liquid nitrogen-chilled isopentane. The collected tissues were stored at −80 °C until use. Liver, gastrocnemius, and brain were collected from “labeled” C57BL/6 mice of each generation (F0, F1, and F2) for genotyping and expressed as a percentage of the unlabeled C57BL/6 control mice (18). For differential proteomic analysis between normal and dystrophic mdx mice, tissues were collected from F2-generation labeled C57BL/6 mice and age-matched unlabeled mdx mice. Tissues were collected at 3, 6, and 12 weeks of age from labeled C57BL/6 and mdx mice (n = 2/age/group) for this study, as well as for other projects. In the current study, we analyzed 3-week-old mdx gastrocnemius to identify early changes in the dystrophic skeletal muscle proteome.

*Monitoring Labeling Efficiency (13C-lysine) in SILAC Mouse Tissues—*Protein lysates were prepared from tissue samples harvested from the labeled C57BL/6 at F0, F1, and F2 generations. Aliquots (50 μg) of protein lysate from each extract were separated via SDS-PAGE and stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA). Individual bands were excised and digested with trypsin, and the resulting peptides were analyzed via LC-MS/MS as described below. Raw spectra were analyzed using Integrated Proteomics Pipeline (IP2 software version 1.01), developed by Integrated Proteomics Applications, Inc (San Diego, CA). Labeling efficiency was determined from unlabeled to labeled peptide ratios obtained for all identified proteins in each tissue (liver, brain, and muscle). These ratios were converted into percentages and then averaged to obtain the overall labeling efficiency for each respective tissue at each generation.

*Sample Processing and Mass Spectrometry Analysis to Identify Proteomic Alterations—*Gastrocnemius muscle was collected from 3-week-old F2-generation SILAC-labeled C57BL/6 mice and age-matched unlabeled dystrophic mdx mice. Gastrocnemius muscle was also collected from age-matched unlabeled C57BL/6 mice. Total proteins were extracted from each muscle with RIPA buffer (50 mM...
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Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (Nonidet P-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with protease inhibitors (Halt protease inhibitor mixture 100X). Aliquots of the protein extracts from the muscles of unlabeled C57BL/6 and unlabeled mdx mice were each mixed 1:1 (50 μg) with protein extract from the muscle of a SILAC-labeled mouse. The protein concentration was estimated via BCA protein assay (Pierce). Labeled and unlabeled protein mixtures were further resolved via SDS-PAGE. The gel was stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA), and each lane was cut into 30 to 35 serial slices. Proteins in each gel slice were in-gel digested with trypsin. The resulting peptides from each band were injected via an autosampler (6 μl) and loaded onto a Symmetry C18 trap column (5 μm, 300 μm inner diameter × 23 mm, Waters Milford, MA) for 10 min at a flow rate of 10 μl/min with 0.1% formic acid. The sample was subsequently separated on a C18 reversed-phase column (3.5 μm, 75 μm × 15 cm, LC Packings Sunnyvale, CA) at a flow rate of 250 nl/min using a Nano-HPLC system from Eksigent (Dublin, CA). The mobile phases consisted of water with 1.0% formic acid (A) and 90% acetonitrile (B). A 65-min linear gradient from 5% to 40% B was employed. Eluted peptides were introduced into the mass spectrometer via a 10-μm silica tip (New Objective Inc., Ringoes, NJ) adapted to a nano-electrospray source (ThermoFisher Scientific). The spray voltage was set at 1.2 kV, and the heated capillary at 200 °C. The LTQ-Orbitrap-XL (ThermoFisher Scientific) was operated in data-dependent mode with dynamic exclusion, in which one cycle of experiments consisted of a full MS survey scan in the Orbitrap (300–2000 m/z, resolution = 30,000) and five subsequent MS/MS scans in the LTQ of the most intense peaks, using collision-induced dissociation with the collision gas (helium) and normalized collision energy value set at 35%.

Database Search and SILAC Ratio Measurement—Protein identification and quantification were performed using IP2 software (version 1.01). Mass spectral data were uploaded into the IP2 software. Files from each lane were searched against the forward and reverse Uniprot mouse database (UniProt release 15.15, March 2010, 16,333 forward entries) for partially tryptic peptides, allowing two missed cleavages and the possible modification of oxidized methionine (15.99492 Da) and heavy Lys (6.020 Da). IP2 uses the Sequest 2010 database from rabbit protein database (UniProt release 15.15, March 2010, 16,333 entries) for partially tryptic peptides, allowing two missed cleavages and the possible modification of oxidized methionine (15.99492 Da) and heavy Lys (6.020 Da). IP2 uses the Sequest 2010

Data Validation Using Spike-in Strategy—To increase the robustness and to statistically validate the data obtained in the initial differential SILAC experiments using mdx and wild-type mice, we employed a spike-in SILAC strategy that was previously used in cell culture systems (24, 25). In brief, gastrocnemius muscle lysates were obtained from both unlabeled C57BL/6 (n = 3) and unlabeled mdx (n = 3) mice. These lysates were spiked with equal amounts of lysate from labeled C57BL6 mice that was used as reference. Downstream sample preparation and MS analysis were performed as described above. To identify significant protein alterations between the mdx and wild-type groups, the mean relative ratio (unlabeled/labeled values) was compared for each protein between mdx (n = 3) and control (n = 3) mice using a non-parametric Wilcoxon rank sum test. Significance was set at p < 0.05, and no adjustments for multiple testing were performed.

Ingenuity Pathway Analysis to Determine the Molecular Mechanisms Implicated in Dystrophic Muscle—A bioinformatics approach was used to elucidate the global implications of differentially expressed proteins in dystrophic muscle. Ingenuity computational pathway analysis (IPA) (Ingenuity Systems, Redwood City, CA) software was applied to identify potentially perturbed molecular pathways in dystrophic muscle. The IPA program uses a knowledge database derived from the literature to relate the proteins to each other based on their interaction and function. The knowledge base consists of a high-quality expert-curated database containing 1.5 million biological findings consisting of more than 42,000 mammalian genes and pathway interactions extracted from the literature. In brief, proteins that were confidently identified in at least two samples (of both C57BL/6 and mdx comparisons) were considered for IPA analysis. All proteins that fell into the specified criteria were shortlisted, SILAC ratios were converted to fold changes and uploaded into the IPA software. Ingenuity then used these proteins and their identifiers to navigate the curated literature database and extract the overlapping network(s) among the candidate proteins. Associated networks were generated, along with a score representing the log probability of a particular network being found by random chance. Top canonical pathways associated with the uploaded data were presented, along with a p value. The p values were calculated using right-tailed Fisher’s exact tests.

Validation Using Biochemical Assays—

Immunoblotting—Gastrocnemius protein lysates (25 µg of protein) from dystrophic (n = 3) and control (n = 3) muscles were mixed with 4x NuPage LDS buffer (Invitrogen) supplemented with 50 mM DTT, heated for 5 min at 85 °C, loaded on Novex® 4%–12% Tris acetate mini gels (Invitrogen), and electrophoresed at 150 V in MOPS running buffer (20X) for 90 min at room temperature. Separated proteins were transferred at 300 mA for 90 min at room temperature onto a nitrocellulose membrane (Millipore Billerica, MA). Membranes were blocked in TBS-T (20 mM Tris, 500 mM NaCl, pH 7.5, with 0.1% Tween 20) supplemented with 5% nonfat dry milk (Bio-Rad) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies against desmin (1:2000; Santa Cruz), annexin-2 (1:1000; Santa Cruz), ILK (1:1000; Santa Cruz), vimentin, collagen, and profilin (1:1000; Epitomics Burlington, MA). All antibodies were diluted in TBS-T-5% milk. Membranes were washed three times (for 10 min each time) in TBS-T and incubated with goat anti-rabbit or rabbit anti-mouse secondary antibodies (Dako, Carpinteria, CA) conjugated to horseradish peroxidase (1:3000 in TBS-T-5% milk) for 1 h at room temperature. The protein bands were revealed with ECL chemiluminescence substrate (Amersham Biosciences). Membranes were then stripped and exposed to β-actin antibody as a loading control. For quantification, the x-ray films were scanned, and densitometry analysis was carried out using a BioRad GS-800 calibrated densitometer running Quantity One software (Bio-Rad). Ratios of the optical density of each specific protein to the corresponding β-actin were compared between mdx and C57BL/6 samples in order to determine significant differences.

Lactate Dehydrogenase and Citrate Synthase activity—Lactate dehydrogenase activity was monitored in mdx (n = 3) and C57BL/6 (n = 3) muscle lysates as described earlier (26). In brief, 2.5 μl of protein extract (1.2 dilution) and 225 μl of assay buffer (2.5 ml of 1 M Tris (pH 7.6), 500 μl of 200 mM EDTA, 500 μl of 5 mM NADH,H+, and 48 ml water) were used to measure enzyme activity. The oxidation of NADH,H+ was recorded after pyruvate addition (10 μl, 100 mM). NADH fluorescence was detected using a luminescence/fluorescence analyzer (Mithras LB 940, Berthold Technologies Bad Wildbad, Germany). Lactate dehydrogenase activity was normalized to the protein concentration and expressed as the mean ± S.E.

Citrate synthase activity (EC 4.1.3.7) was measured in mdx (n = 3) and C57BL/6 (n = 3) muscle lysates as described earlier (26). In brief, 2.5 μl of protein extract (1.30 dilution, v/v) was added to 225 μl of
assay buffer (100 mM Tris, pH 8.0, 2 mM EDTA, 1.25 mM L-malate, 0.25 mM NAD), 0.01% Triton X-100 (v/v), and 6 U/ml malate dehydrogenase (Sigma) to monitor the enzyme activity. The production of NADH, recorded after the addition of 5 μl acetyl-CoA (50 μM).

Enzyme activities were fluorometrically measured (excitation, 340 nm; emission, 450 nm) and represented as the mean ± S.E.

Immunohistochemical Staining—Frozen human muscle biopsies of mutation-defined dystrophin-deficient (DMD) (n = 3) and control (normal) (n = 3) tissues were obtained without any identifiers (IRB Protocol No. 2405). Muscle tissues were sectioned and immunostained using rabbit anti-vimentin (Epitomics) and mouse anti-ILK antibodies (Santa Cruz) and HRP-conjugated anti-rabbit or anti-mouse (Dako, Carpinteria, CA) as the primary and secondary antibodies, respectively. As a specificity control, other serial sections were stained with secondary antibody alone.

For all validation assays, the statistical significance between mdx and C57BL/6 parameters was determined using Student’s t test. For all measurements, p < 0.05 was considered a statistically significant difference.

RESULTS

The incorporation of 13C6-lysine Complete by F2 Generation—The efficiency of 13C6-lysine incorporation was monitored in the liver, brain, and muscle tissue of generations F0, F1, and F2. Mice fed with the custom diet showed progressive incorporation of the 13C-Lys into the proteins of different tissues at each generation (Figs. 1A and 1B). Most proteins were fully labeled (labeling efficiency ≥ 96%) in the liver, muscle, and brain by the F2 generation (Fig. 1B). Liver proteins showed the fastest incorporation rates for heavy lysine, as the relative abundance of heavy peptide was higher even at
F0 (Fig. 1A, liver). In contrast, brain and muscle tissues showed slower incorporation rates (Fig. 1A). By F2 generation, the percent labeling efficiency (mean ± S.E.) by heavy lysine was 97.88 ± 0.38, 96.50 ± 0.95, and 98.26 ± 0.38 in liver, brain, and muscle tissues, respectively. Furthermore, we found that the heavy-lysine diet did not affect the overall health of the C57BL/6 mice, including with regard to their body weight and fertility.

Differential Protein Expression in the Gastrocnemius of Dystrophin-deficient mdx Mice Relative to Age-matched C57BL/6 Controls—Signs of muscle necrosis start at about 3 weeks of age in mdx mice; therefore, we investigated the skeletal muscle proteome at this early stage. We performed differential proteome profiling of the skeletal muscle in pairs of SILAC-labeled-C57BL/6 and unlabeled-C57BL/6 mice and pairs of SILAC-labeled C57BL/6 and unlabeled dystrophic mdx mice. The control experiment showed a narrower distribution of unlabeled-to-labeled protein ratios, with an average mean ± S.D. of 0.96 ± 0.5, indicating that there were fewer differences in the skeletal muscle proteome between the two wild-type mice examined (Fig. 2; supplemental Tables S1 and S2). In contrast, the comparison of unlabeled mdx and SILAC-labeled C57BL/6 mice showed a wider distribution of protein ratios, with an average mean ± S.D. of 1.52 ± 1.3, indicating
that there are several proteins whose relative abundances are significantly altered in the skeletal muscle proteome of mdx mice relative to C57BL/6 control mice (Fig. 2).

Furthermore, Figs. 3A and 3B show a representative mass spectrum and extracted ion chromatogram for a peptide belonging to dystrophin protein that is completely absent from mdx muscle but present in C57BL/6 muscle. Figs. 3E and 3F show data for a peptide of talin-1 that was significantly up-regulated in mdx muscle relative to C57BL/6 muscle. In contrast, Figs. 3C and 3D show data for glyceraldehyde phosphate dehydrogenase protein, which remained unchanged between mdx and C57BL/6 muscle. Taken together, these data indicate that the SILAC strategy can efficiently detect specific protein alterations in dystrophin-deficient skeletal muscle relative to normal skeletal muscle.

Differentially Altered Proteins and Pathways Identified in Dystrophic Muscle—In the initial proteomic analysis, 750 to 850 proteins (with 2 unique peptides) were identified and quantified in gastrocnemius muscle. Among these, ~250 proteins were found to be differentially altered in their levels by at least a factor of 1.5 in mdx muscle relative to C57BL6 control muscle. These proteins mainly belonged to the muscle cytoskeleton, mitochondrial energy metabolism, glycolysis, citric acid cycle, sarcoplasmic reticulum, and calcium homeostasis pathways. Further, to validate the initial proteomic data, we used a spike-in strategy in an independent set of mdx mice (n = 3) and C57BL/6 control mice (n = 3) that were maintained on regular feed. These analyses identified ~75 proteins whose abundance was significantly altered (p < 0.05) between the mdx and wild-type mice (Table I; supplemental Table S3). Proteins such as vimentin, annexin-2, and desmin were found to be up-regulated (>2.5-fold) in the muscle of mdx mice relative to C57BL6 mice, whereas proteins such as myosin and tropomyosin were found to be significantly down-regulated (~2.0-fold) in mdx relative to C57BL6 muscle (Table I). IPA of the proteome profiling data revealed significant (p < 0.05) alterations in mitochondrial function, energy metabolism (citrate cycle, glycolysis), actin cytoskeleton signaling, and ILK pathways in dystrophin-deficient skeletal muscle, indicating their potential role in early dystrophic pathology (supplemental Fig. S1).

The ILK Pathway Is Up-regulated in Dystrophic Muscle—Once differential SILAC proteome profiling and IPA identified the involvement of the actin cytoskeleton and ILK pathways in dystrophin-deficient muscle pathology, we sought to validate these findings using independent assays and an independent set of mice that were maintained on regular mouse feed. Proteins involved in this pathway and those that were found to be differentially altered in our proteome profiling experiments, such as vimentin, desmin, and annexin-2, were validated by means of immunoblotting. As expected, these proteins were found to be significantly up-regulated (p < 0.05) in dystrophin-deficient muscle relative to C57BL6 muscle (Figs. 4A, 4B, 4D, and 4E). These results are in agreement with the SILAC proteome profiling data. Furthermore, we validated the significant up-regulation of ILK itself in dystrophin-deficient muscle, even though we did not detect this protein in our profiling studies (Figs. 4A, 4C). In addition, the presence of additional coordinate players of the actin cytoskeletal and integrin linked pathways, such as coflin and profilin, was examined in dystrophin-deficient muscle via immunoblot analysis (supplemental Fig. S2). Profilin showed a 1.6-fold up-regulation in the proteomic analysis, but immunoblotting showed no difference between mdx and C57BL6 muscle. In sum, these results indicate that proteins involved in the actin cytoskeletal and ILK pathways are up-regulated in dystrophin-deficient muscle, indicating their potential role in disease pathology.

Vimentin and ILK Are Elevated in Human DMD Muscle Biopsies—Immunostaining was performed in normal and DMD muscle tissues to validate the data obtained in the mdx mouse model. This analysis revealed increased staining for vimentin and ILK in the muscle biopsies of DMD patients relative to normal tissues (Fig. 5). In the normal skeletal muscle, the vimentin staining was predominantly observed in capillaries; however, in the dystrophic muscle, striking vimentin staining was observed in the capillaries and muscle infiltrates, including staining in some myofibers (Figs. 5A, 5B). ILK staining was observed in the capillaries of normal skeletal muscle; however, in dystrophic muscle, ILK staining was striking in both the blood vessels and the myofibers (Figs. 5C–5E). These results indicate that the altered pathways identified in
the muscle of mdx mice are also perturbed in the muscle of human DMD patients.

Mitochondria and Metabolic Enzymes Are Affected in Dystrophin-deficient Muscle—Differential SILAC proteome profiling identified various mitochondrial and metabolic enzymes, including hydroxyacyl-coenzyme A dehydrogenase, trifunctional enzyme subunit beta, aconitate hydratase, isocitrate dehydrogenase [NAD] subunit $\alpha$, and lactate dehydrogenase, as down-regulated in dystrophin-deficient muscle (Table I). Further IPA analyses of the data pointed to mitochondrial and metabolic dysfunction in dystrophin-deficient muscle; we sought to validate these findings by means of independent biochemical assays. The activities of two mitochondrial respiratory chain enzymes (lactate dehydrogenase and citrate synthetase) were monitored in normal and dystrophin-deficient muscle (Fig. 6). Dystrophin-deficient muscle showed a significant decrease (~2-fold) ($p < 0.05$) in lactate dehydrogenase activity relative to C57BL/6 muscle ($p < 0.05$). In contrast, no difference was observed in citrate synthase activity between the dystrophic and C57BL/6 muscle (data not shown). Interestingly, no difference was observed in the levels of other mitochondrial proteins, including cytochrome c and transcription factor A mitochondrial, monitored via immunoblotting analysis of dystrophin-deficient and normal muscle (data not shown). In sum, these results indicate that there might be subtle differences in some of the mitochondrial respiratory chain enzymes between mdx and C57BL/6 mice that cannot be detected via immunoblotting but which can be detected with the use of a sensitive proteome profiling method employing a stable isotope labeling strategy.

**DISCUSSION**

Initially, proteome profiling using the SILAC strategy could be implemented only in *in vitro* cell-culture-based systems or, to some extent, *in vivo* using nematodes and drosophila (13, 17, 27, 28). More recently, this versatile technique has been extended to the metabolic labeling of whole mammals with stable isotopes using either $^{13}$C$_6$-Lys-supplemented feed (SILAC mouse) or $^{15}$N-supplemented feed (SILAM). These strategies have allowed accurate proteome profiling of tissues and organs in *in vivo* systems under different physiological conditions (18, 19). In the current study, we extended the application of the SILAC mouse strategy to obtain insights into proteomic alterations in dystrophin-deficient skeletal muscle at the early stage of the pathology. Examination of the gastrocnemius muscle of dystrophic mdx mice at the early
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| Accessiona | Protein nameb | Fold changec |
|------------|--------------|--------------|
| P20152     | Vimentin     | 3.51         |
| P07356     | Annexin A2   | 3.18         |
| P31001     | Desmin       | 2.69         |
| Q62WV3     | 60S ribosomal protein L10 | 2.46 |
| P35979     | 60S ribosomal protein L12 | 2.25 |
| P19253     | 60S ribosomal protein L13a | 2.18 |
| P62242     | 40S ribosomal protein S8  | 2.09         |
| P35980     | 60S ribosomal protein L18 | 2.03         |
| P62908     | 40S ribosomal protein S3  | 2.02         |
| Q62WVN5    | 40S ribosomal protein S9  | 1.99         |
| Q62WV4     | 60S ribosomal protein L11 | 1.99         |
| P67984     | 60S ribosomal protein L22 | 1.96         |
| P14131     | 40S ribosomal protein S16 | 1.95         |
| P68040     | Guanine nucleotide-binding protein subunit beta-2-like 1 | 1.94 |
| Q6BTM8     | Filamin-A    | 1.88         |
| Q68990     | Alpha-actin-3 | 1.79         |
| Q64727     | Vinculin 1.17 |               |
| P20152     | Thioredoxin-dependent peroxide reductase, mitochondrial | 1.71         |
| Q8BMS1     | Long-chain fatty-acid-CoA ligase 1 | 1.71 |
| Q5WUZ7     | SH3 domain-binding glutamic acid-rich protein | 1.71 |
| Q5WUZ7     | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | 1.71 |
| P51174     | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | 1.71 |
| Q8WUZ7     | Trifunctional enzyme subunit beta, mitochondrial | 1.71 |
| P51201     | Aspartate aminotransferase, cytoplasmic | 1.71 |
| P13412     | Troponin I, fast skeletal muscle | 1.71 |
| P16125     | Troponin I, fast skeletal muscle | 1.71 |
| P14131     | Myomesin-1   | 1.71         |
| P62962     | Profilin-1    | 1.71         |
| P07724     | Serum albumin | 1.71         |
| P97457     | Myosin regulatory light chain 2, skeletal muscle isoform | 1.71 |
| P01027     | Complement C3 | 1.61         |
| P14824     | Annexin A6   | 1.55         |
| P99024     | Tubulin beta-5 chain | 1.52         |
| P68372     | Tubulin beta-2C chain | 1.47         |
| P20029     | 78 kDa glucose-regulated protein | 1.46         |
| Q6ERG7     | Tubulin beta-3 chain | 1.43         |
| Q5JK37     | Myozin-1      | 1.42         |
| Q689X6     | Filamin-C     | 1.42         |
| P48036     | Annexin A5   | 1.39         |
| Q68990     | Alpha-actin-3 | 1.39         |
| Q62436     | Liver carboxylesterase N | 1.36         |
| P68234     | Myozin-1      | 1.29         |
| P63101     | 14–3 protein zeta/delta | 1.28         |
| Q9CQZ5     | NADH dehydrogenase (ubiquinone) 1α subcomplex subunit 6 | 1.25         |
| Q3V1D3     | AMP deaminase 1 | 1.25         |
| Q8GZY1     | Eukaryotic translation initiation factor 3 subunit L | 1.23         |
| Q6ERG2     | NADH dehydrogenase (ubiquinone) 1α subcomplex subunit 13 | 1.22         |
| Q62WVB3    | Glycogen phosphorylase, muscle form | 1.21         |
| P20108     | Thioredoxin-dependent peroxide reductase, mitochondrial | 1.18         |
| P99029     | Peroxiredoxin-5, mitochondrial | 1.17         |
| Q64727     | Vinculin      | 1.17         |
| Q65051     | Pyruvate dehydrogenase E1 component subunit β mitochondrial | 1.17         |
| Q6260R2    | Isocitrate dehydrogenase (NAD) subunit α, mitochondrial | 1.12         |
| Q68962     | ADP/ATP translocase 1 | 1.17         |
| Q89K10     | Aconitate hydratase, mitochondrial | 1.17         |
| P47934     | Carnitine O-acetyltransferase | 1.15         |
| P68234     | Liver carboxylesterase N | 1.36         |

a Accession numbers are from the Uniprot database.

b Protein names are from the Uniprot database; significantly different protein modulations (p < 0.05) were determined using the non-parametric Wilcoxon rank sum test.

c Fold changes were calculated using mdx/wt (unlabeled/labeled) ratios quantitated using Integrated Proteomics Pipeline software. Ratios were obtained from n = 3 mice/group.

Table I continued

| Accessiona | Protein nameb | Fold changec |
|------------|--------------|--------------|
| Q68990     | Alpha-actin-3 | 1.36         |
| Q68990     | Alpha-actin-2 | 1.36         |
| P20029     | 78 kDa glucose-regulated protein | 1.36         |
| Q6ERG7     | Tubulin beta-3 chain | 1.36         |
| Q62436     | Liver carboxylesterase N | 1.36         |
| P68234     | Myozin-1      | 1.36         |
| P63101     | 14–3 protein zeta/delta | 1.36         |
| Q9CQZ5     | NADH dehydrogenase (ubiquinone) 1α subcomplex subunit 6 | 1.36         |
| Q3V1D3     | AMP deaminase 1 | 1.36         |
| Q8GZY1     | Eukaryotic translation initiation factor 3 subunit L | 1.36         |
| Q6ERG2     | NADH dehydrogenase (ubiquinone) 1α subcomplex subunit 13 | 1.36         |
| Q62WVB3    | Glycogen phosphorylase, muscle form | 1.36         |
| P20108     | Thioredoxin-dependent peroxide reductase, mitochondrial | 1.36         |
| P99029     | Peroxiredoxin-5, mitochondrial | 1.36         |
| Q64727     | Vinculin      | 1.36         |
| Q65051     | Pyruvate dehydrogenase E1 component subunit β mitochondrial | 1.36         |
| Q6260R2    | Isocitrate dehydrogenase (NAD) subunit α, mitochondrial | 1.36         |
| Q89K10     | Aconitate hydratase, mitochondrial | 1.36         |
| P47934     | Carnitine O-acetyltransferase | 1.36         |
| P68234     | Liver carboxylesterase N | 1.36         |

A stage of disease showed significant alterations in its proteome. Altered pathways included ILK, actin cytoskeleton signaling, mitochondrial energy metabolism, and calcium homeostasis. Some of these pathways were further validated in a separate group of mdx mice, as well as in human DMD biopsies.

Feeding C57BL/6 (normal) mice with a synthetic diet supplemented with 13C6-Lys (heavy) did not affect their growth or reproductive parameters, even after a prolonged feeding period, over a course of two generations. These observations are in accordance with the results of an earlier study using this diet (18). The labeling efficiency in different organs and tissues, including liver, muscle, and brain, by the heavy lysine reached a maximum by the F2 generation. Of the three tissue types, liver had the fastest incorporation rate (~70% of its proteins were 90% to 95% labeled with heavy lysine at about 50 days of feeding in the F0 generation); this is probably due to the rapid protein turnover in the liver. In contrast, terminally

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differentiated tissues such as muscle and brain incorporated heavy lysine at a slower rate, as indicated by the wider distribution of labeling percentages in the F0 and F1 generations. These results suggest that proteomic profiling studies involving muscle need to use tissues from mice fed with a heavy-lysine diet at least for two generations. The overall labeling...
efficiency in the liver, muscle, and brain was ≥96% by the F2 generation, which is in agreement with earlier reports (18, 22, 23).

Comparison of the proteomes of skeletal muscle of the mdx and control mice using the differential SILAC strategy identified previously known, as well as novel, pathways associated with dystrophin deficiency. Approximately 750 to 850 proteins were identified and quantified in the gastrocnemius muscle. These numbers are similar to those previously reported in other skeletal muscle proteome studies (29, 30). However, these numbers are lower than those from a recent study in which a highly sensitive mass spectrometer was used on samples that were subjected to extensive fractionation (31). The identification of a low number of proteins in skeletal muscle can be attributed to the huge dynamic range between high and low abundant proteins in the skeletal muscle tissue (32). Indeed, skeletal muscle is mainly composed of structural proteins such as myosin and actin, which account for more than 40% of the total proteins and thus mask the detection of low-abundant proteins.

Initial SILAC proteome profiling was performed on pairs of labeled-normal and unlabeled-normal mice and of labeled-normal and unlabeled-mdx mice. As expected, we observed a complete absence of dystrophin expression and the down-regulation of dystroglycan complex in the mdx muscle. Furthermore, greater differential protein expression was observed when comparing dystrophin-deficient to normal gastrocnemius muscle, whereas only a few proteins were altered between labeled and unlabeled normal gastrocnemius muscle. To statistically validate the initial findings, we performed spike-in SILAC using an independent set of normal and mdx mice. Of the total number of proteins identified and quantified, ~73 were found to be significantly altered in their levels between dystrophin-deficient and normal (p < 0.05) mice. The top candidate proteins included vimentin, desmin, annexin-II, ribosomal proteins, GRP78, and actinins whose levels were increased in dystrophin-deficient muscle. The enhanced expression of these proteins in dystrophic muscle indicates the perturbation of various signaling mechanisms.

The high levels of vimentin and other extracellular matrix proteins were maintained in the diaphragm of a 22-month-old mdx mouse (33). These data suggest that vimentin is increased very early and stays up-regulated during disease progression. In addition, our results indicate a significant up-regulation of GRP78 (a stress-related protein; chaperone) in dystrophic muscle, suggesting the activation of stress responses early in the disease process. Our profiling results also identified significant down-regulation of the contractile apparatus (e.g. myosin and tropomyosin) and significant down-regulation of proteins involved in mitochondrial energy metabolism such as L-lactate dehydrogenase B chain, 3,2-trans-enoyl-CoA isomerase, and trifunctional enzyme subunit alpha. These results might suggest that the perturbed mitochondrial energy metabolism and the underlying muscle weakness occur very early in the pathogenesis of dystrophin deficiency.

Some earlier studies examined protein changes in dystrophic hind limb muscles, diaphragm, heart, and extraocular muscles of mdx mice at different ages; however, few studies involved dystrophic gastrocnemius muscle (9–12, 34–37). A study using a 2-DE approach reported a 4-fold decrease in adenylate kinase levels in the hind limb muscles of 3-month-old mdx mice relative to C57BL/10 muscles (9). Our proteomic analyses identified adenylate kinase in the gastrocnemius muscle (with at least 12 peptides and 50% sequence coverage); however, no significant difference was observed in the relative abundance of adenylate kinase between mdx and C57BL/6. The disparity in the proteomic alterations observed between these studies can be attributed to the differences in the strain, age, and type of muscles tested. Doran et al. conducted a series of proteomic profiling studies in dystrophic-deficient skeletal muscle and diaphragm (10, 11) and reported a significant decrease in calsequestrin levels in the skeletal muscle of 9-week-old mdx mice relative to controls (11). Those authors also reported the reduced expression of regucalcin and sarcalumenin (an intracellular Ca^{2+} signaling protein) in both young and aged mdx diaphragm, indicating abnormal cytosolic calcium handling in dystrophin-deficient muscle. Even though our proteomic analyses identified calsequestrin (with at least 10 peptides and 20% sequence coverage), no significant difference was observed in its relative abundance between mdx and C57BL/6. We did not detect regucalcin, but we did detect sarcalumenin with good peptide numbers and sequence coverage; however, no significant alteration was noted in the relative abundance of these proteins. The current study used isolated gastrocnemius muscle from perfused mice for the analyses, whereas other studies used either diaphragm or hind limb muscles (a mixture of several muscle groups). It is unclear whether the tissues in other studies were collected from perfused or non-perfused mice. The differences in the tissues and techniques used might be responsible for the discrepant results of these studies. Another study reported an increase in cardiovascular heat...
shock protein in dystrophin-deficient diaphragm (12). Our proteomic profiling did not identify cardiovascular heat shock protein, but several other heat shock proteins were identified. We did not observe significant differences in heat shock proteins between mdx and C57BL/6. However, additional comparative evaluations are needed in order to sort out whether these differences between studies are technical or biological in nature.

IPA was used to delineate the perturbed molecular networks associated with altered protein levels. The significantly altered canonical networks included actin cytoskeleton signaling, ILK pathways, glycolysis, the citrate cycle, and mitochondrial function. These results indicate the usefulness of the current method for identifying multiple perturbed pathways in a single analysis, and suggest that it might be useful for understanding disease processes at the systems level. The validation of data by means of immunoblotting and the complete agreement of these results with the proteomic profiling results indicate the robustness of the SILAC mouse strategy. Furthermore, the utilization of biochemical assays also validated the presence of perturbed mitochondrial function, although no changes in protein levels were detected via immunoblotting in the dystrophic muscle. This suggests that subtle differences can also be measured using the SILAC mouse strategy.

The identification of the involvement of the actin cytoskeletal signaling and ILK pathways in dystrophic pathology early in the disease process is an important finding. Indeed, the silencing of ILK expression in skeletal muscles of mice using a cre/lox system has shown the role of the ILK pathway in causing muscle pathology (38). The deletion of ILK led to the development of progressive muscular dystrophy, which was accompanied by degenerating myofibers and fibrosis, and these features were more severe near the myofascial junctions (39). A subsequent report also showed that skeletal muscle expresses high levels of ILK, predominantly at myotendinous junctions and costameres. Further, it was reported that ILK binds the cytoplasmic domain of beta-1 integrin and mediates the phosphorylation of protein kinase B/Akt, which in turn plays a central role during skeletal muscle regeneration. In addition, an association between beta-1 integrin and insulin-like growth factor 1 receptor was also shown in muscle, and this association is considered critical for insulin-like growth factor 1 receptor/insulin receptor substrate signaling to protein kinase B/Akt during mechanical stress in skeletal muscle (38). Taken together, these results indicate that the up-regulation of the ILK and actin cytoskeletal pathways might be a compensatory mechanism to overcome the loss of dystrophin protein and help protect the susceptible myofiber membrane from contraction-induced damage.

The exploitation of integrin-signaling and related pathways as therapeutic targets for DMD appears promising, as the enhanced expression of beta-1D integrin in dystrophic muscle decreases the damaged myofibers and is attributed to the presence of more functional integrin at the sarcolema (40). The modulation of these pathways in dystrophic mdx muscle

**Fig. 7. Pathways involved in the pathogenesis of dystrophic muscle.**
An absence of dystrophin leads to the compensatory up-regulation of actin cytoskeletal signaling and ILK pathway activation to reduce contraction-induced injury in skeletal muscle. In parallel, reduced mitochondrial function, along with disturbances in calcium homeostasis, exacerbate the disease phenotype.
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via chemical mediators or drugs should provide valuable insight into their specific role in muscle pathology. Furthermore, the validation of some of the candidate proteins in human DMD samples suggests their relevance to human disease.

Our study showed that the levels of several mitochondrial proteins were affected in dystrophin-deficient muscle. A role for mitochondria in dystrophic pathology has been observed previously (41–44). An increase in $\text{Ca}^{2+}$ content has been detected in the sarcoplasmic reticulum and mitochondria of dystrophic skeletal muscle, along with impaired ATP production and metabolic abnormalities (41–44). In addition, we have recently shown (via 10-nonyl acridine orange staining) that mitochondrial mass is decreased in the extensor digitorum longus fibers of mdx mice, indicating that these muscles have a lower capacity to use oxidative energy. We have also shown that the mdx muscle is more fatigable than wild-type muscle, suggesting that dystrophin deficiency leads to significant alterations in mitochondrial function and muscle metabolism (45). In sum, these findings corroborate a role for mitochondria and metabolic pathways in dystrophic pathology.

Based on previously published data and the results of our current study, we propose a model for dystrophic pathology. The primary functional defect of dystrophin deficiency causes susceptibility to contraction-induced damage of the myofiber membranes. The injured fibers cause the compensatory up-regulation of actin cytoskeletal and ILK pathways in order to protect from further damage. In parallel, dystrophin-deficient myofiber might have leaky $\text{Ca}^{2+}$ channels, which would enhance the $\text{Ca}^{2+}$ influx into dystrophic fibers, leading to pro tease activation and free radical formation from cytosolic and mitochondrial sources; this would cause the dysregulation of mitochondria, energy metabolism, and calcium homeostasis. The activation of these pathways can potentially lead to myofiber damage and progression of the dystrophic pathology (Fig. 7). Based on this model, we can speculate that therapies that simultaneously target multiple perturbed pathways might be beneficial for DMD patients and have the potential to ameliorate dystrophic pathology.

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