Restoration of the Dystrophin-associated Glycoprotein Complex After Exon Skipping Therapy in Duchenne Muscular Dystrophy

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We previously conducted a proof of principle; dose escalation study in Duchenne muscular dystrophy (DMD) patients using the morpholino splice-switching oligonucleotide AVI-4658 (eteplirsen) that induces skipping of dystrophin exon 51 in patients with relevant deletions, restores the open reading frame and induces dystrophin protein expression after intramuscular (i.m.) injection. We now show that this dystrophin expression was accompanied by an elevated expression of α-sarcoglycan, β-dystroglycan (BDG) and—in relevant cases—neuronal nitric oxide synthase (nNOS) at the sarcolemma, each of which is a component of a different subcomplex of the dystrophin-associated glycoprotein complex (DAPC). As expected, nNOS expression was relocalized to the sarcolemma in Duchenne patients in whom the dystrophin deletion left the nNOS-binding domain (exons 42–45) intact, whereas this did not occur in patients with deletions that involved this domain. Our results indicate that the novel internally deleted and shorter dystrophin induced by skipping exon 51 in patients with amenable deletions, can also restore the dystrophin-associated complex, further suggesting preserved functionality of the newly translated dystrophin.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive, severely disabling, and incurable X-linked recessive inherited disorder that affects 1 in 3,500 newborn boys and leads to death in the second or third decade of life.[1] In patients with DMD, the open reading frame of the X-linked *dystrophin* gene, encoding dystrophin, is disrupted by deletions (~65%), duplications (~10%), point mutations (~10%), or other smaller rearrangements (15%).[1] Dystrophin is a protein with both a structural and signaling role in muscle, localized to the cytoskeleton immediately beneath the sarcolemma. Regarding its important structural role, dystrophin connects the subsarcolemmal F-actin cytoskeleton to β-dystroglycan (BDG),[2] which is associated with α-dystroglycan and in turn connects to proteins of the extracellular matrix (**Figure 1**). Dystrophin consists of four main functional units (**Figure 1**). First the binding site for actin is located at the N terminus.[3] Second the rod domain contains 24 triple helical spectrin repeats with 4 interspersing hinge domains. Third, the cysteine-rich domain required for binding to BDG[4–6] and fourth, the c-terminal domain that contains the syntrophin[7] and dystrobrevin-binding sites[8]. The cysteine-rich domain consists of WW, EF hand, and ZZ domains[4] and all these are required for BDG binding. Dystrophin assembles with other proteins to form the dystrophin-associated glycoprotein complex (DAPC),[3] composed of three subcomplexes: (i) the sarcoglycans (α, β, γ, and δ), (ii) syntrophin, nNOS, and dystrobrevin, and (iii) BDG and α-dystroglycan. The absence of dystrophin and destabilization of the DAPC is thought to render muscle cells susceptible to stretch-induced damage and increased intracellular calcium influx, leading to a series of pathological processes responsible for skeletal and cardiac muscle fiber necrosis,[9] inflammation and replacement of muscle with fibro-adipose tissue. Regarding the signaling function, dystrophin is involved in the localization of neuronal nitric oxide synthase (nNOS) a protein that directly interacts with dystrophin and syntrophin. nNOS regulates the blood flow in skeletal muscle according to its metabolic needs,[10] and its lack at the sarcolemma is associated with ischemia following exercise, which is believed to cause additional muscle tissue damage in DMD.

Becker muscular dystrophy (BMD) is a milder allelic variant with mutations that maintain the dystrophin open reading frame. An internally shortened but partly functional dystrophin is consequently produced and as a result, BMD patients usually remain ambulant into late adulthood and have a normal life span.[11] Using splice switching oligomers (SSOs), out-of-frame mutations in DMD can be transformed at the mRNA level by exon skipping to in-frame BMD mutations.[12–14] This principle has been proven extensively *in vitro* and in various animal models.[12,13,15] Predominantly, two chemical classes of SSOs are currently being used: 2′O-methyl-ribooligonucleoside-phoshophorothioate (2′OMe) and phosphorodiamidate morpholino oligomers (PMOs).[16,17] Using the *mdx* mouse model of DMD, it has been shown *in vivo* that PMO-based

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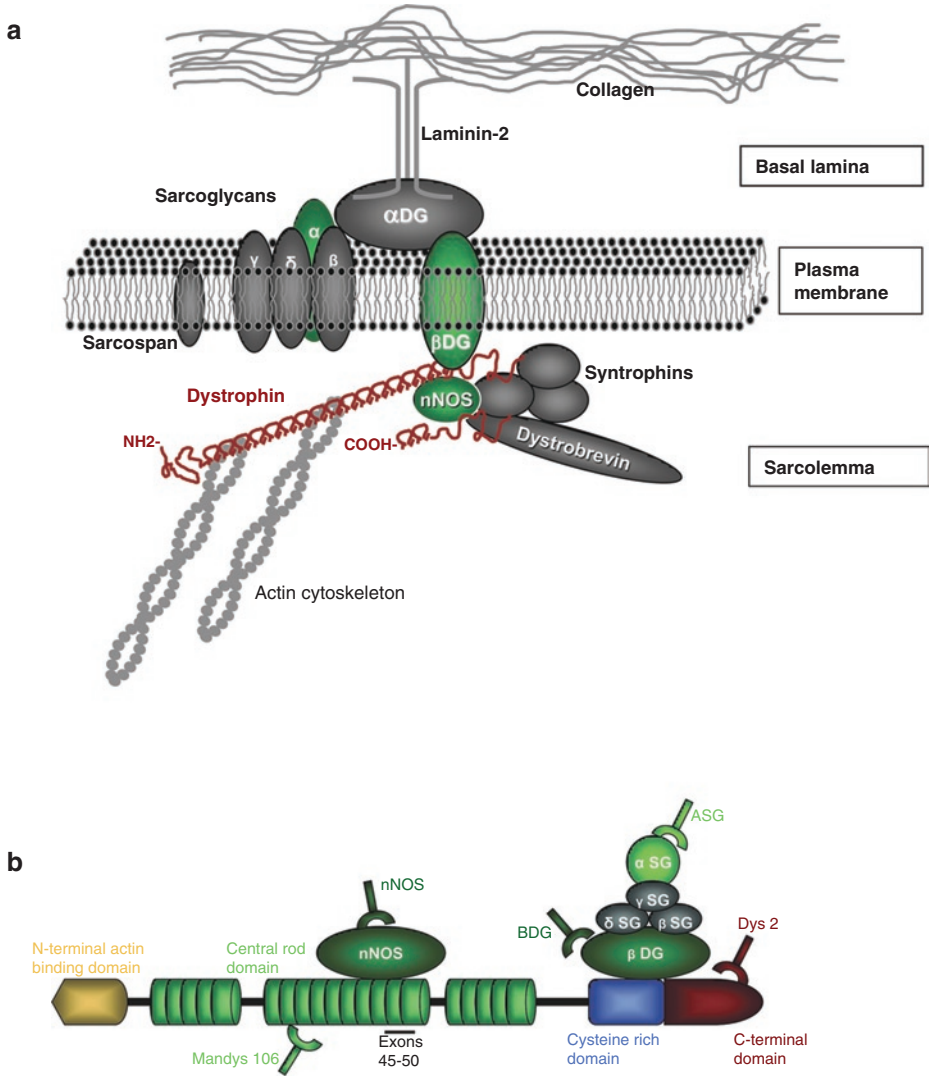

Figure 1 Dystrophin-associated glycoprotein complex. (**a**) This illustration shows the schematic organization of the dystrophin-associated glycoprotein complex (DAPC) at the sarcolemma of myofibers. It shows the DAPC linking the cytoskeleton to the extracellular matrix. ADG, α-dystroglycan; BDG, β-dystroglycan, and the corresponding sarcoglycans. (**b**) Functional dystrophin units and the localization of antibody-binding domains. We show the four functional units of the dystrophin protein, first the N-terminal actin-binding domain, second the central rod domain with the spectrin repeats, third the cysteine-rich domain and fourth the c-terminal domain. Furthermore, it shows where neuronal nitric oxide synthase (nNOS) and α-DG binds to dystrophin. α-SG does not bind directly to dystrophin.

SSOs are more effective than 2′OMe SSOs in restoring the reading frame by exon skipping.[18–20] PMOs have also been administered in the X-linked muscular dystrophy dog, with successful dystrophin restoration and apparent clinical benefit without adverse reactions.[15] We previously performed a proof-of-principle single-blind, controlled, two-dose escalation study of a morpholino SSO (AVI-4658, recently named eteplirsen) which induced skipping of exon 51 in dystrophin mRNA in seven patients with DMD.[16] This morpholino SSO was injected into one extensor digitorum brevis muscle whereas the contralateral muscle received a saline injection. In patients receiving the higher dose, the number of dystrophin-positive fibers ranged between 44% and 79% of total fibers, with dystrophin intensity levels on immunostained fibers reaching up to 42% (range 11–42) of the amount in healthy muscle. We reasoned that for the restored dystrophin to be functional, it should stabilize the assembly of proteins of the DAPC including nNOS, but the experiments that could confirm this presumption were outside the scope of the trial. We now report on the increased expression of DAPC proteins (ASG, BDG) and of nNOS in dystrophin-positive fibers in treated muscles of these patients, each of which is a component of a different subcomplex of the DAPC. This is despite the variable levels of dystrophin present in these fibers.

RESULTS

We studied semiquantitatively three DAPC proteins, each one representative of one subcomplex of the DAPC, in four DMD patients, who received a single intramuscular (i.m.) administration of AVI-4658 at the higher dose of 0.9 mg. From the five patients treated at the higher dose, two with a missing nNOS-binding domain (carrying a deletion of exons 45–50) and two (carrying a deletion of exons 48–50) with an intact nNOS-binding domain in dystrophin, were selected for the study.

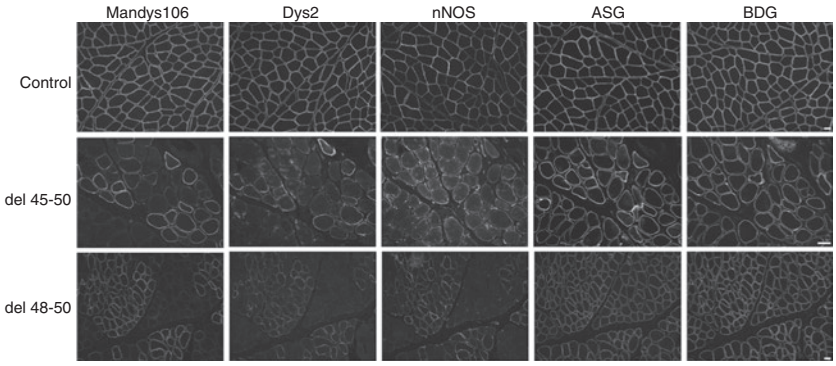

Figure 2 Immunoflourescence analysis of dystrophin-associated proteins after treatment with AVI-4658. Representative images from control and treated extensor digitorum brevis (EDB) muscles from two subjects (del 45–50 and del 48–50) stained with Mandys106, Dys2, neuronal nitric oxide synthase (nNOS), ASG, and BDG. Both patient panels show a fascicle that has restored dystrophin protein after treatment with AVI-4658, adjacent to dystrophin-negative fibers. However, even within the positive fascicle, fibers express variable amounts of dystrophin. nNOS restoration at the sarcolemma of dystrophin-positive fibers of subject with del 48–50 is clearly detected, whereas in subject with del 45–50 a relatively weak staining of nNOS at the sarcolemma is detected. This observation is in concordance with the fact that that the nNOS-binding site (exons 42–45) is deleted in subjects with a 45–50 deletion. Bar = 20 µm.

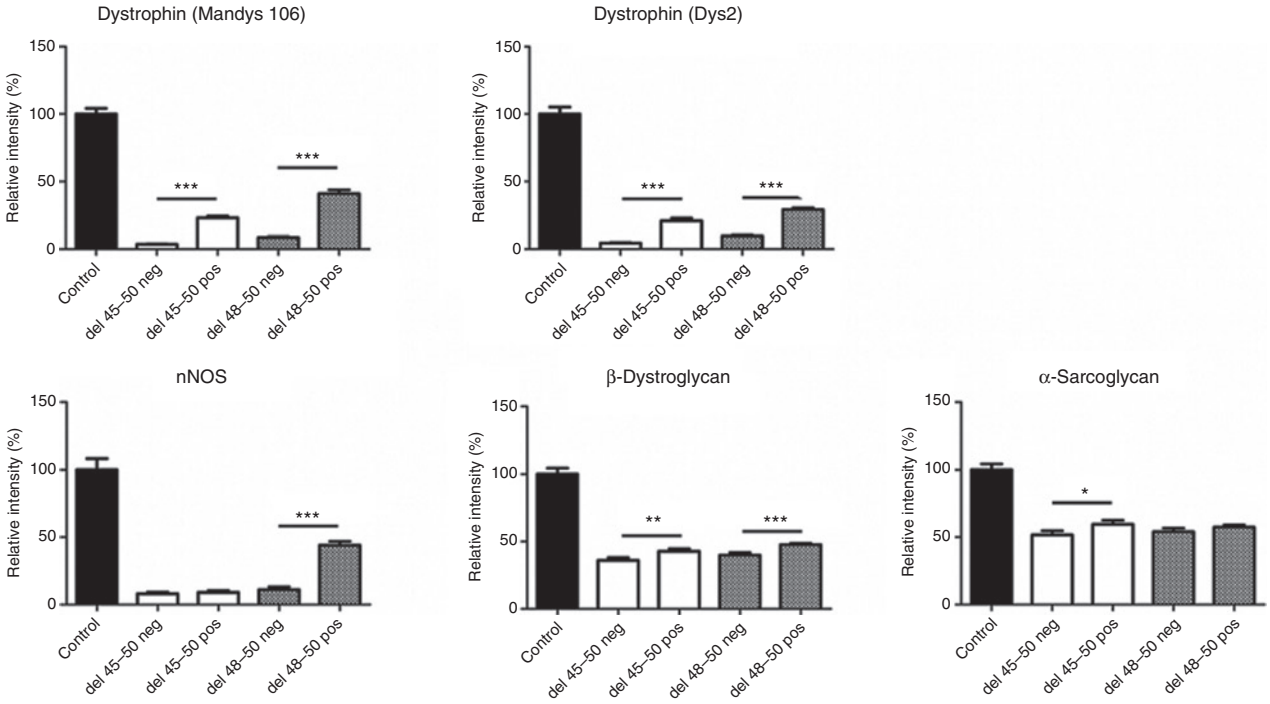

Figure 3 Semiquantitative analysis of dystrophin-associated glycoprotein complex restoration following intramuscular treatment with AVI-4658. Muscle biopsies from the treated extensor digitorum brevis (EDB) muscles of two subjects carrying a del 45–50 and two subjects carrying del 48–50 were analyzed. The intensity of the same 80 selected areas from dystrophin-positive or -negative fibers were recorded. Expression was normalized to β-spectrin and expressed relative to control. Data are presented as percent of control. The differences in sarcolemmal neuronal nitric oxide synthase (nNOS) restoration between subjects carrying deleted exons 48–50 (from 11% ± 19.4 in dystrophin-negative fibers to 44% ± 26.7, $P < 0.0001$) and deleted exons 45–50 (from 8% ± 11.8 in dystrophin-negative fibers to 9% ± 12.6) were expected according to the domain structure of dystrophin, as nNOS binds to spectrin repeats 16/17 (exons 42–45). Significance was set at $P \leq 0.05$, paired two-tailed *t*-test, ns $^*P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, errors bars represent the SE of the mean.

All four patients demonstrated increased dystrophin expression after i.m. injection of the PMO SSO as reported previously.[16] Dystrophin expression was not uniform in the biopsied muscle, being more pronounced in fascicles presumably adjacent to the needle track, as shown by adjacent positive and negative fascicles (**Figure 2**). In addition to the dystrophin staining identified by the antibody MANDYS106 (epitope in exon 43), these patients had clear expression of dystrophin using Dys2, which recognizes an epitope located in the last 17 amino acids of dystrophin. Therefore, the internally deleted dystrophin that was induced after treatment is expected to also contain exons 60–69, which contribute to the cysteine-rich domain and the ZZ domain, necessary for the BDG binding.[4] By visual inspection, we observed that dystrophin-positive fibers did indeed display increased ASG and

BDG expression in comparison to dystrophin-negative fibers (**Figure 2**), indicating a restored DAPC complex. Upon quantification (**Figure 3**), a significant increase in ASG expression was observed in the dystrophin-positive fibers of the 45–50 deleted patients (paired two-sided *t*-test, $P = 0.0141$, from 52% ± 28 to 60% ± 27) and an increase was also observed in the dystrophin-positive fibers of patients with 48–50 deletions (paired two-sided *t*-test, $P=0.2460$, from 54% ± 24 to 57% ± 15). Furthermore, we demonstrated significant increases in BDG expression in both exon 45–50 and 48–50 deleted patients (paired two-sided *t*-test, $P = 0.0076$, from 36% ± 18 to 43% ± 16 and $P = 0.0009$, from 40% ± 17 to 48% ± 10, respectively).

We also studied the localization of nNOS after skipping of exon 51; where its restoration was markedly increased in patients with a 48–50 deletion (from 11% ± 19.4 in dystrophin-negative fibers to 44% ± 26.7 in dystrophin-positive fibers, $P < 0.0001$) but not in those with a 45–50 exon deletion (from 8% ± 11.8 in dystrophin-negative fibers to 9% ± 12.6 in dystrophin-positive fibers, $P = 0.4967$) (**Figure 3**).

DISCUSSION

Skipping of dystrophin exon 51 has been shown to restore dystrophin expression in a subset of myofibers after a single i.m. injection of 2′OMe or PMO SSO in proof-of-principle clinical trials in DMD patients.[16,17] After successful dystrophin restoration the important questions to address are the functional properties of the new-shortened dystrophin protein induced by exon skipping in the human. The lack of dystrophin in DMD leads to reduced levels of all DAPC proteins and even to the absence of nNOS from the sarcolemma.[21–25] The importance of a dystrophin protein containing its functional domains is highlighted by patients with the missense mutation (C3340Y)[4,26] within the BDG binding domain that develop a DMD phenotype even though considerable levels of dystrophin staining was visible at the sarcolemma. Another patient harboring a missense mutation (D3335H)[27] had dystrophin protein expression, but despite this, a DMD phenotype. Similarly in-frame deletions affecting the actin-binding domain also cause a DMD phenotype.[4,28,29] Therefore the functional properties of the restored dystrophin could be partly assessed by the quantification of the DAPC complex. This was shown briefly in animal models of DMD, as successful exon skipping with functional rescue in an exon 52 deficient mouse and in the *mdx* mouse model (which carries a nonsense mutation in exon 23)[30] was accompanied with a substantial increase in DAPC and the restoration of nNOS to the sarcolemma, although the skipping in the *mdx* mouse only lacks the epitopes encoded by exon 23. None of these models have deletions equivalent to those studied in our patients in this study.

Here, we show that restoration of the DAPC can be observed in the subset of myofibers showing dystrophin expression in DMD boys receiving i.m. treatment with SSOs. In particular we observed a significant increase in the sarcolemmal expression of BDG and ASG expression after a single i.m. injection of the PMO AVI-4658 in all four patients studied (**Figure 3**). Importantly, nNOS expression was also significantly restored at the sarcolemma (**Figures 2** and **3**) but to a greater extent in patients with a deletion of exons 48–50 (33% increase), compared to patients with deletion of exons 45–50 (2% increase). The differences in sarcolemmal nNOS restoration between subjects carrying del 48–51 and del 45–50 are in keeping with the domain structure of dystrophin, as nNOS requires the intact spectrin repeats 16/17 on the dystrophin molecule (encoded by exons 42–45) to be properly targeted to the sarcolemma.[31–33] Patients with deletions of exons 45–50 lacks part of this region and the failure to restore nNOS at the sarcolemma is equivalent to that described in BMD patients with the equivalent in-frame deletions.[32,33] These data are encouraging, as they indicate that the shortened dystrophin proteins produced by skipping exon 51 recapitulate protein expression observed in BMD patients with the relevant deletions.

The restoration of sarcolemmal nNOS observed here may have further important implications, as nNOS expression counteracts vasoconstriction and focal ischemia following exercise, which cause additional damage in DMD muscle.[10,34] Additionally, nNOS delocalization leads to nitrosative inhibition of muscle force in *mdx* mice,[35] and this may cause further damage to the muscle. Therefore the restoration of nNOS to the sarcolemma in DMD patients should lead to additional benefit, as their muscles are expected to be better protected from additional damage by these two nNOS-related mechanisms.[34,35] Further clinical research on BMD and other neuromuscular disorders with a loss of nNOS is needed in order to quantify the clinical impact of nNOS loss in humans.[36,37] While the clinical benefit of the increased expression of ASG and BDG in the human needs to be further investigated, the functionality of internally deleted dystrophins produced by exon skipping is already indicated by BMD patients carrying these in-frame deletions.[32,33,38–40] The increased expression of DAPC proteins is an indirect sign of the appropriate assembly of the protein complex and suggests that restored dystrophin retains its fundamental functions. These data are also supported by limited data we have obtained in a recent open label, systemic intravenous dose escalation study using the PMO AVI-4658 (0.5, 1.0, 2.0, 4.0, 10, and 20 mg/kg) for exon 51 skipping in ambulant DMD patients.[27,41] In two of the patients treated with AVI-4658 from whom there was sufficient muscle, we also demonstrated increased α-sarcoglycan and nNOS expression (this latter protein only in the patient with a deletion 49–50, not in the one with a deletion of exons 45–50), as also found in this study. However, we did not quantify BDG and patients with a deletion 48–50 in the previous study.[32]

In summary, we prove now in a comprehensive study of four patients, two carrying a deletion of exons 45–50 and two of 48–50, that the shorter novel dystrophin induced by exon 51 skipping in DMD can restore components of the DAPC as indicated by the increase in ASG and BDG in dystrophin-positive fibers and the genotype-specific relocalization of nNOS to the sarcolemma. These data confirm that restored dystrophin maintains essential domains for its function and further suggest the potential of the PMO AVI-4658 to become a disease modifying drug for DMD.

MATERIALS AND METHODS

The muscle biopsies were obtained during a single-blind, placebo-control dose escalation study (ClinicalTrials.gov, number NCT00159250), with primary outcome to test the safety and efficacy of an i.m. administration of a morpholino AO [eteplirsen (AVI-4658)] designed to skip dystrophin exon 51 in DMD patients.[16] Seven DMD patients harboring deletions

responsive to exon 51 skipping were investigated. Two patients received a dose of 0.09 mg eteplirsen into the extensor digitorum brevis whereas five patients received 0.9 mg; the contralateral muscle received an equivalent volume of normal saline for both dose cohorts. Bilateral extensor digitorum brevis biopsies were obtained 3–4 weeks later. AVI-4658 induced exon 51 skipping and increased expression of dystrophin with correct sarcolemmal localization. The two patients at the low dose showed exon skipping but not increased dystrophin expression. Following the 0.9 mg dose, muscle biopsies of cases 3–6, who received the high dose, demonstrated 44–79% of dystrophin-positive muscle fibers, relative to contralateral muscle background,[16] and were subsequently analyzed for the restoration of the DAPC.

For immunohistochemical detection, unfixed frozen serial transverse sections (7 μm) were incubated with monoclonal antibodies against dystrophin (Dys2, dilution 1:20; Vector Laboratories, Peterborough, UK), MANDYS106[42] (dilution 1:100, gift from Prof G. Morris, Oswestry, UK), α-sarcoglycan (dilution 1:25, Vector Laboratories) and BDG (dilution 1:20; Vector Labs). For nNOS, a polyclonal rabbit antibody (dilution 1:50; Santa Cruz, Santa Cruz, CA) was used. Sections were incubated with biotinylated secondary anti-mouse or anti-rabbit antibodies (1:200; Amersham, Buckinghamshire, UK) for 30 minutes at room temperature. Samples were then incubated with streptavidin conjugated to Alexa 594 (1:1,000; Invitrogen, Paisley, UK) for 15 minutes at room temperature and washed in phosphate-buffered saline before mounting in Hydromount (National Diagnostics, Atlanta, GA). Images were captured using a Leica DMR microscope linked to Metamorph (Molecular Devices, Sunnyvale, CA).[42] Semiquantitative measurements of dystrophin and the DAPC proteins expression levels were carried out as previously described.[42] β-Spectrin immunolabeling (NCL Spec-2, dilution 1:20; Vector Laboratories) was used as a marker of membrane integrity. In brief, images were randomly captured from muscle sections after carefully setting for each of the antibodies the acquisition parameters relative to the control sections. Later the sarcolemmal intensity of 40 different positive or negative fibers was quantified relative to control and normalized to β-spectrin expression using a Leica DMR microscope interfaced to MetaMorph (Molecular Devices).

The intensity of the same 80 selected areas from dystrophin-positive or -negative fibers were recorded. Expression was normalized to β-spectrin and expressed as percent relative to control. Statistical calculations were carried out using the GraphPad Prism 4.0 Software (La Jolla, CA). For comparison of fluorescence levels for each antibody between dystrophin-positive and -negative fibers a two-sided paired *t*-test was used.

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