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1 **Live-imaging of revertant and therapeutically restored dystrophin in the**  
2 ***Dmd*<sup>EGFP-mdx</sup> mouse model for Duchenne muscular dystrophy**

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21 **Short title:** *Dmd*<sup>EGFP-mdx</sup> reporter mouse

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23 fusion protein; revertant muscle fibre, tcDNA, CrispRCas9

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31 **Abstract**

32 **Background:** *Dmd*<sup>mdx</sup>, harbouring the c.2983C>T nonsense mutation in *Dmd* exon 23, is a  
33 mouse model for Duchenne muscular dystrophy (DMD), frequently used to test therapies  
34 aimed at dystrophin restoration. Current translational research is impeded methodologically  
35 because dystrophin visualization requires antibody-mediated signal amplification.

36 **Methods:** We generated a *Dmd*<sup>EGFP-mdx</sup> reporter allele carrying in *cis* the *mdx*-23 mutation  
37 and a C-terminal EGFP-tag. This mouse model allows direct visualization of spontaneously  
38 and therapeutically restored dystrophin-EGFP fusion protein either after natural fibre rever-  
39 sion, or for example, after splice modulation using tricyclo-DNA to skip *Dmd* exon 23, or after  
40 gene editing using AAV-encoded CrispRCas9 for *Dmd* exon 23 excision.

41 **Results:** Intravital microscopy in anaesthetised mice allowed live-imaging of sarcolemmal  
42 dystrophin-EGFP fusion protein of revertant fibres as well as following therapeutic restora-  
43 tion. Dystrophin-EGFP-fluorescence persisted *ex vivo*, allowed live-imaging of revertant and  
44 therapeutically restored dystrophin in isolated fibres *ex vivo*. Expression of the shorter dys-  
45 trophin-EGFP isoforms Dp71 in brain, Dp260 in the retina, and Dp116 in the peripheral nerve  
46 remained unabated by the *mdx*-23 mutation.

47 **Conclusion:** Intravital imaging of *Dmd*<sup>EGFP-mdx</sup> muscle permits novel experimental approaches  
48 such as the study of revertant and therapeutically restored dystrophin *in vivo* and *ex vivo*.

49

## 50 Introduction

51 The X-chromosome-linked muscular dystrophy (*mdx*) mouse is the most frequently used  
52 animal model in preclinical research for Duchenne muscular dystrophy (DMD), a devastating  
53 and the most frequent muscle disease of childhood. The classical *mdx* mouse, also referred  
54 to as *Dmd*<sup>*mdx*</sup>, carries a c.2983C>T nonsense mutation in exon 23 of the *Dmd* gene (also  
55 called the *mdx*-23 allele), resulting in a termination codon (TAA) in place of a glutamine  
56 codon (CAA) [1,2]. The mutation causes complete absence of the *Dmd*-encoded full-length  
57 dystrophin isoforms Dp427, which in wild-type is expressed in skeletal, smooth, cardiac mus-  
58 cle, and in the central nervous system [1]. All other shorter dystrophin isoforms (Dp260,  
59 Dp140, Dp116, and Dp71) are transcribed from more distal promoters located downstream  
60 of exon 23 and are thus normally expressed in the *mdx* model [2–5]. Of note, exon 23 is one  
61 of many exons that encode part of the core region of the dystrophin protein, and its removal  
62 would not shift the reading frame.

63 Indeed, deletion of *Dmd* exon 23 or exclusion of the mutated exon by alternative splicing  
64 restores the open reading frame and entails expression of truncated dystrophin products  
65 that ameliorates the pathological *mdx* phenotype [6–8]. Interestingly, in *mdx* mice, similarly  
66 to other DMD animal models as well as in human patients, some myofibres spontaneously  
67 overcome the null mutation [9]. Such so-called “revertant fibres” express internally trun-  
68 cated dystrophin that excludes at least the exon 23 encoded protein domain [10]. The pres-  
69 ence of a nonsense mutation in an “in frame” exon makes *Dmd*<sup>*mdx*</sup> an ideal animal model for  
70 testing therapeutic strategies that aim at restoring the function of the mutated *Dmd* gene.  
71 Such approaches would comprise (i) antisense-induced splice modulation to skip the mu-  
72 tated exon 23 during RNA maturation using antisense oligonucleotides (AON) such as tricy-  
73 clo-DNA (tcDNA) [11], (ii) stop codon read-through during translation, and (iii) CRISPR/Cas9-  
74 induced genomic deletion of *Dmd* exon 23 [12].

75 Hitherto, no *Dmd*<sup>*mdx*</sup> reporter mouse models are available allowing the visualization of dys-  
76 trophin re-expression in an otherwise dystrophin negative background. Therefore, current  
77 imaging of restored dystrophin in *mdx* mice relies entirely on immunostaining. However,  
78 antibody dependent protocols have several methodological limitations: (i) they do not allow  
79 intravital imaging or live-cell imaging in cell cultures, (ii) it is difficult to quantify signals due  
80 to antibody-mediated signal amplification, (iii) non-specific immunostaining by cross-

81 reacting antibodies is aggravated by the chronic inflammation and antibody deposition in the  
82 *mdx* muscle, and (iv) using epitope specific anti-dystrophin antibodies, certain dystrophin  
83 isoforms might go undetected.

84 We recently generated the *Dmd*<sup>EGFP</sup> reporter mouse line that allows localizing, tracing, and  
85 analysing dystrophin *in* and *ex vivo* by means of native EGFP-fluorescence. We generated  
86 *Dmd*<sup>EGFP</sup> mice through modification of the *Dmd* locus by in-frame insertion of a FLAG-EGFP  
87 sequence downstream of the last *Dmd* exon 79. The *Dmd*<sup>EGFP</sup> reporter mice express a fluo-  
88 rescently labelled dystrophin-EGFP fusion protein from the endogenous locus, which does  
89 not interfere with normal dystrophin function [13]. *Dmd*<sup>EGFP</sup> mice show strong native dystro-  
90 phin-EGFP-fluorescence in skeletal and smooth muscle, heart, brain, and the eye that co-  
91 localizes with known subcellular sites of dystrophin expression, suggesting proper tagging  
92 and distribution of the major dystrophin isoforms. Isolated myofibres and satellite cell-  
93 derived myotubes express dystrophin-EGFP *in vitro* [13].

94 Here we describe the generation of a novel dystrophin reporter mouse model by crossing  
95 the *Dmd*<sup>EGFP</sup> allele into the *mdx* background *in cis*. The so generated *Dmd*<sup>EGFP-mdx</sup> mice carry  
96 the *mdx* exon 23 nonsense mutation and the *EGFP* sequence on the same allele. We show  
97 that dystrophin-EGFP is re-expressed in the dystrophin negative background of the *Dmd*<sup>EGFP-</sup>  
98 *mdx* muscle in revertant fibres, after therapeutic restoration of the dystrophin reading frame  
99 as well as in tissues that express short dystrophin isoforms that are not affected by the *mdx*-  
100 23 mutation.

101

## 102 **Materials and Methods**

### 103 **Animal experiments**

#### 104 **Generation and genotyping of transgenic mice**

105 We conducted all animal experiments according to national and European legislation as well  
106 as to institutional guidelines for the care and use of laboratory animals as approved by the  
107 local authorities (LaGeSo Berlin, T 0222/13) and approved by the French government (Min-  
108 istère de l'Enseignement Supérieur et de la Recherche, autorisation APAFiS).

109 To generate the  $Dmd^{EGFP-mdx}$  mice, we crossed the  $Dmd^{EGFP}$  mouse line, previously generated  
110 in our laboratory on the C57BL/6N background [13], with the conventional  $Dmd^{mdx}$   
111 (C57BL/10ScSn- $Dmd^{mdx}/J$ ) mouse line [14]. We achieved our goal to introduce the  
112  $Dmd^{EGFP}$  allele into the  $mdx$  background in *cis* through natural recombination during meiosis  
113 in female F1 mice that are heterozygous for both the  $Dmd^{EGFP}$  and the  $mdx-23$  allele. We  
114 screened for successful crossing over in males of the F2 generation by genotyping. We then  
115 backcrossed  $Dmd^{EGFP-mdx}$  mice over 9 generations using C57BL/6J mice.

116 Genotyping was performed on genomic DNA isolated from ear clippings. A three-primer PCR  
117 allowed the distinction between animals that were heterozygous or homozygous for the  
118  $EGFP$  sequence using following primers: FW#1: 5'-TGA CTC CCA ATA GTG GCA ACC-3', FW#2  
119 5'-GAG CAA AGA CCC CAA CGA GA-3', REV: 5'-CCA TGC GGG AAT CAG GAG TT-3' (wild-type =  
120 202 bp,  $Dmd^{EGFP}$  = 304 bp). Sanger sequencing was done to screen for the  $mdx$  mutation us-  
121 ing oligonucleotide primers flanking the exon  $mdx-23$  mutation (FW: 5'-AAC TCA TCA AAT  
122 ATG CGT GTT AGT-3', REV: 5'-CTC AAT CTC TTC AAA TTC TG-3').  $Dmd^{EGFP}$  and  $Dmd^{EGFP-mdx}$   
123 mice were bred and maintained in a standard 12 h light/dark cycle and had *ad libitum* access  
124 to food and water. Mice were weaned at four weeks of age and were kept in small colonies  
125 of two to five animals per cage.

#### 126 **Antisense oligonucleotide treatment**

127 The tcDNA-AON PS M23D 15-mer (+2-13) (5'-pAAC CTC GGC TTA CCT-3') targeting the donor  
128 splice site of exon 23  $Dmd$  pre-mRNA was synthesised by SYNTHENA (Berne, Switzerland) as  
129 previously described [11]. Three 6-week-old  $Dmd^{EGFP-mdx}$  mice were injected intravenously  
130 into the retro-orbital sinus under general anaesthesia using 1.5%–2% isoflurane once a week  
131 with the tcDNA compound for an extended period of 20 weeks and at a dose of 200

132 mg/kg/week. *Intravital* imaging was done one week after the last injection as described be-  
133 low. The treated mice were sacrificed three weeks following the last injection.

#### 134 **AAV-vector cloning**

135 **pAAV-SaCas9-sgRNA22:** The *Staphylococcus aureus* Cas9 (saCas9)-3HA-U6-sgRNA scaffold  
136 cassette from the pX601 plasmid (Addgene 61591, Watertown, MA, USA) was cloned into an  
137 AAV-2 plasmid under the control of the C5.12 promoter. The target sequence TAC ACT AAC  
138 ACG CAT ATT TG [15] of intron 22 of the murine *Dmd* gene, was subsequently cloned 5' of  
139 the sgRNA scaffold to obtain the plasmid pAAV-SaCas9-sgRNA22.

140 **pAAV-Cherry-sgRNA23:** The U6-sgRNA scaffold cassette from the pX601 plasmid (Addgene  
141 61591) was cloned into an AAV-2 plasmid downstream of a C5.12 Strep Tag mCherry cas-  
142 sette. The target sequence CAT TGC ATC CAT GTC TGA CT [15] of intron 23 of the murine  
143 *Dmd* gene, was subsequently cloned 5' of the sgRNA scaffold to obtain the plasmid pAAV-  
144 SaCas9-sgRNA23.

#### 145 **AAV production**

146 Adenovirus free rAAV2/9 viral preparations were generated by packaging AAV2-ITR recom-  
147 binant genomes in AAV9 capsids, using a three plasmid transfection protocol as previously  
148 described [16]. Briefly, HEK293 cells were co-transfected with the pAAV-transgene (pAAV-  
149 SaCas9-sgRNA22 or pAAV-Cherry-sgRNA23), an AAV9 RepCap plasmid (pAAV2.9, Dr J. Wil-  
150 son, UPenn) and an adenoviral helper plasmid (pXX6) at a ratio of 1:1:2. Crude viral lysate  
151 was harvested at 60 h post-transfection and lysed by freeze-and-thaw cycles. The viral lysate  
152 was purified through two rounds of CsCl ultracentrifugation followed by dialysis. Viral ge-  
153 nomes were quantified by a TaqMan real-time PCR assay using primers and probes corre-  
154 sponding to the inverted terminal repeat region (ITR) of the AAV vector genome [17]. The  
155 primer pairs and TaqMan probes used for ITR amplification were: 1AAV65/Fwd: 5'-CTC CAT  
156 CAC TAG GGG TTC CTT G-3'; 64AAV65/rev: 5'-GTA GAT AAG TAG CAT GGC-3'; and  
157 AAV65MGB/taq: 5'-TAG TTA ATG ATT AAC CC-3'.

158 50 µl containing both AAVs (*AAV-SaCas9-sgRNA22* and *AAV-Cherry-sgRNA23*) at a titre of  
159 4.6e10 vg of each were injected into the anterior muscle compartment of the lower leg of 6-  
160 week-old *Dmd*<sup>EGFP-mdx</sup> mice and sacrificed 5 months post injection.

#### 161 **Intravital confocal and multi-photon scanning microscopy**

162 Mice were anaesthetised with isoflurane. After one longitudinal incision of the skin at the  
163 lateral side of the lower hindlimb, the exposed muscle was stably positioned on a cover slip  
164 that was fixed to the sample holder, thereby minimizing motion artefacts and enabling  
165 high-resolution microscopy over several hours. Mice were kept at 37°C in the microscope's  
166 incubation chamber during the entire imaging procedure. Muscles were imaged with a com-  
167 bined confocal/multi-photon point-scanning microscope (SP8 MP-Leica microsystem,  
168 Wetzlar, Germany) equipped with a Ti:Sapphire femto-second laser (Mai Tai Deep see-  
169 Spectra Physics) allowing a tuning range for excitation between 690 and 1040 nm. A long-  
170 working distance 25x numerical aperture (NA) 0.95/water (Leica) lens was used for intravital  
171 deep sectioning up to 300 µm depth at 5-10 µm Z-step size. For the confocal imaging, EGFP  
172 was excited with a 488 nm laser line and the fluorescence was collected using classical pho-  
173 tomultiplier detectors (PMT). Their spectral detection window was set between 496-546 nm.  
174 Multi-photon laser was used to excite EGFP with a wave length of 900 nm during live-  
175 imaging. The emission photons where spectrally separated by a dichroic beam splitter  
176 (DM484) and collected on three non-descanned photomultiplier detectors (NDD-PMT),  
177 the band pass filter 500-550 nm was used for EGFP signal, the band pass filter 565-605nm  
178 for mCherry signal and the band pass filter 625-675 nm was used to register the tissue  
179 autofluorescence signal. The 3D videos were obtained using Arivis Software.

#### 180 **Serum CPK analysis**

181 Blood samples were collected from the facial vein of 8-10-month-old wild-type (C57BL/10)  
182 and *Dmd*<sup>EGFP-mdx</sup> mice. Serum creatine phosphokinase (CPK) levels were measured based on  
183 the quantification of NADPH formation derived from the action of CPK on creatine phos-  
184 phate as recommended by the International Federation of Clinical Chemistry in an auto-  
185 mated biochemistry analyser (AU680, Beckman Coulter, Brea, CA, USA) by the pathology  
186 laboratory at Mary Lyon Centre, Medical Research Council, Harwell, Oxfordshire, UK.

#### 187 **Western blot analyses**

188 Total protein was extracted from cryo-sections of different skeletal muscles (*biceps brachii*,  
189 *tibialis anterior*, *triceps brachii*, *quadriceps*, *gastrocnemius* and diaphragm) and from cardiac  
190 muscle of 4-month-old wild-type *Dmd*<sup>EGFP</sup> and *Dmd*<sup>EGFP-mdx</sup> mice. Protein extracts were ob-  
191 tained from pooled muscle sections in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl,  
192 1% NP-40, 1% sodium deoxycholate) with 5% SDS and 1x pierce protease inhibitor (Thermo



193 Fisher, Waltham, MA, USA). Samples were denatured in 1x NuPAGE reducing agent (Thermo  
194 Fischer: NP0009) with 1x NuPAGE LDS Sample Buffer (Thermo Fischer: NP0008) at 100°C for  
195 3 min. Total protein concentration was determined with BCA protein assay kit (Thermo  
196 Fisher) and 25 µg of proteins were loaded onto NuPAGE 3-8% TRIS-acetate protein gels (Invi-  
197 trogen, Carlsbad, CA, USA) in 1x NuPAGE TRIS-acetate SDS Running Buffer (Thermo Fisher)  
198 following manufacturer's instructions.

199 Dystrophin protein was detected by probing the nitrocellulose membrane with the primary  
200 NCL-DYS1 mouse monoclonal antibody (1:130 dilution, NCL-DYS1; Novocastra, Leica Biosys-  
201 tems, Wetzlar, Germany). Vinculin as internal loading control was detected by the primary  
202 monoclonal anti-vinculin (mouse IgG1 isotype) antibody (1:10,000 dilution, Sigma Aldrich, St.  
203 Louis, MO, USA). EGFP protein was detected with a primary anti-GFP rabbit serum polyclonal  
204 antibody (1:700 dilution, Thermo Fisher). Incubations with primary antibodies were followed  
205 by incubation with a goat anti-mouse secondary antibody IRDye® 800CW (Li-Cor, Lincoln, NE,  
206 USA) for dystrophin and vinculin detection and with a goat anti-rabbit secondary antibody  
207 IRDye® 700CW (Li-Cor) for GFP, using the iBind Flex Western Device. Membrane fluores-  
208 cence was converted to value images using the Odyssey® CLx imaging system (Li-cor). Band  
209 fluorescent densities were analysed using the Image Studio™ software (Li-cor). For the pur-  
210 pose of visual presentation of the infrared fluorescent protein bands we allocated green col-  
211 our to visualize the signal from the goat anti-mouse secondary antibody IRDye® 800CW  
212 which bound to anti-dystrophin and anti-vinculin primary antibodies, and red color to visual-  
213 ize the signal from the goat anti-rabbit secondary antibody IRDye® 700CW which bound to  
214 the anti- GFP primary antibody.

### 215 **Histological analyses**

216 *Triceps brachii (Tri)*, *tibialis anterior (TA)* muscles, diaphragm, heart, and *ileum* were har-  
217 vested from 5- and 12-month-old *Dmd*<sup>EGFP</sup> and *Dmd*<sup>EGFP-mdx</sup> mice. The tissues were mounted  
218 using 6% Tragacanth gum 292 (Sigma-Aldrich) or were mounted in OCT and snap-frozen in  
219 liquid nitrogen-cooled isopentane and stored at -80°C. The tissues were then processed for  
220 cryo-sectioning. 8-10 µm cross sections were collected and directly mounted with PBS for  
221 EGFP visualization or directly fixed with 4% PFA and stained. For imaging of innervating pe-  
222 ripheral motor nerve branches, *EDL* muscles were dissected and directly fixed with 4% PFA;  
223 subsequently, smaller bundles were teased/peeled from the muscle and stained.

224 Immunohistological analyses were performed using primary antibodies against dystrophin  
225 (1:50 dilution, DYS2, mouse IgG1, Novocastra), CD31 (1:25 dilution, rabbit, Abcam, Cam-  
226 bridge, UK), MANDYS19 (1:10, mouse IgG1, DSHB, Iowa City, IO, USA), GFP (1:500, rabbit IgG,  
227 Thermo Fisher),  $\beta$ -tubulin III (1:500, mouse IgG2a, Sigma-Aldrich) and laminin (1:500, rabbit,  
228 Sigma-Aldrich), followed by incubation with fluorochrome-labelled secondary antibodies  
229 (1:400, ALEXA Fluor<sup>®</sup> goat anti-mouse IgG1-568, IgG2a-568 or IgG (H&L)-568 and goat anti-  
230 rabbit 488/647, Thermo Fisher) and mounted using Vectashield<sup>®</sup> Antifade Mounting Me-  
231 dium with DAPI (Vector Laboratories, Burlingame, CA, USA). For all sections incubated with  
232 mouse monoclonal antibodies an additional blocking step was performed: after fixation, sec-  
233 tions were blocked with mouse IgG blocking solution from the M.O.M. kit (Vector Laborato-  
234 ries). Fluorescence was visualised using a Zeiss Axio Imager with an ORCA camera  
235 (Hamamatsu, Japan) and AxioVision software or images were recorded with an inverted  
236 fluorescent microscope (Leica DMI4000).

237 Confocal images were taken at the AMBIO facility at the Charité University Hospital, Berlin  
238 using a Nikon Scanning Confocal A1Rsi+ system, with a 40x-Apo-DIC N2  $\lambda$  S objective. Stacks  
239 (316.2 x 316.2 x 7.2  $\mu$ m) of images 0.8  $\mu$ m apart were captured at 1,024 x 1,024 pixel resolu-  
240 tion. Images were analysed and displayed as videos using the Fiji software.

241 Haematoxylin-eosin staining was undertaken according to standard procedures, and the im-  
242 ages were recorded using a digital slide scanner (Leica) and analysed with the ImageScope  
243 software.

#### 244 **Isolation of EDL single myofibres**

245 *Extensor digitorum longus* (EDL) muscles were dissected and digested in 0.2% collagenase  
246 type I (Sigma-Aldrich) in FluoroBrite DMEM media (GIBCO, Thermo Fisher) supplemented  
247 with 1% penicillin/streptomycin, 4 mM L-glutamine (GIBCO), and 1% sodium pyruvate  
248 (GIBCO). Individual, viable, and undamaged myofibres were isolated by gently passing them  
249 through Pasteur pipettes with different sized apertures as described in detail elsewhere [18].  
250 The isolated living myofibres were either mounted on slides with PBS for direct EGFP-  
251 fluorescence detection or cultured for 40 hours in the above mentioned isolation medium  
252 that was additionally supplemented with 10% horse serum (GIBCO) and 0.5% chicken em-  
253 bryo extract (MP Biomedicals, Santa Ana, CA, USA). Myofibres were viewed using SteReo

254 Lumar.V12 stereoscope and Zeiss Axio Imager microscope equipped with an Orca® camera  
255 (Hamamatsu). AxioVision software was used for image acquisition.  
256

257 **Results**

258 **Generation and validation of the  $Dmd^{EGFP-mdx}$  dystrophic reporter mouse model.**

259 For generation of the  $Dmd^{EGFP-mdx}$  mice, we used our previously characterised  $Dmd^{EGFP}$  mouse  
260 line that we crossed with the  $Dmd^{mdx}$  mice. The female F1 mice were heterozygous for  
261  $Dmd^{EGFP}$  and  $Dmd^{mdx}$  alleles. They were crossed with  $Dmd^{EGFP}$  males, and F2 males were  
262 screened for crossover events during meiosis that would recombine both alleles in *cis*. We  
263 had calculated this event to be present in 1:100 oocytes given the genetic distance of  $\approx 1.1$   
264 Mio bp between exons 23 and 79 of the  $Dmd$  gene (**Figure 1A**), which would equal  $\approx 1$  centi-  
265 Morgan (cM). If the *mdx-23* nonsense mutation and the *EGFP*-tag are on the same allele,  
266 male mice will not express the EGFP-tag due to the intervening premature termination  
267 codon, at least not for the full-length muscle isoform. The *EGFP*-tag will be (re-)expressed if  
268 the reading frame of exon 79 is restored, such as observed in revertant fibres.

269 We obtained the first desired crossover in the 163<sup>rd</sup> animal. Further crosses between the  
270  $Dmd^{EGFP-mdx}$  male and heterozygous females led to the establishment and expansion of a sta-  
271 ble line of hemizygous male and homozygous female  $Dmd^{EGFP-mdx}$  mice (**Figure 1B**). The mice  
272 were genotyped for both the presence of the EGFP-sequence by triple oligonucleotide  
273 primer PCR (**Figure 1C**) and for the *mdx-23* mutation by Sanger sequencing (**Figure 1D**). We  
274 then backcrossed  $Dmd^{EGFP-mdx}$  mice into the C57BL/6J line for 9 generations. There was no  
275 significant difference in the postnatal viability until 21 days of age of the new  $Dmd^{EGFP-mdx}$  line  
276 in comparison to the wild-type  $Dmd^{EGFP}$  mice (**Figure 1E**). Since the generation of the mouse  
277 line, many mice lived until 24 months of age with no increased spontaneous death rate dur-  
278 ing ageing.

279 Western blot analysis confirmed the absence of full-length dystrophin-EGFP in protein lys-  
280 ates from  $Dmd^{EGFP-mdx}$  heart, diaphragm, as well as *biceps brachii*, *triceps brachii*, *quadriceps*  
281 *femoris*, *gastrocnemius*, and *tibialis anterior* muscles. Expression of the full-length Dp427-  
282 EGFP isoform was present in positive controls from wild-type  $Dmd^{EGFP}$  muscle lysates (**Fig-**  
283 **ures 2A**). Interestingly, Western blots of wild-type  $Dmd^{EGFP}$  muscle lysates revealed two  
284 separate bands when stained with anti-DYS1 antibody, whereas only the upper band was  
285 EGFP positive.

286 When performing histology, native dystrophin-EGFP-fluorescence was visible in fresh cryo-  
287 sections at the sarcolemma of *Dmd*<sup>EGFP</sup> skeletal, heart and in smooth muscle of the *ileum*,  
288 but absent in *Dmd*<sup>EGFP-mdx</sup> mice, except for sporadic revertant fibres (**Figure 2B**).

289 Skeletal muscle from adult *Dmd*<sup>EGFP-mdx</sup> mice showed the same histopathological alterations  
290 as observed in dystrophic *Dmd*<sup>mdx</sup> mice. These changes comprised increased variation of  
291 myofibre size, myofibre necrosis, internally positioned myonuclei, fatty replacement, mono-  
292 nuclear cell infiltrates, as well as fibrosis (**Figures 2C**). Serum levels of creatine phos-  
293 phokinase were up to 20-fold elevated in *Dmd*<sup>EGFP-mdx</sup> mice, thereby confirming the presence  
294 of muscular dystrophy (**Figure 2D**).

### 295 **Revertant dystrophin-EGFP co-localizes with anti-dystrophin immunostaining**

296 Revertant myofibres expressing native dystrophin-EGFP were easily identified by epifluores-  
297 cence microscopy after cross sectioning (**Figure 3**). For this, a coverslip was mounted on the  
298 slide using a drop of PBS, allowing microscopic inspection immediately after sectioning. Dys-  
299 trophin-EGFP negative myofibres were invisible when fluorescence intensities of revertant  
300 myofibres were set at comparable levels to those of myofibres from wild-type *Dmd*<sup>EGFP</sup> mice.  
301 Furthermore, we compared dystrophin-EGFP-fluorescence intensity of the identical rever-  
302 tant myofibre clusters before and after immunostaining (**Figure 3**). The native dystrophin-  
303 EGFP-fluorescence of revertant myofibres decreased only slightly during subsequent immu-  
304 nostaining steps and co-localised exactly with the signals from anti-dystrophin and anti-  
305 laminin staining, thereby confirming the presence of revertant dystrophin (**Figure 3**).

### 306 **Expression of non-muscle dystrophin-EGFP isoforms**

307 Moreover, we analysed the expression of shorter dystrophin-EGFP isoforms in non-muscle  
308 tissues, which should have remained unaffected as their initiation codons are located down-  
309 stream of the *mdx*-23 mutation. Indeed, we detected strong native EGFP-fluorescence corre-  
310 sponding to the Dp71 isoform in the cerebral blood vessels, expressing CD31 (**Supporting**  
311 **Figure 1A**). However, we did not detect any native EGFP-fluorescence in neurons (data not  
312 shown).

313 We previously demonstrated that retina from wild-type *Dmd*<sup>EGFP</sup> mice expressed full-length  
314 Dp427 as well as the retina-specific Dp260 dystrophin isoforms at the photoreceptor termi-  
315 nals [13]. Dp427 can be detected specifically using the anti-dystrophin antibody MANDYS19,

316 which recognizes an epitope encoded by exon 21 that is located N-terminally of the prema-  
317 ture termination codon of exon 23. In wild-type *Dmd*<sup>EGFP</sup> mice, we observed strong native  
318 EGFP-fluorescence at the photoreceptor terminals of the retina that co-localised with the  
319 signal obtained by immunofluorescence using the MANDYS19 antibody and which corre-  
320 sponds to Dp427 isoform (**Supporting Figure 1B**). In *Dmd*<sup>EGFP-mdx</sup> mice, however, we did not  
321 detect full-length Dp427 at the photoreceptor terminals using MANDYS19 antibodies,  
322 whereas a native EGFP-signal was still present, corresponding to the Dp260 retina-specific  
323 isoform (**Supporting Figure 1B**).

324 Dp116 is a Schwann cell-specific isoform encoded by *Dmd* exons 56–79 [19]. We confirmed  
325 the presence of Dp116 expression in *Dmd*<sup>EGFP-mdx</sup> mice following co-immunostaining of a pe-  
326 ripheral motor nerve branch and in a transverse section of the sciatic nerve using an anti-  
327 GFP antibody for signal enhancement along with  $\beta$ -tubulin III as neuronal marker (**Support-**  
328 **ing Figure 2A, B, Videos 1 and 2**).

### 329 **Live-imaging of sarcolemmal dystrophin-EGFP**

330 Intravital microscopy of the lateral part of *tibialis anterior* (*TA*) muscle in anaesthetised  
331 *Dmd*<sup>EGFP</sup> mice revealed homogeneous dystrophin-EGFP expression at the sarcolemma (**Figure**  
332 **4A, Video 3**). In *Dmd*<sup>EGFP-mdx</sup> mice, only dot-like autofluorescent structures were visible at  
333 different emission wavelengths, likely corresponding to macrophages, whereas sarcolemmal  
334 dystrophin-EGFP was entirely absent (**Supporting Figure 3A, B and Video 4**). Occasionally,  
335 we found dystrophin-EGFP-expression in single myofibres or in small fibre clusters (**Figure**  
336 **4D**). The dystrophin-EGFP-signal was present along smaller or larger segments of these fibres  
337 at the sarcolemma thus qualifying them as being “revertant”.

338 Next, we isolated whole myofibres from the *extensor digitorum longus* (*EDL*) muscles of  
339 adult wild-type C57BL6, *Dmd*<sup>EGFP</sup>, and *Dmd*<sup>EGFP-mdx</sup> mice. Living myofibres from wild-type mice  
340 and *Dmd*<sup>EGFP-mdx</sup> mice did not show any fluorescence (**Supporting Figure 3C-F**), whereas myo-  
341 fibres from *Dmd*<sup>EGFP</sup> mice expressed dystrophin-EGFP homogeneously along the sarcolemma  
342 (**Figure 4B**). Revertant myofibres from *Dmd*<sup>EGFP-mdx</sup> mice were detectable under the binocular  
343 microscope by their green fluorescence and could be isolated for subsequent live-imaging  
344 experiments using higher resolution microscopy or for subsequent myofibre culture (**Figure**  
345 **4E and Supporting Figure 3G,H**). We next cultured single myofibres from *Dmd*<sup>EGFP</sup> and rever-

346 tant myofibres from *Dmd*<sup>EGFP-mdx</sup> mice and demonstrated that dystrophin-EGFP-expression  
347 persisted at the sarcolemmal position after two days in culture (**Figure 4C,F**).

#### 348 **Live-imaging of therapeutically restored dystrophin**

349 In order to evaluate the efficacy of our mouse model for evaluating dystrophin restoration  
350 we performed two different therapeutic approaches. For the first approach, we targeted  
351 the RNA of dystrophin, by systemically treated adult *Dmd*<sup>EGFP-mdx</sup> mice with weekly doses of  
352 200 mg/kg tcDNA to induce *Dmd* exon-23 skipping, as previously published [11]. Following  
353 20 weeks of tcDNA treatment, dystrophin-EGFP was restored along the entire sarcolemma of  
354 all myofibres as revealed by intravital microscopy, whereas less autofluorescent macro-  
355 phages were visible (**Figure 5A and Video 5**). In the second therapeutic approach, we tar-  
356 geted the DNA by injecting AAV-SaCas9-sgRNA22 combined with AAV-Cherry-sgRNA23 vec-  
357 tors into the anterior muscle compartment of lower legs of adult *Dmd*<sup>EGFP-mdx</sup> mice to induce  
358 *Dmd* exon-23 excision. Five months following AAV injection, a large number of myofibres  
359 expressed mCherry in the cytoplasm, demonstrating successful transduction of many myofi-  
360 bres (**Figure 5C'**). Dystrophin was found only in short sarcolemmal segments of these  
361 mCherry positive myofibres, suggesting that exon 23 excision occurred only in a small per-  
362 centage of myonuclei, thereby preventing widespread dystrophin restoration (**Figure 5C**).

363 Moreover, we isolated whole myofibres from the *extensor digitorum longus* (*EDL*) muscles of  
364 *Dmd*<sup>EGFP-mdx</sup> mice following tcDNA or AAV treatment. As expected from intravital imaging,  
365 single myofibres from tcDNA treated mice expressed widespread dystrophin-EGFP along the  
366 sarcolemma (**Figure 5B**), whereas AAV treated myofibres expressed dystrophin-EGFP only in  
367 short segments (**Figure 5D-D'**).

368

369 **Discussion**

370 Here we describe the generation, the phenotype, and the experimental usefulness of the  
371 first dystrophin-EGFP reporter mouse in the *mdx* context, which we named *Dmd*<sup>EGFP-mdx</sup>  
372 mouse.

373 We designed the *Dmd*<sup>EGFP-mdx</sup> mice by introducing an EGFP-coding sequence fused in-frame  
374 to *Dmd* exon 79 into the *mdx-23* allele, which harbours a nonsense mutation in exon 23.  
375 *Dmd*<sup>EGFP-mdx</sup> mice developed the identical muscular dystrophy phenotype as ordinary *mdx*  
376 mice as evidenced by the presence of typical histopathological hallmarks and increased se-  
377 rum CPK levels. The presence of the *mdx-23* mutation resulted in the absence of full-length  
378 Dp427 dystrophin in skeletal, heart, and smooth muscle. Importantly, we observed sporadic  
379 revertant myofibres and revertant fibre clusters, both in skeletal and heart muscle. Those  
380 fibres expressed the dystrophin-EGFP fusion protein at the expected subsarcolemmal loca-  
381 tion. We were also able to detect the expression of the shorter dystrophin isoforms in the  
382 retina (Dp260), in the peripheral nerve (Dp116), and in the brain (Dp71), which are all unaf-  
383 fected by the *mdx-23* mutation.

384 We demonstrated that dystrophin-EGFP could be restored at its sarcolemmal position by  
385 therapeutic modulation of splicing as well as by genome editing, thereby confirming previ-  
386 ous results in classical *Dmd*<sup>mdx</sup> mice [11,15]. We achieved widespread restoration of dystro-  
387 phin following tcDNA-induced exon 23 skipping, whereas dystrophin expression was patchy  
388 following AAV induced exon 23 excision. It should be noted, however, that we here injected  
389 relatively low viral titres of slightly different AAV constructs as compared to previously pub-  
390 lished data [15], which may be the reason for the failure to achieve widespread dystrophin  
391 restoration.

392 We succeeded in visualising dystrophin in living skeletal muscle without antibody staining at  
393 the correct subcellular position *in vivo* as well as *ex vivo*. During intravital microscopy, dys-  
394 trophin-EGFP-fluorescence persisted without much photobleaching for at least one hour in  
395 anaesthetised mice. Muscle could be imaged for up to a depth of 150 µm maximum follow-  
396 ing cutaneous incision and by leaving the muscle fascia intact. At the end of imaging and  
397 after cutaneous suture, mice recovered rapidly and could be kept for further experiments.  
398 Our future goal is to develop live pharmacodynamics for dystrophin restoration therapies.  
399 For this, we are currently developing a transcutaneous window allowing repetitive live-



400 imaging of skeletal muscle for up to several weeks, similarly as co-authors of this work re-  
401 cently developed for tumour imaging [20]. Such longitudinal observations are essential to  
402 determine dosages and dosage intervals for long-term treatment protocols. Such a study  
403 protocol could also drastically reduce the number of required animals according to the 3R  
404 principles of Russell & Burch [21].

405 We also showed that fluorescence of sarcolemmal dystrophin-EGFP was maintained during  
406 culture of isolated myofibres for up to two days. We are currently testing the maximal dura-  
407 tion of such cultures and we are developing a protocol for time-lapse imaging of individual  
408 fibres at specific subcellular positions.

409 *Dmd*<sup>EGFP-mdx</sup> mice bear some additional advantages over classical *mdx* mice for research on  
410 dystrophin expression. Their use makes immunohistological processing redundant, allowing  
411 fast sample processing, avoiding antibody related signal amplification as well as avoiding  
412 antibody induced non-specific background staining, often being present in inflamed *mdx*  
413 muscle. Of note, non-specific background staining can be reduced by using specific blocking  
414 protocols or by using anti-dystrophin antibodies produced in other species than mice.

415 Disadvantageous is the autofluorescence following oxidation of the EGFP signal, which,  
416 however, only appears after prolonged storage at air oxygen levels before fixation of the  
417 material. We found that rapid tissue processing and immediate viewing after cryo-sectioning  
418 yielded best results.

419 We previously demonstrated the presence of two separate bands at the position of Dp427  
420 full-length dystrophin in Western blots from wild-type and from *Dmd*<sup>EGFP</sup> muscle [13], a re-  
421 sult that we herein reproduced. Whereas both bands were positive for DYS1 antibody stain-  
422 ing (directed against the rod domain), only the upper and more slowly migrating band was  
423 also positive for H4 antibody staining (directed against the C-terminus) and EGFP. This sug-  
424 gests the presence of an alternatively spliced isoform at the C-terminus along with the full-  
425 length isoform, which now becomes further separated due to the larger molecular weight of  
426 the dystrophin-EGFP fusion protein.

427 In conclusion, we show that the *Dmd*<sup>EGFP-mdx</sup> mouse is a very useful model due to its potential  
428 applications for live kinetic and quantitative analyses of spontaneously or therapeutically  
429 restored dystrophin expression *in vivo* and *ex vivo*.

430 **List of abbreviations**

431 AAV, adeno-associated virus; AON, antisense oligonucleotide; bp, base pair; cM, centiMor-  
432 gan; CPK, creatine phosphokinase; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DIC,  
433 differential interference contrast; DMD, Duchenne muscular dystrophy; EGFP, enhanced  
434 green fluorescent protein; FW, forward; H&E, Haematoxylin and Eosin staining; HS, horse  
435 serum; IF, immunofluorescence; kDa, kilo-Dalton; Mio, million; PFA, paraformaldehyde; REV,  
436 reverse; tcDNA, tricyclo-DNA; WT, wild-type. *Bi*, *biceps brachii*; *Dia*, diaphragm; *EDL*, *extensor*  
437 *digitorum longus*; *Gas*, *gastrocnemius*; *Quad*, *quadriceps femoris*; *Tri*, *triceps brachii*; *TA*, *tibi-*  
438 *alis anterior* muscle.

439

440 **Declarations**

441 **Ethics approval and consent to participate**

442 The animal (mouse) experiments were approved by the LaGeSo Berlin (Registration number  
443 T 0222/13) and by the French government (Ministère de l'Enseignement Supérieur et de la  
444 Recherche, autorisation APAFiS).

445 **Consent for publication**

446 All authors have seen the final version of the manuscript and consented to its submission to  
447 *Neuropathology and Applied Neurobiology*.

448 **Availability of data and material**

449 All data generated or analysed during this study are included in this published article.

450 **Competing interests**

451 The authors do not state any financial nor non-financial competing interests.

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456 **Authors' contributions**

457 MVP, AS, HA, and MS designed the study. MVP, SMG, FS performed the molecular genetic  
458 experiments. MVP and MS supervised the breeding program. AS and OP performed the  
459 Western blot experiments. MVP and AS performed the histological analysis. MVP, AS, CLB  
460 and TM performed intravital confocal and multiphoton imaging. AS, AM, MVP and HA per-  
461 formed the single myofibre experiments. AG and LG designed the tcDNA. JBD and IR de-  
462 signed and produced AAV vectors. MVP, AS and HA wrote the first draft of the manuscript.  
463 MVP, HA and MS provided funding. All authors read the final version of the manuscript and  
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472 3xHA-bGHpA;U6::Bsal-sgRNA.

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- 539

540 **Figure Legends**

541 **Figure 1. Generation and genotyping of the  $Dmd^{EGFP-mdx}$  reporter mouse line.** (A) Scheme  
542 depicting the breeding programme to introduce the  $Dmd^{EGFP}$  allele into the  $mdx$  background  
543 in *cis* after natural recombination during meiosis in female F1 mice that are heterozygous for  
544 both alleles. The physical genomic distance between the  $mdx-23$  mutation and C-terminal  
545 EGFP tag is 1.1 Mio bp corresponding to a recombination frequency of  $\approx 1$  centiMorgan (cM).  
546 Successful crossing-over could then be detected in the male progeny of the F2 generation  
547 ( $Dmd^{EGFP-mdx/y}$ ). (B) Schematic pedigree with all possible genotypes illustrating the breeding  
548 strategy. Squares depict male, circles female animals. The *EGFP*-allele is depicted in green,  
549 the *mdx* allele in red. After successful crossing-over, expected in  $\approx 1:100$  progeny from the F2  
550 generation, further breeding with  $Dmd^{EGFP-mdx/y}$  males and  $Dmd^{EGFP-mdx/EGFP}$  females and sub-  
551 sequent genotyping resulted in a pure *mdx-EGFP* line starting from F3. (C) PCR genotyping  
552 for the *EGFP* sequence using a three oligonucleotide primer PCR. Presence of the *EGFP* se-  
553 quence results in a 350 bp band, and its absence in a 202 bp band. Homo/hemizygous ani-  
554 mals can be easily distinguished from heterozygous ones. (D) Sanger sequencing for the *mdx*  
555 mutation leading to a premature TAA termination codon at p.Q995 of the dystrophin pro-  
556 tein. (E) The viability of  $Dmd^{EGFP-mdx}$  neonates, before weaning (21 days) was compared to  
557 wild-type  $Dmd^{EGFP}$  mice. Values are depicted as dot-plots with interquartile range, the differ-  
558 ence of the survival rate was not significant,  $p=0.8304$  ( $n=10$  breeding cages, two-tailed, non-  
559 parametric Mann-Whitney U-test).

560 **Figure 2. Characterization of the  $Dmd^{EGFP-mdx}$  mouse.** The  $Dmd^{EGFP-mdx}$  mouse, like the *mdx*  
561 mouse, does not express Dp427 and develops a muscular dystrophy. (A) Western blot analy-  
562 sis of protein extracts from heart, diaphragm (Dia), *biceps brachii* (Bi), *triceps brachii* (Tri),  
563 *quadriceps femoris* (Quad), *gastrocnemius* (Gas), and *tibialis anterior* (TA) muscles using  
564 DYS1 anti-dystrophin antibody against the rod domain, anti-GFP antibody and anti-vinculin  
565 antibody as internal loading control. Two secondary antibodies labelled with near-infrared  
566 dyes were used and for the purpose of visual presentation of the infrared fluorescent pro-  
567 tein bands, we allocated green colour to visualize dystrophin and vinculin bands and red col-  
568 our to visualize the EGFP-Dp427 band. The full-length Dp427 isoform was present in  $Dmd^{EGFP}$   
569 (D) and wild-type muscle and absent in  $Dmd^{EGFP-mdx}$  (M) muscle. The anti-GFP antibody (in  
570 red) detected the tagged Dp427-EGFP protein only in  $Dmd^{EGFP}$  muscle and co-localised only

571 with the upper band of the two separate bands obtained with the Dys1 antibody (in green).  
572 **(B)** Unstained cross-sections of skeletal, cardiac, and smooth muscles revealed native EGFP-  
573 fluorescence (green) in  $Dmd^{EGFP}$  mice, but not in  $Dmd^{EGFP-mdx}$  mice. A single revertant myofi-  
574 bre expressing native dystrophin-EGFP can be seen on the image of the diaphragm and heart  
575 (arrow). *Ileum* was also shown following Haematoxylin-Eosin (HE) staining. Layers: 1 – *mus-*  
576 *cularis externa*, 2 – *muscularis interna*, 3 – *muscularis mucosa/submucosa*, 4 – *mucosa*. Scale  
577 bar: 20  $\mu$ m. **(C)** HE staining of *triceps brachialis* muscle and *diaphragm* cross-sections of 5-  
578 month-old  $Dmd^{EGFP-mdx}$  mice showed signs of the presence of muscular dystrophy, such as  
579 internalised myonuclei (In), necrosis (Ne), mononuclear cell infiltration (Mo), fatty degenera-  
580 tion (Fa), and fibrosis (Fi) in comparison to age-matched  $Dmd^{EGFP}$  controls. Scale bar: 200  $\mu$ m.  
581 **(D)** Serum creatine phosphokinase (CPK) activities were pathologically increased in sera from  
582  $Dmd^{EGFP-mdx}$  mice (n=5, 9-13 month-old mice) as compared to wild-type mice (n=6, 8-10  
583 month-old mice). Values are depicted as dot-plots with mean  $\pm$ SEM, the difference was sig-  
584 nificant with  $p < 0.01$  (two-tailed, non-parametric Mann-Whitney U-test).

585 **Figure 3. Revertant dystrophin-EGFP co-localizes with the anti-dystrophin immunosignal.**  
586 Cross sections of the *tibialis anterior* muscle from  $Dmd^{EGFP-mdx}$  mice showing dystrophin-EGFP  
587 expression at the level of the sarcolemma of a revertant myofibre cluster before and after  
588 immunostaining. We stained serial sections from the same region of interest with antibodies  
589 against the C-terminal dystrophin domain (DYS2, red) in conjunction with laminin, a base-  
590 ment membrane protein (magenta). We observed exact co-localization between the native  
591 dystrophin-EGFP-fluorescence and the anti-dystrophin immunosignal. Merged images addi-  
592 tionally depict the nuclei stained with DAPI (blue). Native dystrophin-EGFP-fluorescence  
593 does not fade during subsequent immunostaining procedures. Scale bar: 10  $\mu$ m.

594 **Figure 4. Live-imaging of sarcolemmal dystrophin-EGFP.** **(A)** Confocal intravital microscopy  
595 of  $Dmd^{EGFP}$  *tibialis anterior* muscle revealed homogeneous fluorescence of dystrophin-EGFP  
596 protein at the sarcolemma of superficial myofibres. Scale bar: 100  $\mu$ m. **(B-C)** Isolated myofi-  
597 bres from  $Dmd^{EGFP}$  EDL muscle. Native dystrophin-EGFP was homogeneously expressed  
598 along the sarcolemma when live-imaged by epifluorescence microscopy directly after isola-  
599 tion (T0; image **B**) or after 40 h in culture (T40; image **C**). Scale bar: 50  $\mu$ m. **(D)** Confocal in-  
600 travital microscopy of  $Dmd^{EGFP-mdx}$  *tibialis anterior* muscle revealed patchy dystrophin-EGFP  
601 expression at the sarcolemma of two revertant myofibres. Revertant dystrophin-EGFP was



602 not expressed homogeneously, but in a segmental fashion. Scale bar: 100  $\mu\text{m}$ . **(E-F)** Isolated  
603 revertant myofibres from  $Dmd^{EGFP-mdx}$  EDL muscle. Native dystrophin-EGFP was expressed  
604 along the sarcolemma of a short revertant myofibre segment when viewed using epifluores-  
605 cence microscopy directly after isolation (T0; image E) or after 40 h in culture (T40; image F).  
606 Scale bar: 50  $\mu\text{m}$ .

607 **Figure 5. Live-imaging of therapeutically restored dystrophin.** **(A)**  $Dmd^{EGFP-mdx}$  mice were  
608 systemically treated for 5 months with 200 mg/kg/week tcDNA. Multi-photon intravital mi-  
609 croscopy of TA muscle revealed homogeneous restoration of dystrophin-EGFP protein at the  
610 sarcolemma of superficial myofibres. N=3. Scale bar: 100  $\mu\text{m}$ . **(B)** Isolated myofibre from EDL  
611 muscle from tcDNA treated  $Dmd^{EGFP-mdx}$  mice. Native dystrophin-EGFP was seen to be ex-  
612 pressed along the sarcolemma by live-imaging using epifluorescence microscopy directly  
613 after isolation. N>20. Scale bar: 50  $\mu\text{m}$ . **(C and C')** Anterior muscle compartment of the lower  
614 leg of  $Dmd^{EGFP-mdx}$  mice was injected with AAV-SaCas9-sgRNA22 along with AAV-Cherry-  
615 sgRNA23 vectors. Confocal intravital microscopy of TA muscle was performed after 5 months  
616 of transduction. Restoration of dystrophin-EGFP was found in short segments of transduced  
617 myofibres. Successful transduction of myofibres with AAV-Cherry-sgRNA23 was revealed by  
618 cytoplasmic mCherry fluorescence. N=5. Scale bar: 100  $\mu\text{m}$ . **(D and D')** Isolated myofibre of  
619 EDL muscle from AAV injected mice was live-imaged using epifluorescence microscopy di-  
620 rectly after isolation. Native fluorescence of restored sarcolemmal dystrophin-EGFP was  
621 found in a short fibre segment, whereas mCherry was expressed throughout the cytoplasm.  
622 Hoechst showing myonuclei. N>20. Scale bar: 50  $\mu\text{m}$ .

623 **Supporting Figure 1. Detection of the dystrophin isoforms Dp71 and Dp260 in  $Dmd^{EGFP-mdx}$**   
624 **mice.** **(A)** The left image depicts a brain cross-section emitting native EGFP-fluorescence  
625 (green). Scale bar: 100 $\mu\text{m}$ . A higher magnification of a brain blood vessel (green) shows par-  
626 tial co-localization of the Dp71 EGFP positive isoform with the endothelial cell marker CD31  
627 (red). Scale bar: 10  $\mu\text{m}$ . **(B)** Wild-type  $Dmd^{EGFP}$  retina was labelled with a MANDYS19 anti-  
628 body to visualise full-length dystrophin in photoreceptor terminals. The MANDYS19 signal  
629 (red) and the native fluorescence of the dystrophin-EGFP fusion protein (green) co-localised  
630 at the same photoreceptor terminals (yellow), whereas the Dp260 isoform corresponds to  
631 the EGFP-only signal. The lower three images depict the retina from  $Dmd^{EGFP-mdx}$  mice. The  
632 MANDYS19 signal is absent, whereas the native EGFP-fluorescence persisted, corresponding

633 to the Dp260 retinal isoform. Right images depicts a magnified section of the adjacent im-  
634 age. Scale bar: 10  $\mu\text{m}$ .

635 **Supporting Figure 2. Detection of the dystrophin isoform Dp116 in  $Dmd^{EGFP-mdx}$  mice. (A)**

636 The confocal microscopy images (one representative image from the Z-stack) depict a bun-  
637 dle of myofibres from  $Dmd^{EGFP-mdx}$  EDL muscle together with their innervating peripheral mo-  
638 tor nerve branch. The double immunolabelling with anti-GFP (green) and anti- $\beta$ -tubulin III  
639 antibodies (red) reveals co-expression of Dp116 and  $\beta$ -tubulin III at peripheral motor nerve  
640 branches (yellow). The striation of skeletal muscle fibres is visible with Nomarski imaging  
641 (grey). **(B)** Staining of a sciatic nerve cross section with anti-GFP (green) and anti- $\beta$ -tubulin III  
642 (red) confirms the expression of Dp116 in the peripheral nerve.  $\beta$ -Tubulin III expression can  
643 be observed in the central neurofilaments that are surrounded by the Dp116 signal from the  
644 Schwann's cells. The right image depicts a magnified section of the adjacent image. Scale  
645 bar: 100  $\mu\text{m}$ .

646 **Supporting Figure 3. Live-imaging of therapeutically restored dystrophin. (A,B)** Intravital

647 multi-photon microscopy of TA muscle from control  $Dmd^{EGFP-mdx}$  mice using a 500-550 nm  
648 band pass filter did not reveal any sarcolemmal dystrophin-EGFP-fluorescence **(A)**. Only dot-  
649 like structures were visible, which were also visible using the 625-675 nm band pass filter  
650 **(B)**, thus qualifying them as autofluorescent signals, likely originating from inflammatory cell  
651 infiltrates that are characteristic of dystrophic muscle. Scale bar: 200  $\mu\text{m}$ . **(C,D)** Isolated myo-  
652 fibre from EDL muscle from wild-type C57BL6 mice did not show any signal in the GFP chan-  
653 nel under live-imaging using epifluorescence microscopy directly after isolation, whereas the  
654 myofibre was visible with Nomarski imaging. Scale bar: 50  $\mu\text{m}$ . **(E,F)** Isolated myofibre of EDL  
655 muscle from control  $Dmd^{EGFP-mdx}$  mice did not show any signal in the GFP channel under live-  
656 imaging using epifluorescence microscopy directly after isolation (applying same imaging  
657 parameters as for  $Dmd^{EGFP}$  myofibres), whereas the myofibre was visible with Nomarski im-  
658 aging. N>20. Scale bar: 50  $\mu\text{m}$ . **(G,F)** Floating isolated myofibres of EDL muscle from control  
659  $Dmd^{EGFP-mdx}$  mice were live imaged using a stereomicroscope immediately after isolation.  
660 EGFP-fluorescence of revertant fibre segments was easily detectable, whereas the non-  
661 revertant fibre was only visible by dark-field microscopy.

662 **Video legends**

663 **Videos 1 & 2.** The videos are the recordings of two confocal microscopy stacks of bundles of  
664 myofibres from  $Dmd^{EGFP-mdx}$  EDL muscle together with their innervating peripheral motor  
665 nerve branches. The double immunolabelling with anti-GFP (green) and anti- $\beta$ -tubulin III  
666 antibodies (red) confirms co-expression of Dp116 and  $\beta$ -tubulin III in the peripheral motor  
667 nerve (yellow). The striation of skeletal muscle fibres is visible in the images recorded by  
668 Nomarski imaging in grey.

669 **Video 3.** 3D-volume representation of a multi-photon scanning image stack from a wild-type  
670  $Dmd^{EGFP}$  mouse. The mouse was anaesthetised and the lateral aspect of the lower leg mus-  
671 cles was imaged *in vivo*. Native dystrophin-EGFP-expression can be observed along the sar-  
672 colemma of the muscle fibres.

673 **Video 4.** 3D-volume representation of a multi-photon scanning image stack from a  $Dmd^{EGFP-}$   
674  $mdx$  mouse. The mouse was anaesthetised and the lateral aspect of the lower leg muscles was  
675 imaged *in vivo*. Native dystrophin-EGFP-fluorescence was absent at the sarcolemmal level of  
676 the muscle fibres. However, green fluorescent signals appeared as scattered spots. These  
677 spots were positive for red fluorescence as well, thereby qualifying them as autofluores-  
678 cence (see green/red overlay at the end of the video). Most likely the signal represents  
679 macrophages, which are known to be autofluorescent and abundant in the *mdx* dystrophic  
680 muscle.

681 **Video 5.** 3D-volume representation of a multi-photon scanning image stack from a dys-  
682 trophic  $Dmd^{EGFP-mdx}$  mouse treated with tcDNA for 20 weeks. The mouse was anaesthetised  
683 and the lateral aspect of the lower leg muscles were imaged *in vivo*. Restoration of native  
684 dystrophin-EGFP was observed at the sarcolemma. Autofluorescent cells were less abun-  
685 dant (green, red, and yellow overlay signal at the end of the video) than in  $Dmd^{EGFP-mdx}$  con-  
686 trol mice (for comparison see **video 4**).