<Supplementary material>

+A: **Supplementary materials and methods**

All reagents/equipment were acquired through Life Technologies (Grand Island, NY, USA) or affiliated product brands unless stated otherwise.

+B: Animals

Male wild-type (WT; *C57BL/10ScSn/J*) and *mdx* (*C57BL/10ScSn-mdx/J*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). They were housed and bred at the Veterans Affairs Animal Facility or the Children's National Medical Center (CNMC) Research Animal Facility (Washington, DC, USA) in an individually vented cage system with a 12 h light–dark cycle, receiving standard mouse chow and purified water *ad libitum*. All animal experiments were conducted in accordance with our IACUC guidelines under approved protocols.

+B: Experimental design in animal studies and tissue preparation

All studies involving mice followed a common schedule for treatment, performance of assays, and collection of tissues with variations in the number and types of assays performed and the age of the mice at commencement of treatment (Figure 1). In the main study (Study 1), treatment groups were as follows: vehicle (VEH), intraperitoneal water and oral cherry syrup; intraperitoneal CpdA (a kind gift from Professor Guy Haegeman, Gent University, Belgium), 3.75 mg/kg/day or 7.5 mg/kg/day, dissolved in water plus oral cherry syrup; oral PNSL, 5.0 mg/kg/day (CNMC pharmacy suspension in cherry syrup) plus intraperitoneal water. The study consisted of three WT groups (VEH, intra-peritoneal CpdA 7.5 mg/kg/day, oral PNSL 5.0 mg/kg/day and four *mdx* groups (VEH, intra-peritoneal CpdA 3.75 mg/kg/day and CpdA 7.5 mg/kg/day, and oral PNSL 5.0 mg/kg/day), with n = 15 per group. A second study (Study 2), where treatment was commenced at 4 weeks, consisted of a VEH-treated WT group and three *mdx* groups: a

VEH, an intra-peritoneal CpdA 7.5 mg/kg/day, and an oral PNSL 5.0 mg/kg/day group, with n = 10 per group. Study 2 was performed in the context of a validation study comparing oral CpdA at 2.5 mg/kg/day, 5.0 mg/kg/day, and 7.5 mg/kg/day with intraperitoneal CpdA at 7.5 mg/kg/day. Outcome measures were limited to H&E analysis and cytokine protein levels in the gastrocnemius muscles. This study was performed in order to confirm bioactivity of intraperitoneal CpdA prior to the conduct of a more comprehensive study (Study 1). The mice were sacrificed and tissue samples were immediately collected and weighed. The right TA was stored in 10% neutral buffered formalin before paraffin embedding for H&E staining. All additional tissues collected were embedded in O.C.T. compound and placed in foil, and then snap-frozen in isopentane chilled in liquid nitrogen and stored at –80 for subsequent assays. For the correlation of gene expression and protein content in the mice in gastrocnemius muscle, frozen tissues were pulverized in liquid nitrogen, using a mortar and pestle, and divided for RNA extraction and protein lysates.

+B: Grip strength measurement (GSM)

Forelimb and hindlimb GSMs were performed as previously described [1] but with an increase in the acclimation period from three to five consecutive days. Briefly, the mice were allowed to grip the horizontal forelimb mesh or an angled hindlimb mesh (Columbus Instruments, Columbus, OH, USA) before a gentle and constant pull of the tail exerted to generate a peak force measurement via a transducer. The GSMs were collected in the morning hours over a 5-day period, with maximum values for each day over this period averaged to obtain absolute GSM values (Kgf) and then divided by body weight (recorded at the beginning of the 5-day period) for normalized GSM values (Kgf/kg).

+B: Open-field behavioural activity (voluntary locomotor) measurements (Digiscan)

Voluntary locomotor activity was measured using an open-field Digiscan apparatus (Omnitech Electronics, Columbus, OH, USA) as previously described [1] except that the

mice were acclimatized for 4 days before data were collected every 10 min over a 1 h period each day for 4 consecutive days. Acclimation and collection of data was performed in the morning for all mice. Briefly, the three-dimensional movements of the mice within the apparatus were detected via lasers and quantified using associated software. Total Distance, Horizontal Activity, and Vertical Activity (all expressed as units), as well as Rest Time and Movement Time (seconds) were analysed.

+B: *Ex vivo* force contraction

The extensor digitorum longus (EDL) muscle of the right hindlimbs were removed from anaesthetized mice and placed vertically in a bath containing buffered mammalian Ringer solution (25°C) and bubbled with 95% O_2/5% CO_2. The distal tendon of the muscle was tied securely to the lever arm of a servomotor/force transducer (Model 305B, Aurora Scientific, Aurora, ON, Canada) and the proximal tendon was fixed to a stationary post in the bath. The muscles were stimulated between two stainless steel plate electrodes. At optimal muscle length, the force developed was measured during trains of stimulation (300 ms) with increasing frequencies until the highest plateau was achieved. The force generated to obtain the highest plateau was established as the maximal isometric force (MIF) generated by the muscle and is expressed in milliNewtons (mN). Further, the specific force (SF) was obtained by dividing the MIF with the physiological cross-sectional area of muscle and is expressed as mN/mm^2. The physiological cross-sectional area was calculated by dividing the muscle mass with the fibre length and density (1.056 kg/m^3) of muscle tissue. The fibre length is established as 0.71 of muscle length for the EDL muscle [2]. For lengthening–contraction studies, the muscle is electrically stimulated with tetanic contractions following the establishment of optimal muscle length, for 300 ms duration. The tetanic contractions, with 2 min rest intervals, are given with increasing frequencies of stimulation until a plateau is reached, normally at 250 Hz. Then the muscle is subjected to a protocol of 10 lengthening contractions separated by 1 min rest intervals. The muscle is stimulated at 250 Hz for 300 ms, allowing the muscle to generate force, immediately followed by a lengthening over 10% of muscle length at a

velocity of two fibre lengths/s. Muscle length is returned to the optimal length immediately after the forced lengthening.

+B: Histopathology scoring

Assessment of pathology in WT and *mdx* mice following treatment was performed in paraffin-embedded right tibialis anterior (TA) cross-sections (8 μm), using H&E staining. Histological evaluations were performed in a blinded manner using coded slides. Quantitative stereology was performed on a section of the entire TA (non-overlapping fields captured by ×40 objective) using an Olympus BX51 microscope (Olympus America, Center Valley, PA, USA). Assessment following accepted criteria (Treat-NMD SOP No. DMD_M.1.2.007) included: assessment of total fibres present; total fibres with central nuclei; total peripheral nuclei; total central nuclei; regenerating fibres (basophilic fibres); degenerating fibres; and inflammation (an interstitial group of 10 smaller inflammatory cell dark blue nuclei in a high-power field). Nuclei located more than one nuclear diameter from the fibre border were deemed 'central'.

+B: Quantitative polymerase chain reaction (qPCR)

RNA was isolated from cells or frozen tissue using an integrated Trizol and RNeasy minikit (Qiagen, Valencia, CA, USA) protocol with DNase I (Qiagen) treatment. cDNA was obtained using Reverse Transcription System (Promega, Madison, WI, USA). All PCR reactions were carried out using Taqman Gene Expression Master Mix except *Spp1* and *Hprt* gene analysis (SYBR Green PCR Master Mix), with the following thermal cycle conditions performed on the 7900HT Fast Real-Time PCR System: initial steps (UNG activation, 50°C for 2 min; DNA polymerase activation, 95°C for 10 min) and 40 cycles with a melting temperature of 95°C for 15 s and an annealing/extension temperature of 60°C for 60 s.

Hprt was used as the housekeeping gene in all qPCR studies. Relative quantification was used in all studies except in the atrophy studies where relative expression software tool (REST) was used. Comparisons between WT and *mdx* mice

were performed in vehicle-treated groups. Within both WT and *mdx* groups, comparisons were made between the vehicle group and other treatment groups. For studies of the expression of genes determining muscle mass, gastrocnemius muscle was used for all genes except *Foxo3*, where the quadriceps was used due to inadequate mRNA from gastrocnemius to complete all studies.

The following Taqman Gene Expression Assays were used: *Hprt* (Mm01545399_m1), *Ccl2* (Mm00441242_m1), *Tnf* (Mm00443260_g1), *Il6* (Mm00446190_m1), *Foxo1* (Mm00490672_m1), *Fbxo32* (Mm00499523_m1), *Trim63* (Mm01185221_m1), *Mstn* (Mm01254559_m1), *Igf1* (Mm00439560_m1), *Pomc* (Mm00435874_m1), and *Sgk1* (Mm00441387_g1). The primer pairs (5′–3′) for the SYBR Green PCR reactions were: *Spp1* (forward) CTTTACAGCCTGCACCCAGA, (reverse) GCCACAGAATGCTGTGTCCT; and *Hprt* (forward) AGCCTAAGATGAGCGCAAGT, (reverse) TTACTAGGCAGATGGCCACA.

+B: Flow cytometry

Protein lysates (supernatant) were acquired from pulverized gastrocnemius (GA) muscles using RIPA buffer (Teknova, Hollister, CA, USA) and Protease Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) before centrifugation at 13 000 rpm for 10 min at 4°C. The fluorescence-activated cell sorting (FACS) system (BD Biosciences, San Jose, CA, USA) was utilized to quantify cytokines with the Cytometric Bead Array Mouse Inflammation Kit, measured according to the manufacturer’s instructions. Cytokines of interest were interleukin (IL)-6, IL-10, chemokine (C–C motif) ligand 2 (Ccl2), tumour necrosis factor (TNF), interferon (IFN)γ, IL-12p70. Measurements were performed using the FacsCalibur and analysed using CellQuest Pro software (v. 6.0). Cytokine quantification was achieved using generated standard curves and normalized to protein concentration determined using a Bio-Rad Microplate Protein Assay (Bio-Rad, Hercules, CA, USA).

+B: Serum creatine kinase (CK) measurements

The blood was collected via retro-orbital bleed under ketamine anaesthesia. 250 μl of blood was collected into Eppendorf tubes, allowed to clot, and kept at room temperature to allow clot contraction prior to centrifugation (10 000 rpm for 10 min at 4°C) and serum collection. CK determination was performed using a standard spectrophotometric method with enzyme-coupled assay reagent, CK10 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Absorption at 340 nm was measured every minute for 2 min at 37°C to calculate enzyme activity. Duplicate measurements were done on each serum sample, and the data expressed as units/litre (U/l).

+B: Cell culture

Commercially available mouse skeletal muscle C2C12 stable cells (ATCC, Manassas, VA, USA) that have an integrated chromosomal reporter consisting of six tandem NF-κB response elements coupled to luciferase were cultured with DMEM containing penicillin/streptomycin (1%), hygromycin (100 μg/ml) and 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 in 96-well plates at 1×10^4 cells/well. These myoblasts were differentiated to myotubes by reducing serum concentrations (replacing 10% FBS with 2% horse serum) and allowing them to remain in culture for a minimum of 96 h. Conditionally immortalized H-2K *mdx* myoblasts were obtained by crossing *mdx* mice and the H-2Kb-tsA58 immortomouse, as previously described [3,4]. These cells were cultured in growth medium containing DMEM, 20% FBS, 2% chick embryo extract (US Biologicals, Boston, MA, USA), 2% L-glutamine, 1% penicillin/streptomycin and 0.02 μg/ml IFNγ (Millipore, Billerica, MA, USA) at 33°C with 10% CO_2 in six-well plates at 5×10^5 cells/well. Differentiation into myotubes was achieved by removing the chick embryo extract, replacing the 20% FBS with 5% horse serum, and incubating at 37°C with 5% CO_2 for at least 96 h. Matrigel (BD Biosciences, San Jose, CA, USA) coating of culture flasks was used to optimize proliferation and differentiation of these H-2K *mdx* myogenic cells. AtT-20/D16v-F2 murine pituitary corticotrophs (ATCC, Manassas, VA, USA) were cultured in DMEM containing 1% penicillin/streptomycin and 10% FBS at 37°C with 5% CO_2 in six-well plates at 2.5×10^5 cells/well. Adherence of the cortiotrophs was improved by coating flasks with Matrigel.

+B: NF-κB luciferase reporter assay

The comparative effects of PNSL and CpdA on NF-κB activity was assessed using a luciferase reporter assay, as previously described [5]. C2C12-NF-κB luciferase myoblasts were plated into 96-well plates at 1×10^4 cells/well and allowed to adhere overnight. They were then pretreated with 1% DMSO or 11 different ½-log incremental concentrations of either PNSL (Sigma-Aldrich, St. Louis, MO, USA) or CpdA (ranging from 0.0001 to 10 μM) for 4 h before stimulation with 10 ng/ml recombinant mouse TNF (R&D Systems, Minneapolis, MN, USA) for 1 h. The cell contents were then extracted using reporter lysis buffer with one freeze–thaw cycle before the lysate was transferred to Costar white plates and the luciferase activity measured, using a single luciferase reporter assay system with a Berthold Centro LB 960 Luminometer. Raw luciferase values were normalized as a percentage of the TNF-treated group, which represented 100% induction. This study was repeated using myotubes.

Cell viability (myoblasts and myotubes) was assessed using a spectrophotometer-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, in which cells were incubated in 96-well plates under conditions similar to the luciferase reporter assay, the medium then removed, and formazan crystals dissolved in DMSO (100%) were added before the plate was read in an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA) at 570 nm. Percentage cell viability was calculated relative to untreated controls.

+B: Immunofluorescence

H-2K *mdx* myoblasts were plated into eight-well Lab-tek chamber slides precoated with Matrigel at 2×10^4 cells/well and allowed to adhere overnight. These cells were pretreated with 1% DMSO, CpdA (4 μM) or PNSL (10 μM) for 4 h, followed by stimulation with 1 ng/ml or 10 ng/ml TNF for 30 min (non-stimulated DMSO-treated groups were also included). Myotubes were treated for 24 h before stimulation for 24 h. Cells were fixed and the following reagents used for immunostaining: primary rabbit

polyclonal antibody to NF-κB p65 (1:100; Abcam, Cambridge, MA, USA); secondary goat anti-rabbit IgG AlexaFluor 568 (1:1000), and DAPI for nuclear detection. A Zeiss fluorescent/apotome inverted Axiovert 200M microscope was used to acquire images.

+B: Western blot

H-2K *mdx* myoblasts were plated into six-well plates precoated with Matrigel at 5×10^5 cells/well and allowed to adhere overnight. Treatments and TNF stimulation for both myoblasts and myotubes were similar to that in the immunofluorescence studies. Following TNF stimulation, nuclear and cytoplasmic fractions from each well were obtained using a p65(RelA) Translocation Assay Kit (Five Photons Biochemicals, San Diego, CA, USA). 10 µl of nuclear fraction and 15 µl of cytoplasmic fraction from each treatment were used. Proteins were separated on a denaturing 4–12% Bis–Tris SDS–PAGE gel and transferred onto PVDF membranes. Following blocking with 3% milk, membranes were immunoblotted overnight at 4°C with the following antibodies (in 5% milk): rabbit polyclonal IgG to p65 (1:400; Five Photons Biochemicals), goat polyclonal IgG to Actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal IgG to Lamin A (1:1000; Abcam). After washing, membranes were probed with HRP-conjugated anti-rabbit (1:2500 dilution; GE Healthcare, Piscataway, NJ, USA) or anti-goat (1:2500; Santa Cruz Biotechnology) secondary antibody (in PBS). The membranes were incubated with ECL Western Blotting Substrate (GE Healthcare) and processed on X-ray film (Denville Scientific, South Plainfield, NJ, USA). Densitometry analysis of p65 nuclear and cytoplasmic blots, normalized to both Actin and Lamin A, was used to assess p65 nuclear:cytoplasmic ratios.

+B: Pituitary corticotroph studies

AtT-20/D16v-F2 murine pituitary corticotrophs were plated into Matrigel-precoated six-well plates at 2.5×10^5 cells/well. They were then treated with 1% DMSO, PNSL (1 or 10 µM) or CpdA (1 or 4 µM) for 24 h before stimulation with 100 nm Corticotrophin-releasing Factor (Sigma-Aldrich) for 3 h. The medium from each well was then collected

for adrenocorticotropic hormone (ACTH) measurement with Mouse/Rat ACTH Ultra Sensitive lumELISA (Calbiotech, Spring Valley, CA, USA) according to the manufacturer's instructions. The concentrations of ACTH in the samples were calculated from the linear range of the standard curve and were normalized to protein concentration, determined using a Bio-Rad Microplate Protein Assay (Bio-Rad, Hercules, CA, USA). Each well was then washed with PBS before the addition of Trizol for RNA extraction, as outlined previously.

+B: Live animal optical imaging

Quantification of inflammation in forelimb and hindlimb muscles of mice was achieved using live-animal optical imaging of cathepsin-B (CTSB) enzyme activity, using a caged near-infrared substrate (ProSense 680) as previously described [6]. Briefly, hair covering areas to be scanned was removed with Nair and mice with significant skin pigmentation were excluded. 24 h prior to acquisition of data, the mice underwent intraperitoneal injection with 2.0 nM ProSense 680 (Perkin-Elmer, Waltham, MA, USA), both prepared in a total volume of 150 µl PBS. Areas of scanning were defined with a polygonal selection tool, then a 670 nm excitation laser was used for probe excitation at a 1.0 mm camera resolution in skeletal limbs (n = 7–10/group); emissions were collected from a 700 nm long-pass filter. Optical imaging intensities from all experiments were normalized to account for differences in scanning laser power, integration times and scanning area, selected between mice using Optiview 2.0 software.

+B: Osteopontin (OPN) ELISA assay

Quantification of OPN in protein lysates from GA muscles was performed using a 96-well plate rat/mouse OPN Quantikine ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, samples of serially diluted recombinant mouse OPN standards and muscle lysates (50 µl) were incubated for 2 h in the wells, after which the samples were removed and the wells washed and then incubated with mouse OPN conjugate (100 µl). Following further washing and incubation

for 30 min with substrate solution, the reaction was terminated and the absorbance at 450 nm was read using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The concentrations of OPN in the samples were calculated from the linear range of the standard curve and were normalized to protein concentration determined using a Microplate Protein Assay (Bio-Rad).

+B: Statistical analysis

For all proposed experiments and procedures, one-way ANOVA with Dunnett's post-test comparison against a control was used for experiments involving more than two groups. Two-tailed Student's *t*-test was used for experiments using only two groups. For qPCR, Relative Expression Software Tool (REST) or non-parametric one-way ANOVA with a Kruskal–Wallis comparison of relative quantification (RQ) values was used. Two-way ANOVA with Bonferroni post-test comparison with VEH-treated controls was performed in weekly body weight comparisons and lengthening–contraction studies where different time-points were involved. The relationship between OPN levels and percentage necrotic fibres in the GA muscles of *mdx* mice were analysed using a Pearson correlation. Significance was defined as a p value < 0.05. Prism 5 statistical software was used for analyses.

+A: **References**

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