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1 **TRPC3, but not TRPC1, as a good therapeutic target for standalone or complementary**
2 **treatment of DMD**

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19

20 **Abstract:**

21 **Background:** Duchenne muscular dystrophy (DMD) is an X-linked inherited disease caused
22 by mutations in the gene encoding dystrophin that leads to a severe and ultimately life
23 limiting muscle-wasting condition. RAAV vector-based gene therapy is a promising
24 approach but is limited by the size of the full-length dystrophin cDNA that greatly
25 exceeds the packaging capacity of a single rAAV. It is thus urgent to find therapeutic
26 strategies alternative or complementary that could treat DMD patients. It is generally
27 assumed that an intracellular calcium overload due to a sarcolemma permeability to
28 calcium (SPCa) increase is an early and critical step of DMD pathogenesis.
29 Nevertheless, the identity of the calcium channels involved in this process is still
30 unclear. In the present study, we aimed to determine whether TRPC1 and/or TRPC3
31 calcium channels may be involved in skeletal muscle SPCa alterations and could
32 represent therapeutic targets to treat DMD.

33 **Methods:** All experiments were conducted in the *DMD^{mdx}* rat, an animal model that closely
34 reproduces the human DMD disease. We assessed $[Ca^{2+}]_c$ and SPCa in EDL (*Extensor*
35 *Digitorum Longus*) muscle fibers from age-matched WT and *DMD^{mdx}* rats of 1.5 to 7
36 months old. TRPC1 and TRPC3 expressions were measured in the EDL muscles at both
37 the mRNA and protein levels, by RT-qPCR, western-blot and immunocytofluorescence
38 analysis.

39 **Results:** We demonstrated early increases in $[Ca^{2+}]_c$, SPCa and TRPC3 protein expression
40 in the *DMD^{mdx}* rat EDL muscles. TRPC1 protein level was also higher in *DMD^{mdx}* rats as
41 compared to WT ones but only 7 months after birth, at an age when the disease is
42 already well established. We thus further focused our attention on TRPC3. Application
43 of Pyr10, a specific inhibitor of TRPC3 abolished the differences between SPCa values
44 measured in WT and *DMD^{mdx}* muscle fibers. Finally, we showed that a rAAV-MD based
45 treatment induced a high MD expression level that accompanied with significant but
46 only partial prevention of calcium homeostasis alterations, skeletal muscle force and
47 TRPC3 protein overexpression.

48 **Conclusions:** All together our results show that correcting TRPC3 channel expression
49 and/or activity appear to be a promising approach as a single or a as a rAAV-based
50 complementary therapy to treat DMD.

51

52 **Keywords:**

53 DMD; Calcium; TRPC1; TRPC3; *DMD^{mdx}* rat; Gene Therapy; skeletal muscle

54

55 **Introduction**

56 Duchenne muscular dystrophy (DMD) is an X-linked inherited disease affecting ~1:5,000 male
57 births and leading to a severe, highly debilitating and ultimately life limiting muscle-wasting
58 condition(1). DMD is caused by mutations in the gene encoding dystrophin, a critical protein
59 for the stability and function of skeletal myofibers and cardiomyocytes(2, 3). Dystrophin
60 establishes a mechanical link between the actin cytoskeleton and the extracellular matrix in
61 muscle fibers through the dystrophin-associated protein complex. DMD-affected boys
62 develop muscle weakness during the first years of life. During teenagehood, they generally
63 become wheelchair-bound and exhibit life-threatening complications caused by respiratory
64 muscle wasting and dilated cardiomyopathy. DMD patients rarely survive into their fourth
65 decade(4). Gene therapy to restore dystrophin expression is a promising approach for the
66 treatment of DMD. Recombinant adeno-associated virus (rAAV) vectors are particularly
67 efficient in transducing skeletal muscle fibers and cardiomyocytes when packaged with the
68 appropriate capsid(5-7), and allow long-term *in vivo* transgene expression(8). However, the
69 full-length dystrophin complementary DNA (cDNA) is 14 kb in length and greatly exceeds the
70 packaging capacity of a single rAAV vector (< 5 kb)(9). Therefore, shortened transgenes, coding
71 for partially functional microdystrophins (MD) that contain essential domains of the
72 dystrophin protein have been generated. The principle of using MDs as therapeutic transgenes
73 arose from the concept that Becker Muscular Dystrophy (BMD) patients with natural in-frame
74 deletions/mutations in their *DMD* gene exhibit a milder dystrophinopathy(10). Our group
75 participated in the first study describing long-term functional rescue after a gene therapy
76 treatment based on rAAV-MD systemic delivery in the Golden Retriever Muscular Dystrophy
77 (GRMD) dog, a large animal model of DMD (11). Three clinical trials using this strategy have
78 been launched in 2018, and the first reported results are very promising(12). In BMD patients,
79 the disease is milder and more heterogeneous compared to DMD patients. Nevertheless,
80 muscle weakness is often noticed in adolescence or young adulthood(13). Additionally,
81 current rAAV-MD trials are based on MDs that are ~40% smaller than the smallest naturally
82 truncated dystrophin reported in a patient with BMD(14). It is thus urgent to find therapeutic
83 strategies alternative or complementary to MD-based gene therapy that could treat both
84 DMD and BMD patients. Such strategies have to target significant and primordial events of
85 the DMD pathogenesis.

86 Calcium plays a critical role in the pathogenesis of DMD as skeletal muscle necrosis is mainly
87 caused by intracellular Ca^{2+} overload(15). Calcium alterations are very early events: they have
88 been detected in muscle fibers of DMD boy fetuses and measured in not fully differentiated
89 human DMD myotubes (16, 17). Intracellular Ca^{2+} overload in DMD is mainly related to an
90 increase of the sarcolemma permeability to Ca^{2+} (SPCa) through the accumulation of Ca^{2+}
91 permeable ion channels(18). The identity of the channels involved in the SPCa increase is still
92 unclear but members of the Transient Receptor Potential (TRP) family have been proposed as
93 possible candidates(18). The mammalian TRP channel superfamily encompasses 28 members
94 that are subdivided into 6 subfamilies according to their sequence homology(19). Two TRP
95 channels caught our attention in the context of the DMD: TRPC1 and TRPC3. The expression
96 of these channels are increased in skeletal, cardiac and smooth muscles in absence of
97 dystrophin expression in the *mdx* mouse(20-22). TRPC3 acts as a positive regulator of reactive
98 oxygen species, its increased expression leading to a fibrotic response in cardiomyocytes(20).
99 TRPC3 has been also proposed to participate to the massive and sustained cytosolic Ca^{2+}
100 increase taking place in skeletal muscle cells during malignant hyperthermia (MH) episode and
101 numerous MH like episodes have been reported in human DMD patients (23, 24). The two
102 channels are involved in myogenesis and regulate cytosolic Ca^{2+} levels in skeletal muscle
103 fibers(25, 26). Targeting TRPC1 and TRPC3 to reduce Ca^{2+} alterations in DMD muscles could
104 thus represent relevant targets for alternative or complementary treatment to MD-based
105 gene therapy.

106 Nevertheless, most of the studies concerning the involvement of Ca^{2+} homeostasis alterations
107 and the TRP channels in the pathogenesis of DMD were conducted in the *mdx* mouse. This
108 animal model of DMD exhibits a very mild muscle dystrophy as compared to DMD patients(27,
109 28). This reduces the scope of the results obtained in *mdx* mice in the understanding of the
110 disease and the development of treatments in human DMD patients. Our team participated
111 to the generation of the *DMD^{mdx}* rat model(27), which more closely reproduces the human
112 DMD disease with progressive and severe skeletal muscle replacement by fibrosis, significant
113 reduction in muscle strength, a decrease in spontaneous motor activity and cardiac
114 involvements.

115 In the present study, we aimed to determine whether TRPC1 and/or TRPC3 channels may be
116 involved in skeletal muscle SPCa alterations in the *DMD^{mdx}* rat and may represent therapeutic

117 targets. We assessed $[Ca^{2+}]_c$ and SPCa in mechanically isolated and fura-2 loaded fibers of the
118 EDL (*Extensor Digitorum Longus*) fast-twitch muscle from age-match wild-type (WT) and
119 *DMD^{mdx}* rats of 1.5 to 7 months old. In this time window, rats undergo puberty and reach
120 adulthood(29) and *DMD^{mdx}* animals progressively display necrosis and regeneration in limb
121 and diaphragm muscles that evolves to severe fibrosis and adipose tissue infiltration(27).
122 TRPC1 and TRPC3 expressions were measured in the EDL muscles at both the mRNA and
123 protein levels, by RT-qPCR and western-blot analysis, respectively. The subcellular localization
124 of the two channels was assessed by immunocytofluorescence and confocal microscopy.
125 Finally, we determined the impact of a rAAV-MD based treatment on Ca^{2+} homeostasis, force
126 development and TRPC expression in *DMD^{mdx}* rat skeletal muscles.

127

128 **Material and Methods**

129 *Animals*

130 A total of 69 *DMD^{mdx}* rats and 59 Sprague Dawley WT rats (littermates) were used in this study.
131 They were obtained, handled and housed from the UTE IRS-UN (University de Nantes, France)
132 and the Boisbonne Center for Gene Therapy (ONIRIS, Nantes, France). The Institutional Animal
133 Care and Use Committee of the Région des Pays de la Loire (University of Angers, France) as
134 well as the French Ministry for National Education, Higher Education and Research approved
135 the protocol (authorizations #2016070618053653 and 2017040616371353). Before sacrifice,
136 animals received a subcutaneous injection of Buprenorphine (0.04 mg/kg, Vetergesic, Ceva
137 Santé Animale, Libourne, France), after 30 min rats were anesthetized by intraperitoneal
138 injection with etomidate (16 mg/kg, Hypnomidate, Janssen-Cilag, Issy Les Moulineaux,
139 France), delivered in 2 or 3 injections separated by 3 min, and ketamine (20 mg/kg, Imalgene
140 1000, Merial, Lyon, France). Animals dedicated to *ex vivo* skeletal muscle contractility analysis
141 and Ca^{2+} measurements were euthanized by heart excision. Other animals were euthanized
142 by intravenous injection of pentobarbital sodium (Dolethal, Vetoquinol, Paris, France).

143 *Histology*

144 Some rats that died prematurely during this study were necropsied. A few tissues and organs
145 (including heart, lung, kidney, liver, spleen, skeletal muscle and brain) were then obtained for
146 immediate fixation in formalin. After paraffin embedding, 4 μ m-thick sections were further

147 stained using Hemalun-Eosin-Saffran routine protocol. Additional tissue staining (Picrosirius
148 red and von Kossa for Ca^{2+}) were performed on skeletal muscle tissues when needed. These
149 tissue samples were observed by a veterinary pathologist to determine the cause of death.

150

151 *Ex-vivo skeletal muscle contractility*

152 Isometric contractile properties of the EDL muscles were evaluated according to methods
153 previously described(30). Briefly, muscles were removed from the hindlimb of anesthetized
154 rats and mounted in an *in vitro* muscle test system (1205A model; Aurora Scientific, Aurora,
155 Canada). Muscles were placed between two platinum electrodes in a muscle bath containing
156 100 ml of bubbled mammalian Ringer solution at 25°C. After a 5 min equilibration period,
157 optimum muscle length was determined by gradual muscle length adjustments and eliciting
158 isometric contractions (supramaximal square-wave pulses of 0.2 ms duration) until the
159 maximum twitch tension was reached. After 5 min of rest, muscles were stimulated at 10, 20,
160 40, 60, 80, 100, 120 Hz for 500 ms at each frequency. Stimulus trains were separated by 1 min
161 interval. Maximum isometric tetanic force was determined from the plateau of this force
162 frequency curve. Following force testing, muscles were removed from the bath, trimmed of
163 tendons, and weighed. Muscle mass was then be used to calculate maximum tetanic specific
164 force in g/g.

165 *Dissection of native muscle fibers*

166 For *in vitro* experiments, EDL muscles were removed from the animal under deep anesthesia
167 and were pinned in a dissecting dish containing physiological solution (NPS) at room
168 temperature (22°C) for further dissection. NPS contained the following: 140 mM NaCl (VWR
169 International, Fontenay sous Bois, France), 5 mM KCl (VWR International, Fontenay sous Bois,
170 France), 1 mM MgCl_2 (VWR International, Fontenay sous Bois, France), 10 mM HEPES (Sigma-
171 Aldrich, Saint Quentin Fallavier, France), 10 mM glucose (Sigma-Aldrich, Saint Quentin
172 Fallavier, France), and 1.8 mM CaCl_2 (Sigma-Aldrich, Saint Quentin Fallavier, France) at pH
173 7.35. Contralateral EDL muscles of some animals were snap-frozen and stored at -80°C for
174 biochemical and molecular biology as described below. Skeletal muscle fibers from EDL muscle
175 of the different groups of rats were dissected intact. Small bundles of 10–15 fibers arranged
176 in a single layer were dissected lengthwise, tendon to tendon, with the use of microscissors,

177 as described elsewhere(31). Part of the bundles were used for Ca²⁺ measurements and the
178 others were kept for immunofluorescence experiments.

179 *Cytosolic Ca²⁺ measurement*

180 EDL muscle bundles were incubated in NPS containing 5 µmol/l Fura-2 AM (Molecular Probes,
181 OR, USA) for 1 h at RT, rinsed twice, and let 30 min before use to ensure complete
182 desesterification. Ratiometric Fura-2 fluorescence measurements were made using an
183 integrated IonOptix (IonOptix, Amsterdam, Netherlands) device and excitation filters of 360
184 and 380 nm. Emitted fluorescence (510 nm) was background subtracted. The cytosolic Ca²⁺
185 concentration ([Ca²⁺]_c) was calculated from ratiometric measurements according to a
186 modified method from Grynkiewicz and colleagues(31, 32).

187 *Determination of sarcolemmal permeability to divalent cations*

188 The manganese quenching technique was used to determine the sarcolemmal permeability
189 to divalent cations (SPCa). Muscle preparations were first perfused for 2 min with NPS
190 containing 0.5 mM Mn²⁺ as a surrogate of Ca²⁺ (quenching solution). Then, the quenching
191 solution was applied to muscle fibers for 2-4 min. During the whole quenching protocol, the
192 fluorescence of Fura-2 excited at 360 nm was acquired at 1 Hz. The quench rates were
193 determined using linear regression analysis of fluorescence signal and expressed as the
194 decline per minute of the initial fluorescence intensity. For experiments dedicated to evaluate
195 Pyr10 Cc1ccc(cc1)C(=O)Nc2cc(C(F)(F)F)nn2 (–N-(4-(3,5-bis(trifluoromethyl)-1H-pyrazole-1-yl)phenyl)-4-
196 methylbenzenesulfonamide), quench rate was first measured in quenching solution. Muscle
197 fibers were washed and then incubated 10 min in NPS without Ca²⁺ but containing 3 µM of
198 Pyr10(33). Quench rate was then newly measured in previous fibers in quenching solution
199 containing 3 µM Pyr10.

200 *Immunofluorescence*

201 EDL muscle fiber bundles containing 5 to 10 fibers were dissected with microscissors in
202 skinning solution and were immunolabelled according to a method adapted from Liu and
203 colleagues(34). Briefly, muscle fiber bundles were stretched in Sylgard 184 silicone elastomer
204 (Sigma, France) molded chambers designed to maintain the muscle fibers at tension allowing
205 to obtain a sarcomere length close to that found in vivo (~2.5 µm) during the whole
206 immunolabelling process. Muscle bundles were then fixed using PBS containing 4% PFA
207 (ThermoFisher, Massachusetts, USA) for 1 hour, then permeabilized using 2% TritonX100

208 diluted in PBS for another hour under agitation. After washing, muscle bundles were
209 incubated during 48 h under agitation in a PBS solution containing goat polyclonal anti-
210 caveoline-3 (1 :50, Bio-Techne, Minnesota, USA) and mouse anti-Ryanodine Receptor (1:100,
211 DSHB, Iowa, USA) antibodies. Donkey serum was added at 5% in this latter solution. After
212 washing muscle bundles were newly incubated for 48 h under agitation in a PBS solution
213 containing 5% of donkey serum, donkey anti-goat Alexa Fluor 647 conjugated (1:400) and
214 donkey anti-mouse Cyanine-3 conjugated (1:400) (Jackson ImmunoResearch, UK). After a new
215 wash, muscle fibers were newly fixed using 4% PFA PBS for 15 min. Finally, muscle fibers were
216 incubated in a PBS solution containing mouse anti-TRPC1 or anti-TRPC3 antibodies Alexa Fluor
217 488 conjugated (1:50, Santa Cruz Biotechnology, Texas, USA) for 48 h under agitation. After
218 final wash, muscle preparations were mounted in ProlonGold (ThermoFisher,
219 Massachusetts, USA) between glass cover slip and slide. Image Z-stacks were acquired using
220 a Nikon confocal A1 N-SIM microscope with a Plan Apo X60 objective (Nikon France Sas,
221 Champigny-Marne, France).

222 *TRPC1 and TRPC3 submembrane distribution analysis*

223 Images of the acquired stack corresponding to the center of muscle fibers were used for
224 analysis. A macro was written under ImageJ macro language to routinely and semi-
225 automatically analyze the images. Briefly, experimenter was asked to enter the number of the
226 cells to analyze in a rectangle Region Of Interest (ROI) and to delimitate the peaks
227 corresponding to Cav-3 labelling. Maximum peak was then automatically calculated and
228 defined as sarcolemma position. This position was then used to calculated into the stack
229 channel corresponding to TRPC1 or TRPC3 labeling the areas of the fiber corresponding to the
230 5 μm spaces beneath sarcolemma and to the cell center, and their corresponding integrated
231 signal density.

232 *Relative quantification of TRPC1, TRPC3 and MD messengers by RT-qPCR*

233 Total RNA was extracted from pieces of EDL muscles from the different groups of rats with
234 QIAzol Lysis Reagent (Qiagen, Germany) according to the manufacturer's instructions. Then,
235 1000 ng of total RNA was treated with RNase-free DNase I (ezDNase from ThermoFisher,
236 Massachusetts, USA) and then reverse transcribed using SuperScript IV Vilo reverse
237 transcriptase (ThermoFisher, Massachusetts, USA) and random primers (ThermoFisher,

238 Massachusetts, USA) in a final volume of 20 μ L. qPCR analysis were then performed on cDNA
239 (diluted 1/40 for TRPC1 and 1/80 for TRPC3) using different primers designed to amplify a
240 specific region of the TRPC1 messenger (Forward: TTCCAAAGAGCAGAAGGACTG and Reverse:
241 AGGTGCCAATGAACGAGTG according to Sabourin and collaborators(35)), the TRPC3
242 messenger (Forward: ACGCTTCTCACCTGACATCA and Reverse: CTGGACAGCGACAAGTATGC)
243 or the MD messenger (Forward: CCAACAAAGTGCCCTACTACATC, Reverse:
244 GGTTGTGCTGGTCCAGGGCGT, and Probe: CCGAGCTGTACCAGAGCCTGGCC). As an internal
245 control, HPRT1 messenger was used to normalize the mRNA concentration (Forward:
246 GCGAAAGTGGAAAAGCCAAGT, Reverse: GCCACATCAACAGGACTCTTG TAG, Probe:
247 CAAAGCCTAAAAGACAGCGCAAGTTGAAT). Results were expressed in relative quantities
248 (RQ): $RQ = 2^{-\Delta Ct} = 2^{-(Ct_{target} - Ct_{endogenous\ control})}$. For each RNA sample, the absence of DNA
249 contamination was also confirmed by analysis of “cDNA liked samples” obtained without
250 addition of reverse transcriptase in the reaction mix.

251 *rTRPC3 cDNA amplification and sequencing*

252 PCR amplification of the coding region around the exon 9 of the TRPC3 cDNA was performed
253 on total cDNA from EDL of WT and DMD^{mdx} rats. PCR were performed using LA Taq polymerase
254 (Takara, Kusatsu, Japan) and the primers published by Kim and collaborators(36): Forward:
255 CAGTGATGTAGAGTGGAAAGTTTGC, Reverse: CTCCTCATTACACCTCAGC. The amplification
256 products were loaded on a 2% agarose gel. The amplification of the full-size cDNA of TRPC3
257 were performed using the following primers: Forward: ACGCAGTACGGCAACATCC, and
258 Reverse: CATTACACCTCAGCGCACT. The amplification products were then sequenced with
259 Sanger method (Genewiz, South Plainfield, USA).

260 *TRPC1 and TRPC3 expression analysis using western-blot*

261 In order to extract EDL muscle total proteins, muscles were homogenized using TissueLyser II
262 (Qiagen, Germany) in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich,
263 Missouri, USA). 50 μ g of protein extracts, denatured 10 minutes at 70°C with Laemmli (Biorad,
264 California, USA), were loaded on a 10% Tris-Glycine Precast polyacrylamide gels
265 (ThermoFisher, Missouri, USA). After 2 hours of migration at 100 volts, and Red Ponceau
266 staining, membranes were blocked over-night at 4°C (PBS-Tween 0.1%, non-fat dry milk 5%
267 and NP40 1%). Then, membranes were incubated 1 hour at room temperature (RT) with
268 mouse anti-TRPC1 antibody (1:50 000, sc-133076; Sant Cruz Biotechnology, Texas, USA),

269 mouse anti-TRPC3 (1:500, sc-514670; Sant Cruz Biotechnology, Texas, USA) or goat anti-
270 GAPDH (1:10 000, Novus Biologicals, Colorado, USA). After washing with PBD Tween 0.1%
271 membranes were incubated with secondary rabbit anti-mouse HRP (1:5000) or rabbit anti-
272 goat HRP (1:2000; Agilent Technology, California, USA). After washing with PBS Tween 0.1%,
273 ECL (ThermoFisher, Massachusetts, USA) was applied on membranes and films were exposed
274 (Amersham Hyperfilm™). MD protein expression in EDL was analyzed as previously
275 published(11). Relative protein expressions were calculated by normalizing signal intensity
276 measured using ImageJ software by GAPDH signal of the corresponding lane and the signal of
277 an experimental sample that was loaded on every gels (ImageJ). This latter allowed us the
278 comparison between gels.

279 *TRPC3 deglycosylation analysis*

280 Deglycosylation analysis of the TRPC 3 protein was performed using Protein Deglycosylation
281 Mix II kit (New England Biolabs, Ipswich, USA). Briefly, 40µg of total protein extract were
282 diluted in water. Then, 2 µL of Deglycosylation Mix Buffer 2 were added to the proteins and
283 the mix was incubated at 75°C for 10 min. Then, 2 µL of the enzyme Protein Deglycosylation
284 Mix II were added to the proteins, followed by incubations 30 min at room temperature and
285 1 hour at 37°C. Positive control (Fetuin) was provided in the kit. The results were analyzed by
286 Western-blot as described previously.

287 *TRPC3 dephosphorylation analysis*

288 Dephosphorylation analysis of the TRPC3 protein was performed using Fast AP
289 Thermosensitive Alkaline Phosphatase kit (ThermoFisher, Massachusetts, USA). The reaction
290 was performed on 30µg of total protein extract diluted in water. Briefly, a mix of 8 µL of 10X
291 FastAP buffer and 60 µL of FastAP Phosphatase were added to proteins. The reaction mix was
292 incubated 1 hour at 37°C. The proteins were then concentrated by adding 320 µL of acetone
293 to the reaction mix, followed by an overnight incubation at -20°C, and a centrifugation at 13
294 000 rpm during 15 min at 4°C. The pellet was then resuspended in water, and analyzed by
295 western-blot as described previously.

296 *Apparent molecular weight analysis*

297 In order to accurately calculate the apparent molecular weight (AMW) and allows comparison
298 between lanes a dedicated macro was built under ImageJ and dedicated western blots were
299 ran. For these latter, four experimental samples were surrounded by two ladder samples to

300 allow robust size determination and limit separation artefacts. The lamgeJ macro was
301 designed to automatically determine the relation between distance of migration and ladder
302 sizes surrounding the size of interest according to a Boltzmann curve fit. The parameters of
303 the Boltzmann equation were then used to calculate the AMW protein of interest starting
304 from distance migration.

305 *Vector production*

306 Murine-specific cDNA sequences of optimized MD version 1 has been previously described(37,
307 38). This MD cDNA is deleted of spectrin-like repeat domain 4 to 23 and CT domain (exons 71–
308 78) and contains the last three amino acids of exon 79 of dystrophin followed by three stop
309 codons(37). MD cDNA sequence was subcloned into a pAAV plasmid that contained the 323bp
310 muscle-synthetic Spc5.12 promoter(39), a synthetic polyadenylation signal of 49bp obtained
311 from the pCI-neo plasmid (Promega, Madison, WI), and two flanking inverted terminal repeat
312 (ITR) sequences of 130 pb from AAV serotype 2. The size of the resulting MD expression
313 cassette (including Spc5.12 promoter, pCI-Neo polyA, and ITRs) was 4 538bp. Recombinant
314 pseudo typed AAV2/9-MD vectors were produced by the Vector Core of the UMR 1089 (CPV,
315 INSERM and University of Nantes) by transient transfection of HEK293 cells followed by
316 purification on cesium chloride density gradients. Final vectors were concentrated and
317 formulated in Dulbecco's phosphate-buffered saline (DPBS, Fisher Scientific, Illkirsch, France),
318 sterile filtered, aliquoted and frozen at ≤ -70 °C. Vector genome titers (vg/mL) were
319 determined using a qPCR assay specific for ITR264(40).

320 *rAAV-MD injection in DMD^{mdx}rats*

321 Prior injection, the rAAV vectors were diluted in Dulbecco's phosphate-buffered saline (DPBS)
322 vehicle solution to obtain a fixed total volume corresponding to 15 mL of perfusate per kg of
323 animal. Injections were performed without anesthesia but under analgesic premedication,
324 performed at least 30 min before injection by subcutaneous injection of Buprenorphine
325 (Véteergésic, Ceva Santé Animale, Libourne, France) at 0.04 mg/kg. Vector or its vehicle was
326 administered at the age of 1 month by the intravenous route in a tail vein at a fixed flow rate
327 of 0.5 mL/min. Animals were sacrificed after 3 months of follow-up.

328 *Diaphragm ultrasonography in vivo*

329 The technique was adapted from Withehead and colleagues(41). Briefly, ultrasonography was
330 performed using a Vivid 7 ultrasound unit (GE Healthcare, Velizy Villacoublay, France)
331 associated to a 14 Mhz M12L probe. First animals, animals received a subcutaneous injection
332 of Buprenorphine (0.04 mg/kg, Vetergesic, Ceva Santé Animale, Libourne, France), after 30
333 min rats were anesthetized by intraperitoneal injection with etomidate (16 mg/kg,
334 Hypnomidate, Janssen-Cilag, Issy Les Moulineaux, France), the hair on the chest and abdomen
335 was removed using hair-removal cream. Once anaesthetized, the rat was placed supine on the
336 imaging platform and the four limbs. The platform was pre-heated to maintain the core body
337 temperature at 37°C, which was monitored with a temperature-sensitive rectal probe.
338 Ultrasound gel was applied to the area overlying the diaphragm and liver. The probe was
339 manually positioned 120° relative to the rat platform. The probe was placed along the
340 transverse mid-sternal axis of the rat, in order to locate the diaphragm on both sides of the
341 body. According to Whitehead and colleagues in the mouse the liver and portal vessels were
342 used as landmarks. M-mode was used to measure the diaphragm movement during normal
343 breathing cycles. The M-mode image window was positioned on the left side of the sternum,
344 over a flat region of diaphragm. Images were then recorded during at least 15 breathing cycles.
345 In order to avoid experimenter and probe positioning artifacts, the probe was removed and
346 replaced 2 times to allow acquisition of 3 records. A semi-automatic analysis method was then
347 applied to measure the amplitude of the diaphragm movement during each inspiration. Each
348 recorded image was filtered using ImageJ (Gaussian Blur>Threshold>Find Edges) and
349 transformed in (x,y) calibrated curves using GetData Graph Digitizer 2.26. A macro was built
350 under ImageJ to automatically calculate the amplitude of diaphragm contraction (the
351 difference in mm between baseline and the peak of the contraction) of 5 consecutive cycles.

352 *Statistics*

353 All statistical analyses were performed using XLStat software (Addinsoft, Paris, France). P-
354 values <0.05 were considered statistically significant. The statistical tests are specified in the
355 text and were choose depending on data number in each group and the number of groups to
356 be tested for a given parameter.

357 **Results**

358 *Malignant hyperthermia episode*

359 At the beginning of this study, some few *DMD^{mdx}* rats were anesthetized using an halogenated
360 agent (isoflurane) in order to obtain control blood samples. Unexpectedly, 3 out of 16 rats
361 died during the course of anesthesia. These rats were necropsied and main organs and tissues
362 were analyzed by a pathologist to determine the cause of death. All these rats typically
363 exhibited similar myopathic lesions typical of the model (27). These lesions included isolated
364 hyalin fibers, small clusters of degenerative fibers associated with muscle fiber regeneration
365 foci, centro-nucleated fibers and anisocytosis with a few inflammatory cells in a slightly
366 increased endomysial space corresponding to mild fibrosis. In addition to these classical DMD
367 lesions, these rats displayed (i) large clusters of round hypereosinophilic fibers
368 (hypercontracted fibers) corresponding globally to half of the total number of fibers. Some of
369 them displayed fragmented cytoplasm and/or hyperchromatic condensed or fragmented
370 nucleus indicative of necrosis (severe rhabdomyolysis), (ii) huge optically empty space
371 between fibers corresponding to massive edema. Using Picrosirius and von Kossa stainings,
372 both specific for Ca^{2+} , an increased concentration of Ca^{2+} was identified in hypercontracted
373 fibers (Figure 1). The association of these lesions are typical of malignant hyperthermia (MH)
374 or MH-like reaction. This syndrome is responsible for the immediate death of the animal
375 notably due to neuronal death associated to severe hyperthermia, as evidenced in some of
376 these animals in which brain tissue conservation was good. Interestingly, similar findings were
377 observed in 5 rats of another study that were found dead during or immediately after an
378 experiment requiring long-lasting posture constraint. As developed in the discussion, these
379 MH events were first clues indicating muscle Ca^{2+} homeostasis alterations in the *DMD^{mdx}* rats.

380 *Ca^{2+} homeostasis is dysregulated in *DMD^{mdx}* EDL muscles*

381 From the age of 1.5 to 7 months, resting $[\text{Ca}^{2+}]_c$ and SPCa were determined in each single fura-
382 2-loaded fiber constituting the bundles dissected from the EDL muscles of WT and *DMD^{mdx}*
383 rats. During this period, resting $[\text{Ca}^{2+}]_c$ slightly but significantly increased in WT EDL muscle
384 fibers (Figure 2A). A similar increase was observed in *DMD^{mdx}* muscles, but at each time point
385 the mean $[\text{Ca}^{2+}]_c$ was ~20 to 30% significantly higher than in WT muscle fibers. In the same
386 muscle bundles, we used the Mn^{2+} quenching technique in order to determine whether resting
387 $[\text{Ca}^{2+}]_c$ rise was associated with an increase in the sarcolemmal permeability to Ca^{2+} (SPCa). In
388 contrast with $[\text{Ca}^{2+}]_c$, SPCa progressively and significantly decreased in muscle fibers from WT
389 rats from 1.5 to 7 months of age (Figure 2B). Age-dependent decrease of SPCa was also

390 observed in *DMD^{mdx}* muscle fibers but the mean values were always significantly higher in
391 dystrophic fibers than in age-matched WT controls. This difference was particularly high at 1.5
392 months of age (~50%) and progressively decreased to reach ~35% at 7 months. In order to
393 assess whether the elevation of $[Ca^{2+}]_c$ and SPCa in *DMD^{mdx}* EDL were associated with changes
394 in TRPC1 and/or TRPC3 expression we conducted a series of experiments to measure mRNA
395 and protein levels of the two channels.

396

397 *Expression of TRPC1 and TRPC3 channels are modified over the course of the DMD pathology*
398 *in DMD^{mdx} rats*

399 Muscle homogenates from WT and *DMD^{mdx}* rats of 1.5 to 7 months of age were analyzed by
400 RT-qPCR and western blot (Figure 3). In WT rats, TRPC1 mRNA expression level was stable over
401 ages (Figure 3A). TRPC1 protein expression level was heterogeneous among ages with a
402 tendency at overexpression at 1.5 months of age, but without statistical significance (Figure
403 3C). Similar results were obtained for *DMD^{mdx}* rats except at 7 months of age, where TRPC1
404 mRNA and protein expression levels were significantly higher than in WT controls (Figures 3A-
405 3C). Results were quite different for TRPC3 (Figures 3B-3D). In WT EDL muscles, both mRNA
406 and protein expression levels were the highest at 1.5 months of age and significantly
407 decreased thereafter to reach a steady-state level at 3 months of age. In *DMD^{mdx}* EDL muscles,
408 TRPC3 mRNA expression levels were significantly lower to that of WT ones at 1.5 months of
409 age. From 3 to 7 months of age, TRPC3 mRNA expression level was stable and similar to that
410 measured in WT animals. In contrast to mRNA, TRPC3 protein expression level was ~2 fold
411 higher in *DMD^{mdx}* than in WT rats at 1.5 months of age (Figure 3D). Although TRPC3 expression
412 decreased with age in both *DMD^{mdx}* and WT rats, it always remained ~2 fold higher in
413 dystrophic animals. As TRPC translocation from intracellular membrane compartments (e.g.
414 sarcoplasmic reticulum) to the sarcolemma could lead to an increase in SPCa. We thus studied
415 the TRPC subcellular localization in muscle.

416 *Subcellular localization of TRPC1 and TRPC3 is not modified in DMD^{mdx} muscles*

417 We investigated the subcellular localization of TRPC1 and TRPC3 proteins in WT and *DMD^{mdx}*
418 muscle fibers by immunofluorescence and confocal microscopy to determine the proportion
419 of TRPC channels expressed at the peripheral sarcolemma compared to the center of the cells.

420 Caveoline-3 (Cav-3) and the Ryanodine Receptors (RyR) were used as sarcolemma and
421 sarcoplasmic reticulum markers, respectively (Figure 4A-4B). Despite an heterogeneous and
422 partly punctiform location, TRPC1 was mostly observed at the peripheral sarcolemma where
423 it seemed to colocalize with Cav-3 (Figure 4A). Similar sarcolemmal location was seen for
424 TRPC3 with a more homogeneous labelling (Figure 4B). In the center of the cells, the
425 immunofluorescence signals for both channels appeared in a typical striated pattern.
426 Comparison of Cav-3 and RyR immunolabeling to that of TRPC1 and TRPC3 did not allowed us
427 to clearly determine the cellular membrane compartments where TRPCs were expressed. This
428 may be due to the high entanglement of t-tubules and sarcoplasmic reticulum (SR) terminal
429 cisternae that form highly specialized ultrastructure in muscle fiber cells, called triads(42). In
430 order to further analyze the potential difference in location between WT and *DMD^{mdx}* muscle
431 fibers, the density signal ratios of TRPC1 and TRPC3 between the peripheral sarcolemma and
432 the center of the cells were calculated (Figure 4C-D, see material and methods section for
433 details). No significant difference was observed between dystrophic and healthy muscle fibers.
434 Considering the late expression alteration of TRPC1 and the earliest one for TRPC3, without
435 significant location change of the two channels, we then focused our attention on TRPC3.

436

437 *Post-translational TRPC3 modification changes in DMD^{mdx} rat skeletal muscle*

438 TRPC3 protein expression was increased in *DMD^{mdx}* muscles whereas the mRNA expression
439 level was rather decreased as compared to WT. This apparent discrepancy may rely on post-
440 translational modifications altering TRPC3 protein turn-over. As shown in Figure 5A, we
441 observed a slight but reliable increase in the apparent molecular weight (AMW) of TRPC3
442 bands revealed on the western blot for *DMD^{mdx}* rats. This was observed at the four ages tested
443 (data not shown). We performed additional western blots specifically designed to measure
444 the AMW. Two consecutive lanes containing WT and *DMD^{mdx}* EDL muscle homogenates were
445 surrounded by lanes in which a pre-stained protein standard was loaded. After blotting and
446 revelation the AMW of the bands was determined using scanned blots and a homemade
447 ImageJ macro. As shown in Figure 5C, we found that TRPC3 AMW was ~2 kDa higher in *DMD^{mdx}*
448 EDL muscles than in WT control muscles. This may be the result of different mRNA transcript
449 variants generated by alternative splicing, or of post-transcriptional modifications.
450 Interestingly, Kim and colleagues reported the alternative splicing of the TRPC3 mRNA exon 9

451 in the brain of mice, rats, and guinea pigs(36). They showed that the resulting protein was
452 around 3 kDa smaller than the full-length protein. We compared the size of the amplicons
453 corresponding to a region surrounding TRPC3 exon 9 by RT-PCR (Figure 5D). There was no
454 significant difference between the amplicons of the two genotypes. We also amplified and
455 sequenced the whole TRPC3 cDNA obtained from EDL, and saw no difference between
456 *DMD^{mdx}* and WT rats (data not shown), suggesting that no differential splicing of the TRPC3
457 mRNA occurs in *DMD^{mdx}* rats. Beside mRNA alternative splicing TRPC3 AMW differences
458 between WT and *DMD^{mdx}* rats may be due to post-transcriptional modifications. It has been
459 reported that TRPC3 presents one N-glycosylation and several phosphorylation sites(43). After
460 enzymatic N-deglycosylation treatment, no significant change in TRPC3 AMW was observed
461 in muscle homogenates from *DMD^{mdx}* rats. However, in these conditions, TRPC3 AMW in WT
462 rats was shifted towards higher molecular weight values, such as the difference in TRPC3
463 AMW between the two genotypes was no longer observed (Figure 5 B). We also assessed the
464 possibility that TRPC3 may be differently phosphorylated in WT and *DMD^{mdx}* muscles. As the
465 experimental kit we used needed high protein concentration, we used the acetone method to
466 concentrate our muscle homogenate. This treatment led to a similar TRPC3 shift in WT muscle
467 homogenates that after deglycosylation treatment. The dephosphorylation treatment *per se*
468 did not further modified TRPC3 AMW (Figure 5B).

469

470 *SPCa inhibition by Pyr10 a TRPC3 specific inhibitor*

471 In order to further assess the possibility that TRPC3 was involved in the SPCa increased of the
472 *DMD^{mdx}* EDL muscle fibers, Pyr10, a specific inhibitor of the channel was applied during SPCa
473 measurements (Figure 5D). For both WT and *DMD^{mdx}* application of NPS containing 3 μ M of
474 Pyr10, a significant inhibition of SPCa was observed. This was more pronounced in dystrophic
475 fibers such as the residual mean SPCa values measured in WT and *DMD^{mdx}* EDL muscle fibers
476 were finally no more different.

477

478 *rAAV microdystrophin gene transfer benefits on Ca²⁺ alterations, TRPC3 expression and*
479 *skeletal muscle force* One main objective of the present study was to determine whether
480 TRPC3 could be a therapeutic target to sustain rAAV-MD treatment. In order to assess this

481 hypothesis a series of experiments was conducted in 4 months old *DMD^{mdx}* rats that received
482 systemic IV injections of a therapeutic dose of rAAV2/9-MD (3E13 vg/kg; MD-*DMD^{mdx}*) at 1
483 month of age. Results obtained were compared to age-matched WT (Vehicle-WT) and *DMD^{mdx}*
484 (Vehicle-*DMD^{mdx}*) littermate rats treated in the same conditions excepted that injections
485 contained only vector formulation buffer (vehicle). As previously observed in untreated
486 animals, when compared to Vehicle-WT, EDL muscle of Vehicle-*DMD^{mdx}* rats exhibited fibers
487 with higher resting $[Ca^{2+}]_c$ and SPCa, similar TRPC3 mRNA level and higher TRPC3 protein
488 expression (Figure 6 A-E). In EDL and diaphragm muscles, endogenous dystrophine was
489 detected by western blot in Vehicle-WT rats but not in Vehicle-*DMD^{mdx}* nor MD-*DMD^{mdx}*
490 animals (Figure 6C). As expected, MD protein was highly expressed in EDL and diaphragm
491 muscles of MD-*DMD^{mdx}* rats 3 months after rAAV2/9-MD injection (Figure 6C). MD expression
492 in EDL muscle was associated with a significant preservation of $[Ca^{2+}]_c$ and SPCa at the fiber
493 level (Fig 6A-B). Nevertheless, MD-associated preservation of Ca^{2+} homeostasis was only
494 partial and the $[Ca^{2+}]_c$ and SPCa mean values were still significantly higher in MD-*DMD^{mdx}* EDLs
495 compared to Vehicle-WT controls. We next assessed the impact of rAAV-MD injection on
496 skeletal muscle function *in vivo* and *in vitro*. Diaphragm contraction amplitude was measured
497 by ultrasound in the three groups of rats prior to sacrifice, together with EDL maximal
498 isometric tension (Figure 6 F-G). When compared to Vehicle-WT counterparts, a significant
499 decrease of diaphragm contraction amplitude and EDL maximal isometric tension were
500 observed in Vehicle-*DMD^{mdx}* rats. In MD-*DMD^{mdx}* rats, diaphragm contraction amplitude was
501 significantly preserved as compared to Vehicle-*DMD^{mdx}* animals, although it was still lower to
502 that of Vehicle-WT rats (Figure 6F). Similar results were observed for EDL maximal isometric
503 tension (Figure 6G). Both parameters were significantly decreased in Vehicle-*DMD^{mdx}* rats
504 compared to Vehicle-WT controls, and this was partially corrected by MD gene therapy (Figure
505 6F-G). In parallel, the increase in TRPC3 protein levels was also partly prevented in MD-
506 *DMD^{mdx}* EDL, without a significant change in TRPC3 mRNA (Figure 6D-E).

507

508 Discussion

509 The main aims of the present study were to assess the involvement of TRPC1 and TRPC3
510 channels in the DMD pathogenesis by participating in the Ca^{2+} homeostasis alterations taking
511 place in the skeletal muscles. The experiments were conducted during the post-natal

512 development of the *DMD^{mdx}* rat, an animal model that closely reproduces the human DMD
513 disease with, in particular, a progressive and severe skeletal muscle necrosis and fibrosis, with
514 significant reduction in muscle strength, and a decrease in spontaneous motor activity(27).
515 Considering that the rAAV-MD based treatments of DMD are currently promising but would
516 rather lead to a milder BMD-like muscular dystrophy, we evaluated the potential of TRPC1 and
517 TRPC3 to represent alternative or complementary therapeutic targets to rAAV-MD based
518 treatments of DMD.

519 Most of the pathogenesis and the preclinical studies concerning DMD were carried out in the
520 *mdx* mouse. This animal model presents a much milder muscular dystrophic phenotype than
521 human DMD patients (28). This could be part of the reason for the poor translation of the
522 findings achieved with these animals and this is why the *DMD^{mdx}* rat was generated (27). The
523 milder phenotype of the *mdx* mouse model probably depends on scale and cell proliferation
524 differences allowing a better compensation of muscle fibers necrosis (44). On the other hand,
525 the cascade of cellular events induced by the lack of dystrophin expression leading to muscle
526 fiber necrosis likely follows a similar scheme in *mdx* mice and DMD patients. In particular, Ca^{2+}
527 alterations have been reported to be very early events in the DMD pathology. Calcium
528 overload has been reported in muscle fibers of DMD boy fetus, measured in not fully
529 differentiated human DMD myotubes and observed in the *mdx* mouse(16, 17, 31). The
530 mechanisms leading to a Ca^{2+} overload have been mostly deciphered in the *mdx* mouse due
531 to the difficulty to work with fully differentiated living human cells. It has been shown that
532 intracellular Ca^{2+} overload is mainly related to an increase of the SPCa through the
533 accumulation of Ca^{2+} permeable ion channels(18). One of the first aims of the present study
534 was thus to assess whether the dystrophic phenotype of the *DMD^{mdx}* was associated with
535 $[Ca^{2+}]_c$ and SPCa increases in skeletal muscle fibers. From 1.5 to 7 months of age WT rats
536 undergo puberty and reach adulthood(29). In this time window, we found that in WT fast-
537 twitch muscle cells $[Ca^{2+}]_c$ and SPCa were not finite values. The progressive increase of $[Ca^{2+}]_c$
538 and the parallel decrease of SPCa in WT EDL muscle fibers sustain the important role of Ca^{2+}
539 homeostasis in EDL muscle fibers during post-natal development. One may be surprised by
540 the apparent lack of correlation between the two processes. Nevertheless, SPCa is solely
541 related to Ca^{2+} influx through Ca^{2+} permeable channels whereas $[Ca^{2+}]_c$ is the instant image of
542 an equilibrium that depends not only on SPCa but also on numerous other processes, like the

543 balance between SR Ca²⁺ leaks and SR Ca²⁺ re-uptake, mitochondria or Ca²⁺-pumps and Na/Ca
544 exchanger at the sarcolemma(45). Interestingly, during *DMD^{mdx}* rat model characterization,
545 we faced to MH-like episodes upon isoflurane-induced anesthesia that forced us to further
546 adapt anesthesia protocols when using this animal model. Some MH-like episodes were also
547 observed in some animals that were submitted to a long-lasting posture constraint, which was
548 a source of stress for the animals. MH is a pharmacogenetic disorder that manifests as a
549 hypermetabolic cascade initiated at the skeletal muscle cell mainly on exposure to
550 halogenated anesthetics, like isoflurane(46). Numerous MH like episodes have been reported
551 in human patients after exposure to inhaled halogenated anesthetics included isoflurane,
552 halothane, and sevoflurane(24), but that can be also triggered by stress. MH is mainly due to
553 mutations in RYR1 and CACNA1S genes, that code for crucial Ca²⁺ channels, the ryanodine
554 receptor from the sarcoplasmic reticulum and the Ca²⁺ voltage-gated channel subunit alpha1S
555 located at the sarcolemma, respectively. No mutation in those genes have been reported in
556 DMD patients exhibiting MH-like syndrome and although the underlying mechanisms are still
557 not clear they likely depend on Ca²⁺ handling alterations(24). MH-like events observed in the
558 *DMD^{mdx}* rats were thus clues sustaining a Ca²⁺ homeostasis alteration in *DMD^{mdx}* skeletal
559 muscle fibers. In order to further assess this hypothesis, we compared [Ca²⁺]_c and SPCa of EDL
560 muscle fibers from the *DMD^{mdx}* rats to that of the WT ones. As soon as 1.5 months of age, both
561 characteristics were significantly higher in the *DMD^{mdx}* rats. In particular, the SPCa was found
562 to be around the value of WT in *DMD^{mdx}* rat muscle fibers. From 1.5 to 7 months of age, [Ca²⁺]_c
563 and SPCa progressively decreased in dystrophic muscle fibers as observed in WT muscle fibers,
564 but were always higher in the *DMD^{mdx}* cells. These results demonstrated that in *DMD^{mdx}* rat
565 skeletal muscle fibers Ca²⁺ homeostasis is altered with a higher Ca²⁺ influx through the
566 sarcolemma and intracellular Ca²⁺ overload.

567 One of the main objectives of the present work was to determine whether TRPC1 and TRPC3
568 channels could represent alternative or complementary therapeutic targets to rAAV-MD
569 based treatments of DMD. For TRPC1, this was observed only after 7 months of age when the
570 dystrophic rats already exhibit a marked phenotype(27). On the other hand, as soon as 1.5
571 months of age, when the dystrophic is milder, *DMD^{mdx}* rat muscle TRPC3 protein expression
572 was already 2-fold higher than in WT rats. Interestingly, in both genotypes TRPC3 protein level
573 decreased and stabilized between 3 to 7 months of age. During this period of age, the

574 expression of TRPC3 was still around 2-fold higher in dystrophic rats than in healthy ones.
575 These results showed, firstly that TRPC3 plays an important role in the post-natal development
576 of the rat EDL muscle, and secondly that TRPC3 is involved in the SPCa increase we observed
577 in EDL muscle fibers from the *DMD^{mdx}* rat model. This is reinforced by the very similar
578 evolution of the SPCa and TRPC3 protein expression in WT and *DMD^{mdx}* rats aged of 1.5 to 7
579 months. It is well documented that trafficking is a critical mode by which plasma membrane
580 localization and surface expression of TRPC channels are regulated(47). Thus, beside
581 expression modification, involvement of TRPCs in SPCa increase may depend on TRPCs
582 subcellular translocations from intracellular. However, conflicting results have been obtained
583 concerning the subcellular localization of TRPCs in striated muscles(48, 49). Moreover, in
584 striated muscle cells the plasma membrane is not restricted to the periphery but also forms
585 intracellular invaginations called T-tubules. Therefore, in immunofluorescent confocal images,
586 a channel revealed solely in the center of the cell could be expressed in the T-tubule walls and
587 be directly involved in ion influx. In the present study, we focused our analysis on the
588 comparison between WT and *DMD^{mdx}* muscle fibers. The objective was to determine if there
589 was a difference between the 2 genotypes in the proportion of TRPC channels expressed at
590 the level of the peripheral sarcolemma compared to the center of the cells. But we did not
591 find any subcellular localization differences of TRPC1 nor TRPC3 between dystrophic and
592 healthy EDL muscle fibers.

593 Thus, SPCa increase may not due to translocation of TRPC3 from intracellular vesicles to
594 sarcolemma, but rather relies mainly on TRPC3 protein level and/or activity increases. The
595 involvement of TRPC3 to SPCa was reinforced by the inhibition of divalent cation entrance by
596 the specific inhibitor Pyr10(33). Although SPCa was more than 2-fold higher in dystrophic
597 muscle fibers as compared to WT ones at 4 months of age, a similar SPCa proportion was
598 inhibited in both genotypes. This indicated that the main part of SPCa increase in *DMD^{mdx}* rat
599 muscle fibers was related to TRPC3. This leads to suggest that TRPC3 channels were involved
600 in the pathogenesis process itself. This is reinforced by the noteworthy work published by
601 Millay and colleagues in 2009, showing that overexpression of TRPC3 specifically in skeletal
602 muscle induced muscular dystrophy in WT mouse(50). Therefore, inhibition of TRPC3 activity
603 and/or expression could lead to reduce the dystrophic process in DMD.

604 One striking result of the present study was that TRPC3 protein expression increased in
605 *DMD^{mdx}* rat skeletal muscles whereas the level of the mRNA coding for this channel rather
606 decreased, in particular at 1.5 months of age. Although further experiments are needed to
607 clarify this apparent discrepancy, it may be explained by 2 main but nonexclusive hypotheses:
608 (i) the expression of different isoforms that may be detected at the protein level but not at
609 the mRNA one, and (ii) the existence of post-translational protein changes that may modify
610 TRPC3 turn-over.

611 Importantly, the primers we used for RT-qPCR analysis were designed to amplify part of exon
612 2 of the TRPC3 mRNA from rat. This part of the unspliced TRPC3 mRNA is fully retrieved in
613 predicted and isolated TRPC3 mRNA isoforms that have been reported up to now (36, 51, 52).
614 Similarly, the monoclonal antibody from that was used in our study is directed to a peptide
615 constituted of the N-terminal one hundred first amino acids of the human TRPC3 protein. This
616 part of the protein is highly conserved between humans and rats whatever the splicing of the
617 coding mRNA. It is therefore likely that in the present study all the TRPC3 isoforms were
618 measured, at both the mRNA and the protein levels.

619 Our results clearly demonstrated that the *DMD^{mdx}* TRPC3 apparent MW (AMW) was 2 kDa
620 higher than the WT one. This difference of AMW may be due to the expression of different
621 TRPC3 isoforms. In the cerebellum from humans, guinea pigs, mice and rats, the team of Gary
622 D Housley identified a short isoform of TRPC3 (TRPC3c), resulting from alternative splicing of
623 exon 9(36, 51). They showed that TRPC3c mRNA was predominant in the cerebellum of these
624 species as compared to the full TRPC3 mRNA (TRPC3b). Interestingly, recombinant TRPC3c and
625 TRPC3b proteins expressed in HEK293 cells exhibited AMWs that were different about 3 to 4
626 kDa, a difference value of apparent AMW that is very closed to that we measured herein
627 between *DMD^{mdx}* and WT. However, gel electrophoresis of the amplicon corresponding to the
628 8 to 10 exons showed that TRPC3 mRNA transcripts from *DMD^{mdx}* and WT rat muscles both
629 conserved the exon 9. Thus, the difference in AMW of TRPC3 in *DMD^{mdx}* and WT muscles did
630 not depend on exon 9 splicing. Moreover, we saw no difference between EDL TRPC3 whole
631 cDNA *DMD^{mdx}* and WT rats, suggesting that the variation in AMW was not related to
632 differential TRPC3 mRNA splicing.

633 Post-translational modifications, such glycosylation and phosphorylation, may influence the
634 stability of proteins but also their western blot AMW. One N-glycosylation site and several

635 phosphorylation sites have been identified in TRPC3 protein(43). Such modifications may
636 influence TRPC3 stability, but they rather seem to regulate the channel basal activity(43).
637 Although further experiments are needed, the results obtained in the present study suggest
638 that TRPC3 is N-glycosylated in healthy muscle rat fibers and un-glycosylated in the *DMD^{mdx}*
639 ones. The higher AMW observed after the deglycosylation may be surprising at first glance,
640 since one may expect a decrease in protein weight. Nevertheless, WB protein separation not
641 only depends on the protein size but also on its conformation. Indeed, it has been previously
642 reported that adding N-glycosylation may induce a decrease in the AMW of a protein despite
643 an increased molecular weight (53). As TRPC3 unglycosylation has been reported to increase
644 channel activity(54), this is something than can explained the increased expression of TRPC3
645 and the subsequent dysregulations of Ca^{2+} homeostasis in EDL muscle fibers lacking
646 dystrophin expression.

647 One key objective of the present study was to determine whether TRPC3 could be a
648 therapeutic target to elaborated DMD treatment complementary to rAAV-based MD therapy.
649 In such a case, the uncomplete correction of TRPC3 expression and activity by the rAAV-based
650 MD therapy is a necessary condition to observe additive benefits. Gene transfer therapy based
651 on rAAV-MD systemic delivery is a promising approach and clinical trials using this strategy
652 are ungoing(12). The MD transgene we used in the present study is very closed to those used
653 in the three ongoing clinical trials. For instance, both MD and the Sarepta micro-dystrophin
654 transgenes contain N-terminus for binding to f-actin; spectrin repeats 1 to 3 and 24; hinges 1,
655 2, and 4; and the cysteine-rich domain(12). Both transgenes present skeletal muscle specific
656 promoters, and systemic injections of rAAV2/9.SP5.12-MD, *DMD^{mdx}* rat herein, and
657 rAAVrh74.MHCK7.micro-dystrophin, in humans for Sarepta, resulted in a MD expression in 80
658 to 90% of skeletal muscle fibers. Therefore, it could be concluded that the rAAV-MD based
659 treatment we used, was very closed to those used in ungoing clinical trials. In the present
660 study, we assessed for the first time the benefits of a rAAV-MD systemic delivery on the Ca^{2+}
661 homeostasis in skeletal muscle fibers lacking dystrophin expression. As excepted from the
662 intimate role of Ca^{2+} homeostasis alterations in DMD pathogenesis, we found that rAAV-MD
663 systemic injections significantly counteracted $[Ca^{2+}]_c$ and SPCa in skeletal muscle fibers.
664 Nevertheless, the benefit was only partial, and both $[Ca^{2+}]_c$ and SPCa were still be higher
665 despite MD expression. Interestingly, similar results were observed when comparing TRPC3

666 expression level between skeletal muscle fibers lacking dystrophin, WT or expressing MD. In
667 particular, TRPC3 expression was still elevated in skeletal muscle fibers transfected by rAAV-
668 MD. These results first reinforced the relation between DMD pathogenesis and TRPC3
669 expression alteration. On the other hand, it also led to suggest that pharmacological or
670 molecular strategies dedicated to inhibit TRPC3 channel expression and/or activity could be
671 effective after MD expression.

672

673 **Conclusion**

674 In the present study, we demonstrated early increases of $[Ca^{2+}]_c$ and SPCa in the EDL fast-
675 twitch muscles from DMD^{mdx} rats. This was accompanied by an increase in TRPC3 expression
676 at the protein level. Finally, we showed that rAAV-MD based treatment induced a high MD
677 expression level, but that was accompanied with significant but only partial prevention of
678 calcium homeostasis alterations, skeletal muscle force and TRPC3 protein overexpression.
679 These results show that correcting TRPC3 channel expression and/or activity appears to be a
680 promising approach as a single or as a rAAV-based complementary therapy to treat DMD.

681

682 **List of abbreviations**

683 **[Ca²⁺]_c**: Cytosolic calcium concentration

684 **AM**: Acetyl-methyl ester

685 **AMW**: Apparent molecular weight

686 **BMD**: Becker muscular dystrophy

687 **Cav-3**: Caveoline 3

688 **cDNA**: complementary DNA

689 **DMD**: Duchenne muscular dystrophy

690 **EDL**: *Extensor digitorum longus*

691 **MD**: Microdystrophin

692 **MH:** Malignant hyperthermia
693 **mRNA:** Messenger ribonucleic acid
694 **NPS :** Normal physiological solution
695 **PBS:** Phosphate buffer saline
696 **PFA :** Paraformaldehyde
697 **Pyr10:** N-(4-(3,5-bis(trifluoromethyl)-1H-pyrazole-1-yl)phenyl)-4-
698 methylbenzenesulfonamide
699 **rAAV:** Recombinant adeno-associated virus
700 **RT-qPCR:** Reverse transcription quantitative polychain reaction
701 **RyR:** Ryanodine receptor
702 **SPCa:** Sarcolemmal permeability to calcium
703 **SR:** Sarcoplasmic reticulum
704 **TRP:** Transient receptor potential
705 **TRPC:** Canonical transient receptor potential
706 **WT:** Wild-type

707

708

709 **Declarations**

710 **Ethics approval and consent to participate:** not applicable

711 **Consent for publication:** not applicable

712 **Availability of data and materials:** The datasets used and/or analysed during the current
713 study are available from the corresponding author on reasonable request.

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721 VF, MA, ML, GT, AL, and BF; Methodology: AC and BF; Project administration: AC, CLG, and BF;
722 Software: BF; Resources: IA; Supervision: TL, CH, CLG and BF; Validation: CLG and BF;

723 Visualization: AC and BF; Writing – original draft: AC, CLG and BF; Writing – review & editing:
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733

734 **References**

- 735 1. Mendell JR, Lloyd-Puryear M. Report of MDA muscle disease symposium on newborn
736 screening for Duchenne muscular dystrophy. *Muscle Nerve*. 2013;48(1):21-6.
- 737 2. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of
738 the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD
739 gene in normal and affected individuals. *Cell*. 1987;50(3):509-17.
- 740 3. Blake DJ, Weir A, Newey SE, Davies KE. Function and genetics of dystrophin and dystrophin-
741 related proteins in muscle. *Physiol Rev*. 2002;82(2):291-329.
- 742 4. Eagle M, Bourke J, Bullock R, Gibson M, Mehta J, Giddings D, et al. Managing Duchenne
743 muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving
744 survival. *Neuromuscul Disord*. 2007;17(6):470-5.
- 745 5. Blankinship MJ, Gregorevic P, Chamberlain JS. Gene therapy strategies for Duchenne muscular
746 dystrophy utilizing recombinant adeno-associated virus vectors. *Mol Ther*. 2006;13(2):241-9.
- 747 6. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of
748 immunocompetent mice by adeno-associated virus vector. *J Virol*. 1996;70(11):8098-108.
- 749 7. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1-9 mediated gene
750 expression and tropism in mice after systemic injection. *Mol Ther*. 2008;16(6):1073-80.
- 751 8. Rivera VM, Gao GP, Grant RL, Schnell MA, Zoltick PW, Rozamus LW, et al. Long-term
752 pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene
753 transfer. *Blood*. 2005;105(4):1424-30.
- 754 9. Athanasopoulos T, Graham IR, Foster H, Dickson G. Recombinant adeno-associated viral (rAAV)
755 vectors as therapeutic tools for Duchenne muscular dystrophy (DMD). *Gene Ther*. 2004;11 Suppl
756 1:S109-21.
- 757 10. Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, Phelps SF, et al. Modular flexibility
758 of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. *Nat Med*.
759 2002;8(3):253-61.
- 760 11. Le Guiner C, Servais L, Montus M, Larcher T, Fraysse B, Moullec S, et al. Long-term
761 microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nat*
762 *Commun*. 2017;8:16105.
- 763 12. Mendell JR, Sahenk Z, Lehman K, Nease C, Lowes LP, Miller NF, et al. Assessment of Systemic
764 Delivery of rAAVrh74.MHCK7.micro-dystrophin in Children With Duchenne Muscular Dystrophy: A
765 Nonrandomized Controlled Trial. *JAMA Neurol*. 2020;77(9):1122-31.
- 766 13. Bushby KM, Goodship JA, Nicholson LV, Johnson MA, Haggerty ID, Gardner-Medwin D.
767 Variability in clinical, genetic and protein abnormalities in manifesting carriers of Duchenne and Becker
768 muscular dystrophy. *Neuromuscul Disord*. 1993;3(1):57-64.
- 769 14. Verhaart IEC, Aartsma-Rus A. Therapeutic developments for Duchenne muscular dystrophy.
770 *Nat Rev Neurol*. 2019;15(7):373-86.
- 771 15. Allen DG, Whitehead NP, Froehner SC. Absence of Dystrophin Disrupts Skeletal Muscle
772 Signaling: Roles of Ca²⁺, Reactive Oxygen Species, and Nitric Oxide in the Development of Muscular
773 Dystrophy. *Physiol Rev*. 2016;96(1):253-305.
- 774 16. Emery AE, Burt D. Intracellular calcium and pathogenesis and antenatal diagnosis of Duchenne
775 muscular dystrophy. *Br Med J*. 1980;280(6211):355-7.
- 776 17. Harisseh R, Chatelier A, Magaud C, Deliot N, Constantin B. Involvement of TRPV2 and SOCE in
777 calcium influx disorder in DMD primary human myotubes with a specific contribution of alpha1-
778 syntrophin and PLC/PKC in SOCE regulation. *Am J Physiol Cell Physiol*. 2013;304(9):C881-94.
- 779 18. Gailly P. TRP channels in normal and dystrophic skeletal muscle. *Curr Opin Pharmacol*.
780 2012;12(3):326-34.
- 781 19. Inoue R, Kurahara L-H, Hiraishi K. TRP channels in cardiac and intestinal fibrosis. *Seminars in*
782 *Cell & Developmental Biology*. 2019;94:40-9.

- 783 20. Numaga-Tomita T, Oda S, Shimauchi T, Nishimura A, Mangmool S, Nishida M. TRPC3 Channels
784 in Cardiac Fibrosis. *Front Cardiovasc Med.* 2017;4:56.
- 785 21. Gervasio OL, Whitehead NP, Yeung EW, Phillips WD, Allen DG. TRPC1 binds to caveolin-3 and
786 is regulated by Src kinase - role in Duchenne muscular dystrophy. *J Cell Sci.* 2008;121(Pt 13):2246-55.
- 787 22. Lopez JR, Uryash A, Faury G, Esteve E, Adams JA. Contribution of TRPC Channels to Intracellular
788 Ca(2+) Dyshomeostasis in Smooth Muscle From mdx Mice. *Front Physiol.* 2020;11:126.
- 789 23. Lopez JR, Kaura V, Hopkins P, Liu X, Uryach A, Adams J, et al. Transient Receptor Potential
790 Cation Channels and Calcium Dyshomeostasis in a Mouse Model Relevant to Malignant Hyperthermia.
791 *Anesthesiology.* 2020;133(2):364-76.
- 792 24. Gurnaney H, Brown A, Litman RS. Malignant Hyperthermia and Muscular Dystrophies.
793 *Anesthesia & Analgesia.* 2009;109(4):1043-8.
- 794 25. Cheung KK, Yeung SS, Au SW, Lam LS, Dai ZQ, Li YH, et al. Expression and association of TRPC1
795 with TRPC3 during skeletal myogenesis. *Muscle Nerve.* 2011;44(3):358-65.
- 796 26. Woo JS, Lee KJ, Huang M, Cho CH, Lee EH. Heteromeric TRPC3 with TRPC1 formed via its
797 ankyrin repeats regulates the resting cytosolic Ca²⁺ levels in skeletal muscle. *Biochem Biophys Res*
798 *Commun.* 2014;446(2):454-9.
- 799 27. Larcher T, Lafoux A, Tesson L, Remy S, Thepenier V, Francois V, et al. Characterization of
800 dystrophin deficient rats: a new model for Duchenne muscular dystrophy. *PLoS One.*
801 2014;9(10):e110371.
- 802 28. Kornegay JN. The golden retriever model of Duchenne muscular dystrophy. *Skelet Muscle.*
803 2017;7(1):9.
- 804 29. Sengupta P. The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med.* 2013;4(6):624-
805 30.
- 806 30. Moorwood C, Liu M, Tian Z, Barton ER. Isometric and eccentric force generation assessment
807 of skeletal muscles isolated from murine models of muscular dystrophies. *J Vis Exp.* 2013(71):e50036.
- 808 31. Fraysse B, Liantonio A, Cetrone M, Burdi R, Pierno S, Frigeri A, et al. The alteration of calcium
809 homeostasis in adult dystrophic mdx muscle fibers is worsened by a chronic exercise in vivo. *Neurobiol*
810 *Dis.* 2004;17(2):144-54.
- 811 32. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved
812 fluorescence properties. *J Biol Chem.* 1985;260(6):3440-50.
- 813 33. Schleifer H, Doleschal B, Lichtenegger M, Oppenrieder R, Derler I, Frischauf I, et al. Novel
814 pyrazole compounds for pharmacological discrimination between receptor-operated and store-
815 operated Ca(2+) entry pathways. *Br J Pharmacol.* 2012;167(8):1712-22.
- 816 34. Liu JX, Høglund AS, Karlsson P, Lindblad J, Qaisar R, Aare S, et al. Myonuclear domain size and
817 myosin isoform expression in muscle fibres from mammals representing a 100,000-fold difference in
818 body size. *Exp Physiol.* 2009;94(1):117-29.
- 819 35. Sabourin J, Bartoli F, Antigny F, Gomez AM, Benitah JP. Transient Receptor Potential Canonical
820 (TRPC)/Orai1-dependent Store-operated Ca²⁺ Channels: NEW TARGETS OF ALDOSTERONE IN
821 CARDIOMYOCYTES. *J Biol Chem.* 2016;291(25):13394-409.
- 822 36. Kim Y, Wong AC, Power JM, Tadros SF, Klugmann M, Moorhouse AJ, et al. Alternative splicing
823 of the TRPC3 ion channel calmodulin/IP3 receptor-binding domain in the hindbrain enhances cation
824 flux. *J Neurosci.* 2012;32(33):11414-23.
- 825 37. Foster H, Sharp PS, Athanasopoulos T, Trollet C, Graham IR, Foster K, et al. Codon and mRNA
826 sequence optimization of microdystrophin transgenes improves expression and physiological outcome
827 in dystrophic mdx mice following AAV2/8 gene transfer. *Mol Ther.* 2008;16(11):1825-32.
- 828 38. Koo T, Malerba A, Athanasopoulos T, Trollet C, Boldrin L, Ferry A, et al. Delivery of AAV2/9-
829 microdystrophin genes incorporating helix 1 of the coiled-coil motif in the C-terminal domain of
830 dystrophin improves muscle pathology and restores the level of alpha1-syntrophin and alpha-
831 dystrobrevin in skeletal muscles of mdx mice. *Hum Gene Ther.* 2011;22(11):1379-88.
- 832 39. Li X, Eastman EM, Schwartz RJ, Draghia-Akli R. Synthetic muscle promoters: activities
833 exceeding naturally occurring regulatory sequences. *Nat Biotechnol.* 1999;17(3):241-5.

- 834 40. D'Costa S, Blouin V, Broucque F, Penaud-Budloo M, Francois A, Perez IC, et al. Practical
835 utilization of recombinant AAV vector reference standards: focus on vector genomes titration by free
836 ITR qPCR. *Mol Ther Methods Clin Dev.* 2016;5:16019.
- 837 41. Whitehead NP, Bible KL, Kim MJ, Odom GL, Adams ME, Froehner SC. Validation of
838 ultrasonography for non-invasive assessment of diaphragm function in muscular dystrophy. *J Physiol.*
839 2016;594(24):7215-27.
- 840 42. Treves S, Jungbluth H, Voermans N, Muntoni F, Zorzato F. Ca²⁺ handling abnormalities in early-
841 onset muscle diseases: Novel concepts and perspectives. *Seminars in Cell & Developmental Biology.*
842 2017;64:201-12.
- 843 43. Liu X, Yao X, Tsang SY. Post-Translational Modification and Natural Mutation of TRPC Channels.
844 *Cells.* 2020;9(1).
- 845 44. Partridge TA. The mdx mouse model as a surrogate for Duchenne muscular dystrophy. *FEBS J.*
846 2013;280(17):4177-86.
- 847 45. Berchtold MW, Brinkmeier H, Muntener M. Calcium ion in skeletal muscle: its crucial role for
848 muscle function, plasticity, and disease. *Physiol Rev.* 2000;80(3):1215-65.
- 849 46. Rosenberg H, Pollock N, Schiemann A, Bulger T, Stowell K. Malignant hyperthermia: a review.
850 *Orphanet J Rare Dis.* 2015;10:93.
- 851 47. Souza LBd, Ambudkar IS. Trafficking mechanisms and regulation of TRPC channels. *Cell*
852 *Calcium.* 2014;56(2):43-50.
- 853 48. Berbey C, Weiss N, Legrand C, Allard B. Transient receptor potential canonical type 1 (TRPC1)
854 operates as a sarcoplasmic reticulum calcium leak channel in skeletal muscle. *J Biol Chem.*
855 2009;284(52):36387-94.
- 856 49. Tajeddine N, Zanou N, Van Schoor M, Lebacq J, Gailly P. TRPC1: subcellular localization? *J Biol*
857 *Chem.* 2010;285(5):1e1; author reply 1e2.
- 858 50. Millay DP, Goonasekera SA, Sargent MA, Maillet M, Aronow BJ, Molkenkin JD. Calcium influx is
859 sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. *Proceedings of the*
860 *National Academy of Sciences.* 2009;106(45):19023-8.
- 861 51. Cederholm JME, Kim Y, von Jonquieres G, Housley GD. Human Brain Region-Specific
862 Alternative Splicing of TRPC3, the Type 3 Canonical Transient Receptor Potential Non-Selective Cation
863 Channel. *Cerebellum.* 2019;18(3):536-43.
- 864 52. Sayers EW, Cavanaugh M, Clark K, Ostell J, Pruitt KD, Karsch-Mizrachi I. GenBank. *Nucleic Acids*
865 *Research.* 2019;48(D1):D84-D6.
- 866 53. Tams JW, Vind J, Welinder KG. Adapting protein solubility by glycosylation. N-glycosylation
867 mutants of *Coprinus cinereus* peroxidase in salt and organic solutions. *Biochim Biophys Acta.*
868 1999;1432(2):214-21.
- 869 54. Dietrich A, Mederos y Schnitzler M, Emmel J, Kalwa H, Hofmann T, Gudermann T. N-Linked
870 Protein Glycosylation Is a Major Determinant for Basal TRPC3 and TRPC6 Channel Activity. *Journal of*
871 *Biological Chemistry.* 2003;278(48):47842-52.

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878 **Figure legends**

879 **Figure 1: Histopathological evaluation of skeletal muscles from *DMD^{mdx}* rats died after**
880 **malignant hyperthermia syndrome.** After specific staining for calcium (left panel: alizarin red
881 and right panel: von Kossa), we observed differences of dye affinity between normal
882 appearing fibers and round hypercontracted fibers that unlighted disruption of calcium
883 concentrations between intracellular and extracellular compartments. Note the severe
884 endomysial edema typical of hyperthermia syndrome. Bar = 100 μm .

885

886 **Figure 2: Evolution of resting calcium homeostasis in EDL muscle fibers from WT and *DMD^{mdx}***
887 **rats during post-natal development. A-** Resting cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$) **B-**
888 Resting sarcolemmal permeability to calcium (SPCa). $[\text{Ca}^{2+}]_c$ and SPCa were measured in Fura2
889 loaded single fibers from mechanically isolated bundles of EDL muscles in WT and *DMD^{mdx}* rats
890 of 1.5 to 7.0 months of age. SPCa was measured using the manganese quenching of Fura2
891 technique. Each bar corresponds to mean value \pm SEM measured in n single fibers from N
892 animals (n/N are indicated at the bottom the bars). *: significantly different from mean value
893 measured in age-matched WT animals. \$: significantly different from mean value measured in
894 animals from the same genotype of 1.5 months of age. $P < 0.05$, Two-way ANOVA and Fisher-
895 LSD post-hoc test for pairwise comparisons.

896

897 **Figure 3: Evolution of TRPC1 and TRPC3 mRNA and protein expression levels in EDL muscles**
898 **from WT and *DMD^{mdx}* rats during post-natal development. A and B:** Relative levels of TRPC1
899 and TRPC3 mRNAs expression in EDL muscle extracts from WT and *DMD^{mdx}* rats of 1.5 to 7

900 months of age. Results were normalized to HPRT1 mRNA expression. **C and D:** Relative levels
901 of protein expression of TRPC1 and TRPC3 in EDL muscle homogenates from WT and *DMD^{mdx}*
902 rats of 1.5 to 7 months of age. Representative films exposed to immunoblots are reported
903 below histograms (obtained for muscle extracts from 6 WT and 6 *DMD^{mdx}* rats of 1.5 months
904 of age). **A, B, C and D:** Each bar represents mean value \pm SEM calculated for N animals (N
905 indicated at the bottom of the corresponding bar). For each age and genotype, immunoblots
906 were realized at least in 3 replicates. *: significantly different from mean value measured in
907 age-matched WT animals. \$: significantly different from mean value measured in animals from
908 the same genotype of 1.5 months of age. $P < 0.05$, Kruskal-Wallis and bilateral Conover-Iman
909 post-hoc test.

910

911 **Figure 4: Subcellular localization of TRPC1 and TRPC3 expression in EDL muscle fibers from**
912 **WT and *DMD^{mdx}* rats. A and B:** Confocal images acquired in longitudinal axis and at the largest
913 diameter of EDL muscle fibers from WT and *DMD^{mdx}* rats of 4.5 months of age. Fibers were
914 fixed, permeabilized and co-labeled by immunofluorescence to highlight TRPC1 or TRPC3
915 protein expression and compare it to RyR and Cav-3 ones. A and B are montages of pictures
916 cropped from original images acquired using a X60 objective. Scale bar are reported in white
917 and corresponds to 14 μm . **C and D:** TRPC1 and TRPC3, respectively, sub-peripheral
918 sarcolemma area (peripheral sarcolemma labeled using Cav-3 antibodies and 5 μm beneath
919 area) to fiber center (the rest of the intracellular area) density signal ratio. Each bar represents
920 mean \pm SEM value of density ratios measured in n muscle EDL muscle fibers and N animals
921 (n/N are noted at the bottom of each bar). Potential significant differences were evaluated
922 using Student's unpaired *t*-test.

923

924 **Figure 5: TRPC3 apparent molecular weight (AMW) and Pyr10-induced SPCa inhibition**

925 **changes between WT and DMD^{mdx} rats in EDL muscle. A:** Immunoblot of TRPC3 of EDL muscle

926 homogenates from 8 different rats (4 WT and 4 DMD^{mdx}) of 4.5 months of age. WT and DMD^{mdx}

927 homogenates were alternated in the different lanes to underline the difference in AMW. **B:**

928 Relation between glycosylation and phosphorylation status and AMW. Immunoblots of TRPC3

929 of EDL muscle from the same DMD^{mdx} and WT rats in Control conditions (Control) or after

930 deglycosylation (Deglyco), acetone treatment only (Acetone) and, acetone treatment and

931 dephosphorylation (Dephospho). **C:** AMW of TRPC3 measured in immunoblots fo EDL muscle

932 homogenates from 4 WT and 4 DMD^{mdx} rats. Measures were made for at least two replicates

933 for each rat. Bars represent mean \pm values. *: significantly different from WT value, Mann-

934 Whitney test, $P < 0.05$. **D:** Agarose gel electrophoresis showing RT-PCR amplicons corresponding

935 to the exon 8 to exon 10 of TRPC3 mRNA from EDL muscles of WT and DMD^{mdx} rats, and a

936 plasmid expressing the full-length rat TRPC3 mRNA isoform. *bp*: size in base pairs. **E:** Pyr10-

937 induced SPCa inhibition expressed as fold change of the mean value of SPCa measured in

938 control conditions in WT EDL muscle fibers. Bars represent mean \pm values of SPCa measured

939 in n EDL muscle fibers from N WT and DMD^{mdx} rats aged of 4.5 months (n/N are indicated

940 under brackets) before (filled bars) and after (empty bars) application of Pyr10 compound. *:

941 significantly different from WT value measured in the same conditions, Two-way ANOVA and

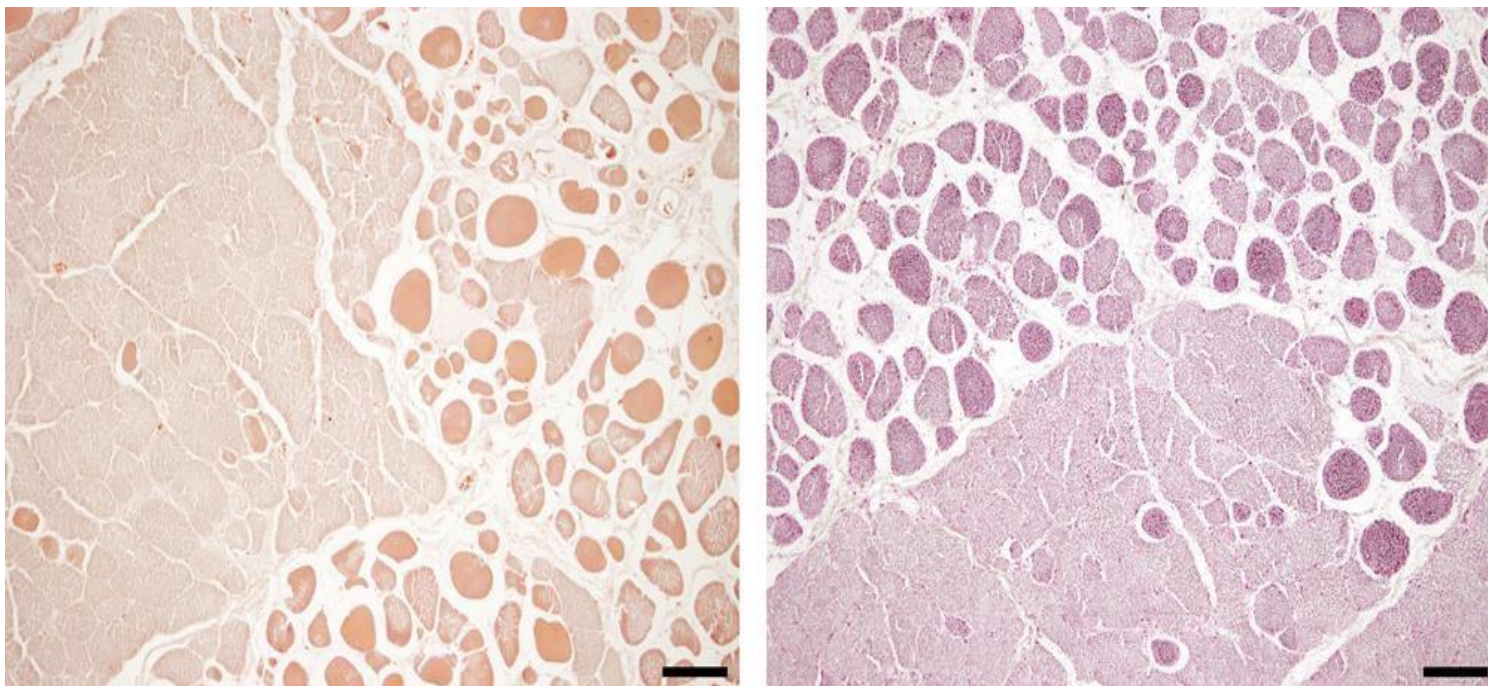
942 Fisher post-hoc test, $P < 0.05$. \$: significantly different from mean SPCa value measured in

943 control conditions in the same genotype, Paired Student's *t*-test, $P < 0.05$.

944

945 **Figure 6: Alterations of calcium homeostasis, TRPC3 mRNA and protein expression levels,**
946 **and skeletal muscle contraction before and after AAV2/9-MD treatment in DMD^{mdx} rats. A:**
947 Typical immunoblot labeled to reveal WT dystrophine (Dyst), MD, TRPC3 and GAPDH in EDL
948 and diaphragm (Dia) muscle homogenates obtained from Vehicle-WT, Vehicle- DMD^{mdx} and
949 MD- DMD^{mdx} rats. **B:** Resting cytosolic calcium concentration ($[Ca^{2+}]_c$) and **C:** Resting
950 sarcolemmal permeability to calcium (SPCa). $[Ca^{2+}]_c$ and SPCa were measured in Fura2 loaded
951 single fibers from mechanically isolated bundles of EDL muscles in vehicle-treated WT and
952 DMD^{mdx} rats, and AAV2/9-MD treated DMD^{mdx} rats (MD- DMD^{mdx}). Bars represent mean \pm SEM
953 values of $[Ca^{2+}]_c$ and SPCa in n EDL muscle fibers from N animals (n/N are indicated at the
954 bottom of the bars). *: significantly different from mean value measured in WT muscle fibers;
955 \$: significantly different from mean value measured in Vehicle- DMD^{mdx} muscle fibers; One-way
956 ANOVA and Fisher LSD post-hoc test, $P < 0.05$. **D:** Maximal diaphragm contraction amplitude
957 measured *in vivo* by echography. **E:** Whole muscle maximal tetanic tension measured *in vitro*
958 in EDL from Vehicle-WT, Vehicle- DMD^{mdx} and MD- DMD^{mdx} rats. **F and G:** Expression of TRPC3
959 mRNAs and proteins, measured by RTq-PCR and western blot, respectively, in EDL muscle
960 extracts from Vehicle-WT, Vehicle- DMD^{mdx} and MD- DMD^{mdx} rats. **D, E, F and G:** bars represent
961 \pm SEM values measured in N animals (N are noted at the bottom of the bars). *: significantly
962 different from mean value measured in WT muscle; \$: significantly different from mean value
963 measured in Vehicle- DMD^{mdx} muscles; Kruskal-Wallis test and Conover-Iman post-hoc test,
964 $P < 0.05$.
965

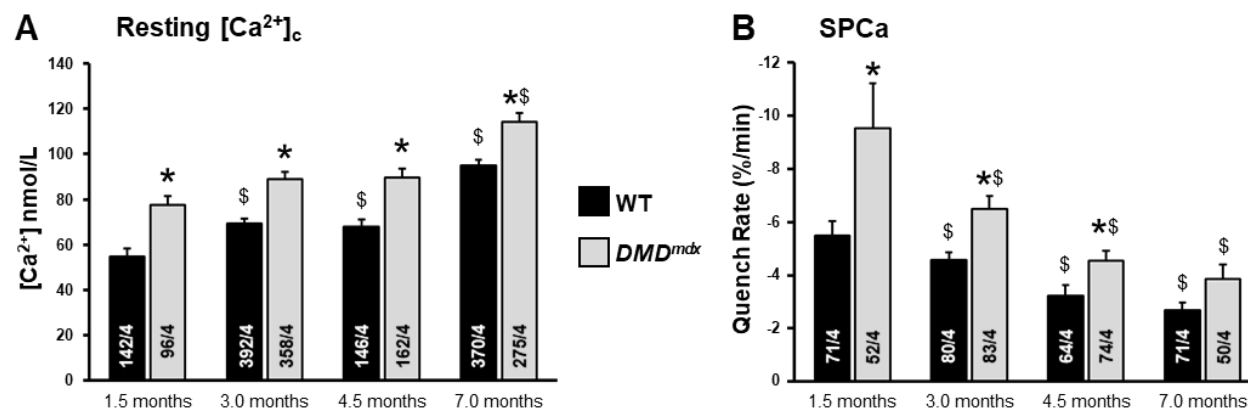
966 **Figure 1**



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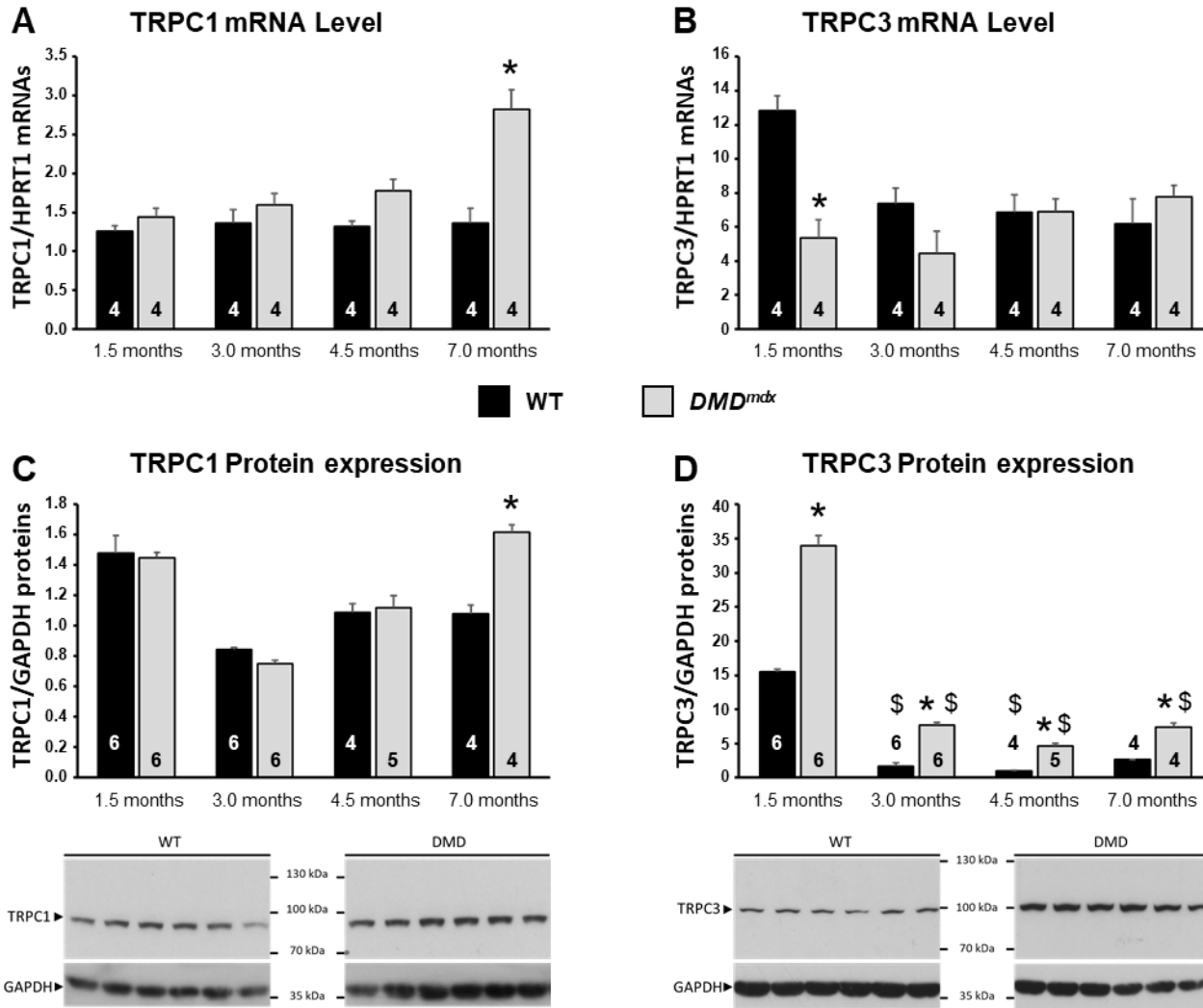
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969 Figure 2



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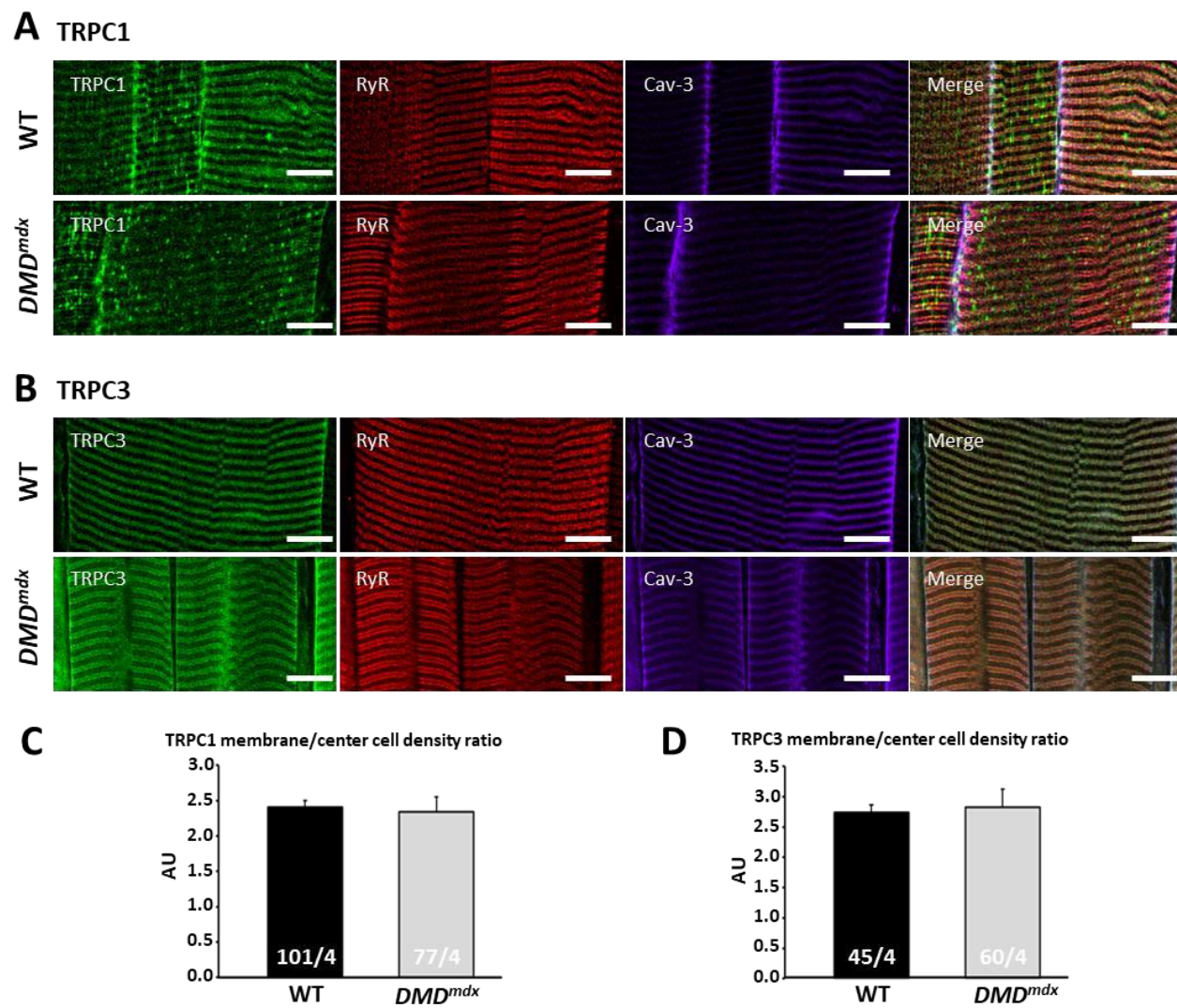
978 Figure 3



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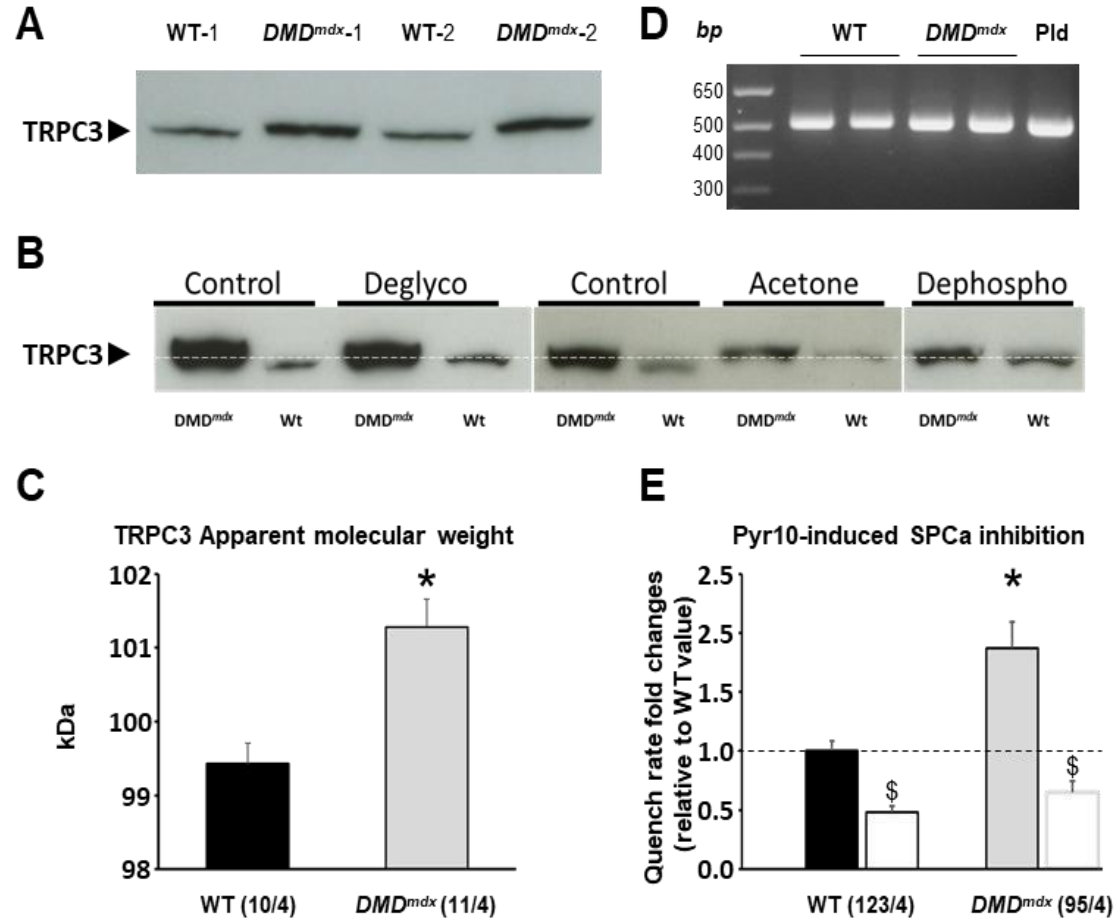
981 Figure 4



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Figure 5

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987 Figure 6

