

1 **Evaluation of the dystrophin carboxy-terminal domain for micro-dystrophin gene therapy in cardiac and**
2 **skeletal muscles in the DMD^{mdx} rat model**

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31 **ABSTRACT**

32 Duchenne muscular dystrophy (DMD) is a muscle wasting disorder caused by mutations in the gene
33 encoding dystrophin. Gene therapy using micro-dystrophin (MD) transgenes and recombinant adeno-
34 associated virus (rAAV) vectors hold great promise. To overcome the limited packaging capacity of
35 rAAV vectors, most MD do not include dystrophin carboxy-terminal (CT) domain. Yet, the CT domain
36 is known to recruit α 1- and β 1-syntrophins and α -dystrobrevin, a part of the dystrophin-associated
37 protein complex (DAPC), which is a signaling and structural mediator of muscle cells. In this study, we
38 explored the impact of inclusion of the dystrophin CT domain on Δ R4-23/ Δ CT MD (MD1), in DMD^{mdx}
39 rats, which allows for relevant evaluations at muscular and cardiac levels. We showed by LC-MS/MS
40 that MD1 expression is sufficient to restore the interactions at a physiological level of most DAPC
41 partners in skeletal and cardiac muscles, and that inclusion of the CT domain increases the
42 recruitment of some DAPC partners at supra-physiological levels. In parallel, we demonstrated that
43 inclusion of the CT domain does not improve MD1 therapeutic efficacy on DMD muscle and cardiac
44 pathologies. Our work highlights new evidences of the therapeutic potential of MD1 and strengthens
45 the relevance of this candidate for gene therapy of DMD.

46 INTRODUCTION

47 Duchenne Muscular Dystrophy (DMD) is a fatal X-linked degenerative neuromuscular disease
48 affecting $\approx 1:5,000$ male births(1). DMD is caused by mutations in the *DMD* gene coding for dystrophin,
49 a large sub-sarcolemmal protein (427 kDa) essential for the integrity of muscular fibers in skeletal and
50 cardiac muscles(2). Dystrophin comprises four structural regions: an actin-binding N-terminal (NT)
51 domain, a central rod domain with 24 spectrin-like repeats and four hinges, a cysteine rich (CR)
52 domain, and a carboxy-terminal (CT) domain. In myofibers, dystrophin plays a central role in the
53 dystrophin-associated protein complex (DAPC) by maintaining the structural link between the actin
54 cytoskeleton and the extracellular matrix, and by recruiting regulatory and signaling proteins
55 (**Fig. 1A**)(3). Its loss leads to DAPC disruption(4), susceptibility to contraction-induced injuries and
56 replacement of muscle cells by connective (fibrosis) and adipose tissue, resulting in progressive loss
57 of muscle function and cardiac involvement(5).

58 Recombinant adeno-associated virus vectors (rAAVs) hold great promise for DMD gene therapy, as
59 they allow efficient transduction of skeletal and cardiac muscles(6) and a long-term *in vivo* transgene
60 expression(7). One caveat is that rAAVs have limited packaging capacity of <5 kb(8) while full-length
61 dystrophin cDNA is >14 kb in length. To overcome this DNA packaging limitation of rAAVs, several
62 micro-dystrophin (MD) transgenes containing essential dystrophin functional domains have been
63 developed(9,10). The rationale for the use of MDs to treat DMD patients is that Becker Muscular
64 Dystrophy (BMD) patients who carry in-frame deletions that naturally produce MDs, exhibit a milder
65 dystrophinopathy(11). Several studies have shown body-wide expression and therapeutic efficacy of
66 MDs in DMD animal models (*mdx* mice, DMD^{*mdx*} rats and DMD dogs) following a single systemic
67 administration of rAAV-MD vectors(12–24). Based on these data, three clinical trials of systemic rAAV-
68 MD gene therapy were initiated in DMD patients in the USA in 2017/2018. Three different MD
69 transgenes are used in these clinical trials: $\Delta R4-23/\Delta CT$, $\Delta R3/\Delta R4-21/\Delta CT$ and $\Delta R2-15/\Delta R18-$
70 $22/\Delta CT$ (18). First results of the clinical trials using the $\Delta R4-23/\Delta CT$ MD and the $\Delta R3/\Delta R4-21/\Delta CT$ MD
71 showed safe and efficient expression of the transgene, associated with clinical improvements at one
72 year post-injection(25,26).

73 Notably, none of these constructs contain the dystrophin CT domain, which constitutes a large portion
74 of the native protein (325 aa long and ≈ 36 kDa), and which is highly conserved between species(27).
75 It is known to interact directly with $\alpha 1$ - and $\beta 1$ -syntrophins(28) (SNT) (binding site encoded by exon 74

76 of the *DMD* gene) and α -dystrobrevins(29) (α -DTN) (binding site on the helix 1 of the coiled-coil
77 domain encoded by exons 74 and 75 of the *DMD* gene). These proteins play a major role in DAPC as
78 α -DTN helps to strengthen the link between dystrophin and the sarcolemma through the sarcoglycan
79 complex(30), while the SNT adaptor proteins help localize intracellular signaling proteins(31) such as
80 sodium voltage-gated (NaV)(32) channels, transient receptor potential (TRP)(33–35) channels and
81 neuronal nitric oxide synthase (nNOS)(36) (**Fig. 1A**). However, it was shown that the expression of a
82 mini-dystrophin without CT domain in a transgenic *mdx* mouse allows restoration of expression of SNT
83 and α -DTN at the sarcolemma of skeletal muscles, even in the absence of a direct link to
84 dystrophin(37). As a consequence, and due to the DNA packaging limitation of rAAV, inclusion of the
85 975 bp CT coding sequence in MD transgenes was dropped early in the development of many
86 variants. However, regions in the CT domain encoded by exons 71 to 74 seem required to restore a
87 normal expression level of α 1-SNT(37). In addition, one study showed that inclusion of helix 1 of the
88 CT coiled-coil domain in MD1 facilitates increased expression of α 1-SNT and α -DTN at the
89 sarcolemma of skeletal muscle and increased muscle resistance to lesions induced by
90 contraction(38). To date, investigations on the role of the dystrophin CT domain has focused on
91 skeletal muscles as the DMD animal model for these studies was the common *mdx* mouse, which
92 shows minimal and late cardiac pathology(39). Nevertheless, a recent genotype/phenotype correlation
93 study conducted in 274 patients raised the possibility that the dystrophin CT domain may have a
94 cardioprotective role(40). Proteomics analysis also showed differences in DAPC composition,
95 especially for some SNT and DTN isoforms, between skeletal muscle and heart in mouse(41).
96 Therefore, the precise impact of the inclusion of the dystrophin CT domain in a MD construct, remains
97 to be explored, especially in the heart. The efficacy of MD expression on the DAPC recovery is
98 classically evaluated by immunolabeling of the known DAPC partners. However, this method only
99 provides information on the sarcolemmal colocalization of known proteins of the DAPC. Mass
100 spectrometry (MS) analysis of MD enriched fractions by co-immunoprecipitation (co-IP) allows the
101 identification of the MD interactome with DAPC partners whether known or not(41).
102 Moreover, the availability of the newly developed *DMD^{mdx}* rat model, which exhibits systematic and
103 stepwise severe phenotypes in both skeletal and cardiac muscles, close to what occurs in DMD
104 patients(42), enables the functional exploration of new MD variants in a relevant model of DMD.

105 In this study, we evaluated for the first time the impact of inclusion of the dystrophin CT domain to
106 $\Delta R4-23/\Delta CT$ MD (also called "MD1") when using rAAV-based gene transfer. Specifically, we
107 evaluated, in both skeletal and cardiac muscles, (i) the DAPC composition and (ii) the therapeutic
108 efficacy after injection of rAAV-MD1+/-CT in DMD^{mdx} rats. We show by liquid chromatography coupled
109 to tandem mass spectrometry (LC-MS/MS) analysis that, despite the absence of the dystrophin CT
110 domain, MD1 can restore a normal level of interaction with most DAPC partners (including SNT and α -
111 DTN) in both skeletal and cardiac muscles of DMD^{mdx} rats. Surprisingly, the partial or complete
112 inclusion of the CT domain to MD1 results in a supra-physiological association with some of these
113 partners. At the functional level, we were unable to demonstrate that incorporation of the coil-coiled
114 motif of the dystrophin CT provides a therapeutic benefit to MD1 in DMD^{mdx} rats. This work
115 strengthens the highly therapeutic potential of MD1 for systemic rAAV-MD gene therapy for DMD.

116

117 **MATERIALS AND METHODS**

118 ***Vector design and production***

119 Murine-specific cDNA sequences of optimized MD version 1 and 2 (MD1 and MD2) have been
120 previously described(13,38). The versions 3 and 4 (MD3 and MD4) were generated from these
121 previous constructs(27). The MD1 cDNA is deleted of spectrin-like repeat domain 4 to 23 and CT
122 domain (exons 71–78) and contains the last three amino acids of exon 79 of dystrophin followed by
123 three stop codons(13). Based on this MD1 construct, the MD2 cDNA contains helix 1 of the coiled-coil
124 motif of the CT domain of dystrophin(38), the MD3 cDNA contains the entire the coiled-coil motif (helix
125 1 and 2) and the MD4 cDNA contains the entire CT domain of dystrophin. Each murine MD cDNA
126 sequence was subcloned into a pAAV plasmid that contained the 323 bp muscle-synthetic Spc5.12
127 promoter(43), a synthetic polyadenylation signal of 49 bp obtained from the pCI-neo plasmid
128 (Promega, Madison, WI), and two flanking inverted terminal repeat (ITR) sequences of 130 pb from
129 AAV serotype 2. The sizes of the resulting MD1, MD2, MD3 and MD4 expression cassettes (including
130 Spc5.12 promoter, pCI-Neo polyA, and ITRs) were 4,538 bp, 4,721 bp, 4,833 bp and 5,114 bp,
131 respectively (**Fig. 1C**). Recombinant pseudotyped AAV2/9-MD vectors were produced by the Vector
132 Core of the UMR 1089 (CPV, INSERM and University of Nantes) by transient transfection of HEK293
133 cells followed by purification on cesium chloride density gradients. Final vectors were concentrated
134 and formulated in Dulbecco's phosphate-buffered saline (DPBS, Fisher Scientific, Illkirsch, France),

135 sterile filtered, aliquoted and frozen at ≤ 70 °C. Vector genome titers (vg/mL) were determined using a
136 qPCR assay specific for ITR2(44). Infectious particles (ip) titer (ip/mL) were determined using an
137 Infectious Center Assay (ICA)(45).

138

139 ***Animals and vector delivery protocols***

140 Study to assess the impact of the dystrophin CT domain on the DAPC composition

141 A total of 21 DMD^{mdx} rats and 4 Sprague Dawley WT rats (littermates) were used in this study. Animals
142 were randomly assigned to the different experimental groups. They were obtained from the UTE IRS2
143 (University of Nantes, France). They were handled and housed in the UTE IRS2. The Institutional
144 Animal Care and Use Committee of the Région des Pays de la Loire (University of Angers, France) as
145 well as the French Ministry for National Education, Higher Education and Research approved the
146 protocol (authorization #2018102616384887). Each vector (MD1, MD2, MD3 and MD4) was
147 administrated without anesthesia to 4 newborn DMD^{mdx} rats (between 4 and 6 days of age) by
148 intraperitoneal (IP) injection of 1.2E14 vg/kg (for rAAV-MD1, MD2 and MD3 vectors) or 2.4E14 vg/kg
149 (for rAAV-MD4 vector). Prior injection, the rAAV vectors were diluted in DPBS vehicle solution to
150 obtain a fixed total volume corresponding to 20 mL of perfusate per kg of animal. These animals were
151 euthanized 4.5 months post-injection by CO2 inhalation. Three 2-month-old DMD^{mdx} rats were also
152 injected intramuscularly in the *tibialis anterior* muscle with a total of 1E12 vg of the rAAV/MD4 vector,
153 after anesthesia. These animals were euthanized 7.5 months post-injection by CO2 inhalation.

154

155 Study to assess the impact of the coil-coiled motif of the CT domain of dystrophin in DMD^{mdx} rats

156 A total of 40 DMD^{mdx} rats and 10 Sprague Dawley WT rats (littermates) were used in this study.
157 Animals were randomly assigned to the different experimental groups. They were obtained from the
158 Boisbonne Center for Gene Therapy (ONIRIS, Nantes, France). They were handled and housed in the
159 Boisbonne Center for Gene Therapy. The Institutional Animal Care and Use Committee of the Région
160 des Pays de la Loire (University of Angers, France) as well as the French Ministry for National
161 Education, Higher Education and Research approved the protocol (authorization
162 #2017040616371353). Prior injection, the rAAV vectors were diluted in DPBS vehicle solution to
163 obtain a fixed total volume corresponding to 15 mL of perfusate per kg of animal. Injections were
164 performed without anesthesia but under analgesic premedication. Vector or its vehicle was

165 administered at the age of 1 month by the intravenous route in a tail vein. Animals were sacrificed
166 after 3 months of follow-up. After anesthesia and incision of the abdomen, whole blood was obtained
167 at the level of the vena cava and euthanasia was performed by intravenous injection of pentobarbital
168 sodium (Dolethal, Vetoquinol, Paris, France). For animals dedicated to *ex-vivo* skeletal muscle
169 contractility analysis and calcium measurements, euthanasia was performed by section of the heart,
170 after anesthesia.

171

172 **Co-immunoprecipitations**

173 Antibody production from hybridomas (DSHB, University of Iowa), protein extractions and
174 immunoprecipitations were performed as previously described(41). Briefly, MANEX1011B (clone 1C7,
175 developed by Glenn E. Morris, Center for Inherited Neuromuscular Disease, RJA Orthopedic
176 Hospital, Oswestry, UK) or control MW8 (developed by Paul H. Patterson, Division of Biology,
177 California Institute of Technology, Pasadena, CA) antibodies were incubated with magnetic
178 Dynabeads protein G (Invitrogen, Illkirsch-Graffenstaden, France; 1.2 mg antibody per 1 mL beads) in
179 100 mM sodium phosphate (pH 5.0) overnight at 4 °C. Antibodies were cross-linked to the beads by
180 incubation in 0.2 M Triethanolamine (Fisher Scientific, Illkirsch, France) containing 20 mM dimethyl
181 pimelimidate (Fisher Scientific, Illkirsch, France) for 30 min at 20 °C.

182 Right after sacrifice, *pectoralis* muscles (for rats injected *via* the IP route and non-injected rats) or
183 *tibialis anterior* muscles (for rats injected *via* the IM route) and heart were dissected and 100 mg of
184 tissues were homogenized for protein extraction (**Fig. S2**). This amount of tissue provided enough
185 material for one experimental co-IP and one control co-IP from the same protein homogenate. Tissues
186 were homogenized 1:10 w/v in ice cold extraction Buffer (1% digitonin (Fisher Scientific, Illkirsch,
187 France), 0.05% NP-40 (IGEPAL CA-630, Sigma Aldrich, Saint Quentin Fallavier, France), NaCl
188 150 mM (VWR International, Fontenay sous Bois, France), Tris 50 mM, pH 7.4 (VWR International,
189 Fontenay sous Bois, France)) with Complete Protease Inhibitors and PhosSTOP (Roche Diagnostics,
190 Meylan, France) using the PowerGen 125 homogenizer (Fisher Scientific, Illkirsch, France). Proteins
191 were extracted on ice for 1 hour, centrifuged at 80,000g for 30 min and supernatant was pre-cleared
192 with protein G agarose beads (Invitrogen, Illkirsch-Graffenstaden, France). Protein concentration was
193 determined with the DC Protein Assay (Bio-Rad, Marnes-la-Coquette, France). Antibody conjugated
194 beads were incubated with 2–2.5 mg protein at +4 °C for 3 hours, washed in ice cold extraction buffer

195 without digitonin, and proteins were eluted in 2% SDS (Sigma Aldrich, Saint Quentin Fallavier,
196 France), 100 mM DTT (Sigma Aldrich, Saint Quentin Fallavier, France) for LC-MS/MS analysis.

197 **LC-MS/MS**

198 15 μ L of the eluents were taken for trypsin digestion. First, 9 μ L of 10% SDS (Affymetrix, Cleveland,
199 OH) in 100 mM TEAB (Triethylammonium bicarbonate, Sigma, Saint Louis, MO) was added to the
200 samples to make the final concentration of SDS at 5%. 5 μ L of 5 μ g/ μ L DTT (FisherScientific,
201 Pittsburg, PA) in 50 mM ammonium bicarbonate (ABC, J.T Baker, purchased through FisherScientific,
202 Pittsburg, PA) solution was added and the sample was incubated at 65 °C for 15 min followed by
203 addition of 5 μ L of 50 mM ABC containing 15 μ g/ μ L iodoacetamide (Acros, purchased through
204 FisherScientific, Pittsburg) followed by incubation at RT for 30 min in the dark. Samples were then
205 acidified by adding 12% phosphoric acid (Sigma, St. Louis, MO) (1:10 v/v acid to sample). 165 μ L of
206 TEAB (Triethylammonium bicarbonate, 1 M)/MeOH ((FisherScientific, Pittsburg, PA) (10:90 v/v) was
207 added to the samples. Samples were then loaded to S-trap (Protifi, Farmingdale NY) and centrifuged
208 at 4,000g for 3 min (at +4 °C) to remove supernatant. 150 μ L of TEAB (Triethylammonium bicarbonate,
209 1 M)/MeOH (10:90 v/v) was added to the trap as wash solution and the trap was washed 5 times. After
210 the final wash, 30 μ L of sequencing grade trypsin (33 ng/ μ L, Promega. Madison, WI) dissolved in
211 50 mM TEAB was added and the digestion was carried on O/N at 37 °C. The following day, peptides
212 were eluted from the trap by adding 40 μ L of 50 mM TEAB, 0.1% formic acid (FA, FisherScientific,
213 Pittsburg, PA) in water and 0.1% FA in Acetonitrile (FisherScientific, Pittsburg, PA) (50:50),
214 sequentially. The eluted peptides were pooled together and dried in a vacufuge and resuspended in
215 20 μ L of 50 mM acetic acid solution (FisherScientific, Pittsburg, PA).

216 Nano-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) of protein
217 identification was performed on a Thermo Scientific orbitrap Fusion mass spectrometer equipped with
218 an nanospray FAIMS Pro™ Sources (Thermo Fisher Scientific, Waltham, MA) operated in positive ion
219 mode. Samples (6 μ L) were separated on an easy spray nano column (Pepmap™ RSLC, C18
220 3 μ 100A, 75 μ m X150 mm Thermo Fisher Scientific, Waltham, MA) using a 2D RSLC HPLC system
221 (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was injected into the μ -Precolumn
222 Cartridge (Thermo Scientific) and desalted with 0.1% Formic Acid in water for 5 minutes. The injector
223 port was then switched to inject and the peptides were eluted off of the trap onto the column. Mobile
224 phase A was 0.1% Formic Acid in water and acetonitrile (with 0.1% formic acid) was used as mobile

225 phase B. Flow rate was set at 300 nL/min. mobile phase B was increased from 2% to 16% in 105 min
226 and then increased from 16-25% in 10 min and again from 25-85% in 1 min and then kept at 95% for
227 another 4 min before being brought back quickly to 2% in 1 min. The column was equilibrated at 2% of
228 mobile phase B (or 98% A) for 15 min before the next sample injection.

229 MS/MS data was acquired with a spray voltage of 1.95 KV and a capillary temperature of 305 °C is
230 used. The scan sequence of the mass spectrometer was based on the preview mode data dependent
231 TopSpeed™ method: the analysis was programmed for a full scan recorded between m/z 375-1500
232 and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive
233 scans starting from the most abundant peaks in the spectrum in the next 3 seconds. To achieve high
234 mass accuracy MS determination, the full scan was performed at FT mode and the resolution was set
235 at 120,000 with internal mass calibration. Three compensation voltage (cv=-50, -65 and -80 v) were
236 used for samples acquisition. The AGC Target ion number for FT full scan was set at 4 x 10E5 ions,
237 maximum ion injection time was set at 50 ms and micro scan number was set at 1. MSn was
238 performed using HCD in ion trap mode to ensure the highest signal intensity of MSn spectra. The HCD
239 collision energy was set at 32%. The AGC Target ion number for ion trap MSn scan was set at 3.0E4
240 ions, maximum ion injection time was set at 35 ms and micro scan number was set at 1. Dynamic
241 exclusion is enabled with a repeat count of 1 within 60 s and a low mass width and high mass width of
242 10 ppm.

243

244 ***Peptide sequence Analysis***

245 Data were searched using Mascot Daemon by Matrix Science version 2.7.0 (Matrix Science, Boston,
246 MA) via ProteomeDiscoverer (version 2.4 Thermo Fisher Scientific, Waltham, MA) against the most
247 recent Uniprot rat databases. Customized database was constructed by replacing dystrophin with the
248 MD sequences to specifically identify and quantify the synthetic MD. The mass accuracy of the
249 precursor ions was set to 10 ppm, accidental pick of 1 ¹³C peaks was also included into the search.
250 The fragment mass tolerance was set to 0.5 Da. Carbamidomethylation (Cys) was used as a fixed
251 modification and considered variable modifications were oxidation (Met) and deamidation (N and Q).
252 Four missed cleavages for the enzyme were permitted. A decoy database was also searched to
253 determine the false discovery rate (FDR) and peptides were filtered according at 1% FDR. Proteins

254 identified with at least two unique peptides were considered as reliable identification. Any modified
255 peptides are manually checked for validation.

256

257

258 ***Label-free quantification***

259 Label-free quantitation was performed using the spectral count approach. Scaffold (Proteomic
260 Software, Inc., Portland, OR) was used for data analysis and calculation of emPAI (Exponentially
261 Modified Protein Abundance Index) values to estimate abundance of each identified protein within the
262 total set of identified proteins(46,47). This index is an exponential form of the number of observed
263 peptides divided by the number of all possible tryptic peptides from a particular protein.

264 Two Scaffold files were generated for DAPC selection and to remove contaminating proteins from the
265 MS analysis of co-IP samples from skeletal muscles and heart. Control co-IP samples using the MW8
266 antibody were grouped in the IgG_co-IP category (n=20 for skeletal muscle and n=19 for heart). Co-IP
267 samples from non-injected WT rats using MANEX1011B antibody were grouped in “WT_DYS_co-IP”
268 category (N=4). Finally, co-IP samples from rAAV9_MD injected DMD^{mdx} rats using MANEX1011B
269 antibody were grouped in four categories: MD1_DYS_co-IP (N=4), MD2_DYS_co-IP (N=4),
270 MD3_DYS_co-IP (N=4) and MD4_DYS_co-IP (N=4 for skeletal muscles and N=3 for heart). In these
271 two scaffold files, emPAIs (non-normalized) were calculated for each identified protein within the total
272 set of identified proteins. Then, a quantitative profile was generated by a variance analysis (ANOVA).
273 Proteins with no significant change of quantitative profile between the different categories and a
274 significant enrichment in IgG_co-IP were considered as background proteins and removed from DAPC
275 selection. Proteins with a significant enrichment in the WT_DYS_co-IP and/or MD_DYS_co-IP
276 categories were selected as potential DAPC proteins. emPAI values of each selected protein were
277 used to perform a 2-way ANOVA followed by a multiple comparison post-hoc “original FDR method of
278 Benjamini and Hochberg” test between WT_DYS_co-IP and IgG_co-IP samples. Proteins with
279 significant difference (Q-value or corrected P-value ≤ 0.05) between WT_DYS_co-IP and IgG_co-IP
280 categories were identified as specific DAPC proteins. Statistics were performed using GraphPad
281 Prism 8.

282 Two new Scaffold files were generated for the comparison of selected DAPC proteins abundance
283 between WT_DYS_co-IP, MD1_DYS_co-IP, MD2_DYS_co-IP, MD3_DYS_co-IP and MD4_DYS_co-

284 IP experimental groups. In these files, IgG_co-IP samples were removed and normalized emPAI
285 values were calculated for each previously selected DAPC proteins of WT_DYS_co-IP,
286 MD1_DYS_co-IP, MD2_DYS_co-IP, MD3_DYS_co-IP and MD4_DYS_co-IP samples. Then, relative
287 abundance of these DAPC partners were calculated using corresponding MD/dystrophin normalized
288 emPAI. Relative abundances of each protein were compared between WT_DYS_co-IP,
289 MD1_DYS_co-IP, MD2_DYS_co-IP, MD3_DYS_co-IP and MD4_DYS_co-IP experimental groups by a
290 2-way ANOVA followed by a multiple comparison post-hoc “original FDR method of Benjamini and
291 Hochberg”. Statistics were performed using GraphPad Prism 8. Q-values (corrected P-value) ≤ 0.05
292 were considered statistically significant.

293 Individual values of emPAI (non-normalized, normalized, and relative to MD/dystrophin normalized
294 emPAI), as well as percentages of coverage, and numbers of unique peptides obtained for each
295 analyzed sample, are available in **Tables S4 to S7**.

296

297 **Vector biodistribution analysis**

298 Just before sacrifice, whole blood was collected from anesthetized rats in tubes containing EDTA as
299 an anticoagulant, aliquoted in human DNA-free, RNase/DNase-free, and PCR inhibitor-free certified
300 microtubes, and stored at ≤ -70 °C before DNA extraction. Genomic DNA (gDNA) was extracted from
301 200 μ L of whole blood using the NucleoSpin® Blood kit (Macherey Nagel, Hoerd, France) according
302 to the manufacturer’s instructions, which were adapted for a Microlab STAR Line liquid handling
303 workstation (Hamilton, Villebon sur Yvette, France). gDNA was extracted from snap-frozen *biceps*
304 *femoris*, *pectoralis* and EDL muscles, heart, diaphragm and liver as previously described (16). Duplex
305 Q-PCR analyses were conducted on a C1000 touch thermal cycler (Bio-Rad, Marnes-la-Coquette,
306 France) using 50 ng of gDNA in duplicate and premix Ex taq (Ozyme, Saint-Cyr-L’Ecole, France).
307 Vector copy numbers were determined using primers and probe specifically designed to amplify both
308 MD1 and MD3 transgenes. Primers and probe designed to amplify the rat *HPRT1* sequence were
309 used to determine the endogenous gDNA copy numbers. For each sample, cycle threshold (Cq)
310 values were compared with those obtained with different dilutions of linearized standard plasmids
311 (containing either the mMD1 expression cassette or the rat *HPRT1* gene).

312

313 **Western blot analysis**

314 Total proteins extraction from snap-frozen *biceps femoris* and heart samples, and MD1/MD3
315 expression analysis by western blot were performed as previously described (16). Protein loading
316 control analysis was assessed using a mouse anti-rat α -tubulin antibody (1:10,000, Sigma-Aldrich
317 T5168, Saint Quentin Fallavier, France) and a secondary anti-mouse IgG HRP-conjugated antibody
318 (1:5,000, Dako P0447, Les Ulis, France).

319

320 ***Histopathological analysis***

321 Samples including *biceps femoris*, *pectoralis* and cardiac muscles were fixed in 10% neutral buffered
322 formalin, embedded in paraffin wax. Sections were cut and stained with hematoxylin eosin saffron
323 (HES) for histopathological evaluation. To avoid bias in the analysis, experiments were done in a blind
324 fashion. Muscular lesions were then semi-quantitatively scored (as described in **Fig. 4** legend), to
325 identify any improvement of the histopathological pattern between the different experimental groups.

326

327 ***MD/dystrophin positive fibers and connective tissue quantification***

328 Samples including *biceps femoris*, *pectoralis* and cardiac muscles were snap-frozen in isopentane
329 cooled in liquid nitrogen and stored at ≤ -70 °C until processing. Sections of 10 μ m from frozen
330 samples were permeabilized in PBS tween 0.2% (Sigma-Aldrich, Saint Quentin Fallavier, France) and
331 saturated in 5% goat serum (Sigma-Aldrich, Saint Quentin Fallavier, France) in PBS tween 0.2%.
332 Sections were then immunolabelled for detection of MD/dystrophin and connective tissue using the
333 mouse monoclonal anti-dystrophin antibody NCL-DYSB (1:50, Novocastra Laboratories, Newcastle
334 upon Tyne, UK) and Alexa Fluor 555 Wheat Germ Agglutinin (WGA) conjugate (1:500, Molecular
335 Probes, Eugene, OR), diluted in 5% rat serum (Sigma-Aldrich, Saint Quentin Fallavier, France) in PBS
336 tween 0.2%. Goat anti-mouse Alexa 488 IgG (H+L) (1:300, Invitrogen A28175, Illkirsch-Graffenstaden,
337 France) was used as secondary antibody, diluted in 5% rat serum in in PBS tween 0.2%. Nuclei were
338 stained with DAPI (1:1000, Invitrogen, Illkirsch-Graffenstaden, France). Fluorescent acquisitions of the
339 entire sections were performed using the slide scanner Axio Scan.Z1 (Zeiss, Marly le Roi, France).
340 For each section, 3 captures were randomly chosen to finally observe a minimum of 250 fibers. In
341 these 3 captures, DYSB positive fibers and WGA positive areas (except for the heart) were quantified
342 using ImageJ open-source image processing software (v 2.0.0-rc-49/1.51a). Additional sections from
343 paraffin embedded samples of heart was stained with picosirius red F3B (Sigma-Aldrich, Saint

344 Quentin Fallavier, France) for collagen visualization. Picrosirius-positive areas in heart sections were
345 quantified using Nikon Imaging software (Nikon, Champigny sur Marne, France).

346

347 ***Calcium measurements***

348 Cell isolation

349 EDL muscles were harvested from animals just after euthanasia. Small bundles were dissected
350 longwise tendon to tendon as described previously(48). Cardiomyocytes were isolated by using the
351 retrograde perfusion method through the aorta with enzyme-containing solutions(49). Briefly, hearts
352 were excised from heparinized (5,000 U/kg body, Heparine choay, Sanofi, Gentilly, France) and
353 anesthetized rats, mounted in a Langendorff perfusion apparatus, and perfused with a Ca^{2+} -free NPS
354 (Normal Physiological Solution) at 37 °C for 3 min. Perfusion was then switched to the same solution
355 containing 300 U/ml Collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) for
356 20 min. The left ventricular tissue was excised, minced, and gently pipette dissociated. Collagenase
357 activity was stopped by the addition of 4% bovine serum albumin (Sigma-Aldrich, Saint Quentin
358 Fallavier, France). At this stage, cardiomyocytes were photographed with an inverted microscope, in
359 bright field and with a X20 objective. Pictures were then used in ImageJ software to measure max and
360 min Feret's diameters to calculate length and width of the cells. Cell suspension was then sequentially
361 washed and resuspended in NPS of increasing $[\text{Ca}^{2+}]$ values.

362 Fura-2 loading and fluorescence measurements

363 EDL muscle bundles, pinned in polydimethylsiloxane coated petri dish (Dow France S.A.S., Saint
364 Denis, France), were incubated with NPS containing 5 μM Fura-2-AM (Molecular Probes, Illkirsch-
365 Graffenstaden, France) at room temperature for 1 h under gentle agitation before washing. Isolated
366 cardiomyocytes were resuspended in NPS containing 3 μM Fura-2-AM for 30 min before washing and
367 Ca^{2+} measurements. Fura-2 fluorescence measurements were made using an integrated IonOptix
368 device (Amsterdam, Netherlands) and excitation filters of 380 and 360 nm. Emitted fluorescence
369 (510 nm) was background subtracted. For resting cytosolic $[\text{Ca}^{2+}]$ measurements, resting fluorescence
370 ratios (360/380 nm) values were measured for each fiber composing EDL muscle bundles or isolated
371 cardiomyocyte. $[\text{Ca}^{2+}]$ values were calculated from the Fura-2 fluorescence ratio using the
372 Grynkiewicz's equation(50). Main parameters of the equation were determined *in situ*(48).

373 Determination of sarcolemmal permeability to Ca^{2+} (SPCa)

374 The manganese quenching technique was used to measure the SPCa in EDL muscle fibers(48).
375 Muscle preparations were perfused by NPS containing 0.5 mM Mn^{2+} as a surrogate of Ca^{2+}
376 (quenching solution). During the whole quenching protocol, the fluorescence of Fura-2 excited at
377 360 nm was acquired at 1 Hz. The quench rates were calculated in each fiber of the preparations
378 using linear regression analysis of fluorescence signal and expressed as the decline per minute of the
379 initial fluorescence intensity.

380 All experiments were done at room temperature. Solutions: NPS contained the following: 140 mM
381 NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 1.8 mM $CaCl_2$ (All chemicals
382 form Sigma-Aldrich, Saint Quentin Fallavier, France) at pH 7.35.

383

384 ***Skeletal muscle force analysis***

385 *Ex-vivo skeletal muscle contractility*

386 Isometric contractile properties of the EDL and *soleus* muscles were evaluated according to methods
387 previously described(51,52). Briefly, muscles were removed from the hindlimb of anesthetized rats
388 and mounted in an *in vitro* muscle test system (1205A model; Aurora Scientific, Aurora, Canada).
389 Muscles were placed between two platinum electrodes in a muscle bath containing 100 mL of bubbled
390 mammalian Ringer solution (Na^+ 140 mM, K^+ 6 mM, Ca^{2+} 3 mM, Mg^{2+} 2 mM, Cl^- 156 mM, pH 7.4,
391 HEPES-buffered) at 25 °C. After a 5 min equilibration period, optimum muscle length was determined
392 by gradual muscle length adjustments and eliciting isometric contractions (supramaximal square-wave
393 pulses of 0.2 ms duration) until the maximum twitch tension was reached. After 5min of rest, muscles
394 were stimulated at 10, 20, 40, 60, 80, 100, 120 Hz for 500 ms at each frequency. Stimulus trains were
395 separated by one-min interval. Maximum isometric tetanic force was determined from the plateau of
396 this force frequency curve. Following force testing, muscles were removed from the bath, trimmed of
397 tendons, and weighed. Muscle mass was then be used to calculate maximum tetanic specific force in
398 g/g. To avoid bias in the analysis, experiments were done in a blind fashion.

399 *In vivo grip test*

400 For all animals included in the study, the grip test was performed, as previously described (42), at
401 least 3 days before the cardiac evaluation at 3 months post-injection. To avoid bias in the analysis,
402 experiments were done in a blind fashion.

403

404 **Assessment of cardiac function**

405 Cardiac function was assessed on anesthetized animals using 2D echocardiography and pulsed
406 Doppler measurements. For all animals included in the study, these tests were performed at least 3
407 days after grip test at 3 months post-injection.

408 2D-echocardiography and pulsed Doppler were performed, as previously described(42), using a Vivid
409 7 ultrasound unit (GE Healthcare, Velizy Villacoublay, France). To avoid bias in the analysis,
410 experiments were done in a blind fashion.

411

412 **RESULTS**

413 **Assessment of the impact of the dystrophin CT domain on the DAPC composition: study**
414 **design**

415 In order to evaluate the impact of inclusion of the dystrophin CT domain on the efficacy of MD1 to
416 recover the DAPC, we used 4 different murine-specific sequence-optimized MD constructs: MD1,
417 MD2, MD3 and MD4. MD2 to 4 were derived from MD1 by progressively including the dystrophin CT
418 domain sequence (**Fig. 1B**): (i) MD1 ($\Delta R4-23/\Delta CT$) is our reference, (ii) MD2 incorporates helix 1 (H1)
419 of the coiled-coil motif, (iii) MD3 includes the entire coiled-coil motif (H1 + H2), and (iiii) MD4 ($\Delta R4-23$)
420 has the entire dystrophin CT domain.

421 To obtain rat skeletal and cardiac muscles expressing these different MDs, we administered rAAV2/9
422 vectors encoding MD1, MD2, MD3 or MD4 cDNAs (**Fig. 1C**). Each vector was administered to 4
423 newborn DMD^{mdx} rats by intraperitoneal (IP) injection to achieve efficient transduction of skeletal
424 muscles and heart(53,54). As expected, the expression of the different MDs was detected by western
425 blot in the *pectoralis* and cardiac muscles, except for MD4 that was weakly expressed in *pectoralis*
426 muscle in 3 out of 4 animals (**Fig. S1**). The rAAV-MD4 vector carries an expression cassette that
427 exceeds the DNA packaging capacity of rAAV (< 5 kb, **Fig. 1C**)(8) thus limiting its infectivity.
428 Supplemental skeletal muscles expressing MD4 were obtained after intramuscular (IM) injection in the
429 *tibialis anterior* muscles of 3 DMD^{mdx} rats at 2 months of age (**Fig. S1**).

430 Based on the methodology of Johnson *et al.* (41), we compared the composition of protein complexes
431 assembled by full-length dystrophin (N=4), MD1 (N=4), MD2 (N=4), MD3 (N=4) and MD4 (n=4 and
432 N=3 for skeletal muscle and for heart, respectively) in skeletal and cardiac muscles of wild-type (WT)
433 and rAAV-MD treated DMD^{mdx} rats. To identify proteins that selectively associate with MD/dystrophin,

434 we performed a co-IP on proteins extracted from 50 mg of muscle using the MANEX1011B
435 monoclonal antibody (DYS_co-IP) or an isotype-matched control antibody (MW8 antibody) (IgG_co-
436 IP) (**Fig. S2**). LC-MS/MS analysis was performed on all co-IP samples and the abundance of each
437 identified protein within the total set of identified proteins was estimated in Scaffold (Proteomic
438 Software, Inc., Portland, OR) by calculation of the emPAI (Exponentially Modified Protein Abundance
439 Index)(46,47).

440

441 ***Identification of dystrophin protein partners in rat skeletal and cardiac muscles***

442 We first confirmed that we can reliably identify proteins known to associate with dystrophin in rat
443 skeletal and cardiac muscles co-IPs by MS analysis. Proteins that specifically co-purify with full-length
444 dystrophin were identified based on significantly higher emPAI values in WT_DYS_co-IP compared to
445 IgG_co-IP (**Table S1**). We noticed that utrophin, a homolog of dystrophin, was also detected in
446 WT_DYS_co-IP, indicating that the MANEX1011B antibody slightly cross-reacts with utrophin. Since
447 utrophin and dystrophin can both bind to DAPC proteins, we cannot exclude that we might have
448 detected some proteins that bind to utrophin in our MD/dystrophin co-IPs. However, utrophin
449 abundance in WT_DYS_co-IP remained minimal when compared to dystrophin, with levels much
450 lower than dystrophin for skeletal muscles and heart (39- and 18-fold respectively) (**Table S1**). This
451 means proteins detected in WT_DYS_co-IP were mainly associated with dystrophin. Furthermore,
452 utrophin contamination was low in all co-IP samples and did not significantly differ between the WT co-
453 IP and MD co-IP samples (one-way ANOVA, data not shown). Therefore, utrophin did not interfere
454 with our analysis of DAPC composition.

455 Proteins found to be significantly enriched in WT_DYS_co-IP compared to IgG_co-IP are shown in
456 **Table S1**. Known DAPC components such as dystrophin, dystroglycan, α -, β -, δ -, and γ -
457 sarcoglycans, α 1- and β 1-SNT, α -DTN and sarcospan were consistently and specifically detected in
458 WT_DYS_co-IPs from both skeletal and cardiac muscles. As previously described in mice(41), nNOS
459 was found binding to dystrophin in skeletal muscle but not in heart, confirming that this protein is also
460 not part of the DAPC in cardiac tissue of rats. Cavin-1 and β 2-SNT, that have been described as
461 heart-specific DAPC partners(41), were detected here in association with both skeletal and cardiac
462 muscle dystrophins. While we cannot rule out that these proteins may be present in skeletal muscle
463 WT_DYS_co-IPs due to binding to utrophin, the fact that we are using LC-MS/MS instrumentation with

464 higher sensitivity than in the previous study(41) could result in the detection of less abundant DAPC
465 partners. Interestingly, additional proteins were specifically enriched in WT_DYS_co-IP compared to
466 IgG_co-IP. Calmodulin-1 (known to associate with skeletal muscle dystrophin(55,56)), pikachurin (a
467 known extracellular ligand of dystroglycan(57)), β -DTN and perilipin-4 were all detected in skeletal
468 muscle WT_DYS_co-IPs. The caveolar proteins caveolin-1 and cavin-2 were instead detected in
469 cardiac muscle WT_DYS_co-IPs. Finally, cavin-3 was detected in association with both skeletal and
470 cardiac muscle dystrophins.

471

472 ***Inclusion of the dystrophin CT domain in MD1 increases recruitment of some DAPC proteins***

473 We next evaluated how the inclusion of progressively larger segments of the dystrophin CT domain
474 impacted DAPC composition in DMD^{mdx} rats. We reasoned that addition of the coil-coiled domain H1,
475 known to bind to SNT and DTN, should lead to improved binding of MD2, MD3 and MD4 to these
476 proteins, when compared to MD1. Scaffold was used to generate normalized emPAI values of each
477 protein of the DAPC, within the total set of identified proteins for WT_DYS_co-IP, MD1_DYS_co-IP,
478 MD2_DYS_co-IP, MD3_DYS_co-IP and MD4_DYS_co-IP experimental groups. We noticed that our
479 western blot analyses indicated differences in abundance between MD/dystrophins (**Fig. S1**) that were
480 also reflected in our mean emPAI values for MD/dystrophins co-purified from 50 mg of starting
481 material (**Fig. S3**). Therefore, to be able to compare DAPC protein abundance between experimental
482 groups, we calculated the ratio of the emPAI of each individual DAPC protein to the emPAI of MD or
483 dystrophin in the same sample.

484 As shown in **Table 1** and **Fig. 2**, we found several significant differences in the abundance of DAPC
485 proteins that co-purify with different MDs. Some protein associations were significantly disrupted with
486 all MD constructs in cardiac and/or skeletal muscles, suggesting that they require domains absent in
487 our MDs or that these associations are decreased below our limit of detection. Protein associations
488 falling in this category include perilipin-4, cavin-3, and caveolin-1 and nNOS, whose binding to
489 dystrophin is known to be absent in our MDs (R16/17)(19). Interestingly, in cardiac muscle where
490 cavin-1 strongly associates with full-length dystrophin, we saw a significantly impaired association with
491 MD1 and MD2 that was progressively rescued with MD3 and MD4 suggesting that it requires an intact
492 coil-coil CT domain.

493 Overall, in both skeletal and cardiac muscles, MD1 had a protein interaction profile that most closely
494 matched that of full-length dystrophin. The only proteins that were significantly more abundant in MD1
495 co-IPs compared to full-length dystrophin were β - and γ -sarcoglycans in skeletal muscle (1.7- and 2.2-
496 fold, respectively), and β -sarcoglycan (1.8-fold) in cardiac muscle. Interestingly, SNT and DTN that
497 bind dystrophin's CT, co-purified in similar amounts with MD1 and full-length dystrophin. By contrast,
498 sequential addition of parts of the CT sequence in MD2, MD3 and MD4 constructs resulted in
499 significant increases in their associations with several sarcoglycans, SNT and DTN compared to MD1
500 and to full-length dystrophin. In skeletal muscle, these involve α -, β -, δ - and γ -sarcoglycans, and α 1-
501 and β 2-SNT. In cardiac muscles, proteins that are more abundant in MD2, MD3 and MD4 co-IPs are
502 α - and β -sarcoglycans, and α 1- and β 1-SNT. Additionally, a few DAPC proteins were more abundant
503 in co-IPs for specific MDs relative to all other MDs and to dystrophin. These are β 1-SNT in MD4
504 skeletal muscle co-IPs; α -DTN in MD3 skeletal muscle co-IPs; and γ -sarcoglycan in MD3 cardiac
505 muscle co-IPs. In general, the significant differences we found in protein associations depend not only
506 on the MD sequence but also on the type of muscle, suggesting tissue-specific differences in the
507 regulation of protein associations with MDs.

508

509 ***Assessment of the impact of the coil-coiled motif of the dystrophin CT domain on the***
510 ***therapeutic efficacy of a MD transgene: study design***

511 To evaluate the impact of the dystrophin CT domain on the therapeutic efficacy of MDs in both skeletal
512 and cardiac muscles, 1 month old male DMD^{mdx} rats were treated systemically with the rAAV2/9-MD1,
513 or the rAAV2/9-MD3 construct. MD3 was chosen because it contains the entire coil-coiled motif
514 (H1+H2) of the dystrophin CT domain, while not exceeding the <5 kb DNA packaging limitation of
515 rAAV(8) (**Fig. 1C**). Both rAAV2/9-MD1 and -MD3 vector batches exhibited similar vg/mL and infectious
516 titers (**Table S2**), implying that their efficacies can be confidently compared. The study design is
517 summarized in **Table S3**. A group of 11 DMD^{mdx} rats were injected with rAAV2/9-MD1 at a dose of
518 3E13 vg/kg, which we know as therapeutic (Bourdon *et al*, unpublished data). To compare the
519 potential therapeutic benefit between MD3 and MD1, additional DMD^{mdx} rats were injected with
520 1E13 vg/kg (N=9 and N=11 for rAAV2/9-MD1 and rAAV2/9-MD3, respectively). Groups of 10 WT and
521 10 DMD^{mdx} control rats were also included and received buffer. All rats were followed for 3 months.
522 Right before euthanasia, muscle function (evaluated by grip test) and cardiac function (evaluated by

523 electrocardiography, echocardiography and pulsed Doppler) were evaluated. After sacrifice, 4 animals
524 from each group were dedicated to assessments of *ex-vivo* muscle contractility analysis and calcium
525 measurements. The other animals were dedicated to post-mortem sampling required for histological
526 and molecular/biochemical analyses.

527

528 ***The inclusion of the coil-coiled motif of the dystrophin CT domain in MD does not affect gene***
529 ***transfer efficacy and MD expression patterns***

530 Using a quantitative PCR (qPCR) assay specific for both MD1 and MD3 sequences, vector genome
531 copy numbers per diploid genome (vg/dg) were assessed in whole blood, skeletal muscles, heart,
532 diaphragm and liver samples obtained at sacrifice (**Fig. 3A**). No qPCR signal was detected in whole
533 blood, confirming that vector genome detection in rAAV-injected animals was restricted to the tissue
534 itself. Both vectors had the same transduction pattern, with the highest vg/dg numbers detected in liver
535 and heart, as expected after IV injection in rodents(58). Lower vg/dg numbers levels (but similar
536 between the different muscles) were detected in skeletal muscles. In correlation with the injected
537 vector dose, significantly more vg/dg were found in tissues of rats injected with 3E13 vg/kg than with
538 1E13 vg/kg of rAAV2/9-MD1 (2.7- to 7.8-fold respectively, depending on the tissue). Instead, the vg/dg
539 values in tissues of rats injected with 1E13 vg/kg of rAAV2/9-MD1 or rAAV2/9-MD3 vector were
540 similar, confirming that both vectors exhibited the same tropisms and infectivities. MD1/MD3
541 expression in skeletal muscles and heart was evaluated by both immunohistochemistry (IHC) staining
542 (**Fig.3B** and **Fig.3C**) and western blot (**Fig.3D**). Again, a dose effect between the two groups treated
543 with 3E13 vg/kg or 1E13 vg/kg of rAAV2/9-MD1 was observed, with a mean of detected MD1-positive
544 fibers of $85.7 \pm 3.5\%$ to $86.6 \pm 2.7\%$ *versus* $33.6 \pm 7.8\%$ to $40.2 \pm 4.3\%$ respectively in skeletal
545 muscles, and of $96 \pm 0.8\%$ *versus* $86 \pm 2.4\%$ in heart. Similar levels of MD1 or MD3 positive fibers
546 were detected in animals injected with 1E13 vg/kg of rAAV2/9-MD1 or rAAV2/9-MD3 ($33.6 \pm 7.8\%$ to
547 $40.2 \pm 4.3\%$ of MD1-positive fibers *versus* $28.5 \pm 4.8\%$ to $43.5 \pm 6.4\%$ of MD3-positive fibers in skeletal
548 muscles, and $86 \pm 2.4\%$ of MD1-positive fibers *versus* $82 \pm 0.8\%$ of MD3-positive fibers in heart).
549 Immunolabeling confirmed the proper localization of both MD1 and MD3 at the sarcolemma in biceps
550 femoris muscle (**Fig. 3C**) and correlated with the detection of the 138 kDa MD1 and 160 kDa MD3
551 protein bands by western blot analysis (**Fig. 3D**). Moreover, the RTqPCR analysis showed that the
552 MD1 and MD3 mRNA are equally expressed in skeletal muscle and heart samples when the two

553 vectors are injected at the same dose. Only low levels of MD mRNA were detected in liver despite the
554 presence of high vg/dg values, confirming the muscle restrictive activity of the Spc5.12 promoter (**Fig.**
555 **S4**). Taken together, these results confirm that both MD1 and MD3 are similarly and properly
556 expressed in skeletal muscles and heart after systemic injection, meaning that their therapeutic
557 efficacies can be compared in a relevant manner.

558

559 ***The inclusion of the coil-coiled motif of the dystrophin CT domain in MD1 does not improve its***
560 ***effectiveness to restore histopathological and calcium homeostasis alterations in DMD^{mdx} rats***

561 Similar to muscle damage in DMD patients, the histopathological pattern of the DMD^{mdx} rats is
562 characterized by increased variation in fiber size due to the simultaneous presence of hypertrophic
563 fibers and small centronucleated regenerating fibers in skeletal muscles, and individual fiber necrosis
564 associated with inflammatory cell infiltration and fibrosis in both skeletal muscle and heart(42). To
565 investigate the effect of MD1 and MD3 expression on skeletal and cardiac muscle histopathology, we
566 performed a quantification of fibrotic connective tissue and a semi-quantification of muscle lesions in
567 these tissues obtained at sacrifice. As shown in **Fig. 4a**, connective tissue represented 16.1 ± 0.7 to
568 $19.4 \pm 0.6\%$ of skeletal muscle area in WT rats *versus* $29.1 \pm 1.8\%$ to $30.5 \pm 2.1\%$ in untreated
569 DMD^{mdx} rats, showing an increase in connective tissue that corresponded to fibrosis. In all rAAV-
570 treated groups, connective tissue amount was significantly reduced between $20.1 \pm 0.4\%$ and $23.7 \pm$
571 1.1% . In heart, connective tissue levels were $2.7 \pm 0.5\%$ in WT rats *versus* $10.2 \pm 2.2\%$ in untreated
572 DMD^{mdx} rats and were also significantly reduced between $3.7 \pm 0.8\%$ and $4.2 \pm 1\%$ after rAAV2/9-MD
573 administration. No significant difference was observed between the different rAAV-injected groups,
574 whatever the injected dose or the nature of the transgene (MD1 or MD3). Semi-quantitative analysis of
575 histological muscular lesions (scored according to **Fig. S5**) revealed typical myopathic lesions in both
576 skeletal muscles and heart of untreated DMD^{mdx} rats, resulting in a significantly higher score
577 compared to WT rats (**Fig. 4B**). This score was improved after injection of rAAV2/9-MD, except with
578 the MD3 vector in the *pectoralis muscle*. The improvement reached statistical significance in *biceps*
579 *femoris* and heart of animals injected with $3E13$ vg/kg of the MD1 vector. When comparing the impact
580 of MD1 and MD3 vectors injected at $1E13$ vg/kg, no significant difference was observed between
581 these groups. However and as reported in a previous study(59), we observed a few isolated ring fibers
582 with sarcomere disorientation (**Fig. S6**) in the skeletal muscles of 6/12 rats expressing MD1 (2/5 rats

583 treated with 1E13 vg/kg and 4/7 rats treated with 3E13 vg/kg). None was observed in the muscles of
584 the 6 rats expressing MD3.

585 In correlation with muscle degeneration and necrosis and with what was described in *mdx* mice(60),
586 alterations in Ca^{2+} homeostasis were observed in skeletal muscles and isolated cardiomyocytes of
587 DMD^{mdx} rats (**Fig. 5**; Fraysse *et al.*, unpublished data). Lack of dystrophin expression in DMD^{mdx} rats
588 was associated with significant higher resting cytosolic Ca^{2+} levels as compared to WT rats in both
589 *extensor digitorum longus* (EDL) muscle (**Fig. 5A**) and cardiomyocytes (**Fig. 5B**; increase of ≈49% in
590 EDL muscle and of ≈31% in isolated cardiomyocytes). Treatment with the rAAV2/9-MD1 vector at
591 3E13 vg/kg showed a significant decrease in resting $[Ca^{2+}]$, although not completely rescued to WT
592 levels. No statistically significant decrease in resting $[Ca^{2+}]$ was observed after injection of rAAV2/9-
593 MD1 or -MD3 at 1E13 vg/kg. We also evaluated the sarcolemmal permeability to Ca^{2+} (SpCa) in EDL
594 muscles using the manganese quenching technique(61), to assess if resting Ca^{2+} overload could be
595 related to a greater Ca^{2+} influx. As shown in **Fig. 5C**, the mean quench rate of untreated DMD^{mdx} rats
596 was significantly higher than in WT rats, reflecting an increase in the permeability of the sarcolemma.
597 This permeability was significantly decreased in the 3 rAAV2/9-MD treated groups, but without any
598 difference between the different doses or transgenes. Overall, these data indicate that the inclusion of
599 the coil-coiled motif of the dystrophin CT domain in MD1 doesn't improve its effectiveness to restore
600 the histopathology and the calcium homeostasis alterations of the DMD^{mdx} rat.

601

602 ***The inclusion of the coil-coiled motif of the dystrophin CT domain in MD1 doesn't improve its***
603 ***effectiveness to restore muscle and cardiac functions in DMD^{mdx} rat.***

604 *Ex-vivo* force measurements were performed on isolated EDL and *soleus* muscles obtained at
605 sacrifice. We evaluated the maximum contractile response amplitude following a series of electrical
606 stimulations (tetanic) (**Fig. 6A** and **Fig. 6B**). When compared to WT rats, both EDL and *soleus*
607 muscles of DMD^{mdx} rats showed a decrease in maximum tetanic amplitude (decrease of ≈20% in EDL
608 and significant decrease of ≈56% in *soleus*). In animals treated with rAAV2/9-MD1 at 3E13 vg/kg, a
609 nearly complete normalization was observed in the EDL muscle and a partial one (≈48%) in the
610 *soleus*. No effect was observed for the two other experimental groups.

611 We also evaluated *in vivo* forelimb force using the grip test. Five trials were performed and the highest
612 grip value obtained during these trials was defined as maximum forelimb grip force. As illustrated in

613 **Fig. 6C**, a reduction in maximum forelimb grip force was observed for untreated DMD^{mdx} rats
614 compared to WT rats. This value was fully normalized after injection of rAAV2/9-MD1 at 3E13 vg/kg,
615 but only partially improved after injection of rAAV2/9-MD1 and rAAV2/9-MD3 at 1E13 vg/kg. The
616 beneficial effect was slightly higher but not statistically significant with rAAV2/9-MD3 than with
617 rAAV2/9-MD1.

618 Analysis of forelimb strength over the course of the test (**Fig. 6D**) showed progressive and marked
619 decrease of forelimb strength in untreated DMD^{mdx} rats, contrary to WT rats and to DMD^{mdx} rats
620 injected with rAAV2/9-MD1 at 3E13 vg/kg. This highlights the high efficiency of the MD1 vector when
621 delivered at this dose. Animals treated with rAAV2/9-MD1 or rAAV2/9-MD3 vectors at 1E13 vg/kg
622 show a delayed curve of force decrease. We can point out that, even if the final forelimb grip force at
623 trial 5 is similar for these two groups, the significant difference of force compared to the WT rats
624 appears later for the rats treated with the MD3 vector (trial 5) than for those treated with the MD1
625 vector (trial 4).

626 Since the majority of DMD patients die from heart failure(62), the cardiac dysfunction described in the
627 DMD^{mdx} rat model(42,63) is a critical feature to evaluate the therapeutic potential of MD. Rapid
628 concentric remodeling associated to an alteration of diastolic function, which progressed unfavorably
629 with age towards systolic dysfunction are characteristics of this model (Toumaniantz, Le Guiner et al.,
630 unpublished data). In this study, cardiac function was assessed on anesthetized animals using
631 electrocardiography (ECG), 2D echocardiography and pulsed Doppler measurements at 3 months
632 post-injection. ECG analysis revealed no significant electrical conduction abnormality whatever the
633 experimental group (data not shown). However, as shown in **Fig. 7A**, the left ventricular wall end-
634 diastolic diameter measured by 2D-echocardiography tended to thicken in untreated DMD^{mdx} rats
635 compared to WT rats and is associated with a decrease in left ventricular diameter, reflecting the
636 concentric hypertrophy of the heart in DMD^{mdx} rats. This structural remodeling was prevented with
637 injection of rAAV2/9-MD1 (whatever the dose), but not with rAAV2/9-MD3. The morphology of isolated
638 cardiomyocytes was also assessed by measuring their length/width *ratio*. This ratio significantly
639 decreased in untreated DMD^{mdx} rats compared to WT rats, indicating a thickening of the isolated
640 cardiomyocytes and correlating with the concentric hypertrophy pattern observed by 2D-
641 echocardiography. This *ratio* was normalized in rats treated with rAAV2/9-MD1 at both doses, but not
642 modified in DMD^{mdx} rats treated with rAAV2/9-MD3 vector. As shown in **Fig. 7B**, ejection fraction,

643 measured using pulsed Doppler, was significantly higher in untreated DMD^{mdx} rats than in WT rats,
644 indicating an adaptation of the systolic function explained by the hypertrophic process previously
645 described. This parameter was almost normalized in the 3 groups of treated animals. Finally, diastolic
646 dysfunction of the untreated DMD^{mdx} rat was also shown in this study *via* the significant increase of
647 deceleration time, the increase of IVRT and the decrease of E/A ratio, when compared to WT rats.
648 Again, a clear trend of normalization of deceleration time and IVRT was observed in all the treated
649 groups, whatever the injected dose and the transgene (**Fig. 7C**). This normalization was not observed
650 with the E/A ratio parameter.

651 These data showed no clear impact of the inclusion of the coil-coiled motif in MD1 on its efficacy to
652 improve muscle and cardiac function in DMD^{mdx} rats. Interestingly, MD3 expression in the heart do not
653 seem to improve structural remodeling (in contrast to MD1) but still seems to prevent systolic and
654 diastolic dysfunctions.

655

656 **DISCUSSION**

657 Report of ≈46% shortened dystrophin in a 61-year-old ambulatory patient with Becker muscular
658 dystrophy (BMD)(64) led to the design of MD transgenes that are compatible with additive gene
659 therapy using rAAV vectors. Following preclinical success of systemic rAAV-MD gene therapy(12–
660 20,20–23), 3 independent clinical trials have been recently started(18). However, due to the limitation
661 of rAAV packaging capacity(8), current MD candidates cannot include all major domains of the full-
662 length dystrophin. In this study, we evaluated if the inclusion of the dystrophin CT domain in MD1
663 could improve (i) its efficacy at recovering DAPC interactions, and (ii) its therapeutic potential, in both
664 skeletal and cardiac muscles of DMD^{mdx} rats.

665 Using dystrophin co-IP and LC-MS/MS analyses, we were first able to identify in the rat model most of
666 the DAPC components that were previously identified in a study done in mice(41). Only CRYAB,
667 isoform 3 of cypher and ahnak1 were not identified as specific DAPC partners in our study since they
668 were also detected in IgG_co-IP control group. However, we found that Cavin-1 and β2-SNT,
669 previously described as heart specific DAPC partners, can be also detected at low levels in skeletal
670 muscle DAPC, which is likely due to an increased sensitivity of our MS-based approach compared to
671 this previous study. Calmodulin-1, was not identified as part of the DAPC in the Johnson *et al.*
672 study(41), but was found associated with dystrophin in skeletal muscle of rabbits(55,56). We

673 confirmed here this association in skeletal muscle in the rat. Finally, new DAPC partners were
674 identified: β -DTN, Pikachurin and Perilipin-4 were specifically found in skeletal muscle DAPC;
675 Caveolin-1 and Cavin-2 were specifically found in heart DAPC; and Cavin-3 was found in DAPC of
676 both tissues.

677 We know from the literature that dystrophin CT domain is not critical for the expression of SNT and α -
678 DTN partners at the membrane of skeletal muscle fibers(37). In addition, always in skeletal muscle,
679 MD1 (Δ R4-23/ Δ CT) was shown to restore the expression of these proteins through the
680 dystroglycan/sarcoglycan complex(37,38,65). These observations agree with the fact that
681 sarcoglycans interact with α -DTN(30) which itself interacts with SNT(29,66), and that MD1 restores the
682 dystroglycan/sarcoglycan complex through the ZZ domain present in the cysteine-rich domain(67).
683 Previous studies also shown that helix 1 of the CT coiled-coil domain is required to restore normal
684 expression levels of SNT and α -DTN at the membrane of skeletal muscle fibers, suggesting a lower
685 recruitment of these partners by MD1 compared to the full-length dystrophin(37,38). We hypothesized
686 that addition of the dystrophin CT domain in MD1 should thus improve binding of these DAPC
687 partners(29,31). In our study, we confirmed that MD1 is able to recruit SNT and α -DTN in skeletal
688 muscle but also in heart. However, when compared to MD1, the results also showed that MD2, MD3
689 and MD4 increase the recruitment of proteins on the sarcoglycan and of the SNT complexes in heart
690 and skeletal muscles, and of α -DTN in heart. Surprisingly, this is also the case when compared to full-
691 length dystrophin. The abundances of these partners are equivalent in the complex associated to MD1
692 or to dystrophin, meaning that MD1 is sufficient to restore the physiological interactions of SNT and α -
693 DTN in skeletal and cardiac muscles, and that supra-physiological levels of these partners are
694 obtained when expressing MD2, MD3 or MD4. These results raise the question about the true path for
695 the recruitment of SNT and α -DTN by the full-length dystrophin and whether it is mediated *via* the CT
696 domain, *via* the sarcoglycan complex or both? Due to the different structures of MD, the affinity
697 between the sarcoglycan complex and DTN could be modified. It is also possible that a recruitment *via*
698 the sarcoglycan complex occurs only in MD associated protein complex, due to structure modification
699 of MD compared to the native dystrophin. In that case, the inclusion of the CT domain would add a
700 recruitment site for SNT and α -DTN, explaining the supra-physiological levels observed with MD2,
701 MD3 and MD4 variants. We also showed that the recruitment of cavin-1 in heart reached equivalent
702 levels than those observed with the full-length dystrophin only when the CT domain is present in MD,

703 suggesting that this domain is required for an optimal recruitment of cavin-1 in heart. In skeletal
704 muscle, it has been described that cavin-1 is rather a dystroglycan partner, independently to
705 dystrophin, and that only a low amount of β -dystroglycan/cavin-1 complex is directly associated with
706 dystrophin(68). Such a mechanism probably occurs also in MD1-expressing heart, in which we
707 observed partial recruitment of cavin-1 despite the absence of the dystrophin CT-domain. Knowing
708 that the absence of cavin-1, which is required for caveolae formation(69), was linked to
709 cardiomyopathic phenotypes(70,71), further investigations are required to assess the functional
710 consequence of a sub-physiological level of cavin-1 in MD1 associated protein complex in heart.

711 Beyond its efficacy to restore the DAPC, we further investigated the impact of the dystrophin CT
712 domain through the comparison of the therapeutic efficacy between MD1 and MD3 (containing the
713 coil-coiled motif of the dystrophin CT domain) in the DMD^{mdx} rat model. It is the first time that the
714 therapeutic impact of the dystrophin CT domain has been assessed on both the muscular and cardiac
715 pathology of a relevant DMD model and after the systemic administration of rAAV-MD. Crawford *et al.*
716 showed that this domain was not critical for dystrophin functionality in the skeletal muscle of
717 transgenic mice(37). But this kind of approach did not allow evaluation of the therapeutic efficacy on
718 an established pathology, representative to that which would be seen in DMD patients. In addition,
719 until now, therapeutic efficacy of MD on the DMD related cardiomyopathy was only poorly described,
720 as the most commonly used DMD animal models, shows minimal and late cardiac
721 features(16,22,39,72). The recently developed DMD^{mdx} rat model gives now the opportunity to
722 evaluate new MD designs on both skeletal muscles and cardiac features of the DMD pathology(42).

723 Since MD1 construct already shows a significant therapeutic effect on animal models(13,16,38), we
724 chose to compare MD1 and MD3 at a known sub-therapeutic dose for MD1 (1E13 vg/dg), to evaluate
725 and potentially highlight a difference in efficacy between these two products. Whereas the 1E13 vg/kg
726 dose was found to be sub-therapeutic in skeletal muscle, this was not fully the case in the heart,
727 because of the preferential tropism of AAV9 serotype for this organ in rodents(58). Similar efficacy of
728 the MD1 product was observed at the 1E13 and 3E13 vg/kg vector doses for some of the criteria
729 assessed, such as heart fibrosis, heart remodeling and heart function. Therefore, it would have been
730 interesting to compare our two vectors at a lower dose, in order to detect in a more refined way the
731 potential benefits of the CT domain specifically in the heart. However, the relevance of the DMD^{mdx} rat
732 model still allowed us to evaluate a wide range of parameters as the histological aspect, the calcium

733 homeostasis in skeletal muscle and heart, the *ex vivo* and *in vivo* muscle strength and the cardiac
734 function. Across all the assessed *criteria*, the inclusion of the coil-coiled motif of the dystrophin CT
735 domain within MD1 did not show any clear therapeutic benefit. At the same dose, both MD constructs
736 showed similar effects on fibrosis, histopathological scores and muscle force. One pointed difference
737 is that cardiac structural parameters appeared to be less corrected with MD3 treatment than with MD1.
738 Still, the DMD^{mdx} rats treated with rAAV2/9-MD3 showed improved systolic and diastolic functions,
739 suggesting that MD1 and MD3 could act on the DMD cardiac pathology *via* different mechanisms. The
740 potential involvement of cavin-1 in this mechanism could be further investigated. Finally, as previously
741 described(59,73,74), we observed some isolated ring fibers in some skeletal muscles expressing
742 MD1, but not in the ones expressing MD3. This is quite surprising considering that formation of ring-
743 shaped myofibers have been associated to the presence of the polyproline site in hinge 2 in this
744 MD(59,73,74). Formation of these ring fibers could also be prevented in MD3 expressing fibers by a
745 change of conformation compared to MD1. Given the fact that these ring fibers were not observed in
746 all MD1-expressing muscles, we cannot exclude that we could have detected some if we had injected
747 more animals with rAAV-MD3. In any case, these ring fibers, in a very low number compared to other
748 myopathies in which they are also described (e.g. myotonic myopathy and some mitochondrial
749 myopathies(75)), do not seem to have any deleterious impact on muscle function.

750 Taken together, our results indicate that the inclusion of the CT domain does not provide a therapeutic
751 benefit to MD1, at least on skeletal and cardiac muscles functions and after 3 months of treatment in
752 DMD^{mdx} rats. On the other hand, we cannot exclude that the dystrophin CT domain could be
753 necessary to more effectively control disease progression at long-term, and/or useful in other organ
754 like brain(76) and smooth muscles(77).

755 Importantly, this work also strengthened the evidence of the therapeutic efficacy of MD1. Our results
756 indicate indeed that MD1 restores the DAPC at a physiological level, with the exception of Cavin-1 in
757 the heart. We also confirmed in a relevant model of DMD (DMD^{mdx} rats) that systemic administration of
758 a rAAV-MD1 vector at 3E13 vg/kg significantly improved histological pattern, muscle force and DMD-
759 related cardiac pathology. We also showed for the first time that MD1 significantly improves DMD-
760 related calcium homeostasis alterations in skeletal muscles and heart. Very recently, a phase 1/2a
761 nonrandomized controlled trial testing the safety and biological efficacy of a single systemic infusion of
762 rAAV-MD1 in 4 DMD patients revealed encouraging results with marked reductions in creatine kinase

763 levels and improvement of muscle histological and functional patterns such as NSAA (North Star
764 Ambulatory Assessment) score(25). These results will obviously have to be confirmed in a randomized
765 clinical trial including more DMD patients, but our study shows new evidence that despite the absence
766 of the dystrophin CT domain, rAAV-MD1 offers high therapeutic potential for a systemic gene therapy
767 for DMD, both at the muscle and cardiac levels.

768 **DATA AVAILABILITY**

769 The data that support the findings of this study are available from the corresponding authors on
770 reasonable request.

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982

983

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1003

1004 **AUTHOR CONTRIBUTIONS**

1005 Study design and reviewing of the data: A.B., V.F., F.M., C.L.G.;

1006 Experimental investigation: A.B., L.Z., A.L., B.F., G.T., T.L., T.G., M.L., C.L., A.H., A.C.;

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1008 Writing – original draft: A.B., L.Z., A.L., B.F. and C.L.G.;

1009 Writing – Review & Editing: all authors;

1010 Supervision: C.L.G. and O.A.;

1011 Funding Acquisition: C.L.G., P.M., G.D. and O.A.

1012

1013 **COMPETING INTERESTS**

1014 P.M., G.D. and C.L.G. are co-authors of a patent for systemic treatment of dystrophic pathologies
1015 (EP3044319A1, dated 27 June 2014). G.D. is an inventor on a PCT for production of large-sized
1016 micro-dystrophins in an AAV-based vector configuration (PCT/EP2016/060350, dated 17 May 2015).

1017 The remaining authors declare no competing financial interests.

1018

1019 **FIGURE LEGENDS**

1020 **Figure 1: Structure of the Dystrophin Associated Protein Complex and of the different micro-**
1021 **dystrophin constructs evaluated in this study.**

1022 (A): Schematic representation of the Dystrophin Associated Protein Complex (DAPC). Dystrophin plays a
1023 central role in the maintenance of muscle membrane integrity and the recruitment of regulatory and signaling
1024 proteins. (B): Schematic representation of the dystrophin protein structure (with focus on the CT domain) and
1025 of the different micro-dystrophin (MD) constructs evaluated in this study. MD1 is deleted of CT domain
1026 (exons 71–78) except that it contains the last three amino acids of exon 79 of dystrophin. MD2 contains helix
1027 1 (H1) of the coiled-coil motif of CT domain, with the interaction domains for α 1- and β 1- SNT and α -DTN,
1028 MD3 contains the entire coiled-coil motif (H1 + H2) of CT domain, and MD4 contains the entire dystrophin
1029 CT domain. (C): Schematic representation of the structure and size of the expression cassettes generated to
1030 produce rAAV2/9-MD vectors. Each MD cDNA sequence was subcloned into a pAAV plasmid that contained
1031 the 323bp muscle-synthetic Spc5.12 promoter, a synthetic polyadenylation signal of 49 bp, and two flanking
1032 inverted terminal repeat (ITR) sequences of 130 pb from AAV serotype 2. The size of the resulting MD1,
1033 MD2, MD3 and MD4 expression cassettes were 4,538 bp, 4,721 bp, 4,833 bp and 5,114 bp, respectively.

1034

1035 **Figure 2: Comparison of DAPC proteins abundance relative to MD/dystrophin between co-IP from rat**
1036 **skeletal and cardiac muscles obtained from the different experimental groups.**

1037 Relative abundance to MD/dystrophin were calculated by dividing the normalized emPAI of each DAPC
1038 partner by the normalized emPAI of the MD/dystrophin expressed in the same sample. Relative abundances
1039 of (A) α -Sarcoglycan, α -DTN, α 1-, β 1- and β 2-SNT in co-IP from skeletal muscle, and of (B) α -Sarcoglycan,
1040 α 1-SNT and Cavin-1 in co-IP from heart were compared between MD1_DYS_co-IP (N=4), MD2_DYS_co-IP
1041 (N=4), MD3_DYS_co-IP (N=4), MD4_DYS_co-IP (N=4 for skeletal muscles and N=3 for heart) and
1042 WT_DYS_co-IP (N=4) experimental groups. Data are presented as mean \pm SEM. Statistics were done using
1043 a 2-way ANOVA followed by a multiple comparison post-hoc “original FDR method of Benjamini and
1044 Hochberg”. Q-values (corrected P-value) ≤ 0.05 were considered statistically significant. Values sharing the
1045 same letter are not statistically different from each other.

1046

1047 **Figure 3: Vector biodistribution and expression in tissues of DMD^{mdx} rats injected with rAAV2/9-MD1**
1048 **and rAAV2/9-MD3 vectors.**

1049 (A): Vector genome copy number per diploid genome (vg/dg) in tissues of the different experimental groups
1050 at euthanasia, determined by qPCR analysis. For each group, the analysis was performed on whole blood

1051 (N=6 animals per group), liver (N=4 animals for the DMD^{mdx} + MD1 1E13 group; N=6 animals for the other
1052 groups), diaphragm (N=6 animals per group), heart (N=5 animals for the DMD^{mdx} + MD1 1E13 group; N=6
1053 animals for the other groups) and pectoral (N=6 animals per group), *biceps femoris* (N=6 animals per group)
1054 and EDL (N=5 animals per group) muscles. The limit of quantification (LOQ) was 0.004 vg/dg. Data are
1055 presented as mean ± SEM. *P < 0.05 (ANOVA test, followed by post-hoc Tukey multiple comparison test).
1056 (B): Percentage of MD/Dystrophin positive fibers determined at euthanasia by immunolabelling in heart (N=5
1057 animals per group for the DMD^{mdx} + MD1 1E13 group; N=7 animals for the DMD^{mdx} + MD1 3E13 group; N=6
1058 animals for the other groups), *pectoralis* muscle (N=7 animals for the DMD^{mdx} + MD1 3E13 group; N=6
1059 animals for the other groups), and *biceps femoris* muscle (N=7 animals for the DMD^{mdx} + MD1 3E13 group;
1060 N=6 animals for the other groups). Data are presented as mean ± SEM. * p<0.05 (ANOVA test, followed by
1061 post-hoc Tukey multiple comparison test). (C): Immunolabelling of transverse sections of *biceps femoris*
1062 muscle with DYSB antibody for MD1/MD3/dystrophin staining (green) and with WGA conjugate for
1063 connective tissue (red). Representative images corresponding to samples collected at euthanasia for each
1064 experimental group. Scale bar, 100 µm. (D): Western-blot analysis of total proteins (50 µg) extracted from
1065 *biceps femoris* muscle and heart samples collected at euthanasia for each rAAV injected group (N=4
1066 animals per group). The blots were stained with MANEX-1011C to reveal the presence of the 138 kDa MD1
1067 and the 160 kDa MD3 protein. α-tubulin antibody as a loading control.

1068

1069 **Figure 4: Histopathological analysis in muscles of DMD^{mdx} rats injected with rAAV2/9-MD1 and**
1070 **rAAV2/9-MD3 vectors.**

1071 (A): Connective tissue area (in %) was assessed using WGA for skeletal muscles and a specific picrosirius
1072 staining for the heart. Analysis were performed at sacrifice on heart (N=5 animals for the DMD^{mdx} + MD1
1073 1E13 group; N=7 animals for the DMD^{mdx} + MD1 3E13 group; N=6 animals for the other groups), *pectoralis*
1074 muscle (N=7 for the DMD^{mdx} + MD1 3E13 group; N=6 animals for the other groups) and *biceps femoris*
1075 muscle (N=7 animals for the DMD^{mdx} + MD1 3E13 group; N= 6 animals for the other groups). Data are
1076 presented as mean ± SEM. * p<0.05 (Parametric ANOVA test, followed by post-hoc Tukey multiple
1077 comparison test). (B): Muscle histopathological score in skeletal muscles and heart samples. Muscles
1078 lesions were scored during histopathological observation of the each muscle by a DVM, European-board
1079 certified anatomopathologist and using the following system: 0 = absence of lesions, 1 = presence of some
1080 regenerative activity as evidenced by centronucleated fibers, 2 = presence of degenerated/necrotic fibers, 3
1081 = tissue remodeling and fiber replacement by fibrosis. In the heart, scoring was based on the presence of
1082 inflammatory and fibrotic areas (1 = 1 area, 2 = multiple isolated areas, 3 = extensive/coalescing multiple

1083 areas). Analysis were performed at sacrifice on heart (N=5 animals for the DMD^{mdx} + MD1 1E13 group; N=7
1084 animals for the DMD^{mdx} + MD1 3E13 group; N=6 animals for the other groups), *pectoralis* muscle (N=7
1085 animals for the DMD^{mdx} + MD1 3E13 group; N=6 animals for the other groups) and *biceps femoris* muscle
1086 (N=7 animals for the DMD^{mdx} + MD1 3E13 group; N=6 animals for the other groups). Data are presented as
1087 mean ± SEM. * $p < 0.05$ (Nonparametric Kruskal-Wallis test, followed by a post-hoc Dunn's multiple
1088 comparison test).

1089

1090 **Figure 5: Analysis of calcium homeostasis in skeletal muscles and isolated cardiomyocytes of**
1091 **DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3 vectors.**

1092 Resting intracellular-free calcium concentration ($[Ca^{2+}]_c$) of EDL muscle fibers (A) and isolated
1093 cardiomyocytes (B) obtained from animals from the different experimental groups. $[Ca^{2+}]_c$ was calculated in
1094 fura-2-loaded isolated muscle fibers using calibration parameters of Grynkiewicz's equation determined *in*
1095 *situ*. A calibration was performed for each muscle used. Data are presented as mean ± SEM from n muscle
1096 fibers and N animals (n/N). * $p < 0.05$ (ANOVA test, followed by post-hoc Bonferroni multiple comparison test).
1097 (C): Sarcolemma permeability to calcium ions (SPCa) in EDL muscle fibers obtained from animals of the
1098 different experimental groups. The sarcolemma permeability to Ca^{2+} was evaluated by measuring the
1099 quench rate of fura-2 fluorescence induced by Mn^{2+} influx. Data are presented as mean ± SEM from n muscle
1100 fibers and N animals. * $p < 0.05$ (ANOVA test, followed by post-hoc Bonferroni multiple comparison test).

1101

1102 **Figure 6: Analysis of muscle force in DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3**
1103 **vectors.**

1104 Maximum tetanic amplitude force of isolated *soleus* (A) and EDL (B) muscles of the different experimental
1105 groups at sacrifice (N=3 animals for the *soleus* of the DMD^{mdx} + MD1 1E13 group; N=4 animals for the other
1106 groups). Data are presented as mean ± SEM in grams (g) normalized to the muscle weight (g/g). * $p < 0.05$
1107 (Nonparametric Kruskal-Wallis test, followed by a post-hoc Dunn's multiple comparison test). (C): Maximum
1108 forelimb grip force of the different experimental groups at 3 months post-injection (N=11 animals for the
1109 DMD^{mdx} + MD1 3E13 group; N=9 animals for the DMD^{mdx} + MD1 1E13 group; N=10 animals for the other
1110 groups). Data are presented as mean ± SEM in grams (g) normalized to the body weight (g/g BW). * $p < 0.05$
1111 (Nonparametric Kruskal-Wallis test, followed by a post-hoc Dunn's multiple comparison test). (D): Cross-trial
1112 evolution of forelimb grip force of the different experimental groups at 3 months post-injection (N=11 animals
1113 for the DMD^{mdx} + MD1 3E13 group; N=9 animals for the DMD^{mdx} + MD1 1E13 group; N=10 animals for the
1114 other groups). Data are presented as mean ± SEM in grams (g) normalized to the body weight (g/g). *

1115 $p < 0.05$ vs WT + Vehicle group; α $p < 0.05$ vs DMD^{mdx} + Vehicle group; \S $p < 0.05$ vs trial 1 of the same group
1116 (Friedman test followed by Dunn's post hoc test).

1117

1118 **Figure 7: Analysis of cardiac function in DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3**
1119 **vectors.**

1120 (A): Structural remodeling of the heart: free wall diastolic thickness and left ventricle diameter were assessed
1121 by Two-dimensional (2-D) echocardiography in the different experimental groups at 3 months post-injection
1122 (N=11 animals for the DMD^{mdx} + MD1 3E13 group; N=9 animals for the DMD^{mdx} + MD1 1E13 group; N=10
1123 animals for the other group). Cardiomyocytes morphology was assessed by the measuring length/width ratio
1124 of isolated cardiomyocytes obtained at sacrifice. Data are presented as mean \pm SEM from n cardiomyocytes
1125 and N animals. * $p < 0.05$ (ANOVA test, followed by post-hoc Bonferroni multiple comparison test). Systolic
1126 (B) and diastolic (C) Functions of the heart: left ventricular ejection fraction, the early diastolic (E), the late
1127 diastolic (A), the E/A ratio, deceleration time and isovolumetric relaxation time was assessed by pulsed
1128 Doppler in the different experimental groups at 3 months post-injection (N=11 animals for the DMD^{mdx} + MD1
1129 3E13 group; N=9 animals for the DMD^{mdx} + MD1 1E13 group; N=10 animals for the other group). Left
1130 ventricular end-diastolic and end-systolic volumes (LVEDV and LVESV, respectively) were calculated from
1131 bi-dimensional long-axis parasternal views by means of the single-plane area-length method. These indexes
1132 were calculated in accordance with the standard, widely accepted formulas to study left ventricular
1133 dysfunction (78). LV ejection fraction (EF) was calculated as [(LV end-diastolic volume - LV end-systolic
1134 volume)/LV end-diastolic volume] \times 100. Systolic function was further assessed by calculation of LVEF using
1135 averaged measurements from 3-5 consecutive cardiac cycles in accordance with the guidelines of the
1136 American Society of Echocardiography. Data are presented as mean \pm SEM. * $p < 0.05$ (Nonparametric
1137 Kruskal-Wallis test, followed by a post-hoc Dunn's multiple comparison test).

1138 **TABLES**

1139 **Table1: Comparison of the relative abundance of each DAPC partners in skeletal and heart samples**
1140 **expressing MD1, MD2, MD3, MD4 or dystrophin.**

1141

1142 **FIGURES**

1143 **Figure 1: Structure of the Dystrophin Associated Protein Complex and of the different micro-**
1144 **dystrophin constructs evaluated in this study.**

1145

1146 **Figure 2: Comparison of DAPC proteins abundance relative to MD/dystrophin between co-IP from rat**
1147 **skeletal and cardiac muscles obtained from the different experimental groups.**

1148

1149 **Figure 3: Vector biodistribution and expression in tissues of DMD^{mdx} rats injected with rAAV2/9-MD1**
1150 **and rAAV2/9-MD3 vectors.**

1151

1152 **Figure 4: Histopathological analysis in muscles of DMD^{mdx} rats injected with rAAV2/9-MD1 and**
1153 **rAAV2/9-MD3 vectors.**

1154

1155 **Figure 5: Analysis of calcium homeostasis in skeletal muscles and isolated cardiomyocytes of**
1156 **DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3 vectors.**

1157

1158 **Figure 6: Analysis of muscle force in DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3**
1159 **vectors.**

1160

1161 **Figure 7: Analysis of cardiac function in DMD^{mdx} rats injected with rAAV2/9-MD1 and rAAV2/9-MD3**
1162 **vectors.**

1163 **SUPPLEMENTARY INFORMATION**

1164 - **Supplementary material and method**

1165 ***Anesthesia protocol***

1166 ***Primers and probes designed for duplex qPCR analysis***

1167 ***MD mRNA-level analysis***

1168

1169 - **Supplementary data**

1170 **Table S1:** Comparison of emPAI means obtained for proteins identified as part of the DAPC in skeletal
1171 muscle or heart samples in WT rats.

1172 **Table S2:** Characteristics of the rAAV2/9-MD1 and MD3 vector batches used to assess their therapeutic
1173 efficacy in DMD^{mdx} rats.

1174 **Table S3:** Characteristics of the different groups of rats included in this study to assess the therapeutic
1175 efficacy rAAV2/9-MD1 versus -MD3.

1176 **Table S4:** Individual values of non-normalized emPAI obtained for each analyzed sample.

1177 **Table S5:** Individual values of percentages of coverage obtained for each analyzed sample.

1178 **Table S6:** Individual values of numbers of unique peptides obtained for each analyzed sample.

1179 **Table S7:** Individual values of normalized emPAI and relative to MD/dystrophin normalized emPAI obtained
1180 for each analyzed sample.

1181 **Figure S1:** Representative MD/dystrophin expression in skeletal muscles and heart of WT and rAAV2/9-
1182 injected DMD^{mdx} rats included in the study to assess the impact of the dystrophin CT domain on the DAPC
1183 composition.

1184 **Figure S2:** Experimental procedure used for the identification, by LC-MS/MS analysis, of DAPC members in
1185 co-IP samples obtained from skeletal muscles and heart of WT rat.

1186 **Figure S3:** MD/dystrophin abundance in co-IP samples from skeletal muscle and heart samples obtained
1187 from the different experimental groups.

1188 **Figure S4:** Relative quantification of MD mRNA levels in tissues of the different experimental groups.

1189 **Figure S5:** Representative muscle lesions that were semi-quantified for the determination of the muscular
1190 histopathological score after hemato-eosin-saffron staining.

1191 **Figure S6:** Example of a ring fiber (black arrowhead) observed on a transverse section of pectoralis muscle
1192 of a DMD^{mdx} rat expressing MD1 (hemato-eosin-saffron staining).

Figure 1

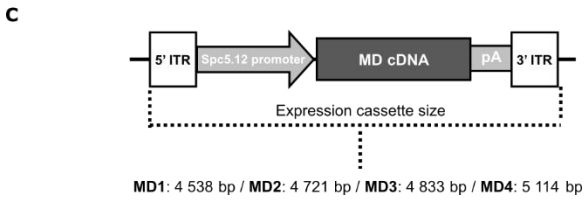
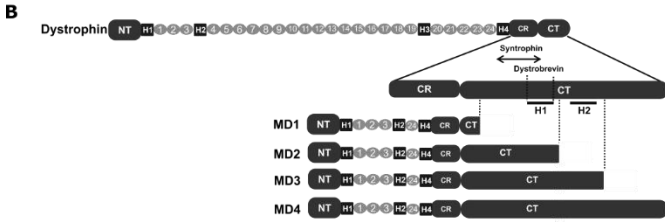
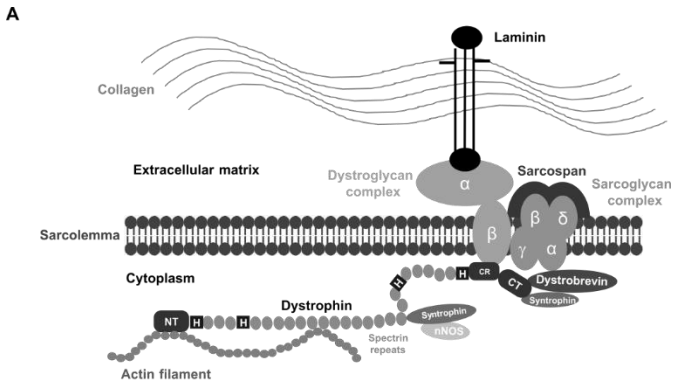
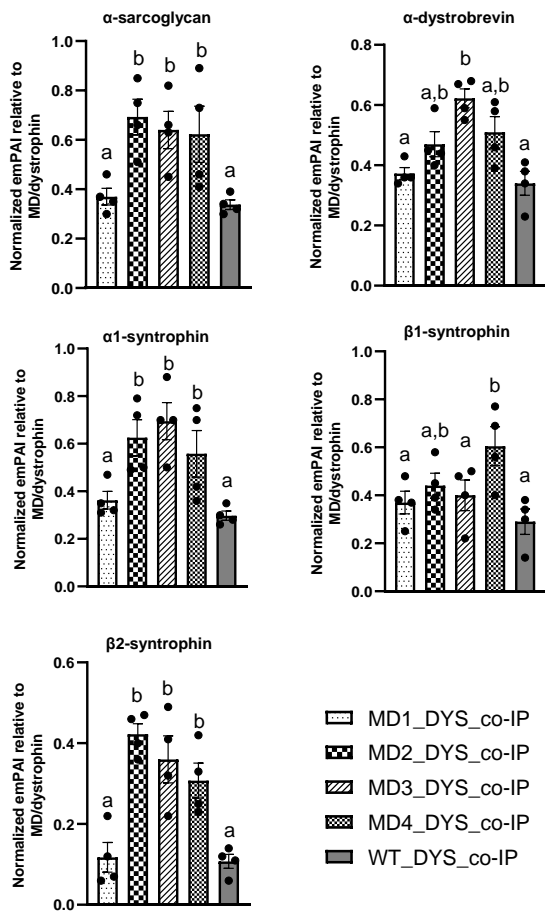


Figure 2

A

Skeletal muscle



B

Heart

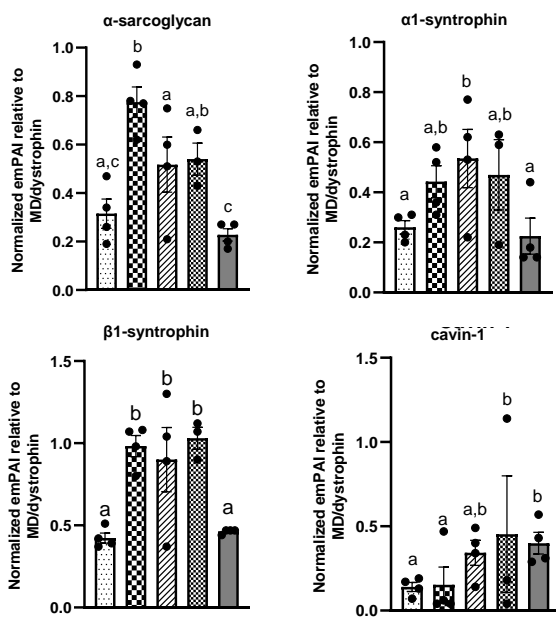


Figure 3

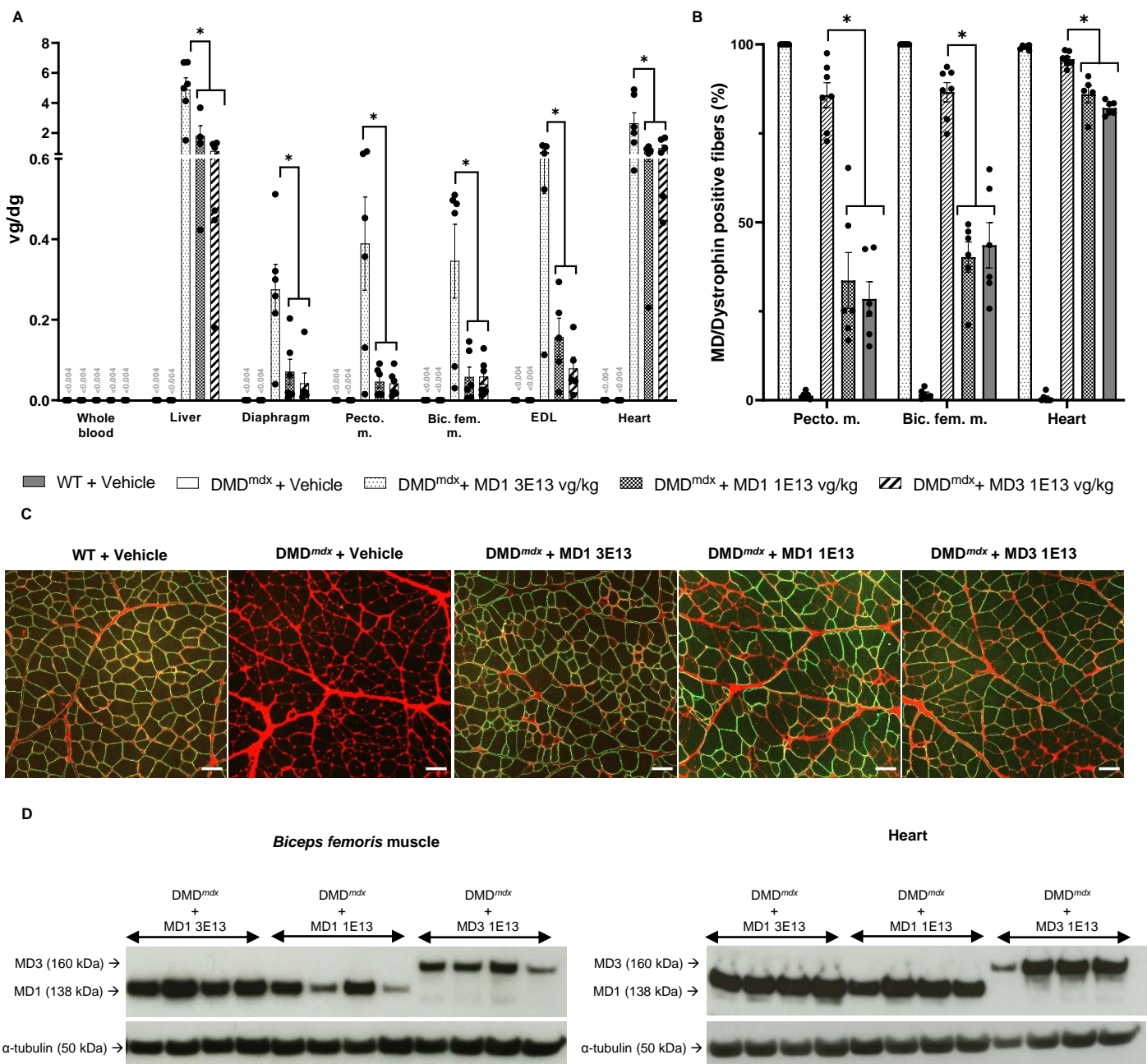


Figure 4

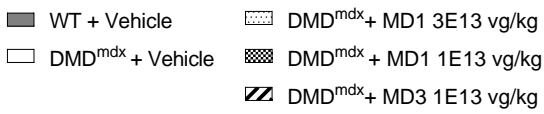
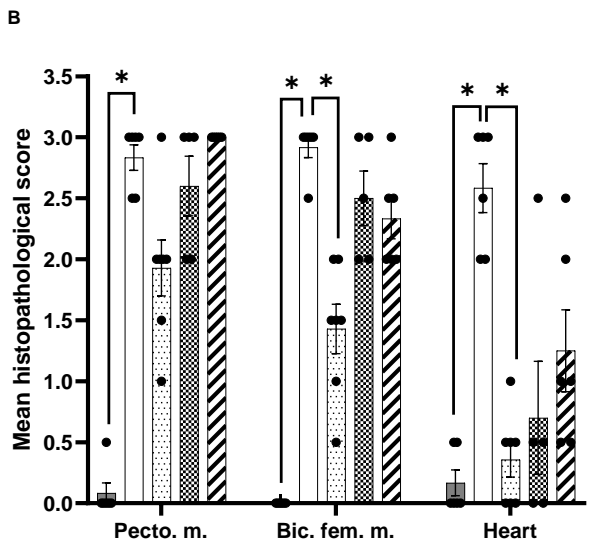
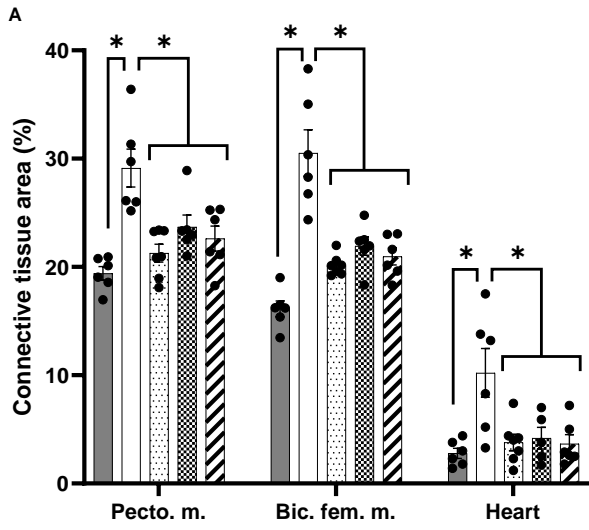


Figure 5

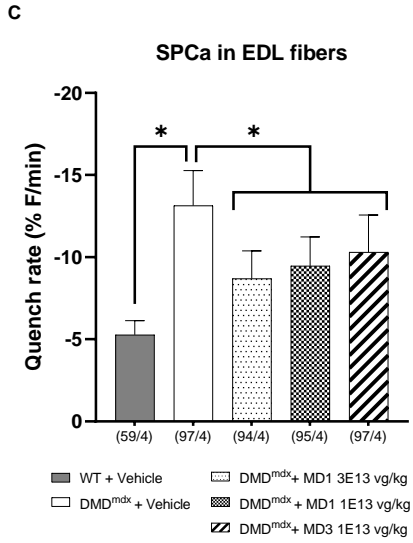
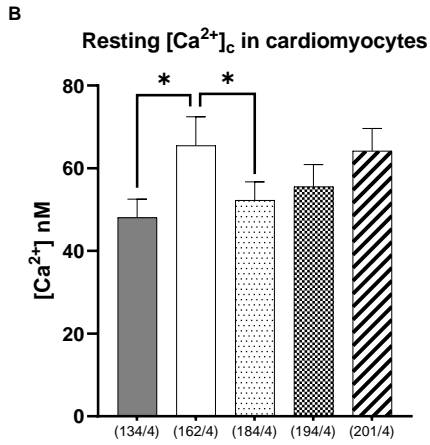
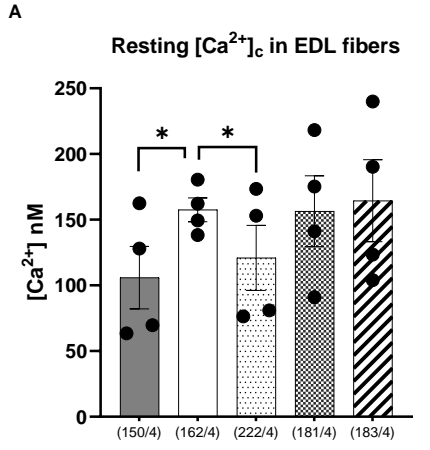


Figure 6

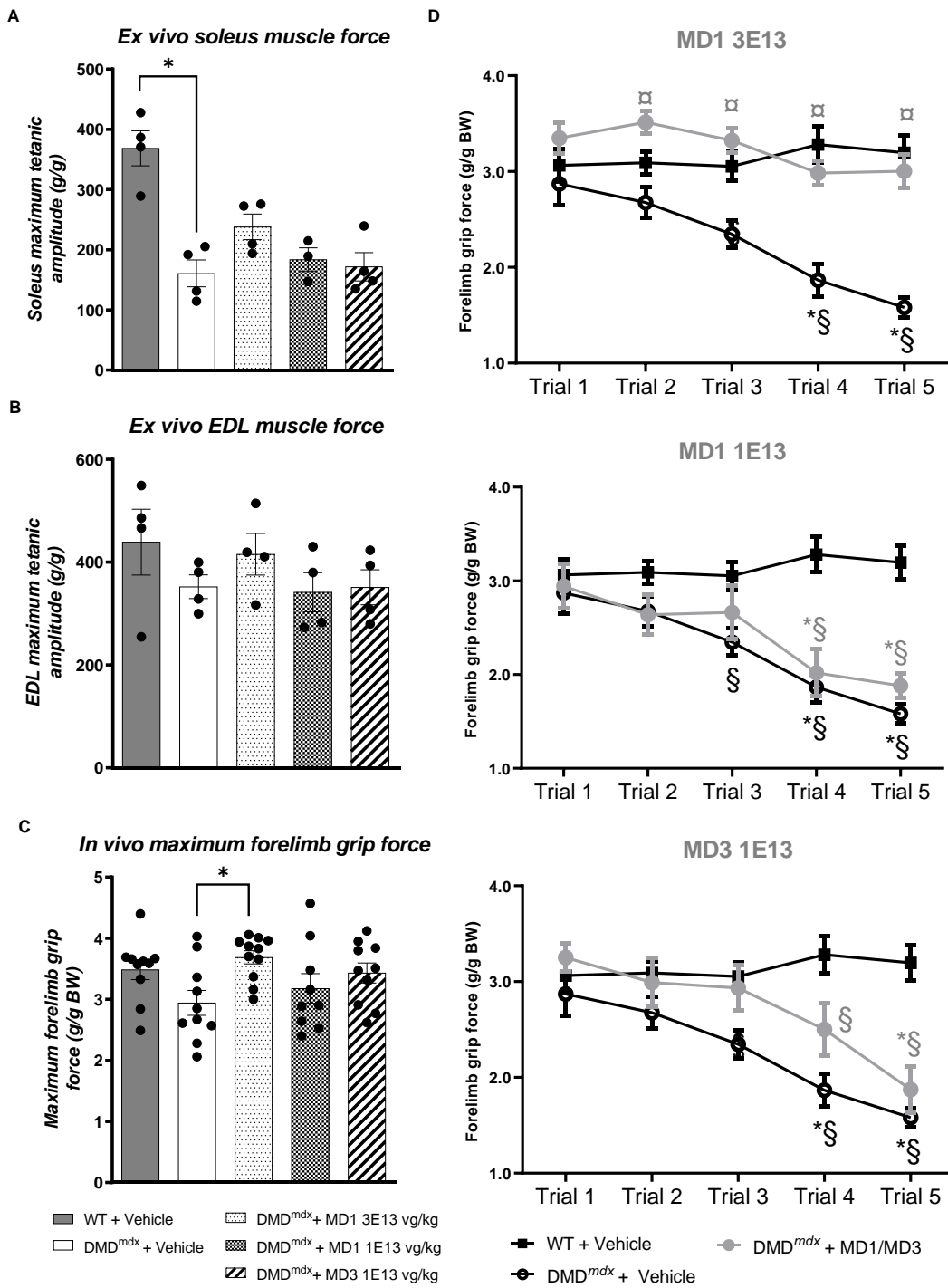


Figure 7

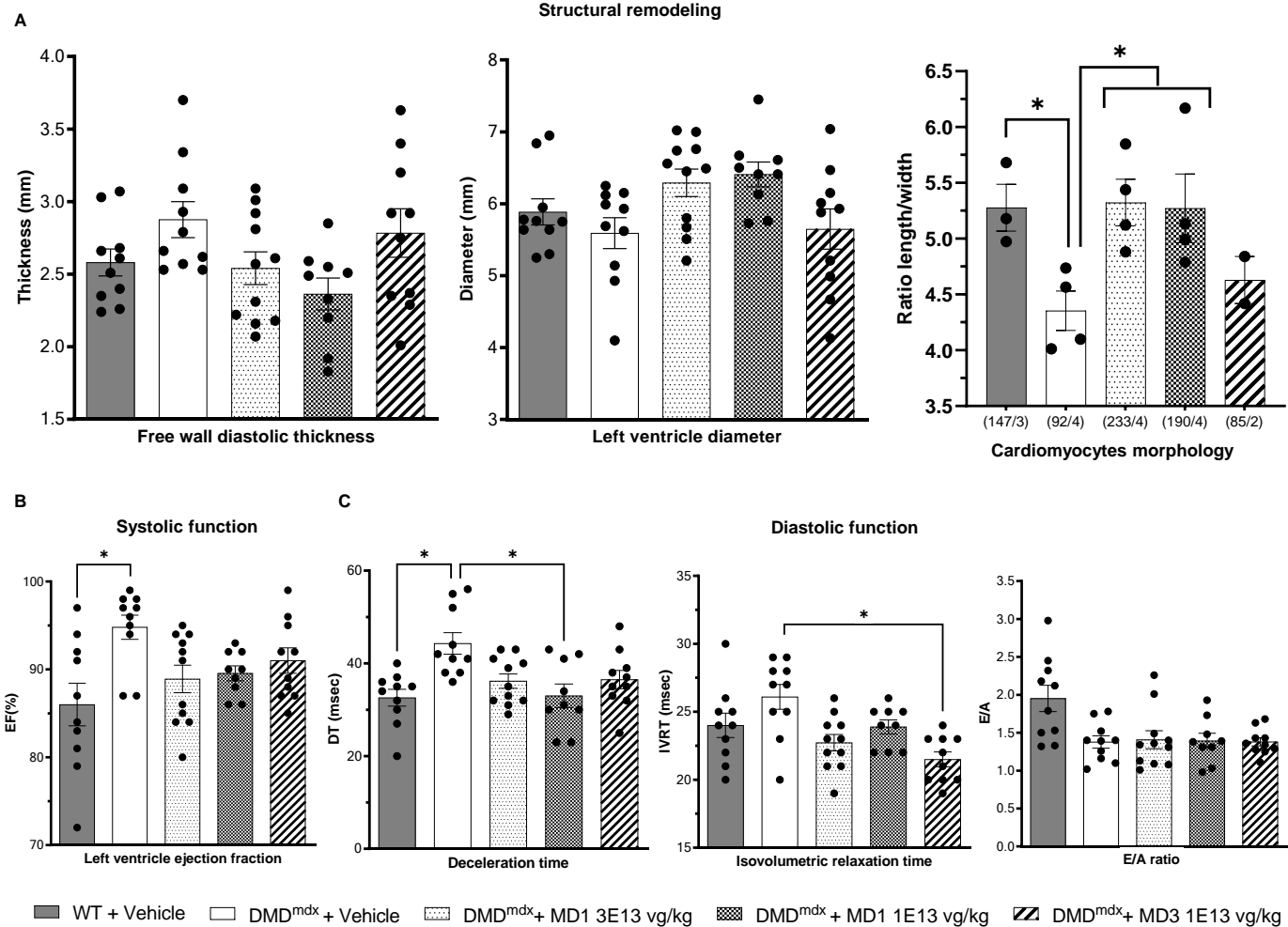


Table 1: Comparison of the relative abundance of each DAPC partners in skeletal and heart samples expressing MD1, MD2, MD3, MD4 or dystrophin.
 Data are presented as mean \pm SEM. Statistics were done using a 2-way ANOVA followed by a multiple comparison post-hoc "original FDR method of Benjamini and Hochbe
 Q-values (corrected P-value) ≤ 0.05 were considered statistically significant. Values sharing the same letter are not statistically different from each other.
 Blank cell = not include in statistics. ND = not detected

Uniprot ID	Protein Name	Skeletal muscles normalized emPAI relative to MD/dystrophin (means)					Heart muscles normalized emPAI relative to MD/dystrophin (means)				
		MD1_DYS co-IP	MD2_DYS co-IP	MD3_DYS co-IP	MD4_DYS co-IP	WT_DYS co-IP	MD1_DYS co-IP	MD2_DYS co-IP	MD3_DYS co-IP	MD4_DYS co-IP	WT_DYS co-IP
D3ZDQ9	α -Sarcoglycan	0.37 ^a	0.69 ^b	0.64 ^b	0.62 ^b	0.34 ^a	0.31 ^{a,c}	0.77 ^b	0.52 ^a	0.54 ^{a,b}	0.23 ^c
D4A5E5	β -Sarcoglycan	0.68 ^a	1.05 ^b	0.79 ^a	0.95 ^b	0.41 ^c	0.65 ^a	0.89 ^b	0.62 ^a	0.52 ^{a,c}	0.37 ^c
F1LYS7	δ -Sarcoglycan	0.34 ^{a,c}	0.55 ^b	0.56 ^b	0.41 ^{a,b}	0.22 ^c	0.21 ^a	0.46 ^a	0.30 ^a	0.24 ^a	0.20 ^a
Q5XID6	γ -Sarcoglycan	0.35 ^a	0.35 ^a	0.35 ^a	0.32 ^a	0.16 ^b	0.33 ^a	0.30 ^a	0.60 ^b	0.37 ^{a,b}	0.25 ^a
B5DFL0	$\alpha 1$ -Syntrophin	0.36 ^a	0.62 ^b	0.69 ^b	0.56 ^b	0.30 ^a	0.26 ^a	0.44 ^{a,b}	0.53 ^b	0.47 ^{a,b}	0.23 ^a
D3ZWC6	$\beta 1$ -Syntrophin	0.37 ^a	0.44 ^{a,b}	0.40 ^a	0.60 ^b	0.29 ^a	0.42 ^a	0.98 ^b	0.90 ^b	1.03 ^b	0.46 ^a
F1M8K0	$\beta 2$ -Syntrophin	0.12 ^a	0.42 ^b	0.36 ^b	0.31 ^b	0.11 ^a	0.12 ^a	0.25 ^a	0.25 ^a	0.24 ^a	0.09 ^a
D4A772	Dystroglycan	0.14 ^a	0.20 ^a	0.21 ^a	0.19 ^a	0.08 ^a	0.10 ^a	0.16 ^a	0.15 ^a	0.10 ^a	0.07 ^a
P84060	α -Dystrobrevin	0.37 ^a	0.47 ^{a,b}	0.62 ^b	0.51 ^{a,b}	0.34 ^a	0.37 ^a	0.34 ^a	0.54 ^a	0.50 ^a	0.32 ^a
D4ABT9	β -Dystrobrevin	0.08 ^a	0.09 ^a	0.22 ^a	0.04 ^a	0.06 ^a					
P29476	Sarcospan	0.17 ^a	0.21 ^a	0.19 ^a	0.19 ^a	0.07 ^a	0.11 ^a	0.20 ^a	0.11 ^a	0.12 ^a	0.05 ^a
P0DP29	nNOS	ND	ND	ND	ND	0.05					
P0DP29	Cavin-1	ND	0.01	0.02	0.01	0.03	0.14 ^a	0.15 ^a	0.34 ^{a,b}	0.45 ^b	0.40 ^b
P41350	Calmodulin-1	0.04	0.07	0.18	ND	0.05					
P41350	Caveolin-1						ND	ND	ND	ND	0.04
P85125	Cavin-2						0.04 ^a	0.06 ^a	0.16 ^a	0.11 ^a	0.14 ^a
Q66H98	Cavin-3	ND	ND	ND	ND	0.04	0.03	ND	0.10	0.06	0.19
Q9Z1H9	Pikachurin	0.02 ^a	0.03 ^a	0.03 ^a	0.04 ^a	0.03 ^a					
D3ZMX6	Perilipin-4	ND	ND	ND	ND	0.02					

rg".