Association of dystrophin-related protein with dystrophin-associated proteins in *mdx* mouse muscle

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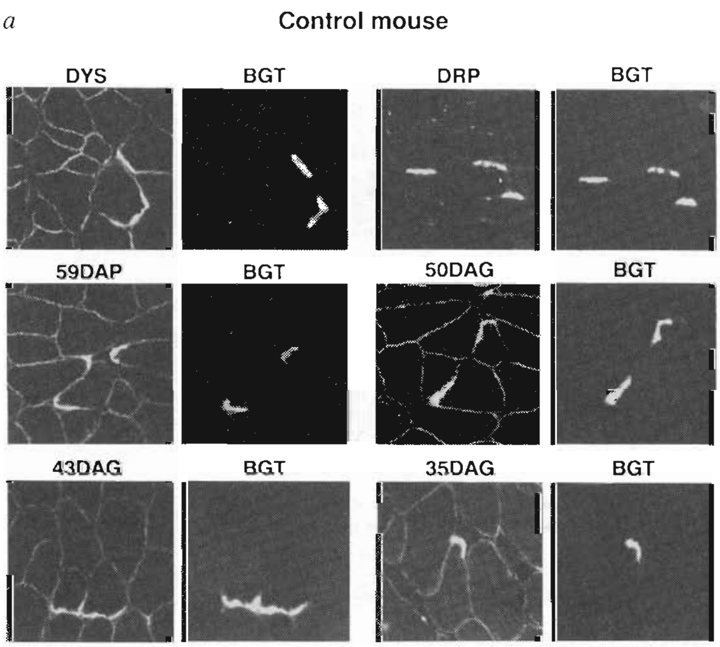
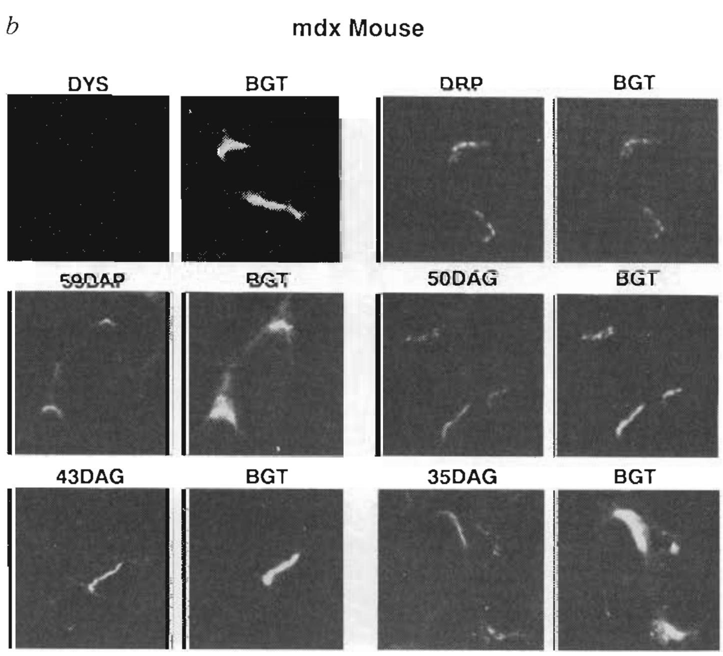
DYSTROPHIN is associated with a complex of muscle membrane (sarcolemmal) glycoproteins that provide a linkage to the extracellular matrix protein, laminin[1–8]. The absence of dystrophin leads to a dramatic reduction of the dystrophin-associated proteins (156DAG, 59DAP, 50DAG, 43DAG and 35DAG) in the sarcolemma of patients with Duchenne muscular dystrophy and *mdx* mice[2,6–8]. Here we demonstrate that dystrophin-related protein (DRP, utrophin), an autosomal homologue of dystrophin[9–17], is associated with an identical or antigenically similar complex of sarcolemmal proteins and that DRP and the dystrophin/DRP-associated proteins colocalize to the neuromuscular junction in Duchenne muscular dystrophy and *mdx* muscle. The DRP and dystrophin/DRP-associated proteins are found throughout the sarcolemma in small-calibre skeletal muscles and cardiac muscle of adult *mdx* mice. Because these muscles show minimal pathological changes[18–20], our results could provide a basis for the upregulation of DRP as a potential therapeutic approach.

The immunofluorescence localization of DRP and dystrophin-associated proteins (DAPs) in adult control mouse, adult *mdx* mouse and Duchenne muscular dystrophy (DMD) quadriceps muscle are shown in Fig. 1. In control mouse, dystrophin and DAPs were localized throughout the sarcolemma but were enriched at the neuromuscular junction (NMJ). DRP was localized to the NMJ in control and *mdx* mouse and DMD muscle. As previously reported[2,6–8], DAPs were greatly reduced in the





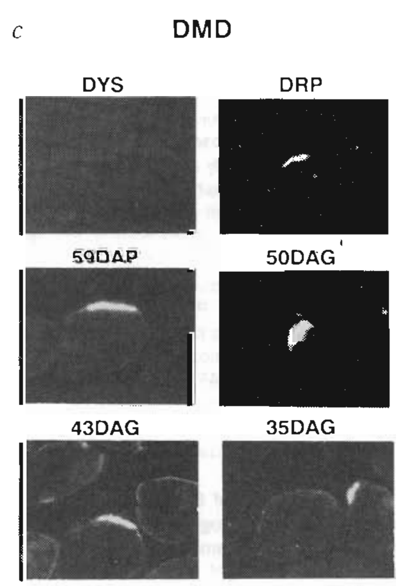
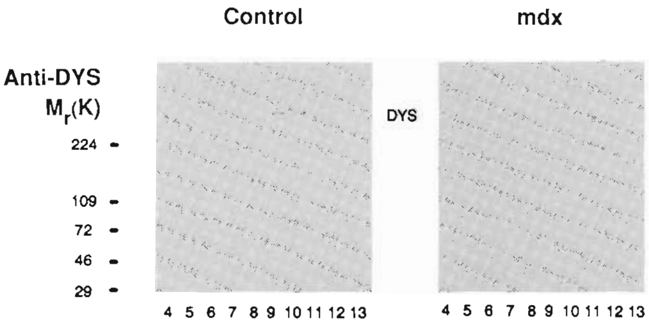


FIG. 1 Immunohistochemical localization of dystrophin (DYS), DRP and DAPs in control mouse (*a*), *mdx* mouse (*b*) and DMD (*c*) skeletal muscle. 156DAG showed the same distribution as the other DAPs (not shown).

METHODS. Cryosections (7 μm) of quadriceps muscle of adult control mouse, adult *mdx* mouse and DMD were stained with an antibody against dystrophin, DRP or DAPs which was detected with FITC-conjugated secondary antibody[2,4–8,14]. NMJs were identified by double-staining with rhodamine-labelled α-bungarotoxin (BGT) in control and *mdx* muscles. NMJs in DMD muscle were identified by double staining with BGT or the staining of the serial sections by the Koelle's method.

extrajunctional sarcolemma of *mdx* mouse and DMD muscle. However, near-normal intensity of DAP staining was observed at the NMJ in *mdx* mouse and DMD muscle (Fig. 1*b*, c).

The colocalization of DRP and DAPs at the NMJ in mouse and DMD muscle suggested a possible association of DRP with DAPs. To test if DRP exists in a complex similar to the dystrophin–glycoprotein complex, isolation of a DRP complex was attempted from both control and *mdx* skeletal muscle membranes using the methods described previously for the isolation of dystrophin–glycoprotein complex: wheat-germ agglutinin–Sepharose column chromatography of digitonin-solubilized skeletal muscle microsomes followed by DEAE-cellulose column chromatography[1,2]. DEAE-cellulose eluates were then loaded on 5 to 20% sucrose density gradients for sedimentation analysis (Fig. 2). Because of the much lower relative abundance of DAPs in the *mdx* muscle, three times more *mdx* material was loaded on the sucrose gradient than for the control mouse gradient. Even with the increased sample loading, the immunoblot analysis of the *mdx* sucrose gradient profile required a much longer development time in order to



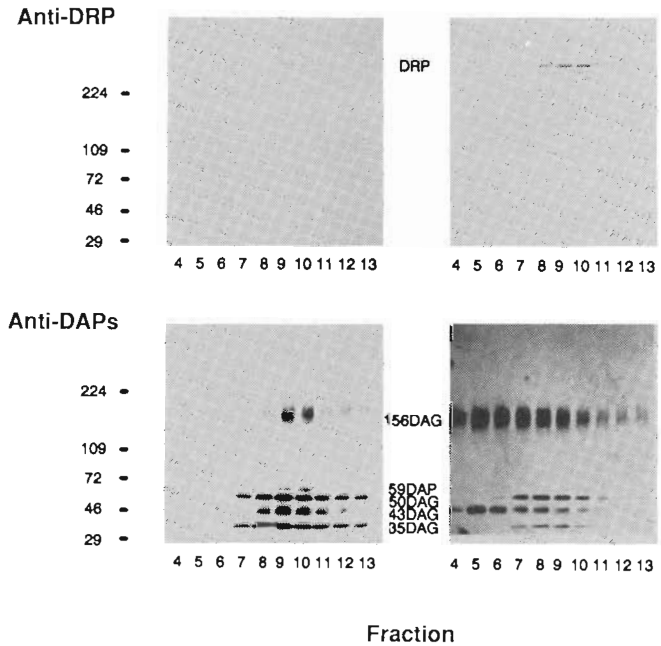


FIG. 2 Sedimentation of dystrophin, DRP and DAPs of control or *mdx* mouse through 5 to 20% linear sucrose density gradients. Shown are nitrocellulose transfers of sucrose gradient fractions 4–13 separated by 3–12% SDS-PAGE and stained with an antibody against dystrophin (Anti-DYS), an antibody against DRP (Anti-DRP) or a cocktail of antibodies against DAPs (Anti-DAPs). Molecular mass standards ($\times 10^{-3}$) are shown on the left. Note that three times more *mdx* sample was applied to the sucrose density gradient than for control sample. In addition, the nitrocellulose transfer of *mdx* sample was developed longer than that of control sample and the photograph of *mdx* sample was overexposed.

METHODS. KCl-washed heavy microsomes (300 mg) prepared from limb and back muscles of adult control or *mdx* mice were solubilized with 280 ml of a solution containing 1% digitonin and 0.5 M NaCl[1–3]. The solubilized proteins were circulated overnight on a 20 ml wheat-germ agglutinin–Sepharose column. After washing, the column was eluted with 60 ml of a solution containing 0.3 M *N*-acetylglucosamine. The eluate was applied to a 1.5 ml DEAE-cellulose column and eluted with increasing concentrations of NaCl[1–3]. The 175 mM NaCl eluate was concentrated and layered onto a 12.5 ml 5–20% (w/v) linear sucrose density gradient[2,3]. After centrifugation in a Beckman VTi65.1 vertical rotor at 200,000*g* for 90 min at 4 °C, 0.6 ml fractions were collected from the top of the gradients as described[2,3].

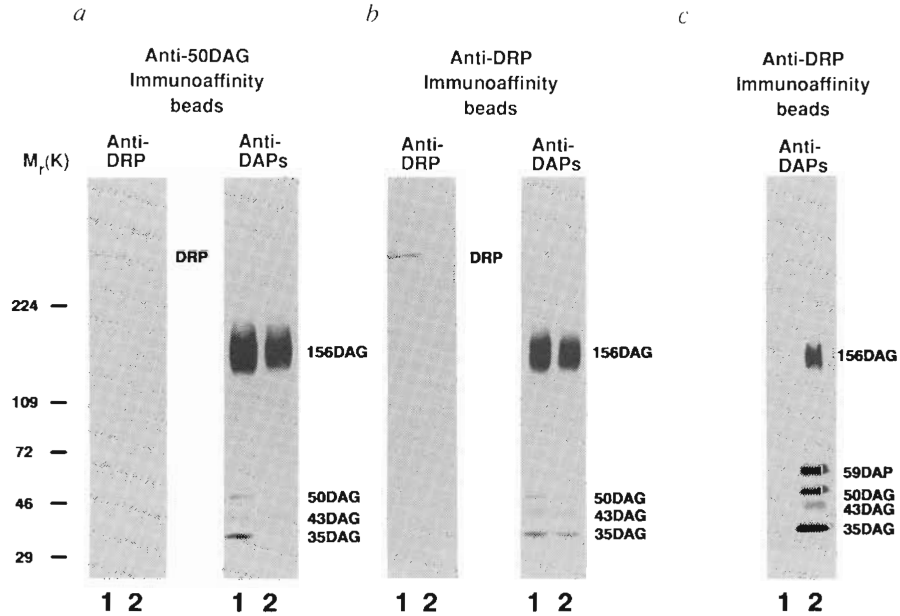


FIG. 3 *a*, Immunoadsorption of DRP and DAPs by anti-50DAG immunoaffinity beads. Anti-50DAG immunoaffinity column (goat anti-mouse IgG-Sepharose coupled with anti-50DAG antibody) void (lane 2) or control column (goat anti-mouse IgG-Sepharose) void (lane 1) immunostained with an antibody against DRP (Anti-DRP) or a cocktail of antibodies against DAPs (Anti-DAPs). *b*, Immunoadsorption of DRP and DAPs by anti-DRP immunoaffinity beads. Anti-DRP immunoaffinity column (protein A-Sepharose coupled with anti-DRP antibody) void (lane 2) or control column (protein A-Sepharose) void (lane 1) immunostained with an antibody against DRP or a cocktail of antibodies against DAPs. The 59DAP (which is not glycosylated) is not identified clearly in (*a*) and (*b*) owing to its extremely low abundance in the wheat-germ agglutinin (WGA) eluate. *c*, Immunoadsorption of DAPs by anti-DRP immunoaffinity beads. Proteins adsorbed by anti-DRP immunoaffinity column (lane 2) or control column (lane 1) immunostained with a cocktail of antibodies against DAPs. 59DAP is clearly detected because it was concentrated on the immunoaffinity beads saturated with the WGA eluate. Molecular mass standards ($\times 10^{-3}$) are shown on the left.

METHODS. For *a* and *b*, 100 μl of 0.3 M *N*-acetylglucosamine eluate from WGA–Sepharose column chromatography of *mdx* mouse (0.2 mg ml^{-1}) was incubated with 30 μl of either immunoaffinity or control beads[2] in the presence of 0.5 M NaCl overnight at 4 °C. After centrifugation, proteins remaining in the supernatants were analysed by 3-12% SDS–PAGE and immunoblotting. For *c*, the beads (100 μl) were incubated with a saturating volume (3 ml) of the same material. After centrifugation and extensive washing, proteins adsorbed by the beads were analysed by 3–12% SDS-PAGE and immunoblotting.

detect DAPs. The control mouse sucrose gradient profile demonstrated that dystrophin, DRP and DAPs cosedimented with a peak in fractions 9 and 10. The *mdx* mouse sucrose gradient profile revealed that DRP and 59DAP also cosedimented with a peak in fractions 9 and 10. In contrast, 156DAG and 43DAG cosedimented in a broad range of fractions, including fractions 9 and 10, whereas 50DAG and 35DAG cosedimented with a peak in fractions 8 and 9. These results suggest that there are two populations of DAPs in *mdx* muscle: a small fraction of DAPs associated with DRP to form a complex similar in size to dystrophin-glycoprotein complex and a large fraction of free DAPs which fail to form a complex in the absence of dystrophin and therefore sediment as much smaller entities.

To test the possible association of DRP with DAPs more directly, immunoadsorption experiments of wheat-germ agglutinin eluate from solubilized *mdx* muscle membranes were done using immunoaffinity beads prepared with antibodies against 50DAG or DRP (Fig. 3). Proteins remaining in the supernatants after centrifugation of the beads were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Coomassie blue-staining of the gel revealed that the overall protein composition in the supernatants was indistinguishable between immunoaffinity and control beads in both anti-50DAG and anti-DRP immunoprecipitation (not shown). Immunoblot analysis revealed that anti-50DAG beads precipitated all of the 50DAG, 35DAG and DRP as well as a small fraction of 156DAG and 43DAG (Fig. 3*a*). Conversely, anti-DRP beads precipitated all DRP and a small fraction of DAPs (Fig. 3*b*). Autoradiography of the immunoblots using ^{125}I-labelled protein A revealed that about 30% of DAPs were precipitated by the anti-DRP beads except 59DAP, which was completely precipitated. Immunoblot analysis of anti-DRP beads incubated with a volume of wheat-germ agglutinin eluate sufficient to saturate all DRP antibody-binding sites confirmed that all of the DAPs could be precipitated by anti-DRP beads (Fig. 3*c*). Similar results were obtained using the wheat-germ agglutinin eluate from solubilized control mouse muscle membranes: (1) anti-50DAG beads precipitated both dystrophin and DRP together with all DAPs and (2) anti-DRP beads precipitated DRP together with a small fraction of DAPs (not shown).

Thus far, our results indicate that there are two populations of DAPs in *mdx* muscle: a small fraction associated with DRP at the NMJ and an uncomplexed fraction which, we believe, would normally associate with dystrophin. The components of

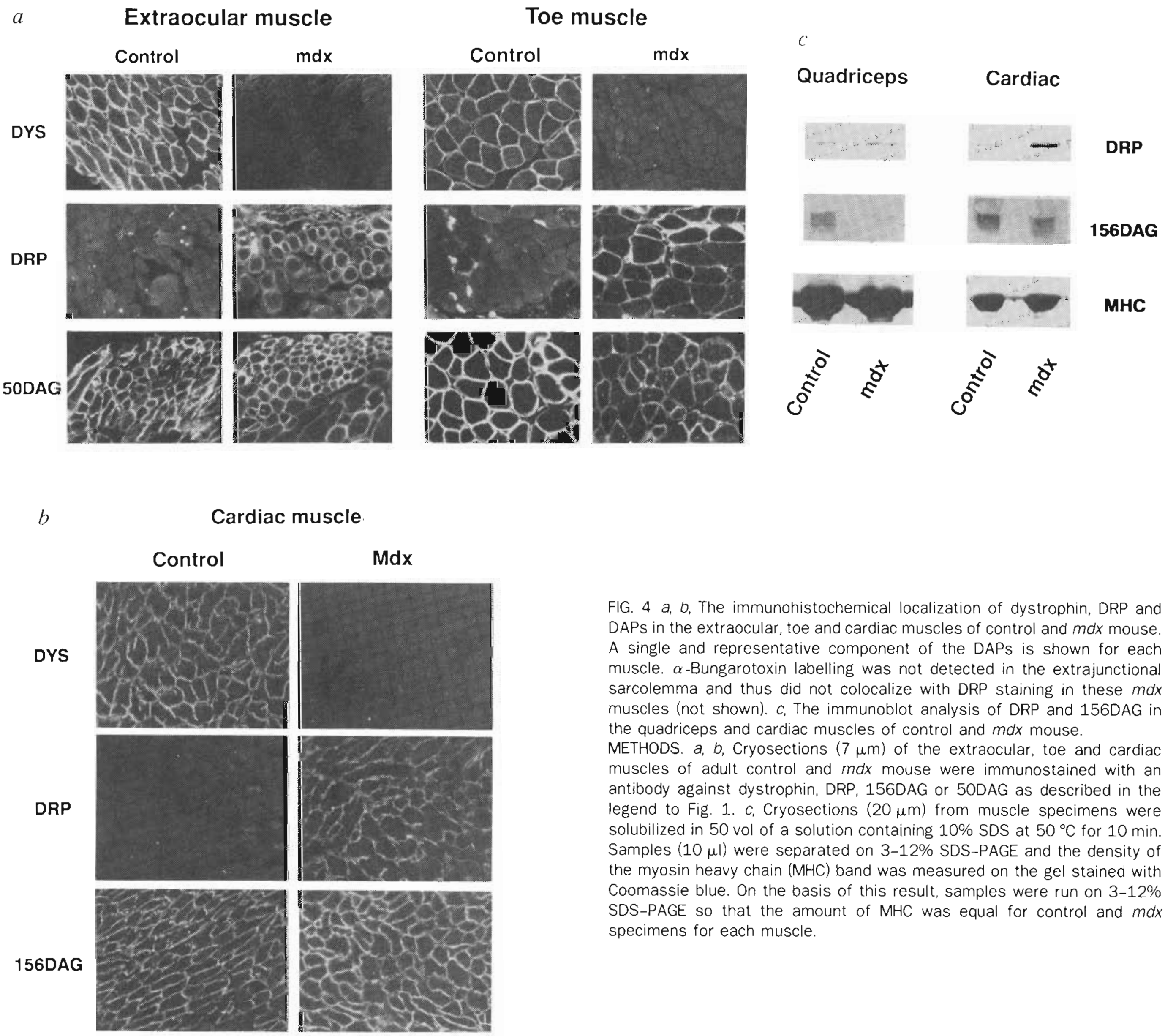


FIG. 4 *a*, *b*, The immunohistochemical localization of dystrophin, DRP and DAPs in the extraocular, toe and cardiac muscles of control and *mdx* mouse. A single and representative component of the DAPs is shown for each muscle. α-Bungarotoxin labelling was not detected in the extrajunctional sarcolemma and thus did not colocalize with DRP staining in these *mdx* muscles (not shown). *c*, The immunoblot analysis of DRP and 156DAG in the quadriceps and cardiac muscles of control and *mdx* mouse.
METHODS. *a*, *b*, Cryosections (7 μm) of the extraocular, toe and cardiac muscles of adult control and *mdx* mouse were immunostained with an antibody against dystrophin, DRP, 156DAG or 50DAG as described in the legend to Fig. 1. *c*, Cryosections (20 μm) from muscle specimens were solubilized in 50 vol of a solution containing 10% SDS at 50 °C for 10 min. Samples (10 μl) were separated on 3–12% SDS-PAGE and the density of the myosin heavy chain (MHC) band was measured on the gel stained with Coomassie blue. On the basis of this result, samples were run on 3–12% SDS-PAGE so that the amount of MHC was equal for control and *mdx* specimens for each muscle.

these two populations could be identical gene products or antigenically cross-reactive isoforms or homologues.

It is well known that all muscles in DMD or in *mdx* mouse are not affected to the same degree although dystrophin is deficient in all muscles[18-21]. For instance, dysfunction of small-calibre skeletal muscles is minimal in both DMD and *mdx* mouse[18,19] and dysfunction of cardiac muscle is absent in *mdx* mouse[20]. The association of DRP with DAPs, together with the recent finding that DRP has been detected in the sarcolemma outside the NMJ in DMD or *mdx* mouse[15-17], suggest the possibility that DRP could compensate for dystrophin deficiency by retaining DAPs in extrajunctional regions of the sarcolemma. To investigate this possibility, we did immunohistochemical analysis of small skeletal muscles and cardiac muscle of adult *mdx* mouse (Fig. 4*a*, *b*). DRP and DAPs are expressed throughout the sarcolemma (compare Fig. 1*b*), whereas in the same muscles in control mouse only dystrophin and DAPs are expressed throughout the sarcolemma. In addition, immunoblot analysis of the crude muscle extracts and its quantitation by autoradiography show a fourfold increase of DRP and a near-normal level of 156DAG in *mdx* cardiac muscle, whereas there is a 1.3-fold increase of DRP and a drastic reduction of 156DAG in *mdx* quadriceps muscle compared to the respective control muscles (Fig. 4*c*). The preservation of DAPs in small skeletal and cardiac muscles of *mdx* mouse suggests that altered expression of DRP could lead to the retention of DAPs. □

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