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Desmin prevents muscle wasting, exaggerated weakness and fragility, and fatigue in dystrophic *mdx* mouse

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Key points

Desmin is like dystrophin associated to costameric structures bridging sarcomeres to extracellular matrix.

A deletion of the desmin gene in *mdx* mice (DKO mice) induces a marked muscle weakness and fatigue resistance, as compared to *mdx* mice.

Muscle fragility (higher susceptibility to contraction-induced injury) was also aggravated in DKO mice as compared to *mdx* mice.

In contrast to *mdx* mice, the DKO mice did not undergo muscle hypertrophy.

Desmin cDNA transfer with adeno-associated virus in newborn *mdx* mice improved muscle weakness.

Overall, desmin plays important and beneficial roles on muscle wasting, performance, and fragility in dystrophic muscle.

Abstract

Duchenne muscular dystrophy (DMD) is a severe neuromuscular disease, caused by dystrophin deficiency. Desmin is like dystrophin associated to costameric structures bridging sarcomeres to extracellular matrix that contribute to muscle function. In the present study, we wanted to gain further insight into the roles of desmin which expression is increased in the muscle from the mouse *mdx* DMD model. We show that a deletion of the desmin gene (*Des*) in *mdx* mice (DKO, mdx:desmin-/-) induces a marked muscle weakness, i.e, a reduced absolute maximal force production, and an increased fatigue as compared to *mdx* mice. Fragility, i.e., higher susceptibility to contraction-induced injury, was also aggravated in DKO mice as compared to *mdx* mice, despite promotion of supposedly less fragile muscle fibres in

DKO mice, and this worsening of fragility was related to a decreased muscle excitability. Moreover, in contrast to *mdx* mice, the DKO mice did not undergo a muscle hypertrophy because of smaller and less numerous fibres, with reduced percentage of centronucleated fibres, thus explaining the severe muscle weakness. Interestingly, Desmin cDNA transfer with adeno-associated virus in newborn *mdx* mice improved specific maximal force normalized to muscle weight. Overall, desmin plays important and beneficial roles on muscle wasting, performance, and fragility in dystrophic *mdx* mice, that differ, at least in large part, from those observed in healthy muscle.

Introduction

Duchenne muscular dystrophy (DMD) is a severe and lethal neuromuscular disease, caused by mutations in the dystrophin gene leading to the absence or reduced function of dystrophin protein in particular in skeletal muscle. Dystrophin is a part of the dystrophin-glycoprotein complex, with dystroglycan and sarcoglycan proteins, and localizes primarily in skeletal muscle to costameres that link intracellular matrix, i.e., F-actin and desmin intermediate filaments, to the sarcolemma and extracellular matrix (Ervasti, 2003; Hughes et al., 2015). Costameres play important role in muscle function since they stabilize the sarcolemma during contraction and are involved in lateral force transmission (Bloch and Gonzalez-Serratos, 2003; Ervasti, 2003; Hughes et al., 2015; Monti et al., 1999). In the absence of dystrophin, the muscle is more fragile, i.e., more susceptible to contraction-induced injury, and weak (reduced maximal force production)(Head et al., 1994; Moens et al., 1993), together with a reduced lateral force transmission (Ramaswamy et al., 2011). A recent study supports a role for the R1-3 region of dystrophin in modulating lateral force transmission and fragility (Nelson et al., 2018). A rapid loss of dystrophin labelling has also been reported following contraction-induced injury (Lovering and De Deyne, 2004). The exaggerated fragility in DMD muscle leads to cycles of degeneration and regeneration with increased percentage of centronucleated muscle fibres and progressive muscle wasting that worsens weakness. In contrast, the *mdx* mouse, a well-known murine model of DMD with exon 23 mutation in the dystrophin gene (*Dmd*), does not present a marked reduction of lifespan (Chamberlain et al., 2007) and muscle wasting but instead shows a surprising muscle hypertrophy (Duddy et al., 2015; Faber et al., 2014; McGreevy et al., 2015).

Together with the dystrophin-glycoprotein complex, integrins are also located to costamere and bridge the laminin of the extracellular matrix to F-actin and desmin intermediate filaments (Hughes et al., 2015). The knockout of γ-actin, one constituent of F-actin gene, in healthy mice causes muscle fragility and weakness (Sonnemann et al., 2006). The knockout of desmin gene in healthy mouse induces muscle weakness, variable effects on fragility, sarcomere disorganization, atrophy and increased percentage of centronucleated muscle fibres (a marker of degeneration-regeneration)(Agbulut et al., 2001; Boriek et al., 2001; Li et al., 1997; Lovering et al., 2011; Meyer et al., 2013; Sam et al., 2000), indicating that desmin also plays important beneficial roles in healthy muscle, such as dystrophin, and γ-actin. Moreover, desmin immunostaining is rapidly loss after contraction-induced injury in healthy mice (Lieber et al., 1996). Desmin forms intermediate filaments that encircles Z-disks and help to maintain sarcomere structure. They also interconnect sarcomeres to integrin-based focal adhesion. It is not yet known whether the roles of desmin on muscle performance and fragility are different in murine DMD models.

Like DMD modifiers such as the dystrophin paralogue utrophin and α7-integrin (Hightower and Alexander, 2018; McGreevy et al., 2015), desmin expression is increased in the *mdx* mouse (Banks et al., 2014). A DMD modifier which expression is increased in the absence of dystrophin, to likely partly compensate dystrophin deficiency, can be characterized by the additional following points: (i) constitutive inactivation of the gene worsens the dystrophic features, and (ii) its experimental induced-overexpression improves dystrophic features (McGreevy et al., 2015). It is not yet known whether desmin could be considered also as *bona fide* DMD modifier.

A recent study reported that the absence of desmin reduces the percentage of centronucleated

muscle fibres in the *mdx*^{4cv} mice showing a mutation in *DMD* exon 53 (Banks et al., 2014), supporting the idea that desmin aggravates some dystrophic histologic features. Thus, the aim of this study was to gain further insight into the physiological roles of desmin in the absence of dystrophin, and also to know if desmin is a DMD modifier, in regard to functional dystrophic features. First, we generated *mdx*:desmin double knock-out mice (DKO), to test the hypothesis that constitutive inactivation of the desmin gene aggravates functional dystrophic features, i.e., weakness and fragility, as compared to *mdx* mice. Secondly, we wanted to determine whether desmin haploinsufficiency (*mdx*:desmin+/- mice) rescues the potential severe DKO phenotype, as compared to DKO mice. Thirdly, we tested the hypothesis that the delivery of desmin gene (*Des*) using adeno-associated virus (AAV) vector (AAV-Des) into DKO muscle improves the potential severe dystrophic features, as compared to DKO mice. Lastly, we examined the possibility that AAV-Des injected into *mdx* muscle improves the mild functional dystrophic features of the *mdx* mice. Together, our results indicated that desmin have beneficial roles on locomotor muscle performance and fragility in dystrophic mdx mice, that differ, at least in large part, from those in healthy mice.

Methods

Ethical approval

Breeding and housing of the animals was carried out in the departmental animal facility, with free access to water and rodent laboratory chow. The animal facility was specific pathogen free, with 12h-light/12h-dark cycle and mice were 3-5 per cage. All procedures were performed in accordance with National and European legislations, under the license A751315 (French Ministry of National Education, Higher Education and Research) and the approved guidelines and the policy and regulations on animal experimentation of The Journal of Physiology (Grundy, 2015). To examine the role of desmin in *mdx* mice, we generated desmin-deficient *mdx* mice by crossing previously generated desmin knock-out (DesKO) (Li et al., 1996, 1997) with *mdx* mice (C57BL⁄10ScSn-mdx⁄J). We first crossed male DesKO mice (C57Bl/6 strain) with female *mdx* mice (C57Bl/10 strain) to obtain double heterozygous *mdx*:desmin (*mdx*:Des+/-) female and *mdx*:heterozygous desmin (*mdx*:Des+/-) male mice with hybrid background (C57Bl/6 x C57Bl/10). The resulting mice were inbred for at least five generations to obtain parents mice. Double knock-out (DKO) mice, lacking both dystrophin and desmin (*mdx*:Des-/-), were obtained by inbreeding *mdx*:Des+/- mice. The genotype of mice was determined using standard PCR as described previously (Li et al., 1996; Shin et al., 2011). The three types of new-born mice (*mdx*:Des+/+, *mdx*:Des+/- and DKO)(all hybrid background C57Bl/6 x C57Bl/10) were obtained in the expected Mendelian ratio. DKO mice are viable but smaller than the two other genotypes and with reduced lifespan. We also used wild-type mice expressing both dystrophin and desmin (C57Bl/10 strain) obtained from Jackson Laboratories (C57). In some experiments, to evaluate muscle regeneration,

cardiotoxin (10 µM, 50 µl), a myotoxic agent, was injected into Tibialis anterior (TA) muscles from 1-month old DKO, and mice were studied 1 month after (Mouisel et al., 2010). We used a pool of males and females DKO mice and the other genotypes are males. Mice from the different genotypes are age-matched. After contractile measurements, the animals were killed by cervical dislocation and muscles were weighed.

Weakness and fragility against lengthening contractions

Maximal tetanic isometric force (weakness) and susceptibility to contraction-induced injury (fragility) were evaluated by measuring the *in-situ* TA muscle contraction in response to nerve stimulation, as described previously (Ferry et al., 2015). Mice were anesthetized using pentobarbital (60 mg/kg ip). Body temperature was maintained at 37°C using radiant heat. The knee and foot were fixed with pins and clamps and the distal tendon of the muscle was attached to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific, Aurora, Canada) using a silk ligature. The sciatic nerve was proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 ms duration (10 V). We measured the absolute maximal force that was generated during isometric tetanic contractions in response to electrical stimulation (125 Hz, 500 ms). Absolute maximal force was determined at L0 (length at which maximal tension was obtained during the tetanus) (P0). Absolute maximal force was normalized to the muscle weight as an estimate of specific maximal force (absolute maximal force/muscle weight)(sP0).

Susceptibility to contraction-induced injury was estimated from the force drop resulting from lengthening contraction-induced injury. The sciatic nerve was stimulated for 700 ms

(frequency of 125Hz). A maximal isometric contraction of the TA muscle was initiated during the first 500 ms. Then, muscle lengthening (10% L0) at a velocity of 5.5 mm/s (0.85 fibre length/s) was imposed during the last 200 ms. Nine lengthening contractions of the muscle were performed, each separated by a 60-s rest period. All contractions were made at an initial length L0. Maximal isometric force was measured 60 s after each lengthening contraction and expressed as a percentage of the initial maximal force.

In some experiments, stimulating electrodes were also applied directly on the muscle, after nerve stimulation. Direct muscle stimulation (125 Hz) was performed in order to evaluate neuromuscular transmission (Ferry et al., 2014a). Comparisons between nerve (10 V) and muscle (80 V) stimulations were made to evaluate nerve-muscle communication. A lower force produced in response to nerve stimulation versus muscle stimulation was indicative of a defect in neuromuscular transmission (neuromuscular failure).

Data was acquired with a sampling rate of 100 kHz (Powerlab 4/25, ADInstrument, Oxford, United Kingdom).

Electromyography

Electromyography was performed with anesthetized mice (pentobarbital, 60 mg/kg ip) in order to evaluate muscle excitability (Delacroix et al., 2018; Roy et al., 2016). For compound muscle action potential (CMAP) recordings, 2 monopolar needle electrodes were inserted into the belly of TA muscle. The recording (cathode) and the reference (anode) electrodes were inserted respectively into the proximal and the distal portion of the muscle. A third monopolar electrode was inserted in the contralateral hindlimb muscle to ground the system. Data was

amplified (BioAmp, ADInstrument), acquired with a sampling rate of 100 kHz and filtered at 5kHz low pass and 1 Hz high pass (Powerlab 4/25, ADInstrument). Recording electrodes were positioned to achieve maximal CMAP amplitude. CMAP were recorded during lengthening contractions and we calculated the root mean square (RMS) of CMAP, as an index of CMAP amplitude. RMS of each CMAP corresponding to each contraction was then expressed as a percentage of the first contraction, used as a marker of muscle excitability.

AAV-desmin gene delivery

Des (cDNA) transfer with adeno-associated virus (AAV) was used to experimentally express and overexpress desmin in TA muscle from DKO and *mdx* mice respectively. Desmin AAV2/1 vector (AAV-Des) was prepared as described previously (Joanne et al., 2013). Briefly, first the full-length human desmin cDNA's were cloned into pSMD2 plasmid (promoter CMV and human β-globin pA) using Xho 1 restriction site. To distinguish the desmin transgene from the endogenous form a c-myc tag was introduced at the 5' of the desmin cDNA. It should be noted that the presence of c-myc at the N-terminal of desmin does not disturb filament assembly or cellular localization (Chourbagi et al., 2011). The plasmid was purified using the PureYield™ endotoxin-free Plasmid Maxiprep System (Promega, Lyon, France) and then verified by restriction enzyme digestion and by sequencing (Eurofins MWF Operon, Ebersberg, Germany). The AAV-Des was produced in human embryonic kidney 293 cells by the triple-transfection method using the calcium phosphate precipitation technique. The virus was then purified by two cycles of cesium chloride gradient centrifugation and concentrated by dialysis. The final viral preparations were kept in PBS solution at -80°C. The number of viral genomes was determined by a quantitative PCR. AAV-Des was injected into TA muscles

from DKO and *mdx* mice, as previously described (Joanne et al., 2013). Titer for AAV-Des was 9 x 10^{11} vector genomes (vg).ml^{-1}. Briefly, 1-month-old mice were anesthetized (2-4% isoflurane) and TA muscles of the right hindlimb were injected (10 µl/10 mg, 5.0 x 10^{10} vg). The right hindlimb muscles of newborn *mdx* mice were also injected by AAV-Des. Control muscle was obtained from left hind limb injected with saline solution only. Muscles from mice were collected 4 weeks or 8 weeks after injection, at the age of 2 months.

Histology of whole muscle (fibres)

Transverse serial sections (8 µm) of TA muscles from 2-month old mice were obtained using a cryostat, in the mid-belly region. We also performed transverse serial section of hindlimb muscles from newborn mice. Some of sections were processed for histological analysis according to standard protocols (Hematoxylin-Eosin, Sirius red, and succinate dehydrogenase, SDH). Other sections were processed for immunohistochemistry as described previously (Ferry et al., 2014b). For determination of muscle fibre diameter and fibre expressing myosin heavy chain (MHC), frozen unfixed sections were blocked 1h in PBS plus 2% BSA, 2% sheep serum. Sections were then incubated overnight with primary antibodies against, laminin (Sigma, France), MHC-2a (clone SC-71, Developmental Studies Hybridoma Bank, University of Iowa), MHC-2b (clone BF-F3, Developmental Studies Hybridoma Bank), MHC-1 (clone BA-D5, Developmental Studies Hybridoma Bank). After washes in PBS, sections were incubated 1 h with secondary antibodies (Alexa Fluor, Invitrogen). Slides were finally mounted in Fluoromont (Southern Biotech). Images were captured using a digital camera (Hamamatsu ORCA-AG) attached to a motorized fluorescence microscope (Zeiss AxioImager.Z1), and morphometric analyses were made using the software ImageJ and a

homemade macro. MHC-2x fibres were identified as fibres that do not express MHC-2b, MHC-2a or MHC-1. For determination of the percentage of fibre type and diameter (minimum feret), we attempted to analyse all the fibres of a cross-section. Data presented correspond to pure MHC-2b, MHC-2x and MHC-2a expressing fibres (without MHC coexpression). Total fibre number was counted at the widest cross-section. To determine the number of muscle fibres transduced following AAV-Des injection, sections were incubated with c-myc antibody (1:1000, rabbit polyclonal, Sigma-Aldrich).

Electronic microscopy of muscle fibres

To evaluate fibre ultrastructure integrity, electron microscopy was performed on TA muscles as described previously (Joanne et al., 2013; Roy et al., 2016). Muscles were fixed in 2 % glutaraldehyde and 2% paraformaldehyde in 0.2M phosphate buffer at pH 7.4 for 1h at room temperature. After 1 h, the muscle was dissected and separated in three by a short-axis section, then fixed overnight at 4°C in the same fixative. After washing, specimens were post-fixed for 1h with 1% osmium tetroxide solution, dehydrated in increasing concentrations of ethanol and finally in acetone, and embedded in epoxy resin. The resin was polymerized for 48h at 60°C. Ultrathin sections (70 nm) were cut with an ultramicrotome (Leica UC6, Leica Microsystems, Nanterre, France), picked-up on copper rhodium-coated grids and stained for 2 min with Uranyl-Less solution (Delta Microscopies, Mauressac, France) and 2 min with for 2 min 0.2 % lead citrate before observation at 80 kV with an electron microscope (912 Omega, Zeiss, Marly le Roi, France) equipped with a digital camera (Veleta 2kx2k, Emsis, Münster, Germany).

Histology of neuromuscular junction

Neuromuscular junction immunostaining was performed on isolated muscle fibres as previously described with minor modifications (Messéant et al., 2015; Ueberschlag-Pitiot et al., 2017). TA muscles were dissected and fixed in 4% PFA for 1 hour at room temperature. Muscle fibres were isolated and incubated for 30 min with 100 mM glycine in PBS. After washing in PBS 3 times, samples were permeabilized and blocked in PBS/3% BSA/5% goat serum/0.5% Triton X-100. Muscle fibres were then incubated at 4°C overnight with rabbit polyclonal antibodies against synaptophysin (Syn, 1/500; ThermoFisher Scientific) and neurofilament 68 kDa (1/500; Millipore Bioscience Research Reagents). After three 1-h washing in PBS/0.1% Triton X-100, muscles were incubated overnight at 4°C with Cy3-conjugated goat anti-rabbit IgG (1/1000, Jackson Immunoresearch Laboratories) and α-bungarotoxin (BTX) Alexa Fluor 488 conjugate (1/500, Life Technologies). Isolated muscle fibres were washed three times with PBS/0.1% Triton X-100 and then flat-mounted in Vectashield (Vector Laboratories) mounting medium. All images were collected on a Zeiss ApoTome.2 microscope with a 63X Plan Apochromat 1.4NA oil objective (Carl Zeiss). Zen.2 microscope software (Carl Zeiss) was used for acquisition of z-serial images. Image analysis was performed using FIJI (National Institutes of Health) as previously (Messéant et al., 2015). Images presented are single-projected image derived from z-stacks. At least, 40 isolated muscle fibres of each genotype were analysed and quantified.

Immunoblotting (desmin, LC3-II)

Immunoblotting was carried out as described previously (Delacroix et al., 2018; Li et al.,

2014). TA muscles were snap-frozen in liquid nitrogen immediately after dissection. Frozen muscles were placed into an ice-cold homogenization buffer containing: 50 mM Tris (pH 7.6), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP40, 2 mM dithiothreitol, 10 mM sodium orthovanadate, 10 mM NaF, 10 mM glycerophosphate and 2% of protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) for LC3-II and ß-tubulin immunoblotting and 7 M Urea, 2 M thiourea, 2% CHAPS, 50 mM dithiothreitol, l0 mM sodium orthovanadate, 10 mM NaF, 10 mM glycerophosphate and 2% of protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) for desmin and GAPDH immunoblotting. Samples were minced with scissors and homogenised using plastic pestles, incubated 30 min on ice and then centrifuged at 12,000 g for 30 min at 4°C. Protein concentration was measured using the Bradford method with bovine serum albumin as a standard. Equal amounts of protein extracts (25 µg) were separated by SDS-PAGE before electrophoretic transfer onto a nitrocellulose membrane (GE Healthcare, Velizy-Villacoublay, France). Western-blot analysis was carried out using anti-LC3-II (1:1000, rabbit polyclonal, Sigma-Aldrich, Saint-Quentin Fallavier, France), anti-ß-tubulin (1:1000, mouse monoclonal, Sigma-Aldrich, Saint-Quentin Fallavier, France), anti-desmin (1:1000, mouse monoclonal, Dako-Cytomation, Trappes, France) and anti-GAPDH antibody (1:5000, mouse monoclonal, Santa Cruz Biotechnology, Heidelberg, Germany). Proteins bound to primary antibodies were visualised with peroxidase-conjugated secondary antibodies (Thermo-Fisher Scientific, Brebières, France) and a chemiluminescent detection system (ECL-Plus, GE Healthcare, Velizy-Villacoublay, France). Bands were quantified by densitometric software (Multi Gauge, Fujifilm). The levels of activation of autophagy were calculated by quantification of the LC3-II band (Sarkar et al., 2009).

qPCR

Total RNA was extracted from TA muscles using TRIzol Reagent (Thermo Fisher Scientific, Saint-Herblain, France) following the manufacturer's instructions. From 250 ng of extracted RNA, the first-strand cDNA was then synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Saint-Herblain, France) with anchored-oligo(dT)18 primer and according to the manufacturer's instructions. Using the Light Cycler® 480 system (Roche Diagnostics), the reaction was carried out in duplicate for each sample in a 6 µl reaction volume containing 3 µl of SYBR Green Master Mix, 500 nM of the forward and reverse primers each and 3 µl of diluted (1:25) cDNA. The thermal profile for SYBR Green qPCR was 95°C for 8 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. To exclude PCR products amplified from genomic DNA, primers were designed, when possible, to span one exon-exon junction. The mean gene expression stability of 2 genes, Sdha (Succinate dehydrogenase complex, subunit A, flavoprotein), Hmbs (Hydroxymethylbilane synthase), was used as the reference transcript. Data were collected and analysed using the LightCycler® 480 software release 1.5.0 (Roche Diagnostics). Primers sequences used in this study are shown in Table 1.

Proteasome activity

Proteasome containing TA muscle homogenates were prepared just after dissection using ice-cold homogenization buffer containing: 20 mM Tris–HCl (pH 7.6), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA and 2 mM DTT. Samples were minced with scissors and homogenised using plastic pestles, incubated 30 min on ice, then centrifuged at 12,000 g for 15 min at 4°C.

Protein concentration was measured using the Bradford method with bovine serum albumin as a standard. The proteasomal chymotrypsin-like, trypsin-like and caspase-like activities of the 20S catalytic core were assayed (Hovhannisyan et al., 2019) using the fluorogenic substrates *N*-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC, Enzo Life Sciences, Villeurbanne, France), Bz-Val-Gly-Arg-7-amino-4-methylcoumarin (Bz-VGR-AMC, Enzo Life Sciences, Villeurbanne, France) and Z-Leu-Leu-Glu-7-amino-4-methylcoumarin (Z-LLE-AMC, Enzo Life Sciences, Villeurbanne, France), respectively. The mixture, containing 10 µg of total protein in 20 mM Tris (pH 8) and 10% glycerol, was incubated at 37°C with 20 µM peptide substrates in a final volume of 100 µl. Enzymatic kinetics were monitored in a temperature-controlled microplate fluorimetric reader. Excitation/emission wavelengths were 350/440 nm. The difference between assays with or without MG-132, a proteasome inhibitor, represented the proteasome-specific activity.

Statistical analysis

Groups were statistically compared using 1 way-analysis of variance (3 or more groups), 2 ways-analysis of variance (2 or more groups with 2 factors) or T-test (2 groups)(Prism 6 software, GraphPad, San Diego, CA, USA). If appropriate, subsequent post-hoc analysis (Bonferroni) was performed. A p value ≤ 0.05 was considered significant. Values are means ± SD.

Results

1-Endogenous desmin is crucial for maximal force production in *mdx* mice

We constitutively inactivate the gene encoding desmin (*Des*) in *mdx* mice (DKO mice). Since the DKO mice have a reduced lifespan, i.e., most of the mice died before the age of 3 months as described previously for the mdx^{4cv} mice with desmin deficiency (Banks et al., 2014), we studied 2-month-old DKO mice. The genotype was confirmed by immunoblotting analyses of whole muscle that show no expression of desmin in DKO mice (Figure 1A). *In situ* force production of TA muscle in response to nerve stimulation were analysed in adult (2-month-old) DKO mice, to assess muscle weakness, an important functional dystrophic feature. We found that absolute maximal force in *mdx* mice and mice with *Des* deletion (DesKO) was slightly reduced as compared to wild-type mice (C57 mice)($p \leq 0.01$)(Figure 1B). In DKO mice, absolute maximal force was more deeply reduced, and decreased by -67% to -69% as compared to *mdx* and DesKO mice respectively ($p \leq 0.0001$)(Figure 1B).

The reduced absolute maximal force in DKO mice as compared to *mdx* mice was not explained by a decreased specific maximal force but rather by the absence of hypertrophy (see below). Indeed, specific maximal force was not significantly different between DKO and *mdx* mice, although it was reduced in DKO and *mdx* mice as compared to C57 mice ($p \leq 0.0001$) (Figure 1C). Histochemical analyses revealed that fibrosis is increased in DKO mice as compared to *mdx* mice ($p \leq 0.01$)(Figure 1D). Ultrastructural analysis revealed a deterioration of sarcomere organisation and Z-line alignment in DKO mice as compared to *mdx* mice

(Figure 1E). Muscle from DKO and DesKO mice presented perturbation of sarcomere alignment but this perturbation is more pronounced in DKO mice (Figure 1E). Thus, fibrosis and the more marked deterioration of sarcomere integrity in DKO mice as compared to *mdx* mice have no marked effect on the muscle's capacity to produce force (Figure 1E). Together, these results show a weakness in DKO mice as compared to *mdx* mice, indicating that desmin is essential to maintain the capacity of maximal force production, particularly in *mdx* mice.

2- Endogenous desmin prevents the worsening of fragility and decreased fatigue resistance in *mdx* mice

An immediate force drop was observed following lengthening contractions in 2-month-old *mdx* mice ($p \leq 0.0001$)(Figure 2A). There was no such force drop following 9 lengthening contractions in DesKO and C57 mice (Figure 2A). This measure evidenced the higher susceptibility to contraction-induced injury in *mdx* mice, i.e. greater fragility, another important *mdx* dystrophic functional feature. In DKO mice, the force drop was greater as compared to *mdx* and DesKO mice ($p \leq 0.0001$), indicating that desmin plays a role to reduce fragility in *mdx* mice, but not in C57 mice. Since the force drop following lengthening contractions was explained by decreased muscle excitability (Roy et al., 2016), we used electromyography to measure the compound muscle action potential (CMAP) in response to nerve stimulation, a marker of muscle excitability. CMAP (root mean square, RMS) decreased following lengthening contractions in *mdx* mice ($p \leq 0.0001$) similarly to maximal force (Figure 2A). Interestingly, CMAP decreased more following lengthening contractions in DKO mice as compared to *mdx* mice ($p \leq 0.0001$)(Figure 2A), indicating that the worsened fragility in DKO mice as compared to *mdx* mice was related to a reduced excitability.

Together with exaggerated weakness and fragility, we found that muscle fatigue resistance was markedly decreased in DKO mice as compared to *mdx* mice ($p \leq 0.001$)(Figure 2B), indicating that desmin is required to normal fatigue resistance in *mdx* mice, but not in C57 mice.

Using immunohistochemical analysis, we found that the increased fragility and reduced fatigue resistance in DKO mice as compared to *mdx* mice was not related to an increased percentage of fast and less oxidative muscle fibres that were more fragile and less fatigue resistant. In fact, the percentage of muscle fibres expressing myosin heavy chain type 2B (MHC-2b) was decreased although not significantly (Figure 2C), whereas the percentage of highly succinate dehydrogenase (SDH) stained muscle fibres (Figure 2D) were increased in DKO mice as compared to *mdx* mice ($p \leq 0.0001$), without change in the percentage of fibre expressing MHC-2a (Figure 2E) and MHC-2x (Figure 2F).

Together, these results show a worsened fragility in DKO mice as compared to *mdx* mice, indicating that desmin is essential to protect the *mdx* muscle from contraction-induced injury, and maintain excitability and fatigue resistance independently from the promotion of more slow and oxidative fibres.

3-Endogenous desmin is indispensable for hypertrophy in *mdx* mice

Another well-known dystrophic *mdx* feature is muscle hypertrophy (Pastoret and Sebille, 1995). Indeed, the weight of the TA muscle was increased (+29%) in 2-month-old *mdx* mice as compared to C57 mice ($p \leq 0.0001$), whereas it was slightly reduced in DesKO mice ($p \leq$

0.01)(Figure 3A). In contrast, the muscle weight from DKO mice is reduced as compared to *mdx* (-59%), DesKO (-37%) and C57 (-46%) mice ($p \leq 0.0001$)(Figure 3A). The reduced muscle weight in DKO mice is also observed when it is normalized to body weight ($p \leq 0.0001$) (Figure 3A) and tibia length ($p \leq 0.0001$)(Figure 3B), indicating that desmin is indispensable to hypertrophy in *mdx* mice, and thus is required to prevent atrophy. Of note, the reduced muscle weight explained the lower absolute maximal force in DKO mice as compared to *mdx* mice ($p \leq 0.0001$)(Figure 1B).

The muscle atrophy (reduced muscle weight) in DKO mice was not apparently related to change in autophagy and ubiquitin proteasome system (Figure 3C-E). Indeed, the level of LC3-II protein (Figure 3C), the trypsin-, chemotrypsin- and caspase-like activity (Figure 3D) and the mRNA levels of *Atrogin-1*, *Murf1* and *Mstn* (Figure 3E) did not differ between DKO and *mdx* mice. Moreover, the increase in mRNA level of *Fst* encoding follistatin, an important factor stimulating muscle growth, did not explain the reduced muscle weight in DKO mice as compared to *mdx* mice ($p \leq 0.01$)(Figure 3E).

Immunohistochemical analyses were performed to gain further insight into the role of desmin concerning muscle size in *mdx* mice. Having in mind that the fibers are branched, they revealed that the apparent number of muscle fibres from DKO mice was reduced as compared to *mdx* mice ($p \leq 0.01$)(Figure 4A). Moreover, we found that the diameter of the fibres was decreased in DKO mice as compared to *mdx* mice ($p \leq 0.0001$ for all the fibres, $p \leq 0.0001$ for fibres expressing MHC-2b, $p \leq 0.05$ for fibres expressing MHC-2a and $p \leq 0.0001$ for fibres expressing MHC-2x)(Figures 4B-E). It was the diameter of the fibres expressing MHC-2b that was the more reduced in DKO mice (Figure 4C). Together, these results indicate that

desmin is required to maintain the number and size of muscle fibres in *mdx* mice, in particular those expressing MHC-2b, and this is more marked in mdx mice than in C57 mice.

Since reduction in nerve-evoked activity results in reduced muscle weight (Agbulut et al., 2009), we next assessed whether neuromuscular junctions (NMJ) morphology is altered in DKO mice. Muscles were stained whole-mount with with α-bungarotoxin (α-BTX) to detect acetylcholine receptors clusters (AChR) and a mixture of antibodies against neurofilament and synaptophysin to label axons and nerve terminals, respectively. NMJ of *mdx* and C57 mice formed a continuous branched postnatal structure exhibiting a typical pretzel-like topology (Figure 4F). In contrast, the postsynaptic shape of NMJ in DKO mice was particularly disorganized with smaller and isolated AChR clusters, suggesting a fragmentation of the NMJ (Figure 4F). Indeed, the number of fragments per AChR clusters was increased by almost 8-fold in DKO mice as compared to *mdx* mice ($p \leq 0.001$)(Figure 4G). AChR area was also reduced in DKO mice as compared to *mdx* mice ($p \leq 0.05$), but not different from to C57 mice (Figures 4H). However, the overlap area between presynaptic and postsynaptic elements was similar between DKO (0.81 ± 0.05), *mdx* (0.88 ± 0.03) and C57 (0.82 ± 0.08) mice, suggesting that NMJ were properly innervated. Since our findings indicate a severe dismantlement of NMJ in DKO mice, we next determined whether neuromuscular transmission failure (inactivity) contributes to reduced muscle weight in DKO mice, by performing electrical TA muscle stimulation that can directly initiate muscle action potentials, without the need of neuromuscular transmission. Stimulating electrodes were positioned on the mid belly of the muscle and the muscle was stimulated with a high strength voltage (80V). We found that absolute maximal forces in response to muscle and nerve stimulations were not different in DKO mice, indicating that there is no neurotransmission failure involved in the

reduced muscle weight and exaggerated weakness in DKO mice (Figure 4I).

4-Endogenous desmin is linked to the high percentage of centronucleated muscle fibres in *mdx* mice

An additional well-known *mdx* dystrophic feature is the high percentage of centronucleated fibres in the hypertrophic *mdx* muscle (Pastoret and Sebille, 1995). Indeed, there are 69.4 % centronucleated muscle fibres in 2-month-old *mdx* mice, as compared to 2.5% in C57 mice ($p \leq 0.0001$)(Figure 5A). In contrast, this percentage is reduced to 18.1% in DKO mice as compared to *mdx* mice ($p \leq 0.0001$)(Figure 5A), although it was increased as compared to DesKO ($p \leq 0.01$) and C57 mice ($p \leq 0.0001$). The lower number of centronucleated muscle fibres and reduced muscle weight in DKO mice suggest an impaired muscle regeneration as compared to the robust muscle repair in *mdx* mice (Faber et al., 2014; Pastoret and Sebille, 1995; Mouisel et al., 2010). To test this hypothesis, cardiotoxin (10 µM, 50 µl), a myotoxic agent was injected into TA muscles from 1-month old DKO, and mice were studied 1 month after. We found no difference concerning absolute maximal force (Figure 5B), specific maximal force (Figure 5C), fragility (Figure 5D), muscle weight (Figure 5E) and fibre diameter (Figure 5F) between cardiotoxin-injected muscle and not cardiotoxin-injected muscle from DKO mice. In addition, there was also no difference between cardiotoxin-injected and not cardiotoxin-injected muscle in *mdx* mice (Figure 5B-F). These results indicate that muscle regeneration is not impaired in the DKO mice and also suggest that the absence of muscle hypertrophy in the DKO mice is likely not related to defective satellite cell function.

5-Endogenous desmin has a role in early stages of postnatal development in *mdx* mice

We next examined the dystrophic phenotype in younger DKO mice, at the age of 1 month. We found that reduced absolute maximal force ($p \leq 0.05$)(Figure 6A), similar specific maximal force (Figure 6B), increased fragility ($p \leq 0.0001$)(Figure 6C), reduced muscle weight ($p \leq 0.0001$)(Figure 6D), smaller percentage of centronucleated muscle fibres ($p \leq 0.0001$)(Figure 6E) and greater fibrosis ($p \leq 0.001$)(Figure 6F) were already observed in the 1-month old DKO mice, as compared to age-matched *mdx* mice. Moreover, fragility (Figure 6C) from the 1-month-old DKO and *mdx* mice were not different from that of 2-month-old DKO and *mdx* mice respectively, in contrast to absolute maximal force ($p \leq 0.01$ for DKO mice and $p \leq 0.0001$ for mdx mice)(Figure 6A), muscle weight ($p \leq 0.0001$)(Figure 6D) and fibrosis ($p \leq 0.0001$)(Figure 6F). These latter results indicate that the degree of weakness (lower specific maximal force) and fragility observed in 2-month-old DKO and *mdx* mice are already acquired at the age of 1 month, suggesting no progression of the disease in DKO mice between 1 and 2 months of age, at least using functional dystrophic features.

We also analysed newborn DKO mice. Hematoxylin and eosin stained muscle cross-sections from newborn DKO mice revealed numerous abnormalities such as higher heterogeneity of fibre diameter and spaces between fibres containing infiltrating cells as compared to newborn *mdx* mice (Figure 6G). Moreover, the expression of *Ccl2* encoding monocyte chemoattractant protein-1 (MCP-1/CCL2), a key chemokine that regulate migration and infiltration of monocytes/macrophages, was 4x higher in newborn DKO mice (4.1 ± 2.4 AU) than in newborn *mdx* mice (1.0 ± 0.6 AU)($p < 0.04$). Thus, the severe phenotype in the DKO mice

was already present in newborn DKO mice.

Together, these results indicated that desmin plays an early role in *mdx* mice.

6- Desmin haploinsufficiency is enough to prevent a severe phenotype in *mdx* mice (*mdx*:desmin+/- mice)

To determine the impact of desmin haploinsufficiency in *mdx* mice, we examined 2-month-old *mdx*:desmin+/- mice. Using qPCR and western blot analyses, we found that desmin mRNA (Figure 7A)($p \leq 0.0001$) and protein level (-31%)(Figure 7B)($p \leq 0.01$) were reduced in *mdx*:desmin+/- mice as compared to that observed in *mdx* mice. Absolute maximal force (Figure 7C), fragility (Figure 7D), muscle weight (Figure 7E) and the percentage of centronucleated muscle fibres (Figure 7F) from *mdx*:desmin+/- mice were not different from that of *mdx* mice. Accordingly, absolute maximal force ($p \leq 0.0001$)(Figure 7C), muscle weight ($p \leq 0.001$) (Figure 7E) and the percentage of centronucleated muscle fibres ($p \leq 0.0001$)(Figure 7F) were increased in *mdx*:desmin+/- mice as compared to DKO mice, whereas fragility ($p \leq 0.0001$) (Figure 7D) was reduced in *mdx*:desmin+/- mice.

Together, these results suggest that 69% of the *mdx* desmin level is enough to rescue DKO phenotype.

7-Exogenous desmin does not play a role in adult DKO mice (DKO+AAV-Des)

We next examined the role of desmin in adult *mdx* mice, using *Des* transfer with adeno-associated virus (AAV-Des) in adult DKO mice, to rescue desmin expression in DKO mice. This allowed for distinguishing the functional role of desmin in adult *mdx* muscle from the developmental consequences of desmin deficiency in *mdx* muscle. We performed intramuscular TA injection with an AAV-Des from 1-month old DKO mice to increase the expression of desmin in muscle (DKO+AAV-Des mice). DKO mice were studied at the age of 2 month. The expression of desmin protein was confirmed by western blot ($p \leq 0.01$)(Figure 8A), and the level of desmin protein in DKO+AAV-Des muscle was not different from that of *mdx* muscle (Figure 8A). We found that specific maximal force (Figure 8B), absolute maximal force (Figure 8C), muscle weight (Figure 8D), the fibre diameter (Fibre 8E) and the percentage of centronucleated fibres (Figure 8F) were not increased in DKO+AAV-Des muscle as compared to DKO muscle.

Thus, there was no role of desmin when its expression started at the adult stage (DKO+AAV-Des muscle), contrary to the case where its expression started at the embryonic stage.

8- *Des* transfer is beneficial in *mdx* mice

Since we showed that exogenous desmin plays beneficial roles in *mdx* mice and desmin is upregulated in *mdx* mice (Banks et al., 2014) possibly by a compensatory mechanism, we tested the possibility that *Des* transfer in adult *mdx* mice would be beneficial. We performed intramuscular injection with an AAV-Des into muscle of 1-month old *mdx* mice to further more increase the expression of desmin in *mdx* muscle (*mdx*+AAV-Des), and muscle was

studied 1 month after. Western blot analyses confirmed that desmin protein level was already increased by 82% in *mdx* mice as compared to C57 mice ($p \leq 0.01$)(Figure 9A), without increased mRNA level of *Des* (Figure 7A), suggesting a better stability and posttranscriptional mechanisms. Desmin level was further increased by 225% in 2-month-old *mdx*+AAV-Des muscle, as compared to *mdx* muscle ($p \leq 0.05$)(Figure 9B). To distinguish the *Des* transgene from the endogenous form a c-myc tag was introduced at the 5' of the *Des* cDNA. The expression of c-myc tag was observed in 43.5 ± 10.5% of the fibres of *mdx*+AAV-Des muscle, indicating that about half of the fibres were transduced. However, we found that absolute maximal force (Figure 9C), specific maximal force (Figure 9C), fragility (Figure 9D) and muscle weight (Figure 9E) from *mdx*+AAV-Des muscle were not different from that of *mdx* muscle.

Since numerous studies demonstrate that the rescue of the dystrophic phenotype by the use of dystrophin independent based gene therapy is more successful when the treatment is performed in younger versus older *mdx* mice (Deol et al., 2007; Squire et al., 2002), *Des* transfer was also performed in newborn *mdx* mice (*mdx*n+AAV-Des mice). Two months after injection, we found that absolute and specific maximal forces were increased by 22% ($p \leq 0.05$) and 36% ($p \leq 0.01$) respectively in *mdx*n+AAV-Des muscle as compared to *mdx*n muscle (Figure 9F). In contrast, fragility (Figure 9G) and weight (Figure 9H) from *mdx*n+AAV-Des muscle were not different from that of *mdx* muscle.

Thus, this result indicates that *Des* transfer was beneficial in *mdx* mice, in regard to muscle weakness.

Discussion

The crucial roles of endogenous desmin in *mdx* mice

Desmin is already known to be important for normal muscle function of healthy muscle (Agbulut et al., 2001; Boriek et al., 2001; Li et al., 1997; Lovering et al., 2011; Meyer et al., 2013; Sam et al., 2000). However, a recent study found that dystrophic histopathology is improved in the absence of desmin in the *mdx*^{4cv} mouse lacking dystrophin (Banks et al., 2014). In the present study, we found for the first time that **endogenous desmin is necessary to maintain hindlimb muscle performance** in the absence of dystrophin. Indeed, desmin increases by 203% absolute maximal force in *mdx* mice as compared to DKO mice, via its beneficial effect on muscle growth leading to muscle hypertrophy in *mdx* mice (see below). Thus, **desmin prevents exaggerated weakness** in *mdx* mice, and we found that this was not related to a possible role of desmin on neuromuscular transmission. Since specific maximal force is not more significantly deteriorated in DKO mice as compared to *mdx* mice, this result could indicate that desmin has no additional apparent role to that of dystrophin in lateral force transmission via the costameres. However, this is not so clear since we found that *Des* transfer in newborn *mdx* mice increased specific maximal force.

Moreover, we reported that endogenous **desmin surprisingly increases by 39% fatigue resistance** in *mdx* mice as compared to DKO mice, and our results indicate that this beneficial role of desmin is not related to increased percentage of slower and more oxidative muscle fibres that were known to be more fatigue resistant. Possibly, endogenous desmin helps to maintain muscle excitability during contraction leading to fatigue in *mdx* mice, since desmin

has beneficial effect on excitability during lengthening contractions in *mdx* mice (see below). Membrane excitability is known to be a critical step in muscle fatigue (McKenna et al., 2008).

Another original and important result of the present study is that **endogenous desmin prevents extreme fragility** in the absence of dystrophin. This role of desmin is crucial since it is thought that fragility initiates repeated cycles of degeneration/regeneration, and thus progressive muscle wasting and weakness. Remarkably, endogenous desmin also reduces the decrease in muscle excitability following lengthening contractions in *mdx* mice, confirming the causal relation between susceptibility to contraction-induced muscle injury and impaired excitability (Delacroix et al., 2018; Roy et al., 2016). Our results exclude the possibility that this beneficial role of desmin is related to the promotion of less fragile muscle fibres in *mdx* mice (Chan et al., 2007; Head et al., 1994; Moens et al., 1993), since we found increased percentage of slower and more oxidative muscle fibres and reduced percentage of centronucleated muscle fibres in DKO mice. Furthermore, the beneficial effect of desmin on fragility is likely not explained by a greater level of utrophin which is known to improve fragility in utrophin transgenic *mdx* mice (Deconinck et al., 1996; Squire et al., 2002) since utrophin is in reality decreased by desmin in *mdx* mice (Banks et al., 2014).

Another result that strikes the attention is that **endogenous desmin is required to muscle hypertrophy** in the absence of dystrophin, independently of the prevention of neuromuscular transmission failure (inactivity) despite neuromuscular junction morphology abnormality. Thereby, desmin allows the hypertrophic dystrophic *mdx* muscle to not exhibit an exaggerated weakness. We found that desmin increases by 141% muscle weight and muscle fibre diameter in *mdx* mice as compared to DKO mice, regardless of change in two important proteolytic

processes, i.e., autophagy and ubiquitin-proteasome system. The altered signalling pathways involved in fibre growth remain to be further investigated, however, a recent study shows that desmin and synemin regulate ERK and JNK signalling pathways, at least in healthy muscle (Li et al., 2014; Palmisano et al., 2015). Our results does not support the possibility that desmin plays a direct role in fibre growth via the functioning of satellite cells, since we found that regeneration was not affected, confirming and extending a previous study (Banks et al., 2014). However, this remains to be confirmed. Moreover, we demonstrated that desmin increases the (apparent) number of muscle fibres by 55% in *mdx* mice. Since the supernumerary muscle fibres per section in *mdx* mice are likely due to fibre branching resulting from abnormal myoblast fusion rather than fibre splitting (Duddy et al., 2015; Faber et al., 2014), our results suggest that desmin is also required for the formation of new segments of muscle fibres, centronucleated, physically connected to a pre-existing fibre. This possibly partly results from a robust regeneration since desmin promotes the necrosis of muscle fibres in *mdx* muscle, via utrophin-independent and -dependent mechanisms (Banks et al., 2014). Thus, the muscle hypertrophy induced by endogenous desmin appears to be related to bigger fibres and increased occurrence of new segments of muscle fibres that are centronucleated in *mdx* mice.

Together, our analyses of the DKO mice revealed that endogenous desmin plays important and beneficial roles in muscle performance, fragility, growth and the occurrence of centronucleated muscle fibres in *mdx* mice when its expression started during embryogenesis. Interestingly, these roles of endogenous desmin in *mdx* mice markedly differ from those we and others observed in healthy mice, indicating that one cannot extrapolate its roles on the basis of what we know in the healthy mice. In healthy mice, we found no marked weakness,

reduced fatigue resistance, increased fragility, dramatic atrophy, and increased or decreased occurrence of fibres with high SDH staining or centronucleated fibres respectively, with the absence of desmin. Moreover, we found that only 69% of the level of *mdx* endogenous desmin is required, since *mdx*:desmin+/- mice do not exhibit severe dystrophic functional features. Furthermore, we observed no effect of desmin when expression starts later, at the adult period, in DKO and *mdx* mice. In contrast to *Des*, the KO of γ-actin gene has surprisingly no effect in *mdx* mice (Prins et al., 2008), indicating that desmin intermediate filament and F-actin have distinct roles. As for desmin, the roles of α7-integrin are important since its constitutive absence in *mdx* mice induce severe dystrophic features in *mdx* (Guo et al., 2006; Rooney et al., 2006). Moreover, our results indicate that the endogenous desmin and the endogenous dystrophin paralogue utrophin appear to have similar beneficial roles in *mdx* mice, since both prevent exaggerated weakness, fragility and atrophy, however endogenous utrophin has no apparent influence on the occurrence of centronucleated muscle fibres in *mdx* mice (Deconinck et al., 1998; Grady et al., 1997; Judge et al., 2011; Lowe et al., 2006). Concerning the fibre centronucleation, it would be of interest to understand the differential effect of endogenous desmin and utrophin on this marker of cycle of fibre degeneration-regeneration, keeping in mind that endogenous desmin plays a role in the peripheral localization of myonuclei (Staszewska et al., 2015).

Desmin is also a DMD phenotype modifier

Utrophin and α7-integrin are identified as DMD modifiers, which can alleviate the dystrophic functional features (Hightower and Alexander, 2018; McGreevy et al., 2015). Since the level of desmin as utrophin is increased in *mdx* mice (the present study, Banks et al., 2014) and that

our results indicate that endogenous desmin absence worsens the mild *mdx* weakness and fragility, we tested the hypothesis that a more marked desmin overexpression would reduce *mdx* dystrophic characteristics. We found that *Des* transfer in *mdx* mice improve the mild dystrophic phenotype when performed in new-born *mdx* mice, i. e., improved weakness, but has limited effect in 1-month-old *mdx* mice. In line, numerous studies demonstrate that the rescue of the dystrophic phenotype by the use of a gene therapy approach not based on dystrophin restoration is more successful phenotypic benefit when the treatment is performed in younger versus older *mdx* mice (Deol et al., 2007; Squire et al., 2002). For example, the transfer of *Utrn* encoding utrophin improves weakness and fragility when injected into the muscle of newborn *mdx* mice but not of adult *mdx* mice (Deol et al., 2007). Therefore, our results show for the first time that desmin is also a DMD modifier in regard to functional features, such as utrophin and α7-integrin (Hightower and Alexander, 2018; McGreevy et al., 2015), indicating that it would be interesting to continue to explore the potential of *Des* transfer as a dystrophin-independent gene therapy for DMD in regard to skeletal muscle function. In line, it might be interesting to study the effect of overexpression of desmin in older mdx mice, i.e. at a more advanced stage of the disease.

It remains to be determined whether the increased level of endogenous desmin observed in *mdx* mice compensates, partly, the absence of dystrophin. Some of our results do not support this, since desmin haploinsuffiency does not worsen *mdx* phenotype. However, the level of desmin in *mdx*:desmin+/- mice is still elevated as compared to C57 mice (about 25%). It is not excluded that the small upregulation of desmin in *mdx*:desmin+/- mice, as compared to C57 mice, may provide some beneficial effect.

The DKO mice is a useful murine DMD model

mdx mice exhibit a mild weakness since they compensate a lower specific maximal force by the mean of hypertrophy. This aspect is problematic for preclinic studies aiming to improve this dystrophic feature via the stimulation of muscle growth, independently of the restoration of dystrophin expression. Thus, a good DMD murine model should exhibit a marked reduced muscle weight as we observed in the DKO mice but not reported in the DBA2-*mdx* mice (Coley et al., 2016; Hakim et al., 2017). Moreover, it should not be based on the inactivation of the *Utrn* as in the DMD murine model consisting of the deletion of both *Dmd* and *Utrn* (*mdx*:utrophin-/-) (Deconinck et al., 1998; Grady et al., 1997) since therapeutic strategies may also aim to target the upregulation of *Utrn* (Guiraud et al., 2015; Miura and Jasmin, 2006). Therefore, our DKO mice could be a useful model since it induces severe dystrophic features like the *mdx*:utrophin-/- mice. In contrast, the *mdx*:desmin+/- mice does not offer any advantage over the *mdx* mice, since its dystrophic features are similar to the *mdx* mice, such as the *mdx*:utrophin+/- mice that exhibit similar weakness and fragility than *mdx* mice (Boulanger Piette et al., 2018). It will be interesting to study the roles of desmin in cardiac muscle from our DKO mice, to further explore the potential of this DMD model regarding the cardiomyopathic aspects of DMD since cardiomyopathy is also mild and late in *mdx* mice.

Conclusion

Our results indicate that endogenous desmin plays important and beneficial roles on muscle performance, fragility and wasting in dystrophic *mdx* mice. Interestingly, endogenous desmin roles differ in large part from those observed in healthy muscle. Thus, important mechanisms that could be dissected to better understand the DMD pathophysiology in the future is how

endogenous desmin contributes to hypertrophy and a large number of centronucleated muscle fibres, and prevents exaggerated fragility by maintaining excitability and fatigue. Moreover, our results suggest the need of future studies to advance further in the study of *Des* transfer as a potential dystrophin based independent therapeutic strategy for DMD, that could be used in conjunction with dystrophin restoration therapy.

Author contributions

AF and OA conceived, coordinated and designed the study.

ZL and OA generated the DKO mice.

AF, JM, AP, ML, PR., ZL and OA. performed and analyzed animal experiments.

PR and CL performed gene and protein expression analyses, with contribution from AL.

PR and ML performed and analyzed histology measurements.

YH performed and analyzed proteasome activity measurements.

AF and ML performed and analyzed skeletal muscle function measurements.

AP performed and analyzed the newborn mice experiments and corresponding gene expression data.

JM performed and analyzed neuromuscular junction experiments.

AF, JM, AP, DF, AK, ZL and OA. wrote the manuscript.

All authors reviewed the results and approved the final version of the manuscript.

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References

Agbulut, O., Li, Z., Périé, S., Ludosky, M.A., Paulin, D., Cartaud, J., and Butler-Browne, G. (2001). Lack of desmin results in abortive muscle regeneration and modifications in synaptic structure. Cell Motil. Cytoskeleton *49*, 51–66.

Agbulut, O., Vignaud, A., Hourde, C., Mouisel, E., Fougerousse, F., Butler-Browne, G.S., and Ferry, A. (2009). Slow myosin heavy chain expression in the absence of muscle activity. Am. J. Physiol., Cell Physiol. *296*, C205-214.

Banks, G.B., Combs, A.C., Odom, G.L., Bloch, R.J., and Chamberlain, J.S. (2014). Muscle structure influences utrophin expression in mdx mice. PLoS Genet. *10*, e1004431.

Bloch, R.J., and Gonzalez-Serratos, H. (2003). Lateral force transmission across costameres in skeletal muscle. Exerc Sport Sci Rev *31*, 73–78.

Boriek, A.M., Capetanaki, Y., Hwang, W., Officer, T., Badshah, M., Rodarte, J., and Tidball, J.G. (2001). Desmin integrates the three-dimensional mechanical properties of muscles. Am. J. Physiol., Cell Physiol. *280*, C46-52.

Boulanger Piette, A., Hamoudi, D., Marcadet, L., Kyomi Labelle, F., Ovidiu David, R., Bossé, S., Argaw, A., and Frenette, J. (2018). Utrophin haploinsufficiency does not worsen the functional performance, resistance to eccentric contractions and force production of dystrophic mice. PLoS ONE *13*, e0198408.

Chamberlain, J.S., Metzger, J., Reyes, M., Townsend, D., and Faulkner, J.A. (2007). Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. Faseb J *21*, 2195–2204.

Chan, S., Head, S.I., and Morley, J.W. (2007). Branched fibers in dystrophic mdx muscle are associated with a loss of force following lengthening contractions. Am J Physiol Cell Physiol *293*, C985-92.

Chourbagi, O., Bruston, F., Carinci, M., Xue, Z., Vicart, P., Paulin, D., and Agbulut, O. (2011). Desmin mutations in the terminal consensus motif prevent synemin-desmin heteropolymer filament assembly. Exp. Cell Res. *317*, 886–897.

Coley, W.D., Bogdanik, L., Vila, M.C., Yu, Q., Van Der Meulen, J.H., Rayavarapu, S., Novak, J.S., Nearing, M., Quinn, J.L., Saunders, A., et al. (2016). Effect of genetic background on the dystrophic phenotype in mdx mice. Hum. Mol. Genet. *25*, 130–145.

Deconinck, N., Ragot, T., Marechal, G., Perricaudet, M., and Gillis, J.M. (1996). Functional protection of dystrophic mouse (mdx) muscles after adenovirus-mediated transfer of a dystrophin minigene. Proc Natl Acad Sci U S A *93*, 3570–3574.

Deconinck, N., Rafael, J.A., Beckers-Bleukx, G., Kahn, D., Deconinck, A.E., Davies, K.E., and Gillis, J.M. (1998). Consequences of the combined deficiency in dystrophin and utrophin on the mechanical properties and myosin composition of some limb and respiratory muscles of the mouse. Neuromuscul Disord *8*, 362–370.

Delacroix, C., Hyzewicz, J., Lemaitre, M., Friguet, B., Li, Z., Klein, A., Furling, D., Agbulut, O., and Ferry, A. (2018). Improvement of dystrophic muscle fragility by short-term voluntary exercise through activation of calcineurin pathway in mdx mice. Am. J. Pathol.

Deol, J.R., Danialou, G., Larochelle, N., Bourget, M., Moon, J.-S., Liu, A.-B., Gilbert, R., Petrof, B.J., Nalbantoglu, J., and Karpati, G. (2007). Successful compensation for dystrophin deficiency by a helper-dependent adenovirus expressing full-length utrophin. Mol. Ther. *15*,

1767–1774.
Duddy, W., Duguez, S., Johnston, H., Cohen, T.V., Phadke, A., Gordish-Dressman, H., Nagaraju, K., Gnocchi, V., Low, S., and Partridge, T. (2015). Muscular dystrophy in the mdx mouse is a severe myopathy compounded by hypotrophy, hypertrophy and hyperplasia. Skelet Muscle *5*, 16.
Ervasti, J.M. (2003). Costameres: the Achilles' heel of Herculean muscle. J. Biol. Chem. *278*, 13591–13594.
Faber, R.M., Hall, J.K., Chamberlain, J.S., and Banks, G.B. (2014). Myofiber branching rather than myofiber hyperplasia contributes to muscle hypertrophy in mdx mice. Skelet Muscle *4*, 10.
Ferry, A., Joanne, P., Hadj-Said, W., Vignaud, A., Lilienbaum, A., Hourdé, C., Medja, F., Noirez, P., Charbonnier, F., Chatonnet, A., et al. (2014a). Advances in the understanding of skeletal muscle weakness in murine models of diseases affecting nerve-evoked muscle activity, motor neurons, synapses and myofibers. Neuromuscul. Disord. *24*, 960–972.
Ferry, A., Schuh, M., Parlakian, A., Mgrditchian, T., Valnaud, N., Joanne, P., Butler-Browne, G., Agbulut, O., and Metzger, D. (2014b). Myofiber androgen receptor promotes maximal mechanical overload-induced muscle hypertrophy and fiber type transition in male mice. Endocrinology *155*, 4739–4748.
Ferry, A., Parlakian, A., Joanne, P., Fraysse, B., Mgrditchian, T., Roy, P., Furling, D., Butler-Browne, G., and Agbulut, O. (2015). Mechanical Overloading Increases Maximal Force and Reduces Fragility in Hind Limb Skeletal Muscle from Mdx Mouse. Am J Pathol *185*, 2012–2024.
Grady, R.M., Teng, H., Nichol, M.C., Cunningham, J.C., Wilkinson, R.S., and Sanes, J.R. (1997). Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell *90*, 729–738.
Grundy, D. (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. J. Physiol. (Lond.) *593*, 2547–2549.
Guiraud, S., Chen, H., Burns, D.T., and Davies, K.E. (2015). Advances in genetic therapeutic strategies for Duchenne muscular dystrophy. Exp. Physiol. *100*, 1458–1467.
Guo, C., Willem, M., Werner, A., Raivich, G., Emerson, M., Neyses, L., and Mayer, U. (2006). Absence of alpha 7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy. Hum. Mol. Genet. *15*, 989–998.
Hakim, C.H., Wasala, N.B., Pan, X., Kodippili, K., Yue, Y., Zhang, K., Yao, G., Haffner, B., Duan, S.X., Ramos, J., et al. (2017). A Five-Repeat Micro-Dystrophin Gene Ameliorated Dystrophic Phenotype in the Severe DBA/2J-mdx Model of Duchenne Muscular Dystrophy. Mol Ther Methods Clin Dev *6*, 216–230.
Head, S., Williams, D., and Stephenson, G. (1994). Increased susceptibility of EDL muscles from mdx mice to damage induced by contraction with stretch. J Muscle Res Cell Motil *15*, 490–492.
Hightower, R.M., and Alexander, M.S. (2018). Genetic modifiers of Duchenne and facioscapulohumeral muscular dystrophies. Muscle Nerve *57*, 6–15.
Hovhannisyan, Y., Melikyan, G., Mougenot, N., Gao-Li, J., Friguet, B., Paulin, D., Li, Z., Ferry, A., and Agbulut, O. (2019). Effects of the selective inhibition of proteasome caspase-like activity by CLi a derivative of nor-cerpegin in dystrophic mdx mice. PLoS ONE *14*, e0215821.
Hughes, D.C., Wallace, M.A., and Baar, K. (2015). Effects of aging, exercise, and disease on

force transfer in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *309*, E1–E10.
Joanne, P., Chourbagi, O., Hourde, C., Ferry, A., Butler-Browne, G., Vicart, P., Dumonceaux, J., and Agbulut, O. (2013). Viral-mediated expression of desmin mutants to create mouse models of myofibrillar myopathy. Skelet Muscle *3*, 4.
Judge, L.M., Arnett, A.L., Banks, G.B., and Chamberlain, J.S. (2011). Expression of the dystrophin isoform Dp116 preserves functional muscle mass and extends lifespan without preventing dystrophy in severely dystrophic mice. Hum Mol Genet *20*, 4978–4990.
Li, Z., Colucci-Guyon, E., Pinçon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. (1996). Cardiovascular lesions and skeletal myopathy in mice lacking desmin. Dev. Biol. *175*, 362–366.
Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L.E., Babinet, C., and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. J Cell Biol *139*, 129–144.
Li, Z., Parlakian, A., Coletti, D., Alonso-Martin, S., Hourdé, C., Joanne, P., Gao-Li, J., Blanc, J., Ferry, A., Paulin, D., et al. (2014). Synemin acts as a regulator of signalling molecules during skeletal muscle hypertrophy. J. Cell. Sci. *127*, 4589–4601.
Lieber, R.L., Thornell, L.E., and Fridén, J. (1996). Muscle cytoskeletal disruption occurs within the first 15 min of cyclic eccentric contraction. J. Appl. Physiol. *80*, 278–284.
Lovering, R.M., and De Deyne, P.G. (2004). Contractile function, sarcolemma integrity, and the loss of dystrophin after skeletal muscle eccentric contraction-induced injury. Am J Physiol Cell Physiol *286*, C230–C238.
Lovering, R.M., O'Neill, A., Muriel, J.M., Prosser, B.L., Strong, J., and Bloch, R.J. (2011). Physiology, structure, and susceptibility to injury of skeletal muscle in mice lacking keratin 19-based and desmin-based intermediate filaments. Am. J. Physiol., Cell Physiol. *300*, C803-813.
Lowe, D.A., Williams, B.O., Thomas, D.D., and Grange, R.W. (2006). Molecular and cellular contractile dysfunction of dystrophic muscle from young mice. Muscle Nerve *34*, 92–100.
McGreevy, J.W., Hakim, C.H., McIntosh, M.A., and Duan, D. (2015). Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. Dis Model Mech *8*, 195–213.
McKenna, M.J., Bangsbo, J., and Renaud, J.-M. (2008). Muscle K+, Na+, and Cl disturbances and Na+-K+ pump inactivation: implications for fatigue. J. Appl. Physiol. *104*, 288–295.
Messéant, J., Dobbertin, A., Girard, E., Delers, P., Manuel, M., Mangione, F., Schmitt, A., Le Denmat, D., Molgó, J., Zytnicki, D., et al. (2015). MuSK frizzled-like domain is critical for mammalian neuromuscular junction formation and maintenance. J. Neurosci. *35*, 4926–4941.
Meyer, G.A., Schenk, S., and Lieber, R.L. (2013). Role of the cytoskeleton in muscle transcriptional responses to altered use. Physiol. Genomics *45*, 321–331.
Miura, P., and Jasmin, B.J. (2006). Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we? Trends Mol Med *12*, 122–129.
Moens, P., Baatsen, P.H., and Marechal, G. (1993). Increased susceptibility of EDL muscles from mdx mice to damage induced by contractions with stretch. J Muscle Res Cell Motil *14*, 446–451.
Monti, R.J., Roy, R.R., Hodgson, J.A., and Edgerton, V.R. (1999). Transmission of forces within mammalian skeletal muscles. J Biomech *32*, 371–380.
Mouisel, E., Vignaud, A., Hourdé, C., Butler-Browne, G., and Ferry, A. (2010). Muscle

weakness and atrophy are associated with decreased regenerative capacity and changes in mTOR signaling in skeletal muscles of venerable (18-24-month-old) dystrophic mdx mice. Muscle Nerve *41*, 809–818.
Nelson, D.M., Lindsay, A., Judge, L.M., Duan, D., Chamberlain, J.S., Lowe, D.A., and Ervasti, J.M. (2018). Variable rescue of microtubule and physiological phenotypes in mdx muscle expressing different miniaturized dystrophins. Hum. Mol. Genet. *27*, 2090–2100.
Palmisano, M.G., Bremner, S.N., Hornberger, T.A., Meyer, G.A., Domenighetti, A.A., Shah, S.B., Kiss, B., Kellermayer, M., Ryan, A.F., and Lieber, R.L. (2015). Skeletal muscle intermediate filaments form a stress-transmitting and stress-signaling network. J. Cell. Sci. *128*, 219–224.
Pastoret, C., and Sebille, A. (1995). mdx mice show progressive weakness and muscle deterioration with age. J. Neurol. Sci. *129*, 97–105.
Prins, K.W., Lowe, D.A., and Ervasti, J.M. (2008). Skeletal muscle-specific ablation of gamma(cyto)-actin does not exacerbate the mdx phenotype. PLoS ONE *3*, e2419.
Ramaswamy, K.S., Palmer, M.L., van der Meulen, J.H., Renoux, A., Kostrominova, T.Y., Michele, D.E., and Faulkner, J.A. (2011). Lateral transmission of force is impaired in skeletal muscles of dystrophic mice and very old rats. J Physiol *589*, 1195–1208.
Rooney, J.E., Welser, J.V., Dechert, M.A., Flintoff-Dye, N.L., Kaufman, S.J., and Burkin, D.J. (2006). Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. J. Cell. Sci. *119*, 2185–2195.
Roy, P., Rau, F., Ochala, J., Messéant, J., Fraysse, B., Lainé, J., Agbulut, O., Butler-Browne, G., Furling, D., and Ferry, A. (2016). Dystrophin restoration therapy improves both the reduced excitability and the force drop induced by lengthening contractions in dystrophic mdx skeletal muscle. Skelet Muscle *6*, 23.
Sam, M., Shah, S., Friden, J., Milner, D.J., Capetanaki, Y., and Lieber, R.L. (2000). Desmin knockout muscles generate lower stress and are less vulnerable to injury compared with wild-type muscles. Am J Physiol Cell Physiol *279*, C1116-22.
Sarkar, S., Korolchuk, V., Renna, M., Winslow, A., and Rubinsztein, D.C. (2009). Methodological considerations for assessing autophagy modulators: a study with calcium phosphate precipitates. Autophagy *5*, 307–313.
Shin, J.-H., Hakim, C.H., Zhang, K., and Duan, D. (2011). Genotyping mdx, mdx3cv, and mdx4cv mice by primer competition polymerase chain reaction. Muscle Nerve *43*, 283–286.
Sonnemann, K.J., Fitzsimons, D.P., Patel, J.R., Liu, Y., Schneider, M.F., Moss, R.L., and Ervasti, J.M. (2006). Cytoplasmic gamma-actin is not required for skeletal muscle development but its absence leads to a progressive myopathy. Dev. Cell *11*, 387–397.
Squire, S., Raymackers, J.M., Vandebrouck, C., Potter, A., Tinsley, J., Fisher, R., Gillis, J.M., and Davies, K.E. (2002). Prevention of pathology in mdx mice by expression of utrophin: analysis using an inducible transgenic expression system. Hum Mol Genet *11*, 3333–3344.
Staszewska, I., Fischer, I., and Wiche, G. (2015). Plectin isoform 1-dependent nuclear docking of desmin networks affects myonuclear architecture and expression of mechanotransducers. Hum Mol Genet *24*, 7373–7389.
Ueberschlag-Pitiot, V., Stantzou, A., Messéant, J., Lemaitre, M., Owens, D.J., Noirez, P., Roy, P., Agbulut, O., Metzger, D., and Ferry, A. (2017). Gonad-related factors promote muscle performance gain during postnatal development in male and female mice. Am. J. Physiol. Endocrinol. Metab. *313*, E12–E25.

Legends of figures

Figure 1. Muscle weakness (absolute maximal force) is worsened in 2-month-old DKO mice, independently from a reduced maximal specific force. A: Representative immunoblot of desmin. B: Absolute maximal force (P0) measured by electrophysiology. C: Specific maximal force (sP0). D: Fibrosis (area covered by sirius red staining). E: Representative sarcomere abnormalities (electronic microscopy). N = 8-16 per group for electrophysiology for and N = 5-6 for histology. a2, a3, a4: significantly different from C57 mice ($p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively). b2, b4: significantly different from DesKO mice ($p \leq 0.01$, $p \leq 0.0001$ respectively). c2, c4: significantly different from *mdx* mice ($p \leq 0.01$, $p \leq 0.0001$, respectively).

Figure 2. Exaggerated muscle fragility related to reduced excitability and impaired fatigue resistance in 2-month-old DKO mice were independent from the promotion of faster and less oxidative muscle fibres. A: Fragility, i.e., force drop following lengthening contractions, and reduced muscle excitability, i.e., decreased compound muscle action potential (CMAP) following lengthening contractions, evaluated by electrophysiology. B: Fatigue resistance, measured by electrophysiology. C: Percentage of muscle fibres expressing myosin heavy chain type 2b (MHC-2b)(immunohistology). D: Percentage of the muscle area covered by muscle fibres with high succinate deshydrogenase (SDH) staining. E: Percentage of muscle fibres expressing MHC-2a. F: Percentage of muscle fibres expressing MHC-2x. N = 6-14 per group for electrophysiology and N = 4-6 for histology. a1, a3, a4: significantly different from C57 mice ($p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively). b2, b3, b4: significantly different from DesKO mice ($p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively). c3, c4: significantly different from *mdx* mice ($p \leq 0.001$, $p \leq 0.0001$, respectively).

Figure 3. The severe atrophy in 2-month-old DKO mice is not associated with altered markers of autophagy and ubiquitin-proteasome system. A: Muscle weight (mg) and muscle weight relative to body weight (mg/10g of body). B: Muscle weight relative to tibia length (mg/mm). C: LC3-II protein expression, as a marker of autophagy (immunoblotting). D: Ubiquitin proteasome activities, as markers of proteolytic process. E: Expression of genes (mRNA) involved in ubiquitin proteasome system and muscle growth (qPCR). N= 8-16 for muscle weight and N= for 3-6 for qPCR and proteasome activity. a1, a2, a3, a4: significantly different from C57 mice ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively). b3, b4: significantly different from DesKO mice ($p \leq 0.001$, $p \leq 0.0001$, respectively). c2, c4: significantly different from *mdx* mice ($p \leq 0.01$, $p \leq 0.0001$, respectively).

Figure 4. The severe atrophy in 2-month-old DKO mice is attributable to both reduced number and diameter of muscle fibres, but not neuromuscular transmission failure despite a severe dismantlement of neuromuscular junction. A: Number of muscle fibres per cross-section (immunohistology). B: Diameter (minimum feret) of muscle fibres. C: Diameter of muscle fibres expressing MHC-2b. D: Diameter of muscle fibres expressing MHC-2a. E: Diameter of muscle fibres expressing MHC-2x**.** F: Representative images of neuromuscular junctions. G: Number of fragments per AChR clusters, evaluated by immunohistology. H: Area of AChR cluster. I: Maximal force in response to nerve or muscle stimulation (electrophysiology). N = 4-6 for fibre histology, N=3 for neuromuscular junction immunohistology, N = 8-18 for electrophysiology. a1, a2, a3: significantly different from C57 mice ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively). b1, b2, b3, b4: significantly different from DesKO mice ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively). c1, c2, c3, c4: significantly different from *mdx* mice ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively).

Figure 5. The absence of a great occurrence of centronucleated muscle fibres in 2-month-old DKO mice was not related to impaired regeneration after myotoxic (cardiotoxin) injection. A: Percentage of centronucleated muscle fibres (immunohistology). B: Absolute maximal force in cardiotoxin-injected muscle (electrophysiology). C: Specific maximal force in cardiotoxin-injected muscle. D: Fragility in cardiotoxin-injected muscle. E: Muscle weight cardiotoxin-injected muscle. F: Percentage of centronucleated muscle fibres in cardiotoxin-injected muscles. N = 3-7 per group for electrophysiology, N = 3-7 for muscle weight, and N = 3-6 for histology. a1, a4: significantly different from C57 mice ($p \leq 0.05$, $p \leq 0.0001$, respectively). b2, b4: significantly different from DesKO mice ($p \leq 0.01$, $p \leq 0.0001$, respectively). c2**,** c3**,** c4: significantly different from *mdx* mice ($p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively).

Figure 6. Severe muscle weakness, fragility and atrophy were already observed in 1-month-old and newborn DKO mice. A: Absolute maximal force (P0), evaluated by electrophysiology. B: Specific maximal force (sP0). C: Fragility. D: Muscle weight. E: Percentage of centronucleated muscle fibres (immunohistology). F: Fibrosis. G: Representative histological abnormalities (microscopy) in hindlimb from newborn DKO mice at 1 (upper panel) and 3 (lower panel) days post-natal as assessed by Hematoxylin/Eosin staining. Insets show a higher magnification of muscle areas showing infiltrating cells and fibre size heterogeneity in the DKO as compared to *mdx* mice. Bars=100µm for the panels and 20µm for the insets. mu: muscle; bn: bone; dr: dermis. N = 6-14 for electrophysiology, N = 8-13 for muscle weight, and N = 5-6 for histology. a1, a3, a4: significantly different from C57 mice ($p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$, respectively). c1, c2, c3, c4: significantly different from *mdx* mice ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$, respectively). t2, t4: significantly different from 2-months old aged mice ($p \leq 0.01$, $p \leq 0.0001$, respectively).

Figure 7. The dystrophic features are similar in 2-month-old *mdx*:Des+/- and *mdx* mice. A: Expression of desmin gene (mRNA)(qPCR). B: Desmin protein (immunoblotting). C: Absolute (P0) and specific maximal forces (sP0), evaluated by electrophysiology. D: Fragility. E: Muscle weight. F: Percentage of centronucleated muscle fibres (immunohistology). N = 6-10 for electrophysiology, N = 6-8 for muscle weight, and N = 4-20 for qPCR, histology and immunoblotting. a4: significantly different from C57 mice ($p \leq 0.0001$). c2, c4: significantly different from *mdx* mice ($p \leq 0.01$, $p \leq 0.0001$, respectively). d3, d4: significantly different from DKO mice ($p \leq 0.001$, $p \leq 0.0001$, respectively).

Figure 8. Increased expression of desmin via *Des* transfer with adeno-associated virus (AAV-Des) improves weakness in 2-month-old DKO muscle. A: Desmin protein (immunoblotting). B: Specific maximal force (sP0)(electrophysiology). C: Absolute maximal force (P0). D: Muscle weight. E: Diameter (mimimum ferret) of muscle fibres (immunohistology). F: Percentage of centronucleated muscle fibres. N = 4-8 for electrophysiology, N = 7-8 for muscle weight, N = 3-4 for immunoblotting and N= 3-7 for histology. c1, c3, c4: significantly different from *mdx* mice ($p \leq 0.05$, $p \leq 0.001$, $p < 0.0001$, respectively). d2: significantly different from DKO mice ($p \leq 0.01$, respectively).

Figure 9. *Des* transfer with adeno-associated virus (AAV-Des) improves weakness in 2-month-old *mdx* mice when performed neonatally (*mdx*n+AAV-Des), but not at 1 month of age (*mdx*+AAV-Des) muscle. A: Desmin protein (immunoblotting). B: Desmin protein. C: Absolute (P0) and specific maximal forces (sP0)(electrophysiology). D: Fragility in *mdx* mice. E: Muscle weight. F: Absolute (P0) and specific maximal forces (sP0). G: Fragility in *mdx* mice. H: Muscle weight. N = 4-11 for immunoblotting, N = 7-14 for electrophysiology,

and N = 7-16 for muscle weight. a2: significantly different from C57 mice ($p \leq 0.01$). c1, c2: significantly different from *mdx* mice ($p \leq 0.05$, $p \leq 0.01$, respectively).

Table 1. Sequences of primers used.

Gene	Forward	Reverse
Housekeeping gene		
Sdha	5'-TTACAAAGTGCGGGTCGATG-3'	5'-GTGTGCTTCCTCCAGTGTTC-3'
Hmbs	5’-AGGTCCCTGTTCAGCAAGAA-3’	5’-TGGGCTCCTCTTGGAATGTT-3’
Genes of interest		
Des	5'-GTCCTCACTGCCTCCTGAAG-3'	5'-AGCATGAAGACCACAAAGGG-3'
Ccl2	5'-CAC CAG CAA GAT GAT CC-3'	5'-ATA AAG TTG TAG GTT CTG ATC TC-3'
Fst	5'-GGAAAACCTACCGCAACGAA-3'	5'-TGATCCACCACACAAGTGGA-3'
Atrogin1 *FBOX32*	5'-TCACAGCTCACATCCCTGAG-3'	5'- TCAGCCTCTGCATGATGTTC-3'
Mstn	5'-GCTACCACGGAAACAATCAT-3'	5'-CAATACTCTGCCAAATACCA-3'
Murf1 *TRIM63*	5'-TGAGGTGCCTACTTGCTCCT-3'	5'-GTGGACTTTTCCAGCTGCTC-3'