Supplementary Methods

Plasmids to evaluate endogenous dystrophin splicing signals. Following primers were used to amplify the endogenous intron/exon junctions of the dystrophin gene from 293 cell genomic DNA. Primers to amplify exon 53 and intron 53 donor sequence are DL226 (forward, 5'-GCGCGGTACCTTGAAAGAATTCAGAATCAGTGG-3') and DL227 (reverse, 5'-GCGCGGATCCCAGCTTTAACGTGATTTTCTG-3'). Primers to amplify intron 53 acceptor signal and exon 54 are DL228 (forward, 5'-GCGCCTCGAGGGTAGGTTAAAAGCAATCT-3') and DL229 (reverse, 5'-GCGCTCTAGACTTTTATGAATGCTTCTCC-3'). Primers to amplify exon 56 and intron 56 donor sequence are DL214 (forward, 5'-GCGCAAGCTTGACCTCCAAGGTGAAATTG-3') and DL215 (reverse, 5'-GCGCGGTACCGGGATGATTTACGTAGACATG-3'). Primers to amplify intron 56 acceptor signal and exon 57 are DL216 (forward, 5'-GCGCGATATCGTACTTTTCTATGGAATTG-3') and DL217 (reverse, 5'-GCGCCTCGAGCCTATGTACATCGTTCTGC-3'). Primers to amplify exon 60 and intron 60 donor sequence are DL218 (forward, 5'-GCGCGGTACCGCACTTCGAGGAGAAATTGCG-3') and DL219 (reverse, 5'-GCGCGGATCCCTGTAACAAAGGACAACAATG-3'). Primers to amplify intron 60 acceptor signal and exon 61 are DL220 (forward, 5'-GCGCCTCGAGTATAGAATGAGAGAACAT-3') and DL221 (reverse, 5'-GCGCTCTAGATGGAAAGAAAGTGCTGAGATGC-3'). Primers to amplify exon 63 and intron 63 donor sequence are DL222 (forward, 5'-GCGCAAGCTTCCACGAGACTCAAACAACTTGC-3') and DL223 (reverse, 5'-GCGCGGTACCCTTTCACACTGCAAACTAC-3'). Primers to amplify intron 63 acceptor signal and exon 64 are DL224 (forward, 5'-GCGCCTCGAGGGCAAATCACTGGGCGTCGG-3') and DL225 (reverse, 5'-GCGCTCTAGAAGCAAAGGGCCTTCTGCAG-3'). The PCR products were inserted in pcDNA3.1(+)(Invitrogen, Carlsbad, California). In p53/53/54 and p60/60/61, the 5' exon and intron donor sequences were cloned into the Kpn I and the BamH I sites. In p56/56/57 and p63/63/64, 5' exon

and intron donor sequences were cloned into the Hind III and the Kpn I sites. In p53/53/54, p60/60/61 and p63/63/64, the intron acceptor sequence and the 3' exon were cloned into the Xho I and the Xba I sites. In p56/56/57, the intron acceptor sequence and the 3' exon were cloned into the EcoR V and the Xba I sites. Finally a previously characterized dD-ITR junction was inserted in intron between the EcoR I and the EcoR V sites (Fig. 1) [1]. All the constructs were under the transcription regulation of the cytomegalovirus (CMV) promoter and the SV40 polyadenylation signal. All the constructs were validated by sequencing.

Plasmids for generating RPA probe. The RPA probes were designed to hybridize with the 5' exon and the intron donor sequence. Briefly, the corresponding regions from p53/53/54, p56/56/57, p60/60/61 and p63/63/64 were amplified by PCR with the following primers: DL293 (forward primer for p53/53/54 RPA probe, 5'-GCGCGAATTCTGAGTCATGGAAGGAGGGTC-3'), DL294 (reverse primer for p53/53/54 RPA probe, 5'-GCGCGGATCCTTAACGTGATTTTCTGTTAA-3'), DL304 (forward primer for p56/56/57 RPA probe, 5'-GCGCGAATTCGTTACAAAGACGTTTGGATAACATG-3'), DL305 (reverse primer for p56/56/57 RPA probe, 5'-GCGCGGATCCTACGTAGACATGTGAGATACCAG-3'), DL297 (forward primer for p60/60/61 RPA probe, 5'-GCGCGAATTCTACCACTTTGGGCATTCAGC-3'), DL298 (reverse primer for p60/60/61 RPA probe, 5'-GGACTAGTGGATCCCTGTAACAAAGG-3'), DL295 (forward primer for p63/63/64 RPA probe, 5'-GCGCGAATTCCCACGAGACTCAAACAACTTG-3'), and DL296 (reverse primer for p63/63/64 RPA probe, 5'-GCGCGGATCCCTTTCACTGCAAACTACTTTA-3'). PCR products were inserted into the EcoR I and BamH I sites in pGEM3Z (Promega, Madison, Wisconsin). All the constructs were validated by sequencing.

Plasmids for making ΔH2-R19 (ΔH2) mini-dystrophin *trans*-splicing vectors. A pair of *cis* plasmids were generated for making each set of *trans*-splicing vectors, including a *cis* donor plasmid and a *cis* acceptor plasmid. To generate *cis* donor plasmids, we first replaced the region from exon 1 to exon 7 (including the ATG start codon) in the ΔH2 mini-dystrophin gene with a PCR fragment carrying the CMV promoter and the sequence from the start codon to the exon 7. The pcisCMVΔR4/ΔC was used as the PCR template [2]. The forward primer for this PCR reaction is DL307 (5'-GCGCACTAGTGTCGACGCGTTACATAACTTACGGTA-3'). The reverse primer for this PCR reaction is DL308 (5'-GGCGATGTTGAATGCATG-3'). The resulting plasmid carries an efficient Kozak sequence to facilitate gene expression. A Sal I site has also been introduced at the 5' end to help the next cloning step. After optimizing the 5' end of the ΔH2 minigene, the 3' end of the gene was also tailored for splitting the gene at introns 60 and 63, respectively. Briefly, the 3' end of the ΔH2 gene (from the presiding exon to the stop codon at exon 79) was replaced by respective PCR fragments carrying the respective exon and the intron donor sequences and a tail-tagged Sal I site. For pcisDonor.60, the plasmid p60/60/61 was used as the PCR template, DL334 (5'–GCGCCTCGAGAAAGTCAAGGCACTTCGAGGAGAAATTGCGCCTC-3') was used as the forward primer and DL335 (5'-GCGCCATATGGTCGACCTGTAACAAAGGACAACAATGTTTAC-3') was used as the reverse primer. For pcisDonor.63, the plasmid p63/63/64 was used as the PCR template, DL309 (5'-GACAGAGCTCTACCAGTCTTTAGG-3') was used as the forward primer and DL310 (5'-GCGCCATATGGTCGACCTTTCACACTGCAAACTAC-3') was used as the reverse primer. Finally, the respective intermediate plasmids were cloned into the Sal I site in pDD2 to generate pcisDonor.60 and pcisDonor.63 [3]. To generate pcisAcceptor.60 (a *cis* acceptor plasmid to split the gene at intron 60), we first performed a two step PCR to obtain the fragment from the endogenous intron 60 splicing acceptor signal to the stop codon of the minigene. The first step includes two PCR reactions. In one PCR reaction, we used pZX15 (a cloning intermediate carrying exon 60, intron 60 splicing sequences

and exon 61) as template and DL337 (5'-GCGCGGTACCTATAGAATGAGAGAACATAA-3') as the forward primer and DL338 (5'-TGGAAAGAAAGTGCTGAGA-3') as the reverse primer to obtain a fragment from intron 60 to exon 61 and to also introduce a Kpn I site in front of the intron 60 splicing acceptor signal. In the other PCR reaction, we used the ΔH2 minigene as the template and DL336 (5'-CTTTTCCTCCCAGGTGGCCGTCGAGGACCGAG-3') as the forward primer and DL312 (5'-GCGCTCTAGAGCCATGTGGAAAAGACTTCC-3') as the reverse primer to obtain a fragment from the end of intron 60 to the end of stop codon. In the second step, we used the purified PCR products from two PCR reactions of the first step as templates (at a molar ratio of 1:1) and use DL337 as the forward primer and DL312 as the reverse primer to amplify the region from the intron 60 splicing acceptor signal to the end of the minigene. The PCR product obtained from the second step contains a Kpn I site at the 5' end and a Xba I site at the 3' end. Finally, this fragment was inserted between the Kpn I and the Xba I sites in pDD295 [4]. The resulting plasmid is named pcisAcceptor.60. A similar strategy was used to generate pcisAcceptor.63. The primers and the templates used for making pcisAcceptor.63 are as follow. The template for the first PCR fragment in the first step is pZX16 (a cloning intermediate carrying exon 63, intron 63 splicing sequences and exon 64), the forward primer for this PCR reaction is DL313 (5'-GAAAGGGTACCGAGTCGG-3') and the reverse primer for this PCR reaction is DL314 (5'-AGCAAAGGGCCTTCTGCAGTC-3'). The template for the second PCR fragment in the first step is the ΔH2 minigene, the forward primer for this PCR reaction is DL311 (5'-GTTTTCCCTCTTTTCAGCTGACCTGAATAATGTCAGATTC-3') and the reverse primer for this PCR reaction is DL312 (5'-GCGCTCTAGAGCCATGTGGAAAAGACTTCC-3'). All the constructs were validated by sequencing.

RNase Protection Assay (RPA). Four μg of each individual experimental plasmid (including p53/53/54, p56/56/57, p60/60/61 and p63/63/64) and 0.5 μg of a control luciferase plasmid pDD12 [1]

were transfected into 70-80% confluent 293 cells using Lipofectamine and Plus reagent (Invitrogen, Carlsbad, California). At 48 hrs after transfection, cells were harvested and protein was extracted from one fifth of cell pellet for luciferase activity assay while total RNA was isolated from the rest of cells according to a published protocol [5]. Briefly, cells were lysed in 4 M guanidine isothiocyanate and total RNA was pelleted in 5.7 M CsCl by ultra-centrifugation. The antisense RPA probes were synthesized with SP6 RNA polymerase by *in vitro* transcription with [α-^{32}P]-rUTP substrate. For RPA reaction, 10 μg total RNA was hybridized with excessive amount of ^{32}P-labeled probes and unprotected RNA was digested by RNaseT1 and RNaseA at the final concentration of 0.923 μg/ml and 18.46 μg/ml, respectively. To normalize RNA extraction, RPA reaction and loading, we also included a probe for endogenous human β-actin in each RPA reaction as we described before [1]. The protected RNA was separated in 8% denaturing polyacrylamide gel and viewed by autoradiography. The protected RNA signals were quantified with the Molecular Imager FX and Quantity One (version 4.2.2 image software; Bio-Rad, Heracules, California). Relative signal intensity was normalized for transfection efficiency (by luciferase activity), RPA reaction and loading (by the internal human β-actin control), and the number of ^{32}P-labeled uridine in each protected band. The expected unspliced and spliced bands for p53/53/54 are 175 nt and 59 nt, respectively. The expected unspliced and spliced bands for p56/56/57 are 170 nt and 69 nt, respectively. The expected unspliced and spliced bands for p60/60/61 are 122 nt and 79 nt, respectively. The expected unspliced and spliced bands for p63/63/64 are 137 nt and 62 nt, respectively.

Muscle protein extract preparation. Protein from individual TA muscle was extracted with a modified microsome preparation protocol [6]. Briefly, muscle was pulverized in liquid nitrogen by hand grinding and then homogenized in a buffer containing 20 mM $Na_4P_2O_4$, 20 mM $NaHPO_4$, 1 mM $MgCl_2$, 0.5 mM EDTA and 303 mM sucrose. The muscle lysate was then cleared by centrifuging at

15,000 rpm, 4 ºC for 15 min in a Sorval RC-5B supercentrifuge. The supernatant was filtered through six layers of cheese-cloth and centrifuged at 34,000 rpm, 4 ºC for 30 min in a Beckman Optima XL-80K ultracentrifuge. The pellet was resuspended in a wash buffer containing 20 mM Tris (pH 7.0), 60 mM KCl, 303 mM sucrose and centrifuged again at 34,000 rpm. Finally, the muscle membrane fraction (microsome) pellet was dissolved in a buffer containing 20 mM Tris (pH 7.0) and 303 mM sucrose.

Muscle force measurement. At three months post-infection, the EDL muscle was carefully dissected and immediately mounted in a jacket organ bath containing oxygenated Ringer's solution (5% CO_2, 95% O_2, pH 7.5, 30 °C). The proximal tendon was fixed in a clamp and the distal tendon was connected to a dual-mode servomotor transducer (300B or 305B, Aurora Scientific, Inc., Aurora, Ontario, Canada). After a 10 min rest at 1 g preload, muscle was stimulated at 600 mA with a 200 μs pulse duration (701A Stimulator; Aurora Scientific, Inc., Aurora, Ontario, Canada). At first, the optimal length (Lo) was determined as the muscle length at which the maximal twitch force was elicited. We then applied three 500 ms tetanic stimulations at 150 Hz to activate all the muscle fibers. After a 5 min rest, we measured the absolute tetanic muscle force after applying three 500 ms stimulations at 80, 120, and 150 Hz respectively. The specific muscle force (kN/m^2) was obtained after normalizing the absolute tetanic force with muscle cross-sectional area (CSA). Muscle CSA was calculated according to the following equation, CSA = (muscle mass, in g)/[(optimal fiber length, in cm) x (muscle density, in g/cm^3)]. The optimal fiber length (L_f) was determined by multiplying Lo by an L_f/Lo ratio of 0.44 [7, 8]. A muscle density of 1.06 g/cm^3 was used [9]. After another 15 min rest, we performed eccentric contraction assay. Briefly, the EDL muscle was stimulated at 150 Hz for 700 ms. After the first 500 ms, the muscle was lengthened by 10% Lo at 0.5 Lo/s for 200 ms. This stimulation-stretch cycle was repeated every 2 min for a total of 10 cycles. The maximal isometric tetanic force

developed during the first 500 ms of stimulation of the first cycle was designated 100%. The percentage of tetanic force loss at each cycle was determined by comparing with the first stimulation. Data acquisition from the servomotors was conducted with a LabView-based DMC program (Version 3.12, Aurora Scientific, Inc., Aurora, Ontario, Canada). Length and force data was analyzed by a LabView-based DMA program (Version 3.12, Aurora Scientific, Inc., Aurora, Ontario, Canada).

Muscle fiber type determination. Two methods were used to determine muscle fiber type. To differentiate between the slow type fiber (type I) and the fast type fiber (type II), we performed immunostaining with an anti-MyHC I antibody (monoclonal, Sigma, St. Louis; 1:2,000). To distinguish type IIA (fast oxidative) and type IIB (fast glycolytic) muscle fiber, we performed Toludine blue staining, a metachromatic dye-ATPase method for the simultaneous identification of different subtypes of type II muscle fiber [10]. Type IIA fiber stains as light pinkish blue and type IIB fiber stains in blue (Supplementary Fig. 6*b*).

References for Supplementary Methods

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