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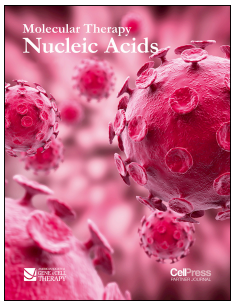

Relationship between myosin heavy chain fibre type and restoration of dystrophin expression and key components of the dystrophin-associated glycoprotein complex by Tricyclo-DNA mediated exon skipping

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Abstract

Exon skipping mediated by tricyclo-DNA (tc-DNA) antisense oligonucleotides has been shown to induce significant levels of dystrophin restoration in *mdx*, a mouse model of Duchenne Muscular Dystrophy. This translates into significant improvement in key disease indicators in muscle, cardio-respiratory function, heart and the central nervous system. Here we examine the relationship between muscle fibre type, based on Myosin Heavy chain profile, and the ability of tc-DNA to restore not only dystrophin but also other members of the dystrophin-associated glycoprotein complex (DAPC). We first profiled this relationship in untreated *mdx* muscle and found that all fibre types support reversion events to a dystrophin positive state, in an unbiased manner. Importantly, we show that only a small fraction of revertant fibres expressed other members of the DAPC. Immunoblot analysis of protein levels, however, revealed robust expression of these components, which failed to correctly localise to the sarcolemma. We then show that tc-DNA treatment leads to nearly all fibres expressing not only dystrophin but also other key components of the DAPC. Of significance, our work shows that MHC fibre type does not bias the expression of any of these important proteins. This work also highlights that the improved muscle physiology following tc-DNA treatment reported previously results from the complete restoration of the dystrophin complex in all MHCII fibres with equal efficiencies.

Introduction

Duchenne Muscular Dystrophy (DMD) affects 1:5000 male births and is the most common fatal childhood muscular disease[1,2]. Mutations in the *DMD* gene affect expression of dystrophin, a protein normally localized to the inner surface of the sarcolemma in muscle fibres[3,4]. Dystrophin together with a number of other proteins that constitute the dystrophin-associated glycoprotein complex (DAPC) act to link the muscle fibre cytoskeleton, the sarcolemma and the extracellular matrix (ECM) into a functional unit that maintains muscle integrity[5,6]. The DAPC is composed of three sub-complexes: (i) the sarcoglycans (α, ß, γ and δ), (ii) syntrophin, nNOS and dystrobrevin and (iii) α and ß dystroglycan. The absence of dystrophin results in a drastic reduction of all components of the DAPC at the sarcolemma and renders muscle cells prone to stretch-induced muscle damage[7].

A number of drug based or surgical procedures have been developed, including the use of corticosteroids or addressing scoliosis, that greatly improve the quality of life for DMD patients or delay disease onset[8,9]. However none have completely halted progression of the disease. Gene-based approaches that aim to restore dystrophin in muscle hold great promise. One attractive approach is to take advantage of the molecular structure of the dystrophin gene and to use antisense oligonucleotides (AON) to promote exon skipping to by-pass mutated stretches of DNA and restore the open reading frame[10]. The aim of the current study is to establish expression of a functional, albeit internally deleted, dystrophin protein. Restoration of dystrophin expression by exon skipping has been proven to be efficacious in-vitro, in animal models and in DMD patients[11-13]. Several classes of chemical modifications have been developed for AON mediated exon skipping, amongst which 2'O-methylribooligonucleoside-phosphorothioate (2'OMe), phosphorodiamidate morpholino oligomers (PMO) and tricyclo-DNA (tc-DNA). The latter has a number of properties that make it an attractive chemistry to exploit for therapeutic uses including high RNA affinity, resistance to nuclease activity and the ability to form nanoparticles that may facilitate uptake into cells[14-16]. We have recently shown, using *mdx* mice as a rodent model for DMD, that tc-DNA mediates unprecedented levels of exon skipping after systemic delivery not only in skeletal muscle but also in the heart and brain[16]. This translated into normalisation of specific force in the tibialis anterior muscle as well as improved cardio-vascular function and the correction of behavioural characteristics[16].

A number of studies using AON in both *mdx* mice and DMD patients have shown restoration of dystrophin in a subset of muscle fibres[13,17,18]. Most skeletal muscles are composed of a heterogeneous population of muscle fibres that differ in their metabolic properties as well as contractile speeds, a feature impacted by the type of Myosin Heavy Chain (MHC) being

expressed. Muscle of adult mice is composed of MHCI, MHCIIA, MHCIIX and MHCIIB fibres. MHCI has the slowest contraction rate and is highly reliant on oxidative phosphorylation for energy production. MHCBIIB is at the other end of the spectrum displaying the fastest contraction rates and highly dependent on glycolytic metabolism. Slow fibres are invested with a higher capillary density as well as thicker ECM compared to fast fibres[19,20]. Fast contracting fibres with its decreased ability to store energy in the ECM is hypothesised to facilitate greater proportion of force transfer to the skeletal elements[21]. A number of studies have shown that slow muscle, based on Myosin Heavy Chain expression profiling as well as physical measures express more dystrophin than fast muscle[22] and that fast muscle fibres are preferentially affected in DMD[23].

Here we examined whether the efficacy of dystrophin exon skipping is influenced by MHC fibre type, possibly due to fibre type differences in ECM thickness impacting on the rate of AON diffusion into the muscle fibre. We first profiled revertant fibres in *mdx* mice with a view of establishing whether their appearance was related to MHC fibre type. We then investigated the relationship between the restoration of dystrophin and of members of the three DAPC sub-complexes by treatment with tc-DNA and by MHC fibre type. Our results demonstrate that revertant fibres caused by splicing events in untreated *mdx* mice develop in a manner independent of MHC fibre type. Importantly we show that only a fraction of revertant fibres also express DAPC members. Treatment with tc-DNA results in over 90% of all fibres expressing all proteins examined, with no observed bias towards any one MHC fibre type. These data demonstrate that tc-DNA treatment is able to induce exon skipping in all MHCII fibres.

Results

We first established the myosin heavy chain (MHC) landscape of the tibialis anterior muscle (TA) and the effect wrought upon it first by the *mdx* mutation, and secondly after treatment with antisense oligonucleotides consisting of tc-DNA. Previous work has reported that the *mdx* mutation affects the MHC fibre profile in a muscle specific manner; with the EDL and soleus unchanged by the mutation[24] whereas the diaphragm contained slower isoforms compared to control[25]. Analysis of the TA of WT mice at its maximum circumference revealed an approximate ratio of 1:3:6 with respect to MHCIIA, MHCIIX and MHCIIB fibres (Figure 1A and B). The same ratios were found in the TAs of *mdx* mice and tc-DNA treated *mdx* mice (Figure 1A and B). Statistical analysis failed to reveal significant differences in the proportions of a particular MHC isoform between the three cohorts. Therefore the MHCII

profile of TA muscle was not affected by the absence of dystrophin or by treatment with tc-DNA.

We next examined the relationship between revertant fibres (dystrophin^{+}), co-expression of one member of each of the three DAPC sub-complexes, and MHCII class in untreated *mdx* mice. There were approximately 60 dystrophin+ fibres in the TA of 20-22 week old mice. The ratio of dystrophin^{+} in relation to MHC fibre type (IIA:IIX:IIB) was approximately 1:3:6 respectively. Therefore the segregation of dystrophin^{+} fibres within MHC subtypes followed the distribution of each isoform. Hence there was no bias towards any one MHC isoform with regards to reversion to a dystrophin positive state (Figure 2A). Profiling the expression of ß-sarcoglycan, nNOS and α-dystroglycan revealed a number of interesting features. First, they were found in all three MHC fibre isoforms and similar to dystrophin, there was no bias towards any one MHCII type (Figure 2A). ß-sarcoglycan, nNOS and α-dystroglycan expressing fibres were a subset of those that expressed dystrophin. However the number of fibres that expressed these three molecules was always lower than the number expressing dystrophin (Figure 2B). Indeed, nNOS positive fibres although being the most frequent of the three, only represented about half of dystrophin positive fibres.

These results show that revertant fibres do not express the full complement of DAPC components.

Thereafter we examined the expression of dystrophin, ß-sarcoglycan, nNOS and α-dystroglycan in relation to MHC fibre type in the TA of tc-DNA treated *mdx* mice. Immunostaining revealed that the majority of fibres were positive for dystrophin after tc-DNA treatment which is in agreement with previous findings of Goyenvalle and colleagues[16] (Figure 3A). Robust expression of dystrophin was found in all MHC fibre types and analysis of frequency with respect to fibre proportion revealed that there was no bias to any one fibre type. Immunostaining for ß-sacroglycan, nNOS and α-dystroglycan revealed the same features as dystrophin; the vast majority of fibres expressed the three proteins and their presence in a particular MHC fibre type was proportional to the frequency of that form (Figure 3A). We then compared the relative frequency of fibres expressing each of the four proteins. We found there were significantly more fibres that expressed dystrophin than the other three components of the DAPC assessed (Figure 3B).

These results show that tc-DNA treatment of *mdx* mice results in the restoration of dystrophin in the majority of muscle fibres. This is also the case for α-dystroglycan, ß-sarcoglycan and nNOS. However the number of fibres expressing ß-sarcoglycan, nNOS and α-dystroglycan were significantly lower compared to dystrophin.

Previous work has shown that contractile properties of a muscle fibre impact both qualitatively and quantitatively on its surrounding extracellular matrix[19,20]. Here we examined the relationship between MHC fibre type and expression of components of the DAPC as well as an ECM component Collagen IV using semi-quantitative techniques. Fluorescence intensity was used as previously described to gain an indication of the amount of protein at the sarcolemma[18]. We first determined the signal intensity for the five proteins in question in relation to MHC fibre type in the revertant fibres from the *mdx* mouse. For each fibre type, the signal intensity was set to a reference value of 1. Thereafter the same procedure was repeated for the tc-DNA treated muscle and intensity compared to that of the untreated TA muscle. The outcome of the process showed that tc-DNA treatment resulted in an increase in the amount of each protein of interest in all fibre types compared to untreated revertant *mdx* fibres (Figure 4). We also measured the thickness of the expression domain for each of the five marker proteins, revealing that each expression domain was thicker in MHCIIA fibres compared to MHCIIB fibres (Figure 4). This relationship persisted following tc-DNA treatment. Secondly, we found that there was an increase in the expression domain following tc-DNA treatment for all fibre types (Figure 4).

These results show that the amount of each component of the DAPC and collagen IV at the sarcolemma were elevated above those found in revertant fibres.

Last, we examined the effect of tc-DNA treatment on the total level of expression of the DAPC components under investigation here. To that end we carried out quantitation of Western blots. Our results showed that there was a 14 fold increase in the amount of dystrophin following tc-DNA treatment (Figure 5A and B). Interesting we found robust expression of ß-sarcoglycan, nNOS and α-dystroglycan in untreated *mdx* muscle and that their levels were not changed significantly by tc-DNA treatment. In summary, components of the DAPC are translated in the absence of dystrophin, but fail to localise to the sarcolemma.

Discussion

Tc-DNA chemistry is an exciting development in the area of molecular medicine. We have previously shown that in the context of a mouse model of Duchenne Muscular Dystrophy that tc-DNA treatment was more efficacious in restoring muscle function than many other approaches[16]. Of particular note was the finding that tc-DNA antisense oligonucleotides spontaneously form nanoparticles which are believed to promote entry into the cell, and may be the reason why these were able to penetrate the heart and the brain[16]. In this current study we investigated the relationship between muscle compositions in terms of MHC fibre type

and dystrophin restoration by tc-DNA AON with a view of developing an understanding of its specificity of action.

We commenced the study by comparing the MHC profile of the TA muscle in the three cohorts under investigation; WT, *mdx* and tc-DNA treated *mdx* mice. We found that all three shared the same MHC profile. Previous studies have shown that the MHC profile of the diaphragm underwent a significant change in its MHC compositions with a decrease in the proportion of MHCIIB$^+$ fibres and a concomitant increase in the number of MHCI$^+$ fibres[25]. The change in MHC profile was proposed to be an adaptive step to preserve contractile function and fibre integrity by lowering energy requirements. In contrast, the EDL and soleus muscles of the *mdx* were shown to be identical to those from WT in terms of MHC profile[24]. Our results here now add the TA to the list of muscles that show normal MHC profile in the *mdx* mouse. Nevertheless, all *mdx* muscles have reduced specific force. We propose that if a change towards a slower MHC profile is an adaptive change to the absence of dystrophin, it must be a secondary step that is dependent on other upstream factors, one of which could be contractile activity which would explain the change in constantly used muscles like that diaphragm and not in limb muscles. A number of studies have proposed mechanisms to explain the reduced specific force in *mdx* including nitrosylation of the contractile machinery which, if it occurred, would lead to long term damage due to low turnover rate of MHC[26,27]. However recent work has shown that the MHC from *mdx* functions normally in terms of cross-bridging which argues against long term effect of altered muscle function[28]. This suggestion is indirectly corroborated by the efficacy of tc-DNA treatment being able to normalise specific force in the TA of *mdx* mice[16].

Our work examining the distribution of the DAPC protein in untreated *mdx* muscle offers interesting insights into the formation of the functional unit. First, we show that there was no bias in terms to MHC fibre type and the appearance of dystrophin. Therefore if metabolic activity were to generate differential cellular stress based on fibre contraction rate, then this metric does not impact on the splicing events that restore dystrophin expression in *mdx* muscle. Furthermore, when serendipitous events lead to the restoration of dystrophin they bring back the protein in a relatively normal manner with respect to MHC fibre type; slower fibres expression more than faster ones[22]. Additionally, there was no bias in terms of MHC fibre type and any of the other components of the DAPC examined here. An interesting feature highlighted by our work in this section was the finding that revertant fibres (dystrophin$^+$) are heterogeneous in terms of their DAPC composition; dystrophin$^+$>nNOS$^+$>αDG$^+$>ßSG$^+$. Our quantifications of DAPC expression in revertant

fibres extends previous findings of Lu et al who showed co-expression of DAPC proteins in clusters of revertant fibres [29]. Interestingly, our Western blotting data agrees with prior reports showing an abundance of nNOS, αDG and ßSG total protein in *mdx* muscle[30]. It follows, therefore, that a mechanism must be active that prevents the translocation of DAPC proteins to the sarcolemma of some revertant fibres. One possibility is that they may not have had sufficient time to correctly translocate. This is, however, unlikely since revertant fibres form from events that occur in muscle precursors[31]. Another possibility is linked to the poor diffusion of dystrophin within the myofibre sarcolemma, limiting membrane expression to spatially confined nuclear domains. Moving out of this dystrophin domain during serial sectioning would affect detection of other components of the DAPC. While this phenomenon can contribute to a decreased co-detection of dystrophin and DAPC proteins, it should be noted that dystrophin expression in revertant fibres has been reported to span membrane segments of 654 ± 409 μm[32] . It is unlikely that exiting a nuclear domain during collection of serial sections over a length of muscle not exceeding 160μm (16 serial sections) could on its own account for over half of the revertant fibres lacking expression of other DAPC proteins (Figure 3b). It is therefore possible that a significant proportion of internally deleted dystrophins generated by revertant fibres is not able to assemble a functional DAPC but can be correctly localized to the membrane. This hypothesis would be consistent with reports of truncated or internally deleted dystrophins that lack the cysteine rich domain required for interaction with the DAPC but can still be correctly localized to the sarcolemma[33]. Overall our results highlight two interesting findings: first, the majority of revertant fibres produce internally deleted forms of dystrophin that cannot functionally contribute to force transduction since they are uncoupled from the dystroglycan and sarcoglycan complexes; and second, *mdx* muscle has a rich pool of DAPC proteins available for recruitment to the sarcolemma upon expression of a functional dystrophin protein.

Restoration of dystrophin expression following tc-DNA treatment resulted in near total coverage of TA fibres, consistent with our previous work[16]. We do not believe that the variation in the affinities of antibodies for their epitopes is a decisive factor in showing a variation in DAPC profile between *mdx* and tc-*mdx* mice. We base this conclusion on the fact that have we have compared the same strain (indeed litter mates) with or without tc-DNA treatment. Therefore the differing affinities between antibodies for their particular epitope would remain a constant factor. Hence the appearance of a molecule at the sarcolemma in tc-*mdx* mice compared to *mdx* must be due to changes in the expression levels of the protein. The results of this current study demonstrate that there is no bias with regards to dystrophin

expression induction following tc-DNA treatment and fibre type. This is, we believe, highly relevant and important for prospective translation into therapies. Previous work carried out in humans revealed restoration of dystrophin in a subset of muscle fibres, a differential restoration that may have been influenced by the structural properties of the muscle[13,17]. Indeed, it is well established that slow muscle fibres have a thicker ECM in comparison to fast fibres[19,20]. It would, therefore, be reasonable to postulate that slow fibres are more resistant to infiltration by tc-DNA AON. However our work shows that, at least in terms of type II fibre sub-types, there is no preference to exon skipping. This bodes well for the use of this chemistry in a spectrum of muscles with differing fibre composition as it seems they are all in principle able to take up the tc-DNA AON. In addition we show that MHC fibre type does not influence the restoration of the other components examined here. Also, tc-DNA treatment leads to more of each component at the sarcolemma compared to revertant *mdx* fibres. Nevertheless it is worth noting that not all the fibres that expressed dystrophin contained the other three components of the DAPC examine here; dystrophin^{+}>nNOS^{+}> ßSG^{+}>αDG^{+}. This again highlights the point that the presence of the DAPC proteins does not necessarily translate into them being assembled into a functional complex. There is a dearth of knowledge regarding mechanisms that regulate the formation of the DAPC, a gap in our understanding that requires urgent attention. One potential consequence of this gap in our understanding is that it is possible that we will develop the means of inducing protein dystrophin expression, but that it may not translocate to the correct sub-cellular region and therefore reduce therapeutic benefit. Insights into this process could be gained by examining revertant fibres. Nevertheless, tc-DNA treatment resulted in over 90% of the fibres having all four of the DAPC components at the sarcoplasm. We believe that this high level of DAPC restoration explains the normalisation of specific force following tc-DNA treatment, and again bodes well for translation into the clinic since previous studies have demonstrated that restoration of dystrophin protein levels to 10-20% of wild type results in improved health[34].

In summary we show that reversion of fibres to a dystrophin positive state in *mdx* mice is a stochastic process with regard to MHC fibre type. However expression of dystrophin in *mdx* revertant fibres only translates into a minority (>25%) of fibres expressing members of the three sub-complexes. Tc-DNA treatment results in over 90% of fibres expression dystrophin as well as members of the three sub-complexes in the TA muscle. Importantly, there is no bias in terms of expression of any component with regards to MHC fibre type. This work shows that in principle tc-DNA treatment is equally efficacious across all type II fibres.

Methods

Animals

Animal procedures were performed in accordance with national and European legislation, approved by the French government (Ministère de l'enseignement supérieur et de la recherche, Autorisation APAFiS #6518). *Mdx* (C57BL/10ScSc-Dmdmdx/J) and C57BL/10 mice were bred in our SOPF animal facility at the Plateform 2Care, UFR des Sciences de la santé, Université de Versailles Saint Quentin and were maintained in a standard 12-hour light/dark cycle with free access to deionized water and standard laboratory chow (*M20*, *Dietex*), ad libitum. Mice were weaned at weeks 4-5 postnatal and 2-5 individuals were housed per cage. Mice were randomly allocated to treatment and control groups ensuring equal numbers of control and treated mice within the same litters.

The tcDNA-AON PS M23D (5′-AACCTCGGCTTACCT-3′) targeting the donor splice site of exon 23 of the mouse dystrophin pre-mRNA, used in this study was synthesised by SYNTHENA (Bern, Switzerland). 6-8 week old male *mdx* mice were injected intravenously in the retro-orbital sinus, under general anaesthesia using 1.5-2% isoflurane, once a week with 200mg/kg/wk of the M23D-tcDNA for a period of 12 weeks. Treated mice were sacrificed 2 weeks after the last injection and muscles and tissues were harvested and snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C before further analysis.

Immunohistochemistry

Dissected and frozen muscles were mounted in Tissue Tech freezing medium (Jung) cooled by dry ice/ethanol. Immunohistochemistry staining was performed on 10 µm cryosections that were dried for 30 minutes at room temperature (RT) prior to three washes in 1 x PBS. Muscle sections were incubated in permeabilisation buffer solution (0.952 g Hepes, 0.260 g MgCl2, 0.584 g NaCl, 0.1 g Sodium azide, 20.54 g Sucrose and 1 ml Triton X-100) for 15 minutes at room temperature, before the application of block wash buffer (PBS with 5% foetal calf serum (v/v), 0.05% Triton X-100) for 30 minutes at room temperature.

Primary antibodies were pre-blocked in wash buffer for 30 minutes prior to incubate onto muscle sections overnight at 4°C. Pre-blocked in wash buffer was performed for all secondary antibodies (in dark) for minimum of 30 minutes prior to their addition onto the slides. Sections were then incubated for 1 hr in the dark at room temperature. Finally, slides were mounted in fluorescent mounting medium, and myonuclei were visualised using

(2.5µg/ml) 4, 6-diamidino-2-phenylindole (DAPI). Details of primary and secondary antibodies are given in supplementary file.

Western blotting

TA proteins from 20-22-week-old male mice (20µg/lane) were separated on 4–12% gradient SDS-PAGE gels (Invitrogen), transferred to nitrocellulose membranes (Whatman), and blocked with 5% skim milk in 0.1% Tween 20/Tris buffered saline. Membranes were cut at appropriate molecular weights in order to allow for simultaneous probing of the exact same samples for dystrophin and multiple DAPC proteins. Membrane strips were then incubated with appropriate primary antibodies overnight at 4^0C, followed by a 1 hour incubation at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Protein bands were visualized using enhanced chemiluminescence reagents (Pierce). Signal was detected on X-Ray film (RPI) at multiple exposures. For densitometric analysis, protein band intensities from multiple non-saturated film exposures were quantified using ImageJ (NIH). Values in the linear range of pixel intensities were selected for quantifications. Signal intensities were normalized to GAPDH, used as an internal loading control and probed on the same membrane. Details of primary and secondary antibodies are given in supplementary file.

Semi-quantitative measures of sarcolemma protein expression

Intensity of signals of protein of interest was measured as previously described[18]. Briefly, membrane signal intensities of approximately 30 muscle fibres of each MHC phenotype (IIA, IIX and IIB) in each TA muscle section from *mdx* and *mdx* treated with tc-DNA- mice were measured. Fiji software was used to measure signal from area of interest after images had being corrected for background to avoid regions of signal saturation. To calculate relative signal intensity levels, individual measurements from treated and control fibres were taken as a percentage of mean of control samples.

Sarcolemma thickness measurement

Connective tissue thickness between approximately 30 fibres of the same MHC phenotypes (IIA-IIA, IIX-IIX and IIB-IIB) of TA muscle sections was measured using Fiji software. One measurement on the constant connective tissue thickness, with multi measurements on the fluctuating connective tissue thickness areas between each two myofibres that expressed

same MHC isoform were taken on all muscle sections of *mdx* and *mdx* treated with tc-DNA-mice.

Imaging and analysis

A Fluorescence microscope (Zeiss AxioImegar A1) was used to examine immunofluorescent stained sections, and images were captured using an Axiocam digital camera with Zeiss Axiovision computer software version 4.8.

Statistical analysis

Data are presented as mean ± SE. Significant differences between two groups were performed by two-tailed Student's t-test for independent variables. Differences among groups were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests as appropriate. Statistical analysis was performed on GraphPad Prism software. Differences were considered statistically significant at p*< 0.05, p**< 0.01 or p***< 0.001.

Legends

Figure 1. Myosin heavy chain profile of the Tibialis anterior muscle. (A) Immunohistochemical images of TA muscle from 20-22-week-old male wild-type, *mdx* and tc-DNA treated *mdx* mice. Green fibres signify the expression of MHCIIA with MHCIIB appearing as red. Non green and red fibres represent MHCIIX. (B) MHC profile in the three cohorts. Results show that WT, *mdx* and tc-DNA treated *mdx* mice have the same proportion of each MHC subtype. (n=4 for each cohort). Statistically analysis performed by one-way ANOVA followed by Bonferroni's correction for multiple comparison.

Figure 2. Expression of Dystrophin, ß-sacroglycan, nNOS and α-dystroglycan in the TA muscle of 20-22-week-old male *mdx* mice. (A) Each row shows the entire TA muscle stained for one of the four molecules together with a magnified detailed image. All positive fibres were correlated to the expression of a MHC isoform and their distribution is give as the total number as well as proportion to the frequency of the MHC isoform. (B) Graph showing the proportion of TA fibres expressing the four investigated molecules. (n=4 for each cohort).

*p<0.05, **p<0.01, ***p<0.001. Statistical analysis performed by one-way ANOVA followed by Bonferroni's correction for multiple comparison.

Figure 3. Expression of Dystrophin, ß-sarcoglycan, nNOS and α-dystroglycan in the TA of 20-22-week-old male tc-DNA treated *mdx* mice. (A) Each row shows the entire TA muscle immune stained for Dystrophin, ß-sarcoglycan, nNOS and α-dystroglycan together with a magnified detailed image. All positive fibres were correlated to the expression of a MHC isoform and their distribution is give as the total number as well as proportion to the frequency of the MHC isoform. (B) Graph showing the proportion of TA fibres from tc-DNA treated *mdx* mice expressing the four investigated molecules. (n=4 for each cohort). *p<0.05, **p<0.01, ***p<0.001. Statistical analysis performed by one-way ANOVA followed by Bonferroni's correction for multiple comparison.

Figure 4. Semi-quantitative analysis of DAPC restoration following tc-DNA treatment of 20-22-week-old male *mdx* mice. Untreated and tc-DNA treated muscle show high levels of individual proteins in MHCIIA fibres (white arrows) compared to MHCIIB fibres (yellow arrows). Intensity from over 30 regions for each fibre type were taken from revertant untreated *mdx* and set to reference value of one. Similar numbers of intensity readings were plotted for each fibre type from tc-DNA treated *mdx* muscle. In all cases there was a significant increase in intensity compared to untreated revertant fibres of the same MHC type. Thickness of expression domain was measured and plotted for each MHC isoform originating from revertant untreated *mdx* and tc-DNA treated *mdx* muscle. MHCIIA fibres had thicker expression domains compared to MHCIIB. Thickness for expression domains irrespective of MHC fibre type was increased by tc-DNA treatment. (n=4 for each cohort). Scale bar applicable to all images equals 50µm. *p<0.05, **p<0.01, ***p<0.001. Statistical analysis performed using 2-tailed t-Test.

Figure 5. Western blot analysis of DAPC proteins 20-22-week-old male mice. (A) Western blot image of DAPC proteins in the TA muscle. (B) Quantification of DAPC proteins relative to GAPDH. Note the robust expression of ß-sarcoglycan, nNOS and α-dystroglycan in untreated *mdx* muscle. (n=3 for each cohort). **p<0.01. Statistical analysis performed using 2-tailed t-Test.

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Author contribution

Conceptualisation KP. Methodology AG, LG. Investigation SO, KLH, HCH, FM. Writing SO, FM, AG, LG, KP. Supervision KP.

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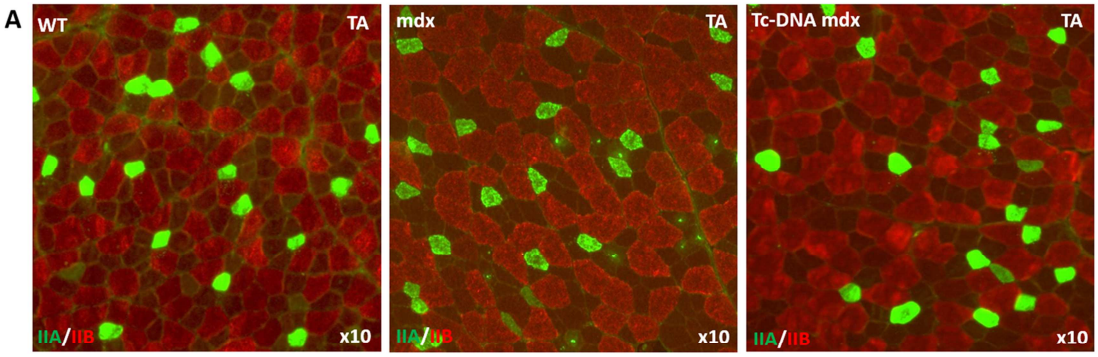
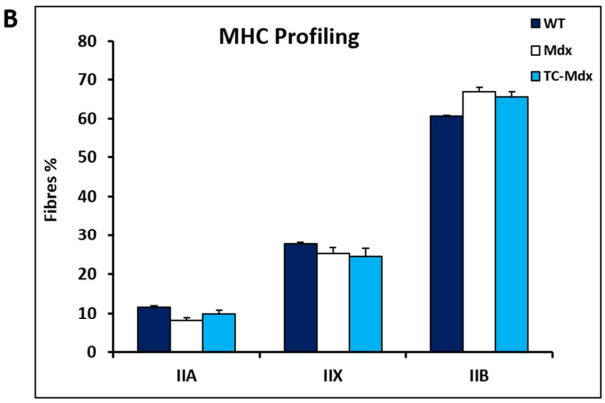
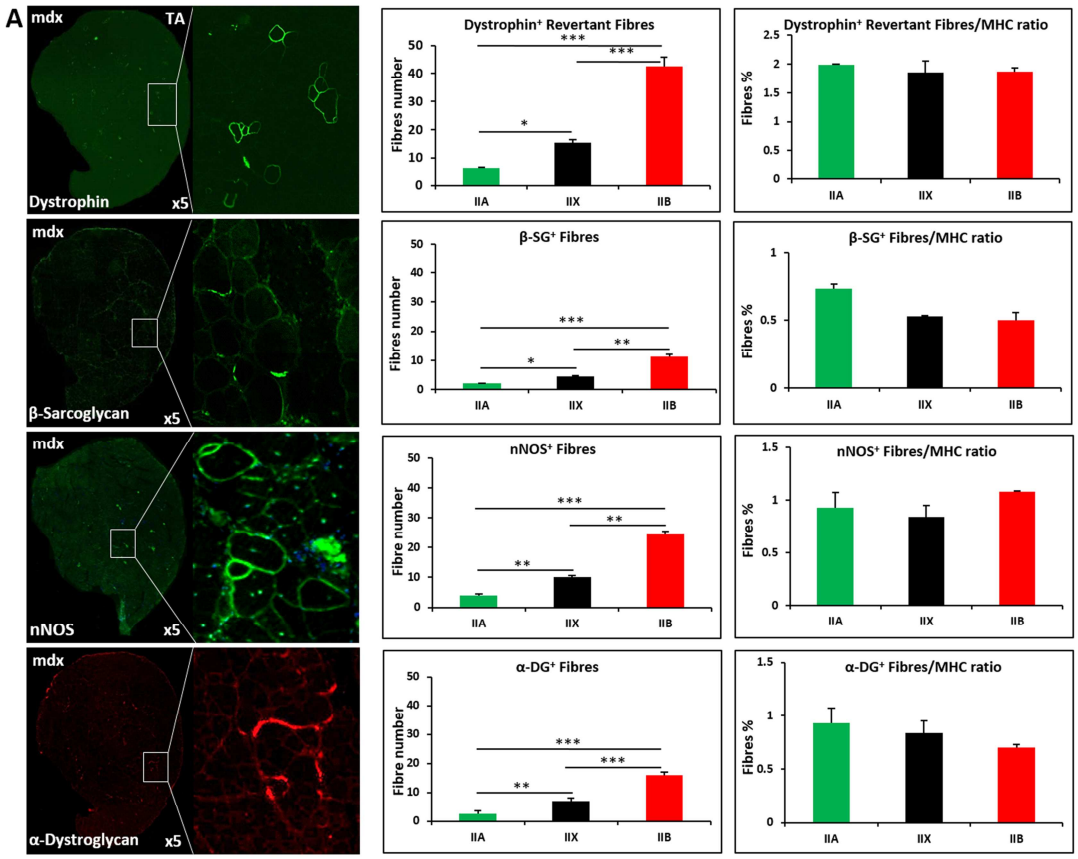
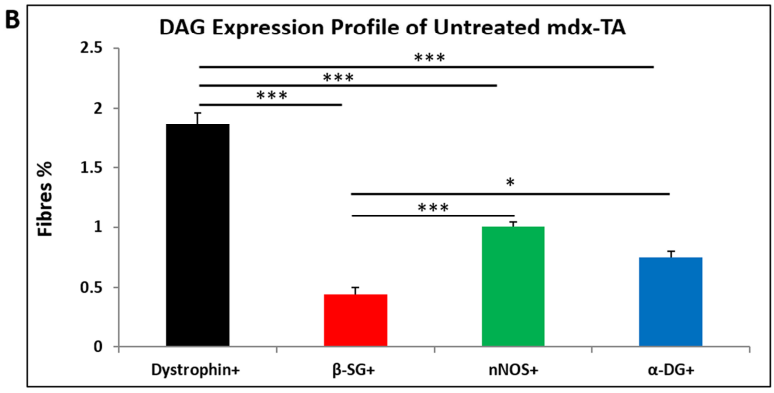
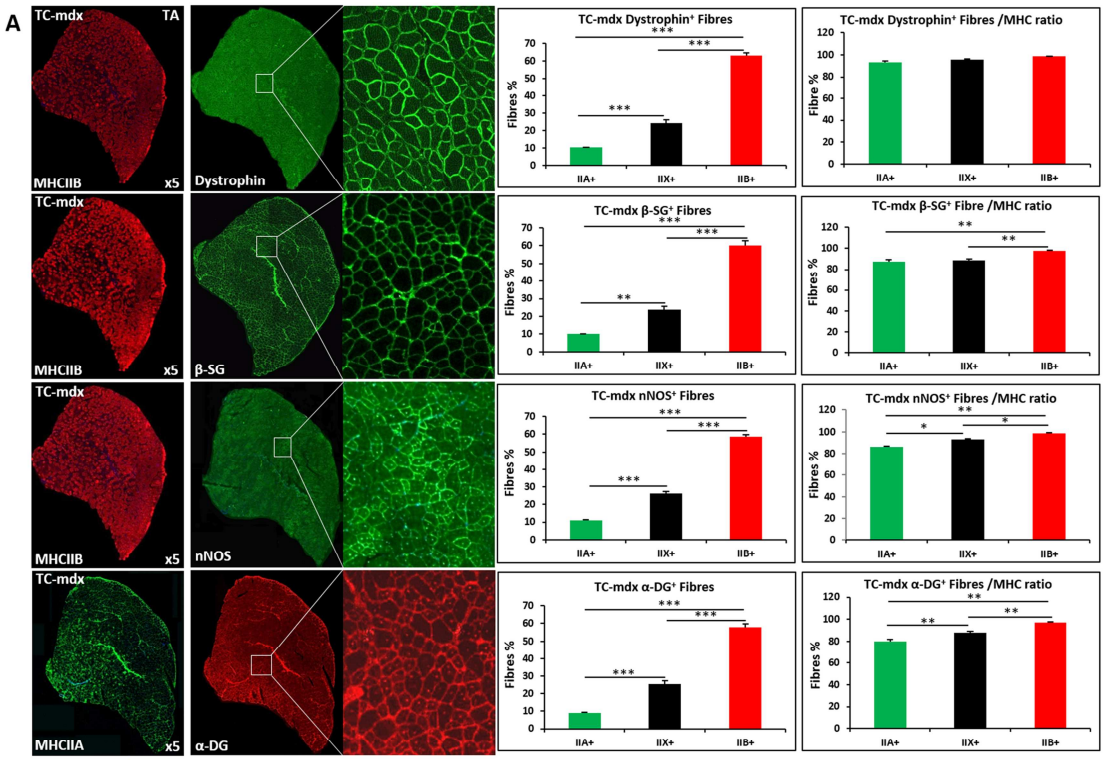
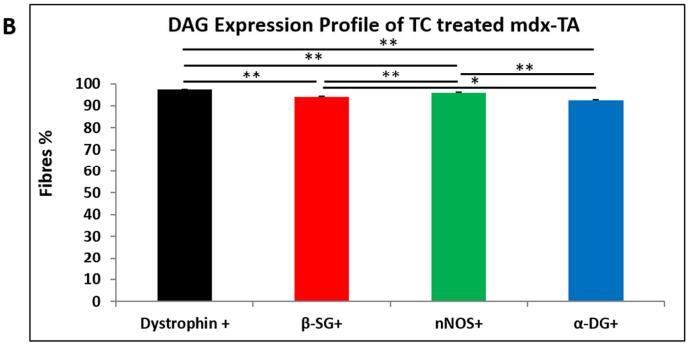


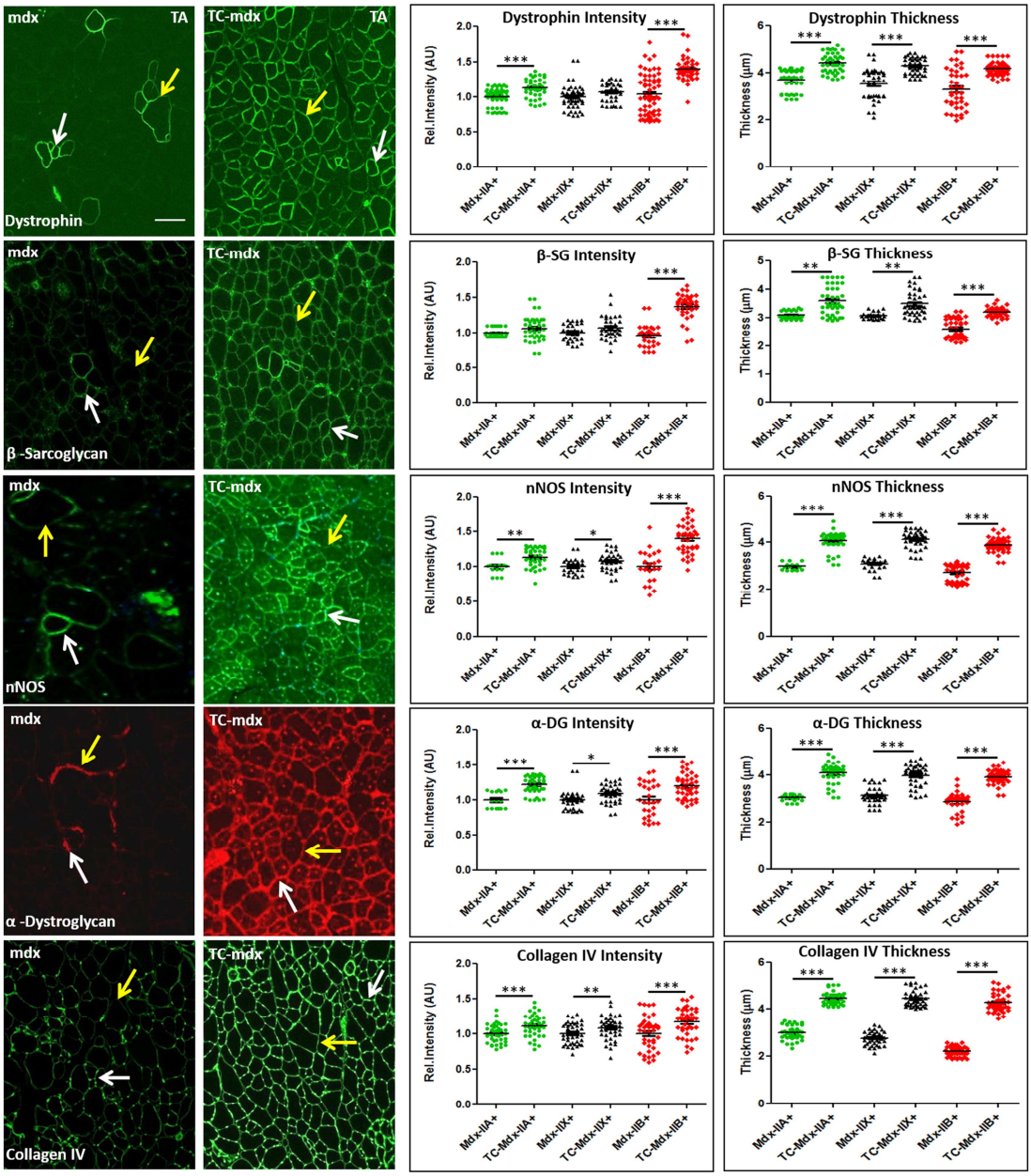



A
mdx
TA
Dystrophin
x5
β-Sarcoglycan
nNOS
α-Dystroglycan
Dystrophin+ Revertant Fibres
Fibres number
IIA
IIX
IIB
Dystrophin+ Revertant Fibres/MHC ratio
Fibres %
β-SG+ Fibres
β-SG+ Fibres/MHC ratio
nNOS+ Fibres
Fibre number
nNOS+ Fibres/MHC ratio
α-DG+ Fibres
α-DG+ Fibres/MHC ratio


B
DAG Expression Profile of Untreated mdx-TA
Fibres %
Dystrophin+
β-SG+
nNOS+
α-DG+


A
TC-mdx
TA
MHCIIB
x5
Dystrophin
β-SG
nNOS
MHCIIA
α-DG
TC-mdx Dystrophin⁺ Fibres
TC-mdx Dystrophin⁺ Fibres /MHC ratio
TC-mdx β-SG⁺ Fibres
TC-mdx β-SG⁺ Fibre /MHC ratio
TC-mdx nNOS⁺ Fibres
TC-mdx nNOS⁺ Fibres /MHC ratio
TC-mdx α-DG⁺ Fibres
TC-mdx α-DG⁺ Fibres /MHC ratio
Fibres %
Fibre %
IIA+
IIX+
IIB+


B
DAG Expression Profile of TC treated mdx-TA
Fibres %
Dystrophin +
β-SG+
nNOS+
α-DG+


mdx
TA
TC-mdx
TA
Dystrophin
Dystrophin Intensity
Dystrophin Thickness
β -Sarcoglycan
β-SG Intensity
β-SG Thickness
nNOS
nNOS Intensity
nNOS Thickness
α -Dystroglycan
α-DG Intensity
α-DG Thickness
Collagen IV
Collagen IV Intensity
Collagen IV Thickness
Rel.Intensity (AU)
Thickness (µm)
Mdx-IIA+
TC-Mdx-IIA+
Mdx-IIX+
TC-Mdx-IIX+
Mdx-IIB+
TC-Mdx-IIB+