Supporting Information

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SI Materials and Methods

Western Blot Analysis. For cell membrane preparations, biglycan null myotubes were washed in PBS, scraped from tissue culture flasks and homogenized in dissection buffer (0.3 M sucrose, 35 mM Tris, pH 7.4, 10 mM EDTA, 10 mM EGTA ,and protease inhibitor mixture; Roche Applied Science). Samples were centrifuged at 7,000 × *g* at 4 °C for 5 min. Membranes were then collected by centrifugation of the supernatants at 38,000 × *g* for 60 min at 4 °C. Protein concentrations were determined by the bicinchoninic acid protein concentration assay (Pierce). For total protein extraction from biglycan null myotubes, cells were washed in PBS and solubilized in RIPA lysis buffer (Santa Cruz Biotechnology) for 25 min, lysates were centrifuged at 10,000 × *g,* and supernatants were collected. Membrane fractions from quadriceps and biceps femoris were prepared as previously described (1).

Cell or muscle fractions were separated by SDS/PAGE and proteins were transferred to nitrocellulose membranes. Total protein staining (SYPRO Ruby; Invitrogen) was visualized on a Storm Imager (Amersham Bioscience). Blots were incubated with primary antibody followed by goat anti-mouse IgG conjugated to HRP (Amersham). Signal was detected with ECL plus (Amersham) using a Storm Imager.

Quantitative RT-PCR. RNA extraction from the biglycan null immortalized muscle cell line and quadriceps femoris muscles from injected mdx animals was performed using the TRIzol method (Invitrogen). Purified RNA was converted to cDNA using the SuperScript III First-Strand Synthesis System Kit (Invitrogen). qPCR reactions were performed using the SYBR-Green method (Invitrogen) on the ABI PRISM 7300 real-time thermocycler. Primers were designed using DS Gene primer design software (Accelrys). ATP synthase was used for normalization. Data analysis was performed using the standard curve method (2).

The primers used were as follows: ATPSase forward: 5′-TGG GAA AAT CGG ACT CTT TG-3′; ATPSase reverse: 5′-AGT AAC CAC CAT GGG CTT TG; Utrophin forward: 5′- TCC CAA GAC CCA TTC AAC CC; Utrophin reverse: TGG ATA GTC AGT GTT TGG TTC C (gi110431377; 3′ UTR between bases 10383–12382).

Animals. Congenic biglycan null mice on a C3H background were generated as described previously (1) and were compared with WT C3H from the Jackson Laboratory. C57BL/10ScSn-mdx/J mice were obtained from Jackson Laboratory; *mdx:utrn*$^{-/-}$ mice were bred as described (3).

Antibodies. The following primary antibodies were used: monoclonal anti-utrophin (Vector Labs), rabbit anti-utrophin (a generous gift of S. Froehner, University of Washington, Seattle, WA), rabbit anti-dystrophin (Abcam), monoclonal anti–γ-sarcoglycan (Vector), rabbit anti-laminin (Sigma), rabbit anti–β2-syntrophin (4), and rabbit anti-nNOS (Invitrogen). The specificity of the monoclonal anti-biglycan (2A5) (1) and rabbit anti-biglycan (5) was established by Western blot (1, 5, 6) and ELISA (*Results*); no reactivity was observed when these reagents were tested on biglycan null samples. The following secondary antibodies were used: Alexa 488 goat anti-mouse IgG and Alexa 555 goat anti-rabbit IgG (Invitrogen), HRP goat anti-mouse IgG, and HRP goat anti-rabbit IgG.

Cell Culture. Immortalized biglycan null cells were grown as previously described (1). Cells were differentiated for 4–5 d and then treated with 1 nm rhBGN in differentiation medium for 8 h.

Serum Chemistries. Blood was collected by cardiac puncture from rhBGN and vehicle injected mice and spun at 3,300 RPM for 10 min to separate serum. Serum creatine kinase, BUN, creatinine, AST, and total bilirubin analyses were performed by the University of Californai–Davis Comparative Pathology Laboratory.

Detection of rhBGN in Serum. Adult C57/B6 mice were injected i.p. with 10 mg/kg rhBGN, and blood was collected by cardiac puncture 30 min, 1 h, and 24 h postinjection (n = 3–4 mice/condition). Control experiments showed that comparable levels of rhBGN were present in plasma (0.12 μg/mL at 1 h postinjection, n = 2). For two-site ELISAs, plates were coated with mouse anti-biglycan antibody, blocked, and incubated with serum samples or standard biglycan dilutions followed by rabbit anti-biglycan and goat anti-rabbit HRP. Sensitivity of the assays was ~5 ng/mL.

1. Mercado ML, et al. (2006) Biglycan regulates the expression and sarcolemmal localization of dystrobrevin, syntrophin, and nNOS. *FASEB J* 20:1724–1726.
2. Biggar WD, et al. (2004) Deflazacort in Duchenne muscular dystrophy: A comparison of two different protocols. *Neuromuscul Disord* 14:476–482.
3. Mann CJ, et al. (2001) Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci USA* 98:42–47.
4. van Deutekom JC, et al. (2007) Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med* 357:2677–2686.
5. Bowe MA, Mendis DB, Fallon JR (2000) The small leucine-rich repeat proteoglycan biglycan binds to alpha-dystroglycan and is upregulated in dystrophic muscle. *J Cell Biol* 148:801–810.
6. Rafii MS, et al. (2006) Biglycan binds to alpha- and gamma-sarcoglycan and regulates their expression during development. *J Cell Physiol* 209:439–447.

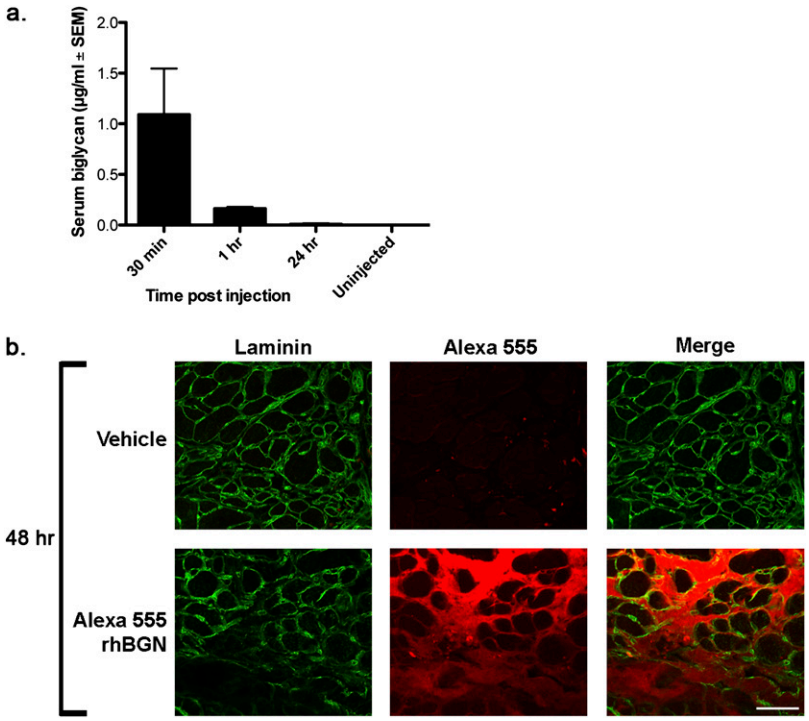

Fig. S1. Systemically delivered rhBGN can be detected in the circulation and becomes localized to muscle. (*A*) Detection of rhBGN in serum following i.p. delivery. Mice were injected i.p. with 10 mg/kg rhBGN, and serum was collected 30 min, 1, and 24 h postinjection (n = 3–4 animals/group). Two-site ELISAs were performed as described in *Materials and Methods*. Biglycan (endogenous) was not detected in serum from uninjected mice. However, rhBGN was readily detected in serum following a systemic injection of the recombinant protein. (Scale bar = 50 μm.) (*B*) Systemically delivered rhBGN becomes stably localized to muscle. Alexa 555-rhBGN (*Materials and Methods*) was injected i.p. into adult mdx mice, and diaphragms were harvested 48 h later. Endogenous laminin was detected by indirect immunofluoresence. Systemically delivered Alexa 555-rhBGN is localized in the extracellular matrix surrounding the myofibers.

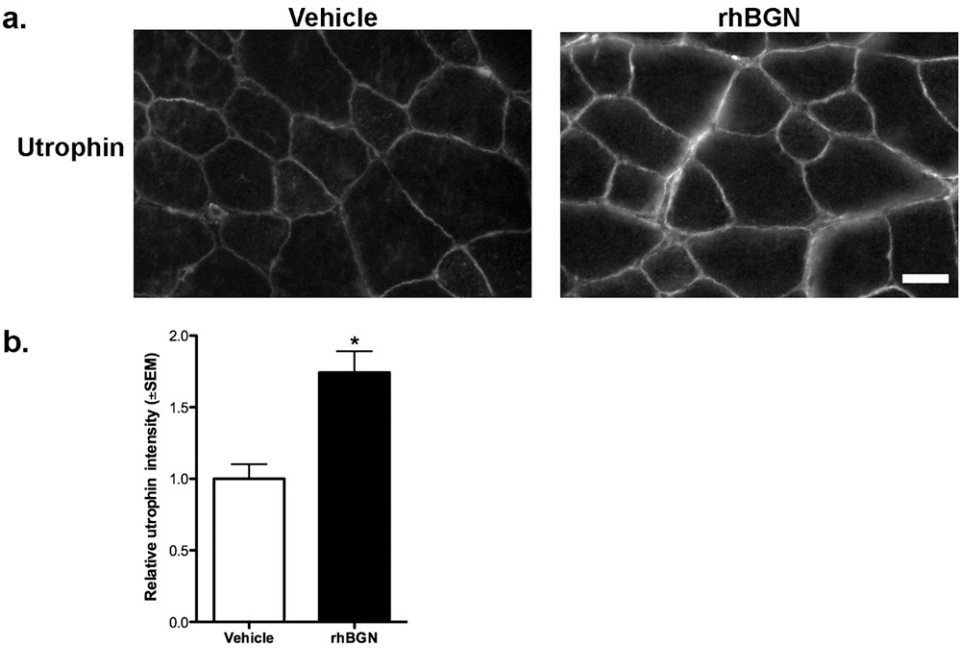

Fig. S2. RhBGN treatment increases sarcolemmal utrophin expression in the tibialis anterior of mdx mice. (*A*) Utrophin immunostaining of TA muscles from mdx mice that received one i.p. injection of rhBGN or vehicle. Systemically delivered rhBGN increased utrophin expression in TAs of mdx mice compared with vehicle-injected mice. (*B*) Quantification of increased utrophin expression in TA muscle from rhBGN treated mice (1.74-fold increase, $*P < 0.001$, Student unpaired t test; n = 300 sarcolemmal segments from three muscles for each group). (Scale bar = 25 μM.)

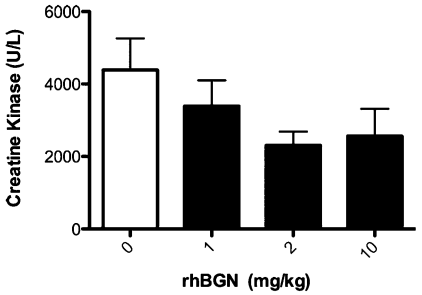

Fig. S3. Creatine kinase levels in rhBGN-treated mdx mice. Creatine kinase levels in ^{32}P mdx mice that received a single injection of 1 mg/kg (n = 23), 2 mg/kg (n = 12), or 10 mg/kg (n = 11) rhBGN or vehicle alone (n = 24) at P18. RhBGN-treated mice showed trends of decreased CK levels, but the results did not reach statistical significance (one-way ANOVA, $P > 0.05$).

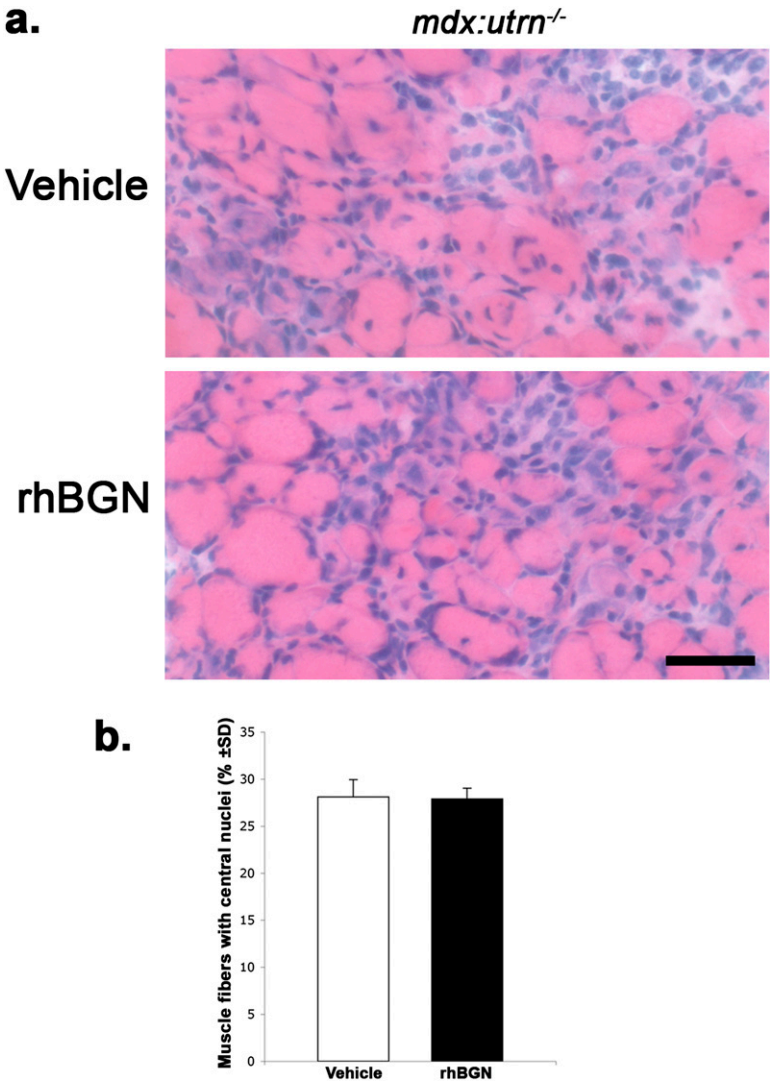

Fig. S4. RhBGN fails to counter dystrophic pathology in *mdx:utrn*$^{-/-}$ double KO animals. (*A*) Mutant mice lacking both dystrophin and utrophin (*mdx:utrn*$^{-/-}$) were injected at P19 with recombinant rhBGN or vehicle. Diaphragms were isolated 3 wk later, sectioned, and stained with H&E. Characteristic extensive muscle pathology of these double KO animals—areas of mononuclear cell infiltration and foci of necrosis/regeneration and centrally nucleated myofibers—was comparable in rhBGN- and vehicle- injected animals. (Scale bar = 50 μm.) (*B*) RhBGN administration does not decrease CNFs in *mdx:utrn*$^{-/-}$ mice. Percentages of centrally nucleated muscle fibers were determined from the H&E-stained diaphragm sections from rhBGN and vehicle injected *mdx:utrn*$^{-/-}$ (n = 2 vehicle-injected and 3 rhBGN-injected mice; unpaired Student t test, P = 0.45).

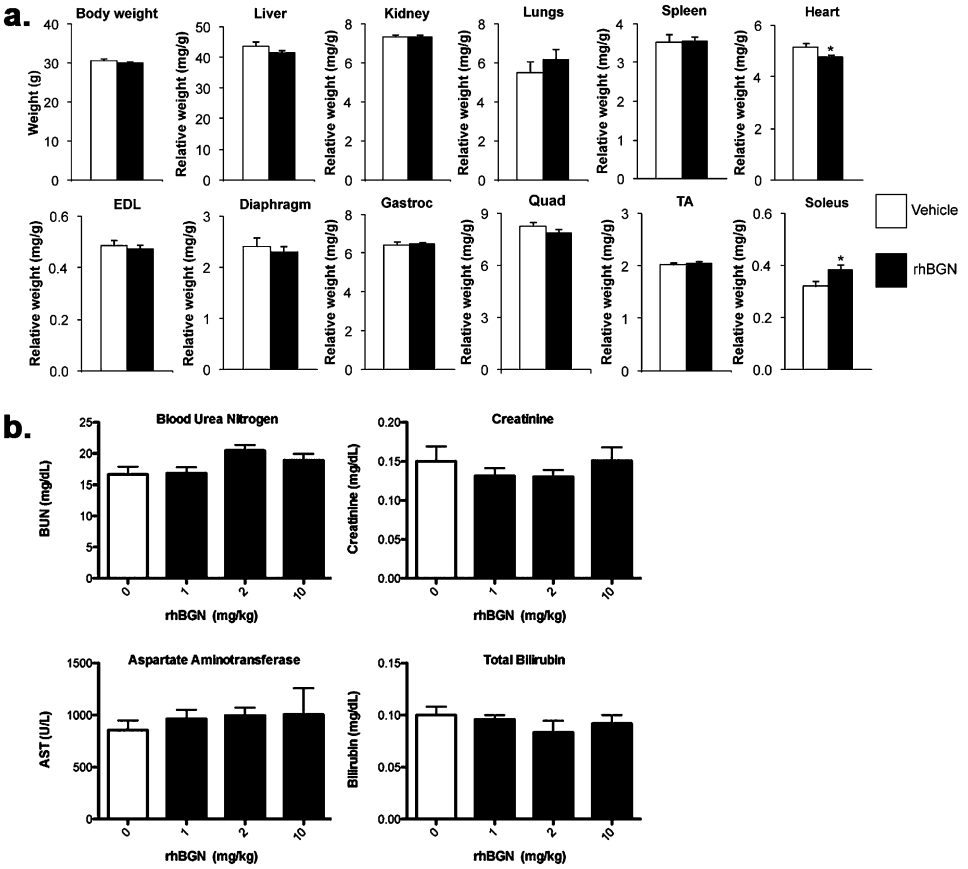

Fig. S5. RhBGN is well tolerated in mdx mice. (*A*) P14 mdx mice were injected at 3-wk intervals for 3 mo with either rhBGN or vehicle. Tissues were harvested at 15 wk and weighed. All organ and muscle weights are plotted relative to total body weight in mg/g ($n = 8$ animals/group; $^*P < 0.05$; unpaired Student t test). (*B*) Liver and kidney function in rhBGN treated mice. Serum was collected from ^{32}P mdx mice that received an i.p. injection of 1, 2, or 10 mg/kg rhBGN or vehicle only. There were no significant changes in serum levels of BUN, creatinine, AST, or total bilirubin.

Table S1. Contractile properties of extensor digitorum longus (EDL) muscles

Parameter	Control mdx (n = 16 muscles)	Treated mdx (n = 16 muscles)
ECC force drop 1–5 (%)	22.2 ± 2.7	14.9 ± 1.2*
Twitch		
Absolute force (mN)	108.9 ± 5.1	107.3 ± 6.2
Specific force (mN/mm^2)	54.0 ± 3.2	56.6 ± 4.0
Tetanus		
Absolute force (mN)	577.5 ± 27.6	559.4 ± 30.5
Specific force (mN/mm^2)	287.0 ± 18.3	295.0 ± 19.7
EDL weight (mg)	14.7 ± 0.7	14.2 ± 0.4
EDL Lo (mm)	12.4 ± 0.2	12.5 ± 0.1
CSA (mm^2)	2.07 ± 0.07	2.00 ± 0.08

Mdx mice were injected at 3-wk intervals starting at P14 with either rhBGN (25 μg/injection, i.p.) or vehicle, and tissue was harvested at 15 wk of age. Data were collected and analyzed as described in *Materials and Methods* and are from the same set of muscles as presented in Fig. 8. CSA, cross sectional area; ECC, eccentric contraction; Lo, muscle length.
*P = 0.02, unpaired Student t test.