MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Total Absence of Dystrophin Expression Exacerbates Ectopic Myofiber Calcification and Fibrosis and Alters Macrophage Infiltration Patterns

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Duchenne muscular dystrophy (DMD) causes severe disability and death of young men because of progressive muscle degeneration aggravated by sterile inflammation. DMD is also associated with cognitive and bone-function impairments. This complex phenotype results from the cumulative loss of a spectrum of dystrophin isoforms expressed from the largest human gene. Although there is evidence for the loss of shorter isoforms having impact in the central nervous system, their role in muscle is unclear. We found that at 8 weeks, the active phase of pathology in dystrophic mice, dystrophin-null mice (*mdx* ^{β geo}) presented with a mildly exacerbated phenotype but without an earlier onset, increased serum creatine kinase levels, or decreased muscle strength. However, at 12 months, *mdx* ^{β geo} diaphragm strength was lower, whereas fibrosis increased, compared with *mdx*. The most striking features of the dystrophin-null phenotype were increased ectopic myofiber calcification and altered macrophage infiltration patterns, particularly the close association of macrophages with calcified fibers. Ectopic calcification had the same temporal pattern of presentation and resolution in *mdx* ^{β geo} and *mdx* muscles, despite significant intensity differences across muscle groups. Comparison of the rare dystrophin-null patients against those with mutations affecting full-length dystrophins may provide mechanistic insights for developing more effective treatments for DMD. (*Am J Pathol* 2020, 190: 190–205; <https://doi.org/10.1016/j.ajpath.2019.09.021>)

Duchenne muscular dystrophy (DMD) is a severely debilitating and invariably fatal X-linked neuromuscular disorder, which results from mutations in the *DMD* gene.¹ *DMD* is the largest human gene known, encoding multiple structurally diverse isoforms of dystrophin.² Three full-length transcripts, comprising 79 exons, encode 427-kDa proteins

while further intragenic promoters³ drive expression of progressively truncated variants (Figure 1A).

The current central hypothesis states that Duchenne muscular dystrophy pathology is caused by the loss of the full-length dystrophin (Dp427) in myofibers, where it anchors the dystrophin-associated protein complex, linking the

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extracellular matrix, the sarcolemma, and the intracellular cytoskeleton. This assembly is considered critical for muscle function and survival. Therefore, all the current preclinical and clinical therapeutic approaches are aimed at dystrophin restoration in differentiated muscle cells.

However, there is growing evidence that DMD mutations produce a range of significant cell-autonomous abnormalities in both human and mouse myogenic cells, suggesting a much earlier onset of pathology and explaining impaired muscle regeneration.^{4–10}

The severity of DMD-associated cognitive impairment correlates with the cumulative loss of dystrophin isoforms expressed in the central nervous system, thus suggesting a prominent functional role for these shorter isoforms in brain cells.^{11,12} However, little attention has been given to the potential role of shorter dystrophins controlled by the intergenic promoters, and few in-depth comparisons between the full-length and the dystrophin-null muscle phenotypes have been undertaken. Interestingly, the proportion of patients with a severe motor and cognitive phenotype has been shown to correlate with mutations affecting all dystrophins.¹³ Gene mutations causing DMD disrupt the reading frame and include large deletions (68%), duplications (11%), and smaller rearrangements and point mutations (20%).¹⁴ Initial analyses indicated that the *DMD* gene mutation hot spots are located in the regions encoding the full-length isoforms. However, although large deletions and duplications have a nonrandom distribution with the two identifiable hot spots, small insertions/deletions and point mutations are distributed along the entire gene,¹⁵ thus affecting multiple isoforms.

Interestingly, there are little data documenting the expression of the so-called nonmuscle dystrophin isoforms in muscle. Given that myogenic cells are affected by *DMD* mutations and are known to express some of these truncated isoforms (eg, Dp71), we hypothesized that null *DMD* mutations may alter functions of myogenic cells and thus affect the phenotype. Therefore, the consequences of total loss of *DMD* expression were investigated. The muscle pathology was compared in the most widely used animal model of DMD—the *mdx* mouse, lacking full-length isoforms because of a point mutation in exon 23,¹⁶ against the *mdx* ^{β geo} dystrophin-null mouse with the reading-frame disruption downstream of exon 63, which is present in all dystrophins and therefore with all isoforms being ablated. This mouse, unlike models generated by chemical mutagenesis, is a true pan-dystrophin knockout with no revertant fibers present.¹⁷

Materials and Methods

Animals

The male *mdx* and *mdx* ^{β geo} wild-type control mice (C57Bl10 and C57Bl6, respectively) were used in

accordance with institutional Ethical Review Board and the Home Office (United Kingdom) approvals. The C57Bl10 and C57Bl6 strains derived from the common origin,¹⁸ and it has been demonstrated that the *mdx* mutation on the C57Bl6 background shows the same pathology as the original Bl10 strain.¹⁹ All mice were maintained under pathogen-free conditions and in a controlled environment (12-hour light/dark cycle, 19°C to 23°C ambient temperature, and 45% to 65% humidity). Mice were euthanized by carbon dioxide inhalation, and cells and muscles were dissected and used for protein extraction or frozen in isopentane prechilled in liquid nitrogen for cryosectioning.

Antibodies and Reagents

The following antibodies were used at 1:1000: anti-dystrophin (ab15277; Abcam, Cambridge, UK), antiactin (A2066; Sigma-Aldrich, Gillingham, UK), anti-F4/80 (ab6640; Abcam), and anti-CD68 (ab125212; Abcam). All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific (Loughborough, UK).

Serum Creatine Kinase Level Measurement

Blood samples were collected, allowed to coagulate, and centrifuged for 10 minutes at 2500 $\times$ g. Sera isolated immediately after centrifugation were analyzed for the creatine kinase levels using the Creatine Kinase Activity Assay Kit (CK-NAC; Randox Laboratories Ltd., Crumlin, UK), according to manufacturer's instructions.

Force Measurements in Diaphragms *ex Vivo*

Whole diaphragms from 4-month-old wild-type and dystrophic mice were excised, and contractile force strength was measured following the Treat Neuromuscular Disorders Neuromuscular Network (TREAT-NMD) standard operating procedures (<https://treat-nmd.org/research-overview/preclinical-research/experimental-protocols-for-dmd-animal-models>, last accessed September 26, 2019) and as previously described.⁸ Essentially, diaphragms were placed into Krebs-Ringer solution. Sutures were tied and muscle then attached to an immobile plastic clamp with the central triangular section of the diaphragm being used for testing. Contractile force was measured using a mechanical force transducer (ADInstruments, Oxford, UK), amplifier, and data acquisition setup. Excitation was achieved via local field potentials through platinum electrodes in oxygenated (95% O₂, 5% CO₂) Krebs-Ringer solution, at a constant temperature (37°C). After incremental stretching to establish the optimal excitation-to-force generation length and confirmation of the appropriate voltage twitch stimulus, diaphragm sections were subjected to a 140-V (2-millisecond) stimulus train at 100-Hz frequency for 0.5 to 1 second. The test regimen involved collecting six twitch responses, followed by six tetanic trains, with a 2-minute rest

Figure 1 Dystrophin isoform expression in *mdx* versus *mdx*^{βgeo} muscle. **A:** DMD mutation location and their effects on predicted isoform expression in *mdx* and *mdx*^{βgeo}. The *mdx* mouse carries a point mutation in exon 23, whereas *mdx*^{βgeo} harbors an insertion disrupting the reading frame downstream from exon 63. **B:** Western blot analysis of dystrophin protein expression in 8-week muscles showing loss of the Dp427 isoform in *mdx* samples and loss of all isoforms from *mdx*^{βgeo}, which confirm it to be a complete dystrophin knockout. Triplicate bands shown represent lysates from three different animals, and actin is shown as a protein loading control.

period between each. All forces were normalized to muscle wet weight and expressed as Newtons per gram of tissue (N/g).

Grip Strength Test

In this and all other *in vivo* tests, investigators (N.C. and Scott Rodaway) were blinded with respect to the sample group allocation. The grip strength test was performed, as previously described²⁰ and according to the TREAT-NMD protocol (http://www.treat-nmd.eu/downloads/file/sops/sma/SMA_M.2.1.002.pdf, last accessed September 26, 2019). Essentially, mice were held by the tail and slowly approached to a metallic grid (6 × 6-cm) connected to a force sensor gauge (FG-5000A; Lutron Electronic, London, UK). Once the animal gripped the grid by its forelimbs, a gentle horizontal traction was applied to the tail until the animal let the grid go. The maximal force was recorded over two trials with a 1-minute intertrial interval. Strength was estimated by the mean of both trials.

RNA-Sequencing Analysis

Total RNA was extracted from tibialis anterior (TA) of 7-week-old C57BL/10 and *mdx* male mice (*n* = 4), quality controlled, and sequenced, as previously described.⁸

Quality control of raw reads was performed using fastQC version 0.11.7 (Babraham Institute, Cambridge, UK; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>, last accessed September 26, 2019). Reads were trimmed using

trim-galore version 0.4.4 (Babraham Institute; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore, last accessed September 26, 2019) with parameters to remove adapter sequence and low-quality sequence tails. Trimmed reads were mapped against the GRCm38 *Mus musculus* genome from Ensembl using the STAR version 2.5.3a modified universal RNA-seq aligner²¹ with the following parameters: `-outSAMmultNmax 300 -outSAMstrandField intronMotif`. Properly paired reads that mapped uniquely to the genome, with a mapping quality >20, were retained for further analyses.

Differential expression analysis was conducted using the DESeq2 package²² in R version 3.5.1 (<http://www.R-project.org>, last accessed September 26, 2019).²³ Gene models were taken from Ensembl version 91, and read counts over unique genes were quantified using the *summarizeOverlaps()* function in the GenomicAlignments package²⁴ using parameters `mode = Union, singleEnd = FALSE, ignore.strand = FALSE, fragments = FALSE, preprocess.reads = invertStrand`. *P* values were adjusted for multiple testing by using the Benjamini and Hochberg false discovery rate correction.²⁵ The whole muscle RNA-seq data can be accessed from Array Express (<https://www.ebi.ac.uk/arrayexpress/experiments>; accession code E-MTAB-7698).

Histologic Stains

Hematoxylin and eosin, Alizarin red (AR), periodic acid–Schiff, Von Kossa, oil red O, and picrosirius red

Figure 2 Morphologic and functional alteration in *mdx* versus *mdx*^{βgeo} muscle. **A–C:** Morphometric analysis of 8-week tibialis anterior muscles reveals a shift in fiber size (**A**) in the order of: wild-type (Wt) > *mdx* > *mdx*^{βgeo}, which was found to be consistent for the average fiber area (**B**) and feret diameter (**C**). **Insets:** Example of fiber threshold and the analysis using ImageJ version 2.0.0-rc-69/1.52p (Fiji). **D:** Numbers of centrally nucleated fibers are significantly elevated: *mdx*^{βgeo} > *mdx* > Wt. **E:** Serum creatine kinase (CK) levels indicative of sarcolemma stability are not significantly altered in *mdx*^{βgeo} compared with *mdx*. **F:** Grip strength at 8 weeks is reduced by approximately 50% in both *mdx* and *mdx*^{βgeo} compared with Wt, and diaphragm contractile force is also low in both *mdx* and *mdx*^{βgeo}. **G:** In contrast, at 12 months, increases in maximum force were recorded in both Wt and *mdx*, but not in *mdx*^{βgeo}, diaphragms, which remain at a basal level. **H and I:** Picrosirius red staining for collagen (**H**) and oil red O staining for fat (**I**) in 8-week versus 12-month diaphragms reveal significant increases in older animals: *mdx*^{βgeo} > *mdx* > Wt. Data are expressed as means ± SD (**B–I**). *n* = 3 to 5 (**A–I**). **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001. Scale bars = 250 μm (**H** and **I**). FOV, field of view.

staining methods followed standard operating procedures from TREAT-NMD—recommended protocols available online (<http://treat-nmd.eu/research/preclinical/dmd-sops>, last accessed September 26, 2019). All staining was performed using cryosections (10 μm thick), air dried onto poly-L-lysine-coated glass slides (Fisher Scientific). Slides were mounted in DPX or aqueous media, coverslipped, and imaged (Axiozoom V.16; Zeiss, Cambridge, UK). Representative images per genotype are shown, whereas

montages, where *n* = 30 to 40, were constructed and assessed using ImageJ version 2.0.0-rc-69/1.52p (Fiji, <https://fiji.sc>, last accessed September 26, 2019) counting tools.²⁶

Whole-Body Tissue Clearing, Imaging, and Analysis

Clearing procedure was performed as described previously.²⁷ Briefly, animals were deeply anesthetized with i.p.

Figure 3 Muscle fiber mineralization is linked to the loss of dystrophin expression in *mdx* and *mdx*^{βgeo}. **A:** Immunohistochemistry staining for dystrophin in 8-week tibialis anterior (TA) muscle sections confirms the *mdx* to express dystrophin in a small number of revertant fibers. In contrast, *mdx*^{βgeo} animals display no revertant fibers, in keeping with the molecular alteration in these animals. **B:** On dissection, significant white striations (**arrows**) are observed in the diaphragms of *mdx*^{βgeo}, which are also found in *mdx*, albeit at much lower levels, but not in controls. Heightened diaphragm hypercontraction is also consistently observed in the order of: *mdx*^{βgeo} > *mdx* > wild-type (Wt), represented by the enlarged region of translucent connective tissue in the center. **C:** Striations are found in all skeletal muscle groups of dystrophic mice but at different levels, with proximal muscles (quadriceps and gluteus) being affected more than distal muscles, such as TA. **D:** Heart muscles are affected, albeit showing slightly different striation patterns than skeletal muscles (Supplemental Figure S1). **Arrows** highlight regions of ectopic calcification (white streaks). Scale bars = 100 μm (A).

injection of lethal dose of sodium pentobarbital (100 mg/kg), subjected to cardiac perfusion, and subjected to fixation followed by 2 to 3 days of clearing with CUBIC reagent-1²⁸ or reagent-1A (Riken, http://cubic.riken.jp/data/CUBIC_clearing_protocol_with_Reagent-1A.pdf, last accessed September 26, 2019) clearing solutions and 1 day of 0.03% (w/v) AR staining dissolved in fresh clearing solution. Finally, specimens were placed for 2 to 3 days of gentle shaking with fresh clearing solution at 37°C in an incubator to remove the excess of unbound AR. Images were collected with customized light-sheet apparatus and analyzed according to a previously described protocol.²⁷

Immunolocalization and Morphologic Analysis

Frozen muscle was transferred to a cryostat chamber and allowed to equilibrate to −20°C. Cryosections (10 μm thick) were then cut from the middle third of the sample and collected on poly-L-lysine (0.5 mg/mL)-coated glass slides. Sections were allowed to air dry for several hours. Samples were fixed in a 2% to 4% w/v paraformaldehyde solution in Tris-buffered saline/Tween 20 for 15 minutes

at 4°C, followed by two washes in phosphate-buffered saline/Tween 20. The primary antibody incubation in phosphate-buffered saline/Tween 20 containing 10% v/v serum was applied for 2 hours at room temperature or overnight at 4°C. Three 5-minute Tris-buffered saline/Tween 20 washes were applied before secondary antibody incubation in phosphate-buffered saline/Tween 20 and 10% v/v serum containing Hoechst (1:1000) fluorescent nuclear counterstain for 1 hour at room temperature. Sections were finally washed three times for 30 minutes before mounting in FluorSave (Merk Millipore, Watford, UK) fluorescence mounting medium. Either entire cross-sections through the midportion of TA muscles were captured in their entirety using Axiozoom V.16 (Zeiss) or whole cross-sections were made of montaged ×20 magnification fields of view. For quantification of immunofluorescence cells, a semiautomated (unbiased) method using a thresholding macro designed in ImageJ software (Fiji) was used. Numbers were then expressed per unit of area. For diaphragms, counts per unit area for each animal were derived by averaging the counts from five fields of view encompassing a significant portion of each diaphragm cross-section. Counts were also made

Figure 4 Histochemical and mineral analyses in 8-week-old *mdx* and *mdx*^{geo} muscles. **A** and **B**: Alizarin red (**A**) and Von Kossa (**B**) staining demonstrates that the white striations in diaphragm sections contain calcium and phosphate, respectively. **C–F**: Electron backscatter diffraction analysis of diaphragm sections from 8-week *mdx*^{geo} (**C**) identifies colocalization of calcium (**D**) and phosphate (**E**) in electron-dense fibers, with the calcium/phosphate ratio of 1.50 (**F**), consistent with the presence of tricalcium phosphate [Ca₃(PO₄)₂] or hydroxyapatite, which has a ratio of 1.6729. *n* = 3 mice (**A–F**). Scale bars = 250 μm (**A–E**).

using the threshold and *analyze particles* functions of ImageJ software.

Western Blot Analysis

Proteins were extracted, resolved, and blotted, as described previously.^{20,29} Blots were blocked in 5% w/v nonfat milk powder in 1× Tris-buffered saline (50 mmol/L Tris, 150 mmol/L NaCl, and 0.01% v/v Tween-20; Sigma-Aldrich), for 1 hour before probing with primary antibody diluted in the same blocking buffer (overnight at 4°C or 2 hours at room temperature), then washed (three times) with 1× Tris-buffered saline/Tween 20 for 10 minutes and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse 1:10,000 (A4416; Sigma-Aldrich) or anti-rabbit 1:5000 (A6154; Sigma-Aldrich) overnight at 4°C or 1 hour at room temperature. Specific protein bands were visualized using Luminata Classico or Forte chemiluminescent substrates (WBLUC0500 or WBLUF0500, respectively; Merck Millipore), and images were obtained using a ChemiDoc MP system (Bio-Rad, Hertfordshire, UK). Densitometric analyses were performed using the integrated density measurement function of ImageJ software. All experiments were repeated at least three times in triplicate, throughout.

X-Ray Micro-Computed Tomography

Quadriceps were placed within a 1.5-mL tube (Eppendorf, Stevenage, UK) and supported by a polyurethane foam

saturated in 70% ethanol. Muscles were imaged using a Zeiss Xradia 520 Versa X-ray microscope (Zeiss) operating at an energy of 50 kV, a power of 4 W, and a tube current of 80 μA; and a Zeiss LE1 filter was positioned directly after the X-ray source. A 0.4× objective lens was used with an X-ray source (sample distance of 20 mm) and a detector (sample distance of 105 mm). A total of 1601 X-ray projection images were collected over 360 degrees at equal intervals with an isotropic voxel size of 11 μm. The exposure time for each projection was 2 seconds. The projections were reconstructed using the manufacturer's integrated software, which uses a filtered back projection reconstruction algorithm. The individual tomography scans were quantified using the threshold function in ImageJ²⁶ and visualized in three dimensions using TXM3DViewer (Zeiss).

Statistical Analysis

Results are reported as means ± SD, where *n* refers to number of independent experiments (3 to 6). Significance scores were based on Kruskal-Wallis with post-hoc Dunn's test for nonparametric multiple comparisons; one-way analysis of variance with post-hoc Tukey test for normal multiple comparisons; and unpaired *t*-tests for individual comparisons, with Mann-Whitney post-hoc test for nonparametric *t*-tests (GraphPad Prism8; GraphPad Software, San Diego, CA). For cumulative frequency distribution, Kolmogorov-Smirnov test was used. Differences were considered statistically significant at *P* < 0.05.

Results

Dystrophic Pathology in *mdx* ^{β geo} Dystrophin-Null Mice

Muscle pathology in the *mdx* muscles begins to present at 2 to 3 weeks, reaching maximum intensity in leg muscles at approximately 8 weeks, before plateauing at approximately 12 to 16 weeks.^{30,31} However, the *mdx* mouse diaphragm shows progressive pathology³² and, therefore, this muscle closely represents the human condition.

To identify the potential phenotypic differences resulting from the absence of all versus full-length isoforms, *mdx* and *mdx* ^{β geo} (dystrophin-null) mice (Figure 1) were compared following the TREAT-NMD standard operating procedures.^{33,34}

At 8 weeks (the peak of pathology), morphologic analysis of leg muscles revealed a significant shift in myofiber cross-sectional area toward smaller fibers in dystrophic muscle in the order of wild-type (BL10) > *mdx* > *mdx* ^{β geo} (Figure 2, A and B). A significant reduction in the average ferret diameter followed the same trend (Kruskal-Wallis with Dunn's test) (Figure 2C). Central nucleation was significantly elevated in *mdx* ^{β geo} compared with *mdx* (Mann-Whitney test, $P = 0.0159$) (Figure 2D). At 8 weeks, serum creatine kinase levels (Figure 2E) were not significantly different (Mann-Whitney test, $P = 0.4127$) between the two dystrophic strains, indicating that loss of short dystrophins did not exacerbate sarcolemma damage. Grip strength *in vivo* (Figure 2F) and diaphragm strength *ex vivo* (Figure 2G) were equally reduced in both dystrophic strains at 8 weeks. Yet, there was an age-dependent difference between *mdx* ^{β geo} and *mdx*: In 12-month-old animals, diaphragm contractile force strength showed small but significant increase in *mdx* preparations (unpaired *t*-test, $t = 6.572$, $df = 4$, $P = 0.0028$), but no increase was found in *mdx* ^{β geo} (unpaired *t*-test, $t = 0.6558$, $df = 4$, $P = 0.5478$) (Figure 2G). Furthermore, fibrosis (Figure 2H) and fat accumulations (Figure 2I) were both found elevated in 12-month-old *mdx* ^{β geo} diaphragms compared with age-matched *mdx*.

Total Dystrophin Loss Exacerbates Ectopic Calcification of Dystrophic Muscle Fibers

Muscles from *mdx* ^{β geo} mice do not express any dystrophin isoforms (Figure 1B) or truncated variants in revertant fibers (Figure 3A). In contrast, at 8 weeks, striking opaque fibers,

particularly prominent in diaphragms but detectable in all major skeletal muscle groups, were found to be notably more abundant in *mdx* ^{β geo} than in *mdx* mice (Figure 3, B–D). The appearance of these fibers closely resembled ectopic calcification reported previously in the *mdx*, *mdx*/*Utrophin* double knockout and the humanized-*mdx* mouse models,^{35–38} in the golden retriever muscular dystrophy dog,^{39,40} and, more important, in DMD patients.⁴¹ To confirm, it was first verified if calcium- and phosphorus-containing deposits were present in these opaque diaphragm fibers using AR (Figure 4A) and Von Kossa (Figure 4B) stains, respectively. Scanning electron microscopy energy-dispersive X-ray spectroscopy electron backscatter analysis (Figure 4C) confirmed the presence of mineral deposits containing both calcium (Figure 4D) and phosphate (Figure 4E) with a molar ratio of 3:2 (Figure 4F), consistent with tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$].⁴²

The striated appearance of calcified fibers showed regions of calcification with distinct patterning, sometimes along the length of almost entire fiber, sometimes in short regions of otherwise unaltered fiber (Figure 3C and Supplemental Video S1). Muscle groups most severely affected with ectopic calcification were diaphragm (Figure 3B) and the proximal limb (quadriceps and gluteus) with a consistently milder phenotype in the distal groups (TA and gastrocnemius) (Figure 3C). More important, this ectopic calcification was also found in cardiac muscles of *mdx* ^{β geo} (Figure 3D and Supplemental Figure S1), which, to our knowledge, is the first demonstration of this abnormality in a DMD model.

Whole-Body Musculature Analysis of Ectopic Calcification in *mdx* and *mdx* ^{β geo}

The initial study revealed significant differences in ectopic calcifications between various muscle groups, indicating the need for systematic comparisons. To screen for and quantify ectopic mineralization in various muscle groups of the entire animal, previously optimized whole-body tissue optical clearing method²⁷ was applied. Such an approach, when combined with AR staining, allowed us to demonstrate excessive accumulation of ectopic calcifications in *mdx* ^{β geo} versus *mdx* and confirm complete absence of these in control animals (Figure 5, A–D). Thereby, calcified deposits were observed particularly abundant within *mdx* ^{β geo} diaphragms (Figure 5B) but also in skeletal muscles of the laryngopharynx, forelimb, lumbar region, pelvic region, and

Figure 5 Whole-body and three-dimensional muscle analysis of ectopic calcifications in *mdx* and *mdx* ^{β geo}. Whole-body tissue clearing and Alizarin red staining show distribution of ectopic calcification across the entire musculature. **A:** Representative bright-field (top panels) and epifluorescent images (bottom panels) reveal sites of myofiber calcification and allow detailed comparative imaging of the affected body regions. **B:** Epifluorescent images of the selected planes from **A** demonstrate higher prevalence of calcifications in *mdx* ^{β geo} versus *mdx* with a complete absence of deposits in the control mouse. **Arrowheads** indicate clusters of calcium deposits in laryngopharynx (1 to 3), forelimb (4 to 6), diaphragm (7 to 9), lumbar region (10 to 12), and hind limb (13 to 15). **C** and **D:** Spinalis pars lumborum from macroscopically prescreened mice was isolated and imaged in crossed polarized light (**C**) and light-sheet fluorescence microscopy (**D**). **E** and **F:** Three-dimensional light-sheet data allow us to reconstruct distribution of sites of ectopic calcification (**E**) and quantify its pattern in muscles of *mdx* ^{β geo} and *mdx*—presented herein as percentage mineralization and cumulative frequency distributions in triceps brachii, spinalis pars lumborum, and biceps femoris (**F**). Unpaired *t*-test and two-sample Kolmogorov-Smirnov test were performed. Data are expressed as means $\pm$ SD (**F**). $n = 3$ mice per group (**F**). * $P < 0.05$. Scale bar = 5 mm (**D**). Original magnification, $\times 4$ (**A–E**).

Figure 6 Quantification of muscle fiber mineralization in *mdx* versus *mdx*^{βgeo}. **A** and **B**: Alizarin red (AR) staining was quantified in *mdx* (**A**) and *mdx*^{βgeo} (**B**) diaphragms (Diap.) at 8 weeks of age. **C** and **D**: Representative images of tibialis anterior sections from *mdx* (**C**) and *mdx*^{βgeo} (**D**) are shown to illustrate the difference in severity between different muscle groups. **E**: Alizarin red images were thresholded, a mask was generated in ImageJ software, and fibers displaying an arbitrarily assigned positive value at or above the threshold level were counted using the ImageJ version 2.0.0-rc-69/1.52p (Fiji) particle analysis function. **F**: A significant increase in absolute numbers and percentage of mineralized muscle fibers is found in *mdx*^{βgeo} compared with age-matched *mdx* diaphragms. **G–J**: Striations along the entire length of fibers were analyzed in whole muscle mineralization analysis using three-dimensional (3D) X-ray imaging. **G** and **H**: Quadriceps (QUAD.) from 8-week-old *mdx* (**G**) and *mdx*^{βgeo} (**H**) in 3D rendering reveals two different patterns of mineralization: one diffuse and globular and the other striated (left and right sides of tissue shown in **H**, respectively). **I** and **J**: Representative Z-sections for *mdx* and *mdx*^{βgeo} are shown, respectively. Data are expressed as means ± SD (**F**). $n = 3$ (**F**). **** $P < 0.0001$. Scale bars = 5 mm (**G–J**). Original magnification, $\times 4$ (**A–E**).

hind limbs. Next, a customized light-sheet setup was used to perform detailed three-dimensional imaging of isolated muscles from three distinct body regions (ie, spinalis pars lumborum, biceps femoris, and triceps brachii) (Figure 5, E and F). When compared with *mdx*, every *mdx*^{βgeo} muscle was characterized by a higher percentage of tissue mineralization, with differences being particularly striking in triceps brachii, where ectopic calcification reached 11.59% in *mdx*^{βgeo} and 0.36% in *mdx* [percentage of mineralization: unpaired *t*-test $t(4) = 5.32$; $P < 0.01$] (Figure 5F). In contrast, the difference was not found to be statistically significant in spinalis pars lumborum [unpaired *t*-test $t(4) = 2.62$; $P = 0.058$] (Figure 5F) and biceps femoris [unpaired *t*-test $t(4) = 0.97$; $P = 0.386$] (Figure 5F). Cumulative frequency distribution analysis showed different

distribution of calcified deposits in triceps brachii and spinalis pars lumborum muscles from *mdx*^{βgeo} mice in comparison to *mdx* mice (Figure 5F).

Further confirmation of muscle fiber calcification was undertaken using AR staining of TA (Figure 6, C and D) and diaphragm (Figure 6, A and B) sections and particle analysis–based quantification of threshold images using ImageJ software (Figure 6E). Significantly elevated numbers and percentages of calcified fibers were confirmed in diaphragms (Figure 6F), whereas TA was confirmed to be less affected by the ectopic calcification. Finally, ectopic calcification in isolated 8-week-old quadriceps *mdx* and *mdx*^{βgeo} muscles were visualized in three dimensions under the X-ray microscope (Xradia; Zeiss) (Figure 6, G–J, and Supplemental Video S1).

Figure 7 Timing and evolution of muscle fiber mineralization in *mdx^{βgeo}* muscles. **A–C:** At 2 weeks, muscles appear normal with no visible striations. **D–F:** By 4 weeks, light striations begin to appear (arrows). **G–I:** After a peak at approximately 2 months (**G**), calcium-containing fibers (arrows) disappear at approximately 10 to 12 weeks (**H**), and are replaced by connective tissue (**I**). Note the increased opacity of the diaphragm with increasing age (**G** to **I** progression). **J:** Quantification of Alizarin red (AR)—positive fibers across ages. **K:** Quantification of AR staining in 2-, 3-, and 6-month-old diaphragm sections, confirming the absence of mineralization. Data are expressed as means $\pm$ SD (**J** and **K**). $n = 3$ (**J** and **K**). **** $P < 0.0001$.

Age of Onset and Evolution of Ectopic Muscle Mineralization in *mdx* and *mdx^{βgeo}*

The onset and progression of muscle pathology in the *mdx* muscle are well documented with cycles of degeneration and regeneration and significant sterile inflammation between 3 and 12 weeks of age, followed by a significant reduction of symptoms from 12 weeks onward. The exception is diaphragm, where the pathology is progressive and thus resembles human disease.^{30,31} Aforementioned exacerbation of ectopic mineralization in *mdx^{βgeo}* led us to assess whether total dystrophin ablation triggers an earlier onset of dystrophic damage with ectopic calcification. To test this hypothesis, AR

staining intensities were analyzed in 2- and 4-week-old *mdx* and *mdx^{βgeo}* diaphragm muscle sections. Two-week-old muscles were found to be visually devoid of detectable calcifications (Figure 7, A–C), but at 4 weeks, white striations were clearly beginning to form in limb and diaphragm muscles (Figure 7, D–F). Quantification of AR staining in diaphragm sections confirmed first calcified fibers to appear somewhere between 2 and 4 weeks of age but equally in both *mdx* and *mdx^{βgeo}* animals (Figure 7J). Given the nature of the ectopic calcification, it could be expected to worsen with age, particularly in diaphragms. However, analyses in 3- and 6-month-old mice showed the calcified fibers could no longer be found (Figure 7K). The diaphragm appearance,

with thickening and opacity (Figure 7, G–I), may be due to ongoing inflammation and emerging fibrosis, which are pathologic hallmarks of 12-month-old diaphragms. Indeed, picrosirius red staining for collagen (Figure 2H) revealed the presence of fibrosis.

Differences in Macrophage Distribution and Association with Mineralized Fibers in *mdx* versus *mdx* ^{β geo} Diaphragms

Inflammation is the well-known pathologic hallmark of DMD. It affects muscle regeneration but also degeneration and fibrosis.^{43,44} Of the inflammatory cells found in *mdx* muscles, macrophages play a significant yet complex role: Their depletion results in the reduction or exacerbation of pathology, depending on the stage of disease.^{35,44,45} The RNA-seq data (Array Express code E-MTAB-7698) identified significant contribution of macrophage genes to the altered inflammatory gene expression profile in *mdx* muscles (Supplemental Figure S2). Furthermore, a recent study has demonstrated that inorganic phosphate (Pi) can specifically activate macrophages to prevent ectopic calcification.⁴⁶ Given that the evolution of calcified muscle fibers mirrored the onset and cessation of inflammation in *mdx* muscle, the immune cells in the muscle sections were analyzed. F4/80 staining for macrophages was markedly different in the two dystrophic strains: *mdx* muscle showed scattered staining with numerous macrophage puncta spread throughout the tissue and only some larger puncta of intense staining (Figure 8, C and G). In contrast, *mdx* ^{β geo} muscles displayed large F4/80-positive puncta, which colocalized perfectly with mineralized fibers and appeared almost uniquely and intricately associated with them (Figure 8, A, B, D–F, and H). Often, macrophages were tightly associated with what appeared to be partially degraded fibers (Figure 8B). The CD68 and osteopontin staining colocalization in these macrophages indicated their predominantly M1 phenotype (Figure 8, I–K). CD68 macrophage targeting of ectopic calcium deposits was also increased in *mdxBgeo*, with significantly increased clustering of CD68 (Figure 8, L and M).

In conclusion, total loss of dystrophin expression in the mouse model of DMD specifically exacerbated ectopic

myofiber calcification, altered macrophage infiltration, and aggravated subsequent fibrosis.

Discussion

There is evidence that absence of the full-length (427-kDa) dystrophin in the fully differentiated myofibers may not necessarily cause the dystrophic phenotype.^{47,48} In contrast, Dp427 has been shown to play a role in satellite cells^{4,5,9,49,50} and there are clear data that a lack of DMD gene expression affects various important functions of myoblasts, including cell proliferation, differentiation, energy metabolism, and signaling.^{6,8,51} These and other findings indicate that dystrophic pathology starts much earlier than has been suggested⁵² and point at the importance of the loss of dystrophin expression in myogenic cells, dysfunction of which determines abnormalities of muscle regeneration and therefore disease progression. Because Dp71 dystrophin has been found in undifferentiated myogenic cells,⁵³ we hypothesized that *Dmd* gene mutations eliminating expression of this isoform may further alter functions of myogenic cells and thus affect the dystrophic phenotype. Therefore, the muscle pathology was compared in the most widely used animal model of DMD—the *mdx* mouse, lacking full-length isoforms because of a stop mutation in exon 23,¹⁶ against the *mdx* ^{β geo} dystrophin-null mouse.¹⁷ The latter DMD model is interesting as it has no observed dystrophin-positive revertant fiber clusters⁴⁵ and allows complex phenotypes to be investigated. Notably, mutation hot spots of large deletions and duplications are located in the regions encoding the full-length isoforms. However, small insertions/deletions and point mutations are distributed along the entire gene¹⁵; and these would affect the full spectrum of dystrophins. However, there are little data evaluating the role of the shorter dystrophin isoforms in muscle.

Our analyses revealed a slightly exacerbated phenotype in *mdx* ^{β geo}, especially in older mice. However, these dystrophin-null mice did not show an earlier onset of the dystrophic pathology, which might have been expected given that Dp71 was found expressed in muscle development.^{17,54} The muscle pathology being similar to that in *mdx* mice was in agreement with the previous study in Cre-

Figure 8 Differential macrophage distribution and association with mineralized fibers in *mdx* and *mdx* ^{β geo} muscles. **A:** Confocal image showing F4/80 (red) macrophage marker and cell nuclei (blue) staining combined with mineral deposits visualized in bright field in *mdx* ^{β geo} diaphragms. **B:** Calcified fibers can be seen saturated with macrophages (arrow). **C and D:** Macrophage distribution differs between *mdx* and *mdx* ^{β geo} muscles: In *mdx* diaphragm (**C**), macrophages can be seen distributed throughout the tissue with some areas of increased infiltration, whereas in *mdx* ^{β geo}, macrophages appear to be predominantly associated with calcified fibers (**D**). **E–H:** Confocal images of F4/80 staining without bright field, corresponding with **A** through **D**, respectively. **I–K:** Higher-magnification images showing CD68 (red) marker colocalization with osteopontin (green), indicating the predominantly M1 phenotype of macrophages associated with calcified fibers. **L:** ImageJ-based (Fiji) quantification of images showing the CD68 staining to localize to fewer (left panel) but larger (right panel) puncta in *mdx* ^{β geo}, confirming macrophage clustering at sites of mineralization. **M:** Significant differences in CD68 puncta number and size are found between *mdx* and *mdx* ^{β geo} (left and middle panels), but total CD68 intensity in *mdx* ^{β geo} is not found to be significantly different to that of *mdx* (right panel). Data are expressed as means $\pm$ SD (**M**). $n = 3$ (**M**). **** $P < 0.0001$. Scale bars: 250 μ m (**A**, **C**, **D**, **E**, **G**, and **H**); 50 μ m (**B** and **F**); 100 μ m (**I–K**). FOV, field of view.

loxP mouse, in which the DMD gene was deleted.⁵⁵ Moreover, no increase in serum creatine kinase levels, indicative of sarcolemma permeability, suggested a different role for this short isoform. Interestingly, the most striking alteration in *mdx* ^{β geo} was the ectopic calcification. Ectopic calcifications have been reported previously in *mdx*^{35,56} and were found particularly abundant in *mdx/Utrrophin* double-knockout, *mdx*/ δ -sarcoglycan double-knockout,⁵⁷ and the humanized-*mdx* mouse models,^{35–38} which all present with an exacerbated dystrophic phenotype. Notably, ectopic calcifications are also found in human DMD patients.⁴¹

One explanation for the different phenotypes could be the protection afforded to the *mdx* muscle by the revertant, dystrophin-positive fibers. These revertants are thought to arise through splicing events, and they occur at varying frequencies in different muscle groups; approximate average values of 2% to 7% in TA and 1% to 4% in diaphragm have been reported previously in the *mdx* mouse.⁵⁸ These fibers are completely absent from *mdx* ^{β geo} muscles. Given that approximately 15% dystrophin-positive fibers appear sufficient to protect against contraction-induced injury,⁵⁹ revertant fibers could perhaps have some impact.

Another important question is the following: which cells are affected by the lack of short dystrophins? Given that myofibers do not express Dp71, it is likely that these are satellite cells or myoblasts. However, the loss of DMD gene expression in nonmuscle cells cannot be excluded, as indicated by the purinergic phenotype found in DMD patients' lymphoblasts.⁶⁰ Therefore, disease phenotypes in DMD patients with mutation affecting all dystrophins should be reevaluated.

More important, there were significant differences in ectopic calcifications between different muscle groups. This finding may shed new light on the mechanism behind the absence of damage in some and the progressive pathology in other muscle groups that are observed in both DMD patients and mouse models of this disease. This diversity in ectopic calcification was confirmed using a combination of methods, including X-ray microscopy, and a newly developed whole-body optical tissue clearing approach.³¹ The latter method allows us to perform highly reproducible and quantitative assessment of mineralization without a need for highly specialized and expensive equipment, thus permitting such unbiased complete comparisons to be performed in numerous laboratories. Scanning electron microscopy energy-dispersive X-ray spectroscopy electron backscatter analysis of mineralized fibers revealed the presence of calcium and phosphate with a molar ratio of 3:2, consistent with tricalcium phosphate [Ca₃(PO₄)₂].³⁵ Nuclear magnetic resonance–based approaches would be more conclusive, but difficulties in obtaining entirely organic-free material without sample damage prevented further investigations. Therefore, although the presence of hydroxyapatite previously described in *mdx* muscle cannot be ruled out, this material was reported between rather than within fibers, what was documented herein.³⁵

Calcified muscles have been linked to increased Pi levels, and serum Pi was found elevated in *mdx* mice.⁴⁶ Furthermore, dietary Pi intake has been shown to increase muscle calcifications, whereas reduced Pi diet alleviated ectopic calcification in *mdx* mouse muscle *in vivo*.^{45,61} In turn, calcium precipitate inhibition with pyrophosphate and bisphosphonate has already shown therapeutic promise in DMD.^{62–64} However, it is not clear whether ectopic calcification is linked to the intracellular calcium accumulation, which resulted in the calcium hypothesis of DMD damage. Assuming that these events are connected, the exacerbated calcification in the dystrophin-null muscle suggests that the calcium influx via permeable sarcolemma solely due to the absence of Dp427 is an insufficient explanation.⁶⁵ Indeed, although elevated calcium levels in muscle fibers are sufficient to induce dystrophic-like changes,⁶⁶ this can occur independently of membrane instability.⁶⁷

Ectopic calcification was also found in *mdx* ^{β geo} hearts but without obvious histologic deterioration compared with *mdx*. These data also agree with observations of cardiac histopathology not being significantly different between *mdx* and the Cre-loxP DMD-null mice.¹² Interestingly, Dp71 in cardiomyocytes is located exclusively in the T-tubules.¹² Given that most of the calcium enters the cell via T-tubules, absence of Dp71 could affect this function and contribute to ectopic calcification.

Ectopic calcifications in dystrophic muscle appeared at 3 to 4 weeks in both *mdx* and *mdx* ^{β geo} mice, increasing in number up until 8 to 12 weeks, beyond which calcified myofibers were replaced by fibrosis, which is one of the hallmarks of this disease. Thus, calcification follows the course of *mdx* pathology in limb muscles and in the diaphragm, one *mdx* muscle that most closely reproduces disease progression in humans. The timing of calcified fibers being replaced by fibrosis was also approximately week 12. Therefore, calcification seems to have the same temporal pattern of presentation and resolution in all dystrophic muscle, despite significant differences in intensity across different muscle groups (Figure 5).

The cycles of degeneration and regeneration in *mdx* muscle are concomitant with immune cell infiltration. These immune cells are attracted by the danger-associated molecular patterns released from damaged muscle, and they play important roles in the pathology: they can contribute to damage but are also involved in clearing the cellular debris and releasing factors facilitating satellite cell activation and therefore promoting muscle regeneration. Moreover, in the chronic disease, inflammation is also linked to fibrosis.^{32,33}

The role of macrophages in these processes has been well documented: Pathology in immunocompromised *mdx* mice that retained functional macrophages was largely unaltered,³⁴ whereas macrophage depletion before the onset of muscle damage resulted in significant improvement^{35,36} and total ablation exacerbated the disease.⁴⁴ Furthermore, a recent study demonstrated that inorganic phosphate can activate macrophages to adopt a phenotype allowing them to

prevent ectopic calcification.³⁷ Given that the evolution of calcified muscle fibers mirrored the onset and cessation of inflammatory cell infiltrates in *mdx* muscle and the important role of macrophages, these cells were analyzed in relation to calcification. The distribution of macrophages was markedly different in *mdx* versus *mdx*^{*βgeo*} muscles, with a close colocalization of F4/80 staining puncta with mineralized fibers in the latter. Moreover, the staining often appeared crescent shaped, around what looked like partially digested fibers (Figure 8B). In view that Pi-induced macrophages can evoke anticalcification actions, which are mediated by increased availability of extracellular ATP and pyrophosphate,³⁷ the dystrophic muscle would offer ideal conditions for their activation. However, markers expressed on cells in *mdx*^{*βgeo*} muscles suggested that these had predominantly the M1 phenotype, whereas the Pi-induced macrophages were shown to adopt a phenotype resembling the M2 subtype.³⁷ Of course, macrophages are known for their ability to change phenotype in response to environmental signals, so functional interplay between populations preventing calcification and eliminating calcified deposits is possible. Manipulating macrophage functions should provide further insight into their role in this process.

Understanding these phenomena may also aid in identifying new therapeutic approaches. Furthermore, ectopic calcification is associated with pathologic outcomes in many human disorders apart from DMD, including osteoarthritis,⁶⁸ atherosclerosis,⁶¹ sarcoma,⁶⁹ renal disease,⁷⁰ fibrodysplasia ossificans progressiva,⁷¹ and soft tissue impact trauma,⁷² where macrophage-specific roles are already established.

Given these mouse model data and the correlation of severity of patients' cognitive impairment with the loss of shorter dystrophins both suggest a prominent functional role for these isoforms, comparison of muscle pathology in dystrophin-null patients against those with mutations affecting full-length dystrophins only is clearly warranted. Mouse with selective ablation of Dp71 is not dystrophic⁵⁴ but presents with retinal channel abnormality,⁷³ early cataract formation,⁷⁴ and vomeronasal nerve defasciculation.⁷⁵ In contrast, transgenic overexpression of Dp71 resulted in more severe muscle disease.^{76,77} Therefore, it may not be the absence of Dp71 but altered expression of dystrophin isoforms at a critical time point or/and at a specific location that causes the pathology.

Understanding the mechanism of this abnormality may contribute to the development of more effective treatments not only for DMD but a range of diseases.

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Supplemental Data

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